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The Role of Intercellular Channels in the Transepithelial Transfer of Water and Sodium in the Frog Urinary Bladder

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Summary. Epithelial cells of frog urinary bladders fixed in different physiological states were examined by electron microscopy. It was shown: (1) that when bladders incubated with a hypotonic mucosal medium are water-permeabilized with oxytoxin, arginine-vasotocin, cyclic 3′,5′-AMP and theophylline, this leads to a cellular swelling and the opening of intercellular channels; (2) that these effects are not observed when the transepithelial net water flow is suppressed by abolishing the external osmotic driving force; and (3) that modifications in the rate of active sodium transport do not change the morphological appearance of intercellular channels.

These results are especially discussed with respect to the localization of the intracellular site of the final effect of antidiuretic hormone on water permeability, and to the role of intercellular channels in the transepithelial transfer of water and sodium.

In *Anurans*, the urinary bladder serves as a reservoir for the very diluted urine formed by the kidneys. This epithelium is able to transport Na^+ actively from urine to body fluids and to reabsorb water during dehydration. The rates of Na^+ and water transfers are increased by antidiuretic hormone. The toad or frog bladder is generally considered one of the most suitable structures for the study of the basic features of water and Na^+ transport by epithelia and for studying the action of antidiuretic hormone at the cellular level.

A functional description of this epithelium in terms of a three-compartment system limited by the apical and basal-lateral membranes of a single

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layer of epithelial cells was derived from a precise examination of the permeability of the amphibian urinary bladder to water and electrolytes (*see*, for instance, Leaf, 1965). In this model, the reabsorption of NaCl by the epithelium is assumed to be the result of the activity of a Na^+ pump located at the basal or basal-lateral membrane, which carries Na^+ ions from the intracellular to the internal medium against an electrochemical gradient. The entry of Na^+ into the cell which is supposed to be rate-limiting for the whole system would result from passive diffusion or carrier-mediated transport down an inward-directed electrochemical gradient. According to the majority of authors on the subject, the hormone would enhance the permeability to the passive movement of Na^+ ions at the apical border. An increase in the Na^+ cellular content will result, so that at the basal border more Na^+ would be available to the active transport mechanism.

On the other hand, it is generally considered that the rate-limiting barrier for the passage of water through the epithelium (which is necessarily also the site of antidiuretic hormone's final action) is a porous layer located at or near the mucosal membrane. The increase in the osmotic permeability of the epithelium in response to antidiuretic hormone would result in an expansion in the diameter of water-filled channels crossing the apical membrane.

Parallel morphological studies by Pak Poy and Bentley (1960), Peachey and Rasmussen (1961), Carasso, Favard and Valérien (1962), Choi (1963), and Jard, Bourguet, Carasso and Favard (1966) have shown the following. (1) The epithelial cells of frog or toad bladders are swollen in preparations incubated with a hypotonic mucosal medium and treated with antidiuretic hormone. This observation most probably indicates that the osmotic pressure of the intracellular medium decreases in the presence of the hormone. (2) Intercellular spaces are enlarged in vasopressin-stimulated bladders, suggesting that intercellular spaces may constitute an important pathway for water transfer through the epithelium.

In relation to the general concepts briefly summarized above, we had two main objectives in this investigation.

(1) *A study, by a morphological approach, of the osmotic behavior of the epithelial cells of the frog's urinary bladder, and its modifications by neurohypophysial hormones and other pharmacological agents such as cyclic 3',5'-AMP and theophylline, which are known to mimic the action of antidiuretic hormone on water permeability* (Orloff & Handler, 1961; Bourguet, 1968). A possible role of intercellular channels as a preferential pathway for water transfer was investigated. This morphological approach takes

into consideration mainly the changes occuring in the cellular volume, the intercellular spaces and the junctions between cells.

(2) *An examination of the possible morphological modifications associated with alterations in the rate of Na^+ transport by the epithelium.*

Materials and Methods

Materials

The animals used were frogs (*Rana esculenta*) kept in tap water at 20 °C, without feeding, for at least a week before the experiments were carried out. Bladders were dissected from the pithed frogs and mounted in a double lucite chamber isolating two independent pieces of the same preparation. The epithelium was maintained in a fixed position against a nylon mesh by applying hydrostatic pressure of 20 cm H_2O to the mucosal compartment.

In all these experiments, except where otherwise specified, the serosal side of the bladder was bathed with an aerated Ringer's solution (Na^+, 114.5 mM; K^+, 5 mM Ca^{++}, 1 mM; Cl^-, 119 mM; HCO_3^-, 2.5 mM, pH 8.1). Depending on the experimental purpose, the mucosal medium was either a Ringer's solution, an isotonic mannitol solution (4% in water), or a hypotonic Ringer's solution in which NaCl concentration is reduced to 1/2 or 1/20 of its normal value.

The products used in this study were synthetic oxytocin (Syntocinon, Sandoz) synthetic Arginine[8]-oxytocin[1], cyclic 3′,5′-AMP (Schwartz Bioresearch Inc.), and theophylline (Mann).

Permeability Measurements

The osmotic flow of water was measured using the automatic device described previously (Bourguet & Jard, 1964). The rate of Na^+ transport was measured, in the absence of an electrochemical gradient, by the short-circuit current technique of Ussing and Zerahn (1951), or, in open-circuit conditions, by the magnitude of the active inward-directed unidirectional flux, determined by means of radioactive ^{24}Na.

To determine the Na^+ permeability of the submucosa (lamina propria and muscular layer), the serosal and mucosal media were replaced by a 10 mM ethylenediamine tetra-acetate Ca^{++}-free Ringer's solution. After a 90-min incubation period, the epithelial cells were removed by vigorous stirring of the mucosal compartment. The absence of macroscopical leaks in the remaining submucosa was tested by measuring permeability to hemoglobin. Permeability coefficients to Na^+ were deduced from measurements of the unidirectional mucosal-to-serosal flux of radioactive Na^+, during five successive 10-min periods.

Electron Microscopy

The preparation of the specimens for electron-microscopic observation was as follows. At the end of the experimental period, the serosal medium was removed, rapidly replaced by a 2% glutaraldehyde solution in a cacodylate buffer (0.11 M, pH 7.4), and the fixation was allowed to proceed for one hour. This procedure causes minimum

1 The authors wish to thank Dr. R. A. Boissonas for supplying them with Arginine[8]-oxytocin.

disturbances of the preparation and the quality of the fixation is similar to that obtained when the fixative is added on the two sides of the epithelium. The bladder was then removed from the chambers and rinsed overnight at 4 °C in the cacodylate buffer. The preparations were post-fixed for 45 min in a 1% OsO_4 solution in a veronal HCl buffer (0.11 M, pH 7.4). They were dehydrated with alcohol and embedded in araldite. Sections perpendicular to the surface were prepared and stained with lead citrate (Venable & Coggeshall, 1965). Tangential sections presented in Figs. 3 and 4 were stained with phosphotungstic acid in absolute alcohol.

The freeze-etched preparations were obtained with a Balzers freeze etcher. The bladders were incubated for 30 min in a Ringer's solution containing 20% glycerol.

Ribbons (1 cm long by 2 mm wide) were cut and rolled up. The roller obtained was set on the specimen holder of the instrument, and then frozen in liquid Freon. After the sections were etched, a replica of the surface was obtained by evaporating carbon and tungsten. This replica was observed in electron microscopy using a RCA EMU 3 C, a Philips EM 200 or a Philips EM 300 microscope.

The thickness of the epithelial layer was measured on electron micrographs by the mean distance between the mucosal border and the basement membrane. For each bladder, a mean value was deduced from measurements on 4 to 10 micrographs.

