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Morphologic Response of the Rabbit Cortical Collecting Tubule to Peritubular Hypotonicity: Quantitative Examination with Differential Interferene Contrast Microscopy

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Summary. The isolated and perfused cortical collecting tubule of the rabbit was examined by differential interference contrast microscopy in order to characterize the morphologic response of this nephron segment to peritubular hypotonicity. Computer-assisted, morphometric procedures were developed to obtain measurements of cell volume and lateral intercellular space geometry from interference contrast images of perfused nephron segments. Following dilution of the bath from 290 to 190 mOsm in the absence of antidiuretic hormone (T = 25°C), the cells swelled rapidly to a new steady-state volume which was maintained for at least 20 to 30 min and which was about 90% of that predicted for ideal osmometric behavior. The increase in cell volume was accomplished entirely by bulging of the cells into the lumen; lateral space width and outside tubule diameter were unaffected by peritubular hypotonicity. In addition, the swelling of the cells was associated with an apparent swelling of intracellular organelles, e.g., nuclei and mitochondria. Our results indicate that cells of the mammalian collecting tubule swell without the capacity for significant volume regulation at 25°C and without the cytoplasmic vacuolation and dilation of the lateral intercellular spaces observed following the onset of antidiuretic hormone-dependent volume reabsorption (E. Ganote, J. Grantham, H. Moses, M. Burg and J. Orloff, *J. Cell Biol.* **36**:355, 1968).

Key Words differential interference contrast microscopy · rabbit cortical collecting tubule · hypotonicity · cell volume regulation · lateral intercellular spaces · antidiuretic hormone

Introduction

Antidiuretic hormone (ADH)-dependent water reabsorption in the collecting duct is the principal means by which the mammalian kidney concentrates the urine and conserves water. Considerable evidence indicates that ADH stimulates water reabsorption by increasing the luminal membrane water permeability of hormone-responsive cells (Ganote, Grantham, Moses, Burg & Orloff, 1968; Schafer & Andreoli, 1972; Harmanci, Kachadorian, Valtin & DiScala, 1978). Our understanding of the pathway for water movement from the luminal membrane to the peritubular space is less clear and derives largely from electron microscopic observations of kidney slices and isolated collecting ducts which suggest that cell swelling, intercellular space-dilation and cytoplasmic vacuolation are the major structural events associated with ADH-stimulated volume reabsorption (Ganote et al., 1968; Grantham, Ganote, Burg & Orloff, 1969; Tisher, Bulger & Valtin, 1971; Woodhall & Tisher, 1973). However, these studies have provided little quantitative information regarding the magnitudes and temporal sequence of the morphologic changes associated with the onset of hormone-dependent flow, information that would be useful in determining the important barriers to transepithelial water movement.

The present studies, summarized in this and the following paper, were designed to provide a detailed, quantitative description of flow-induced structural changes in the rabbit cortical collecting tubule and to contrast these changes to those observed following exposure to peritubular hypotonicity under conditions of zero transepithelial flow. Isolated and perfused collecting tubules were directly visualized with differential interference contrast (DIC or Nomarski) microscopy, a light-microscopic technique which offers high resolution and a depth of focus which is sufficiently shallow to enable the epithelium to be optically sectioned (Allen, David & Nomarski, 1969). Computer-assisted image analysis was used to measure the geometry of the lateral intercellular spaces and to estimate cell and lateral space volumes. In this first of a series of two papers, we present examples of DIC images of isolated and perfused nephron segments and describe in detail the morphometric procedures. Also discussed is the application of these techniques to the characterization of the structural response of the cortical collecting tubule to dilute peritubular

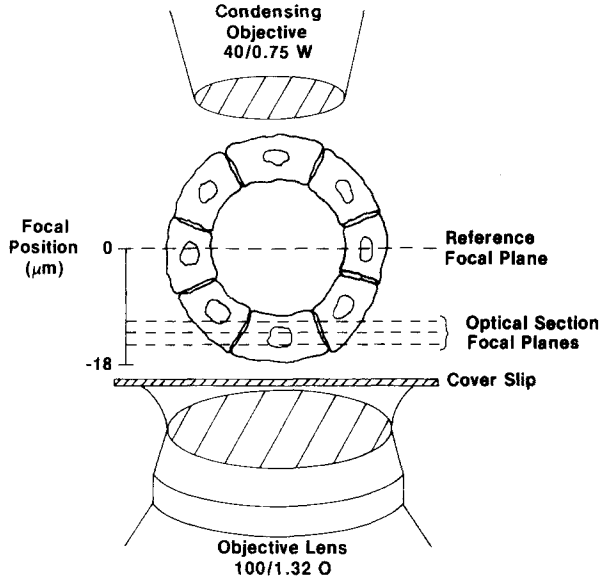

Fig. 1. Schematic representation of the focal positions of the reference focal plane and the optical sections through the epithelial cell layer. The focal position of each optical section was recorded as the displacement (in μm) from the reference focal position, which was defined as that focal plane which was coplanar with the longitudinal axis of the tubule. In this "end-on" view of the tubule the elements of the microscope have not been drawn to scale

media in the absence of ADH. The results demonstrate that under these conditions cells of the collecting tubule swell as osmometers without cytoplasmic vacuolation and without a detectable change in lateral space width. In the following paper we describe experiments which utilized the techniques and data presented in the first paper to provide a complete description of flow-induced structural changes in the collecting tubule and a more detailed understanding of the pathway for ADH-dependent water reabsorption.

