PATHS OF TRANSTUBULAR WATER FLOW IN ISOLATED RENAL COLLECTING TUBULES

JARED J. GRANTHAM, CHARLES E. GANOTE, MAURICE B. BURG, and JACK ORLOFF

From the Laboratory of Kidney and Electrolyte Metabolism, National Heart Institute, and the Laboratory of Experimental Pathology, National Institute of Arthritis and Metabolic Diseases, National Institutes of Health, Bethesda, Maryland 20014

ABSTRACT

The cells of perfused rabbit collecting tubules swell and the intercellular spaces widen during osmotic flow of water from lumen to bath induced by antidiuretic hormone (ADH). Ouabain had no influence on these changes. In the absence of net water flow intercellular width was unaffected when tubules were swollen in hypotonic external media. Therefore, during ADH-induced flow widening of intercellular spaces is not a consequence of osmotic swelling of a closed intercellular compartment containing trapped solutes, but rather is due to flow of solution through the channel. Direct evidence of intercellular flow was obtained. Nonperfused tubules swollen in hypotonic media were reimmersed in isotonic solution with resultant entry of water into intercellular spaces. The widened spaces gradually collapsed completely. Spaces enlarged in this manner could be emptied more rapidly by increasing the transtubular hydrostatic pressure difference. In electron micrographs a path of exit of sufficient width to accommodate the observed rate of fluid flow was seen at the base of the intercellular channel. It is concluded that the intercellular spaces communicate with the external extracellular fluid and that water, having entered the cells across the luminal plasma membrane in response in ADH, leaves the cells by osmosis across both the lateral and basilar surface membranes.

Vasopressin increases the osmotic permeability to water in several receptor tissues (1–3). The hormone stimulates the production of cyclic 3′,5′-adenosine monophosphate, which, in turn, mediates the change in water permeability at the urinary surface of the tissue possibly by increasing the size or number of aqueous pores (1, 2, 4). Implicit in this postulated mechanism has been the view that water flows directly through the cytoplasm and not between adjacent cells. However, recent electron microscope studies have been interpreted as indicating that water may pass across epithelia by an extracellular as well as a cellular route in response to antidiuretic hormone (ADH) (5, 6). The purpose of the present studies was to obtain further evidence in isolated collecting tubules that water flows between as well as through the cells in response to ADH.

MATERIALS AND METHODS

Collecting tubules were removed from the cortex and outer medulla of rabbit kidneys as described previously (3, 6–8). They were preincubated for 3 hr at 23°–25°C in gassed (95% O_2 and 5% CO_2) medium (NaCl, 115 mM; KCl, 5 mM; $NaHCO_3$, 25 mM; Na acetate, 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, Maryland]; and dextrose, 5.5

mM). The final osmolality and pH were 290 milliosmolal and 7.4, respectively. The osmolality of this basic mixture was varied when necessary by removing or adding NaCl.

The tubules were placed in a special chamber mounted on the stage of a Unitron inverted microscope (Unitron Instrument Co., Newton Highlands, Mass.) and were observed directly at high magnification (× 200–600) throughout the course of the experiment, including the period of fixation and dehydration. For illumination the beam from a high intensity Kohler illuminator was directed on a disk of frosted glass placed approximately 5 cm above the tissue. With the diffused light and ordinary bright-field objectives, individual cells and subcellular structures including nuclei, vacuoles, and mitochondria were visible.

Perfused Tubules

The technique of tubule perfusion and measurement of net water absorption and the composition of perfusion solutions have been described previously (6, 8). In the present studies, tubules were perfused with either a 290 or a 125 milliosmolal solution. Test agents and fixatives were added to the outer bathing solution only.

In order to determine whether active sodium transport is a significant factor in initiating the morphologic response of collecting tubules to antidiuretic hormone (ADH) during perfusion with hypotonic solution, ouabain (California Biochemical Corporation, Los Angeles, Calif.) was added to the bath prior to treatment with hormone (two tubules), or during ADH-stimulated maximal net water absorption (one tubule). In a single experiment ouabain alone was added. A concentration of the drug was used (5×10^{-5} M) which is known to completely eliminate the transtubule electrical potential difference of collecting tubules within 20 min (9).

Nonperfused Tubules

Net water flow from lumen to bathing medium was eliminated in some experiments by studying nonperfused tubules with collapsed lumens. The ends of these tubules were occluded tightly. Since the volume of the collapsed lumen was negligible, the tubular volume (cells plus interspaces) of fixed and unfixed specimens was computed from the outer diameter and length measured from photographs of the individual tubules. Several measurements of tubular volume were made on each specimen before and after changes in size were induced by altering the osmolality of the bathing solution. In some experiments an image-splitting measuring eyepiece (Vickers Instruments Inc., Malden, Mass.) was used to make rapid measurements of tubular diameter.

Osmotically Induced Swelling and Shrinking of Tubules

Tubular volume of both perfused (five tubules) and nonperfused (25 tubules) tubules was altered rapidly by changing the tonicity of the outer bathing solution. Solutions with osmolalities covering a range of 117–450 milliosmolal were used. Osmolality was measured with an Aminco-Bowman osmometer. ADH was added in some experiments (25 μU/ml Pitressin; Parke-Davis, Detroit, Mich.). Tubules were fixed for electron microscopy at 1–120 min after the experimental change in tubular volume.

Effect of Luminal Pressure on Intercellular Spaces

The effect of changes in transtubular hydrostatic pressure on the size of intercellular spaces was evaluated in four tubules. The innermost of two concentric glass micropipets used to perfuse tubules (7) was filled with a solution isosmotic with the regular medium in the outer bath. The perfusion pipet had an inside tip diameter of 15 μ. The perfusion pipet was attached via plastic tubing to a 5 ml reservoir which was open to the atmosphere. Tubules were cannulated and perfused with a pressure head of 10 cm H_2O. Once the tubule lumen was distended, the distal end of the specimen was occluded by crimping, and the pressure within the tubule was regulated by varying the height of the reservoir. The pressure was first reduced to zero, and the tubule lumen remained collapsed during the initial 200 min of incubation. The effect of lumen pressure on the size of the lateral channels was evaluated by first loading the intercellular spaces with fluid, by means of a procedure to be described later, and then by determining the relationship between the rate of collapse of the intercellular spaces and the applied hydrostatic pressure. No ADH was present in these experiments.

Tracer Studies

Horseradish peroxidase (Sigma Chemical Co., St. Louis, Mo.) was used as a tracer (10) to determine whether the basilar junction of adjacent cells permits substances of large molecular size to enter the intercellular spaces from the bath. Three nonperfused tubules were incubated for 1, 5, and 15 min, respectively, in regular medium containing horseradish peroxidase (5 mg/ml). The tubules were fixed in 1.1% glutaraldehyde solution, incubated for 15 min in reagent containing 3,3′-diaminobenzidine tetrahydrochloride (DAB) (Sigma Chemical Co., 50 mg DAB/100 ml of 0.05 M, Tris-HCl buffer, pH 7.6) and H_2O_2 (0.01%), and postfixed in osmium tetroxide (11). In three other tubules the enzyme was also included in the glutaraldehyde fixative solution. As a

control the enzyme was omitted, and the tubule was carried through the developing reactions.