Results

General Description of the Epithelium

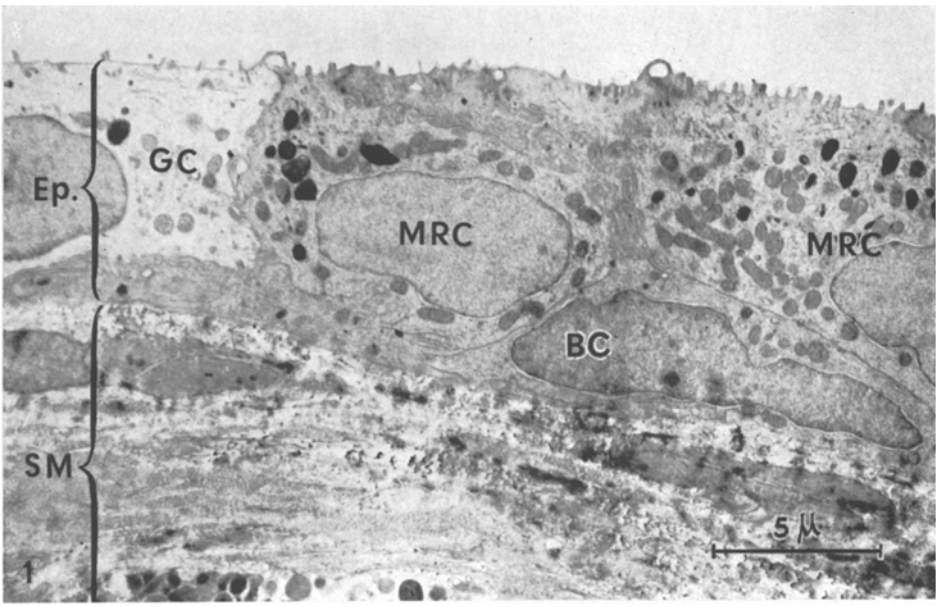
The histological structure of the epithelial layer of the urinary bladder of *Rana esculenta* is similar to that described for *Bufo marinus* (Pak Poy & Bentley, 1960; Peachey & Rasmussen, 1961; Choi, 1963; and Di Bona, Civan & Leaf, 1969*a*), or for *Bufo bufo* (Carasso *et al.*, 1962).

Two major types of cells can be recognized. We shall use the names suggested by Choi (1963) for *Bufo marinus* bladder, i.e., granular and mitochondria-rich cells (Figs. 1 & 2).

A third category must also be added, i.e., basal cells, characterized by a dense cytoplasm and an indented nucleus. This last category probably represents young and indifferentiated epithelial cells.

As is the case for other species, the granular cells represent approximatively 90% of the epithelium's cell population in the *Rana esculenta* urinary bladder. In this simple squamous epithelium, granular and mitochondria-rich cells come into contact both with the urine on the mucosal side and with the basal lamina on the serosal side as has been demonstrated by Di Bona *et al.* (1969*a*) in the toad bladder.

The ultrastructure of the different cell categories will not be described in detail in this paper. Indeed, it is very similar to the ultrastructure of *Bufo bufo* (Carasso *et al.*, 1962) or *Bufo marinus* (Peachey & Rasmussen, 1961; Choi, 1963). Our study is mainly concerned with the morphological changes associated with the variation of the transepithelial transfer of water and Na^+.



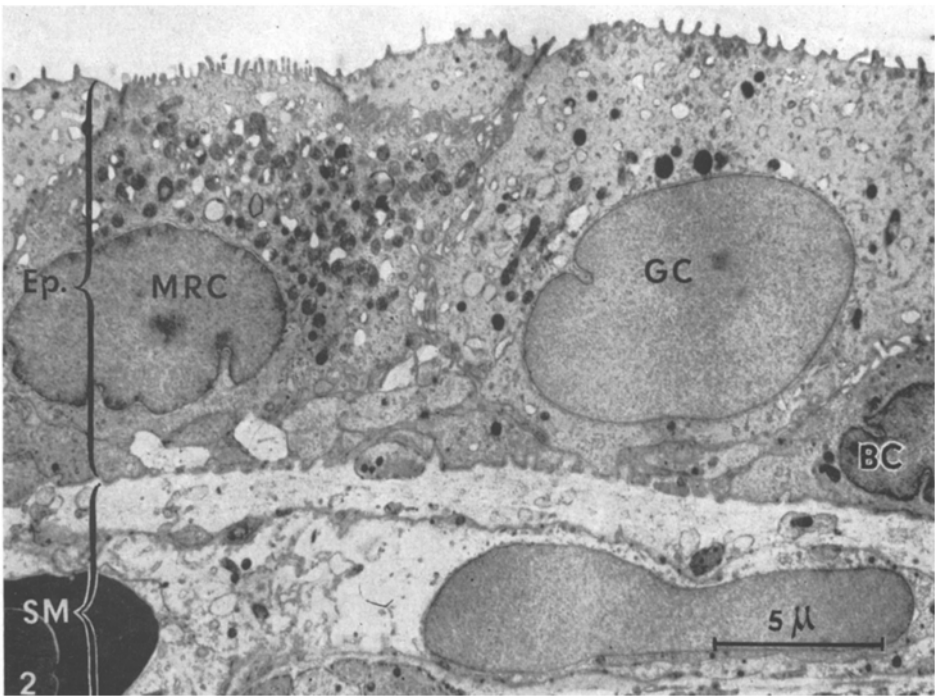
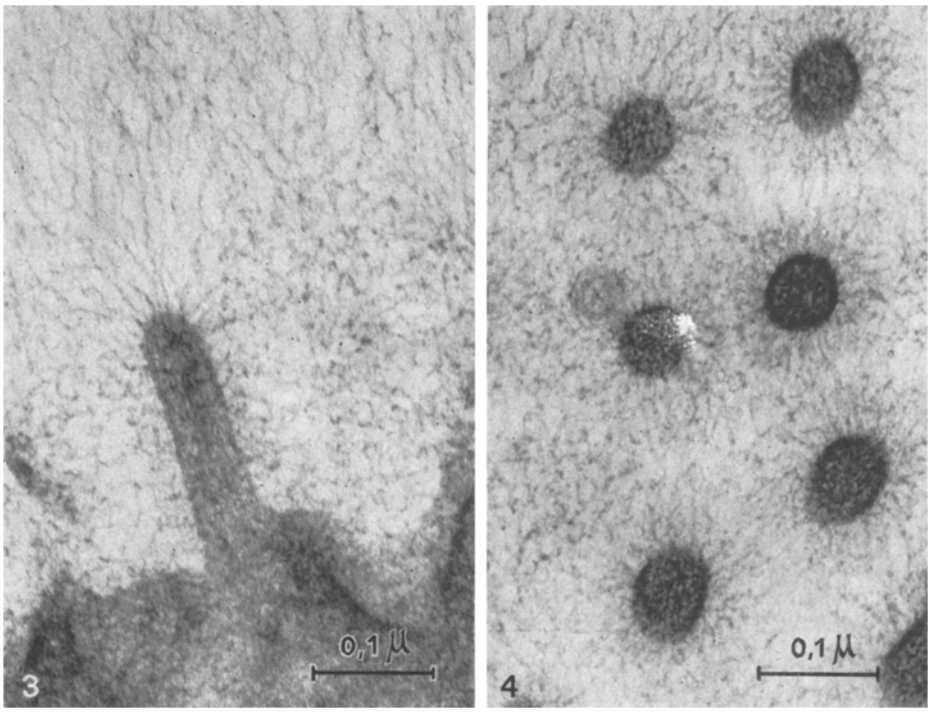


Fig. 1. Epithelial layer (*Ep*) of a frog urinary bladder fixed in a resting state. The three types of cells can be recognized: granular cells (*GC*), mitochondria-rich cells (*MRC*) and basal cells (*BC*). The epithelial cells are supported by the submucosa (*SM*). ×4,500

Fig. 2. Epithelial layer (*Ep*) of a frog urinary bladder stimulated by oxytocin in the presence of an osmotic gradient. Both mitochondria-rich cells (*MRC*) and granular cells (*GC*) are swollen (*compare* with Fig. 1). *SM*, submucosa; *BC*, basal cell. ×4,500

