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Extracellular Ca^{2+} and the Effect of Antidiuretic Hormone on the Water Permeability of the Toad Urinary Bladder: An Example of Flow-Induced Alteration of Flow

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Summary. The extracellular Ca^{2+} requirement for antidiuretic hormone (ADH) stimulation of water permeability in the toad urinary bladder has been critically examined. The polarity of the tissue was maintained with 1 mM Ca^{2+} in the mucosal bathing medium and a serosal bath nominally free of Ca^{2+}. Under these conditions, ADH-induced osmotic water flow was inhibited by more than 60% while enhancement of the diffusional permeability to water was unaffected. Structural studies revealed that low serosal Ca^{2+} led to parallel alterations in epithelial architecture that amounted to a significant distortion of the osmotic water pathway. Prevention of these alterations, or restoration of normal cell-cell contact showed that the reduction of serosal Ca^{2+} did not restrict hormonal action, *per se*, but that it resulted in a weakening of cell-cell junctions such that intercellular space distension during water flow occurred to a point where the geometric conditions for maintenance of osmotic flow were compromised. We conclude that extracellular Ca^{2+} is not a requirement for the molecular aspects of ADH action but that, in its absence, a direct measurement of ADH-induced osmotic flow proves to be an inaccurate index of the hormone-generated changes in epithelial transport characteristics. Under certain conditions the ADH-effect on the tissue's hydraulic permeability is probably best assessed by measurement of the diffusional permeability to water; although accuracy in this determination is difficult, it is not as strongly dependent on tissue geometry.

Key words toad bladder · antidiuretic hormone · water permeability · calcium · epithelial structure

Introduction

It has been reported that toad bladders incubated in low Ca^{2+}, or in its absence, display an irreversible blunting of the hydroosmotic response to ADH [4] and that the absence of Ca^{2+} decreases both basal and ADH-stimulated rates of Na^+-transport [5]. It was later found that removal of Ca^{2+} from the bathing media causes disruption of tight junctions and that the cells eventually break free from the supporting submucosa [29]. Not surprisingly, these structural alterations were coincident with large increases in transepithelial permeability to inulin, water, and other small molecules. Under these circumstances ADH apparently failed to increase the diffusional water permeability [29], but the appearance of large aqueous channels between the cells renders this measurement questionable. However, bladders incubated in the absence of Ca^{2+} were able to respond to ADH with increased oxygen consumption, provided Na^+ was present in the incubation medium [29]. Thus indirect measurements showed that, in the total absence of Ca^{2+}, the hormone was still able to induce an increase in Na^+ permeability which, for obvious reasons, was not demonstrable by conventional techniques. Since the effects of ADH on the transepithelial pathways for water and Na^+ can be dissociated through several maneuvers [1, 19, 36], the results of Hays et al. [29], *per se*, do not prove that the effect of ADH on water permeability is similarly independent of extracellular Ca^{2+}.

Following the communications by Bentley [4, 5] and Hays et al. [29], others have investigated the extracellular Ca^{2+} requirement for the effect of ADH on osmotic permeability. We have summarized reported results in Table 1. Listed are only those studies in which at least a minimal Ca^{2+} concentration was present in either or both of the bathing media. In these experiments no Ca^{2+} chelators were added to the "Ca^{2+}-free media" so that presumably *some* Ca^{2+} contamination persists (*cf.* [31]).[1] In most instances a degree of inhibition of the ADH response has been observed when the Ca^{2+} concentration has been drastically lowered.

As Table 1 illustrates, apparent contradictions in the literature preclude any firm conclusion. Clarification of this dilemma requires consideration of sev-

[1] Table 1 does not include data from the work of Bentley [4] in which the toad bladders were incubated with media nominally free of Ca^{2+} on both sides and in which the hydroosmotic response was inhibited by 55%; we assume that in this case there was a dissipation of the osmotic gradient, due to opening of tight junctions.

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Table 1. Reported effects of low Ca^{2+} concentrations on the ADH-induced J_v in amphibian urinary bladder

Species	Ca^{2+} concentration (mM)		ADH (mU · ml^{-1})	% change of J_v	Reference
	Control	Experimental			
	Mucosa/Serosa	Mucosa/Serosa			
B. marinus	0.54/2.7	0.54/0.27	1	−29	[4]
B. marinus[a]	0.20/2.0	0.20/0.20	20	−47	[20]
B. marinus[b]	0.20/1.0	0.20/0.20	20	−77	[2]
B. marinus	0.40/2.0	0.40/0.0	1	+68	[31]
R. esculenta[c]	0.05/1.0	0.05/0.0	15	−15	[48]
	0.05/1.5	0.05/0.0		+12	
	0.05/3.0	0.05/0.0		+30	
R. grylio[a]	0.20/2.0	0.20/0.20	20	−83	[21]

[a] Serosal Ca^{2+} lowered at the peak of the response. In all other cases Ca^{2+} was lowered before stimulation with ADH.

[b] Change in response to ADH in low Ca^{2+} calculated by comparing with the mean of several control responses in different bladders reported in the same work.

[c] Change in response to ADH in low Ca^{2+} calculated by comparing with different curves reported in one figure of the same work. When the responses in 1.5 and 3.0 mM serosal Ca^{2+} are compared to that in 1.0 mM serosal Ca^{2+}, in the same figure, the responses are inhibited by 25% and 40%, respectively.

eral issues. It has been shown that the complete absence of Ca^{2+} does not interfere with the binding of ADH to the receptor but that a Ca^{2+} concentration of 10^{-6} M is needed for a full activation of adenylate cyclase by the hormone [32]. The inhibitions of the response listed in Table 1 were obtained with Ca^{2+} concentrations greater than 10^{-6} M and therefore are not logically due to inadequacy of the hormone-receptor interaction, or to insufficient accumulation of cyclic AMP.

When cells are incubated in Ca^{2+}-free media, a depletion of the intracellular exchangeable pools of Ca^{2+} may ensue [6]. If intracellular Ca^{2+} is a mediator of the response to ADH [20–24, 31, 33, 37], the depletion of intracellular Ca^{2+} pools may result in an inhibition of the hormonal effect that is not due to a lowering of extracellular Ca^{2+} (*cf.* [6]). In most systems, cytosolic Ca^{2+} concentration has been estimated to be about 10^{-7} M [38]. In the experiments listed in Table 1 the concentrations of extracellular Ca^{2+} are between 20- to 5,000-fold higher than the cytosolic Ca^{2+} concentration. Therefore, it is doubtful that the inhibitions were due to a loss of cellular Ca^{2+}.

Since Ca^{2+} is one of the factors that maintain cell adhesion in the epithelial structure, in question is whether inhibition is due to disruption of intercellular junctions and consequent dissipation of the transmural osmotic gradient. From studies on the surface potential of isolated cells of toad bladder it has been concluded that a Ca^{2+} concentration of 2×10^{-5} M is sufficient to maintain the adhesion of epithelial cells in the intact tissue [34]. The studies cited in Table 1 were performed with medium Ca^{2+} concentrations 10- to 30-fold greater than the minimum needed for tissue integrity. Nonetheless, in these reports there were no studies of tissue structure, passive transepithelial permeability to large molecules, or of maintenance of the osmotic gradient.

The analysis of a flow process across a system with multiple barriers in series provides a readily appreciated biological example of the Heisenberg principle. The many reported instances of rectification by epithelia (e.g., as reviewed in Ref. 30) illustrate this. In the specific case of ADH-induced water flow across the amphibian urinary bladder, it is clear that the pathway for water is modified during osmotic flow. The generally accepted view is that the hormone-triggered facilitation of transmural osmosis appears to be due to a specific action on the granular cells of the epithelium [7, 14], where enhanced water permeability is a consequence of the formation of aqueous pores or channels in the luminal membrane [26, 47]. Water crosses the apical membrane to the serosal side through the intercellular spaces; concomitantly the volume flow induces profound granular cell swelling and expansion of the intercellular spaces [12, 14].

It is logical to assume that the measured rate of water flow is strongly dependent upon details of these complex geometrical modifications. Consequently, any dependence of the structure of the system on extracellular Ca^{2+} (even apart from that of the tight junction) is potentially able to influence water flow determinations. In light of the several considerations raised above, we have designed the present study to reexamine this issue in detail.

Materials and Methods

Urinary bladders were isolated from double-pithed, female toads (*Bufo marinus*) from the Dominican Republic. In all experiments, sac volume was 5 ml and beaker volume was 80 ml, unless otherwise stated.

Solutions

The composition of the standard Ringer's solution was as follows (in mmol/liter): NaCl, 110; KCl, 5; $CaCl_2$, 1; glucose, 5; Tris-HCl, 5; pH 8.0–8.1; osmolality, 233 mOsm/kg H_2O. Mucosa-to-serosa osmotic gradients were generated by lowering the mucosal NaCl concentration to 15 mM (osmolality 58 mOsm/kg H_2O). In all cases "Ca^{2+}-free" saline was made by deleting $CaCl_2$ from the otherwise unchanged solutions. Double-distilled, deionized water was used and plastic (rather than glass) materials were employed throughout to minimize Ca^{2+} contamination (*cf.* Ref. 43). Calcium concentrations in the bathing media were determined with an atomic absorption spectrophotometer (Perkin Elmer, model 107; Norwalk, CT) and Ca^{2+} electrodes (Beckman; Fullerton, CA). ADH, arginine vasopressin (Pitressin, Parke Davis; Morris Plains, NJ), was used at a supramaximal dose of 20 mU · ml^{-1} (7×10^{-8} M).

Gravimetric Determinations of Osmotic Water Flow

Volume flow was determined as described by Bentley [4]. The experiments were performed following a common protocol of three periods. Each period consisted of a single 20-min determination except for specific cases where three 10-min intervals were used in Period 3.

Period 1: To evaluate hydrostatically generated water flow, water shifts were examined in paired hemibladders in the presence of isosmotic solutions in both serosal and mucosal compartments.