Fixation for Electron Microscopy

Previously (6), glutaraldehyde was substituted isosmotically for sodium chloride in the regular medium (final glutaraldehyde concentration, 0.9%). In the present studies it was also necessary to fix tubules which had been swollen in solutions made hypotonic by reducing the concentration of sodium chloride. Invariably, the tissues swelled further when solutions isosmotic with the hypotonic medium and containing 1.1% glutaraldehyde were used to fix swollen tubules. The change in volume during fixation was prevented by adding more sodium chloride to the fixative solution. Empirically, it was found that in hypotonic solutions 10.6 g of glutaraldehyde per liter (Fisher Scientific Company, Pittsburgh, Pa.) were as osmotically effective as about 20 mEq of sodium chloride per liter. This relationship was used when glutaraldehyde was substituted for sodium chloride in different hypotonic fixative solutions. Incubation for 10–15 min in a 1.1% glutaraldehyde solution produced satisfactory fixation. It was observed that tissues fixed even longer in glutaraldehyde or osmium tetroxide retained osmometric properties. Therefore, in order to prevent volume changes during processing of the tissue subsequent to primary fixation, all solutions were kept isosmotic (or, in the case of glutaraldehyde and osmium tetroxide solutions, of equivalent "effective" osmotic activity) to the medium bathing the tubule before initial fixation. In this manner, tubular volume was maintained constant during fixation with glutaraldehyde and osmium tetroxide and rinsing. In some experiments single tubules were stained in 1% aqueous uranyl acetate solution prior to dehydration in alcohol. Dehydration in alcohol always caused the tissue to shrink to approximately 65% of its original volume. For obvious reasons, it would be desirable to prevent shrinkage during dehydration in alcohol, but this was not possible.

After dehydration in alcohol, the tubules were passed through propylene oxide and embedded in Araldite 502 (Ciba Products Co., Fair Lawn, N. J.). Sections were cut with glass or diamond knives, mounted on Formvar-coated copper grids, stained with uranyl acetate and lead citrate (12), and examined in a Philips EM 200 electron microscope.

RESULTS

Effect of Ouabain on Intercellular Spaces

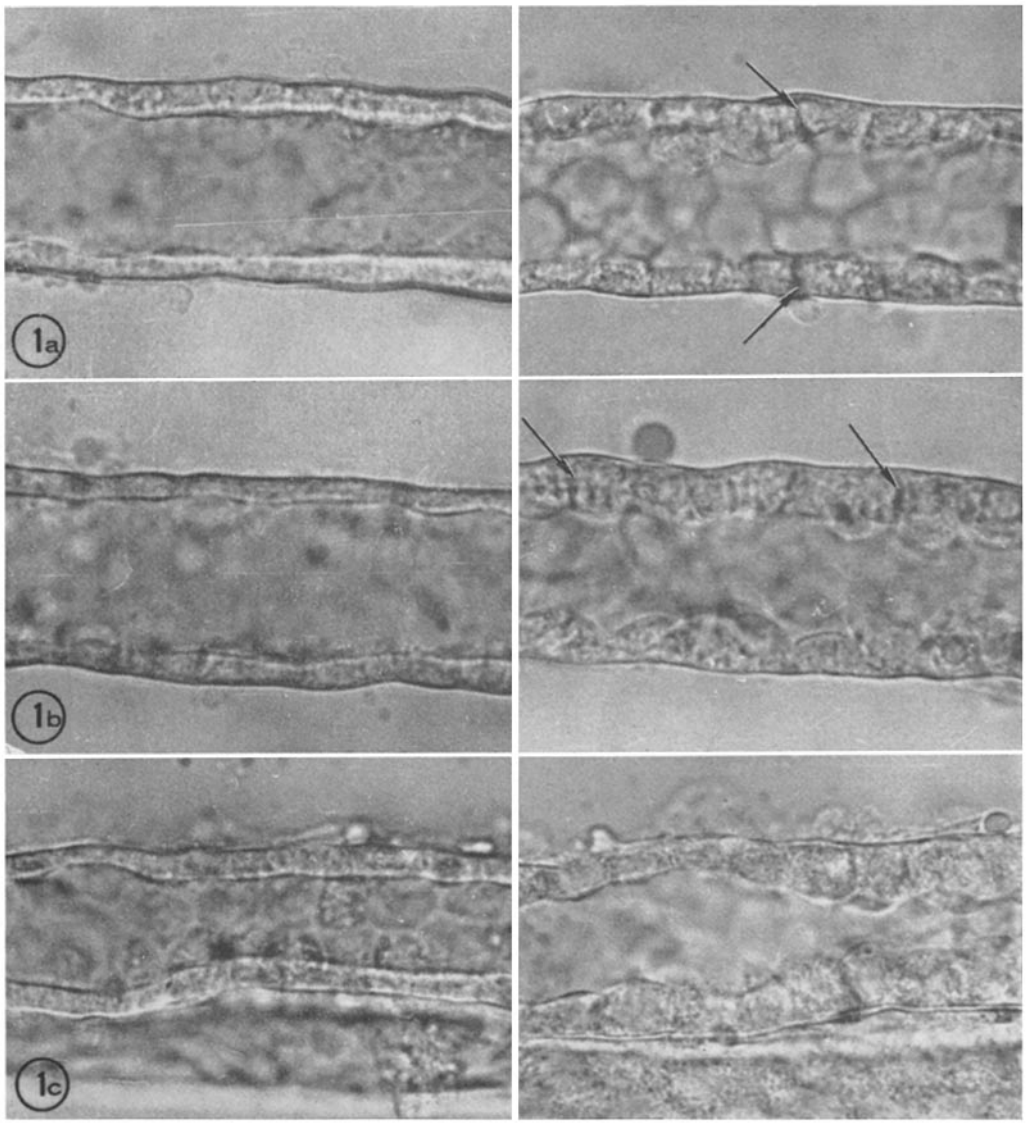
As reported previously (6), tubules perfused with a hypotonic solution respond to ADH by a swelling of individual cells and an increase in the width of the intercellular spaces (Fig. 1). When ouabain was added to inhibit sodium transport, the same changes occurred following treatment with ADH (Figs. 1 and 2). Furthermore, the addition of ouabain after the development of the ADH effect did not alter the morphological response to the hormone. In two experiments net water absorption was measured. An increase in osmotic flow was elicited with ADH (mean increment + 9.8 μL cm^{-2} min^{-1} osmol^{-1}) and maintained despite the presence of ouabain. Ouabain alone had no effect on the width of intercellular spaces.

Effect of Swelling Induced by Hyposmotic External Bathing Solution on Tubular Morphology

In order to determine whether tissue swelling per se is associated with dilation of the lateral intercellular spaces, collecting tubules were removed from the kidney and bathed in regular medium (290 milliosmolal). After 200 min of incubation in the absence of ADH, the outer bathing medium was rapidly replaced with a 210 or 147 milliosmolal solution. In these experiments the volume of the cells of nonperfused tubules increased 30 and 79%, respectively. The time course of change in cell volume of nonperfused tubules during swelling in 117 milliosmolal solution is shown in Fig. 3. ADH had no detectable effect on the rate of tissue swelling in hyposomotic bathing medium or on the final appearance of the tissue. Photomicrographs of functioning tubules in hypotonic media revealed obvious swelling of the cells but no increase in the width of the intercellular spaces (Figs. 1 and 4). Nonperfused as well as perfused tubules both in the presence and in the absence of ADH were fixed for electron microscopy at various times after swelling was initiated. In no tubule was widening of the intercellular spaces detected (Fig. 5).

The Effect on Morphology of Shrinking Swollen Tubules in Regular Medium

The time course of shrinkage in regular medium of nonperfused tubules swollen in 117 milliosmolal solution is shown in Fig. 3. In contrast to the water uptake curve which had only a single recognizable rate component of short duration, the "shrink" curve is biphasic with a large rapid component of water loss and a smaller slow component. Upon inspection of the shrink curves it is apparent that the slow component may account for as much as



FIGURES 1 *a–c* Bright-field microscope photographs (diffused light source) of functioning perfused collecting tubules. The focal plane is at the central axis of each tubule. Fig. 1 *a*: *Perfusate hypotonic.* On the left is the appearance in the absence of ADH. The same portion of the tubule is shown on the right after ADH has maximally increased the permeability to water. The cells are swollen, and the intercellular markings (arrows) are more prominent than in the control. × 620. Fig. 1 *b*: *Perfusate hypotonic.* Control tubule is on the left. On the right is the same portion following treatment with both ADH and ouabain. The cells are swollen with increased prominence of intercellular spaces (arrows) as in tubule 1 *a*. × 620. Fig. 1 *c*: *Perfusate isotonic.* Control tubule is on the left and appears similar to control photographs of tubules *a* and *b*. On the right is the appearance after incubation for 74 min in hypotonic bathing medium (147 milliosmolal). Although the cells are swollen, the intercellular markings are not more prominent than in the control. Adherent thick ascending limb at bottom. × 613.

