THE EFFECT OF SMOOTH MUSCLE ON THE INTERCELLULAR SPACES IN TOAD URINARY BLADDER

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ABSTRACT

Phase microscopy of toad urinary bladder has demonstrated that vasopressin can cause an enlargement of the epithelial intercellular spaces under conditions of no net transfer of water or sodium. The suggestion that this phenomenon is linked to the hormone's action as a smooth muscle relaxant has been tested and verified with the use of other agents effecting smooth muscle: atropine and adenine compounds (relaxants), K^+ and acetylcholine (contractants). Furthermore, it was possible to reduce the size and number of intercellular spaces, relative to a control, while increasing the rate of osmotic water flow. A method for quantifying these results has been developed and shows that they are, indeed, significant. It is concluded, therefore, that the configuration of intercellular spaces is not a reliable index of water flow across this epithelium and that such a morphologic-physiologic relationship is tenuous in any epithelium supported by a submucosa rich in smooth muscle.

INTRODUCTION

Vasopressin stimulates net sodium transport and osmotic water flow across the urinary bladder of the toad (14). Several morphologic studies have demonstrated that vasopressin-induced water flow across this preparation is accompanied by an enlargement of the epithelial intercellular spaces (16, 17, 2, 1, 8). Pak Poy and Bentley (16) have suggested that the spaces in toad bladder increase as a direct consequence of the increased water flow; this view has been supported by data correlating the extent of opening with measured rates of fluid transport in rabbit gallbladder under a variety of experimental conditions (13, 5, 21, 4). However, we have recently noted that enlargement of the intercellular spaces could be produced by vasopressin in the absence of demonstrable net transport of water or, for that matter, of sodium ions (6). At that time it was proposed that the action of vasopressin as a smooth muscle relaxant (9) might be responsible for the observed increase in intercellular spaces and the present study investigates this proposition in detail.

METHODS

Female specimens of the toad, *Bufo marinus*, were obtained from the Dominican Republic (National Reagents, Inc., Bridgeport, Conn.), and were force-fed meal worms upon arrival and maintained at room temperature on moist earth. Urinary hemibladders were excised from doubly pithed toads, rinsed in Ringer's solution, and mounted in Lucite double chambers (19). Appropriate electrical measurements (short-circuit current or electrical potential with or without measurements of resistance) were made in each case and, where desirable, net volume flow was also monitored (20). Ringer's solution of the following composition was used: Na^+, 113; K^+, 3.5; Ca^{++}, 0.9;

Cl^-, 116; HCO_3^-, 2.4 mM; pH, 7.5–8.0; and tonicity, 220 milliosmols/kg H_2O. For experiments where an abolishment of active transport was desired, $choline^+$ was quantitatively substituted for Na^+ ($choline^+$ Ringer's solution); in studies employing K^+ as a muscle contractant, this cation replaced all (K^+ Ringer's solution) or one half (Na^+—K^+ Ringer's solution) of the Na^+. Reagents used included: vasopressin (Pitressin, Parke, Davis & Co., Detroit Mich.), atropine (Inland Alkaloid Co., St. Louis Mo.), adenosine triphosphate (Sigma Chemical Co., St. Louis, Mo.), adenosine (California Corporation for Biochemical Research, Los Angeles, Calif.), and acetylcholine chloride (Merck and Co., Inc., Rahway, N. J.).

Phase microscopy rather than electron microscopy was utilized since the configuration of intercellular spaces was readily observed with this technique, and the amount of tissue that could be sampled was substantially greater. Specimens for microscopy were fixed in the chambers by addition of glutaraldehyde (Fisher Scientific Co., Pittsburgh, Pa.) to a final concentration of 1.0% and allowed to stand for 15–30 min before removal. Rectangles of tissue were excised from the chambers and immersed in 1.0% glutaraldehyde in phosphate buffer to be processed with a postfixation treatment in osmium tetroxide and a final embedment in epoxy as described previously (7). Sections for phase microscopy, 1–1.5 μ thick, were cut on a Reichert OmU2 ultramicrotome (G. Reichert Optische Werke A. G., Vienna, Austria) and examined with Zeiss optics. Phase microscopic examination was performed by one of us (DRDB) without prior knowledge of the experimental protocol to eliminate bias in interpretation. In order to confirm that the spaces studied were indeed intercellular in location, each specimen was also examined with a Philips EM200 electron microscope.