Period 2: The experimental hemibladders were transferred to Ca^{2+}-free solutions. The change to the experimental medium was through two 10-min washing periods. Each hemibladder was suspended 1 min in a beaker where the Ringer was stirred at 100–200 rpm. It was then rinsed in a second beaker for 9 min. This washing procedure was then repeated. At transfer of the hemibladders from one beaker to the next, the hanging drop was blotted with filter paper. When the mucosal bath was changed to a Ca^{2+}-free solution, the same method was followed, except that rinses were with 5-ml aliquots of saline exchanged through the suspending cannula. Control hemibladders were identically treated but with the standard (Ca^{2+}-containing) Ringer's solution. Hydrostatistically-induced water flows were determined by weighing at the beginning and end of this period.

Period 3: An osmotic gradient ($\Delta\Pi$) of 4.3 atm was created by two serial exchanges of the mucosal bath with the hypoosmotic solution. The hemibladders were immediately weighed, placed in fresh medium (with or without ADH), and weighed again after 20 min.

Identical protocols were used to measure osmotic water flow in everted hemibladders.

Flow Calculations

The surface areas of hemibladders were derived from their contained volumes with the assumption that each was a perfect sphere. In the absence of an osmotic gradient, water shifts were minimal (between +2 and $-2\,\mu l \cdot cm^{-2} \cdot hr^{-1}$) so that the effect of hydrostatic pressure on transepithelial volume water flow was assumed to be negligible.

Under these circumstances the net water flow (J_v) is expressed by [1]:

$$J_v = L_p(RT\Delta C_{Na}\sigma_{Na}) \tag{1}$$

in which L_p ($cm \cdot sec^{-1} \cdot atm^{-1}$) is the coefficient of hydraulic conductivity; the expression ($RT\Delta C_{Na}\sigma_{Na}$) is $\Delta\Pi$ (atm), the osmotic pressure gradient, where R is the gas constant, T the absolute temperature, ΔC_{Na} is the transepithelial difference in Na^+ concentration, and σ_{Na} is the reflection coefficient for Na^+ (in this case assumed to be unity).

For comparison with results obtained with measurements of diffusional water permeability, L_p was expressed as a coefficient of osmotic permeability [1]:

$$P_f = L_p RT(\bar{V}_w)^{-1} \tag{2}$$

where P_f (in $cm \cdot sec^{-1}$) is the osmotic permeability and $\bar{V}_w$ is the partial molal volume of water.

Osmolality of the solutions were determined by freezing-point depression (Fiske automatic osmometer, model 130; Uxbridge, MA). In experiments where small samples (10 µl) were taken from the mucosal bath to follow changes in $\Delta\Pi$, equivalent volumes were replaced and osmolality was measured with a vapor-pressure osmometer (Wescor, model 5100A; Logan, UT).

Osmotic Permeability Measurements in Glutaraldehyde-Fixed Bladders

These experiments were performed with a technique similar to that described by Eggena [15, 16]. Before fixation the protocols followed were as described for the gravimetric determination of volume flow except for variation in the time of application of an osmotic gradient in Period 3. At the end of the third period a 50% glutaraldehyde solution (Electron Microscopy Sciences; Ft. Washington, PA) was introduced in the mucosal bath to yield a final concentration of 1%. After 5 min, the mucosal bath was washed twice with fresh solution and the serosal bath changed. *The post-glutaraldehyde mucosal change was always done with isosmotic Ringer, irrespective of the osmolality of the mucosal bath during the fixation period.* After 15 min the mucosal bath was again twice changed for a hypoosmotic Ringer. The bladder was weighed and the water loss assessed every 5 min over a 30 min interval.

Experimental values were plotted as J_v *vs.* time and P_f was computed with Eq. (2) from the intersection on the y axis (J_v) at $t = 0$ min of a regression line.

Determinations of Diffusional Water Permeability

Most of these experiments were performed on everted bladders with a technique modified from one previously described [25, 35]. The experimental protocol, before preparing the hemibladders for the measurement of diffusional permeability, was the same as described for the determination of osmotic permeability.

All of the experiments above were performed with isosmotic media in the mucosal and serosal compartments. In additional experiments with everted bladders, a $\Delta\Pi$ of 4.3 atm was employed for sequential measurement of bulk flow and THO unidirectional flux. The diffusional permeability coefficients for water (P_{D_w}, in $cm \cdot sec^{-1}$) were computed from the Fick equation and the surface area was calculated from volume, assuming a perfect sphere:

$$P_{D_w} = J_{THO}^{M \to S}[\Delta C_{THO}\, 4\Pi(3V/4\Pi)^{2/3}]^{-1} \tag{3}$$

where, $J_{THO}^{M \to S}$ is the net increase of THO, per unit time, in the system (tissue plus serosal bath), ΔC_{THO} is the mean concentration gradient of THO on both mucosa and the system during the period of measurement, and V is the volume, calculated from the weight.

The P_{D_w} obtained in the experiments were expressed as mucosa-to-serosa diffusional water fluxes, in $\mu l\,cm^{-1}\cdot hr^{-1}$ [28]:

$$J_w^{M\to S} = P_{D_w}(C_w \cdot a_w)\,\bar{V}_w \quad (4)$$

where C_w is the molar concentration of water and a_w is the water activity coefficient of the solution, which was taken as 0.99646 [39].

Determinations of Inulin and Mannitol Permeabilities

For these experiments the bladders were filled with 3 ml of Ringer containing 10 μCi of ^{14}C-inulin or ^{14}C-mannitol. The bladders were suspended in media stirred at 300–400 rpm. Tracer permeability (P_{in} or P_{man}) was determined in the mucosa-to-serosa direction in the absence of either serosal or mucosal Ca^{2+} or by withdrawing Ca^{2+} from both compartments. Calcium was removed from the corresponding bath by five rinses with Ca^{2+}-free solution before the addition of radioactive tracer to the mucosa. In experiments with a Ca^{2+}-free mucosal bath, the fifth rinse contained the labeled solute. After 20 min the mucosal and serosal baths were sampled for scintillation counting and the bladders were transferred to fresh bathing medium for an additional 20 min before a second sample was taken. Results were compared to the tracer permeability of paired control hemibladders which were bathed with Ca^{2+} on both sides. P_{in} and P_{man} were computed with Eq. (3).

All radioisotopes and the scintillation fluid (Aquasol) were purchased from New England Nuclear (Boston, MA). Scintillation counting was done in a 1215 Rackbeta (LKB Instruments; Rockville, MD). Standard corrections for efficiency and quenching were used.

Determinations of Na^+ Transport

Net Na^+ trnasport was monitored with a standard short-circuit current technique. Bladders with stable short-circuit currents and potential differences of at least 20 μA/cm^2 and 40 mV were used. The bladders were short circuited throughout the experiment, except for brief periods during which the spontaneous potential difference was measured to derive values for transmural resistance.

Optical and Electron Microscopy

The methodology followed was the same as previously described [14]. At appropriate times a 50% glutaraldehyde solution was added to the mucosal and serosal baths of the hemibladders, to a final concentration of 1%. Stirring of mucosa and serosa were achieved by vigorous aeration of both baths. After 30 min, pieces of approximately 1 cm^2 were removed and left overnight in Sorensen's phosphate buffer with 1% glutaraldehyde. Subsequently, the tissues were washed in Sorensen's buffer, post-fixed in OsO_4, dehydrated, and embedded in an Epon-Araldite mixture. Sections were cut on a Sorvall MT-5000 ultramicrotome (DuPont Co., Newtown, CT). One-micrometer sections for light microscopy were stained with Toluidine Blue; thin sections were stained with uranyl acetate and lead citrate and examined in a JEOL 100CX-TEMSCAN electron microscope (Japan Electron Optics; Peabody, MA). All histologic samples were coded to prevent bias in interpretation. A minimum of five pairs of samples were examined for each experimental condition studied.

Calculations

Data reduction was performed with the Prophet computer system (Biological Handling Program, Division of Research Resources, National Institutes of Health). Results are expressed as mean ± SEM. Standard programs for linear regression and *t*-tests for paired data were used.

Table 2. Effect of Ca^{2+} withdrawal from mucosal and/or serosal solutions on the transepithelial ($M\to S$) ^{14}C-inulin permeability

Ca^{2+} on Mucosa/Serosa	P_{in} ($cm\cdot sec^{-1}\times 10^7$)		n
	20 min	40 min	
+/+	1.0 ± 0.2	0.7 ± 0.3	7
−/+	1.6 ± 0.5	1.8 ± 0.7	
+/+	0.9 ± 0.1	1.0 ± 0.3	7
+/−	2.0 ± 0.4	1.9 ± 0.5	
+/+	0.6 ± 0.3	0.9 ± 0.2	6
−/−	16.3 ± 4.8[a]	67.9 ± 12.5[a]	

[a] $p<0.001$

Results

I. Ca^{2+}-Removal, Solute Permeability, and Tissue Integrity

Since disruption of tight junctions with Ca^{2+} removal depends on the use of Ca^{2+}-chelating agents [31, 48], we attempted to preserve tissue polarity, using only repetitive washing to remove Ca^{2+}. The integrity of epithelia in Ca^{2+}-free media was assessed by transepithelial passive permeability to large molecules and by structural studies.

In the presence of Ca^{2+}, the inulin permeability (P_{in}) of toad bladder is marginally detectable; with complete Ca^{2+} removal from both sides, P_{in} increases some 60-fold, in parallel with the opening of the tight junctions [29]. We measured P_{in} with removal of Ca^{2+} from mucosa, from serosa, and from both sides of the tissue. Results were compared to the P_{in} of paired hemibladders which contained Ca^{2+} on both sides. The results are reported in Table 2. Removal of either mucosal or serosal Ca^{2+} produced small and statistically insignificant increases in P_{in}, probably due to a modest degree of tight junction disruption. However, with Ca^{2+} withdrawal from both sides, P_{in} increased by 27- and 75-fold over 20- and 40-min periods, respectively.

Permeability to mannitol (P_{man}), tested in the mucosa-to-serosa and serosa-to-mucosa directions, after 20 and 40 min of serosal Ca^{2+} withdrawal, was identical in each of the periods studied and in either type of preparation. The mean results for both periods for control and experimental hemibladders, respectively, were (in $cm\cdot sec^{-1}\times 10^7$): mucosa-to-serosa, 3.1 ± 0.4 and 2.8 ± 0.7 ($n=5$, $p>0.5$); serosa-to-mucosa, 3.0 ± 0.7 and 3.3 ± 0.4 ($n=7$, $p<0.2$). As with P_{in} determinations, it seems safe to conclude that no significant paracellular solute pathways are produced by these manipulations.