Materials and Methods

Isolation and Perfusion of Nephron Segments

The methodology for isolating and microperfusing rabbit cortical collecting tubules was similar to that previously described (Burg, Grantham, Abramow & Orloff, 1966; Al-Zahid, Schafer, Troutman & Andreoli, 1977) with the minor modifications noted below. Briefly, female white New Zealand rabbits (1 to 3 kg), which had been allowed free access to food and water, were decapitated and the kidneys removed. Segments of cortical collecting tubules (1 to 3 mm in length) were dissected from kidney slices bathed at room temperature (21 to 23°C) in a Krebs-Ringer's bicarbonate solution pre-equilibrated with 95% O_2/5% CO_2, adjusted to pH 7.4, 290 mOsm, and containing (in mM): 115 NaCl, 25 $NaHCO_3$, 1.2 Na_2HPO_4/NaH_2PO_4, 5 KCl, 1.2 $MgSO_4$, 5.5 glucose, 1.0 $CaCl_2$ and 10 Na-acetate. Bovine serum albumin (6%) was included within the dissection media to prevent the tubules from adhering to the dissection dish or the transfer pipettes. The dissected tubule segments were transferred to a thermoregulated chamber (volume ~ 1.5 ml) mounted on the stage of an inverted microscope (Model IM-35:C. Zeiss, Oberkochen, West Germany). The tubule segments were cannulated using low power (6.3× objective), bright field optics for which the light source was diffused through an opalized glass disc that replaced the condenser assembly of the microscope. Cannulation was achieved by aspirating the free ends of each tubule into holding pipettes and advancing a concentric perfusion pipette from one end approximately 100 to 250 μm into the tubule lumen. Perfusion rates within the range of 10 to 20 nl/min were driven by constant hydrostatic pressures of 20 to 30 cm H_2O. The tubules were perfused with a Krebs-Ringer's phosphate solution adjusted to pH 7.4, 290 mOsm, and containing (in mM): 150 NaCl, 2.5 Na_2HPO_4/NaH_2PO_4, 5 KCl, 1.2 $MgSO_4$ and 1 $CaCl_2$.

The initial bathing solution was the protein-free, Krebs-Ringer's bicarbonate solution (290 mOsm) described above. In some experiments the bathing solution was replaced with a solution identical in composition except the osmolality was reduced to 190 mOsm by the deletion of NaCl. Within the chamber the bath was mixed and aerated by bubbling with 95% O_2/5% CO_2 and continuously perfusing the chamber by means of a syringe pump (Model 255; Sage, Cambridge, Mass.) with fresh solution at a flow rate of 0.3 ml/min. Composition of the bathing solution was changed by flushing the chamber for 45 sec with 10 to 15 ml of the new solution and then perfusing at the normal flow rate.

In order to reduce the initially high water permeability of the isolated and perfused collecting tubule (Al-Zahid et al., 1977), each experimental protocol included an initial incubation period of 60 to 90 min during which the temperature of the bath was maintained at 38°C. The temperature was then lowered to 25°C and maintained at that level throughout the course of the experimental observations.

Microscopic Observation of Isolated Nephron Segments

The microscope used for these studies was fitted with the necessary equipment for transmitted light, differential interference contrast optics (Allen et al., 1969). For experimental observations we used a 100× oil immersion, planachromat lens (1.32 numerical aperture; E. Leitz, Wetzlar) as the objective lens. As reported previously (Kirk, Schafer & DiBona, 1983), a 40× water immersion lens (0.75 numerical aperture; C. Zeiss) replaced the standard condenser assembly because the latter was too large to fit between the holding pipettes when lowered to the proper working distance of less than 2 mm. An infrared reflection filter was placed between the tungsten light source of the microscope and the condenser to prevent heating of the tubule during prolonged experimental observations.

The tubule was positioned in the optical path by moving the stage in the X–Y plane using a coaxial drive. The micromanipulators (Model M2; Narashige, Japan) used for positioning the holding pipettes were mounted directly to the stage so that the entire perfusion assembly (manipulators, pipettes, chamber) moved with the stage as a unit. Focus was adjusted by moving the objective lens up or down independently of the stage. Focal position was recorded with a Z-axis probe (Heidenhain) that was

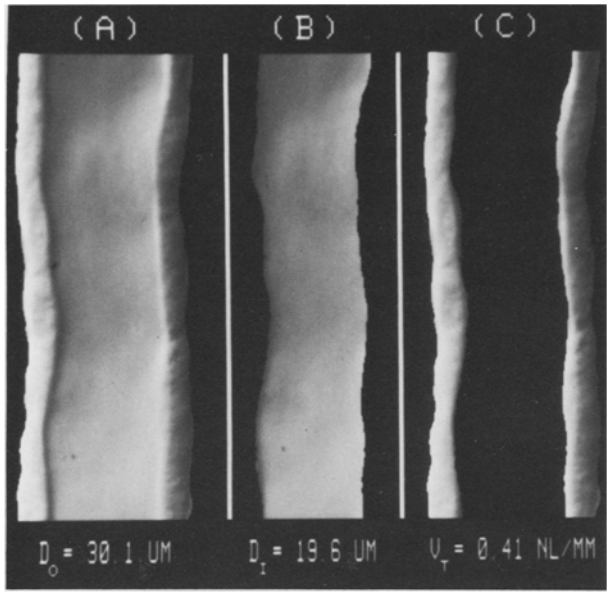

Fig. 2. Method for estimating epithelial volume (V_T). (*A*) Tracing of the outer margins of the tubule observed at the reference focal position. Dividing the surface area of the traced profile by its length (~100 μm) provides an estimate of the mean outside diameter (D_o) of the tubule. (*B*) Tracing of the margin of the tubule lumen to determine the mean inside diameter (D_i) of the tubule. (*C*) Profile of the epithelial cell layer obtained by subtracting profile (*B*) from profile (*A*). V_T was calculated from D_o and D_i according to Eq. (1) in the text

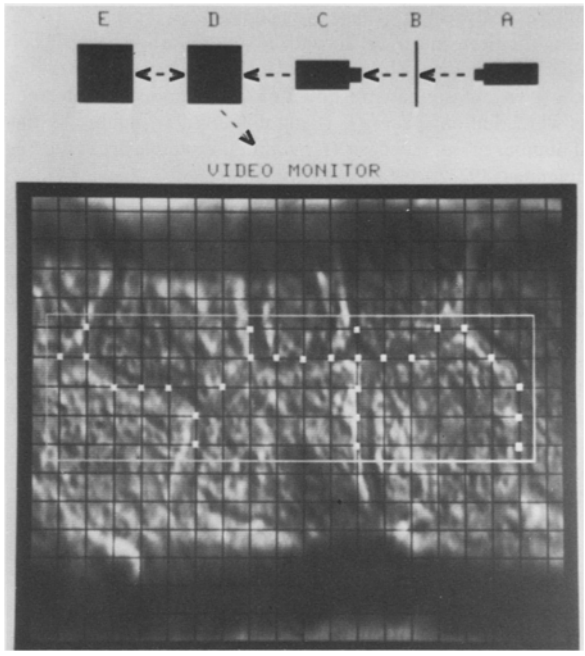

Fig. 3. Procedure for estimating the areal density of lateral intercellular spaces (AD_{ICS}). *A*. film projector; *B*. screen; *C*. video camera; *D*. image analysis system; *E*. computer. *See* text for details of the sampling paradigm