RESULTS

In the course of these and other experiments we had noted that a well defined reference condition with tightly closed intercellular spaces was not always found. In fact, it was clear that our preparations would be relatively open or closed depending on the degree of stretch imposed on the tissue in the process of mounting it on the chamber; i.e., very loosely mounted tissues showed a markedly open intercellular space configuration, while the opposite was found if the tissue were very tightly stretched. Figs. 2 *a* and 3 *a* serve to illustrate this finding. Therefore, to provide an appropriate control where an opening of spaces was the predicted result, experiments were performed on tightly mounted hemibladders; when a reduction in the size of spaces was anticipated, the tissue was mounted loosely. In each case, care was exercised to assure that both control and experimental sides of the individual hemibladders were subjected to the same degree of stretch.

In order to investigate the morphologic effects of vasopressin in the absence of either net salt or water flow, three experiments were performed with $choline^+$- rather than Na^+ Ringer's solution. Hormone was administered to a final serosal concentration of 40–200 mU/ml, and tissues were fixed with glutaraldehyde 15–16 min later. The results of such an experiment are represented in Figs. 1 *a* and 1 *b*. It is clear that the intercellular spaces on the vasopressin-treated side are greatly enlarged compared to those of the control situation which are scarcely resolved at this magnification.

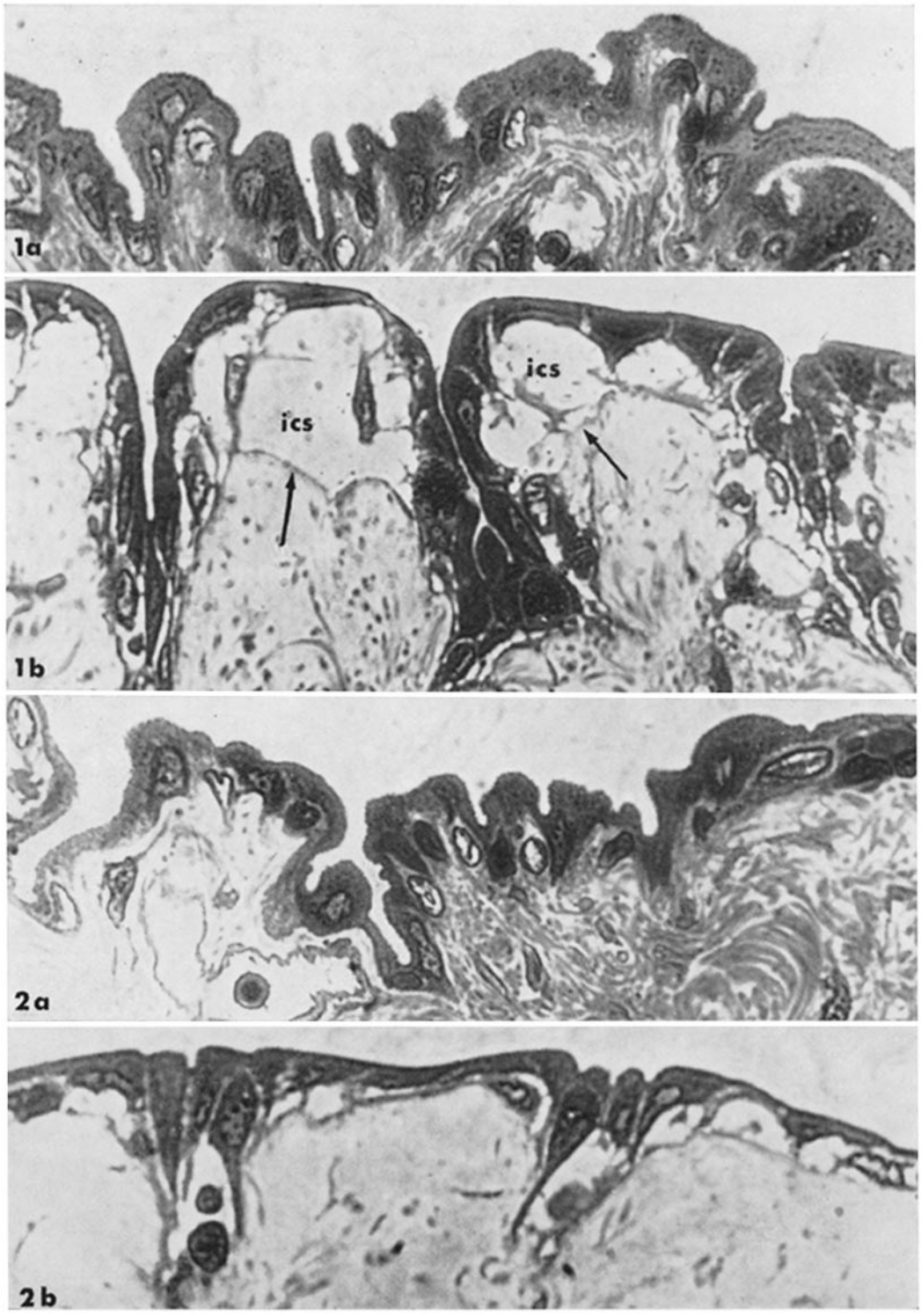
To further dissociate the morphologic from the transport effects of vasopressin, low doses were administered to bladders bathed in Na^+ Ringer's solution; the use of low hormonal concentrations was found to delay the onset of the short-circuit current response by several minutes. In three experiments vasopressin was added to a final serosal concentration of 20 μU/mL and tissue was fixed within 2½ min, before any increase in

