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Pathways for Movement of Ions and Water Across Toad Urinary Bladder

II. Site and Mode of Action of Vasopressin

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Summary. Application of either mucosal hypertonicity or serosal hypotonicity increases the electrical conductance of toad urinary bladder by altering the permeability of the apical intercellular junctions which are rate-limiting to transepithelial flow of ions and water between the cells. Prior addition of vasopressin has been found to inhibit both the electrical and morphologic effects. In the presence of mucosal hypertonicity, the hormone also induces shrinkage of the granular cells, with no perceptible change in the volume of the other epithelial cells. The skin of *Xenopus laevis* is similarly responsive to increases in tonicity of the outer bathing medium but here, where vasopressin exerts a natriferic but not a hydroosmotic effect, hormone administration does not inhibit the osmotically induced electrical and morphologic changes. These results may be interpreted within the framework of current concepts concerning the granular cell response to vasopressin and the response of the limiting junctions to transepithelial osmotic gradients. Vasopressin facilitates hydroosmotic flow in either direction across the epithelium, specifically by increasing the water permeability of the luminal-facing plasma membrane of the granular cells.

It has long been appreciated that sodium may cross biological membranes by a variety of processes including active transport, exchange diffusion and simple diffusion. Some, but probably not all, of these processes may be mediated by the same molecular mechanism, whose transport properties vary with the experimental conditions [26]. Thus, the plasma membrane may constitute a mosaic of parallel pathways for transport.

Epithelial membranes, in particular, provide additional types of heterogeneous pathways for sodium transport [12, 48]. Increasing evidence indicates that sodium and other ions may penetrate the "tight" or "limiting"

junctions between the epithelial cells and subsequently traverse the lateral intercellular spaces [7, 13, 17, 22, 23, 28, 40, 49]. In addition, the variety of cell types within mucosal epithelia suggests a corresponding heterogeneity of transport properties.

The conductance through one or another set of transepithelial pathways can be selectively altered. Osmotic gradients favoring net water movement from serosa-to-mucosa may be established by increasing the tonicity of the mucosal medium or by decreasing the tonicity of the serosal medium ("reverse osmotic gradients"); such gradients raise the electrical conductance across the urinary bladder of the toad [17, 47, 49]. Evidence presented in the first paper of the present series has established that application of gradients increases the size of the space within the limiting junctions, reducing the resistance to transepithelial ionic movement through the intercellular channels [17].

On the other hand, vasopressin increases the tissue conductance by increasing sodium movement through the transcellular active transport pathways. This effect is mediated by a fall in resistance to sodium movement specifically across the apical plasma membrane of the transporting cells [8, 11, 12, 37, 38, 50]. Additional evidence suggests a further effect of vasopressin on the sodium pump [21, 30–32, 35], but the quantitative significance of this effect is yet uncertain.

The results of the current study establish the presence of a strong interrelationship between these separate parallel intercellular and transcellular pathways. This phenomenon appears to arise from the altered osmolality of the intracellular fluids induced by the hydroosmotic action of vasopressin in the presence of reverse osmotic gradients; no direct effect of vasopressin on the limiting junctions need be invoked. In addition, the data provide further information concerning the site and mode of action of vasopressin; the results: (a) confirm that vasopressin exerts a selective hydroosmotic effect on the granular cells [19], (b) provide the first direct evidence that vasopressin enhances osmotically induced water flow, in response to normal and reverse osmotic gradients, through the same transcellular pathway, and (c) confirm that the hormonal natriferic and hydroosmotic actions are entirely separable.

A preliminary report of these investigations has been presented elsewhere [10].

Materials and Methods

Female specimens of the toad *Bufo marinus* were obtained from the Dominican Republic (National Reagents, Inc., Bridgeport, Conn.) and maintained on moist wood

chips. Urinary hemibladders from doubly pithed toads were usually mounted in Lucite double-chambers of 2.5 cm^2 cross-sectional area, providing experimental and control samples from the same tissue. In several experiments, hemibladders were studied in similar double-chambers of 1.2 and 1.3 cm^2 cross-sectional area, respectively. The serosal surface of the tissues was supported by nylon mesh. The volume of the mucosal medium always exceeded that of the serosal medium to maintain a slight gradient of hydrostatic pressure across the preparation.

For reasons described below, it was of interest to study the skin of *Xenopus laevis*. Female specimens were generously provided by Dr. Don P. Wolf. Samples of ventral abdominal skin were excised from doubly pithed specimens and treated exactly as were the urinary hemibladders.

The sodium Ringer's solution consisted of (mM): Na^+, 117.3; K^+, 3.5; Ca^{++}, 0.9; Cl^-, 116.3; HCO_3^-, 2.4; HPO_4^{2-}, 1.8; $H_2PO_4^-$, 0.3; the pH was 7.6 and tonicity 236 mOsm/kg water. The choline Ringer's solution consisted of (mM): choline, 113.0; K^+, 3.6; Ca^{++}, 0.9; Cl^-, 114.8; HPO_4^{2-}, 1.7; $H_2PO_4^-$, 0.2; the pH was 7.7 and tonicity 224 mOsm/kg water. The calcium Ringer's solution consisted of (mM): Na^+, 99.7; K^+, 3.5; Ca^{++}, 10.0; Cl^-, 120.8; HCO_3^-, 2.4; and of similar pH and tonicity.

As previously described [17], the experimental protocol was to clamp the transepithelial potential at 0 mV except for 9-sec intervals every 30 sec when the transepithelial potential was increased to 10 or to 12 mV (serosa positive to mucosa), by means of chlorided silver electrodes in series with 3 M KCl agar bridges. Potentials were monitored by means of calomel electrodes in series with similar salt bridges. Transepithelial electrical current was continuously monitored and displayed on a dual pen recorder.

All electrical measurements were performed using the electrical circuit previously described in detail [17]. By introduction of a suitable feed-back loop to an otherwise standard circuit, it is possible to automatically correct for the resistance of the bathing-media and to control the difference in electrical potential across the tissue itself.

Data reduction was carried out by measuring R_o (the initial tissue resistance just after a steady-state was attained), R_i (the tissue resistance just prior to the final experimental manipulation), and R_e (the final tissue resistance just prior to fixing the preparations). For example, in protocol A, vasopressin (Pitressin; Parke, Davis & Co., Detroit, Mich.) was added to the serosal medium of the experimental quarter-bladder, following which excess solute was added to the mucosal medium of each quarter-bladder, and finally the tissue was fixed. In this case, R_o, R_i and R_e were the resistances before addition of vasopressin, before addition of mucosal solute, and before addition of fixative, respectively.

Protocol B consisted of the addition of vasopressin to the serosal media of both experimental and control hemibladders, followed by the addition of solute to the experimental mucosal medium, and final fixation of both tissues. Protocol C consisted of the initial addition of excess solute to the mucosal media of both experimental and control tissues, and the subsequent addition of vasopressin, with subsequent fixation of both tissues. Addition of solute was performed by addition of small volumes of Ringer's solution containing high concentrations of the test solute. Thus, addition of mucosal solute did not alter the concentrations of any of the other components of the bathing media. In several experiments, prostaglandin E_1 (PGE_1) was added to the serosal bathing medium; the PGE_1 was a generous gift of Dr. Geoffrey W. G. Sharp.

Data were analyzed by calculating the relative fractional change in conductance (RFC), defined as

$$\left(\frac{R_o}{R_e}-\frac{R_o}{R_i}\right)_{\text{Exp}}\Big/\left(\frac{R_o}{R_e}-\frac{R_o}{R_i}\right)_{\text{Control}}.$$

As previously demonstrated [17], this RFC is equal to the ratio of the increment in conductance (per amount of transporting tissue) for the experimental quarter-bladder to that for the control. An absolute value for the RFC of greater than, or less than, one indicates that the change in conductance on the experimental side was greater than, or less than, that on the control side, respectively. Positive values for RFC indicate that the experimental and control conductances changed in parallel, both either increasing or decreasing; negative values indicate that the changes in conductance on the two sides were opposite in sign. Under the condition of the present study, R_o was 1.83 ± 0.07 (mean $\pm$ SEM) $k\Omega\,cm^2$ for tissues bathed with isotonic sodium Ringer's solution.

Tissue fixation was performed by simultaneous addition of suitable volumes of 50% (w/v) glutaraldehyde (Fisher Scientific Co., Pittsburgh, Pa.) to mucosal and serosal solutions to provide a final concentration of 1%, and allowed to stand for 15 to 30 min before excision. Rectangles of tissue excised from the chambers were immersed in 1% glutaraldehyde in phosphate buffer. Tissue samples were post-fixed in osmium tetroxide and embedded in epoxy as previously described [18] but one-half of each sample was also stained *en bloc* with uranyl acetate [20]. Selections were cut with a Reichert OmU2 ultramicrotome (G. Reichert Werke, A. G., Vienna, Austria). Examination of the sections with a Philips EM-200 electron-microscope was initially performed by one of us without prior knowledge of the experimental protocol, to reduce possible bias in interpretation.