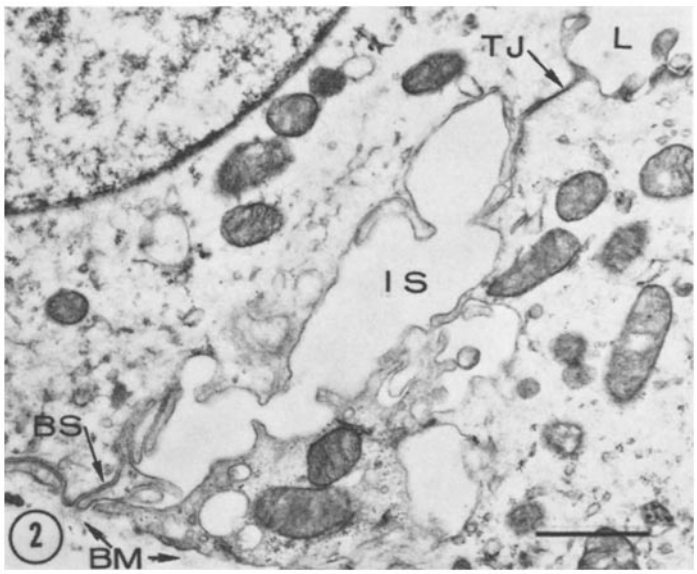


FIGURE 2 An intercellular space (*IS*) of a collecting tubule perfused with hypotonic solution and treated with both ADH and ouabain. The intercellular space is dilated, except for the apical tight junction (*TJ*) and basilar slit (*BS*). *L*, tubular lumen; *BM*, basement membrane. Lead citrate-uranyl acetate staining. Marker, 1 μ. × 15,000.

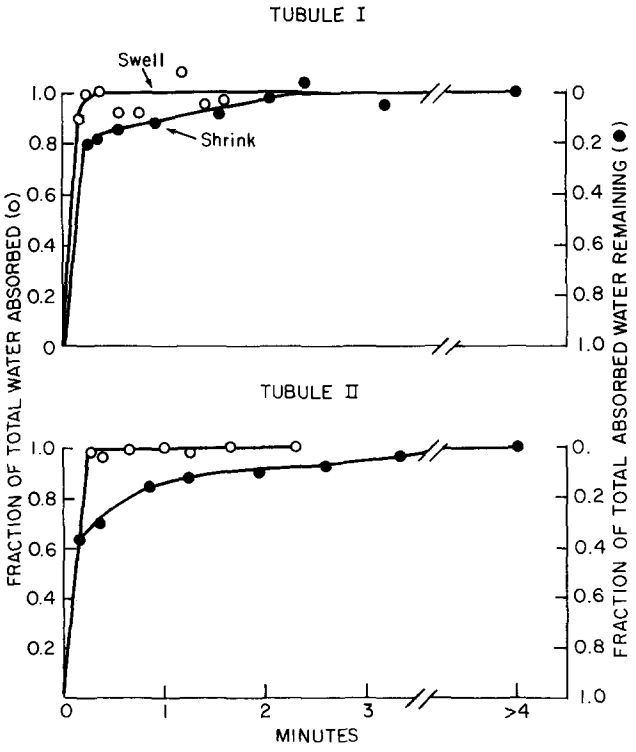
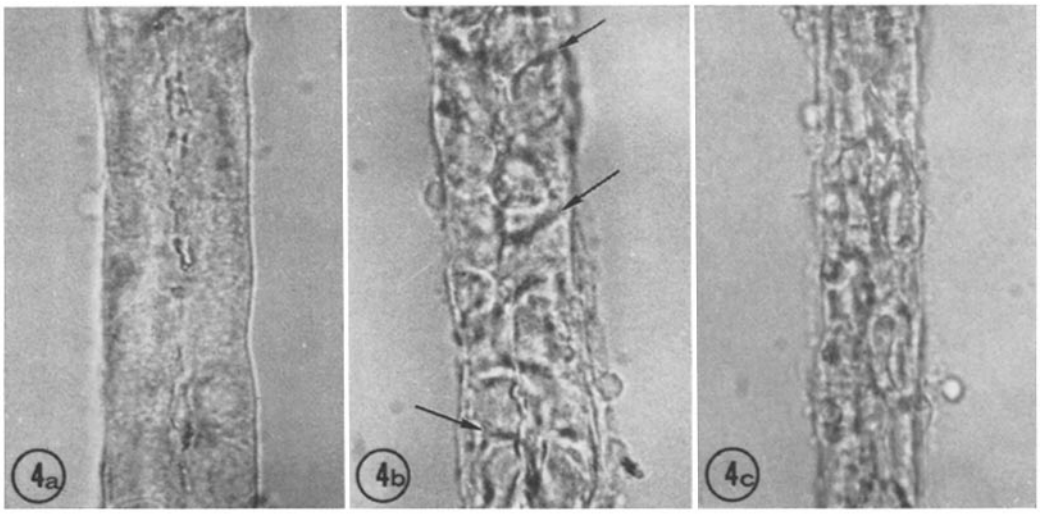


FIGURE 3 The time course of net water gain and loss in nonperfused collecting tubules. The results of two separate studies are shown (Tubules I and II). The curve labeled "swell" depicts water uptake by the tubule following placement in 117 milliosmolal medium. The ordinate on the left is the fraction of total water absorbed, $\frac{Vt - Vo}{Vs - Vo}$, where Vo = steady state tubular volume in regular medium, Vt = volume at time t, Vs = steady state volume in hypotonic medium. The curve labeled "shrink" shows the loss of water from the same swollen specimen following placement back into regular medium. The ordinate on the right, inverted to permit comparison of the two curves, is the fraction of total water absorbed remaining in the tubule, $\frac{Vs - Vt}{Vs - Vo}$. The tubules shrink at a much slower rate than they swell.



FIGURES 4 *a–c* Bright-field microscope photographs of a functioning nonperfused tubule. In Fig. 4 *a* the tubule is shown after incubation in 147 milliosmolal solution. The tissue is swollen, and the intercellular markings are not well defined. In Fig. 4 *b* is the appearance of the tubule 30 sec after having been replaced in regular medium. The tubular diameter is decreased, and the intercellular markings are especially prominent (arrows). Immediately after this photograph was taken the specimen was fixed in glutaraldehyde and OsO_4 without change of tubular volume. In Fig.14 *c* is the appearance after dehydration in alcohol. The tubular diameter is decreased, and there is pronounced irregularity of the basilar surface of the cells. The intercellular spaces remain prominent, however. An electron micrograph of this tubule is shown in Fig. 7. × 640.