fixed directly to the nosepiece of the microscope and which monitored the travel of the objective lens with a precision of ±0.5 μm. Reference for focal position was the longitudinal axis of the tubule (*see* Fig. 1), which was defined operationally as that focal plane at which the opposing walls of the tubular epithelium were narrowest and most sharply defined. This reference focal position could be reproduced subjectively with a level of precision which equaled or exceeded that of the *Z*-axis probe and did not drift during the course of experimental observations. In all experiments in which the tubular epithelium was optically sectioned the focal plane was advanced *downward* into the cell layer nearest the bottom of the chamber and the displacement of focal position (in μm) from reference recorded (*see* Fig. 1). Images were collected and stored on black and white film (Technical Pan 2415; Eastman Kodak, Rochester, N.Y.) using a 35 mm camera (Contax RTS, Yashica, Japan) mounted directly on the microscope.

Morphometric Procedures: Measurement of Epithelial Volume

Figure 2 illustrates the technique we used to determine the volume of the epithelial cell layer (V_T). Photographic prints of DIC images (1500 to 2000×) of isolated tubule segments observed at the reference focal position were fixed directly to an electromagnetic drawing tablet (The Wedge; Talos Systems, Inc., Scottsdale, Ariz.) interfaced to a laboratory computer (PDP 11/23; Digital Equipment Corp., Boston, Mass.). The outlines of each tubule (*A*) and tubule lumen (*B*) were traced and, with the aid of a computer program, the surface areas of the traced profiles were calculated. Dividing the surface areas of profiles (*A*) and (*B*) by the length of the traced image (≅100 μm) provided estimates of mean outside (D_o) and inside (D_i) tubule diameters from which the volume of the epithelial cell layer (V_T) was calculated:

$$V_T \text{ (volume/tubule length)} = \pi(D_o^2 - D_i^2)/4. \quad (1)$$

In order to provide an estimate of the reproducibility of the method, V_T was measured 4 to 8 times for each image and the resulting coefficients of variation ranged from 1 to 2%.

Morphometric Procedures: Areal Density of Lateral Intercellular Spaces

In order to characterize the geometry of the lateral intercellular spaces, we employed a systematic point-counting procedure to determine the areal density of lateral spaces (AD_{ICS}), i.e., the fraction of the total sample area occupied by spaces, within optical sections obtained at varying distances between the tight junction and the basement membrane. The procedure for determining AD_{ICS} is summarized in Fig. 3. The DIC images, stored on negative film, were projected onto a ground glass screen and scanned with a video camera. Data were relayed as voltages to an image processing system (Model GMR-27; Grinnell Systems Corp., San Jose, Calif.) interfaced to the laboratory computer where the resulting digitized images were signal-averaged over 256 successive video frames to improve the signal-to-noise ratio. For mor-

phometric analysis the signal-averaged images were displayed on a video monitor and overlaid with a square lattice, or grid, of specified dimensions. The areal density of the lateral intercellular spaces was determined by counting the number of intersections which fell on spaces (I_{ICS}) and dividing this number by the total number of intersections (I_T) within the sample area (Weibel & Bolender, 1973):

$$AD_{ICS} = I_{ICS}/I_T. \quad (2)$$

A computer program coordinated the sampling of each grid and, on command of a cursor controlled by the investigator, marked each intersection designated as "hitting" a lateral space. This provided a pictorial assessment of sampling accuracy and prevented each intersection that fell on a lateral space from being counted more than once. The reproducibility of the method was determined by sampling each image with the same grid 4 to 8 times (coefficient of variation: 1 to 5%). All reported measurements were performed by a single investigator (KLK) although, in preliminary trials, we compared measurements performed on the same images by different investigators and found good agreement in the results.

Statistical Analysis

Data are expressed as means ± standard error of the mean (SEM). Paired statistical analyses were performed by standard Student *t*-test of the null hypothesis.

Results

DIC Images of the Rabbit Cortical Collecting Tubule

Figure 4 shows a single collecting tubule exposed to isotonic perfusate and bath in the absence of ADH. Four DIC images were obtained at focal positions 0 (longitudinal axis), −12.5, −14.0 and −15.5 μm, respectively. Note that with the focal plane at the level of the longitudinal axis (Fig. 4*a*) the cell profiles from apex to base can be visualized directly, a perspective which differs from the other images and cannot be obtained when observing flat epithelial sheets. The resolving power of DIC microscopy is evidenced in this image by the resolution of a single cilium, approximately 0.5 μm in diameter, which protrudes into the lumen and bends in the direction of perfusion.

The optical sections through the cell layer shown in Figs. 4*b*, *c* and *d* illustrate the shallow depth of focus provided by DIC microscopy. The vertical displacement between successive images is only 1.5 μm, yet the structural differences are marked. Surface microvilli are apparent in the most superficial optical section, and, as the focal plane progresses more deeply into the cell layer, lateral intercellular spaces and intracellular structures such as nuclei can be observed. However, we have not been able to distinguish between the two cell types of the collecting tubule, the principal and intercalated cells, which have been identified in electron microscopic images of this nephron segment (Ganote et al., 1968; Kaissling & Kriz, 1979; LeFurgey & Tisher, 1979).

Influence of Peritubular Hypotonicity on Collecting Tubule Structure

We reasoned that a complete understanding of the transepithelial pathway for ADH-dependent water flow requires an ability to distinguish between those structural events which are specifically induced by flow and those events which are associated with osmotic swelling under conditions of zero transepithelial volume flow. Figure 5 shows the changes in morphology following exposure to dilute bath (190 mOsm) in the absence of ADH. The control images, 2*a* and 2*b*, were obtained in the presence of isotonic perfusate and bath at focal positions 0 and −17.5 μm, respectively. The experimental images, 2*c* and 2*d*, were obtained in the same tubule at the identical focal positions 10 min after changing to the hypotonic bath. Dilution of the bath resulted in marked swelling of the epithelium as evidenced by bulging of the cells into the lumen. The increased thickness of the cell layer was associated with an apparent increase in nuclear volume and a more granular appearance of the cytoplasm which reflects, perhaps, the swelling of mitochondria and other intracellular organelles. Note that cytoplasmic vacuolation and intercellular space dilation were not features of the swelling response, in contrast to the observations during ADH-dependent transepithelial flow (*see* Figs. 1 and 2 in Kirk, Schafer & DiBona, 1984). In parallel experiments dilution of the perfusate alone (290 to 130 mOsm) in the absence of ADH had no effect on epithelial structure (*results not shown*) indicating that, in the absence of hormone, the basolateral cell membranes are considerably more permeable to water than the opposing luminal cell membranes.