Electron microscopic examination of tissues handled in this way was compatible with tracer-permeability determinations. No significant alterations of junction structure were noted with Ca^{2+} removal from either bath, while complete cell-cell separation was common after incubation with Ca^{2+}-free media on both sides. Observations on *in vitro* preparations with differential interference-contrast optics [11, 12] revealed cells floating free in the mucosal medium only minutes after Ca^{2+} removal from both tissue surfaces.

II. Effects of Withdrawal of Serosal Ca^{2+} on Basal and ADH-Stimulated Na^+ Transport

Nine experiments were performed where Ca^{2+} was deleted with five 30-sec washes of the serosal chamber with Ca^{2+}-free Ringer's solution. After 20 min of serosal Ca^{2+} removal, the short-circuit current (I_{sc}) increased from 37 ± 6 to $46 \pm 8\ \mu A \cdot cm^{-2}$, the potential difference (E_t) decreased from 60 ± 9 to 32 ± 10 mV, and the transepithelial conductance (G_t) increased from 0.75 ± 0.09 to 1.25 ± 0.12 mmho $\cdot cm^{-2}$. These changes were statistically significant ($p < 0.05$) when compared either with values obtained prior to Ca^{2+} withdrawal or with those of paired control hemibladders, in which five changes with a Ca^{2+}-containing solution were made.

Addition of ADH to the Ca^{2+}-free serosa produced (after 20 min) an increase in I_{sc} of $22 \pm 7\ \mu A \cdot cm^{-2}$ but no change E_t, thus G_t increased further to 2.25 ± 0.15 mmho $\cdot cm^{-2}$. In control tissues ADH increased I_{sc} by $25 \pm 9\ \mu A \cdot cm^{-2}$ after 20 min; control and experimental hemibladders were statistically indistinguishable in this regard.

III. Effects of Ca^{2+} Withdrawal on Basal and ADH-Stimulated Water Permeabilities

A. Osmotic Permeability

The absence of either mucosal or serosal Ca^{2+} alone did not alter osmotic permeability in the absence of ADH. With hormone addition, permeability in the absence of mucosal Ca^{2+} was significantly higher than in control tissues. Without serosal Ca^{2+}, the ADH-induced increase in osmotic permeability was severly blunted; J_v was diminished by $123 \pm 6\ \mu l \cdot cm^{-2} \cdot hr^{-1}$ ($62 \pm 2\%$ inhibition). The results of these experiments are reported in Table 3. Osmolality determinations assured that no dissipation of the imposed gradient occurred over the course of these experiments.

The Effect of Replacing Serosal Ca^{2+} with Other Divalent Cations: Experimental hemibladders were washed for 20 min in four changes of a serosal bath in which $CaCl_2$ was quantitatively replaced by the chloride salt of each of several divalent cations. Under these conditions, Sr^{2+} was the only substitute cation that effectively replaced Ca^{2+}; Zn^{2+} seemed to have an additional deleterious effect (as reported for Cu^{2+} [*cf.* 25]). The sequence of divalent cations for effective sustenance of the ADH-induced increase in J_v was:

$$Ca^{2+} = Sr^{2+} \gg Ba^{2+} \cong Mn^{2+} > Mg^{2+} \gg Zn^{2+}$$

These results coincide with some observations [29], but conflict with others [4].

Table 3. Effect of Ca^{2+} withdrawal from mucosal and/or serosal solutions on basal and ADH-stimulated volume water flow

Ca^{2+} on Mucosa/Serosa	ADH	J_v ($\mu l \cdot cm^{-2} \cdot h^{-1}$)	P_f ($cm \cdot sec^{-1} \times 10^4$)	n	p
+/+	−	3.8 ± 0.2	3.3 ± 0.2	7	> 0.2
−/+		4.0 ± 0.3	3.5 ± 0.3		
+/+	+	201 ± 17	177 ± 15	9	< 0.05
−/+		217 ± 12	191 ± 11		
+/+	−	4.3 ± 0.4	3.8 ± 0.3	11	> 0.5
+/−		4.2 ± 0.5	3.7 ± 0.4		
+/+	+	192 ± 5	169 ± 5	46	< 0.001
+/−		70 ± 3	62 ± 3		

Table 4. Effect of Ca^{2+} withdrawal from mucosal and/or serosal solutions on basal and ADH-stimulated diffusional permeability to water

Ca^{2+} on Mucosa/Serosa	ADH	P_{D_w} ($cm \cdot sec^{-1} \times 10^4$)	$J_w^{M \to S}$ ($\mu l \cdot cm^{-2} \cdot hr^{-1}$)	n	p
+/+	−	0.9 ± 0.05	321 ± 20	8	< 0.05
−/+		1.1 ± 0.04	404 ± 6		
+/+	+	3.8 ± 0.3	1,365 ± 51	7	< 0.05
−/+		4.0 ± 0.1	1,445 ± 40		
+/+	−	0.9 ± 0.05	333 ± 19	9	> 0.5
+/−		0.9 ± 0.06	337 ± 22		
+/+	+	3.4 ± 0.1	1,237 ± 44	11	> 0.5
+/−		3.4 ± 0.1	1,226 ± 35		

B. Diffusional Permeability

Results of the experiments are shown in Table 4. Withdrawal of mucosal Ca^{2+} produced small but significant enhancements of P_{D_w}. The absence of serosal Ca^{2+} modified neither the basal nor the ADH-stimulated values for this parameter.

Results obtained on the increase in P_{D_w} (Table 4) produced by ADH when serosal Ca^{2+} was withdrawn are strikingly different from the effects on

Table 5. Effect of Ca^{2+} withdrawal from serosal solution on ADH-stimulated net and unidirectional water fluxes across everted bladders

	J_v	$J_w^{M\to S}$	P_f	P_{D_w}
	($\mu l \cdot cm^{-2} \cdot h^{-1}$)		($cm \cdot sec^{-1} \times 10^4$)	
Control	191±23	1,240±30	168±18	3.5±0.1
Experimental	64±12	1,210±40	56± 9	3.4±0.1
p	<0.001	>0.2	<0.001	>0.2

Net and unidirectional fluxes were determined for each preparation concomitantly. ($n=9$)

P_f (Table 3). To account for the possibility that Ca^{2+} contamination (8×10^{-6} M) of the serosal bath of everted bladders might be sufficient to sustain the response to ADH while that of noneverted bladders is not, two types of experiment were performed.

a) P_{D_w} was measured in noneverted hemibladders under the conditions in which an inhibition of P_f was obtained, but with no applied osmotic gradient. Results for six paired hemibladders were: control, 3.5 ± 0.2 cm $\cdot$ sec$^{-1} \times 10^4$ ($1{,}240 \pm 60$ $\mu l \cdot cm^{-2} \cdot hr^{-1}$); experimental, 3.5 ± 0.3 cm $\cdot$ sec$^{-1} \times 10^4$ ($1{,}261 \pm 62$ $\mu l \cdot cm^{-2} \cdot hr^{-1}$). It is apparent that P_{D_w} was unaffected under conditions in which P_f was strongly inhibited.

b) P_f and P_{D_w} were measured serially in individual everted hemibladders, in the presence of an applied osmotic gradient. Results of these experiments are provided by Table 5. In everted hemibladders J_v was inhibited by $66 \pm 7\%$ while $J_w^{M\to S}$ was not modified. Osmolality determinations showed that $\Delta\Pi$ was 4.3 atm throughout the measurements. Although no corrections for solvent drag were made, P_{D_w} in the presence of a gradient (Table 5) was the same as that obtained in its absence (Table 4).

In experiments with everted bladders we observed that, 40 min after Ca^{2+} withdrawal from both baths, $J_w^{M\to S}$ increased from 372 ± 31 to $1{,}143 \pm 72$ ml $\cdot$ cm$^{-2} \cdot$ hr^{-1} ($n=7$, $p<0.001$). To test whether ADH might still increase $J_w^{M\to S}$ with Ca^{2+}-free media on both sides, the following experiment was performed . Ca^{2+} was absent from the mucosal and serosal baths of both control and experimental tissues for 20 min. ADH was introduced for a 20-min interval in the experimental hemibladders while the controls were incubated further in the Ca^{2+}-free media. In this way each tissue was incubated for a total of 40 min in Ca^{2+}-free media, but one had ADH for the last 20 min in order to detect any effect of ADH against the large $J_w^{M\to S}$ of the "background." Under these conditions ADH produced an increase of $J_w^{M\to S}$ from $1{,}139 \pm 68$ to $1{,}344 \pm 70$ ($n=7$, $p<0.001$).

IV. Details of P_f Inhibition

A. Time-Dependent Inhibition of P_f

Experiments were performed with three 10-min determinations in the experimental period (Period 3); results are shown in Fig. 1. The effects of ADH on the P_f of control and experimental hemibladders appear divergent — the inhibition increasing with time (Fig. 1*A*). Fractional change of P_f (P_f experimental/P_f control) was plotted as shown in Fig. 1*B*. Inhibition of the osmotic permeability produced by deletion of serosal Ca^{2+} increases linearly with time. Since Ca^{2+} was withdrawn 20 min before simultaneous imposition of the osmotic gradient and ADH, the result cannot be interpreted as due to a progressive fall in the concentration of serosal Ca^{2+}. Nor was it due to a progressive dissipation of $\Delta\Pi$ since osmolalities were measured as constant throughout the experiments. A line drawn through the experimental points at 10, 20 and 30 min intersects the y axis ($t=0$ min) at a P_f ratio >1. If deletion of serosal Ca^{2+} were to blunt the effect of ADH on P_f it should do so from the onset, and the fractional change at $t=0$ would be <1. It appears that factors other than Ca^{2+} absence generate the inhibition of the ADH-induced flow and that they do so as the response develops.

