J. Membrane Biol. 12, 101–128 (1973)


# Pathways for Movement of Ions and Water across Toad Urinary Bladder

## I. Anatomic Site of Transepithelial Shunt Pathways

Donald R. DiBona and Mortimer M. Civan*

Laboratory of Renal Biophysics and Department of Medicine,
Massachusetts General Hospital, and Departments of Anatomy and Medicine,
Harvard Medical School, Boston, Massachusetts 02114

Received 13 June 1972

*Summary.* Mucosal hypertonicity, produced by the addition of NaCl, KCl, mannitol, urea, sucrose or raffinose, reduced the electrical resistance of toad urinary bladder and induced bullous deformations (blisters) of the most apical junctions of the mucosal epithelium; the smaller solutes were most effective in eliciting both phenomena. Study of the effect of addition and subsequent removal of mannitol from the mucosal medium indicated that both the electrical and morphologic changes are reversible and follow the same time course. Mucosal hypertonicity induced comparable changes in the tissue in the presence or absence of inhibition of active sodium transport by replacement of sodium by choline, or by addition of ouabain or amiloride. Dilution of the tonicity of the serosal medium similarly reduced the tissue resistance and induced blisters within the epithelium, demonstrating that the osmotic gradient, rather than the mucosal hypertonicity itself is the cause of the osmotically-induced resistance change. The data indicate, therefore, that the osmotic gradient reduces the electrical resistance of the tissue primarily by deforming the apical junctions.

The simplest interpretation of the data is that the apical tight junctions are considerably more permeable to water and small solutes than had previously been thought. Addition of solute to the mucosal medium leads to the diffusion of solute into the junctions: the subsequent transfer of water from the lateral intercellular spaces and/or the adjacent cellular cytoplasm, deforms these structures and reduces the resistance to the passage of ions across the tissue. The results suggest that the apical junctions constitute the rate-limiting permeability barrier of the putative parallel shunt pathway across toad bladder.

When urea is added to the outer medium bathing isolated frog skin, rendering the solution hypertonic, the transepithelial electrical resistance falls [31, 35]. This observation, first noted by Ussing, has been subsequently extended to a wide variety of solutes [2, 12, 18, 23, 33, 34] and to other

* *Current address*: Departments of Physiology and Medicine, University of Pennsylvania School of Medicine, Philadelphia, Pa. 19104.

epithelia, including the urinary bladder of the toad [30]. Although of considerable interest, the site and mode of action of mucosal hypertonicity has been unclear, and the physiologic significance, if any, of the effect has been obscure.

Recently, deformations of the apical junctions of toad bladder and skin have been noted to occur when the resistance of these preparations was lowered by application of a mucosal bathing medium made hypertonic with sucrose [9]; Wade and DiScala have independently reported the appearance of these junctional deformations in toad bladder following application of a variety of solutes other than sucrose [36]. The current communication presents the results of a more detailed study of the relationship between the osmotically-induced morphologic and resistance changes. The data demonstrate that the solutes which reduce the tissue resistance also produce blistered junctions, that the two effects are reversible with similar time courses, and that these phenomena are largely or entirely independent of active sodium transport. We conclude that mucosal hypertonicity reduces tissue resistance specifically by reversibly altering the structure of the apical ("tight") junctions. A preliminary report of these results has appeared in abstract form [10]. A subsequent and separate communication will be addressed to the possible physiologic role of this effect.

## Materials and Methods

Female specimens of the toad, *Bufo marinus*, were obtained from the Dominican Republic (National Reagents, Inc., Bridgeport, Conn.) and maintained on moist wood chips. Urinary hemibladders from doubly-pithed toads were mounted in Lucite double-chambers of 2.8 $cm^2$ cross-sectional area, providing experimental and control samples from the same tissue. The serosal surface of the tissues was supported by nylon mesh. The volumes of solution were 6 to 12 and 5 to 9 ml on the mucosal and serosal surfaces, respectively, providing a pressure head of 0.5 to 3.5 cm solution.

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

The experimental protocol was to clamp the transepithelial potential (Fig. 1) at 0 mV (short-circuited state) except for 9-sec intervals every 30 sec when the transepithelial potential was increased to 12 mV (serosa positive with respect to mucosa), by means of chlorided silver electrodes in series with 3 M KCl agar bridges. Potentials were monitored by means of calomel electrodes in series with similar salt bridges. The transepithelial electrical current was continuously monitored and displayed on a dual pen recorder (Model A-22A, Varian Associates, Palo Alto, Calif.).

Under the usually employed experimental conditions, the bathing media are isotonic, and the osmolality of these solutions is not changed during the course of an experiment. In such circumstances it is ordinarily unnecessary to correct for the resistance of the

bathing media. However, in the present series of experiments, the ionic strengths of the bathing media were changed, resulting in marked alterations in the tissue resistance as well. Since the ratio of solution resistance to tissue resistance changed considerably, two potential sources of error were thereby introduced.

First, measurement of the resistance of the entire preparation occasionally included significant contributions from the solution resistances. The resistances of the solutions employed may be easily measured in the identical chambers in the absence of tissue, so that it is a simple matter to subtract the solution resistance from the total measured resistance. Second, short-circuiting the entire preparation does not necessarily short-circuit the tissue so that the baseline transepithelial voltage for measuring resistance will be variable. When a potential $V$ is imposed across the entire preparation (point $S$ with respect to point $M$ of Fig. 1) by passage of a current I from mucosa to serosa, the voltage $V_t$ across the tissue alone (point $s_t$ with respect to point $m_t$ of Fig. 1) will be given by:

$$V_t = V - I(r_m + r_s) \tag{1}$$

where $r_m$ and $r_s$ represent the resistance of solution between voltage sensing bridge and between mucosal and serosal surfaces, respectively. Using a simple equivalent circuit to represent the tissue (as described in Fig. 1),

$$V = E - I(R_t + r_m + r_s) \tag{2}$$

where $E$ is the spontaneous transepithelial potential and $R_t$ is the tissue resistance,

$$\therefore\ V_t = (V)\left[1 + \left(\frac{R_t}{r_m + r_s} + 1\right)^{-1}\right] - (E)\left[1 + \left(\frac{R_t}{r_m + r_s}\right)\right]^{-1}. \tag{3}$$

Thus, the true transepithelial potential $V_t$ is a function not only of $V$, the externally imposed potential, but also of $E$ and $(R_t)/(r_m + r_s)$. This dependence of $V_t$ would be of no concern were the current-voltage relationship across toad bladder perfectly linear. However, depending upon the precise experimental circumstances, the $I-V$ relationship may be far from linear [7, 16]. Therefore, it is highly desirable to be able to control the voltage across the tissue itself, rather than across both tissue and solutions.