FIGURE 1 *a* Control quarter-bladder, tightly mounted, and bathed with isotonic $choline^+$ Ringer's solution on mucosal and serosal surfaces. × 600.

FIGURE 1 *b* Experimental quarter-bladder from the same double-chamber experiment as Fig. 1 *a*, mounted and bathed as above but with the addition of 200 mU/ml of vasopressin to the serosal bath. Intercellular spaces (*ics*) are clearly enlarged, even in regions of epithelial folding. Basement membrane is indicated by arrows. × 600.

FIGURE 2 *a* Control quarter-bladder, tightly mounted, and bathed with isotonic Na^+ Ringer's solution on mucosal and serosal surfaces. × 600.

FIGURE 2 *b* Experimental quarter-bladder from same experiment as Fig. 2 *a*, mounted and bathed as above but with addition of 20 μU/ml of vasopressin to the serosal bath. This sample was fixed before a short-circuit current response was elicited by the hormone and shows marked enlargement of the intercellular spaces. × 600.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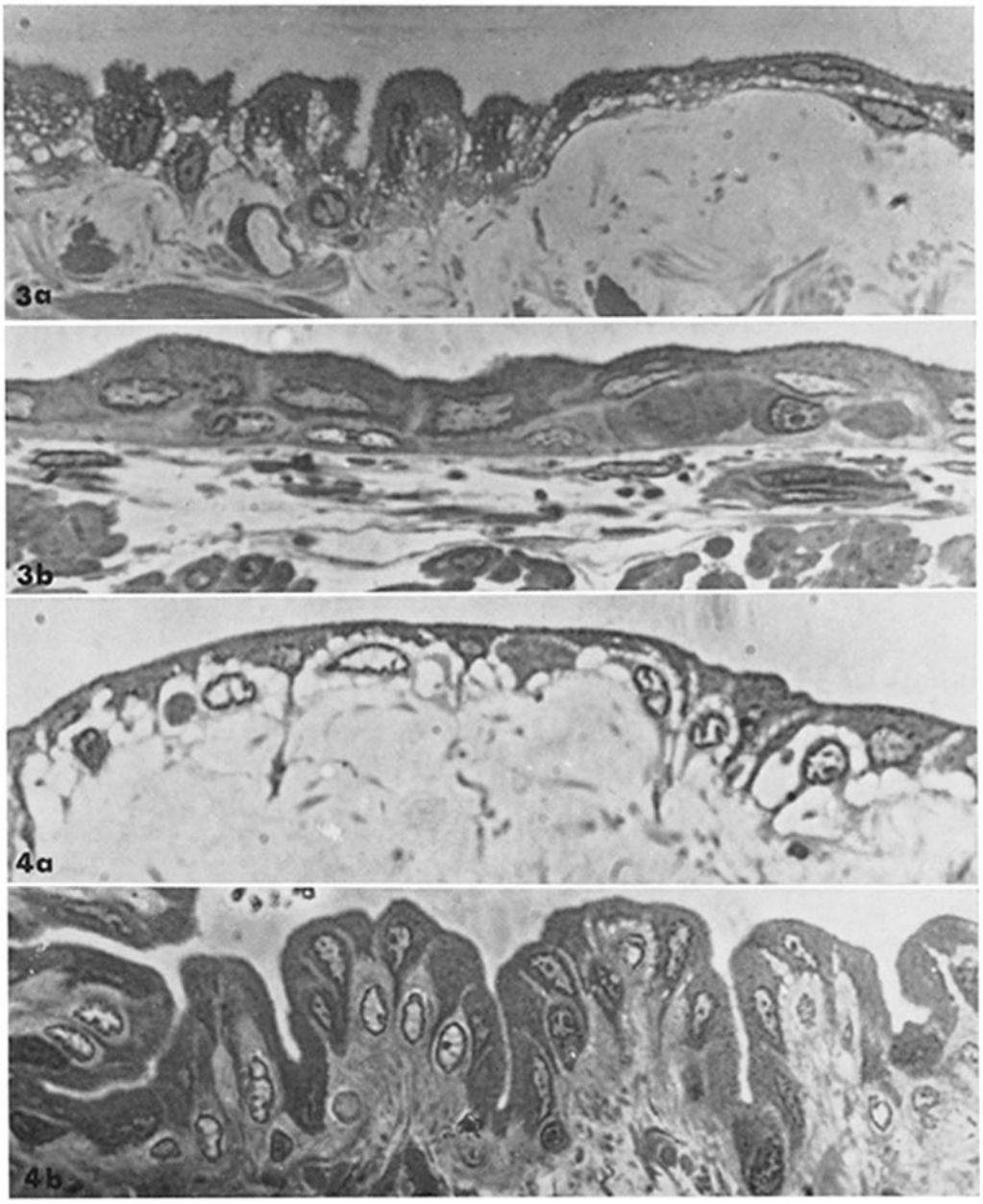

FIGURE 3 *a* Control quarter-bladder, loosely mounted, and bathed with isotonic Na^+ Ringer's solution on mucosal and serosal surfaces. × 600.

FIGURE 3 *b* Experimental quarter-bladder from the same experiment as Fig. 3 *a*, mounted as above but bathed with isotonic Na^+ Ringer's solution on the mucosal surface, and isotonic K^+ Ringer's solution on the serosal surface. The loose aggregation of epithelial cells seen in Fig. 3 *a* is not found here as the spaces between cells have apparently closed. × 600.