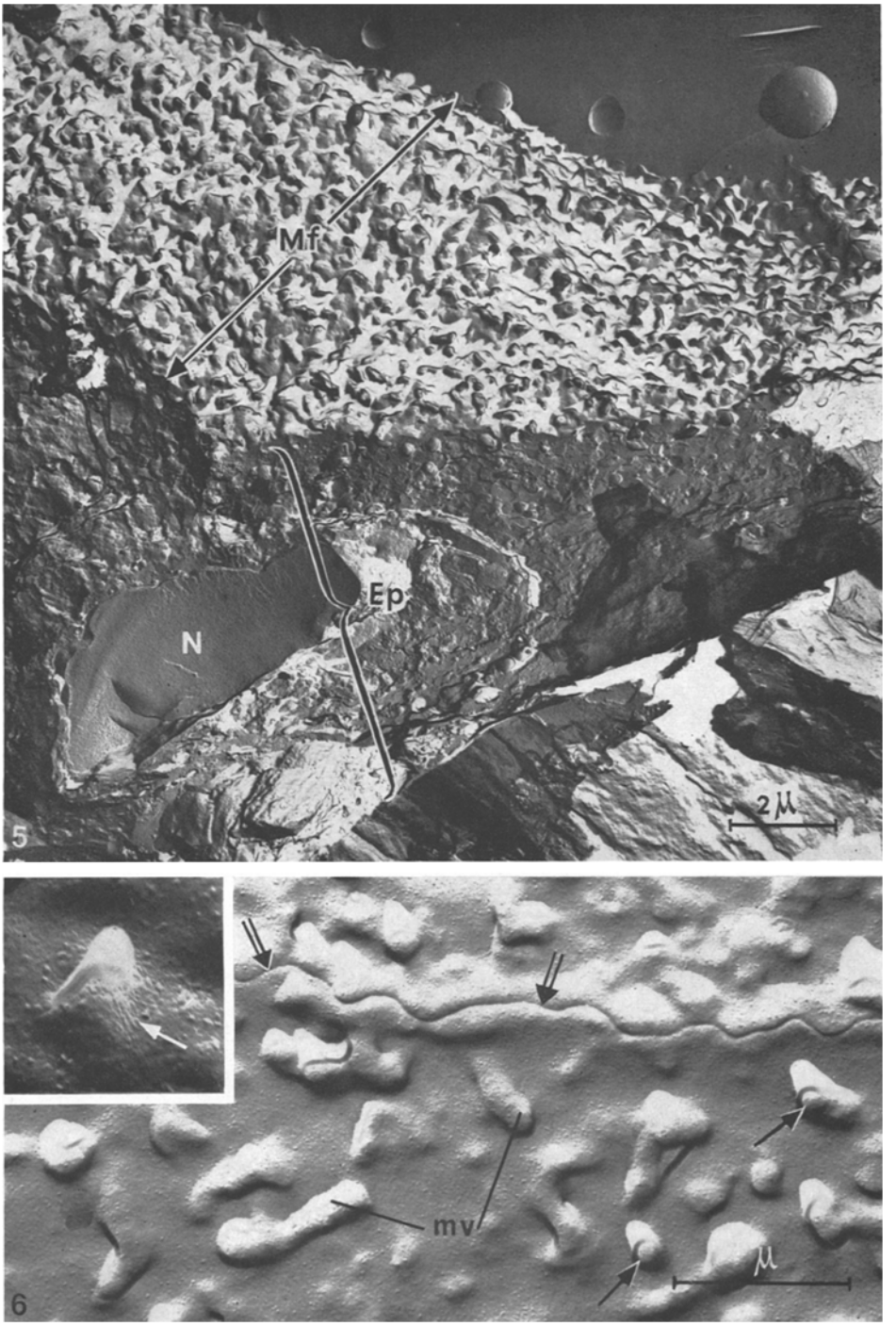


Figs. 3 and 4. Microvilli of the mucosal membrane. Fig. 3. Longitudinal section. Fig. 4. Transversal section. The preparations are stained with phosphotungstic acid. Note the presence of an extracellular coat formed by a dense network of filaments. × 80,000

The mucosal membrane is characterized by the presence of numerous microvilli covered with a dense coat of filaments; these filaments are clearly visible on tangential sections stained with phosphotungstic acid (Figs. 3 & 4). The microvilli are apparent on preparations obtained by the freeze-etching technique (Figs. 5 & 6); the fibrillar structure of the coat is recognizable in some places (Fig. 6, inset).

Adjacent cells are more or less imbricated. In the middle and the basal parts of the epithelium, the epithelial cells show long infoldings. On the mucosal side, however, there are no such infoldings, and this is clearly apparent on the apical views observed on freeze-etched preparations (Fig. 6).

Adjacent cells are held together by tight junctions located near the mucosal membrane (Fig. 7) and by desmosomes (Fig. 9) sparsely distributed over the basal-lateral membranes. On the serosal side, the epithelium is limited by a continuous basal lamina (Fig. 8).