This capability is provided by the simple circuit of Fig. 1. In practice, $R_f$ was made zero before the onset of the experiment. The resistance of each solution to be used was then calculated by measuring the current necessary to apply pulses of 12 mV across the chamber in the absence of tissue. Since the bladder was subsequently mounted symmetrically between the tips of the two voltage sensing bridges, $(r_m + r_s)$ was simply calculated as the sum of the previously measured resistances of the mucosal and serosal solutions divided by two. During the course of the experiment, $R_f$ was then fixed at $(r_m + r_s)$. As will be appreciated from the circuit, this manipulation effectively subtracted off the solution resistances, so that the potential applied by the stimulator was the potential difference applied across the tissue itself.

Following a suitable baseline period of measurement, the standard protocol was to remove a small volume of solution from the mucosal medium, and to replace the Ringer's solution with an identical volume either of experimental solution or of fresh Ringer's solution. Usually, the experimental solution consisted of Ringer's solution containing a high concentration of the solute under consideration; thus, addition of the experimental medium did not change the concentration of any of the components in the mucosal medium, with the exception of the solute under investigation. Care was exercised to change the value of $R_f$ just prior to adding the new solution when the conductance of the new solution exceeded that of the initial bathing medium, while $R_f$ was changed just after adding a new solution of lower conductance. Because of the large capacitance

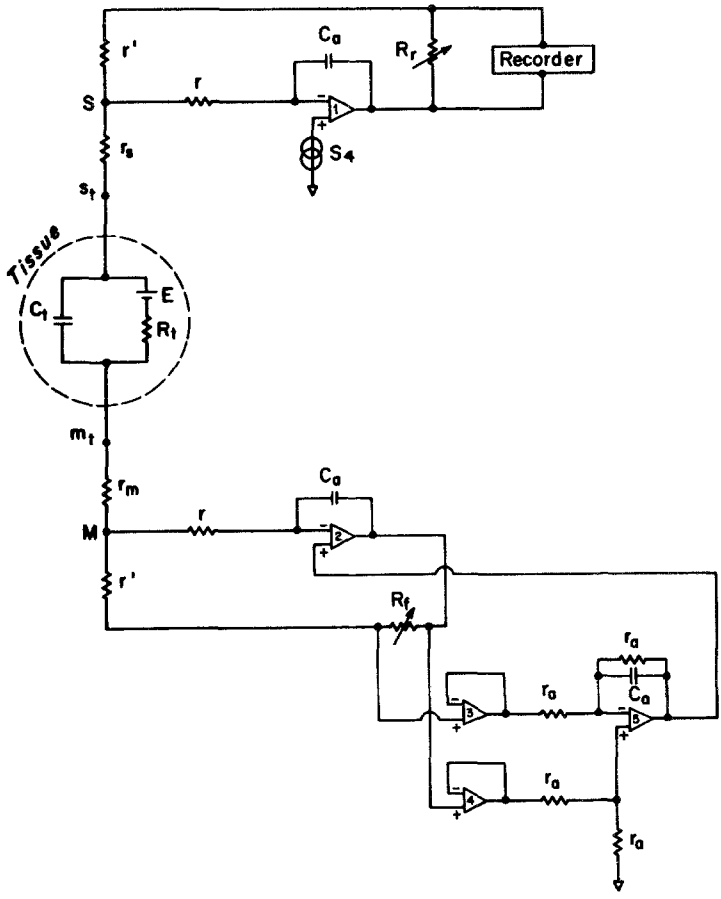


Fig. 1. Electrical circuit of apparatus and simple equivalent circuit of tissue. The bladder is represented by a capacitor $C_t$ in parallel with a battery $E$ in series with a resistor $R_t$. The serosal $s_t$ and mucosal $m_t$ surfaces are separated by the resistances $r_s$ and $r_m$ of the two bathing solutions from the positions $S$ and $M$ of the voltage-sensing leads placed in the serosal and mucosal bathing media, respectively. The resistances $r$ and $r'$ represent the3 M KCl agar bridges leading to the voltage-sensing calomel electrodes and to the current-passing chlorided silver electrodes, respectively. Elements (1) through (5) are operational amplifiers (Teledyne Philbrick Nexus, Dedham, Mass.; Model No. 1026). The values for the other circuit elements are: $C_a = 100$ pF, $r_a = 10$ kΩ, and $R_r$ is chosen for convenience to be 100 to 1,000 Ω. With $R_f = 0$, the circuit is conventional, clamping the potential of point $S$ with respect to that of point $M$ alternately at 0 and at +12 mV, in response to the signal provided by the $S_4$ square-wave generator (Grass Instruments Co., Quincy, Mass.). The transepithelial current $I$ is continuously displayed on a paper chart recorder by monitoring the voltage developed across the series resistor of known resistance $R_r$. When $R_f$ is increased to equal $(r_s + r_m)$, the potential difference between points $s_t$ and $m_t$ becomes equal to the potential difference applied by the $S_4$ stimulator, while the potential difference between the bathing media will exceed this value by $(I)(r_s + r_m)$. Therefore, with this simple circuit, adjusting $R_f$ to equal the resistance of the bathing solutions, it is possible to apply the voltage clamp specifically across the tissue itself

$C_t$ across toad bladder, the high frequency components of the square-wave pulses applied were effectively shunted across the tissue; these components would then produce instabilities of the circuit were $R_f$ to exceed the true solution resistance.

The circuit of Fig. 1 was used in all experiments in which the mucosal medium was made hypertonic with an electrolyte and in most of the experiments where the solute added was a nonelectrolyte.