FIGURE 4 *a* Control quarter-bladder, loosely mounted, and bathed with isotonic Na^+ Ringer's solution on mucosal and serosal surfaces. × 600.

FIGURE 4 *b* Experimental quarter-bladder from the same experiment as Fig. 4 *a*, mounted and bathed as above but with addition of 1 mM acetylcholine to each bath. Intercellular spaces are closed with reference to the control situation. × 600.

short-circuit current or conductance could occur. Note that in Figs. 2 *a* and 2 *b*, where these results are illustrated, the difference between control and experimental quarter-bladders is comparable to that seen in Figs. 1 *a* and 1 *b* where the experimental dose was 10,000 times greater.

If a relaxation of smooth muscle can open the intercellular spaces in a tightly mounted preparation, contraction might be expected to close them in a loosely mounted hemibladder. Substitution of K^+ for Na^+ in the serosal compartment of three preparations, with fixation 7–10 min later, was found to have precisely this effect (Figs. 3 *a* and 3 *b*). The addition of 1 mM acetylcholine to the serosal medium, fixing 12–19 min later, appeared somewhat less effective. Figs. 4 *a* and 4 *b* indicate the extent of the difference between control and experimental that was found with sufficient frequency to permit a morphological determination of which was the experimental quarter-bladder; other sections showed little difference between the two sides.