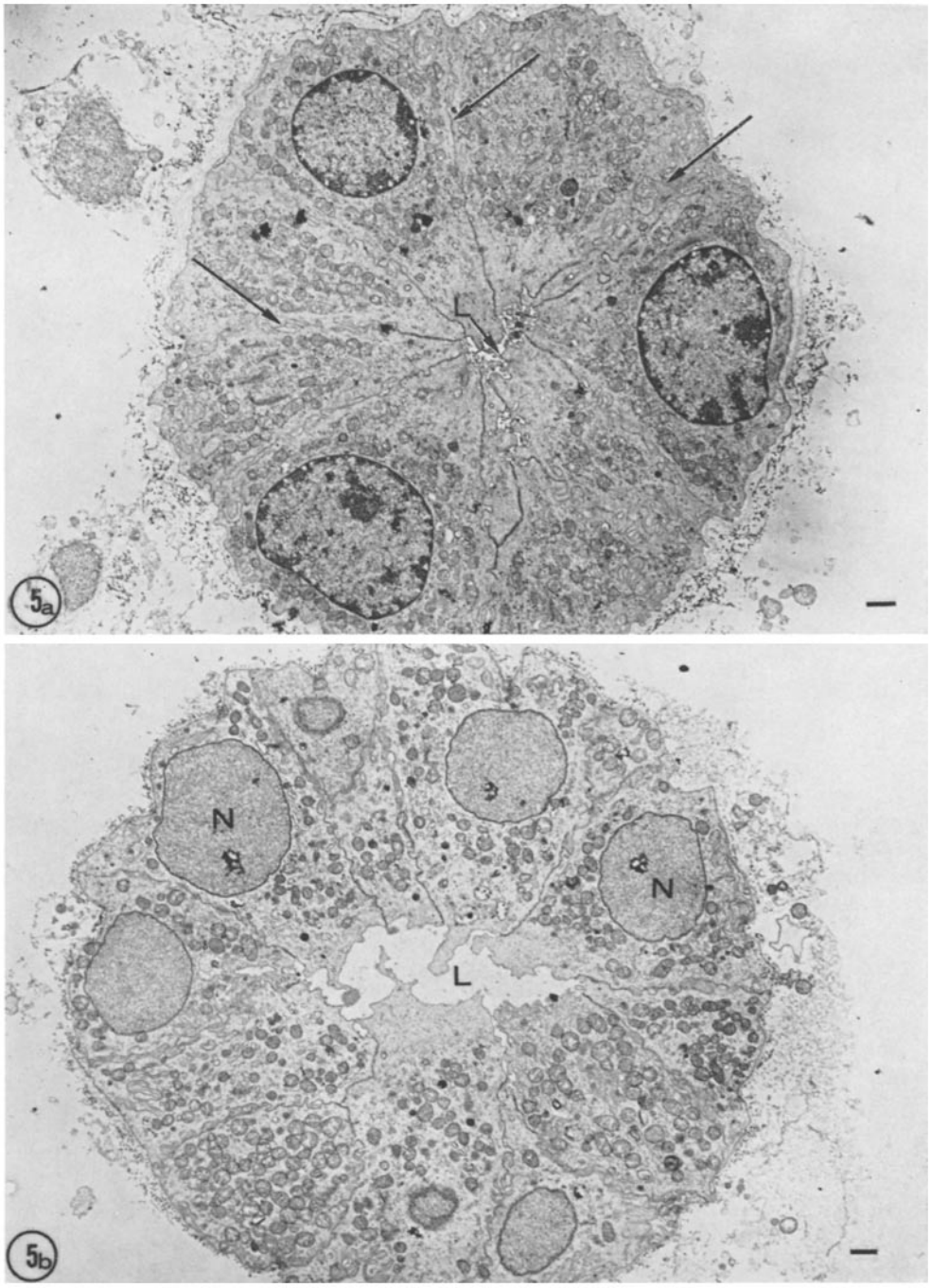
20% of the water lost by the tubule during shrinkage.

In order to determine the effect of the magnitude of osmotic swelling on the subsequent rate of shrinkage in regular medium, tubules were immersed in solutions of differing hypotonicity. The results of two experiments are shown in Fig. 6. In the curve labeled *A* the tubule had been exposed to 117 milliosmolal solution before shrinkage in regular medium; in the curve labeled *B* the tissue had been swollen in 212 milliosmolal solution before shrinkage. For comparison the curves are superimposed so that the labeled point *C* represents the initial relative tubular volume at zero time for curve *B*. In both curves (*A* and *B*) there is a fast component and a slow component of shrinkage in regular medium. By extrapolating to zero time the portion of the curves representing the slow component the initial volume of the slow compartment can be estimated. The initial volume of the slow compartment is much greater in curve *A* than in curve *B*.

Inspection of the tissue in the light microscope during shrinkage revealed widely dilated (up to 3 μ in width) intercellular spaces (Fig. 4) which developed immediately after immersion in regular medium and then gradually disappeared. In eight tubules swollen in 117 or 147 milliosmolal solution the average disappearance time of the intercellular spaces was 200 sec (range 90–350). This time interval approximates that of the slow component of tubule volume contraction shown in Fig. 6. The initial volume of water in the interspaces immediately following immersion of swollen tissue (117–147 milliosmolal) in regular medium was calculated from measurements of light photomicrographs. This volume was as much as 30% of the water lost from the tissue during shrinkage and is sufficient to account for the slow kinetic compartment in curve *A* of Fig. 6.

In tubules initially swollen in 212 milliosmolal solution the intercellular spaces were barely perceptible in the light microscope during shrinkage of the tissues in regular medium. This is compatible with the small initial volume of the slow kinetic compartment defined in curve *B* of Fig. 6.

Ouabain (three experiments) and ADH (two experiments) had no effect on the rate of collapse of the widened intercellular spaces. The swelling and shrinking cycle was repeated as many as 10



FIGURES 5 *a–b* Electron micrographs of nonperfused collecting tubules. In Fig. 5 *a* is the appearance of a tubule in regular bath medium. The lumen (*L*) is not open. The intercellular spaces (arrows) are not dilated. × 4000. In Fig. 5 *b* is the appearance of a tubule incubated in 147 milliosmolal solution for 10 min. The intercellular spaces are the same width as in the control tubule 5 *a*. Dispersion of nuclear chromatin (*N*) was a regular feature of swollen tubules. Although the lumen (*L*) is open in this section, it was rarely seen in nonperfused tubules. Lead citrate and uranyl acetate staining. Marker, 1 μ. × 3,400.

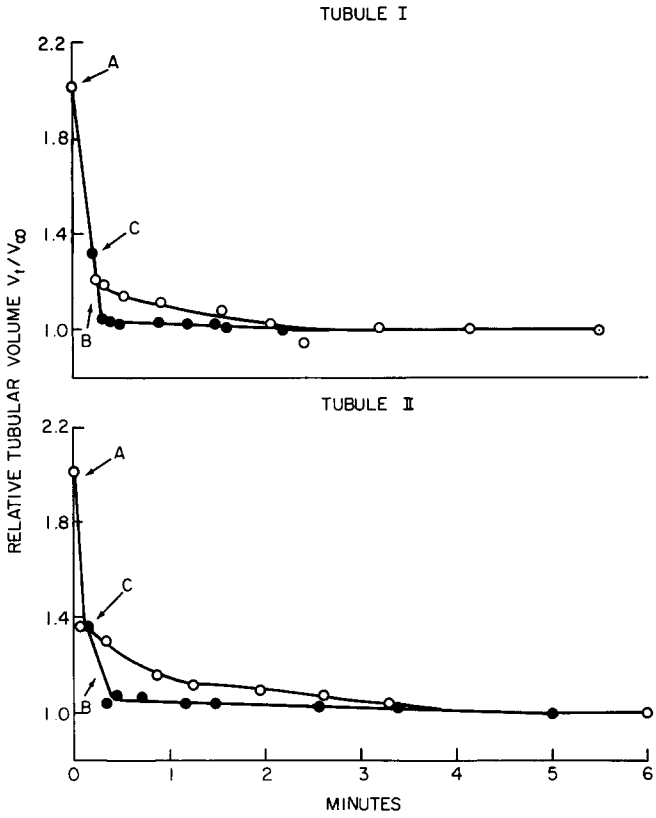


FIGURE 6 The effect on the subsequent rate of tubular shrinkage in regular medium of the initial volume of tubules swollen in differing hypotonic solutions. Two experiments are shown. Tubules I and II are the same tubules depicted in Fig. 3. The relative tubular volume, $\frac{Vt}{V\infty}$, is the tubular volume in regular medium at time t divided by the steady state tubular volume in regular medium, V_∞. In curve *A* the tubule was incubated initially in 117 milliosmolal solution and at $t = 0$ bathed quickly in regular medium. In curve *B* the same tubule was incubated in 212 milliosmolal solution and at $t = 0$ (point *C*) bathed in regular medium. For comparison the curve of experiment *B* has been transposed onto the curve of experiment *A* so that $t = 0$ of experiment *B* falls on the curve of experiment *A* at point *C*. The two curves do not coincide over the interval beyond point C.

separate times in a single tubule without apparent injury to the tissue.