B. Morphological Features

Bladder structure was examined for each of the several experimental manipulations employed. Fig. 2*a–c* provide comparative light microscopic views of the principal findings. Tissue where no Ca^{2+} removal was employed exhibited the expected appearance when fixed at the height of an ADH-induced water-flow response (Fig. 2*a*); granular cells were preferentially swollen and intercellular spaces were noticeably dilated while the contacts between adjacent cells were maintained through fine plicated extensions of the cell membranes. In bladders where a Ca^{2+}-free serosa was employed, the imposition of ADH-induced water flow resulted in a more marked distension of the intercellular spaces (Fig. 2*b*), often resulting in an apparent separation of the epithelium into two discrete "layers" of cells: a superficial layer of granular, mitochondria-rich and goblet cells with maintenance of junctional contacts at the mucosal edge and an incomplete layer of basal cells resting

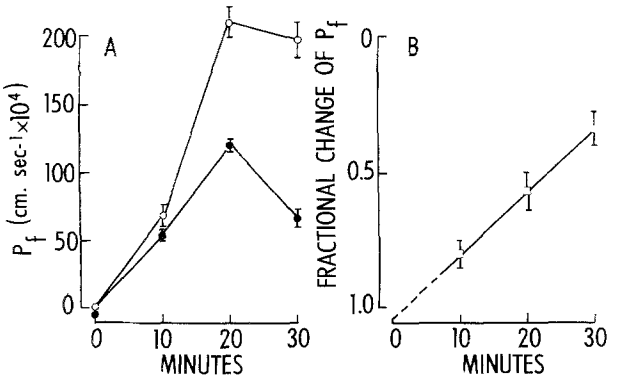


Fig. 1. Effects of withdrawing serosal Ca^{2+} on the ADH-induced osmotic permeability (P_f). (*A*): Experimental hemibladders (○) were incubated with Ca^{2+}-free serosal saline in the absence of an osmotic pressure gradient ($\Delta\Pi$) for 20 min before adding ADH (20 mU · ml^{-1}) and imposing a $\Delta\Pi = 4.3$ atm at $t = 0$ min. Control hemibladders (○) were treated identically but with 1 mM serosal Ca^{2+}. (*B*): P_f data from *A* calculated as fractional inhibition (Experimental P_f/Control P_f). The broken line is the extrapolation to $t = 0$ of the line that connects the experimental points

on the basal lamina. The degree of separation between these layers was highly variable often extending to as much as 75 μm in sectioned views while adjacent regions occasionally looked identical to tissue fixed without prior Ca^{2+} removal. In every case, however, with blind examination positive identification of the experimental tissue (Ca^{2+}-free serosa) was unequivocal on the basis of this difference in the appearence of the intercellular space. Removal of serosal Ca^{2+} did not produce this gross distension of the intercellular space in the absence of an osmotic flow. Figure 2*c* illustrates the appearance found consistently in tissues fixed 20 min after ADH addition to bladders incubated in Ca^{2+}-free serosal medium but with no imposed osmotic gradient; no cell separation of any consequence was found beyond that often seen in these circumstances and attributable to ADH action on the submucosal smooth muscle [13].

Transmission electron microscopy (Fig. 3*a–c*) illustrated the fact that Ca^{2+} removal from the serosa coupled with space distension during water flow resulted in a disruption of desmosomes (and presumably gap junctions) between adjacent epithelial cells. Figure 3*a* shows a portion of the "elevated" layer of surface cells from a preparation where epithelial splitting was most pronounced. (Note the light micrograph insert in Fig. 3*a*.) In Fig. 3*b*, dense thickenings of the basolateral aspect of a granular cell membrane are identified as probable sites of formerly intact desmosomal connections. Similar structures were found on basal cell membranes (on mucosally oriented surfaces). Tight junctions (or *zonulae occludentes*) were almost never affected by this procedure while the *zonulae adherentes* (intermediate junctions) and desmosomes of the apical junctional complex were often at least partially disrupted (Fig. 3*c*).

C. Osmotic Permeability and Structure of Glutaraldehyde-Fixed Bladders

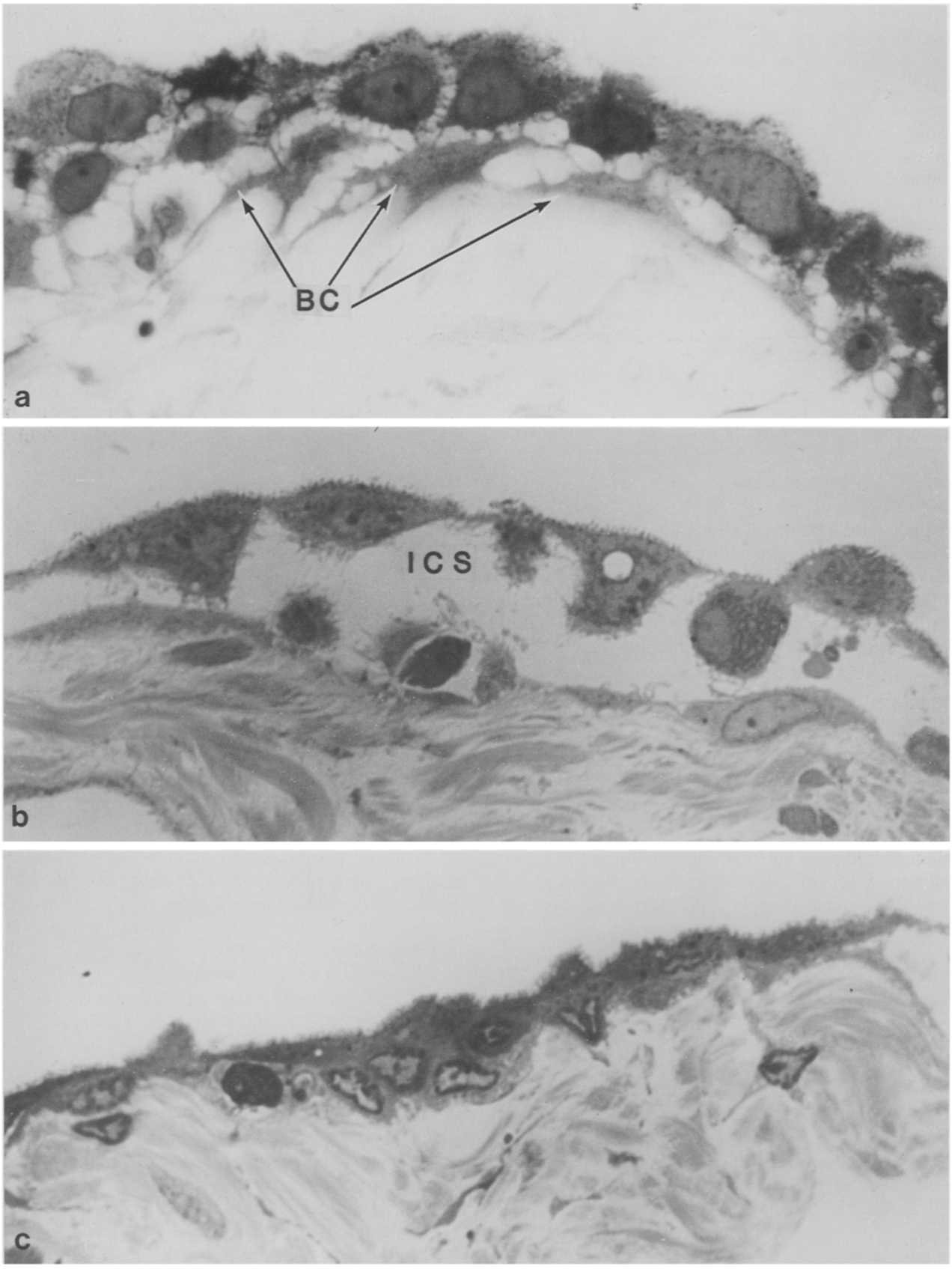
Based on these observations we formulated a working hypothesis to explain why the effect of ADH on osmotic permeability appears to be inhibited in the nominal absence of serosal Ca^{2+}. It may be that ADH produces a normal activation of all of the cellular mechanisms which lead to the increase in water permeability even in the absence of serosal Ca^{2+}. The hormonal effect on the osmotic permeability of the epithelium is *apparently* blunted because the resulting bulk water flow perturbs important geometric relationships among the cellular components of the epithelial system. As a test of this hypothesis, it seemed advantageous to measure the effect of ADH on the P_f of bladders incubated without serosal Ca^{2+} and in the absence of an osmotic gradient.

To do this we turned to a modification of the glutaraldehyde-fixation technique developed originally by Eggena [15, 16]. Tissues were stimulated with ADH in the presence or absence of serosal Ca^{2+}, fixed briefly (*see* "Methods") and only then exposed to a gradient for determinations of the rate of osmotic flow. Intercepts of the regression lines on the *y* axis (P_f) showed that the P_f (in cm · $sec^{-1} \times 10^4$) was 196 ± 10 in the presence of serosal Ca^{2+} and 209 ± 11 in its absence ($n = 9$, $p > 0.05$).

A test of whether or not this finding was artifactual was then performed. The experiment was repeated, but bladders were fixed after a gradient was imposed for 20 min. In this case P_f values were 186 ± 12 cm · $sec^{-1} \times 10^4$ in the presence of Ca^{2+} and 65 ± 9 cm · $sec^{-1} \times 10^4$ in its absence ($n = 7$, $p < 0.001$).

In light of these results additional experiments were performed in which tissues were fixed after imposing the gradient at selected times. Hemibladders were handled as above, but the gradient was created 5, 10 or 15 min before the end of the 20-min period of incubation with ADH. For each experiment the mean percent inhibition of P_f due to Ca^{2+} absence (as compared to the P_f of the Ca^{2+}-containing, paired hemibladder) was plotted as a function of the time during which the osmotic gradient was imposed. Results are shown in Fig. 4.

The amount of inhibition of P_f increased linearly


BC
a
ICS
b
c

between 0 and 10 min after imposing the osmotic gradient. At $t=0$ the P_f of the Ca^{2+}-free hemibladders is $7\pm4\%$ higher than in the presence of Ca^{2+}. After $t=10$ min ($24\pm1\%$ inhibition) there is a sudden jump in the amount of inhibition ($63\pm7\%$ at 15 min and $67\pm2\%$ at 20 min), reaching a level comparable to the 62% inhibition of P_f found in nonfixed bladders after 20 min of gradient application (Table 3). Therefore, it appears that the amount of inhibition of ADH-induced P_f in the absence of serosal Ca^{2+} is more dependent on the magnitude of J_v than on any direct effect of Ca^{2+} withdrawal upon the hormonal action on osmotic permeability.