Smooth muscle relaxants other than vasopressin were also tested. Adenine compounds have been reported to be effective as smooth muscle relaxants (18). In two experiments, 9 mM adenosine and, in one experiment, 5 mM ATP was added to the serosal and mucosal bathing media, followed by glutaraldehyde 6–10 min later; experimental quarter-bladders were easily distinguished from controls by the abundance of enlarged spaces. As suggested by Figs. 5 *a* and 5 *b*, these compounds did not produce a striking effect. Experimental sides showed considerable variability; some regions were clearly open, others closed, and many revealed cells with only very small spaces around them or cells that seemed to be on the boundary between open and closed regions.

A more dramatic method for demonstrating the action of a smooth muscle relaxant on intercellular space enlargement involved bathing tightly mounted hemibladders on mucosa and serosa with Na^+ Ringer's solution + 1 mM acetylcholine. After a period of 16–42 min, the serosal compartment of one half-chamber was rinsed and refilled with Na^+ Ringer's solution + 1 mM atropine. Three experiments performed this way yielded a strong correlation between enlarged spaces and treatment with the smooth muscle relaxant, atropine. In another three experiments done this way, the mucosal compartment of each half-chamber was filled with dilute Ringer's solution (110 milliosmols) and osmotic water flow in response to the established gradient (mucosa to serosa) was monitored. In no case did detectable water flow occur despite the pronounced space enlargement produced by atropine treatment (Figs. 6 *a* and 6 *b*).

To pursue further the relationship between intercellular space size and the rate of transepithelial water flow, we attempted to limit the smooth muscle effect of vasopressin while using the hormone to stimulate water flow. Individual double chamber experiments were carried out in the following way. Control quarter-bladders were exposed to isotonic Na^+ Ringer's solution on both mucosa and serosa. Experimental halves were bathed with Na^+—K^+ Ringer's solution; this solution was applied isotonically (220 milliosmols) to the serosa and diluted (110 milliosmols) to the mucosa.

After administration of vasopressin (200 mU/ml) to both serosal media, a net movement of water (0.6–1.0 μl/min per quarter bladder, mucosa-to-serosa) occurred in the experimental samples without any measurable response on the control side. Examination of these tissues showed that control preparations (Na^+ Ringer's solution, no gradient, no water flow) did not contain swollen cells but had markedly open intercellular spaces (Fig. 7 *a*); the experimental quarter-bladders (Na^+—K^+ Ringer's solution, gradient, substantial water flow) revealed swollen cells but the intercellular spaces, while present (Fig. 7 *b*), seemed less enlarged than those of the control.

All of these phase microscopic results were also quantified (Table I) according to the following protocol. Approximately 1000 cells of the control and experimental side of each preparation were classified "open" or "closed." A cell surrounded by a visibly enlarged intercellular space at a magnification of 2000 was considered "open;" all others were considered "closed."

In the last series of experiments, where it was desirable to determine the relative size of intercellular spaces, the sample populations were classified a second time at a magnification of 250. In these studies of water flow following vasopressin, the experimental side was subjected to an osmotic gradient and a smooth muscle contractant (K^+) and the control side was bathed with isotonic solutions without a contractant. Spaces were significantly less frequent on the experimental side despite the increased water flow. In addition, the

same material was recounted at an eightfold reduction in magnification; here, the differences in frequency distribution of cells in the open configuration was even more striking. Since the difference between the counts obtained at the two magnifications was also significant ($p < 0.05$), it is fair to conclude that the spaces present during water flow under these circumstances were smaller as well as less numerous than those of the control.

The effect of net water flow on the intercellular spaces was also studied with a smooth muscle contractant both on the experimental and on control sides. Isotonic Na^+—K^+ Ringer's solution bathed both surfaces of the control preparation, as well as the serosal surface of the experimental quarter bladder; the mucosal medium of the experimental quarter-bladder was a Na^+—K^+ Ringer's solution diluted with distilled water to a tonicity one-fifth that of the serosal medium. The addition of vasopressin to both quarter-bladders to a final serosal concentration of 200 mU/ml increased transepithelial water movement to 5.3–7.1 μl/min per quarter-bladder on the experimental side, but had no measurable effect on net water transport on the control side. Applying the techniques of data reduction used above to the three hemibladders studied, the intercellular spaces were slightly increased on the experimental side, both at magnifications of 2000 (% Change ± SEM = 9 ± 7) and at 250 (% Change ± SEM = 2 ± 1). Neither these differences nor the difference between the counts obtained at the two magnifications were significant.