In analyzing the data, the tissue resistance $R_e$ at the end of the experiment was normalized by dividing $R_e$ by the tissue resistance $R_i$ just before addition of excess mucosal solute. When the experimental protocol permitted the adjacent quarter-bladder to serve as an appropriate control, the relative change in resistance was calculated as $\frac{(R_e/R_i)\text{ experimental}}{(R_e/R_i)\text{ control}}$ to minimize the effect of time-dependent spontaneous changes in tissue resistance. Under the conditions of the present study, $R_i$ was $1.44 \pm 0.056$ (mean $\pm$ SEM) k$\Omega$ cm$^2$ for tissues bathed with isotonic sodium Ringer's solution.

At the conclusion of each experiment, tissues were fixed by simultaneous addition of suitable volumes of 50% (w/v) glutaraldehyde (Fisher Scientific Co., Pittsburgh, Pa.) to mucosal and serosal solutions to provide a final glutaraldehyde concentration of 1% and allowed to stand for 15 to 30 min before removal. Rectangles of tissue excised from the chambers were immersed in 1% glutaraldehyde in phosphate buffer. Tissue samples were post-fixed in osmium tetroxide and embedded in epoxy as previously described [12] but one-half of each sample was also stained *en bloc* with uranyl acetate [14]. Sections were cut with a Reichert OmU2 ultramicrotome (G. Reichert Werke, A.G., Vienna, Austria). Examination of the sections with a Philips EM-200 electron-microscope was initially performed by one of us without prior knowledge of the experimental protocol to reduce possible bias in interpretation.

## Results

### *Effect of Mucosal Hypertonicity*

Addition of a wide variety of solutes to the mucosal medium resulting in tonicities as high as 672 mOsm/kg water increased the tissue conductance in each of the many preparations studied. As demonstrated in the tracing of Fig. 2, tissue resistance decreased within 30 sec following addition of solute, and continued to decrease monotonically during the period of observation. Although qualitatively similar results were observed following addition of NaCl, KCl, urea, mannitol, sucrose or raffinose, the magnitude of the effect clearly depended upon the concentration and molecular size of the solute applied. For example, raffinose was effective when dissolved in Ringer's solution to a final mucosal tonicity of 650 but not at 430 mOsm/kg water, while addition of KCl to a final tonicity of only 400 mOsm/kg water significantly decreased tissue resistance.

Subsequent electron-microscopic examination of the fixed tissues consistently demonstrated a bullous enlargement or blistering of the space within the apical junctions in every preparation where the resistance had been lowered with mucosal hypertonicity and in none of the control tissues,

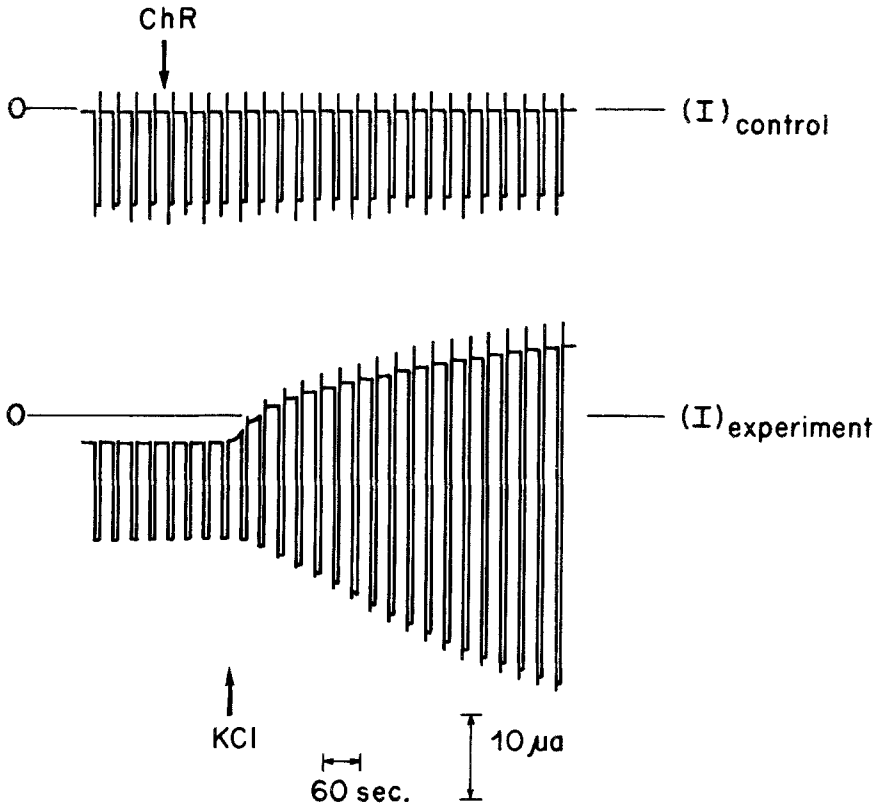


Fig. 2. Responses of short-circuit current and tissue resistance to mucosal hypertonicity. The abscissa is time; the ordinate is the current required to reduce the transepithelial potential either to zero (short-circuit current, upper envelope of each tracing) or to +12 mV (lower envelope of each tracing). The tissue resistance is, therefore, inversely proportional to the displacement of the upper from the lower envelope of each tracing. The hemibladder of this experiment was initially bathed with isotonic choline Ringer's solution on both the mucosal and serosal surfaces. At the time indicated by the lower arrow, a small volume of 1.2 M KCl was added to the mucosal medium of the experimental quarter-bladder (lower tracing), increasing the tonicity to 372 mOsm/kg water. A similar volume of isotonic choline Ringer's solution was added to the mucosal medium of the control quarter-bladder (upper tracing) at the time indicated by the upper arrow. As will be appreciated from the tracing, mucosal hypertonicity induced a prompt fall in the tissue resistance, even in the absence of sodium in the bathing media. The increase in "short-circuit current" is a reflection of the diffusion potential established by the addition of the KCl to the mucosal medium

which had been exposed only to isotonic solutions. Fig. 3 provides an illustration of experimental tissue at low power and demonstrates both the junctional blistering and the consistent accompanying result, a strict closure of the lateral intercellular spaces. Examination of control preparations at high magnification, as in Fig. 4, revealed that the observable space between