Results

Vasopressin and Mucosal Hypertonicity

In an initial series of experiments, one hemibladder from each of three toads was subjected to protocol A, while the paired hemibladder was in each case used for protocol B. Specifically the hemibladders in protocol A were initially bathed with isotonic Ringer's solution until a steady state was reached. At that point, vasopressin was added to the serosal medium of the experimental quarter-bladder to a final concentration of 114 mU/ml, while an identical volume of Ringer's solution was added to the serosal medium of the adjoining control quarter-bladder. Within 3 min after adding vasopressin, the short-circuit of the experimental tissue began to rise. At the peak of response ($7^1/_2$ to $9^1/_2$ min after hormonal administration), small volumes of concentrated solutions of urea were added to the mucosal media of both experimental and control quarter-bladders, raising the total mucosal osmolality to 452 to 489 mOsm/kg water (i.e. approximately to twice that of Ringer's solution). Within 30 sec after rendering the mucosal medium hypertonic, the conductance of the control tissue markedly increased (as previously described [17]), and continued to increase until fixation 4 to 7 min later. On the other hand, the conductance of the experimental side changed very little during the same period of time. The records from a representative preparation are presented in Fig. 1. Calculated as described in Materials and Methods, the relative fractional change in conductance (RFC) ranged from -0.0038 to $+0.038$; a negative value indi-

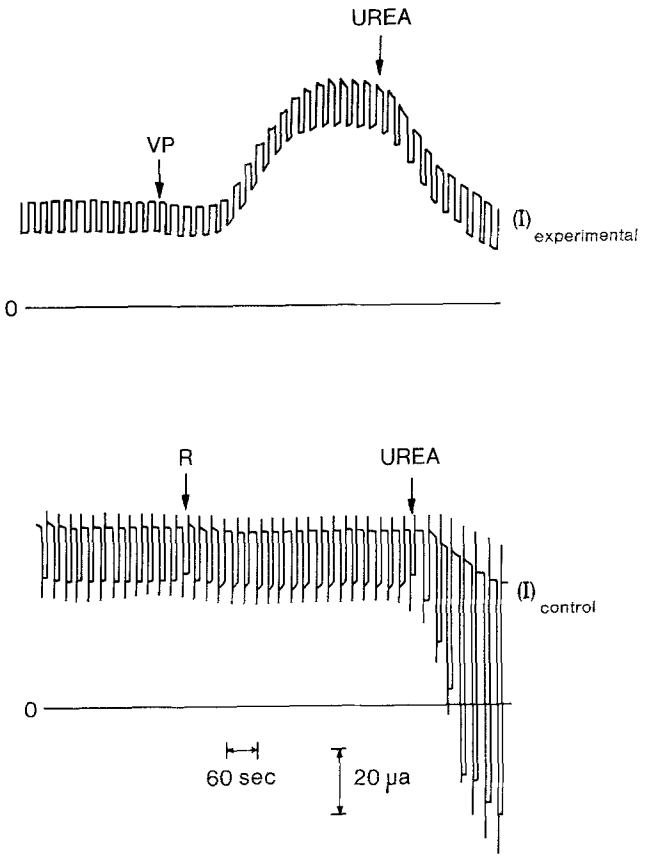

Fig. 1. Hormonal inhibition of the osmotically induced conductance change (Exp. 1A, Table 1). The upper curve was obtained from the experimental side, and the lower curve from the control side, of adjoining quarter-bladders; the cross-sectional area of each side was 2.5 cm^2. The ordinate is the current (I) necessary to reduce the transepithelial potential to zero (upper envelope of each curve) or to (+) 12 mV (lower envelope). The tissue conductance is thus given by the magnitude of the current deflection, divided by 12 mV. The tissues were bathed in isotonic Ringer's solution until a steady-state was reached. Then, at the vertical stroke labeled "VP" in the upper curve, vasopressin was added to the serosal medium of the experimental quarter-bladder to a final concentration of 114 mU/ml. At the vertical stroke marked "*R*" in the lower curve, an identical volume of Ringer's solution was added to the serosal medium bathing the control preparation. Within 3 min, the short-circuit current and conductance of the experimental quarter-bladder increased. At the peak of the hormonal response (i.e., at the vertical strokes labeled "Urea"), urea was added to the mucosal medium of each quarter-bladder to a final osmolality of 452 to 489 mOsm/kg water. The short-circuit current promptly fell on each side, and the conductance of the control tissue markedly increased. However, the conductance of the experimental tissue changed very little, if at all. The electrical resistances of the experimental tissue just before addition of vasopressin (R_o), just before addition of urea (R_i), and just before fixation (R_e) were 668, 566 and 548 Ω, respectively. On the control side, the corresponding values were 771, 811 and 180 Ω, respectively. Calculated as defined in the text, the relative fractional change in conductance (RFC) was (−) 0.0038

Table 1. Summary of relative fractional changes in conductance defined as

$$\left(\frac{R_o}{R_e}-\frac{R_o}{R_i}\right)_{\text{Exp}}\Big/\left(\frac{R_o}{R_e}-\frac{R_o}{R_i}\right)_{\text{Control}}$$

(*see* Materials and Methods) and of percentage blistering under a variety of experimental conditions

Exp.	Protocol	Solutions		Relative fractional change in conductance	% Blistering		
		Experimental	Control		Exp.	Con.	Exp.−Con.
1.	A	M: Hypertonic (urea) S: Isotonic, *Vasopressin*	M: Hypertonic (urea) S: Isotonic No Vasopressin	0.015±0.012 ($p<0.001$)	23±17.1	95±1.1	−72±15.9 (3) ($p<0.05$)
	B	M: *Hypertonic* (urea) S: Isotonic, Vasopressin	M: Isotonic S: Isotonic, Vasopressin	−0.44±1.19 ($p>0.3$)	32±28.3	0.0±0.00	32±28.3 (3) ($p>0.3$)
2.	A	M: Hypertonic (KCl) S: Isotonic, *Vasopressin*	M: Hypertonic (KCl) S: Isotonic, No Vasopressin	0.12±0.072 ($p<0.01$)	34±20.9	93.9±0.93	−59±20.2 (3) ($0.1>p>0.05$)
	B	M: *Hypertonic* (KCl) S: Isotonic, Vasopressin	M: Isotonic S: Isotonic, Vasopressin	−9.9±23.1 ($p>0.6$)	19±12.5	0.0±0.00	19±12.5 (3) ($p>0.2$)

3.	C	M: Hypertonic (KCl) S: Isotonic, *Vasopressin*	M: Hypertonic (KCl) S: Isotonic, No Vasopressin	-0.56 ± 0.39 ($0.1>p>0.05$)	73 ± 9.2	94.1 ± 0.63	-21 ± 9.4 (3) ($p>0.1$)
4.	A	M: Isotonic S: Hypotonic, *Vasopressin*	M: Isotonic S: Hypotonic, No Vasopressin	-1.21 ± 0.58 ($p<0.05$)	$2{,}4\pm0.82$	82 ± 5.8	-80 ± 6.1 (4) ($p<0.001$)
5.	A	M: Hypertonic (mannitol) S: Isotonic, Vasopressin Ca^{++}	M: Hypertonic (mannitol) S: Isotonic, Vasopressin, No Ca^{++} supplement	2.00 ± 0.96 ($p>0.4$)	0.90 ± 0.90	0.30 ± 0.30	0.60 ± 0.61 (3) ($p>0.4$)
6.	A	M: Hypertonic (urea) S: Isotonic, Vasopressin, PGE_1	M: Hypertonic (urea) S: Isotonic, Vasopressin, No PGE_1	-0.13 ± 1.52 ($p>0.5$)	57 ± 11.4	59 ± 17.5	2.2 ± 23.6 (3) ($p>0.9$)
7.	A(X.S.)	O: Hypertonic (KCl) I: Isotonic, *Vasopressin*	O: Hypertonic (KCl) I: Isotonic, No Vasopressin	1.06 ± 0.12 ($p>0.6$)	63 ± 3.5	62 ± 3.5	1.4 ± 4.1 (4) ($p>0.8$)

M and S refer to the mucosal and serosal bathing media, respectively; O and I refer to the outer and inner media, respectively, bathing *Xenopus* skin (X.S.) The differences between the experimental and control solutions are in italics. Numbers in parentheses represent the number of paired quarter-bladders studied during the course of each series of experiments. p represents the probability of the null hypothesis, and has been calculated on the basis of Student's t-test.

cates that the conductance of the experimental quarter-bladder declined, while that of the control rose. The mean ± SEM for the series was 0.015 ± 0.012, (Exp. 1A, Table 1) indicating that vasopressin almost completely abolished the effect of urea on conductance.