Electron micrographs of tubules placed in fixative soon after the spaces appeared (Fig. 7) confirmed the impression that the dark shadows seen between cells in the light microscope (Fig. 4) were dilated intercellular spaces. In tubules fixed immediately after the spaces appeared, the entire length of the intercellular channel was widened. In tubules fixed after 7 min in regular solution, when no spaces could be seen between the cells under the light microscope, electron micrographs also showed collapsed intercellular spaces.

Tubules transferred from regular medium to hypertonic solution (406 milliosmolal) developed widened intercellular spaces. However, in contrast to the experiments discussed above, the spaces never disappeared completely. Fig 8 is an electron micrograph of a widened intercellular space in a tubule incubated for 20 min in hypertonic medium before fixation.

A summary of the results of the swelling and shrinking experiments is shown schematically in Fig. 9.

Anatomy of the Intercellular Space

In electron micrographs a space ranging in width from 70 to 300 A was seen between the membranes of adjacent cells at the basilar portion of the intercellular space, whereas in the region of the apical tight junction the outer leaflets of adjacent membranes appeared fused (Fig. 10). In order to determine more precisely the relative widths of the apical and basilar junctions in electron micrographs, horseradish peroxidase was added to the outer bath as a tracer. Under the conditions of these experiments the horseradish peroxidase could not be fixed in the lateral interspace at a concentration high enough to give unequivocal staining unless the enzyme was also included in the glutaraldehyde fixative solution. When the latter procedure was used, a dense accumulation of the reaction product adherent to the walls throughout the intercellular space was observed (Fig. 10). No accumulation of reaction product was seen in the region of the apical tight junctional complex. The inability to capture the tracer within the interspace without also including

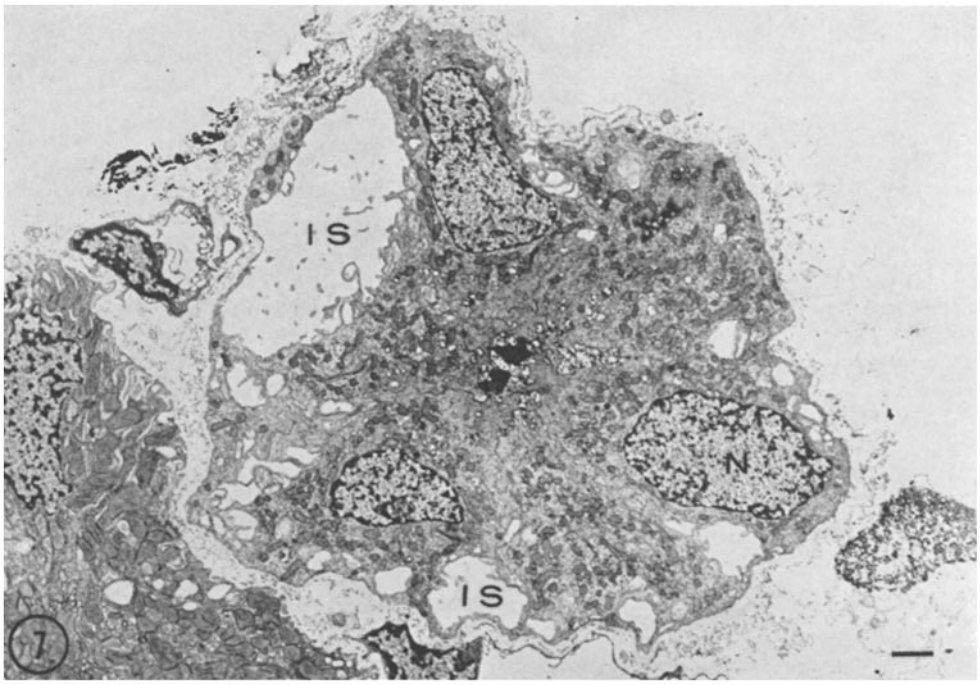


FIGURE 7 Electron micrograph of a nonperfused collecting tubule which had been placed in regular medium following swelling in 147 milliosmolal solution. (The same tubule is shown in Fig. 4 prior to and following fixation and dehydration.) There are marked dilation of the intercellular spaces (*IS*) and irregularity of the basilar surface of the tubule. The nuclear chromatin (*N*) appears to be normally distributed. Fragment of thick ascending limb at lower left. Lead citrate and uranyl acetate staining. Marker 1 μ. × 5,300.

the enzyme in the fixative solution may be related to the fact that complete glutaraldehyde fixation requires several minutes. At these small dimensions in single tubules, conceivably the enzyme diffuses out of the intercellular channel before it can be fixed by the glutaraldehyde.

Influence of Hydrostatic Pressure on the Rate of Disappearance of Dilated Intercellular Spaces

The pressure required to open the lumen of a collapsed tubule ranged from 2 to 5 cm H_2O. Once the lumen was opened, the transtubule pressure and outer tubule diameter were regulated by raising or lowering the fluid reservoir. When the pressure was increased to 10 cm of water the outer tubular diameter increased markedly (Fig. 11). Above 10 cm of water pressure very little change in diameter was produced by relatively large increments in pressure, probably owing to limited extensibility of the basement membrane primarily. When the transtubular hydrostatic pressure difference was then reduced to zero the tubular lumen collapsed.

Collecting tubules absorb virtually no fluid when the perfusion and outer bathing media are isosmotic (3). Since there is no net flow of fluid in the system under these conditions, the hydrostatic pressure (ΔP) at all points within the lumen of the tubule must be virtually the same as that within the perfusion pipet. Widened intercellular spaces were created by shrinking swollen nonperfused tubules in regular medium. The luminal hydrostatic pressure was then increased to a steady value by raising the fluid reservoir and gently infusing solution into one end of the tubule. The other end was blocked. Pressure applied in this manner increased the rate of collapse of dilated intercellular spaces. The average disappearance time of intercellular spaces with no applied ΔP was 200 sec; when the ΔP was increased to 5 cm of water immediately after the spaces were created, the col-