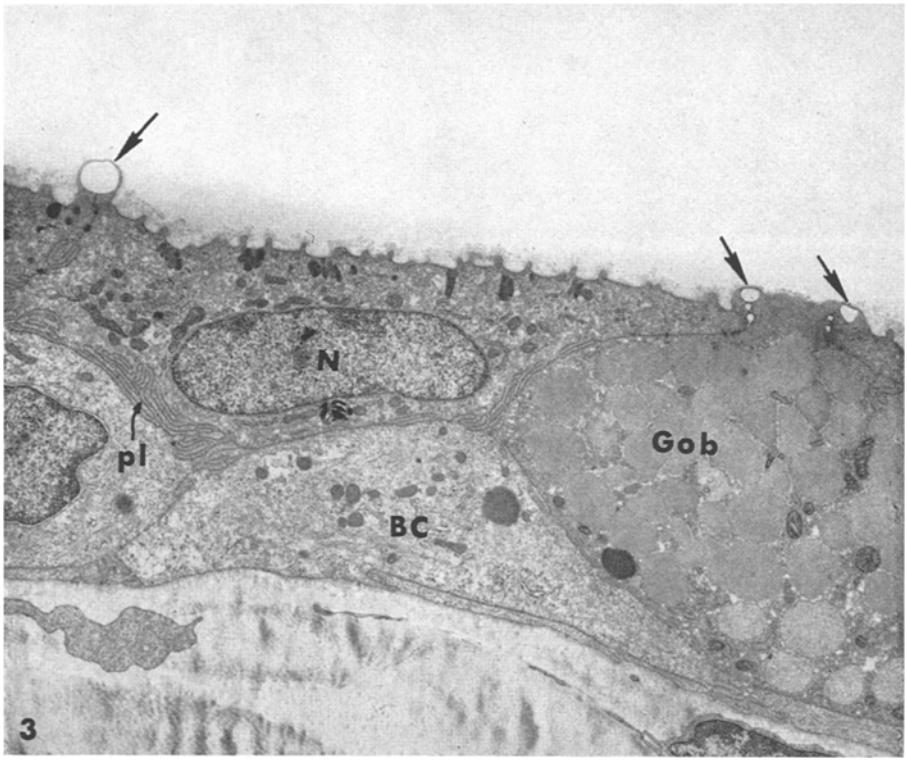


Fig. 3. Electron-microscopic appearance of toad urinary bladder fixed after the mucosal surface (top) has been exposed to a bathing solution made hypertonic with mannitol as described in the text. The epithelial cells do not appear swollen or shrunken by this treatment. Lateral cell margins are thrown into unusually tight plications *pl* obliterating the intercellular space at this magnification except at the luminal end (arrows) where bullous enlargments are seen. This junctional blistering is present in each of the situations shown. On the left, two granular cells comprise the junction; the two instances on the right are of junctions formed by apposition of granular cells to a goblet cell *Gob*. Nucleus *N*; basal cell *BC*. ×7,500

cells at the level of the apical junction was only focally obliterated. This observation was facilitated by *en bloc* staining and is in agreement with the observations of these structures in other tissues (*see*, e.g., [4]). Following application of hypertonic mucosal solutions, the majority of junctional profiles observed were altered as described previously [9] and as shown in Figs. 5 and 6. Considerable variability in the size and appearance of the blisters produced was noted, even within a single preparation. The variability in observed size and distribution of blisters can be more easily appreciated from a consideration of Fig. 7. Oblique sectioning of the tissue demonstrates that the junctional alteration is not continuous but is composed of multiple,