To examine whether vasopressin in fact completely inhibited the osmotically induced conductance change, each of the paired hemibladders from the same three toads was subjected to protocol B. Once again, the tissues were bathed with isotonic Ringer's solution until a steady state was reached. Then vasopressin was added to the serosal media of both experimental and control preparations, to a final concentration of 114 mU/ml. At the peak of the response in short-circuit current $8^1/_2$ to $9^1/_2$ min later, urea was added to the mucosal medium of the experimental tissue alone, raising the osmolality to 452 to 489 mOsm/kg water. A similar small volume of Ringer's solution was added to the mucosal medium of the control tissue. Over the succeeding 9 to 11 min until fixation, the conductance of the control tissue fell slightly, with the waning of the hormonal effect. In one experiment, the conductance of the experimental tissue fell even more (RFC = 1.919). In the remaining two experiments, the conductance of the experimental side increased appreciably (RFC = − 1.842 and −1.393) but not nearly so strikingly as in the control preparations. Because of the scatter of results, the mean ± SEM for the RFC was −0.44 ± 1.19, not statistically significant from +1 (Exp. 1B, Table 1). However, it seems clear from the data obtained in the latter two experiments that vasopressin did not completely abolish the conductance change induced by urea in all preparations, at least in the concentrations used.

Similar results were obtained using KCl to elevate the mucosal tonicity to 474 to 486 mOsm/kg water. At a serosal concentration of 114 mU/ml, vasopressin markedly reduced the magnitude of the conductance change produced by the reverse osmotic gradient (mean RFC ± SEM = 0.12 ± 0.072; Exp. 2A, Table 1).

As in the case of the experiments with urea, each of the paired hemibladders from the same three toads was subjected to protocol B. In each of the three experiments following treatment with vasopressin, administration of KCl to the mucosal medium to a final osmolality of 474 to 486 mOsm/kg water, increased the conductance of the experimental preparation within the 10 to 12 min before fixation. Therefore, although the mean ± SEM for the RFC was −9.9 ± 23.1, it seemed clear that prior addition of vasopressin markedly inhibited but did not entirely abolish the conductance change induced by mucosal KCl.

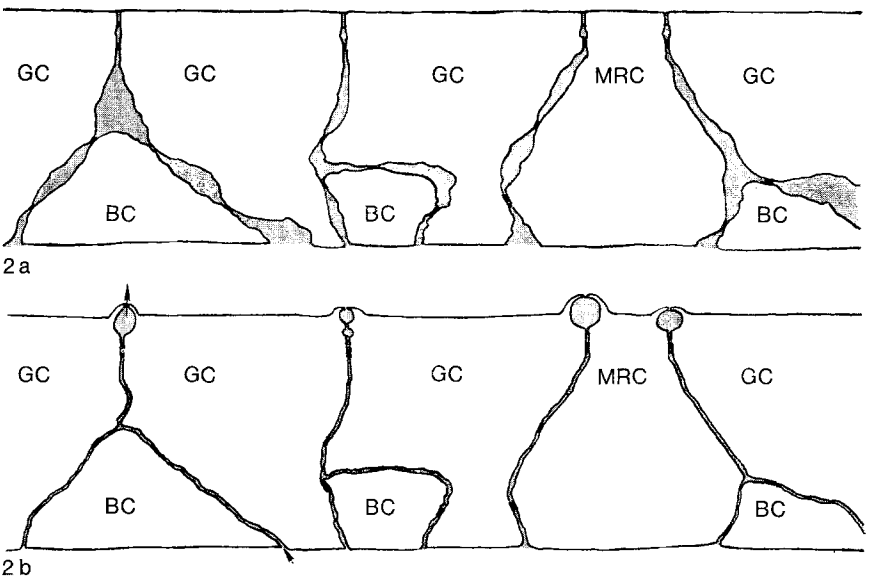

Fig. 2. Schematic representation of osmotically induced changes in bladder epithelium in the absence of vasopressin. (*a*) When both mucosal (upper) and serosal (lower) surfaces are bathed with isotonic Ringer's solution, the profiles of granular (GC), mitochondria-rich (MRC) and basal (BC) cells appear in thin sections as drawn. The spaces between cells are slightly patent (gray zones) and the limiting junctions at the luminal end of the spaces are the regions of closest apposition between adjoining cells. (*b*) After application of a transmural reverse osmotic gradient (by addition either of water to the serosa or of excess solute to the mucosa), the intercellular spaces are considerably narrowed, except at the level of the apical limiting junctions where spherical enlargements are found. Changes in cell volume are unrelated to this phenomenon. Serosal dilution will cause moderate swelling (as depicted), while mucosal hypertonicity is expected to result in cell shrinkage; in either case, similar changes are observed in the junctions and spaces, and are closely associated with the observed increase in passive transmural conductance. Water flow induced by reverse osmotic gradients is presumed to be largely intercellular (arrow) for these reasons. Further details are to be found in the previous paper [17] of this series

Previous studies have strongly suggested that application of reverse osmotic gradients across toad bladder alters the tissue conductance, specifically by altering the structure of the apical limiting junctions. The structural consequences of mucosal hypertonicity as described in the first paper of this series [17] are represented in Fig. 2, which summarizes those findings. It was, therefore, of considerable interest to determine whether the hormonal inhibition of the osmotically induced conductance change was accompanied by a corresponding reduction in the blistering of the apical junctions.

As previously noted [17], rendering the mucosal medium hypertonic in the absence of vasopressin produced bullous enlargements or blisters of the

intercellular space within the apical junctions; mucosal hypertonicity also reduced the size of the lateral intercellular spaces. However, prior addition of vasopressin, as in protocol A, markedly reduced the degree of blistering without increasing the size of these lateral spaces. In fact, the lateral intercellular spaces appeared even more closed in the tissues pretreated with hormone.

To quantify the degree of junctional blistering in all of the experimental samples, the sectioned tissues were examined at an instrumental magnification of 12,000 × ; observations were performed at 120,000 × after binocular magnification. Percentage blistering was calculated as the percentage of the usually 100 or more junctional profiles observed containing at least a single blister [17]. All junctions observed were included in the measurement without any data selection whatsoever. Results were finally expressed as the per cent blistering on the experimental side minus the per cent blistering on the control side (Table 1).

On this basis, prior addition of vasopressin was found to reduce the blistering induced by urea by $72 \pm 15.9\%$, a value statistically different from 0 at the 0.05 probability level (Exp. 1A, Table 1). Vasopressin also reduced the blistering effect of KCl by $59 \pm 20.2\%$, a value not quite significant at the 0.05 level (Exp. 2A, Table 1).

As anticipated from the electrical data, vasopressin also reduced the incidence of blistering noted under protocol B (Exps. 1B and 2B, Table 1). Because of the considerable scatter of the data, the difference in blistering between experimental and control sides was not statistically significant, both following urea and following KCl. However, some blisters were found in two of the three experiments in Exp. 1B, and in all three of the experiments in Exp. 2B, while no blisters can be observed under control conditions without a gradient in osmolality across toad bladder. The sole exception was the preparation of Exp. 1B, where vasopressin completely prevented the subsequent addition of mucosal urea from increasing the tissue conductance; in this case, vasopressin also completely prevented the appearance of blisters.

In addition to the above morphologic effects of vasopressin upon the course of events produced by the subsequent introduction of excess mucosal solute, two further morphologic changes were particularly striking. First, the cell size of specifically the granular cells was markedly reduced. In the absence of vasopressin, application of mucosal hypertonicity produces little shrinkage of the surface epithelial cells. However, in the presence of vasopressin, mucosal hypertonicity causes enormous shrinkage of the granular cells (Figs. 3, 4). This reduction in cell size may be appreciated from:

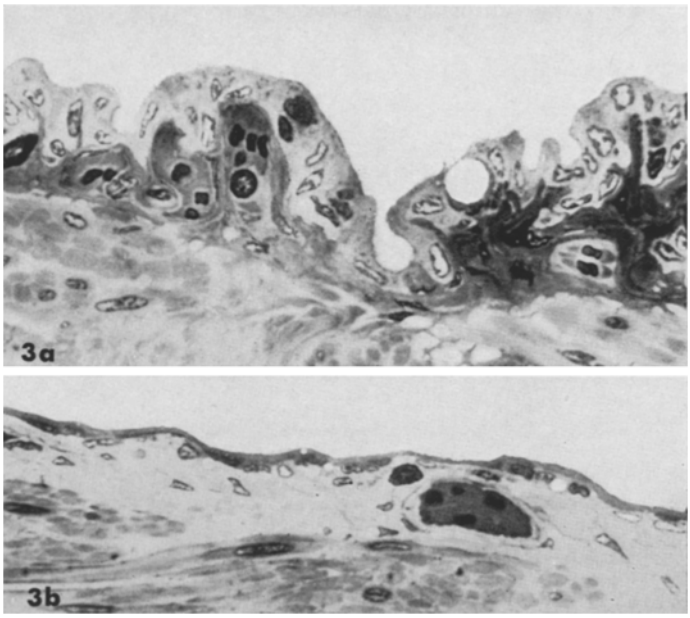

Fig. 3. Phase microscopic presentation of the results of sequential addition of excess mucosal solute and vasopressin. (*a*) The control quarter-bladder had been exposed to a mucosal solution whose tonicity was elevated to 452 to 489 mOsm/kg water by addition of urea; the serosal bath remained isotonic. Junctional blistering, evident by electron-microscopy, cannot be appreciated here, but it is apparent that the intercellular spaces are closed. (*b*) The experimental quarter-bladder was first exposed to mucosal hypertonicity, as in the control (*a*), but vasopressin was subsequently added to the serosal medium (protocol B). The thickness of the epithelium has been strikingly reduced, because of shrinkage of the surface epithelial cells, which now appear considerably more dense. Both micrographs 550 ×

(1) the reduction in cross-sectional area of the cellular profile on random section, (2) the increased density of the nuclear heterochromatin, (3) the more crenated profile of the nucleus, and (4) the increased electron-density of the cytoplasm in comparison to that of the subapical granules. Indeed, the electron-density of the surrounding cytoplasm may exceed that of the granules; this reversal of electron-density has been observed only under conditions of cell shrinkage [17]. As in the case of apical blistering, cell size was estimated without prior knowledge of the experimental protocol.