DISCUSSION

The results suggest that vasopressin can increase the size of the intercellular spaces largely by an effect other than that on transepithelial transport. In the absence of net Na^+ transport (choline$^+$ Ringer's solution) and in the absence of net water flow (no osmotic gradient), the hormonal effect on tissue morphology persists. These observations have been extended by using concentrations of vasopressin some 10^4 times smaller than those usually applied; fixation of the tissues before the appearance of any change in short-circuit current or resistance has permitted the dissociation of the morphologic from the characteristic electrical effects.

This dissociation was particularly striking in those preparations where the experimental side was bathed with a half Na^+ and half K^+ Ringer's solution and where the mucosal medium was diluted with distilled water to create an osmotic

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FIGURE 5 *a* Control quarter-bladder, tightly mounted and bathed with isotonic Na^+ Ringer's solution on mucosal and serosal surfaces. × 600.

FIGURE 5 *b* Experimental quarter-bladder from the same experiment as Fig. 5 *a*, mounted and bathed as above but with addition of 9 mM adenosine to each bath. Regions with some open and some apparently closed intercellular spaces as shown here were quite numerous. × 600.

FIGURE 6 *a* Control quarter-bladder, tightly mounted, and bathed with Na^+ Ringer's solution + 1 mM acetylcholine, isotonic to serosa, hypotonic (half-strength) to mucosa. × 600.

FIGURE 6 *b* Experimental quarter-bladder from the same experiment as Fig. 6 *a*, mounted and initially bathed as above, but 10 min prior to fixation, the serosal compartment was rinsed and refilled with isotonic Na^+ Ringer's solution + 1 mM atropine. While the intercellular spaces are enlarged, no measureable water flow occurred. × 600.

FIGURE 7 *a* Control quarter-bladder, tightly mounted and bathed with isotonic Na^+ Ringer's solution on mucosal and serosal surfaces with addition of 200 mU/ml of vasopressin to the serosal bath. × 600.

FIGURE 7 *b* Experimental quarter-bladder from the same experiment as Fig. 7 *a*, mounted as above, and bathed with Na^+—K^+ Ringer's solution, isotonic to serosa, hypotonic (half-strength) to mucosa. Vasopressin was added to the serosa (200 mU/ml as above) but here, in the presence of a gradient, osmotic water flow occurred, the epithelial cells are quite swollen and, in the presence of a high concentration of K^+ (inducing a smooth muscle contraction) the intercellular spaces are not as enlarged as for the control. × 600.