Morphological features of the bladders used in the glutaraldehyde-fixation technique: Paired hemibladders were incubated without serosal Ca^{2+} and with or without a gradient during ADH stimulation. Twenty min after ADH addition both were fixed with 1% mucosal glutaraldehyde for 5 min and washed with isosmotic medium on both sides. A gradient was imposed across each hemibladder and 30 min later tissues were fixed and processed for electron microscopy.

Hemibladders in which P_f was determined after 20 min of gradient application exhibited, as before, a detachment of the granular and mitochondria-rich cells from the basal cells and supporting tissues. They were the hemibladders that displayed a 67% inhibition of P_f on average (Fig. 4). On the contrary, hemibladders in which the fixation procedure was performed prior to the application of a gradient displayed a maintenance of cell-cell contacts and, in many cases, very little distension of the intercellular spaces (Fig. 5). In these hemibladders the P_f was not different from the P_f of the Ca^{2+}-containing hemibladders (Fig. 4).

VI. Reversibility of the Inhibition of Osmotic Permeability

Based on the findings at this point in the study, we used a pair of protocols to test the reversibility of P_f inhibition. First (Fig. 6, curve *1*), serosal Ca^{2+} was withdrawn for 20 min before an osmotic gradient was imposed, ADH was added for 20 min, and the inhibition of P_f was recorded. The hypoosmotic mucosal bath was changed twice for isosmotic Ringer, ADH was washed, and Ca^{2+} was restored. Four separate experiments were performed in which the hemibladders were allowed to recover for 15, 30, 60 or 90 min. At the end of each recovery period the isosmotic mucosal bath was changed twice for hypoosmotic saline, ADH was added for 20 min, and P_f was recorded. Second (Fig. 6, curve 2), the first challenge was performed in the absence of a gradient, ADH was washed out, Ca^{2+} was restored, and the mucosal bath was changed again for an isosmotic bath. The bladders were left to recover for 15 min in one experiment and for 60 min in another. After the recovery period the mucosal bath was changed twice for the hypoosmotic Ringer, ADH was added for 20 min, and P_f was measured.

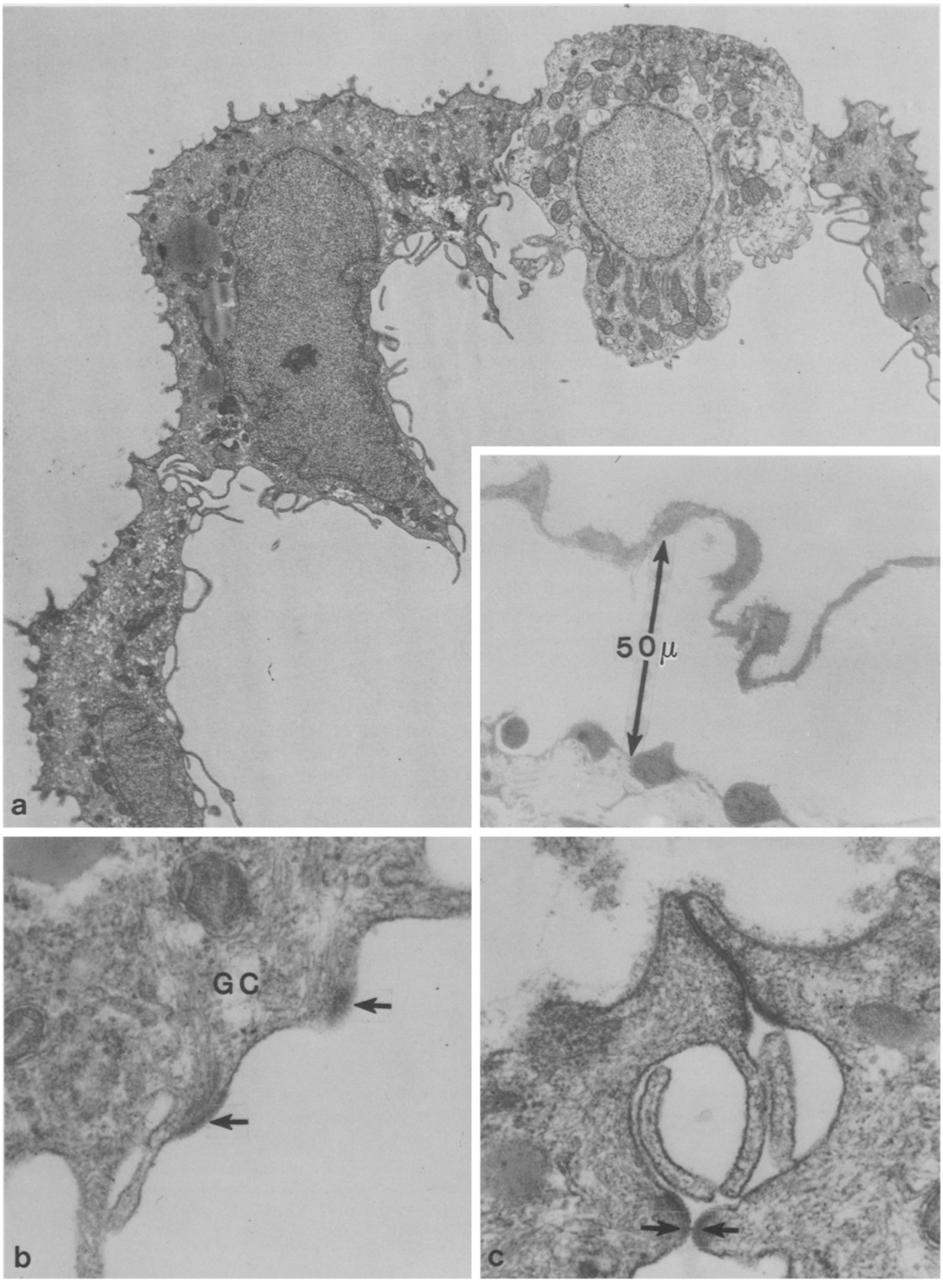
Results (shown in Fig. 6) are expressed as changes in percent inhibition of the experimental P_f as recorded in the second stimulation with ADH as a function of recovery time elapsed between first and second stimulation.[2]

When a gradient was present during each challenge but not during the recovery periods (curve 1 in Fig. 6) inhibition was slowly reversed. While in the first challenge inhibition was $66\pm3\%$ ($p<0.001$, $n=20$), when 30 min had elapsed the inhibition was reduced to $14\pm3\%$ ($p<0.05$, $n=6$). At 60 and 90 min no significant difference remained between control and experimental tissues.

On the contrary, when a gradient was present only during the second ADH stimulation (curve 2 in Fig. 6) no significant differences between the second responses of control and experimental hemibladders were observed even if only 15 min of recovery were allowed. At this point in recovery the experimental hemibladders tested with the first variation were still

[2] It is worth pointing out that in control hemibladders the P_f of the second challenge was the same, or larger, than the P_f of the first challenge. Therefore, it is valid to present the results in Fig. 8 as percent inhibition (or recovery) of the experimental hemibladders.

Fig. 2 (facing page). Comparative light microscopic views of ADH-stimulated hemibladders after exposure to differing experimental conditions. (*a*): Views of bladder epithelium fixed 20 min after ADH stimulation in the presence of a 4.3-atm osmotic gradient with no depletion of normal (1 mM) Ca^{2+} concentration in either bathing medium. The epithelium appears as often described previously with swollen surface (granular) cells, separated from a chain of basal cells (*BC*) by a dilated intercellular space that is transected by an array of slender stalks of attachment between cells. (*b*): When the epithelium is treated as above but with removal of serosal Ca^{2+} (as described in text), the intercellular space (*ICS*) is often distended to an extent that suggests disruption of the attachments between surface and basal cells. This view is representative of these preparations in that it shows an "average" separation of cells into layers as opposed to the often encountered extremes typified in Fig. 3. (*c*): Here the epithelium was fixed 20 min after ADH stimulation in the absence of serosal Ca^{2+} but with no applied osmotic gradient. The intercellular space remained closed here and in the majority of views from these samples. All figures shown are of toluidine blue stained sections at a magnification of 1600×


a
50μ
GC
b
c

inhibited by a mean of 33%. With the second variation, the P_f was $16 \pm 2\%$ higher in the experimental than in the control hemibladders when a 60-min recovery period had elapsed ($n=5$, $p<0.05$).

These results show that full reversibility of inhibition can be obtained but that restoration of Ca^{2+} is not the only requirement. As the development of flow is necessary to obtain inhibition of P_f, its suppression by cancellation of the gradient is essential to the recovery process.

Structural features of the reversibility process: The study was performed in paired hemibladders, following the first variation above. Each was incubated for 20 min in the absence of serosal Ca^{2+} before an osmotic gradient was imposed and the tissue exposed to ADH for 20 min. One of the hemibladders was fixed for structural study; in the other the gradient was eliminated and the tissue was transferred to a serosal solution without hormone and with 1 mM Ca^{2+}, where it remained for 90 min. After this recovery period the osmotic gradient was imposed and the preparation was challenged with ADH for 20 min before fixation and processing for structural study.

The results are presented in Fig. 7. Epithelial geometry, fixed after the first challenge, (Fig. 7*a*) was distorted as previously described (Figs. 4 and 5). After 90 min of recovery and after the bladder responded normally to a second ADH challenge (Fig. 6, curve *1*), epithelial morphology was indistinguishable from that of hemibladders never exposed to Ca^{2+}-free solutions. Recovery of a normal response to ADH appears to go hand in hand with reestablishment of a normal epithelial configuration.