Although, on the basis of these multiple criteria, vasopressin together with mucosal hypertonicity clearly and markedly shrank the granular cells, no effect was noted on the cell size of the other epithelial cells. Of the more than 500 mitochondria-rich cells, 400 goblet cells, and 5,000 basal cells observed under these conditions, none were noticeably shrunken; in each case, the size of the contiguous granular cells was markedly reduced.

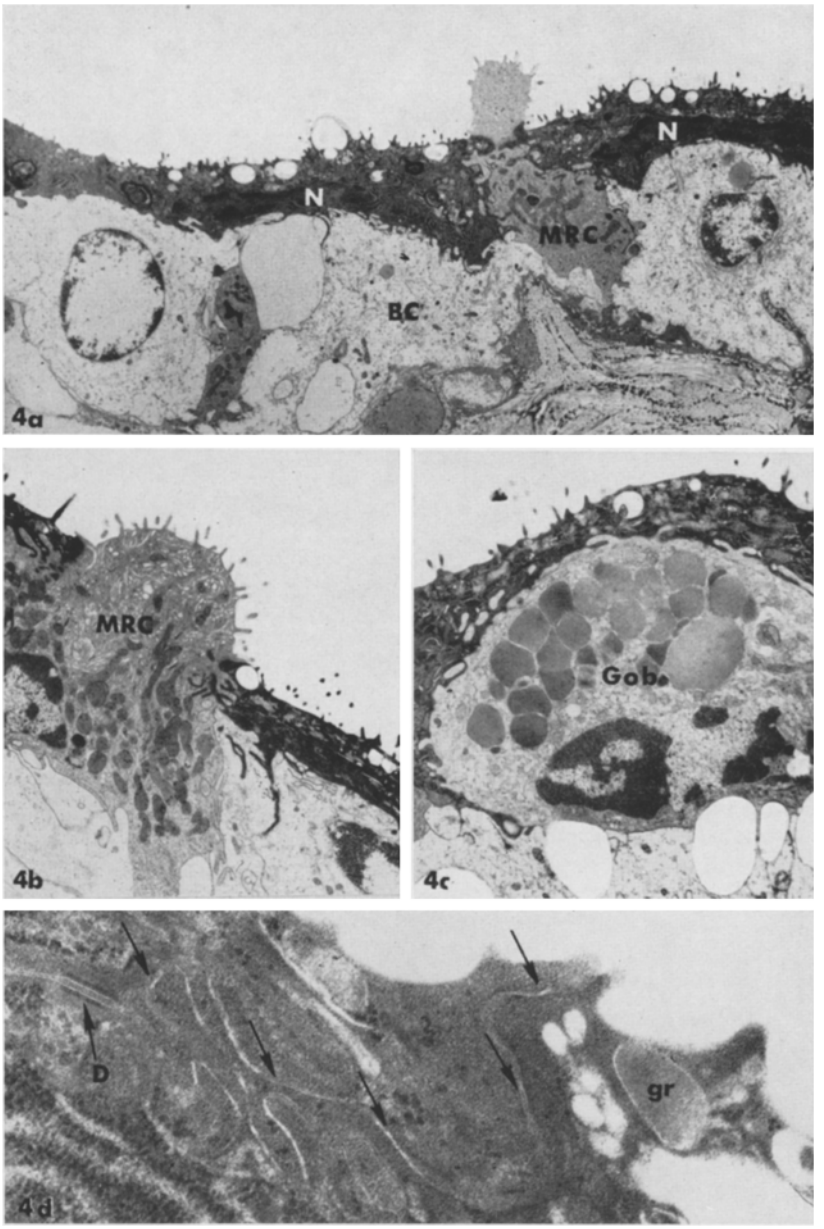

Fig. 4. Electron-microscopic presentation of the results of sequential addition of excess mucosal solute and vasopressin. (*a*) Tissue exposed first to mucosal hypertonicity (achieved by adding urea to a final tonicity of 452 to 489 mOsm/kg water), and subsequently to vasopressin (protocol B). Extremely shrunken, vacuolated granular cells contain flattened, dense nuclear profiles (*N*), and surround a mitochondria-rich cell (MRC) whose cytoplasmic density and mitochondria appear normal. Underlying basal cells (BC) are similarly unaffected. The mucosal aspect of the granular cells is studded with clear vacuoles, but no blistering of the limiting junctions is noted. 4,200 ×. (*b*) Quarter-bladder exposed

Finally, the sequence of hormonal treatment and subsequent addition of mucosal solute caused enormous vacuolation of all cell types of the bladder epithelium (Fig. 5). Although increased endocytosis and exocytosis have been reported to be initiated by neurohypophyseal hormones [41], that degree of pinocytosis is relatively modest. Furthermore, in previous studies of toad bladder with hypertonic solutions on mucosa and serosa, vacuolation was never prominent, and very rare with the solutes used in this study.

Thus, vasopressin administration before application of mucosal hypertonicity results in: (1) reduction of the osmotically induced conductance change, (2) reduction in the degree of blistering of the apical junctions, (3) shrinkage specifically of the granular cells, and (4) vacuolation of all cell types comprising the mucosal epithelium.

It should be noted that similar effects seemed to result from addition of vasopressin after, rather than before, application of mucosal hypertonicity. In each of three experiments, vasopressin was added to the serosal medium of the experimental tissue to a final concentration of 114 mU/ml, after KCl had been added to the mucosal media of both experimental and control tissues, raising the osmolality to 456 to 467 mOsm/kg water. Although the conductance of the control preparation continued to rise, vasopressin reduced the conductance and degree of blistering of the experimental tissue. However, because of the considerable data scatter, the averaged results were not statistically significant (Exp. 3, Table 1).

Vasopressin and Serosal Hypotonicity

Previous studies have established that the tissue conductance can be increased and apical blisters can be induced by serosal hypotonicity, as

to vasopressin and subsequently to mucosal hypertonicity, achieved by adding KCl to a final tonicity of 474 to 486 mOsm/kg water (protocol A). At higher power, it is evident that the mitochondria-rich cell (MRC) is relatively unaffected, in comparison to the dense adjacent granular cell profiles. The intercellular space between adjacent cells is not visible at any point along the cell margins, nor within the junctional regions. 6,000 ×. (*c*) Quarter-bladder treated as in (*a*), illustrating the fact that each of the goblet cells (Gob) examined under these conditions appears normal. 8,500 ×. (*d*) High power view of interface between two granular cells after treatment as in (*b*). Unlabeled arrows point to regions of the intercellular space, which is very tightly closed throughout its length. The greatest separation between adjacent cell membranes occurs where they are adjoined by the extracellular aspect of the desmosomes (D). In these preparations, the cytoplasmic electron density of the granular cells is increased beyond that of their characteristic granules (gr). 42,500 ×