Influence of Peritubular Hypotonicity on Epithelial Volume

Figure 6 shows the time courses of the effects of bath dilution on the mean inside (D_i) and outside (D_o) tubule diameters and the calculated epithelial volume (V_T) for a single collecting tubule. In response to peritubular hypotonicity, D_i rapidly de-

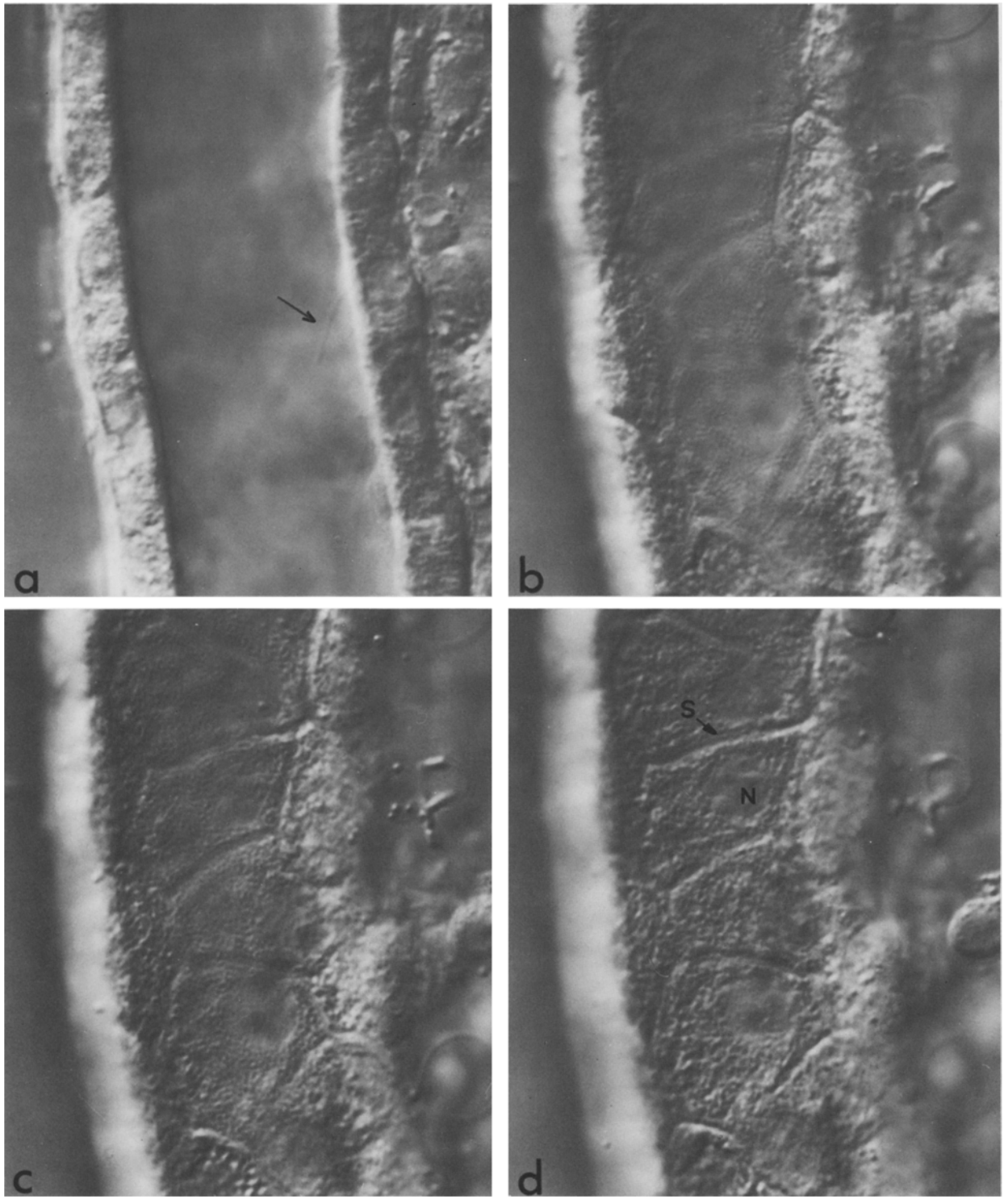

Fig. 4. DIC images of a single rabbit collecting tubule exposed to isotonic perfusate and bath in the absence of ADH. *a*) Reference focal position. Note the clump of interstitial cells adhering to the outside of the tubule and the single cilium (*see arrow*) protruding into the tubule lumen. *b*) Focal position: -12.5 μm. Numerous microvilli can be observed in this "surface view" of the luminal cell membrane. *c*) Focal position: -14.0 μm. The lateral cell borders become apparent as the plane of focus is advanced into the cell layer. *d*) Focal position: -15.5 μm. Nuclei (*N*) and lateral intercellular spaces (*S*) are clearly defined in this "interior view" of the tubular epithelium. 1575×