Figs. 5 and 6

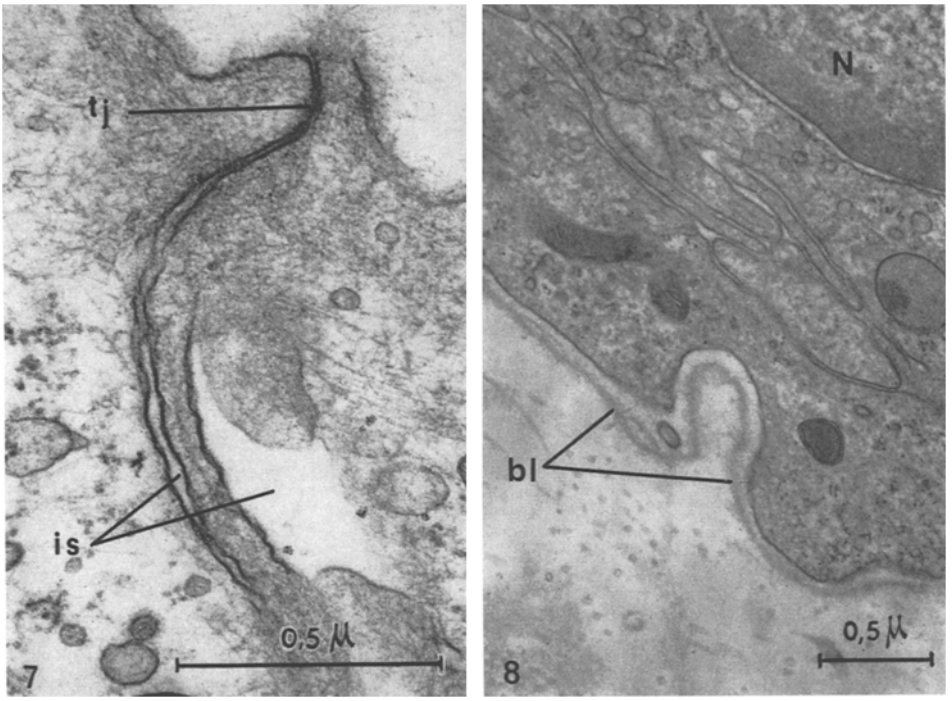


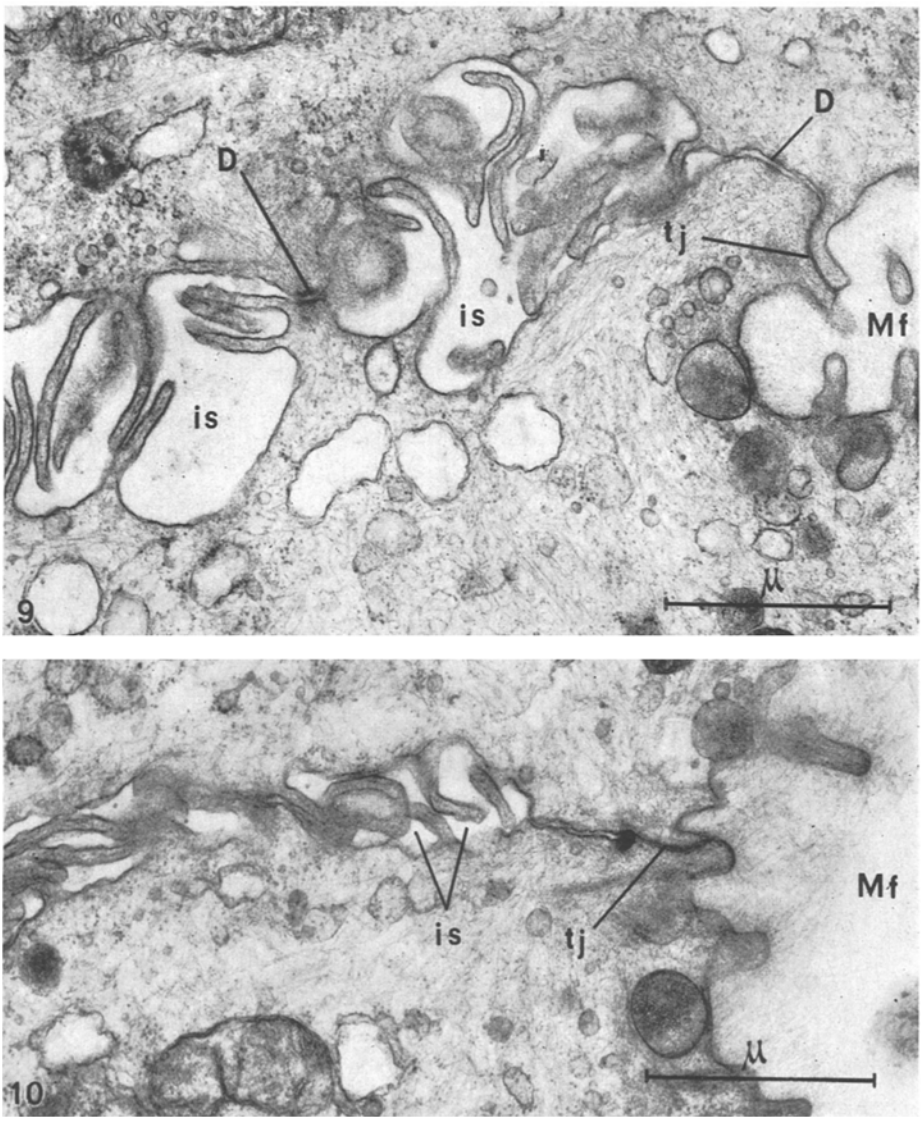
Fig. 7. Junction between two adjacent cells. *tj*, tight junction or *zona occludens*; *is*, intercellular space. × 70,000

Fig. 8. The serosal border of one epithelial cell showing the basal lamina (*bl*). *N* nucleus. × 30,000

As will be seen later, the thickness of the epithelial layer depends on the tonicity of the mucosal and serosal media and on the presence or absence of hormonal stimulation. The mean thickness determined on eight resting bladders incubated with pure Ringer's inside and 50% diluted Ringer's

Fig. 5. One epithelial cell of the frog urinary bladder observed by the "freeze-etching" technique. In the upper part of the figure, the fracture follows the mucosal surface (*Mf*) covered with numerous microvilli. In the lower part, the fracture crosses the cell: the nuclei (*N*) can be recognized. × 7,500

Fig. 6. Part of two adjacent cells observed at the mucosal face level. The lateral faces, which are side by side, do not present deep infoldings at this level but are slightly undulated (double arrows). Several microvilli (*mv*) are etched (single arrows). × 25,000. *Inset.* Microvillus at higher magnification; the fibrillar structure of the coat is recognizable (arrow). × 50,000



Figs. 9 and 10. Intercellular spaces in two stimulated bladders. One is stimulated by oxytocin (Fig. 9) and the other with 3′-5′-cyclic AMP (Fig. 10). *Mf* mucosal surface *tj* tight junction; *D* desmosomes; *is* intercellular spaces. × 30,000

outside was 4.28 ±0.81 μ. The submucosa contains numerous collagen fibers muscular cells and blood vessels (Figs. 1 & 2). Its thickness varies greatly in different regions of the bladder, and ranges from 1.6 to 25.0 μ. The mean ±SD value determined for 38 preparations is 5.66 ±0.73 μ.

Morphological Changes Associated with Water Permeabilization in the Presence of an Osmotic Gradient

In Figs. 1 and 2, the morphological aspects of the epithelial cells of two bladders are compared. Both were incubated with a Ringer's solution on the serosal side and a twofold-diluted Ringer's on the mucosal side. One was stimulated by adding oxytocin [(5 mu/ml) (Concentration expressed as hydrosmotic mu per ml. One hydrosmotic unit is defined as the hydrosmotic activity of one Iu of synthetic oxytocin.)] to the serosal solution, and fixed at the maximum of the hormonal stimulation about 20 min after the addition of hormone. The second was fixed in a resting state. The rates of net water flow measured just prior to fixation were 1.4 μliter/min/cm^2 for the stimulated bladder and 0.2 μliter/min/cm^2 for the control.

When the bladder is stimulated by the hormone, some morphological modifications can be observed (Figs. 2 & 9). The thickness of the epithelium increases, and the granular and mitochondria-rich cells are swollen. In these cells, the cisternae of the endoplasmic reticulum are distended, forming vacuoles of different sizes. Ribosomes can be observed in parts of the limiting vacuolar membrane, but this is not generally the case, and the vacuolar membrane is usually smooth. The other organelles are unchanged after stimulation. Basal cells do not swell and are not modified by the increase in the net water flow. The intercellular spaces are enlarged, except

Table 1. *Influence of oxytocin on the cellular volume of the epithelial cells of bladders incubated in the presence of a 115 mosm osmotic gradient. (Values are means ± SD.)*

Bladder	Net water flow[a] (μliter · min^{-1} · cm^{-2})	Thickness of epithelial layer[b] (μ)
Stimulated[c]	1.70 ± 0.16 (15)[d]	8.16 ± 0.76 (48)[e]
Control	0.14 ± 0.04 (8)[d]	4.28 ± 0.81 (42)[e]
Comparison of stimulated vs. control	$t = 6.796$, $P < 0.001$	$t = 3.233$, $P < 0.001$

[a] Measured just prior to fixation.

[b] Determined on several sections of each individual bladder. All sections were perpendicular to the external surface of the epithelium (the nylon mesh supporting the preparation allows a precise orientation during the embedding). On each section, the thickness of the epithelium was determined from four to five measurements of the distance between the mucosal surface and the basement membrane.

[c] Stimulated by addition of oxytocin (5 to 20 mu/ml) to the Ringer's solution bathing the serosal side.

[d] Number of individual bladders.

[e] Total number of sections used for determination of thickness.

Table 2. *Comparison of the effects of oxytocin and Arginine[8]-oxytocin, 3′,5′-cyclic AM or theophylline on cellular volume and intercellular spaces. Bladders were incubated wit Ringer's solution as the serosal medium and a twofold-diluted Ringer's solution as th mucosal medium*

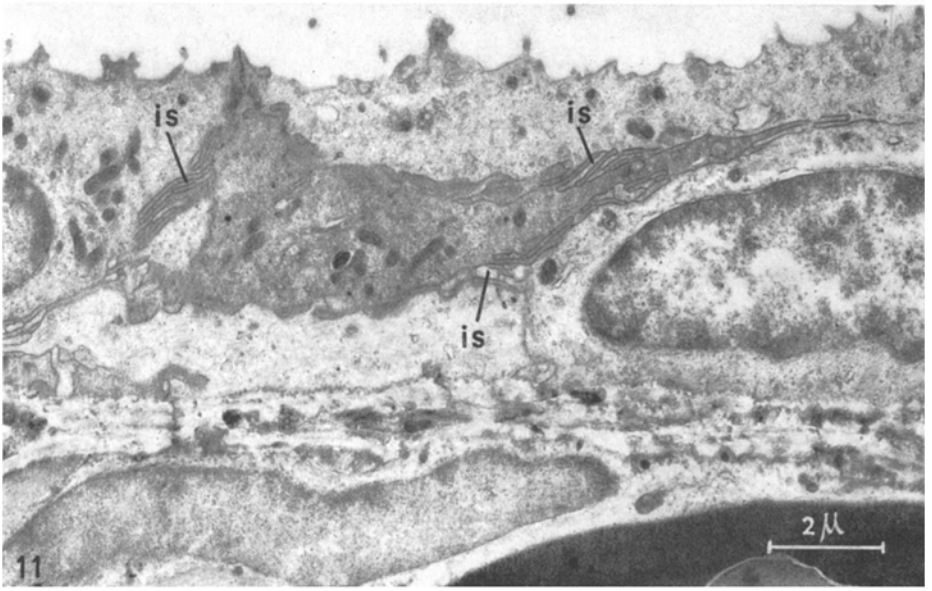
Treatment Exp. no.	Net water flow (μliter · min^{-1} · cm^{-2})	Thickness of epithelial layer (μ)	Enlargement o intercellular spaces
	Experimental hemibladder		
1. A.V.T. (20 mu/ml)[a]	2.06	9.5 (3)[b]	+[c]
2. 3′,5′-cyclic AMP (10^{-3} M)	2.12	11.1 (3)	+
3. "	2.60	5.6 (5)	+
4. Theophylline ($5 \cdot 10^{-3}$ M)	1.19	11.0 (3)	+
	Control hemibladder		
1. Oxytocin (20 mu/ml)[a]	1.50	7.0 (3)	+
2. "	1.56	9.8 (3)	+
3. "	3.0	6.5 (5)	+
4. "	1.32	10.1 (3)	+