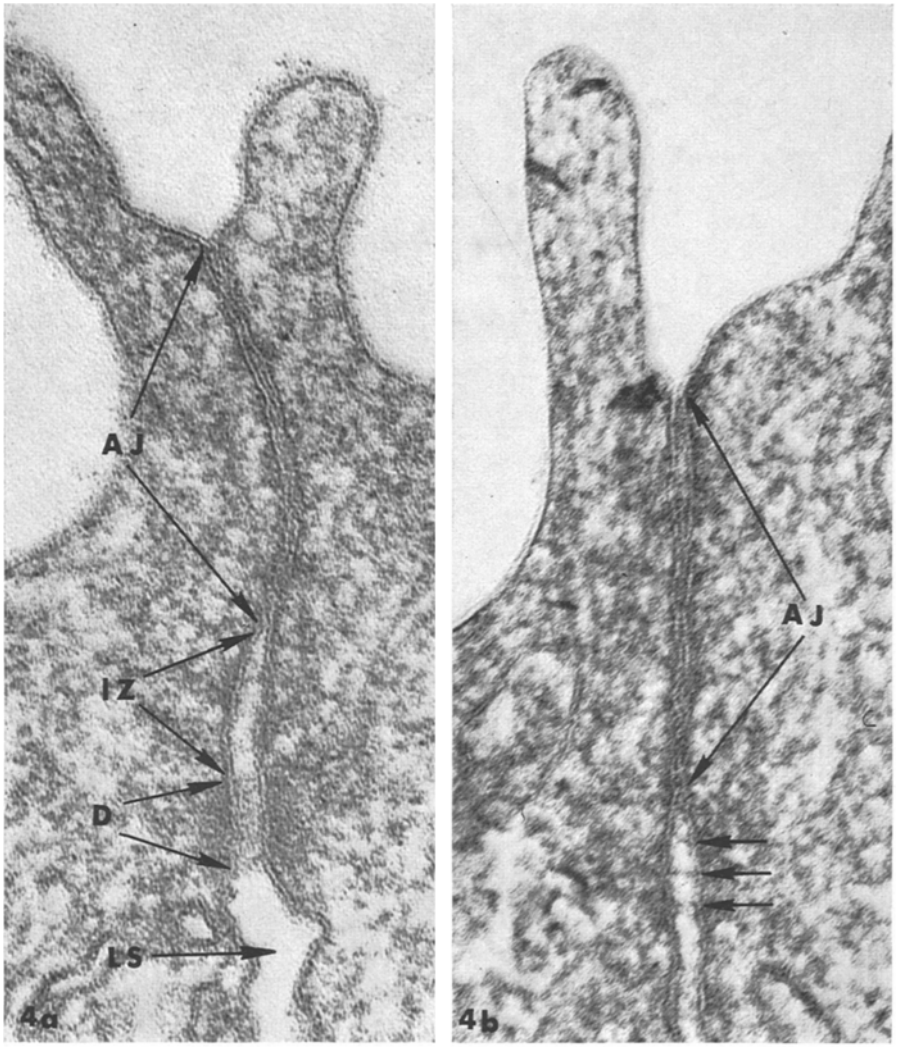


Fig. 4. Junctional complexes from bladder epithelium exposed only to isotonic solutions prior to fixation. (*a*) The apical portion of the complex *AJ* displays the maximum proximity of adjoining cells. Within this region, however, it is observed that the intercellular space is still resolved at several positions. The intermediate zone *IZ* between the apical or "tight" junction and the desmosome approximates a *zonula adhaerens* [14] which is not extensively developed in this tissue, however. The lateral intercellular space *LS* below the desmosome *D* shows considerable variation even when the bladder is exposed only to isotonic solutions, but is generally more open than observed in Fig. 3. (*b*) The apical junction here shows only focal obliteration of the intercellular space. This is better appreciated by tilting the picture to view it along the long axis of the junction. Unlabeled arrows point out electron-dense lines which appear to transect the intercellular space as is often seen in a more clearly defined *zonula adhaerens*. Each $\times 150{,}000$

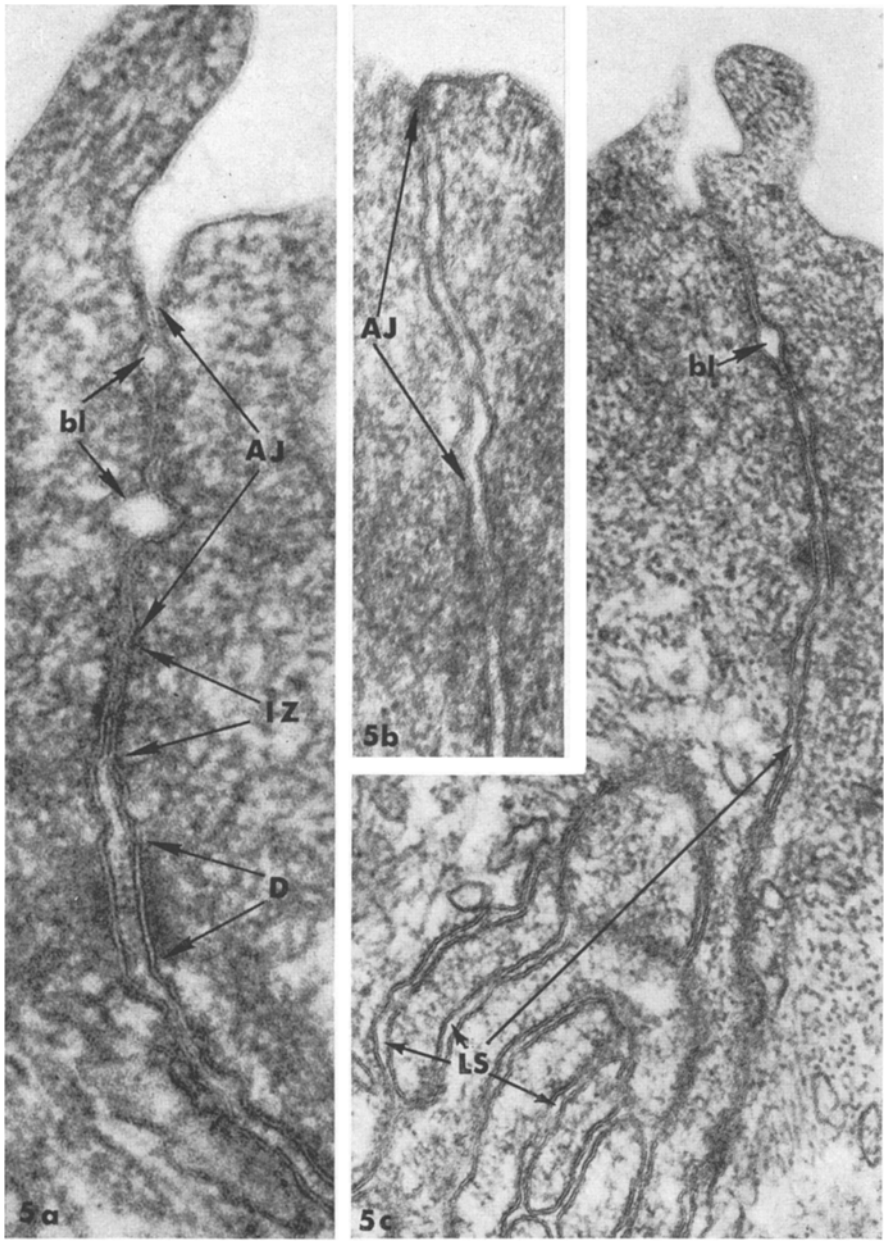


Fig.5. Early deformations of junctional complexes. Tissues were fixed after the mucosal solutions were rendered hypertonic with either mannitol, raffinose, or KCl (*a*, *b*, *c*, respectively). (*a*) Small blisters *bl* are observed within the apical portion of the junctional complex *AJ*; a more closed appearance is noted in the intermediate zone; desmosomes *D* seem unaffected. (*b*) Much less frequently following exposure to an osmotic gradient the junctional profile *AJ* appears more uniformly open. (*c*) Concurrent with junctional blistering *bl* the subjacent lateral intercellular spaces *LS* appear more closed than in control tissues. However, the adjacent lateral plasma membranes are still separated by some 200 Å, so that the rate-limiting barrier to passage of solutes and water between the cells is likely to remain the most apical cell junction; (*a*), (*b*): × 100,000; (*c*): × 35,000