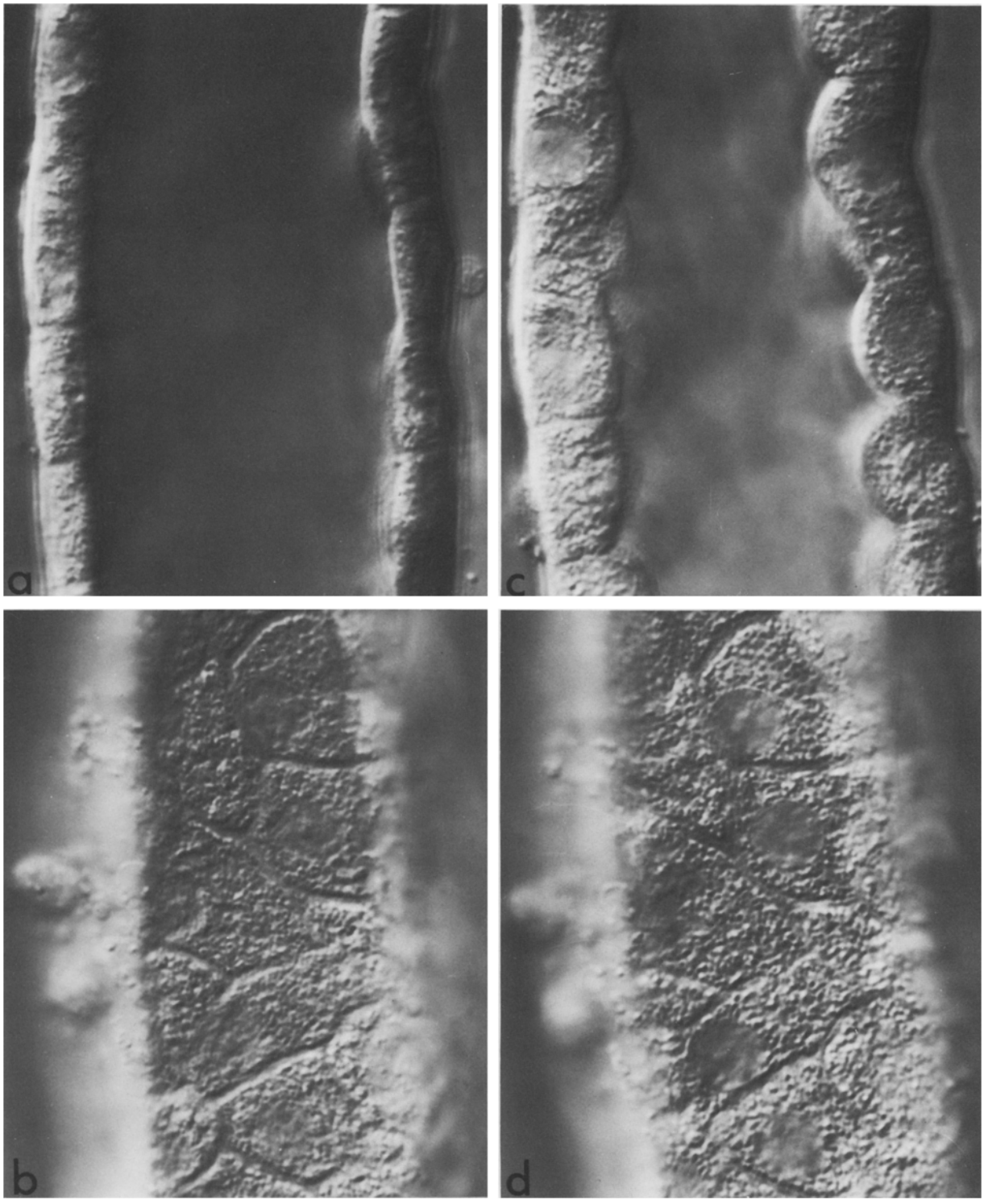

Fig. 5. Influence of peritubular hypotonicity on the morphology of the rabbit cortical collecting tubule. *a*) Reference focal position; 290 mOsm perfusate/290 mOsm bath. *b*) Focal position: −17.5 μm; 290 mOsm perfusate/290 mOsm bath. *c*) Reference focal position; 290 mOsm perfusate/190 mOsm bath. *d*) Focal position: −17.5 μm; 290 mOsm perfusate/190 mOsm bath. ADH was absent throughout the course of the experiment. 1575×

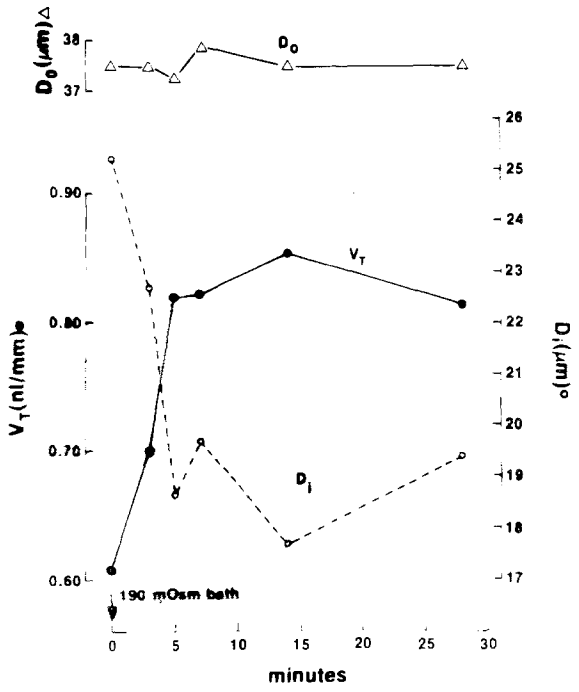

Fig. 6. Time courses of the effects of peritubular hypotonicity on outside tubule diameter (D_o), inside tubule diameter (D_i) and epithelial volume (V_T). Data are derived from a single experiment in which ADH was absent throughout and the tubule was initially perfused and bathed with isotonic solutions (290 mOsm). The data are plotted as functions of time following replacement of the bathing solution with a hypotonic solution (190 mOsm), which was performed 160 min after the rabbit had been sacrificed. V_T was calculated from D_o and D_i according to Eq. (1) in the text

creased whereas D_o remained virtually unchanged.[1] V_T, calculated from D_o and D_i according to Eq. (1), rapidly increased following the osmotic perturbation to a new steady state which was maintained for at least 25 min. The form of this curve, i.e., a rapid monotonic volume increase to a new steady state, implies that cells of the collecting tubule swelled osmotically following bath dilution and without a significant loss or gain of solute, unless such changes in intracellular solute content occurred during the initial rise in cell volume.

The Table summarizes the magnitude of the increase in V_T following bath dilution, as well as the associated changes in D_o, D_i, and the mean thickness of the epithelial cell layer, in four experiments. On the average, V_T and mean cell layer thickness increased by 37 and 47%, respectively. Again, the

[1] The change in D_i (and V_T) was delayed to some extent by the time required to completely change the bathing solution (<45 sec).