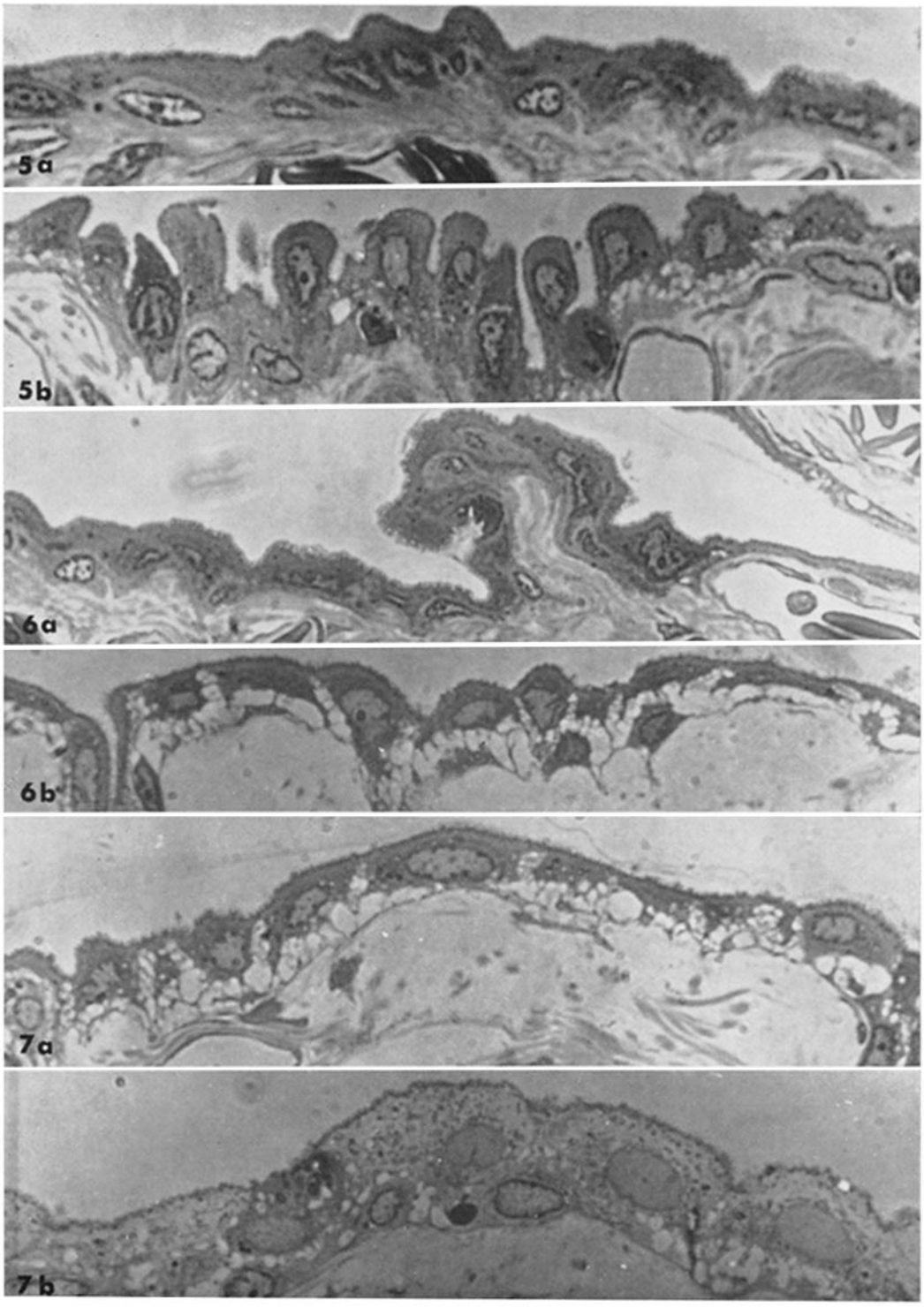
5a
5b
6a
6b
7a
7b

TABLE I

Experiment (n)*	% Change	Probability
Vasopressin (choline$^+$ Ringer's solution) (3)	73 ± 3	<0.001
Vasopressin (low concentration) (4)	54 ± 9	<0.02
Adenosine and ATP (3)	30 ± 12	<0.20
K^+ Ringer's solution (3)	81 ± 4	<0.001
Acetylcholine (4)	44 ± 20	<0.20
Atropine (6)	74 ± 3	<0.001
Na^+–K^+ Ringer's solution (with water flow) at × 2000 (3)	23 ± 4	<0.05
Na^+–K^+ Ringer's solution (with water flow) at × 250 (3)	49 ± 9	<0.05

* (n) = number of experiments performed.
Results are normalized with reference to the control situation; i.e., for relaxants (vasopressin, adenine compounds, atropine): % change = (% open [experimental] − % open [control])/% closed (control); for contractants (K^+, acetylcholine): % change = (% closed [experimental] − % closed [control])/% open (control).

gradient; the control quarter-bladder was bathed with isotonic Na^+ Ringer's solution on both mucosa and serosa. Subsequent addition of vasopressin to both halves of the preparation resulted in increased net water flow on the experimental side, whereas the intercellular spaces were significantly larger and more numerous on the control side.

Since vasopressin is an effective smooth muscle relaxant for toad bladder, other agents effecting smooth muscle tone were also studied. The relaxants, atropine and, to a lesser extent, adenine compounds, produced an increase while the contractants, K^+ and, to a lesser extent, acetylcholine, produced a decrease in the size of the intercellular spaces. The data therefore indicate that vasopressin increases the size of the intercellular spaces largely through its action in relaxing the smooth muscle of the bladder.