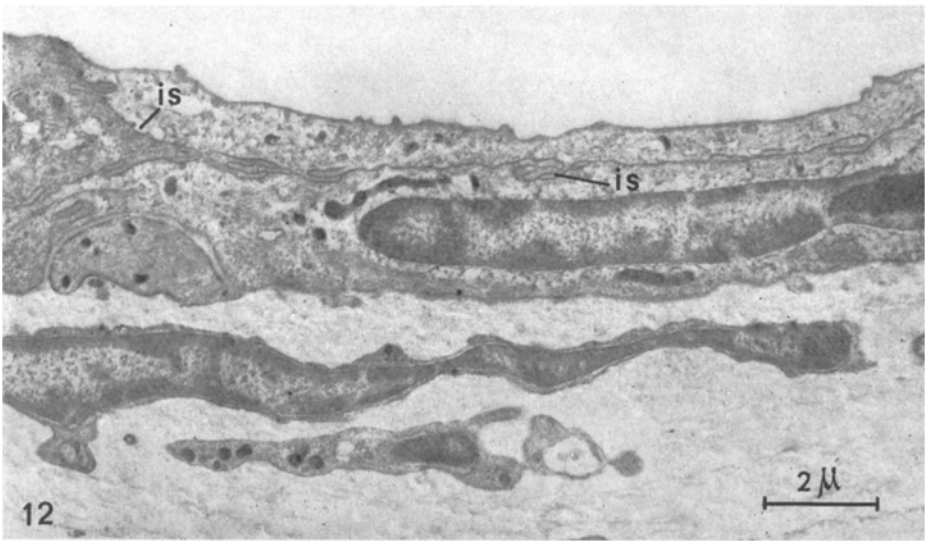
[a] Concentration expressed as hydrosmotic mu per ml. One hydrosmotic unit i defined as the hydrosmotic activity of one IU of synthetic oxytocin.

[b] Number of sections used for the determination of the thickness (*see* Fig. 1 legend)

[c] Plus sign indicates there was enlargement.

in the tight junctions and desmosomes regions (Fig. 9). Table 1 quantitatively summarizes the results of 15 similar experiments. It is shown that th thickness of the epithelial layer of stimulated bladders is about twice tha of the control resting bladders. For these experiments, oxytocin was use in concentrations ranging from 5 to 20 mu/ml. The magnitude of the respons varied from one preparation to another (the increase in net water flo ranging from 0.9 to 4.2 μliters/min/cm^2). However, there is no significan correlation between the thickness of the epithelial layer and the magnitud of the net water flow measured just before fixation ($r=0.362$, $p>0.1$) Of the fifteen stimulated preparations, eleven clearly showed a large increas in intercellular spaces, and three a moderate increase. The cellular swellin measured on 14 sections of a series of 4 stimulated bladders incubated with a 20-fold-diluted Ringer's solution on the mucosal side was similar to tha measured in the preceding series (mean thickness of the epithelial layer 7.88 ± 1.77 μ as compared with 8.16 ± 0.76 μ; *see* Table 1).





Figs. 11 and 12. Aspects of two hemibladders incubated with Ringer's solution on both sides. One of these has been stimulated by oxytocin (Fig. 11) and the other one is in a resting state (Fig. 12). In both cases, the measured net water flow was zero. The intercellular spaces (*is*) are not enlarged in the hemibladder stimulated by oxytocin, the aspect of which is quite similar to the non-stimulated hemibladder

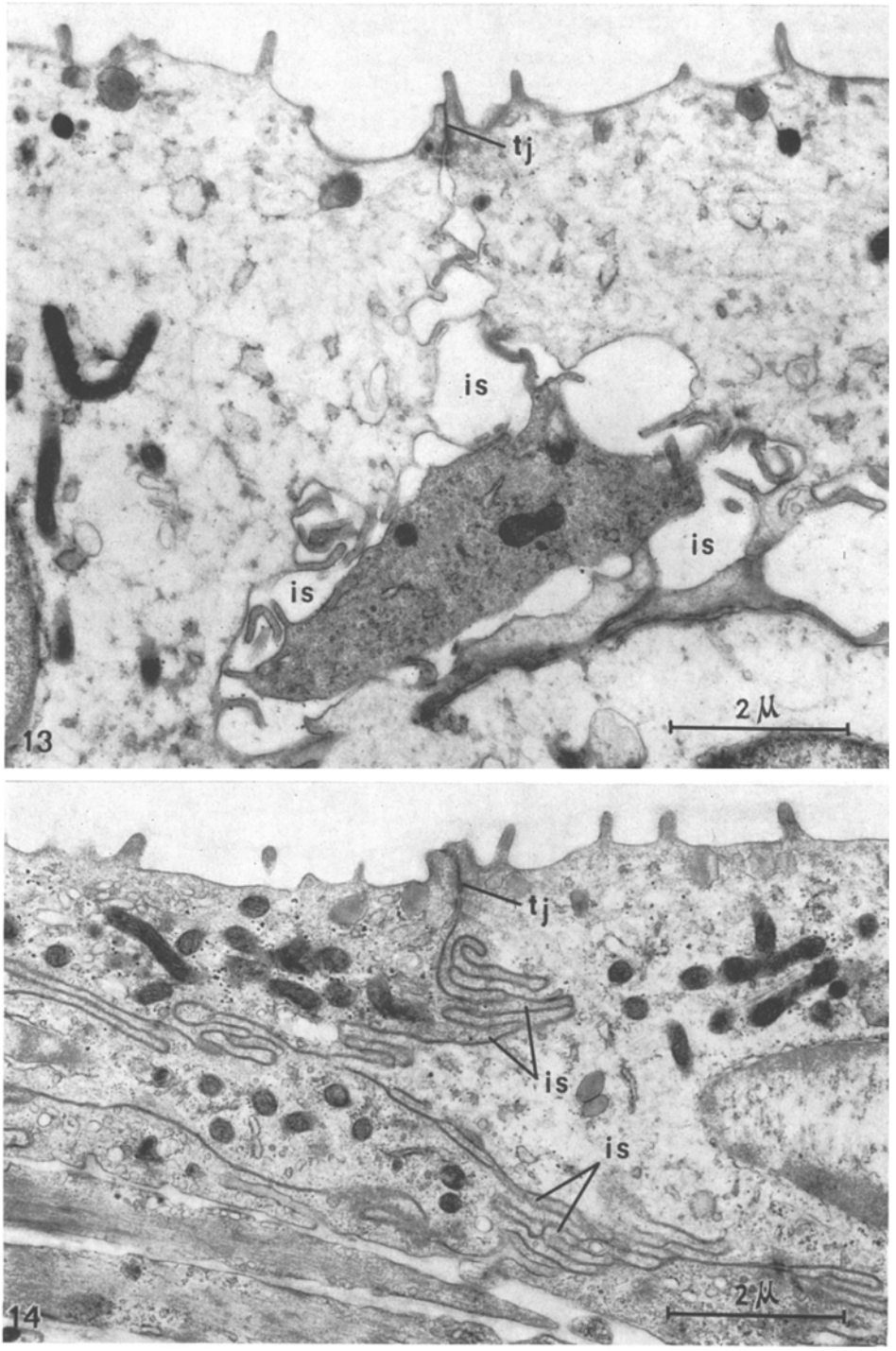
As indicated in Table 2, stimulation of the epithelium with Arginine^8-oxytocin (the hormone occuring naturally in amphibians), or with cyclic 3′,5′-AMP or theophylline produces a cellular swelling and an enlargement

of the intercellular spaces similar to those produced by oxytocin on contro hemibladders (Fig. 10).