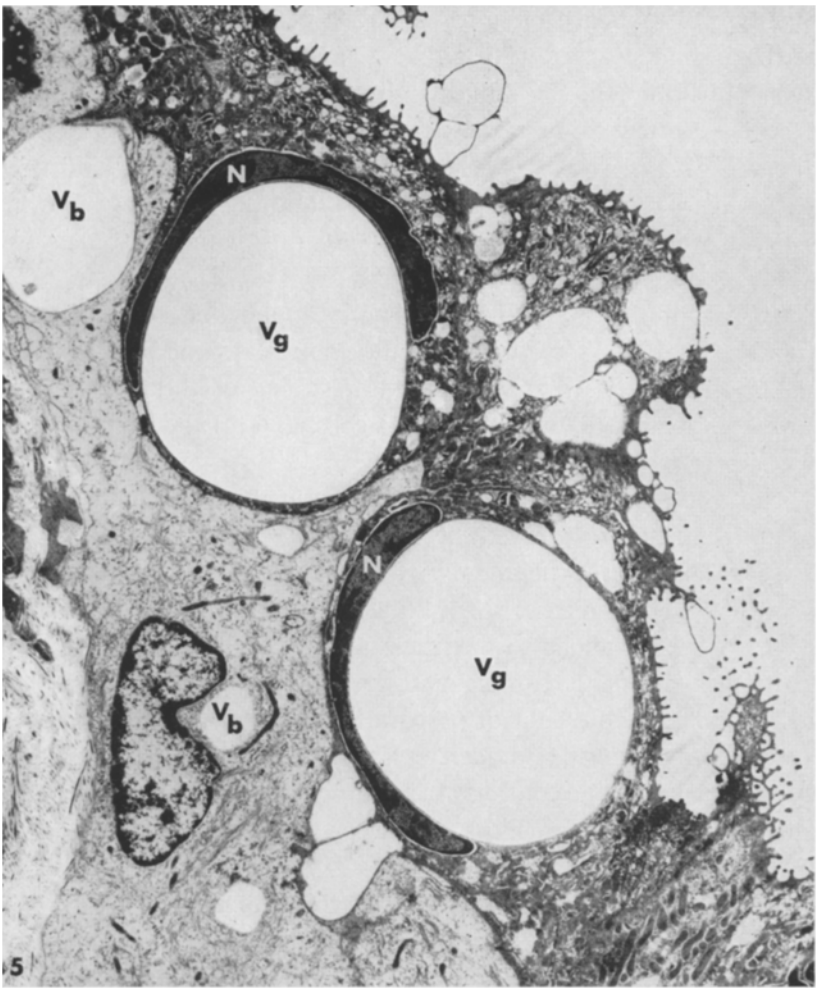

Fig. 5. Electron-micrograph of bladder epithelium fixed after exposure to mucosal hypertonicity (achieved by addition of KCl to 474 to 486 mOsm/kg water) and subsequently to vasopressin (protocol B). In this field, the degree of cytoplasmic vacuolation is particularly striking. Vacuoles are found in the granular cells (V_g) and in the basal cells (V_b), as well as in the other cell types (not shown here). It is apparent from the very dense appearance of the nuclear chromatin and from the flattened observed profiles (N) that the nuclear volume is reduced. 6,000 ×

well as by mucosal hypertonicity [17]. To examine the possible interaction between vasopressin and serosal hypotonicity, four hemibladders were bathed with isotonic Ringer's solution until a steady state was attained. Vasopressin was then added to the serosal medium of the experimental quarter-bladder to a final concentration of 100 to 114 mOsm/kg water, while an identical volume of Ringer's solution was added to that of the control.

At the peak of the vasopressin response 9 to $13^1/_2$ min later, deionized water was added to both serosal media, reducing the tonicity to 115 to 125 mOsm/kg water. The tissues were fixed 24 to 35 min later. In each case, the conductance of the control tissue increased, while that of the experimental tissue decreased. The mean $\pm$ SEM of the RFC for the four preparations was -1.21 ± 0.58, a value significantly different from $+1$ (Exp. 4, Table 1). At the same time, the degree of apical blistering was also reduced by $80 \pm 6.1\%$ ($p < 0.001$). In addition, the experimental quarter-bladders manifested the same selective shrinkage of the granular cells, and the generalized vacuolation of the entire mucosal epithelium described above. Thus, addition of vasopressin prior either to application of serosal hypotonicity or mucosal hypertonicity resulted in similar electrophysiologic and morphologic effects.

Dissociation of Hormonal Natriferic and Hydroosmotic Effects

In two experiments, vasopressin added to the experimental side of hemibladders bathed in choline Ringer's solution followed by mucosal addition of KCl to a final osmolality of 466 to 472 mOsm/kg water, resulted in the same electrophysiologic and morphologic changes noted in tissues bathed with sodium Ringer's solution. Therefore, the interaction between vasopressin and transepithelial osmotic gradients did not seem to require the presence of Na^+. On the other hand, on the basis of the reasoning presented in the Discussion, it seemed likely that the hydroosmotic effect of vasopressin was critical to the development of the characteristic hormonal effects detailed above. Therefore, three different approaches were followed in an effort to specifically eliminate the hydroosmotic effect of vasopressin.

First, under favorable conditions, elevated calcium concentrations have long been known to selectively reduce the hydroosmotic, but not the natriferic, response to vasopressin [1, 3, 4, 45]. Therefore, experimental quarter-bladders were bathed in calcium Ringer's solution containing 10 mM Ca^{++} for 45 to 49 min before addition of vasopressin to a final serosal concentration of 10 mU/ml on both experimental and control sides. At the peak of the vasopressin response 10 to $19^1/_2$ min later, mannitol was added to the mucosal media of experimental and control tissues to a final tonicity of 499 to 505 mOsm/kg water, and the tissues fixed $8^1/_2$ to 10 min later. As will be appreciated from Table 1 (Exp. 5), the presence of elevated levels of Ca^{++} seemed to affect neither the electrical nor the histologic changes characteristically induced by vasopressin plus mucosal hypertonicity.

Second, prostaglandin E_1 (PGE_1) has been reported to selectively enhance the natriferic, and inhibit the hydroosmotic, response of the toad bladder to vasopressin [34]. Therefore, PGE_1 was first added to the serosal medium

of the experimental tissue of three preparations to a final concentration of 3.6×10^{-5} to 3.9×10^{-4} M; an identical volume of solvent (ethanol) alone was added to the serosal solution of the control sides. Although Lipson and Sharp [34] had noted an increase in short-circuit current after addition of PGE_1, this effect was not noted in the present series of experiments. After $16^1/_2$ to 33 min, vasopressin was added to both serosal media to a final concentration of 10 to 29 mU/ml. After 11 to $31^1/_2$ min had elapsed, mannitol was added to both mucosal media to a final osmolality of 499 to 505 mOsm/kg water. The tissues were subsequently fixed $10^1/_2$ to 16 min later. As indicated in Table 1 (Exp. 6), PGE_1 appeared to have no measurable effect either upon the osmotically induced change in conductance or upon the degree of junctional blistering.

Although both the calcium Ringer's solution and PGE_1 have been reported to inhibit the hydroosmotic response of toad bladder to vasopressin, these inhibitions are known to be relative, and not absolute. (Increasing the concentration of Ca^{++} or PGE_1, and lowering the concentration of vasopressin enhance the magnitude of the inhibition.) On the other hand, it was necessary to employ a reasonably high level of vasopressin in order to produce a clearly demonstrable effect upon the osmotically induced conductance change and blistering. To strike a balance between these two opposing considerations, it was impracticable to operate under conditions where the hydroosmotic response was entirely abolished. Therefore, the results might have indicated either that: (1) the hydroosmotic response was not critical to the effects induced by vasopressin and mucosal hypertonicity, or (2) the hydroosmotic response had been insufficiently suppressed to provide an adequate test of the hypothesis.

To distinguish between these possibilities, a third approach was taken in an effort to eliminate the hydroosmotic response of vasopressin entirely. The amphibian *Xenopus laevis* is an aquatic toad, so that this organism's skin would not likely exhibit a hydroosmotic response to vasopressin. In fact, Maetz [39] and Bentley [5] have demonstrated that neurohypophyseal hormones exert a marked natriferic, with little or no hydroosmotic, effect on *Xenopus* skin. Therefore, the ventral abdominal skin was obtained from four female specimens of *Xenopus laevis*, and treated exactly as toad bladder in Exp. 2A (Table 1). Skins were initially bathed in Ringer's solution until a steady state had been attained. Vasopressin was then added to the inner solution bathing the experimental side of each skin to a concentration of 100 mU/ml; this dose appeared to be supramaximal since the subsequent administration of vasopressin to a final concentration of 200 to 300 mU/ml had no additional effect. Within 2 to 5 min, the short-circuit current and

conductance began to increase, and continued to increase with time until KCl was added 58 to 72 min later to the outer solutions bathing both experimental and control halves; the final osmolality of the outer bathing media was 413 to 420 mOsm/kg water. (The natriferic response to vasopressin is clearly far more sustained in *Xenopus* skin than in toad bladder; in an additional unfixed tissue, the short-circuit current and conductance continued to rise with time for at least 94 min.) Finally, the tissues were fixed $5^1/_2$ to 10 min after rendering the outer solutions hypertonic.

As in toad bladder, addition of excess mucosal solute dramatically increased tissue conductance; over the period of time observed, the conductance of the control tissue increased by 88 to 108%. Because of the sustained natriferic response of the experimental side to hormone, it was necessary to slightly modify the technique of data reduction. The initial tissue resistance (R_o) remained, as before, the resistance just prior to the time of vasopressin administration. Similarly, the final tissue resistance (R_e) was still defined as the resistance following addition of mucosal solute and just prior to addition of fixative. However, the resistance (R_i) was now defined as the resistance which *would* have been measured just prior to fixing the tissue, had no excess solute been added to the outer medium. Because the slow sustained response of conductance following vasopressin was linear with time over the 25 to 36 min before adding KCl, it proved simple to graphically extrapolate the value for (R_i) in each case; a similar extrapolation was performed to estimate the value of (R_i) for the control side, as well. As in all protocols of this study, the relative fractional change in conductance (RFC) was defined as

$$\left(\frac{R_o}{R_e}-\frac{R_o}{R_i}\right)_{\text{Exp}}\Big/\left(\frac{R_o}{R_e}-\frac{R_o}{R_i}\right)_{\text{Control}}.$$

The mean $\pm$ SEM for the RFC was 1.06 ± 0.12, a value clearly indistinguishable from $+1$ (Exp. 7, Table 1). Morphologic examination of the *Xenopus* skins indicated the presence of junctional blistering similar to that observed in toad bladder; these blisters were observed, however, exclusively in the limiting junctions between the cells of the outermost living cell layer. Blistering was noted in $63 \pm 3.5\%$ of the limiting junction profiles from the experimental tissue, and in $62 \pm 3.5\%$ of those from the control. This difference in degree of blistering was clearly indistinguishable from zero (*see* Fig. 6).