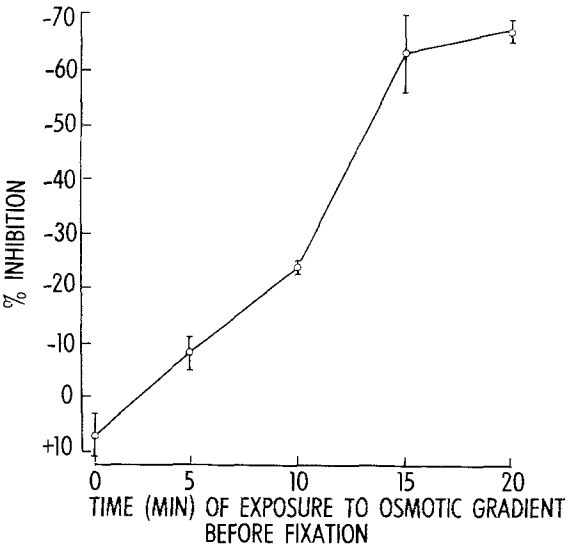


Fig. 4. Volume water flow (J_v)-dependent inhibition of the effects of ADH on osmotic permeability (P_f), determined in glutaraldehyde-fixed paired hemibladders. Serosal Ca^{2+} was removed from the experimental hemibladders for 20 min in the absence of an osmotic pressure gradient ($\Delta\Pi$). Then ADH (20 mU · ml^{-1}) was added for 20 min to both control and experimental hemibladders. The hormonal stimulation was performed in the absence of a $\Delta\Pi$ ($t=0$ min in abscissa), or a $\Delta\Pi=4.3$ atm was imposed during the last 5, 10 or 15 min of the ADH challenge or for the full 20 min of hormonal stimulation ($t=5$, 10, 15 and 20 min, respectively, in abscissa). Control and experimental hemibladders were fixed with 1% mucosal glutaraldehyde at the end of the 20-min ADH period, and P_f was computed as explained in the text. The P_f values of the experimental hemibladders (on the ordinate) are expressed as a percent of the response in the control hemibladders as: [(Experimental P_f/Control P_f) × 100] − 100

Discussion

The ADH-induced cascade of subcellular events that lead to increased water permeability of the toad urinary bladder [1, 19] appears to be substantially independent of extracellular calcium. Support for this conclusion comes from two fundamental observations which have been examined here in some detail. First is the maintenance of an intact ADH response as measured by the hormone's effect on diffusional water permeability. Second is the observation of dramatic morphological changes in the presence of osmotic flow when the serosal bathing medium is nominally free of calcium; the nature of these changes may be interpreted to explain the *measured* inhibition of osmotic permeability under these conditions.

A. Validity of Imposed Experimental Conditions

The principal criterion for examining the question of ADH dependency on extracellular calcium is the structural and functional integrity of the tissue under the conditions selected for investigation of this issue. With 1 mM Ca^{2+} in the mucosal bath and a

Fig. 3 (facing page). Electron microscopy of bladder fixed 20 min after ADH addition in the presence of an osmotic gradient and in the absence of serosal Ca^{2+} (as in Fig. 2*b*). (*a*): Note that the surface cells shown here are not separated one from another and that the basolateral margin is plicated in an extensive array of finger-like projections. The insert (a light micrograph of this preparation at a magnification of 1050×) illustrates that in this tissue the elevation of surface cells as a sheet was of the order of 50 μm or more over broad regions. 6250×. (*b*): The basolateral margin of granular cells (*GC*) in these preparations are studded with membrane thickenings (arrows), probably indicative of former sites of desmosome attachment to basal cells or adjacent granular cells. 62,700×. (*c*): At high magnification, it is clear that tight junction structure is well preserved in these preparations, while the subjacent intermediate junction and desmosome (unlabeled arrows) of the junctional complex show evidence of partial disruption. 62,700×

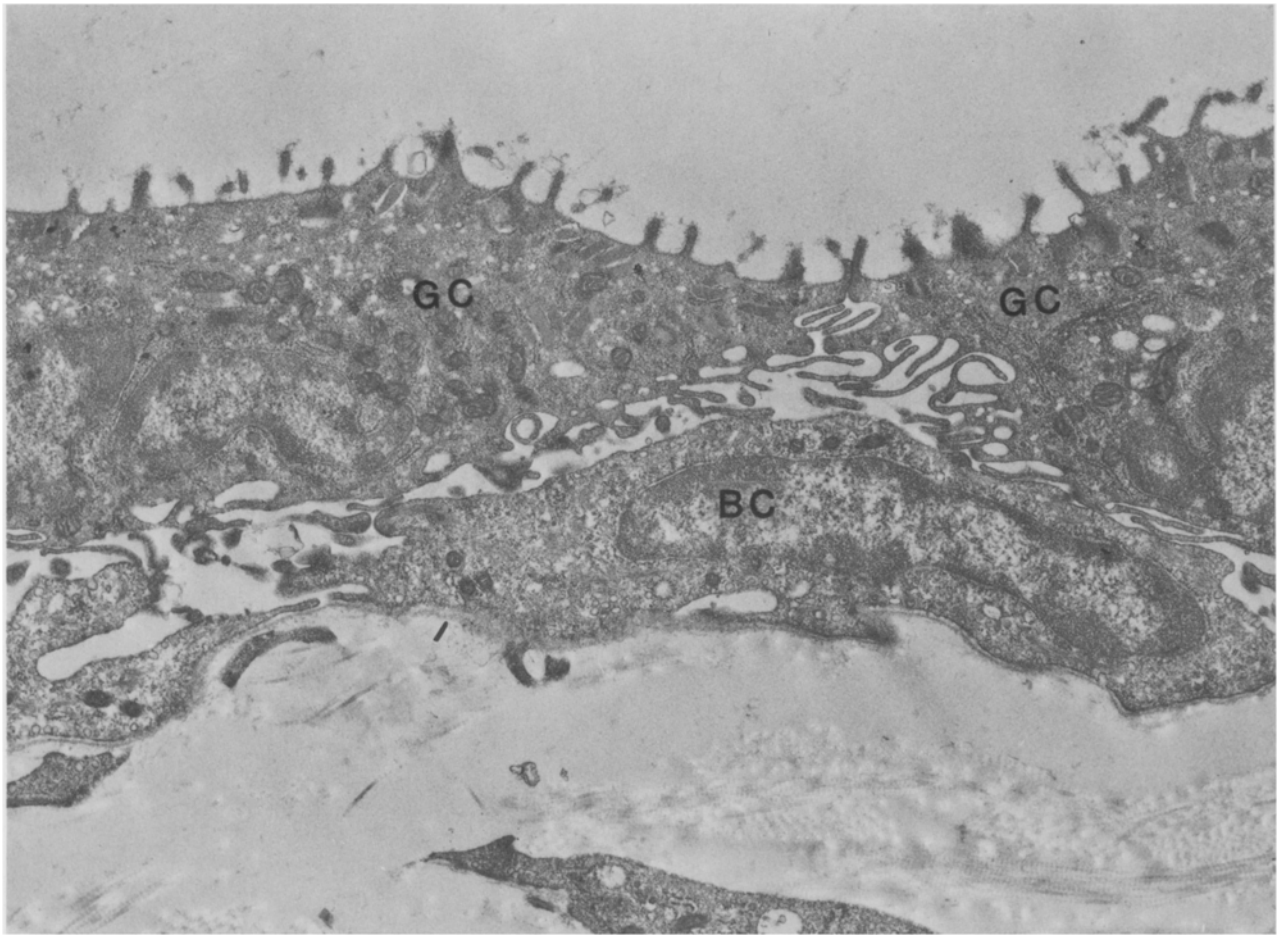


Fig. 5. Electron microscopic view of bladder fixed during peak water flow but where ADH stimulation, in the absence of serosal Ca^{2+}, was performed in the absence of a transmural osmotic gradient. Water flow was assessed here by application of a gradient only after the tissue was exposed briefly to glutaraldehyde as described in the text. Note that granular (*GC*) and basal cells (*BC*) are contiguous in numerous places and that the intercellular space is only slightly patent. 11,200×

Ca^{2+}-free serosal solution, transepithelial permeability to mannitol and to inulin was preserved at levels comparable to values under optimal physiologic conditions. Preservation of imposed osmotic gradients established that there were no prominent water or salt leaks resulting from serosal Ca^{2+} removal and the morphologic integrity of tight junctions and typical cellular ultrastructure was similarly intact. Normal responses to the natriferic action of ADH were largely maintained and, despite small increases in short-circuit current and total tissue conductance, it appeared that the electrical characteristics of the tissue were substantially unchanged. We conclude that Ca^{2+}-removal as we have applied it did not preclude meaningful investigation of the hormone dependence on significant extracellular concentrations of this cation.

B. Dissociation of ADH-Action on Osmotic and Diffusional Permeabilities to Water

It has been well established that, in ADH-sensitive epithelia, the hormone produces an increase in measured osmotic permeability (P_f) far in excess of its effect on diffusional permeability to water (P_{D_w}). The discrepancy has been attributed to cellular diffusion constraints, significant unstirred layers in series with the epithelium, and various "series-barrier" hypotheses [1, 10, 26, 30]. Recently, Rosenberg and Finkelstein [41, 42] have provided a physical-chemical basis for the discrepancy based on single-file diffusion through narrow aqueous channels; the magnitude of the P_f/P_{D_w} ratio after ADH-stimulation is far in excess of values that their reasoning can account for.

In our experiments, as summarized in Tables 3 and 4, ADH increased the ratio of P_f/P_{D_w} from 4 to 50 in the presence of serosal Ca^{2+} but only from 4 to 16 in its absence. This diminution indicates that Ca^{2+}-removal reduces the dissociation between the two permeability coefficients; measured individual values showed that it does so through selective inhibition of P_f. Because of the inherent difficulties in accurate measurement of P_{D_w}, maintenance of the ADH-effect here must be examined closely.