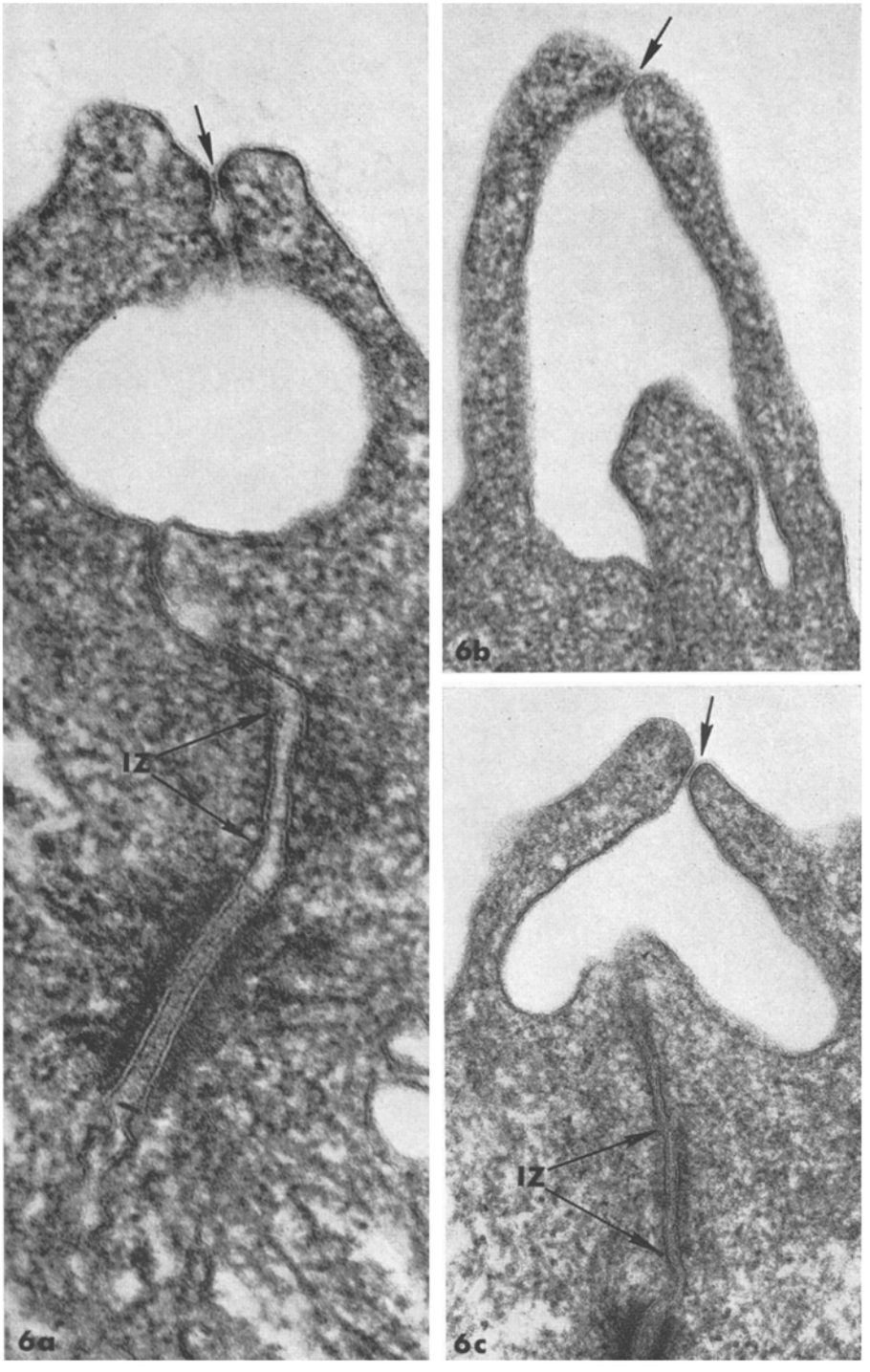


Fig. 6. Advanced deformations of junctional complexes. Tissues were fixed after the mucosal solution was made hypertonic with KCl, NaCl, or sucrose (*a*, *b*, or *c*, respectively). The intercellular space within the apical junction has undergone a prominent bullous enlargement. At the luminal tip of the junction (unlabeled arrows) an area of close contact persists, in general, between apposing cells, while the junction profile below contains one or more large blisters. Continuity of the cell membranes through the blistered region is readily appreciated although portions are obliquely sectioned. The intermediate zone *IZ* of the complex may appear open, as in (*a*), or closed, as in (*c*); both conditions may be found within the same sample. (*a*), (*b*): × 100,000; (*c*): × 75,000