Examination of the junctions from three additional skins bathed with isotonic Ringer's solution on both surfaces indicated that no blisters are present under control baseline conditions. Therefore, in the one experi-

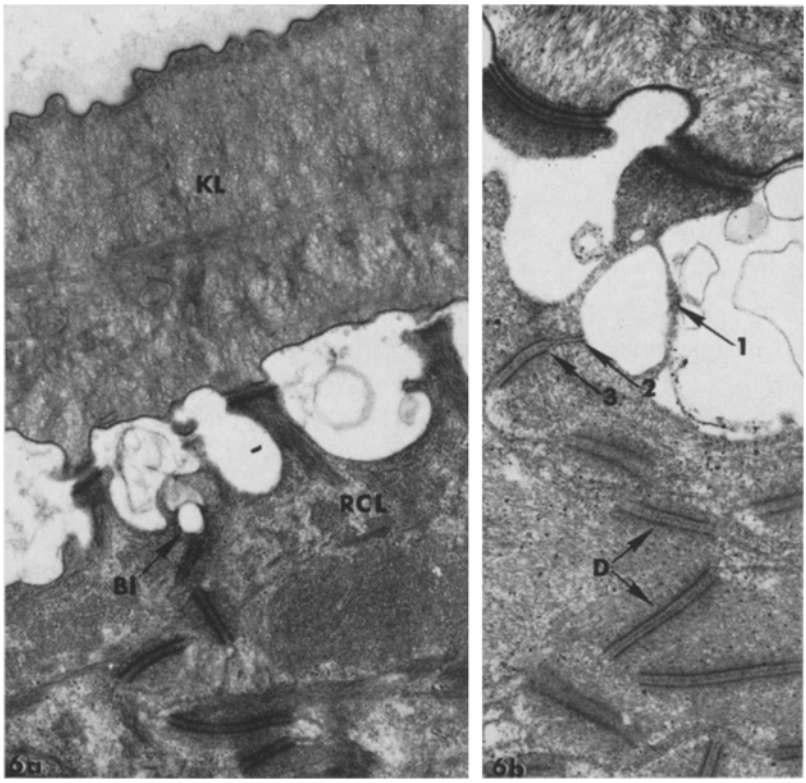

Fig. 6. Electron-micrographs of the outermost region of the abdominal skin of *Xenopus laevis* following exposure to hypertonicity of the outer bathing medium, achieved by addition of KCl to 413 to 420 mOsm/kg water. (*a*) Control tissue in the absence of vasopressin. The keratinized layer (KL) was unaffected by the procedure, while the limiting junctions of the first reactive cell layer (RCL) were blistered (Bl) as in toad bladder. 20,000 ×. (*b*) Experimental tissue treated as in (*a*), but following addition of vasopressin (protocol A). Vasopressin altered neither the change in electrical conductance, nor the appearance of blistering induced by reverse osmotic gradients. Arrows (1) and (2) delineate the blistered region, while arrows (2) and (3) indicate the nonblistered region of a junctional profile. The RCL is characterized by many desmosomes (D) as in the skin of other amphibia. 36,000 ×

mental situation where the hydroosmotic response of vasopressin was eliminated, the hormone altered neither the electrophysiologic nor the morphologic effects of mucosal hypertonicity.

Discussion

Application of reverse osmotic gradients has been thought to increase the electrical conductance of toad bladder by altering the state of the apical

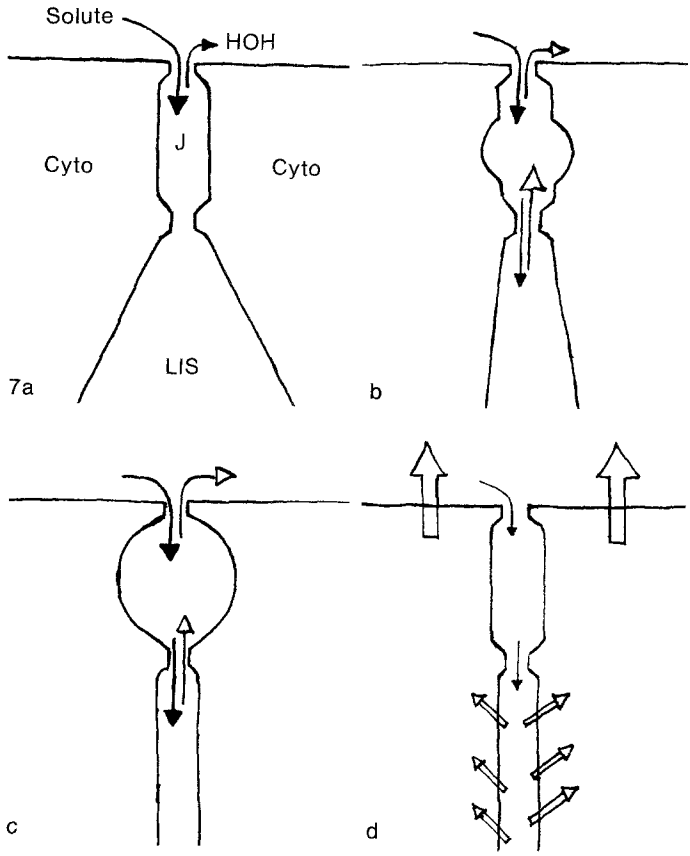

Fig. 7. Schematic presentation of the presumed mode of interaction between vasopressin and reverse osmotic gradients. The open arrows indicate net movements of water, while the closed arrows indicate net movements of solute. The mucosal medium is located at the top of each panel. (*a*) In the absence of vasopressin, addition of excess solute to the mucosal medium establishes a gradient favoring net movement of solute into the extracellular space (J) within the limiting junctions. The osmotic gradient favors movement of water from J into the mucosal medium. The apical plasma membranes of the epithelial cells are considered relatively impermeable to water and to the solute applied. (*b*)–(*c*) As solute continues to diffuse into J from the mucosal medium, some solute will diffuse through the junction into the lateral intercellular space (LIS). A standing gradient will be established, with the osmotic pressure of the fluid within J intermediate between those of the mucosal medium and the LIS. This gradient in osmotic pressure will induce continued movement of water from the LIS and/or the adjacent cytoplasm (Cyto); as long as the rate of entry of water into the junction exceeds its rate of exit into the mucosal medium, the junctional profile will be distended. With increase in volume of J, the resistance to transepithelial passage of solutes and water will diminish. (*d*) If, however, vasopressin is present, mucosal hypertonicity will produce a marked net movement of water across the apical plasma membranes. The tonicity of the intracellular fluids will increase, inducing net water movement from the LIS into the cytoplasm across the basal-lateral plasma membranes. In this case, the difference in tonicity between J and the intracellular fluids will be reduced, or even reversed in sign, so that no enlargement of the limiting junctions will be observed. Thus, vasopressin would be expected to inhibit the osmotically-induced changes in conductance and in junctional profile, even in the absence of a primary hormonal effect upon the limiting junctions

limiting junctions, permitting increased transepithelial fluxes of ions through the intercellular pathways. Vasopressin has been considered to increase tissue conductance by primarily increasing the transepithelial flux of sodium through the transporting cells. However, the results obtained indicate a pronounced interaction between these experimental manipulations, rather than the simply additive effect on tissue conductance which might have been anticipated. This observation raised the question whether or not current concepts concerning the site of action of these two experimental manipulations are well founded.

The first paper in this series [17] seemed to have conclusively demonstrated that reverse osmotic gradients increase conductance specifically by causing bullous deformation or blistering of the limiting intercellular junctions. In that report, the correlation between the two effects was established by: (1) a comparison of the magnitude of each effect through a range of solutes, (2) the strikingly similar time course of the development and reversibility of each effect, (3) the persistence of each effect in the absence of active sodium transport, and (4) the parallel establishment of the two effects when a comparable osmotic gradient was established either by dilution of the serosal medium or by application of mucosal hypertonicity, dissociating the phenomena from possible osmotic effects on the cells themselves.