The mechanism by which the activity of the underlying smooth muscle may affect the size of the intercellular spaces may well be related to the following characteristics of toad bladder: (*a*) The rate-limiting barrier to transepithelial movement of water lies at the urinary surface of the mucosal epithelial cells (17, 2, 8). (*b*) The basement membrane is freely permeable not only to water but also to molecules as large as Thorotrast (3) and horseradish peroxidase (15). (*c*) The submucosa, which contains much solid matter in the form of collagen bundles, capillaries, and the smooth muscle itself, constitutes little resistance to the passage either of electrical current (10) or of radioactive ions (11), and must therefore permit the ready passage of water across it. It should also be noted that, when mounted in a chamber, the edges of the tissue are fixed in place and the contraction of bundles of smooth muscle in series with collagen fibers does not lead to free shortening of the bladder; a contraction or relaxation of the muscle is, therefore, expected to have a more pronounced effect on the over-all tension on the preparation here than it would in vivo or in a cannulated bag preparation.

Smooth muscle contractants cause loosely mounted tissue to visibly contract into a flat sheet. Therefore, the smooth muscle forms a functionally complete sheet; the smooth muscle fibers of the submucosa must be linked either to each other or to the basement membrane through intervening bundles of collagen. When the muscle fibers contract, the bulk material interposed between muscle and basement membrane, including collagen bundles, fibroblasts, and capillaries, will be pressed against the basement membrane (as may be appreciated from a comparison of the contracted and relaxed tissues of Figs. 1–6). To the extent that the urinary surface of the mucosa is not infinitely compliant, the hydrostatic pressure within the mucosa must consequently rise. Intercellular water will therefore be extruded into the submucosa across the porous basement membrane, and the intercellular spaces will appear reduced. When the muscle bundles relax, submucosal fluid will be free to redistribute across the basement membrane, and the intercellular spaces will appear enlarged.

This formulation leads to the expectation that the technique used to mount the bladder on a chamber would markedly affect the size of the intercellular spaces. Mounting the preparations highly stretched would enhance the effect of a

given degree of contraction of smooth muscle, whereas mounting the bladder very loosely would minimize the effect of any given state of contraction. This correlation was indeed observed and provided us with a technique for establishing appropriate pretreatment reference states. (In each case, care was exercised to mount the experimental and control sides of the half-bladder as symmetrically as possible). In another study currently being carried out in this laboratory (M. M. Civan, D. R. DiBona, W. S. Metcalf, and I. Singer. Data in preparation.) we have noted that applied hydrostatic pressure serves to close intercellular spaces in loosely mounted bladders as would be anticipated.

Although the intercellular spaces may be enormously increased in the absence of net salt and water transport, and although dilation of the spaces in the presence of an osmotic gradient (e.g., with atropine) does not measurably increase net transepithelial water movement, the results by no means preclude the possibility that increased water flow may also cause a small increase in size of the intercellular spaces.

Ganote et al. have studied the effect of vasopressin on the isolated collecting duct of rabbit kidney (12). Their observation that the hormone increases the intercellular spaces only in the presence of an osmotic gradient across a preparation free of smooth muscle is the most convincing evidence thus far adduced that increased net water flow *per se* may increase the size of intercellular spaces in epithelia.

Carasso et al. have studied frog bladders supported by nylon mesh and subjected to mucosal hydrostatic pressures of some 20 cm of water (1). Under these conditions, the neurohypophyseal hormone oxytocin, also a smooth muscle relaxant (9), increased the intercellular spaces only in the presence of an osmotic gradient across the tissue. However, the magnitude of the increase was so small that, as opposed to the enlargements noted here, they would probably be imperceptible by phase microscopy; therefore, the increase in intercellular spaces caused by increased water flow *per se* would not have been appreciated under the present protocol. Evidently the hydrostatic pressure caused a general reduction in the size of the intercellular spaces as well as specifically minimizing the effect of changes in smooth muscle tone in their preparations.

It is reasonable to conclude therefore that increased net water flow does generally increase the intercellular spaces of epithelia. However, enormous enlargements can result from relaxation of the submucosal smooth muscle in toad bladder. Caution must therefore be exercised in drawing conclusions concerning the physiologic significance of dilated intercellular spaces in epithelial preparations containing smooth muscle in the supporting tissue.

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