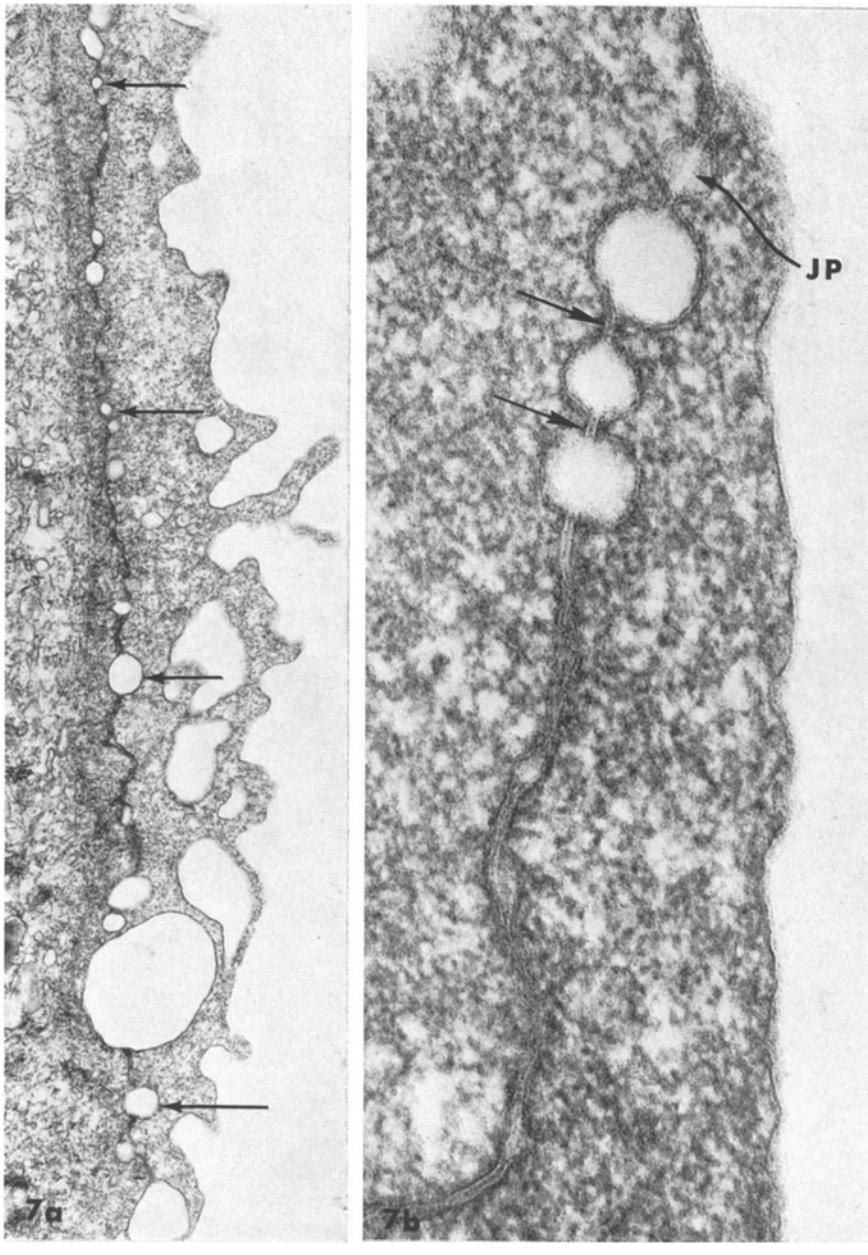


Fig. 7. Oblique sections of junctional zones. Such sections confirm that the deformations are focal in nature. In each view the mucosal edge is at the right of the field. In (*a*) four blisters are pointed out (arrows) and as many as 15 others can be counted along this junction profile. Part (*b*) shows, at higher power, that the junction segments between deformations (at the unlabeled arrows) are regions of apparently close contact. In one position where the section is favorable a closed junction profile *JP* and a small blister profile are, in fact, superimposed. Each view is of tissue treated with hypertonic solution on the mucosa (tonicity elevated to 467 mOsm/kg water with mannitol).
(*a*): ×23,000; (*b*): ×150,000

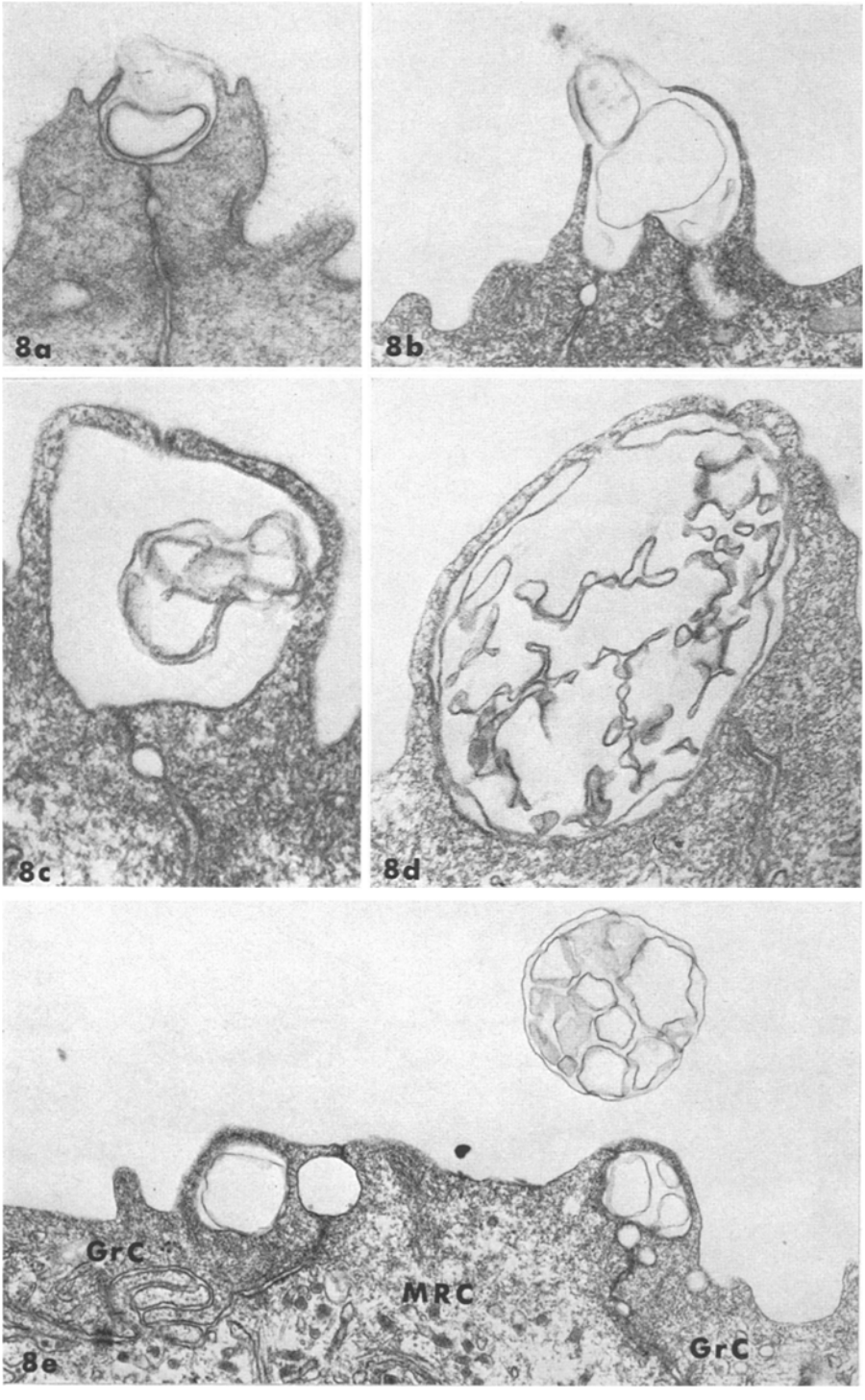


Fig. 8a—e

focal deformations. Occasionally, as in Fig. 8, deformation of the junction may be more extensive, involving rearrangement of membrane components and, more rarely, a direct opening to the mucosal bath.

To quantify the junctional deformation, the fraction of all the observed junctional profiles containing at least a single blister has been determined for each tissue. This approach has proven useful for comparison of samples.