Table. Steady-state changes in inside and outside tubule diameters and epithelial volume following peritubular dilution

	Mean outside diameter (D_o) (μm)	Mean inside diameter (D_i) (μm)	Mean cell layer thickness ($(D_o - D_i)/2$) (μm)	Epithelial volume (V_T) (nl mm^{-1})
(1) 290 mOsm perfusate/ 290 mOsm bath	37.6 ± 1.9	26.0 ± 1.1	5.9 ± 0.4	0.59 ± 0.07
(2) 290 mOsm perfusate/ 190 mOsm bath	38.0 ± 2.3	20.7 ± 1.3	8.7 ± 0.9	0.81 ± 0.11
(2)/(1)	1.01 ± 0.01	0.80 ± 0.03	1.47 ± 0.06	1.37 ± 0.04
P values	>0.20	<0.02	<0.02	<0.01

n = 4; no ADH present.

increase in V_T was due entirely to a reduction in D_i; D_o did not change. Thus, none of the increase in epithelial volume was associated with cell swelling directed outwardly against the basement membrane, a result which implies that this membrane (or the basal cell membrane) is considerably less compliant than the opposing luminal cell membrane.

Influence of Peritubular Hypotonicity on Lateral Intercellular Space Geometry

The areal density of lateral spaces (AD_{ICS}), determined for different zones, or volume elements, within the epithelial cell layer, was utilized to estimate the effects of bath dilution on the geometry of the lateral spaces. AD_{ICS}, the fraction of the image area occupied by lateral spaces, was estimated according to the procedure described in detail in the Materials and Methods section. Briefly, stored images were overlaid with a grid and AD_{ICS} was determined for each image by counting the number of intersections which fell on spaces and dividing this number by the total number of intersections within the sample area. The appropriate grid dimensions were determined by sampling repetitively individual images with grids of different spacing and orientation. Rotating the grid by 11, 22 or 33° had no effect on the AD_{ICS} measurement and the coefficient of variation determined from repetitive measurements using the same grid was smallest when the distance between adjacent grid intersections was less than 3 μm. For the measurements reported here, the distance between adjacent intersections was always less than 2 μm and the total number of intersections within the sample area ranged from 200 to 300 (coefficient of variation: 1 to 5%).

Figure 7*a* summarizes data from four experiments which show that AD_{ICS} does not vary sub-

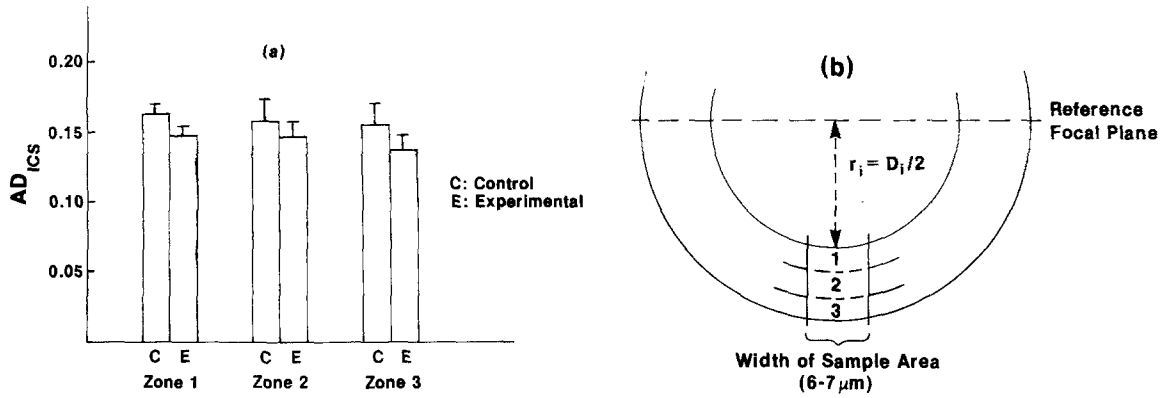

Fig. 7. (a) The influence of peritubular hypotonicity on the areal density of lateral intercellular spaces (AD_{ICS}) determined for three zones within the epithelial cell layer. The control values were derived from optical sections obtained in the presence of isotonic perfusate and bath (290 mOsm). The experimental values were derived from images obtained at the corresponding focal positions 20 to 30 min following replacement of the bath with a hypotonic solution (190 mOsm). Each optical section was grouped into one of three cellular zones according to the difference between the mean lumen radius (r_i), determined for the control case, and focal position (*see* Fig. 7*b* and text for details). Each reported value for AD_{ICS} represents the mean ± SEM for four experiments. (*b*) Schematic representation of the sampling zones used for the determination of areal density of lateral intercellular spaces (AD_{ICS}). Each zone represents a 2-μm-thick section of the epithelial cell layer. Due to the curvature of the epithelium, the width of the sampling area was limited to 7 μm

stantially as the plane of focus is advanced through the cell layer nor does AD_{ICS} change following exposure to the hypotonic bathing solution. For each experiment, AD_{ICS} was determined for six optical sections through the same portion of the epithelial cell layer; three sections were obtained prior to bath dilution and three were obtained at the corresponding focal positions following bath dilution. The identical grid and sample area was used to sample each of the six images obtained in a given experiment.[2] In Figure 7*a* the focal positions for the optical sections have been grouped into three cellular zones, each of which represents a 2-μm-thick section of the cell layer (Fig. 7*b*). Each optical section was grouped into one of these zones according to the difference between the mean lumen radius ($D_i/2$), determined for the control condition, and focal position (reference: longitudinal axis). Thus, zone 1 represents the innermost region of the epithelial cell layer observed before (but not after) osmotic swelling of the epithelium and zone 3 represents the outermost region under both conditions. As shown in Fig. 7*a*, AD_{ICS} did not vary substantially between zones 1, 2 and 3 during isotonic conditions (all P values > 0.10) nor did the 190 mOsm bath significantly influence AD_{ICS} measured in any of the three zones (all P values > 0.10).

Concerning lateral space geometry and its dependence on bath tonicity, we can interpret the data summarized in Fig. 7 in the following way. AD_{ICS} is related to the average width ($\bar{w}$) of the lateral spaces within each image according to:

$$AD_{ICS} = \frac{A_{ICS}}{A_T} = \frac{\bar{w}L_{ICS}}{A_T} \tag{3}$$

where A_T, A_{ICS} and L_{ICS} represent total sample area, lateral space area and lateral space length, respectively. A_T and L_{ICS} are parameters which did not vary between the optical sections obtained for each experiment so that AD_{ICS} provides a relative measure of the average space width, $\bar{w}$, as a function of both focal position and experimental condition. Thus, the data of Fig. 7 support two main conclusions. First, to an initial approximation, average space width is independent of location within the cell layer along the transport axis from lumen to bath. Second, space width does not vary during osmotic swelling of the epithelium, at least, during swelling of the magnitude we observed in these studies. As we will show in the following paper (Kirk et al., 1984), this second point is in contrast to the marked increase in space width which accompanies ADH-induced water flow and which can be observed during an increase in V_T of the same magnitude as that reported in the present studies.