Recently, Bindslev *et al.* [6] have presented two data which they believe dissociate the conductance changes from junctional blistering induced by reverse osmotic gradients. Application of 500 mM sucrose to the serosal surface or passage of large polarizing currents induced conductance changes unrelated to junctional blistering; the latter maneuver was thought to produce minimal areas of focal blistering. However, it is scarcely to be expected that all conductance changes induced in toad bladder are mediated by the same mechanism; their tracer flux data confirm that all three modes of conductance enhancement invoke nonspecific pathways but do not warrant the conclusion that only a single pathway is involved. For example, introduction of sucrose to a slightly lower serosal concentration (400 mM) has been observed to produce very different results [13]. In this respect, change in cell volume is a factor of potential importance since shrinkage increases selective ionic permeability across the plasma membranes of other preparations [33, 44]. The bases, whatever they may be, for the conductance changes noted in their two different experimental manipulations, seem to be distinct from the mechanism by which reverse osmotic gradients increase tissue conductance, i.e. through junctional blistering.

Although some evidence has been adduced suggesting that vasopressin too may actually facilitate intercellular transport across epithelia [24, 25], the great bulk of the literature suggests that vasopressin facilitates movement of Na^+, water, and a restricted group of other molecules, including urea, through transcellular pathways. Two lines of evidence provide particularly strong support for this concept. First, it now seems incontrovertible that the transport effects of vasopressin are mediated by the generation of 3′,5′-cyclic adenosine monophosphate (cyclic AMP) from intracellular ATP [2, 27, 29, 42, 43]. Second, the transepithelial hydroosmotic flow following vasopressin appears to proceed through transporting epithelial cells since: (a) only one (the granular cells) of the four cell types of the mucosal epithelium undergoes cell swelling following addition of hormone to tissues bathed with hypotonic mucosal media [19], and (b) the maximal possible flow rate between the epithelial cells has been calculated by one investigator to be some 30 times smaller than that actually observed [9]. Indeed, one set of experiments performed here provides striking evidence that this is so. In the experiments where vasopressin was added subsequent to elevation of mucosal tonicity with KCl, the hormone produced (a) a fall in conductance, (b) a reduction in junctional blistering, and (c) a shrinkage of the granular cells. It is exceedingly difficult to logically explain these three phenomena if the hydroosmotic action of vasopressin is other than on the granular cell luminal-facing plasma membrane.

Thus, it seems clear that mucosal hypertonicity does increase the conductance of the intercellular pathway, and vasopressin does increase that of the transcellular pathway across toad bladder. It might have been anticipated, therefore, that simultaneous application of both conditions would have elicited an additive increase in tissue conductance. Instead, prior administration of vasopressin markedly inhibited the increase in conductance characteristically elicited by adding urea or KCl to the mucosal media. Vasopressin also reduced the magnitude of the subsequent increase in conductance caused by application of serosal hypotonicity. Electronmicrographs of the same tissues indicated that vasopressin not only inhibited the osmotically induced conductance changes, but also reduced the degree of blistering of the apical junctions.

Since the hormone produced similar inhibitions in two preparations bathed with choline Ringer's solution, it seemed likely that the hydroosmotic, rather than the natriferic, effect of vasopressin was of importance in eliciting these phenomena. Therefore, attempts were made to eliminate the hormonal hydroosmotic effect in three different ways. First, the calcium concentration of the bathing media was increased. Second, in other experi-

ments, prostaglandin E_1 (PGE_1) was added to the serosal media. Both maneuvers are thought to partially, but not completely, inhibit the hydroosmotic response to vasopressin; neither approach measurably affected the interaction between vasopressin and mucosal hypertonicity. The third approach, to utilize the skin of *Xenopus laevis*, proved more successful.

The skin of *Xenopus laevis* responds to vasopressin, oxytocin, and vasotocin with a natriferic, but with little or no hydroosmotic, effect [5, 39]. Administration of vasopressin to *Xenopus* skin in the present study produced a sustained progressive increase in short-circuit current and electrical conductance. However, the later addition of mucosal solute increased the conductance further; the magnitude of this increase was independent of the presence of hormone. Subsequent electron-micrographs of the same tissues indicated that the degree of apical blistering induced by mucosal hypertonicity was also independent of the presence of hormone. Thus, in the one circumstance where the hydroosmotic response to vasopressin could be entirely eliminated, with preservation of the natriferic response, the action of hormone upon the electrical and morphological effects of mucosal hypertonicity was also eliminated. The absence of any interaction here also supports the presumption that the natriferic effect of the hormone is through a cellular rather than a junctional site of action.

The simplest and most direct interpretation (Fig. 7) of the data appears then to be the following. Introduction of excess solute into the mucosal bathing medium establishes a concentration gradient favoring entry of solute both into the cell cytoplasm and into the extracellular fluid within the apical intercellular junctions. If the plasma membrane is relatively impermeable to the solute applied, the gradual diffusion of solute into the apical junctions establishes an osmotic gradient favoring water entry into the junctions from the more basal intercellular spaces and from the cytoplasm of the adjoining cells. In the presence of vasopressin, however, the apical plasma membranes will be far more permeable to water. In this case, mucosal hypertonicity will induce not only an accumulation of solute in the apical junctions, but will also promote a net water flow from the cell interior into the mucosal medium. Under these circumstances, the tonicity of the intracellular fluid in the granular cells increases, reducing or even reversing the osmotic gradient between cytoplasm and the adjoining junction compartment; this interpretation is supported by the increased electron density of the granular cell cytoplasm. The driving force for water uptake by the junction from the adjacent cytoplasm is then reduced. Possible water uptake from the subjacent intercellular space into the function is also reduced by the strict closure of the space. Thus, the apical junctions will be

only minimally blistered, and little conductance change will be observed.

On the other hand, an osmotic gradient must exist between the basal portion of the intercellular spaces and between both the apical junctions and the cell cytoplasm, irrespective of the presence or absence of vasopressin. The resulting movement of interstitial fluid should reduce the size of these intercellular spaces whether or not vasopressin has been administered. Thus, the results obtained in the present study may be explained by, and provide further support for, current concepts of the mode and site of action of both mucosal hypertonicity and vasopressin. The very strict closure of the lateral intercellular spaces produced by vasopressin and mucosal hypertonicity may indeed contribute to the resistance of the intercellular pathway.

Of considerable interest was the observation that, in the presence of mucosal hypertonicity, vasopressin produces a reduction in the cell volume of the granular cells alone. The volumes of the basal cells, mitochondria-rich cells and goblet cells were not measurably affected. Prior control studies have established that serosal hypotonicity can produce swelling of all the cell populations of the mucosal epithelium [19] and that serosal hypertonicity will shrink all of these cellular elements [13, 17].

This observation complements the earlier finding that, in the presence of mucosal hypotonicity, vasopressin produces swelling of the granular cells alone [19]. The datum also constitutes the first direct evidence that when vasopressin enhances osmotic water flow from serosa-to-mucosa the water moves through rather than between the epithelial cells. Thus, the enhancement of hydroosmotic flow in either direction by the hormone is due to a direct or indirect, but highly specific, effect upon the granular cells. Unfortunately, such direct evidence for the localization of vasopressin's natriferic action is lacking. Increases in active Na^+ transport (or for that matter, urea permeability) might well be mediated through other cells; the hormone is even effective on extraepithelial elements within the same preparation [14–16]. It is also possible that the necessary intermediate to vasopressin action, cAMP, is generated in the mitochondria-rich cells, specifically [46] and distributed in a precise way throughout the epithelial cells through intercellular low resistance pathways [36].

Despite the cell selectivity of the hormonal hydroosmotic effect, vasopressin seemed to induce a diffuse and prominent vacuolation when solute was subsequently added to the mucosal medium. This effect is observed neither when vasopressin is added to tissue bathed with isotonic media, nor when tissues are bathed with hypertonic mucosal media in the absence

of vasopressin. The precise basis for this phenomenon remains unclear. Speculation as to the significance of this vacuolation seems unwarranted as there is virtually no basic information about the movement, redistribution or compartmentalization of fluids within epithelial cells that are subjected to osmotic stresses, or of a potential hormonal regulation of these processes.

In summary, the data obtained from studying the interaction of vasopressin and reverse osmotic gradients have suggested the following major conclusions with regard to the site and mode of action of the hormone: (1) Vasopressin inhibits the osmotically induced blistering of the limiting junctions and the related changes in transmural conductance; (2) The basis for these inhibitory effects may be understood to result specifically from the hormone's hydroosmotic activity; its natriferic activity does not interfere with osmotically induced changes in junction conductance or structure; (3) Vasopressin increases hydroosmotic flow from serosa-to-mucosa or mucosa-to-serosa through the same transcellular pathway, the granular cells of the mucosal epithelium; (4) The evidence now seems conclusive that the basis for hormonal enhancement of osmotic water flow is an increase in water permeability of the luminal-facing membrane of the granular cell; and (5) Despite the strong interaction between vasopressin and the osmotically induced changes in the limiting junctions, there is no apparent reason to assign any junctional site of action to the hormone.