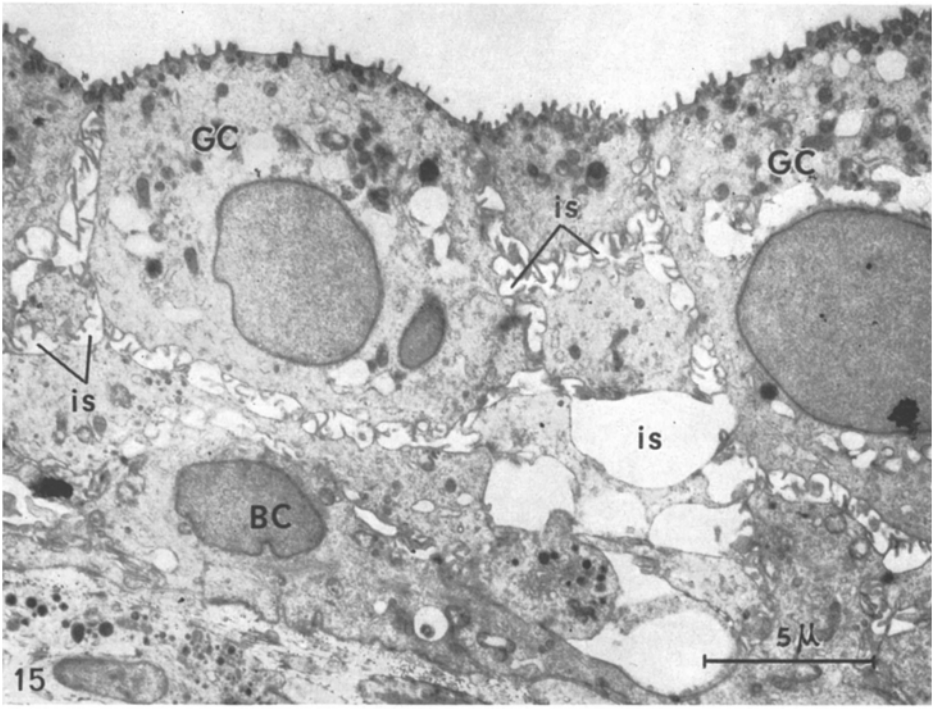
Cellular swelling and enlargement of intercellular spaces in respons to hormonal stimulation appear to be related to the presence of a larg net water flow through the epithelium, rather than to the primary actio of oxytocin on cellular permeability or to an extraepithelial action of th hormone. These effects are absent when the net water flow is suppresse by abolishing the external osmotic driving force (*see* Figs. 11 & 12). Thus the mean thickness of the epithelial layer measured on 16 sections of a series of 5 stimulated bladders incubated with a Ringer's solution on botl sides is 3.17 ± 0.43 μ, a value which is significantly smaller than the corre sponding value (8.16 ± 0.76 μ) determined on stimulated bladders incubate in the presence of an osmotic gradient (*see* Table 1), but not statistically different from that measured on resting bladders incubated under the sam conditions (4.28 ± 0.81 μ; *see* Table 1).

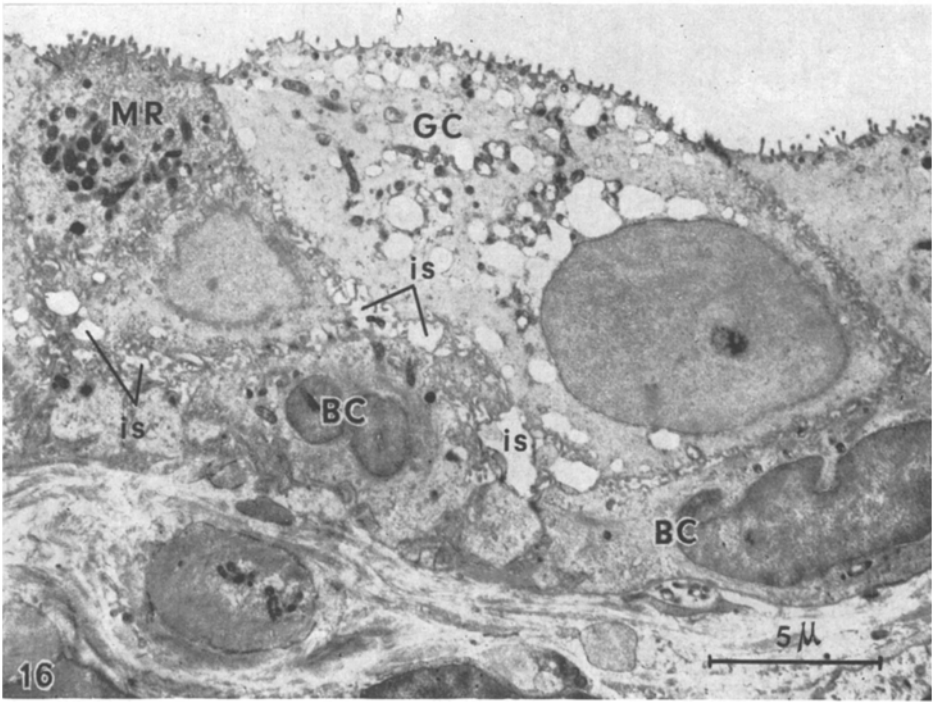
Furthermore, cellular swelling and enlargement of intercellular space are reversible. The morphological appearance of resting bladders is the same whether they are kept in a resting state throughout the experiment, o previously stimulated and fixed at a sufficiently long interval since th washing out of the hormone to ensure complete reversibility of the biologica action (*cf.* Figs. 13 & 14). Cellular swelling can occur very rapidly afte the onset of a large net water flow through the epithelium. To test tha point, experiments were conducted in which bladders were incubated with a Ringer's solution on both sides and stimulated by adding oxytocin to th serosal medium. In the absence of an external osmotic driving force, ther is no net water flow through the epithelium. After about 20 min (the tim required for complete development of the action of oxytocin), the mucosa medium is very rapidly diluted. As a result of the application of an osmoti gradient, a net water flow appears, which reaches its maximum value 1 t 3 min later (Bourguet, *unpublished results*). When fixed 2 or 3 min after th onset of the osmotic gradient, the stimulated bladders show a large cellula swelling, as indicated in Table 3 and Figs. 15 and 16.

Figs. 13 and 14. Reversibility of the enlargement of intercellular spaces produced b oxytocin on bladders stimulated in the presence of an osmotic gradient. Two piece of the same bladder are compared. One (Fig. 13) was fixed at the maximum of the wate permeabilization induced by oxytocin; intercellular spaces (*is*) are open and the cell swollen. The second (Fig. 14) was fixed about 30 min after the washing-out of th hormone from the serosal medium. At that time, the net water flow had returned to it low resting level. Note that the cells are no longer swollen and that intercellular space (*is*) are closed. *tj* tight junctions. × 12,500



Figs. 13 and 14





Figs. 15 and 16

Table 3. *Cellular volume and intercellular spaces in stimulated bladders rapidly fixed after the onset of an osmotic gradient. Bladders were stimulated when incubated with Ringer's solution on both sides. Twenty minutes later, the mucosal medium was rapidly diluted and the preparation fixed 2 or 3 min afterward*

Net water flow [a] (μliter · min^{-1} · cm^{-2})	Thickness of the epithelial layer (μ)	Enlargement of intercellular spaces [b]
1.72	7.10 (3) [c]	0
1.72	9.32 (3)	+
2.96	14.58 (3)	+
1.82	16.67 (7)	+
2.14	6.61 (6)	+
–	7.87 (3)	±
2.07 ± 0.24 [d]	10.19 ± 1.61 [d]	

[a] Measured in the minute which precedes fixation.
[b] Clearcut increase (+); moderate increase (±); no significant increase (0).
[c] Number of sections used for determination of thickness (*see* Fig. 1 legend).
[d] Mean ± SD (compare with values in Table 1).