The AD_{ICS} measurement provides an index, not

[2] The curvature of the renal tubular epithelium complicates the interpretation of the AD_{ICS} measurement, particularly with regard to the relationship between AD_{ICS} and focal position. The plane of focus necessarily transects regions within the epithelial cell layer which are at different distances from the lumen. To minimize this problem the sample areas were chosen not to exceed 6 or 7 μm in width (e.g., Figs. 3 and 7*b*) so that the range in depth within the cell layer for each optical section due to the curvature of the epithelium was not greater than ±0.3 μm.

only of relative space width, but also of the fractional volume within each cellular zone which is occupied by lateral spaces (Weibel & Bolender, 1973). Since AD_{ICS} did not vary between these zones, the total epithelial volume which is occupied by lateral intercellular spaces (V_{ICS}) can be calculated for the control condition as follows:

$$V_{ICS} = AD_{ICS} V_T. \quad (4)$$

And, the volume of the cells (V_{cell}) can be approximated by the remaining epithelial volume[3]:

$$V_{cell} = (1 - AD_{ICS}) V_T. \quad (5)$$

Substituting into Eqs. (4) and (5) the mean values for AD_{ICS} (0.16) and V_T (0.59 nl mm^{-1}) determined for the control condition, we calculate values for V_{cell} and V_{ICS} of 0.50 and 0.09 nl mm^{-1}, respectively.

Because lateral space width did not change during osmotic swelling of the epithelium, the increase in V_T observed following bath dilution largely reflected an increase in V_{cell}. We can estimate the changes in V_{cell} and V_{ICS} associated with bath dilution according to the following arguments. V_{ICS} changed following bath dilution only to the extent that the average height of the spaces (from apex to base) increased. This increase in space height can be expected to be considerably less than the 47% increase in the mean thickness of the epithelial cell layer, since the latter was primarily the result of the cells bulging into the lumen. As an estimate of the degree to which space height increased, we measured, using images obtained at the reference focal position, the increase in distance from the basal surface to the luminal surface determined at the apparent position of the lateral spaces between adjacent cells[4] (*see* Fig. 8). The results showed that the mean height of the lateral spaces increased 24 ± 3% (15 measurements, 3 tubules) following peritubular dilution. Using this value as an estimate of the degree to which V_{ICS} increased following hypotonic stress, we calculate that only about 10% of the observed increase in V_T (~0.02 nl mm^{-1}) could be accounted for by an increase in V_{ICS}. The remaining increase in V_T (~0.20 nl mm^{-1}) represented a 40% increase in cell volume, a value which is somewhat less than the 53% increase predicted by the Boyle-van't Hoff relation for an osmometer in which all of the cell volume participates in the volume adjustment (Hoffman, 1977)[5].

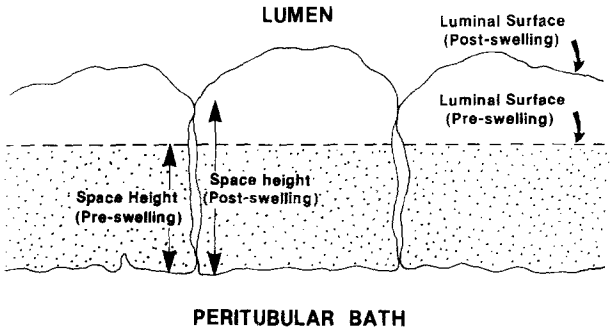

Fig. 8. Method for determining the influence of peritubular hypotonicity on the height of the lateral intercellular spaces. Lateral space height was derived from DIC images obtained at the reference focal position before and after bath dilution (290 to 190 mOsm). The height of the spaces was estimated as the distance from the basal surface to the luminal surface measured at the apparent position of the lateral spaces between adjacent cells

Discussion

Previous investigators have utilized DIC optics to derive meaningful structural data from light microscopic images of nonrenal epithelia (Spring & Hope, 1979; Berglindh, DiBona, Ito & Sachs, 1980; DiBona, 1981; Persson & Spring, 1982). The feasibility of DIC microscopy as a tool for studying nephron structure and function has been discussed before (Horster & Gundlach, 1979) but, until the present work, no attempt had been made to derive quantitative information from DIC images of the mammalian renal tubular epithelium. We chose the isolated and perfused nephron as the specimen for examining renal structure and function with DIC microscopy for two reasons. First, microscopic images of nephron segments are more easily interpreted when the tubule lumen is patent, a condition which does not obtain for nonperfused nephron segments. Second, with the isolated microperfused tu-

[3] We ignore in this calculation the contribution of basilar extracellular spaces, which cannot be observed with DIC microscopy and which presumably occupy little volume.

[4] This is most likely a slight overestimate of space height since we include in this measurement the height of the tight junctions, which are seldom observed in DIC images of the collecting tubule. Nonetheless, because the lateral spaces occupy only a small fraction of the total epithelial volume, small errors in the estimate of the change in space height (or space width) associated with peritubular dilution will not substantially influence the calculated increase in cell volume.

[5] Changes in tubular length could lead to errors in extrapolating the observed increase in cell volume, that was normalized to tubule length, to the average increase in the volume of individual collecting tubular cells. However, we have been unable to detect any changes in the lateral dimensions of collecting tubular cells following osmotic swelling.

bule preparation, optical and physiologic data (e.g., water flow, transepithelial PD) can be collected simultaneously and compared for the same tubule segment (Kirk et al., 1984). The only constraint in combining DIC optics with the perfused tubule preparation was the condenser assembly which had to be narrow enough to allow it to be lowered to the proper working distance without contacting the perfusion pipettes. The condenser of choice provided satisfactory resolution in most respects and, even though the depth of focus was compromised to some extent because of the lesser numerical aperture of this lens, we could still optically section the tubular epithelium in thicknesses considerably less than 1.5 μm.

Morphometric Procedures

The bulk of the work summarized in this paper was dedicated to the development of morphometric procedures which could be used to derive quantitative structural information from light microscopic images of perfused nephron segments. The major advantages of the procedures for estimating epithelial volume and the areal density of lateral intercellular spaces were the rapidity and reproducibility with which these measurements could be performed. In addition, since for each method structural information was derived from sampling relatively large areas of epithelium, these procedures are statistically preferable to measuring structural parameters (e.g., tubule diameter, lateral space width) at a few discrete locations along the length of the tubule.