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References

1. Argy, W. P., Jr., Handler, J. S., Orloff, J. 1967. Ca^{++} and Mg^{++} effects on toad bladder response to cyclic AMP, theophylline, and ADH analogues. *Amer. J. Physiol.* **213**:803
2. Bär, H.-P., Hechter, O., Schwartz, I. L., Walter, R. 1970. Neurohypophyseal hormone-sensitive adenyl cyclase of toad urinary bladder. *Proc. Nat. Acad. Sci.* **67**:7
3. Bentley, P. J. 1959. The effects of ionic changes on water transport across the isolated urinary bladder of the toad *Bufo marinus*. *J. Endocrinol.* **18**:327
4. Bentley, P. J. 1960. The effects of vasopressin on the short circuit current across the wall of the isolated bladder of the toad *Bufo marinus*. *J. Endocrinol.* **21**:161
5. Bentley, P. J. 1969. Neurohypophyseal hormones in amphibia: A comparison of their actions and storage. *Gen. Comp. Endocrinol.* **13**:39
6. Bindslev, N., Tormey, J. McD., Pietras, R. J., Wright, E. M. 1974. Electrically and osmotically induced changes in permeability and structure of toad urinary bladder. *Biochim. Biophys. Acta* **332**:286

7. Boulpaep, E. L. 1967. Ion permeability of the peritubular and luminal membrane of the renal tubular cell. *In*: Symposium über Transport und Funktion Intracellulärer Elektrolyte. F. Kruck, editor. pp. 98–107. Urban und Schwarzenberg, Munich
8. Civan, M. M. 1970. Effects of active sodium transport on current-voltage relationships of toad bladders. *Amer. J. Physiol.* **219**:234
9. Civan, M. M. 1970. Path of bulk water movement through the urinary bladder of the toad. *J. Theoret. Biol.* **27**:387
10. Civan, M. M., DiBona, D. R. 1973. Interaction of trans- and intercellular pathways across toad bladder. *13th Annu. Meet. Amer Soc. Cell Biol., Abstr.* 110, p55a
11. Civan, M. M., Frazier, H. S. 1968. The site of the stimulating action of vasopressin on sodium transport in toad bladder. *J. Gen. Physiol.* **51**:589
12. Civan, M. M., Kedem, O., Leaf, A. 1966. Effect of vasopressin on toad bladder under conditions of zero net sodium transport. *Amer. J. Physiol.* **211**:569
13. DiBona, D. R. 1972. Passive pathways in amphibian epithelia: Morphologic evidence for an intercellular route. *Nature, New Biol.* **238**:179
14. DiBona, D. R., Civan, M. M. 1969. Toad urinary bladder: Intercellular spaces. *Science* **165**:503
15. DiBona, D. R., Civan, M. M. 1970. The effect of smooth muscle on the intercellular spaces in toad urinary bladder. *J. Cell. Biol.* **46**:235
16. DiBona, D. R., Civan, M. M. 1972. Clarification of the intercellular space phenomenon in toad urinary bladder. *J. Membrane Biol.* **7**:267
17. DiBona, D. R., Civan, M. M. 1973. Pathways for movement of ions and water across toad urinary bladder. I. Anatomic site of transepithelial shunt pathways. *J. Membrane Biol.* **12**:101
18. DiBona, D. R., Civan, M. M., Leaf, A. 1969. The anatomic site of the transepithelial permeability barriers of toad bladder. *J. Cell. Biol.* **40**:1
19. DiBona, D. R., Civan, M. M., Leaf, A. 1969 The cellular specificity of the effect of vasopressin on toad urinary bladder. *J. Membrane Biol.* **1**:79
20. Farquhar, M., Palade, G. E. 1963. Junctional complexes in various epithelia. *J. Cell Biol.* **17**:375
21. Finn, A. L. 1971. The kinetics of sodium transport in the toad bladder. II. Dual effects of vasopressin. *J. Gen. Physiol.* **57**:349
22. Frizzell, R. A., Schultz, S. G. 1972. Ionic conductances of extracellular shunt pathway in rabbit ileum. Influence of shunt on transmural sodium transport and electrical potential differences. *J. Gen. Physiol.* **59**:218
23. Frömter, E., Diamond, J. 1972. Route of passive ion permeation in epithelia. *Nature* **235**:9
24. Ginetzinsky, A. G. 1958. Role of hyaluronidase in the reabsorption of water in the renal tubules: The mechanism of action of the antidiuretic hormone. *Nature* **182**:1218
25. Ginetzinsky, A. G., Krestinskaya, T. V., Natochin, Ju. V., Sax, M. G., Titova, L. K. 1960. Evolution of the substrate acted upon by antidiuretic hormone. *Physiol. Bohem.* **9**:166
26. Glynn, I. M., Hoffman, J. F., Lew, V. L. 1971. Some 'partial reactions' of the sodium pump. *Phil. Trans.* **262**:91
27. Handler, J. S., Butcher, R. W., Sutherland, E. W., Orloff, J. 1965. The effect of vasopressin and of theophylline on the concentration of adenosine 3′,5′-phosphate in the urinary bladder of the toad. *J. Biol. Chem.* **240**:4524
28. Handler, J. S., Preston, A. S., Orloff, J. 1972. Effects of ADH, aldosterone, ouabain, and amiloride on toad bladder epithelial cells. *Amer. J. Physiol.* **222**:1071
29. Hynie, S., Sharp, G. W. G. 1971. Adenyl cyclase in the toad bladder. *Biochim. Biophys. Acta* **230**:40

30. Janáček, K., Morel, F., Bourguet, J. 1968. Étude expérimentale des potentiels électriques et des activités ioniques dans les cellules épithéliales de la vessie de Grenouille. *J. Physiol., Paris* **60**:51
31. Janáček, K., Rybová, R. 1967. Stimulation of the sodium pump in frog bladder by oxytocin. *Nature* **215**:992
32. Janáček, K., Rybová, R. 1970. Nonpolarized frog bladder preparation: The effects of oxytocin. *Pflüg. Arch. Ges. Physiol.* **318**:294
33. Kregenow, F. M. 1971. The response of duck erythrocytes to hypertonic media: Further evidence for a volume-controlling mechanism. *J. Gen. Physiol.* **58**:396
34. Lipson, L. C., Sharp, G. W. G. 1971. Effect of prostaglandin E_1 on sodium transport and osmotic water flow in the toad bladder. *Amer. J. Physiol.* **220**:1046
35. Lipton, P., Edelman, I. S. 1971. Effects of aldosterone and vasopressin on electrolytes of toad bladder epithelial cells. *Amer. J. Physiol.* **221**:733
36. Loewenstein, W. R., Socolar, S. J., Higashino, S., Kanno, Y., Davidson, N. 1965. Intercellular communication: Renal, urinary bladder, sensory, and salivary gland cells. *Science* **149**:295
37. Macknight, A. D. C., Leaf, A., Civan, M. M. 1970. Vasopressin: Evidence for the cellular site of the induced permeability change. *Biochim. Biophys. Acta* **222**:560
38. Macknight, A. D. C., Leaf, A., Civan, M. M. 1971. Effects of vasopressin on the water and ionic composition of toad bladder epithelial cells. *J. Membrane Biol.* **6**:127
39. Maetz, J. 1963. Physiological aspects of neurohypophyseal function in fishes with some reference to the Amphibia. *Symp. Zool. Soc. London* **9**:107
40. Mandel, L., Curran, P. F. 1972. Response of the frog skin to steady-state voltage clamping. I. The shunt pathway. *J. Gen. Physiol.* **59**:403
41. Masur, S. K., Holtzman, E., Schwartz, I. L., Walter, R. 1971. Correlation between pinocytosis and hydroosmosis induced by neurohypophyseal hormones and mediated by adenosine 3′,5′-cyclic monophosphate. *J. Cell Biol.* **49**:582
42. Orloff, J., Handler, J. S. 1962. The similarity of effects of vasopressin, adenosine-3′,5′-phosphate (cyclic AMP) and theophylline on the toad bladder. *J. Clin. Invest.* **41**:702
43. Orloff, J., Handler, J. S. 1967. The role of adenosine 3′,5′-phosphate in the action of antidiuretic hormone. *Amer. J. Med.* **42**:757
44. Parker, J. C., Hoffman, J. F. 1965. Independence of cation permeability, cell volume, and metabolism in dog red cells. *Fed. Proc.* **24**:589
45. Petersen, M. J., Edelman, I. S. 1964. Calcium inhibition of the action of vasopressin on the urinary bladder of the toad. *J. Clin. Invest.* **43**:583
46. Scott, W. N., Sapirstein, V. S., Yoder, M. J. 1973. Localization of transport-related enzymes in the toad bladder epithelium. *6th Annu. Meet., Amer. Soc. Nephrol.* p. 93
47. Urakabe, S., Handler, J. S., Orloff, J. 1970. Effect of hypertonicity on permeability properties of the toad bladder. *Amer. J. Physiol.* **218**:1179
48. Ussing, H. H., Windhager, E. E. 1964. Nature of shunt path and active sodium transport path through frog skin epithelium. *Acta Physiol. Scand.* **61**:484
49. Wade, J. B., Revel, J. P., DiScala, V. A. 1973. Effect of osmotic gradients on intercellular junctions of the toad bladder. *Amer. J. Physiol.* **224**:407
50. Yonath, J., Civan, M. M. 1971. Determination of the driving force of the Na^+ pump in toad bladder by means of vasopressin. *J. Membrane Biol.* **5**:366