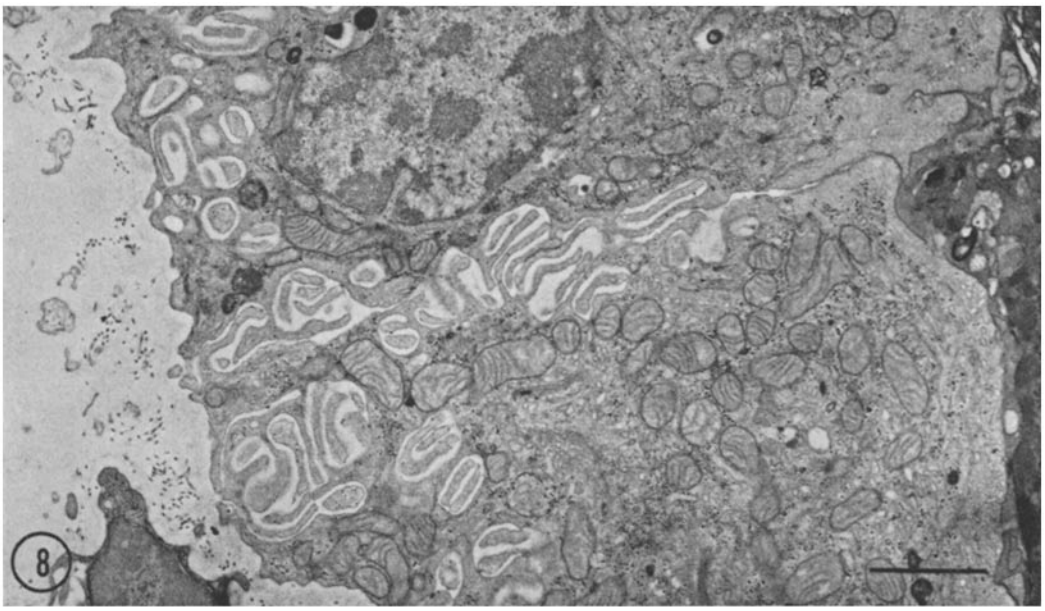


FIGURE 8 Electron micrograph of an intercellular space in a nonperfused collecting tubule which had been placed in hypertonic solution (406 milliosmolal) following incubation in regular bath medium. The interspace is uniformly dilated and contains prominent lateral processes. The cytoplasm is relatively dense. Lead citrate and uranyl acetate staining. Marker 1 μ. × 15,600.

lapse time decreased to 10–30 sec (15 measurements in four tubules).

DISCUSSION

It had been shown previously in isolated collecting tubules of the rabbit that cellular swelling and widening of intercellular spaces accompanied the increased osmotic flow of water caused by ADH (6). It was proposed that the lateral channel as well as a transcellular route serve as pathways of bulk water transport across the tissue. Other possible explanations for the appearance of dilated intercellular spaces were not excluded at that time. For example, if the lateral intercellular spaces were functionally closed and contained solute, they might swell osmotically, as does the cell when ADH increases the permeability of the lumen membrane and permits hypotonic perfusion fluid from the lumen to enter the cell. If this were true, widened intercellular spaces would also be expected to appear in nonperfused tubules swollen in hyposmotic external bathing solutions. In the present studies the intercellular spaces were not dilated in such tubules examined at various times after acutely reducing the tonicity of the external bath. On the basis of this result it is apparent that lateral intercellular spaces do not contain enough trapped solute to dilate osmotically to the extent seen previously during ADH-induced water transport.

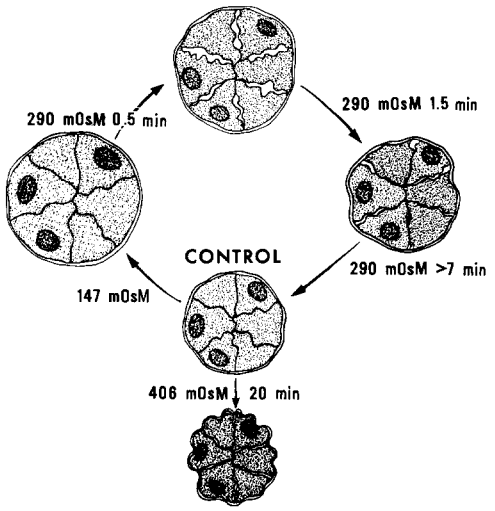
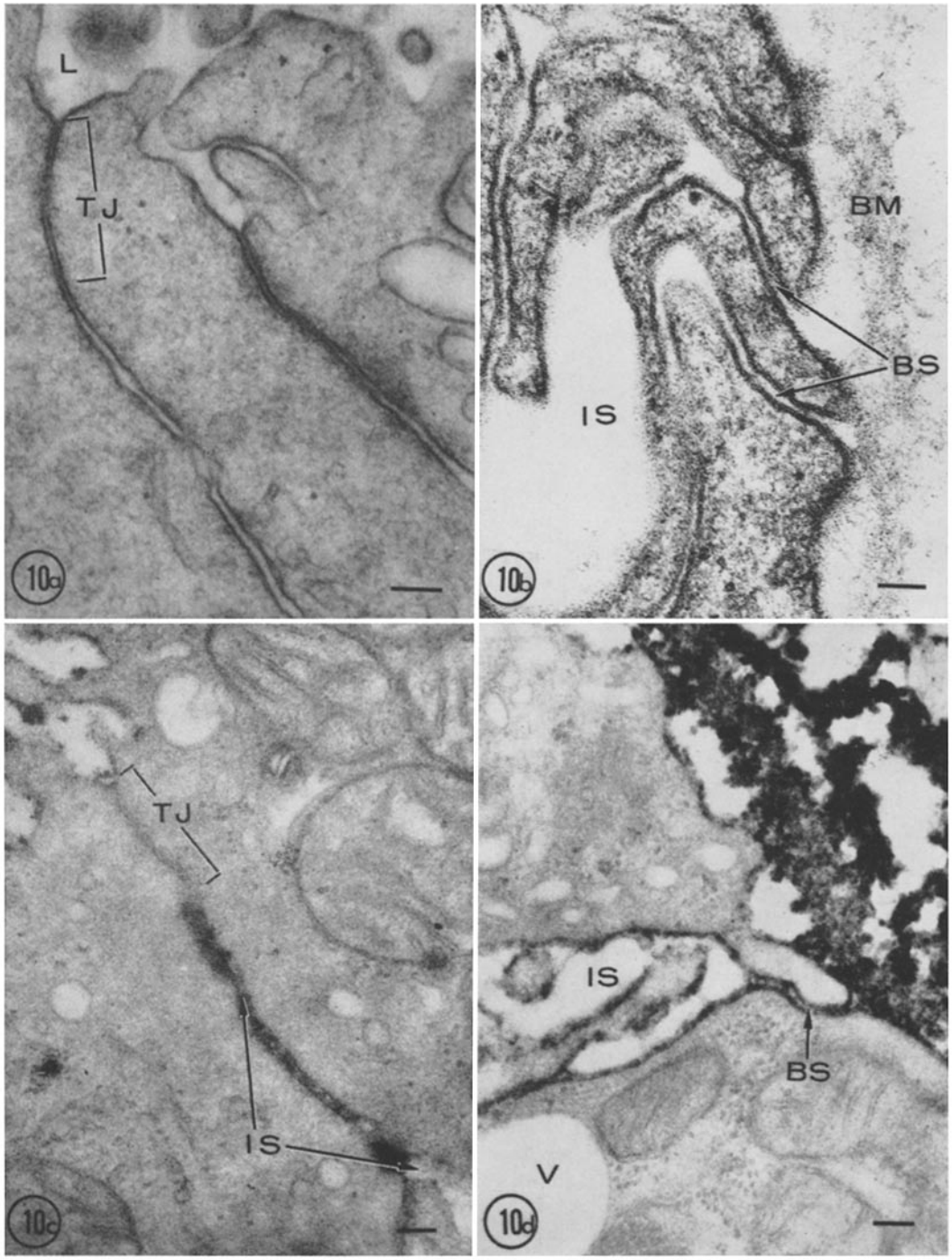


FIGURE 9 Schematic diagram of the results of immersing collecting tubules in anisotropic solutions. Osmolalities of the external bathing solutions and the duration of incubation are noted. Not drawn to scale.