Morphological Changes Associated with Experimental Modifications in the Rate of Na^+ Transport

Taking into account the influence of net water flow on the morphological appearance of the bladder's epithelial cells, we studied possible morphological changes associated with modifications of the Na^+ transport rate, under conditions of a zero net water flow (i.e., in the absence of an osmotic gradient). Three experimental conditions known to modify the Na^+ transport rate were investigated: (1) Na^+ transport was abolished by replacing the mucosal medium with an isotonic Na^+-free mannitol solution; (2) Na^+ transport was stimulated by adding oxytocin; and (3) the epithelium was short-circuited.

The results of these experiments are depicted in Table 4. It can be observed that an increase in the rate of Na^+ transport obtained either by

Figs. 15 and 16. Comparison of two pieces of the same bladder. One (Fig. 15) was incubated with Ringer's on both sides and stimulated by oxytocin. Twenty minutes later, the mucosal compartment was rapidly diluted and the preparation fixed 2 min afterwards at the maximum of the rapid increase of water flow observed in these conditions. The second (Fig. 16) was incubated throughout the experiment with a twofold-diluted Ringer's solution in the mucosal compartment; oxytocin was added and the preparation fixed at the maximum of the hormonal stimulation. On both preparations, the cellular volume is increased and the intercellular spaces (*is*) are enlarged. *BC* basal cells; *GC* granular cells; *MR* mitochondria rich cell. ×4,500

Table 4. *Effects on cellular volume and intercellular spaces of modifications in the rate c Na^+ transport*

Stimulation by oxytocin[a]

Exp. no.	Stimulated			Control		
	Na^+ influx[a] (% increase)	Cellular thickness (μ)	Inter-cellular spaces	Na^+ influx (% increase)	Cellular thickness (μ)	Inter-cellular spaces
1	160	3.24 (3)[b]	0	−10	8.77 (3)	0
2	179	3.32 (4)	0	− 2	4.15 (4)	0
3	255	3.55 (3)	0	−15	5.03 (3)	0

Short-circuit conditions[c]

Exp. no.	Short-circuit		Open-circuit		
	Cellular thickness (μ)	Inter-cellular spaces		Cellular thickness (μ)	Inter-cellular spaces
4	4.45 (3)	0		7.08 (4)	0

Suppression of Na^+

Exp. no.	Incubation with a Na^+-free mucosal medium		Incubation with Ringer's solution on both sides		
	Cellular thickness (μ)	Inter-cellular spaces		Cellular thickness (μ)	Inter-cellular spaces
5	5.18 (4)	0		5.66 (3)	0

[a] The Na^+ influx is measured during four successive 10-min periods. Oxytoci is added at the end of the second period. The increase in Na^+ influx during the fourt period is expressed as a percentage of the mean value measured during the first tw control periods.

[b] Number of sections used for the determination of the cellular thickness (*se* Fig. 1 legend).

[c] The Na^+ net flux measured by the short-circuit current technique was 0.8 μEquiv $hr^{-1} \cdot cm^{-2}$. This value is about nine times larger than the mean value of the Na^+ measure in open-circuit conditions (0.09 μEquiv $\cdot hr^{-1} \cdot cm^{-2}$ in experiments 1, 2 and 3).

short-circuiting the preparation or by adding oxytocin to the serosal com partment does not lead to any significant increase in the size of intercellula spaces. Furthermore, cellular volume decreased as a result of increasin Na^+ transport. The morphological aspect of one hemibladder incubate with a Na^+-free isotonic mannitol solution is indistinguishable from tha of the control hemibladder.

Discussion

The results reported in this paper show that the increase of the osmotic flow of water through the bladder produced by oxytocin, Arginine^{8}-oxytocin, cyclic AMP or theophylline is followed by rapid and reversible modifications of the morphology of the epithelial cells, namely, an increase in cellular volume and enlargement of intercellular spaces. Before we discuss these results with regard to the mode of action of neurohypophysial hormones and similar agents at the cellular level, the origin of the morphological modifications observed must be clarified. The increase in cellular volume can arise either (1) from a dilution of the intracellular medium by osmotic movements of water, or (2) from an increase in the cellular pool of osmotically active solutes, the osmotic pressure of the cellular compartment remaining unchanged. According to the former hypothesis, one can expect the cellular swelling to depend on the tonicity of the media in contact with the mucosal and basal lateral plasma membranes, and on their relative water permeabilities. According to the latter hypothesis, the cellular swelling would arise from a modification by the hormone of the rates of solute transfer through the cellular plasma membranes. It can reasonably be assumed that this effect is independent of the presence of an osmotic gradient between the two sides of the epithelium. Therefore, the absence of cellular swelling when the net water flow through the epithelium is suppressed by abolishing the external osmotic driving force is a strong argument in favor of the first hypothesis. In fact, it was observed on a few preparations that the cellular volume of the epithelial cells decreases when the rate of Na^+ transport increases in the absence of a net water flow. These results probably reflect the same phenomenon noted by MacRobbie and Ussing (1961). In some but not in all their experiments, these authors observed a shrinkage of the frog skin epithelium after short-circuiting the preparation.

Similarly, the enlargement of intercellular spaces can be related to osmotic movements of water, to an increased rate of solute transport from the cell to intercellular spaces through the lateral plasma membranes, or even to an extraepithelial action of the hormone as recently suggested by Di Bona and Civan (1969). The role of solute movements through the lateral membranes must be carefully discussed in relation to observations obtained from other epithelia such as the gallbladder. In the rabbit gallbladder, it has been shown (*see*, for instance, Diamond & Tormey, 1966) that solute transport from an intracellular compartment of intercellular spaces facing the blood is able to create a local osmotic gradient responsible for a flow of water from the cell to the intercellular spaces.

If the enlargement of intercellular spaces in the frog bladder is caused by an increase in the rate of solute transport through the lateral membrane in response to hormonal stimulation, one can also expect this effect to be present in preparations stimulated under conditions of a zero net water flow. The observation that intercellular spaces remain closed in all the stimulated bladders incubated with a Ringer's solution on both sides of the preparation strongly suggests that modifications in the rate of solute transport through the lateral membranes do not play a significant role in the enlargement of intercellular spaces. On the other hand, several arguments indicate that the enlargement of intercellular spaces observed in our experiments cannot be caused by the relaxing action of the hormone on the muscular cells present in the submucosa, as recently suggested by Di Bona and Civan (1969, 1970) for the toad bladder: (1) the effect is not observed on stimulated bladders fixed under conditions of a zero net water flow (2) recent observations (Parisi, Ripoche, Bourguet, Favard & Carasso, 1969) indicate that removal by microdissection of the greater part of the submucosa does not suppress either the enlargement of intercellular spaces or cellular swelling linked to water flow; and (3) as reported by Grantham *et al.* (1969), the opening of intercellular spaces can be observed in isolated rabbit kidney collecting tubules when osmotic reabsorption of water is increased by antidiuretic hormone. Thus, it seems reasonable to conclude that under our experimental conditions both cellular swelling and opening of intercellular spaces linked to water flow are primarily caused by osmotic water movements.

The difference between our results and those of Di Bona and Civan (1969, 1970) can perhaps be explained by a difference in the technique used for mounting the epithelium. In our technique, the preparation is submitted to a 20-cm H_2O hydrostatic pressure, which compresses the serosal surface of the bladder against a nylon mesh. This hydrostatic pressure is most probably sufficient to completely mask an additive action of oxytocin on the intercellular spaces resulting from relaxation of the muscular fibers of the submucosa.