The presence of mucosal and serosal unstirred layers produce an underestimation of the real P_{D_w} of the tissue [1, 10, 11, 26, 30]. With the stirring conditions of our experiments (300–400 rpm)[3] we have obtained a P_{D_w} (in $cm \cdot sec^{-1} \times 10^{-4}$) of 0.9 ± 0.02 ($n = 24$) without ADH and 3.9 ± 0.08 ($n = 33$) with ADH. To analyze the contribution of the unstirred layers to the P_{D_w} values we have obtained in the presence of ADH, we will start by assuming that $3.9 \cdot 10^{-4}\, cm \cdot sec^{-1}$ is the P_{D_w} of an unstirred layer comprised of tissue, mucosal and serosal components. The operative thickness of this total (equivalent) unstirred layer (α_t) can be computed from the following Eq. (5):

$$\alpha_t = D_w^o R_{D_w}^a \tag{5}$$

where $D_w^o = 2.4 \times 10^{-5}\, cm^2 \cdot sec^{-1}$ is the free diffusional coefficient of water in water [39] and $R_{D_w}^a$ is the reciprocal of the experimental (apparent) coefficient of water diffusional permeability after ADH (2,654 $sec \cdot cm^{-1}$ in our experiments).

According to Eq. (5), α_t is 615×10^{-4} cm. To calculate the functional thickness of each of the three layers in this composite value we must make two more assumptions: (i) The value of the mucosal unstirred layer (α_a) will be taken as 30×10^{-4} cm from data obtained in frog skin with stirring rates of 300–500 rpm [9]. (ii) The thickness of the serosal unstirred layer (α_s) will be computed from the P_{D_w} obtained in the supportive tissues of toad bladder devoid of the epithelial cell layer [27]. Then, computed with Eq. (5), the value is $\alpha_s = 250 \times 10^{-4}$ cm. The mucosal and serosal unstirred layers would have a functional thickness $(\alpha_a + \alpha_s) = 280 \times 10^{-4}$ cm. The third layer is the tissue ("unstirrable") layer (α_e). Then,

$$\alpha_e = \alpha_t - (\alpha_a + \alpha_s) = 335 \times 10^{-4}\ \text{cm}. \tag{6}$$

In the presence of ADH and in the absence of unstirred layers it would be expected from previous assumptions that the diffusional permeability to water of the epithelium would be similar to an equivalent unstirred layer whose operative thickness for water is 335×10^{-4} cm. The P_{D_w} can be computed from Eq. (5) and is $7.2 \times 10^{-4}\, cm \cdot sec^{-1}$. This P_{D_w} is equal to $R_{D_w} = 1{,}389\, sec \cdot cm^{-1}$, which is similar to $R_{D_w} = 1{,}408\, sec \cdot cm^{-1}$ ($P_{D_w} = 7.1 \times 10^{-4}\, cm \cdot sec^{-1}$) found by Hays [26] through the zero intercept when R_{D_w} is plotted against the reciprocal of the stirring

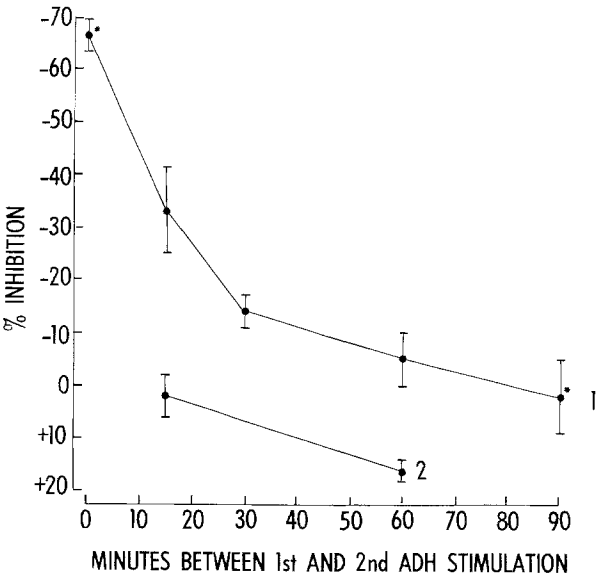


Fig. 6. Reversibility of the inhibition of the effects of ADH on osmotic permeability (P_f) produced by withdrawal of serosal Ca^{2+}. Paired hemibladders were incubated for 20 min with (control) and without (experimental) 1 mM serosal Ca^{2+} in the absence of an osmotic pressure gradient ($\Delta\Pi$) before stimulating with ADH (20 $mU \cdot ml^{-1}$). *Curve 1:* A $\Delta\Pi$ (=4.3 atm) was imposed at the time of ADH addition. After determining P_f, the hormone was washed out, the hypoosmotic mucosal saline was replaced with an isosmotic solution (abolishing $\Delta\Pi$) and 1 mM Ca^{2+} was readded to the serosa. The control and experimental hemibladders were allowed to recover under these conditions for 15 ($n=5$), 30 ($n=6$), 60 ($n=5$), and 90 ($n=4$) min. After the recovery period a $\Delta\Pi = 4.3$ atm was again imposed, ADH readded and the second P_f response determined. *Curve 2:* Conditions as for Curve *1*, except that the first stimulation with ADH was done in the absence of a $\Delta\Pi$. The bladders were allowed to recover (ADH washed and 1 mM Ca^{2+} readded) for 15 ($n=4$) and 60 ($n=5$) min. The second hormonal stimulation was done with a $\Delta\Pi = 4.3$ atm. *Ordinate:* [(Experimental P_f/Control P_f) × 100] − 100; *Abscissa:* time allowed for recovery between first and second ADH stimulations. The experimental point at $t=0$ in Curve *1* is the mean ± SEM of the inhibition of P_f in the first ADH challenge in all hemibladders ($n=20$). No P_f could be determined at $t=0$ min for Curve *2* since there was no $\Delta\Pi$. According to the experiments in glutaraldehyde-fixed bladders (Fig. 4), during the first ADH stimulation the P_f of the experimental hemibladders would be $7 \pm 4\%$ higher than that of the control hemibladders. The asterisks in Curve *1* denote the experimental periods in which paired hemibladders were processed for structural observations (Fig. 7)

[3] Low stirring rates were used in order to avoid damage to the weakened structure of Ca^{2+}-deprived tissues.

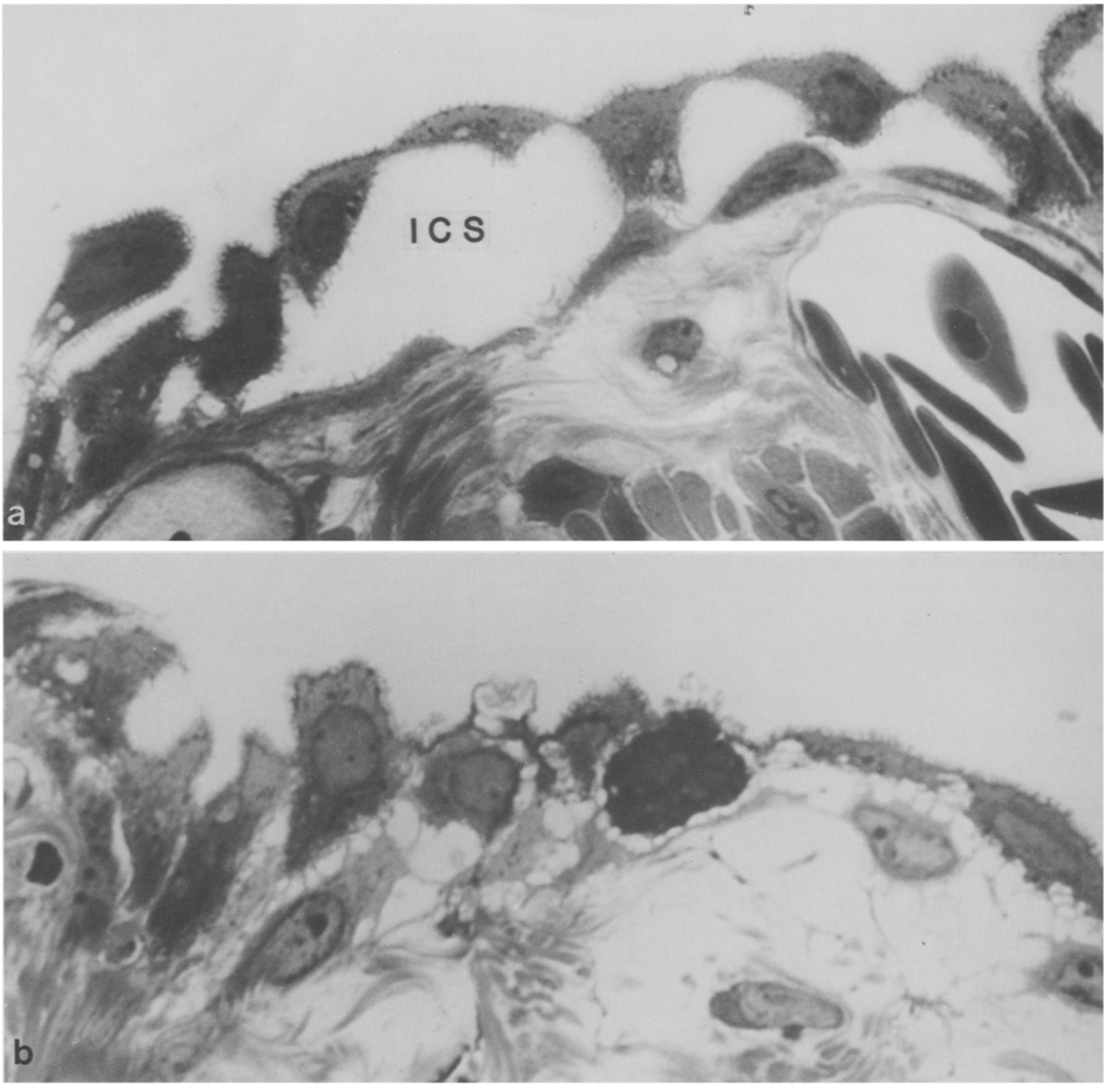


Fig. 7. Comparative light microscopic views of paired hemibladders to illustrate reversibility of Ca^{2+}-depletion effects on epithelial geometry. (*a*): Epithelial structure when tissue was fixed 20 min after imposition of ADH and an osmotic gradient with Ca^{2+}-free serosal conditions displays markedly expanded intercellular spaces (*ICS*) with frequent sites where the surface layer of cells is lifted considerably from the basal cells and basal lamina. (*b*): This tissue was subjected to the same procedure as that in *a* but allowed to recover under isomotic conditions with replenishment of medium Ca^{2+} for 90 min. Subsequently, the tissue was exposed to an osmotic gradient and ADH for 20 min before fixation. Resulting morphology is indistinguishable from that of tissue never exposed to Ca^{2+}-free serosal incubation as was shown in Fig. 3*a*. Each view 1600×

rate. Presumably $P_{D_w} = 7.2 \times 10^{-4}\,\mathrm{cm \cdot sec^{-1}}$ is $P^r_{D_w}$ = the real diffusion coefficient of water in the tissue in the absence of unstirred layers. (In fact, in other experiments with everted bladders and with stirring rates of 800–1,000 rpm we obtained values of $7.3 \times 10^{-4}\,\mathrm{cm \cdot sec^{-1}}$.)