FIGURES 10 *a–d* Electron micrographs of apical and basilar intercellular junctions. Fig. 10 *a* shows the apical tight junctions (*TJ*) of an interspace in a permeable perfused tubule. The adjacent membranes appear fused in the region indicated. *L*, lumen. Uranyl acetate stain. × 77,500. Fig. 10 *b* shows the basilar slit (*BS*) of a permeable perfused tubule which had been soaked in aqueous 1% uranyl acetate. Several views of basilar slits are present in this section. There appears to be direct communication between the dilated portion of the intercellular space (*IS*) and the basement membrane (*BM*). × 69,900. Figs. 10 *c* and *d* reveal the appearance of horseradish peroxidase reaction product in the intercellular space of a nonperfused tubule. In Fig. 10 *c* the reaction product is deposited most heavily in the mid-portion of the interspace (*IS*) up to the region of the tight junction (*TJ*). A few small deposits may be present along the lumen membrane. Unstained. × 59,400. Fig. 10 *d* shows the base of the same interspace as that shown in 10 *c*. Dense reaction product is present in and adherent to the basement membrane, the basilar slit (*BS*), and the body of the intercellular space (*IS*). *V*, intracellular vacuole. Unstained. Markers, 0.1 μ. × 59,800.

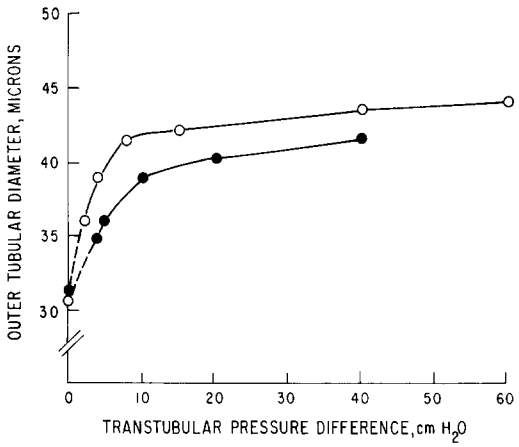


FIGURE 11 The relationship between luminal hydrostatic pressure and outer tubular diameter in two collecting tubules. Positive luminal pressure was applied through the perfusion pipet. The pressure required to open the tubular lumens was 2 and 4 cm of water.

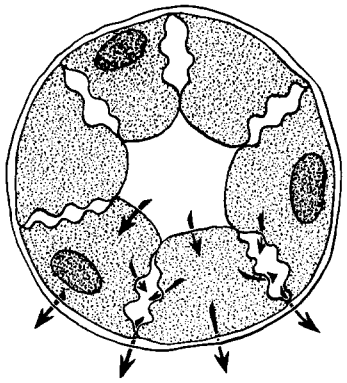


FIGURE 12 Proposed pathways of osmotically induced net water movement in collecting tubules treated with antidiuretic hormone. The arrows indicate the paths of bulk water flow.

Another possible explanation for dilation of the intercellular spaces is that ADH-induced water movement through the lateral interspaces is coupled to active solute transport as has been postulated for other tissues (13–15). Although this seems unlikely in view of the fact that ADH does not cause the intercellular spaces to widen in the absence of an osmotic gradient (6), as an additional test ouabain, an inhibitor of sodium transport, was added to tubules transporting water at a maximal rate along an osmotic gradient. The drug, whether introduced before the addition of ADH or after, had no effect on the appearance of the intercellular spaces. Thus, distention of the spaces in the presence of ADH is not related to active sodium transport.

In view of the foregoing observations, it is concluded that in perfused permeable tubules the lateral intercellular spaces widen as a consequence of the passage of water through the channels. If the lateral interspaces remain dilated owing to the continuous entry of water from the cell by passive flow along an osmotic gradient, then the osmolality of the interspace must be maintained higher than that of the cell or lumen. For this to occur, solute must diffuse into the lateral intercellular space. An obvious route connecting the lateral intercellular spaces with the outer bath and its infinite supply of solute is the gap between adjacent cell membranes at the base of each intercellular space. These gaps are wide enough for horseradish peroxidase, and presumably smaller molecules such as sodium chloride, to diffuse into the intercellular space from the external bath.

It was observed that nonperfused tubules swollen in hypotonic solutions and then reimmersed in the original regular medium also developed widely dilated intercellular spaces. As in perfused tubules water apparently leaves the hypotonic cells by osmosis across the lateral membranes, causing the intercellular spaces to enlarge. In nonperfused tubules the supply of dilute solution in the cell is quickly exhausted; consequently, net flow of water from the cell into the intercellular space is of short duration. In the absence of continued inflow of fluid, the intercellular spaces collapse as fluid leaks into the external extracellular medium. The force causing the fluid to leak from the intercellular space into the bath is probably a difference in hydrostatic pressure.

The pressure difference required to evacuate the fluid from a single dilated interspace through the basilar slit in a given period of time can be calculated from an equation based on Poiseuille's Law (16). For this calculation it is assumed that in a nonperfused tubule (*a*) the apical junction is closed, (*b*) the basilar intercellular junction is a fixed rectangular slit constituting the major resistance to hydrodynamic flow in the lateral intercellular space, and (*c*) the basement membrane has no hydraulic resistance; then,

$$\frac{\dot{Q}_f}{\Delta P} = \frac{2L}{3\eta\Delta x}\left(\frac{w}{2}\right)^3$$

where $\dot{Q}_f$($\dot{Q}_f = 0.42 \times 10^{-12}$ cm^3 sec^{-1})[1] is the quantity of filtrate moved out of a single intercellular space in the collapse time of 200 sec. ΔP is the mean difference in pressure between the intercellular space and the external bath, and η (0.009 dyne sec cm^{-2}) is the viscosity of the protein-free filtrate at 25°C. The dimensions of the basilar slit are mean values obtained from electron micrographs (corrected for tissue shrinkage during dehydration) and light microscope photographs. L (10.6×10^{-4} cm) is the length of an individual basilar slit between two adjacent cells, Δx (5×10^{-5} cm) is the path length (or height of the slit), and w (150×10^{-8} cm) is the width of the slit. It is calculated that, in an average tubule shrinking from a swollen volume to baseline in regular medium, a pressure difference of only 0.6 cm of water is required to collapse the intercellular spaces in 200 sec. We believe that the small pressure required to evacuate the intercellular space of fluid during osmotic shrinkage is supplied by compression of the cells by an extensible elastic casing, specifically the basement membrane. The basement membrane is probably at or very near its relaxed state in nonperfused tubules in regular medium. In tubules swollen above their baseline volume the basement membrane is taut, and tissue pressure and presumably the pressure in the intercellular spaces are increased. In contrast, the basement membrane does not compress the cells and intercellular spaces in tubules shrunken below baseline volume in hypertonic medium. According to this view the intercellular spaces remain open in tubules shrunken below their normal size in hypertonic media because the basement membrane is flaccid, whereas, in swollen tubules immersed in regular medium the intercellular spaces collapse because the basement membrane compresses the cells and intercellular spaces, providing a driving force for the removal of fluid from the lateral channel. The thesis was tested by applying a hydrostatic pressure of 5 cm of water in the lumen to previously nonperfused tubules with dilated intercellular spaces. It can be calculated, as discussed previously, that with an imposed pressure difference of 5 cm of water the spaces should collapse in approximately 25 sec. The experiment yielded results in accordance with theory; application of 5 cm H_2O caused the spaces to collapse in 10–30 sec. This provides direct evidence in functioning tubules that the intercellular spaces communicate with the external extracellular compartment and that the hydraulic resistance of the basilar slit is an important factor determining hydrodynamic flow. Thus, extending this view, in tubules perfused with hypotonic solution and treated with ADH the width of the lateral intercellular space during osmotic removal of water from the lumen probably depends upon the magnitude of the tubular perfusion pressure as well as on the absolute rate of fluid transport through the extracellular channel.