At least three mechanisms could account for the osmotic swelling of the epithelial cells of the bladder in response to an increased net water flow. They are described in Fig. 17. In the resting state, the osmotic permeability of the apical barrier is assumed to be very low compared to that of the internal barrier. This means that the cellular compartment and intercellular spaces are in an almost complete osmotic equilibrium with the isotonic internal compartment. In a first hypothesis, we considered the flow of water capable of maintaining a hypotonic medium in the submucosa

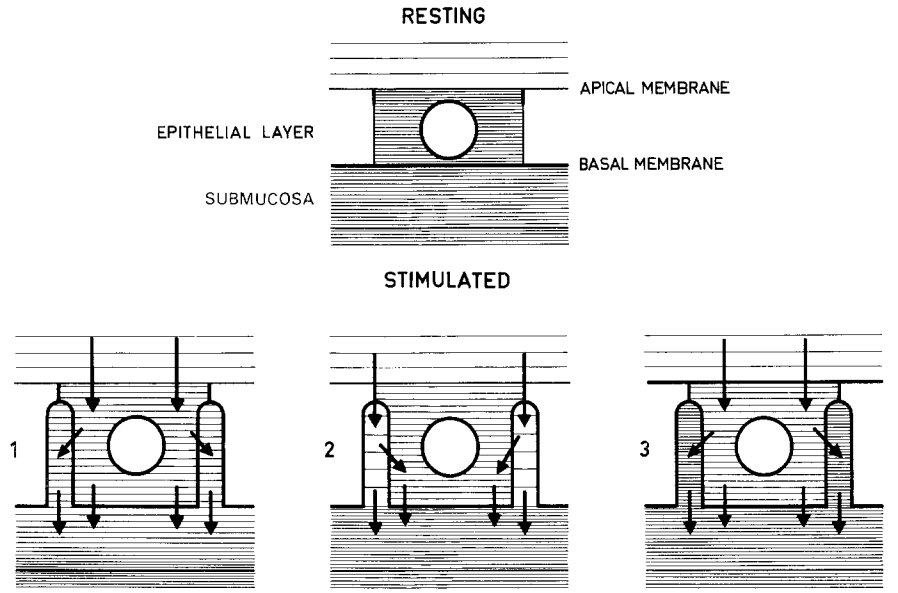


Fig. 17. Schematic representation of epithelial cells of the frog urinary bladder. Upper part: resting bladders. Lower part: water-permeabilized bladders, showing swollen cells and open intercellular channels. The thicknesses of the lines which represent the apical membrane and the basal lateral membrane are an indication of their relative water permeabilities. The relative osmotic pressures of the different compartments are indicated by the density of hatching. Arrows show the different possible paths for the transepithelial water flow. For comments, *see* text

by sweeping away the solutes from the basal membrane, this movement of solutes being incompletely compensated for by backward diffusion from the serosal medium. Such hypotonicity of the submucosal compartment would contribute to the cellular swelling. This hypothesis seems unlikely in view of the relative values of the linear velocity of flow through the epithelium (JW) and of the diffusion coefficient of NaCl in the submucosa (DS). Using equations similar to those derived by Dainty and House (1966) for the measurement of unstirred layers in frog skin, the concentration of solutes at the level of the basal membrane of the epithelial cells (C_s^m) can be calculated as follows:

$$C_s^m = C_s^i \exp(-JW\,\delta_0/DS) \tag{1}$$

in which C_s^i is the absolute concentration in the internal medium, and δ_0 is the thickness of the submucosa. Introducing in Eq. (1) 2.84×10^{-5} cm · sec^{-1} for JW [the linear velocity corresponding to the mean value of the

net water flow measured on the stimulated bladders, i.e., 1.7 μliter·min^{-1} cm^{-2} (*see* Table 1)], 5.7×10^{-4} cm for δ_0, and 3×10^{-7} cm^2·sec^{-1} for D, (the experimental value measured), the expected value for C_s^m/C_s^i is 0.95 Thus, despite the fact that the submucosa does offer slight resistance to th diffusion of NaCl, it seems unlikely that dilution of the submucosal com partment by the flow of water can account for the cellular swelling observed Furthermore, as mentioned above, removal by microdissection of the sub mucosa does not suppress cellular swelling linked to water flow.

According to the second hypothesis (Fig. 17, no. 2), water would flow directly through the tight junctions from the external medium into inter cellular spaces. If the linear velocity of flow in intercellular spaces is high enough to counteract solute diffusion from the internal medium, steady state conditions will be achieved in which intercellular spaces would contain a hypotonic medium allowing swelling of epithelial cells by osmotic water movements through their lateral plasma membranes. In a first approximation, Eq. (1) can be used to calculate the linear velocity of flow necessary to maintain, at the level of the tight junctions, a solute concentration equal to 50% of that present in the internal medium. Using the mean thickness of the epithelial cells of the stimulated bladders (8.2 μ—*see* Table 1—for δ_0 and 1.48×10^{-5} cm^2·sec^{-1} for DS, the diffusion coefficient of NaCl in a 0.1 M aqueous solution at 25 °C), the corresponding value of JW is 1.23×10^{-2} cm·sec^{-1}. For a net water flow through the epithelium of 1.7 μliters·min^{-1}·cm^{-2}, the value of 1.29×10^{-2}·cm^{-2}·sec^{-1} can be attained if the area of the intercellular spaces projected onto the basement membrane represents less than 0.25% of the total epithelial area. If one assumes that the tangential section of one epithelial cell is a square of 10 μ this would correspond to a mean width of the intercellular spaces of about 125 Å. This later value is obviously less than that which can be measured on electron micrographs (0.1 to 0.5 μ). Moreover, if tight junctions are believed to constitute a preferential path in transversal exchanges (*see*, for instance, Loewenstein, Socolar, Higashino, Kanno & Davidson, 1965) there is no experimental data revealing longitudinal permeability.

Finally, the most likely explanation for the cellular swelling observed is shown in Fig. 17, no. 3. According to this third hypothesis, water flows through all the surface of the cell's apical plasma membrane. The primary action of the hormone results in an increase in the osmotic permeability coefficient of the apical barrier so that the osmotic equilibrium of the cell shifts toward the diluted external medium. From this model, the cellular volume can be expected to depend on the ratio of the osmotic permeability coefficients of the apical and basal plasma membranes, and, for a given

value of this ratio, on the osmotic pressure of the diluted Ringer's at the mucosal border. In order to account for the twofold increase in cellular volume frequently observed in stimulated bladders bathed with half Ringer's outside, one has to assume that the ratio of the osmotic permeability coefficients of the apical and basal plasma membranes is increased by the hormone to a value much larger than unity. Furthermore, it was not possible to demonstrate a clear-cut correlation between the cellular swelling in stimulated bladders and the magnitude of the net water flow through the epithelium of the magnitude of the osmotic gradient between 115 and 215 mosm/liter. These results might indicate independent regulation by the hormone of the osmotic permeability of the apical membrane controlling the magnitude of the net water flow, and of the ratio of the osmotic permeabilities of the basal and apical membranes which control the cellular volume as a function of the tonicity of the external medium.

The hypothesis we are discussing cannot explain directly the enlargement of intercellular spaces. The dilution of intracellular fluid may be expected to lead to an osmotic flow of water from the cell to the intercellular spaces. The maximum increase in the size of these spaces which can be achieved by osmotic equilibrium alone without solute movements cannot exceed a twofold increase, and does not account for the experimental observation. However, an inward diffusion of solutes in intercellular spaces can help to maintain, in steady state conditions, an osmotic gradient between the diluted intracellular compartment and the intercellular compartment. Part of the osmotic flow through the epithelium can therefore be diverted toward intercellular spaces. The resulting effect corresponds to a large increase in the surface available for osmotic equilibration with the isotonic inside medium. It can be considered as a regulatory mechanism which allows the cell to counteract too large a cellular swelling resulting from osmotic permeabilization of the apical plasma membrane in contact with a diluted medium. This effect also helps to maintain a high osmotic gradient between the two sides of the limiting apical barrier.

On the other hand, this morphological approach does not allow any relevant evaluation of the structural changes in the apical plasma membrane involved in its permeabilization by antidiuretic hormone, especially with respect to the well-documented concept of the functional porosity of the rate-limiting permeability barrier at the mucosal surface of the epithelium. Fixation of the epithelium partly preserves the permeability changes induced by antidiuretic hormone (Carasso *et al.*, 1966), but treatment of fixed bladders with the concentrated alcohol solutions used for dehydration of microscopic samples leads to a very large degree of permeabilization to

water and electrolytes and to the complete disappearance of the differenc between control resting bladders and previously stimulated bladders.

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