Using the $P^r_{D_w}$ value derived here (or in Ref. [26]), the assumption of the thickness of the unstirred layers can be verified with the equation for calculation of the permeability of barriers in series [1]:

$$R^a_{D_w} = R^r_{D_w} + [\alpha (D^o_w)^{-1}]. \tag{7}$$

Where $R^a_{D_w}$ and $R^r_{D_w}$ are the reciprocals of the apparent (experimental) and real (calculated) P_{D_w}, respectively; α is the operative thickness of the unstirred layer ($\alpha_a + \alpha_s$). Then,

Table 6. Effect of low pH, colchicine, and lanthanum on ADH-stimulated diffusional permeability to water

	pH		Colchicine		La^{3+}	
	8.1	6.2	−	+	−	+
P_{D_w} ($cm \cdot sec^{-1} \times 10^4$)	3.9± 0.2	2.6± 0.2	4.2± 0.3	2.9± 0.2	4.4± 0.3	2.8± 0.1
$J_W^{M \to S}$ ($\mu l \cdot cm^{-2} hr^{-1}$)	1,392 ±32	939 ±37	1,518 ±60	1,024 ±34	1,561 ±54	993 ±31
n	6		8		6	
p	<0.001		<0.001		<0.001	

Low pH (6.2 *vs.* 8.1) and $LaCl_3$ (10^{-3} M) were introduced 10 min before ADH; colchicine (10^{-4} M) was introduced 240 min before. ADH (20 mU · ml^{-1}) was applied for 20 min in each case.

$$\alpha_a + \alpha_s = D_w^o(R_{D_w}^a - R_{D_w}^r) = 2.4(2{,}564 - 1{,}389) \times 10^{-5} = 282 \times 10^{-4}\ \text{cm}. \quad (8)$$

The $P_{D_w}^{\alpha}$ of an unstirred layer of this magnitude is 8.6×10^{-4} cm · sec^{-1} [Eq. (6)]. The maximal $P_{D_w}^r$ that would be measured in the epithelium with ADH is 7.2×10^{-4} cm · sec^{-1}. The $P_{D_w}^{\alpha}$ in series with $P_{D_w}^r$ yields the $P_{D_w}^{\alpha}$ that we have obtained experimentally: 3.9×10^{-4} cm · sec^{-1}. From these calculations we conclude that the P_{D_w} of the unstirred layers is higher than the maximal P_{D_w} that can be obtained in the tissue. It is inferred, therefore, that any modification of the ADH effect on the diffusional permeability barrier of the epithelium can be detected with the stirring rates used in our experiments.

To test this assertion beyond theoretical considerations, other experimental maneuvers known to inhibit the effect of ADH on P_f were performed to assess the ability to detect a modified $P_{D_w}^a$ in the conditions we have used above. The treatments were: low serosal pH [22], serosal La^{2+} [24, 48] and colchicine [25, 46]. As shown in Table 6 these three conditions decreased the effects of ADH on $P_{D_w}^a$ by 31 to 36%.

Theoretical considerations and these experimental results strongly suggest that $P_{D_w}^{\alpha}$ did not significantly interfere with the measurements, such that changes in $P_{D_w}^a$ could not be detected. Consequently, we conclude that the absence of serosal Ca^{2+} does not modify the action of ADH on the rate-limiting barrier to water diffusion.

C. Structural Alterations and the Inhibition of Osmotic Permeability

With confirmation that the measured lack of an effect of Ca^{2+} removal on the ADH-induced increase in P_{D_w} was not due to experimental limitations, the demonstrated inhibition of measured P_f remains to be explained. P_f is derived from the measured value of water flow (J_v) and the applied transmural osmotic gradient ($\Delta\Pi$). If J_v is diminished (as seen here) and $\Delta\Pi$ does not change over the course of the experiment (as also seen) then P_f is inhibited by definition. This implies — but does not assure — that ADH induction of "osmotic water permeability" has been reduced while "diffusional water permeability" (P_{D_w}) was unchanged. Do these coefficients represent separate pathways or has there been some secondary phenomenon that has affected the significance of a direct measurement of J_v as a means for estimation of ADH action? Postulation of completely separate pathways for diffusional and osmotic flow is not warranted. However, there are strong reasons to expect that the apparent Ca^{2+} dependence of ADH-induced volume flow is due to an epiphenomenon related to the structural integrity of the tissue.

The depression of measured osmotic flow observed in the absence of serosal Ca^{2+} was, in every case, accompanied by a gross distortion of the epithelial architecture. This pattern of alterations might well result in obligatory establishment of a diminished steady-state flow rate, while ADH action, *per se*, may have been completely unaffected. The actual mechanics of flow inhibition are questionable; potential explanations are discussed below. Notable, nonetheless, is the fact that the epithelial distortion was seen only when J_v was allowed to proceed through the Ca^{2+}-deprived structure. The amount of J_v inhibition was directly related to the magnitude of J_v itself; this was demonstrated with measurements of osmotic permeability in glutaraldehyde-fixed bladders (Fig. 4) and by analysis of the time course of inhibition (Fig. 1). Structural studies in the experiments on the reversibility of inhibition (Figs. 6 and 7) revealed that restoration of the normal response was coincident with a reestablishment of cell-cell contacts.

This leads us to conclude that the inhibition of ADH-induced flow is due more to a distortion of the pathways for osmotic flow than to an intrinsic inhibition of ADH action. Bulk water flow from granular cells to the intercellular spaces creates an intraepithelial hydrostatic pressure which tends to separate granular cells from each other and from the rest of the structure. When Ca^{2+} is lowered below a

certain threshold, the cell-cell contacts are weakened so that hydrostatic pressure elevation produces the disruption of the system observed. In these circumstances, J_v decreases by more than 60% (Tables 3 and 5), while the unidirectional water flux ($J_w^{M\to S}$) remains unmodified (Table 5). This suggests that the permeability of the apical membrane remains as high as in a structurally intact bladder. Why then is the osmotic flow across the tissue inhibited when the mucosa-to-serosa osmotic gradient is not cancelled? Hypothetical explanations are best framed with close consideration of the structural specializations of this epithelium, some of which have not been considered important in transmural osmotic flow previously.

The most obvious explanation for inhibition is based on the observation of accumulated fluid in Ca^{2+}-deprived tissues. Volume "retained" by the epithelium during the 20-min period of measurement was between 19 and 72 $\mu l \cdot cm^{-2}$. If this volume is considered as that contained between two parallel planes (the surface cells in one and the basal cells and supportive tissues in the other) each of 1 cm^2 area, the distance between the planes would be between 20 and 70 μm. These distances are within the range of observed separation between the layers of cells. This convenient explanation seems untenable, however, because of our findings in everted hemibladders where volume flow was measured as accumulated weight; in these experiments the degree of inhibition (66%) was virtually identical to the inhibition obtained in noneverted bladders (62%).

The significance of accumulated intraepithelial fluid is in doubt, and speculation is unwarranted without quantitative estimation of its extent compared to that contained in the distended spaces of control tissues.

Alternative schemes might be based on current information about the special characteristics of basal cells which, traditionally, are not regarded as important in the water flow process. Wade [47] has described two features of this cell type which are worth noting here. First, the basal cell plasma membrane reveals (by freeze-fracture techniques) that it contains intramembranous particle aggregates very similar, if not identical, to those described as operative in ADH action at the granular cell apical membrane. These particulate structures in basal cells do not vary in their distribution with ADH application, however; consequently, they may be viewed, conceivably, as "water channels" always present in the basal cell membrane. If they are instrumental in the transmural movement of water, reduced hydrostatic pressure in the intercellular space might limit their use as water channels.

Wade [47] has also found that gap junctions in this epithelium are associated invariably with basal cells. Granular and mitochondria-rich cells do not appear to be directly coupled to each other or among themselves. Gap junctions have only been found between adjacent basal cells and between basal cells and granular cells. He speculated that ionic coupling within the epithelium may be operative exclusively through these cells. Is it possible that some fraction of the water crossing the epithelium passes through these structures from granular to basal cells enroute to the submucosa? Is it possible, on the other hand, that ADH-induced production of cyclic AMP is in the basal cell and that the nucleotide diffuses into the granular cell to elicit the hydroosmotic response? Either of these possibilities would help to explain why mechanical uncoupling of these cells results in an inhibition. Chemical uncoupling should also produce an inhibition then; for example, low pH is a known junction uncoupler [40, 44] and, indeed, low pH is also a known inhibitor of water flow [22]. Against this view, however, is the fact that glutaraldehyde-fixed bladders seemed to retain the permeability level of the water pathway, and this agent presumably increases the resistance of gap junctions [3]. The final resolution of the exact mechanism of this inhibition will necessarily follow a more precise description of the permeability barriers and pathways for osmotic water flow.

We conclude that extracellular Ca^{2+} has no direct modulatory role in ADH stimulation of water permeability. Of course, the participation of membrane-bound or intracellular Ca^{2+} in the mediation of hormonal effects on target cells [8, 20–24, 31, 33, 37, 45, 48] is not challenged by these experimental results. Maintenance of a full ADH response in terms of diffusional permeability to water stands as the strongest argument for uninhibited ADH action. The suppression of volume flow appears to be due to volume flow itself which, with reduced Ca^{2+}, serves to disrupt the epithelial architecture. House [30] has discussed a collection of processes which produce "flow-induced deformation of the permeability barrier", and the phenomenon observed here comfortably fits into that classification. As discussed by others [17, 18], it seems fair to speculate that flow-induced alteration of flow occurs to some extent even under optimal physiologic conditions in this tissue.

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