In the present studies evidence is presented in support of the view that water traverses the intercellular channel in transit through the tissue. This does not exclude the possibility that water also passes out of the cytoplasm in a parallel path across the basilar surface of the tubule cells as well. If it is assumed that in the slow phase of volume contraction in nonperfused tubules in regular medium, as in the experiments discussed above (Figs. 3 and 6), all of the water exits through the intercellular basilar slit, then it is possible to deduce whether any fluid passed across the basilar surface of the cells during the preceding rapid phase of shrinkage. As discussed previously, it is possible to determine the hydraulic conductivity of the basilar slit during the loss of a definite volume of solution from the interspaces only. If the same volume of solution is placed entirely within the cells, then its subsequent rate of loss from the tissue will be determined by the hydraulic conductivity of the basilar slit, provided the solution exits by that path exclusively. In other words, regardless of the site of origin of the fluid, if the intercellular channel is the final path the basilar slit is rate limiting. In order to "load" individually the intercellular spaces or the cells and compare the relative rates of fluid loss from these compartments,

[1] If it is assumed that the lateral intercellular spaces fill with fluid instantaneously after immersion of swollen tissue in isotonic medium, then the mean rate of filtration, $\dot{Q}_f$; through the basilar slit during the subsequent collapse of the intercellular space can be calculated by dividing the initial volume of the main body of the interspace by the collapse time. In order to simplify the calculation, it is assumed that the intercellular space between two adjacent cells is rectangular; thus,

$$\frac{\text{length} \times \text{width} \times \text{height}}{\text{collapse time}} =$$

$$\frac{(10.6 \times 10^{-4}\text{ cm}) \times (10^{-4}\text{ cm}) \times (8 \times 10^{-4}\text{ cm})}{200\text{ sec}}$$

$$= 0.42 \times 10^{-12}\text{ cm}^3/\text{sec}.$$

the following experiment was done. A swollen tubule in 117 milliosmolal solution was quickly bathed in regular medium (Fig. 6), and the slow component (intercellular space washout) of tissue shrinkage was identified (curve *A*). The tubule was then immersed in another hypotonic solution (curve *B*) chosen to give an increase in tissue volume approximately equal to the calculated initial volume of the slow compartment in the preceding experiment. Thus, at point *C* the tissue contained equal volumes of "extra" solution. In *A* nearly all of the extra solution was located in the interspaces, whereas in *B* the amount of extra water was located primarily in the cells (the interspaces were collapsed). At point *C* it is assumed that in *A* and *B* at equal tubular volume the tissue pressure was also equal. Given these conditions and assumptions, if water was lost in curve *B* only by passing sequentially from the cells, through the interspaces, across the basilar slits, and into the external medium, then the tissue should not shrink at a rate exceeding that of the slow compartment in curve *A*. Obvious from Fig. 6 is the fact that less time was required for the same amount of water to leave the tissue when it originated mostly within the cells, than when it originated within the interspaces alone. Therefore, in the rapid phase of tissue shrinkage water escapes by another route, namely across the basilar surface of the cells.

In this tissue the junction of adjacent cells in the apical region of the intercellular spaces can probably be excluded as a site of transepithelial water flow or ADH action. In electron micrographs the opposing cellular membranes appear fused, and the junction does not readily admit the tracer horseradish peroxidase. In addition, the junction is highly impermeable to even smaller water-soluble substances such as urea, thiourea, and acetamide (17). These considerations, plus the fact that the combined plane luminal area of the intercellular spaces available to osmosis is so much less than that of the luminal cellular membrane area, militate against significant bulk flow of water across the apical tight junction during transepithelial water flow.

In summary, the pathways of transtubular water movement in collecting tubules are diagrammed in Fig. 12. In agreement with other workers it is proposed that antidiuretic hormone has a single effect on water movement, i.e. to increase the permeability to water of the luminal plasma membrane. Water enters the cells by osmosis and causes them to swell. Since the osmolality of the swollen cell is less than that of the external bath or communicating lateral intercellular space, water flows from the cells into these extracellular compartments. Initially, water enters the lateral channel faster than it leaves through the basilar slit, thereby creating increased pressure in the intercellular space. The volume of the space increases until the pressure within the channel is sufficient to drive the water entering from the lateral membranes across the hydraulic resistance of the basilar slit. With the inception of bulk flow through the narrow passage at the base of the interspace the amount of solute that can diffuse "upstream" into the channel is probably diminished. Thus, the volume flow through the intercellular channel is determined in part by the amount of solute diffusing into the intercellular space from the external bath.

Received for publication 30 October 1968, and in revised form 9 December 1968.

REFERENCES

1. KOEFOED-JOHNSEN, V., and H. H. USSING. 1953. The contribution of diffusion and flow to the passage of D_2O through living membranes. *Acta Physiol. Scand.* **28**:60.
2. HAYS, R. M., and A. LEAF. 1962. Studies on the movement of water through isolated toad bladder and its modification by vasopressin. *J. Gen. Physiol.* **45**:905.
3. GRANTHAM, J. J., and M. B. BURG. 1966. Effect of vasopressin and cyclic AMP on permeability of isolated collecting tubules. *Amer. J. Physiol.* **211**:255.
4. ORLOFF, J., and J. HANDLER. 1967. The role of adenosine 3′,5′-phosphate in the action of antidiuretic hormone. *Amer. J. Med.* **42**:757.
5. CARASSO, N., P. FAVARD, and J. VALERIEN. 1962. Variations des ultra structures dans les cellules epitheliales de la vessie du crapaud apres stimulation par l'hormone neurophypophysaire. *J. Microsc.* **1**:143.
6. GANOTE, C., J. J. GRANTHAM, H. L. MOSES, M. B. BURG, and J. ORLOFF. 1968. Ultrastructural studies of vasopressin effect on isolated perfused renal collecting tubules of the rabbit. *J. Cell Biol.* **36**:355.
7. BURG, M. B., J. J. GRANTHAM, M. ABRAMOW, and

J. ORLOFF. 1966. Preparation and study of fragments of single rabbit nephrons. *Amer. J. Physiol.* **210**:1293.

8. GRANTHAM, J. J., and J. ORLOFF. 1968. Effect of prostaglandin E_1 on the permeability response of the isolated collecting tubule to vasopressin, adenosine 3′,5′-monophosphate and theophylline. *J. Clin. Invest.* **47**:1154.
9. BURG, M. B., L. ISAACSON, J. J. GRANTHAM, and J. ORLOFF. 1968. Electrical properties of isolated perfused rabbit renal tubules. *Amer. J. Physiol.* **215**:788.
10. KARNOVSKY, M. J. 1967. The ultrastructural basis of capillary permeability studied with peroxidase as a tracer. *J. Cell Biol.* **35**:213.
11. MILLONIG, G. 1961. Advantages of a phosphate buffer for OsO_4 solutions in fixation. *J. Appl. Phys.* **32**:1637. (Abstr.)
12. REYNOLDS, E. S. 1963. The use of lead citrate at high pH as an electron-opaque stain in electron microscopy. *J. Cell Biol.* **17**:208.
13. KAYE, G. I., H. O. WHEELER, R. T. WHITLOCK, and N. LANE. 1966. Fluid transport in the rabbit gallbladder. *J. Cell Biol.* **30**:237.
14. TORMEY, J. MCD., and J. DIAMOND. 1967. The ultrastructural route of fluid transport in the rabbit gallbladder. *J. Gen. Physiol.* **50**:2031.
15. BERRIDGE, M. J., and B. L. GUPTA. 1967. Fine structural changes in relation to ion and water transport in the rectal papillae of the blowfly, Calliphora. *J. Cell Sci.* **2**:89.
16. PAPPENHEIMER, J. R., E. M. RENKIN, and L. M. BORRERO. 1951. Filtration diffusion and molecular sieving through the peripheral capillary membranes. A contribution to the pore theory of capillary permeability. *Amer. J. Physiol.* **167**:13.
17. BURG, M. B., S. HELMAN, J. GRANTHAM, and J. ORLOFF. Effect of vasopressin on the permeability of isolated rabbit cortical collecting tubules to urea, acetamide and thiourea. Excerpta Medica. In press.