The areal density of lateral spaces is a particularly versatile structural parameter in that it provides information on both fractional lateral space volume and relative lateral space width as functions of focal position and experimental condition. In order to maximize the precision of the method for estimating the areal density of lateral spaces, we chose a systematic point-counting procedure as opposed to a random sampling method because, in general, the former will yield more precise estimates of areal density (Ebbesson & Tang, 1967). The accuracy of our estimates of lateral space geometry can only be inferred by critically examining the morphometric procedures we utilized and by comparing our results to similar data derived from electron microscopic images of the rabbit cortical collecting tubule. On the basis of two arguments one might reason that our measurements of the fractional epithelial volume occupied by lateral spaces are slight overestimates. First, DIC microscopy cannot resolve the small, lateral cell membrane processes which protrude into the lateral spaces, as suggested by electron microscopy (Welling, Evan & Welling, 1981). Second, although our data indicate that lateral space width is relatively constant along the transport axis, narrowing of the spaces at discrete locations within the cell layer (e.g., near the tight junctions and basilar slits) could not have been detected. In spite of these potential errors, the areal density measurement would be expected to provide a reasonable index of relative changes in lateral space geometry. Moreover, the fractional epithelial volume occupied by lateral spaces we estimated for the control condition (0.16) is within the range reported for the fractional contribution of extracellular spaces to epithelial volume in fixed cortical collecting tubules observed with electron microscopy (Welling et al., 1981). It should be emphasized, however, that the latter estimate included the contribution of basilar spaces to the extracellular space volume (these spaces presumably occupy a small volume). In addition, the diuretic states of the rabbits prior to fixation of the kidneys were unknown in the studies of Welling et al. (1981) and, as shown in the following paper (Kirk et al., 1984), the volume of the lateral spaces increases markedly during ADH-induced antidiuresis.

Structural Response of the Mammalian Collecting Tubule to Peritubular Hypotonicity

Our DIC microscopic observations of the living and perfused rabbit collecting tubule confirm previous electron microscopic observations of the nonperfused rabbit collecting duct (Grantham et al., 1969) which indicated that the morphologic response of this nephron segment to peritubular hypotonicity in the absence of ADH differs qualitatively from the changes associated with the onset of ADH-dependent flow. Specifically, swelling of the epithelial cell layer under zero transepithelial flow conditions was not associated with dilation of the lateral intercellular spaces or vacuolation of the cytoplasm. Thus, these structural changes, which are clearly observed with DIC optics during the onset of hormone-dependent flow (Kirk et al., 1984), are events specifically induced by transepithelial volume flow and do not reflect simple, osmotic swelling of compartments within the epithelium.

The quantitative information derived from these experiments supports two major conclusions. First, the luminal cell membranes of the cells of the collecting tubule are considerably more compliant than either the basement (and/or basal cell) membranes or the lateral cell membranes. Second, under the conditions of our experiments, the cells of the

collecting tubule swell but do not exhibit any regulatory decrease in volume. The first conclusion is supported by the observations that the volume increase following bath dilution was accomplished entirely by a reduction in inside tubule diameter (i.e., cells swelled into the lumen, not outwardly against the basement membrane) and the insensitivity of lateral space width to cell swelling. The relative noncompliance of the basement membrane is consistent with the data of Welling and Grantham (1972) who reported that the isolated basement membrane of the collecting tubule is highly nondistensible over a fourfold range of applied intraluminal pressure (~4 to 16 cm H_2O). The second conclusion is supported by the time course of the epithelial volume increase. The form of this curve, rapid swelling to a new steady state, is, in fact, contrary to the data of Dellasega and Grantham (1973) who reported for the nonperfused cortical collecting tubule incubated at 37°C and exposed to one-half isotonic bath a gradual relaxation of epithelial volume toward the control (isotonic) value following the initial volume increase. The discrepancy probably results from the different temperatures at which the two studies were conducted and suggests that, under some conditions, cells of the mammalian collecting tubule have the capacity to "dump" solute in order to regulate their volume back toward that observed in the presence of isotonic media.

The estimated fractional increase in cell volume associated with bath dilution was less than that predicted theoretically for an osmometer bounded by a completely solute-impermeant membrane and for which all of the cell volume participates in the adjustment to peritubular hypotonicity. We have no evidence for solute loss or for a significant luminal membrane water permeability under these conditions, factors which could attenuate the osmotic swelling of tubular cells. Therefore, in order to account for the discrepancy between the predicted and observed osmometric behavior of the collecting tubule, we can reasonably model the cells as osmometers that contain a significant volume fraction which is insensitive to hypotonicity, such as that occupied by dry matter and nonsolvent water. Rearranging the original equation of Ege (1927), we can calculate the fraction of the control cell volume which is osmotically inactive (F_I) from the relative increase in cell volume (α) observed following bath dilution and the ratio of the control (π_c) and experimental (π_e) peritubular osmotic pressures:

$$F_I = \frac{1 - \alpha\left(\frac{\pi_e}{\pi_c}\right)}{1 - \frac{\pi_e}{\pi_c}}. \quad (6)$$

Inserting the appropriate values for α(1.40) and π_e/π_c (0.66) into Eq. (5), we calculate a value for F_I of 0.22. Thus, when exposed to a hypotonic perturbation of this magnitude, cells of the cortical collecting tubule swell as if 22% of their volume is osmotically insensitive. This value for F_I is within the range reported for mammalian cells (Hoffman, 1977), including cells within outer medullary slices of the rat kidney bathed in sucrose-containing media (Law, 1975), and, as will be shown in the next paper (Kirk et al., 1984), is an important piece of information relating the cell volume increase associated with ADH-dependent flow to the relative hydraulic conductivities of the opposing cell membranes.

In summary, we visualized the isolated and perfused rabbit cortical collecting tubule with DIC microscopy and developed morphometric procedures useful for characterizing experimentally induced perturbations in cell volume and lateral intercellular space geometry. This paper described in detail the development of the optical and morphometric procedures and the application of these techniques to the analysis of the structural response of the mammalian collecting tubule to peritubular hypotonicity. In the following paper we present a series of experiments in which we utilized these techniques to characterize the morphologic changes associated with ADH-induced volume reabsorption in the mammalian collecting tubule.

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