### *Reversibility of Osmotically-Induced Effects*

The correlation between the osmotically-induced decrease in resistance and the appearance of blistering was strengthened by an examination of the reversibility of the two effects. The following protocol was used in three paired experiments. After a suitable control period, mannitol was added to the mucosal solution of one quarter-bladder (*A*) raising the tonicity to 450 to 467 mOsm/kg water; an equivalent volume of isotonic Ringer's solution was added to the adjoining quarter-bladder (*B*). When the resistance of *A* had fallen to $48.0 \pm 0.63\%$ of its baseline value, the serosal and mucosal chambers of both *A* and *B* were drained, washed three times, and refilled with isotonic Ringer's solution. At this point the mucosal solution of quarter-bladder *B* was made hypertonic with mannitol (450 to 467 mOsm/kg water) and Ringer's solution was added to *A*; in the ensuing 23 to 26 min, the resistance of *A* returned to $95 \pm 6.5\%$ of its initial value while that of *B* fell to $54 \pm 2.7\%$ of its initial value. Both quarter-bladders were fixed at this time. Subsequent examination demonstrated blisters in $89 \pm 3\%$ of the junctional profiles in quarter-bladders *B* and only $9 \pm 4\%$ in quarter-bladders *A*, indicating a reversal of junctional deformation after restoration of isotonic conditions and a reversal of the mannitol-induced fall in resistance.

### *Independence from Active Sodium Transport*

To determine whether the changes in tissue conductance and blistering were dependent upon active sodium transport, three series of studies were performed, using ouabain, amiloride and choline replacement for sodium to reduce or abolish net sodium movements.

Fig. 8. Elaborations of membrane profiles occasionally observed within deformed junctions. In (*a*)–(*e*) the solutes used to render the mucosal solution hypertonic were NaCl, KCl, mannitol, sucrose and raffinose, respectively. The profiles in (*a*) and (*b*) show regions where the outer boundary of the junction is quite disrupted. In (*c*) and (*d*) membrane profiles within the blisters are continuous with the inner facing membrane while in (*e*) a large membranous profile seems to be removed from the epithelial surface, probably having a connection out of the plane of section. (*a*), (*b*): $\times 40{,}000$; (*c*), (*d*): $\times 100{,}000$; (*e*): $\times 25{,}000$

In a preliminary series of three experiments, hemibladders were initially immersed in choline Ringer's solution for 15 to 20 min, and subsequently mounted and bathed with choline Ringer's solution on both surfaces. KCl was added to the mucosal solution of the experimental side, raising the tonicity to 372 mOsm/kg water. After 8.5 to 11.5 min, when the experimental tissue resistance had fallen to 0.26 to 0.42 of its initial value (mean $\pm$ SEM $= 0.37 \pm 0.052$) the tissues were fixed. Subsequent electron-micrographs demonstrated blistering in $90 \pm 1\%$ of observed junction profiles from the experimental side and no blistering whatsoever on the control side (Table 1).

Although it was thus clear that sodium was not necessary for mucosal hypertonicity to decrease tissue resistance, it was of interest to determine whether the presence or absence of sodium markedly affected the magnitude of the osmotically-induced resistance change. Therefore, another series of three experiments was performed. The tissues were preincubated once again for some 15 min in choline Ringer's solution. Once the hemibladders were mounted, the control side was bathed on both surfaces with sodium Ringer's solution whereas the experimental quarter-bladder was bathed with choline Ringer's solution. The addition of sodium reduced the tissue resistance by $31 \pm 19\%$, consistent with previous observations [7]. KCl was subsequently added to each mucosal medium, raising the tonicity to 385 to 406 mOsm/kg water. In each case, mucosal hypertonicity reduced the resistance both of the experimental and control quarter-bladders.

Since the addition of sodium altered the tissue resistance prior to the application of mucosal hypertonicity, data reduction in terms of the fractional change in resistance, as used above, would be inappropriate. Rather, the data were expressed as the fractional change in conductance of the experimental preparation with respect to that of the control,

$$\left(\frac{R_0}{R_e} - \frac{R_0}{R_i}\right)_{\text{exp}} \Big/ \left(\frac{R_0}{R_e} - \frac{R_0}{R_i}\right)_{\text{control}};^{1}$$

1 That this expression is the appropriate form for the fractional change in conductance may be appreciated from the following reasoning. The tissue conductances $g_0$, $g_i$ and $g_e$ may be defined as the reciprocals $1/R_0$, $1/R_i$ and $1/R_e$, respectively of the values for the tissue resistances. Dividing these extensive parameters by $m$, the amount of transporting tissue, defines the intensive parameters $\bar{g}_0$, $\bar{g}_i$ and $\bar{g}_e$:

$$\begin{aligned} \bar{g}_0 &= g_0/m = 1/R_0 m \\ \bar{g}_i &= g_i/m = 1/R_i m \\ \bar{g}_e &= g_e/m = 1/R_e m. \end{aligned} \qquad \text{(a)}$$

$R_0$, $R_i$ and $R_e$ are the tissue resistances just prior to addition of sodium, just prior to application of mucosal hypertonicity, and at the end of the experiment following application of mucosal hypertonicity, respectively. Since addition of ouabain or of amiloride also induced resistance changes in the tissue, the results were calculated in terms of fractional changes in conductance in these two additional series of experiments as well (*see below*).

Calculated in this fashion, KCl caused a fractional change in conductance of the preparation bathed in choline Ringer's solution to that bathed in sodium Ringer's solution of $0.78 \pm 0.24$, a value insignificantly different from 1 at the 0.5 probability level (Table 2). Tissue examination revealed $92 \pm 3\%$ blistered profiles without sodium and $92 \pm 1\%$ with sodium.

Substitution of choline for sodium was accompanied by a change in the cytoplasmic appearance of the granular cells. Irregular masses of electron-transparent material, as shown in Fig. 9, were found in each of the choline-treated samples. Such alteration of cytoplasmic appearance did not seem to affect the structure of organelles, however, since nuclei, mitochondria, endoplasmic reticulum, Golgi apparatus and granules appeared as they did in the absence of choline. This cytoplasmic change did not seem to have any effect on the tissue's response to mucosal hypertonicity since neither the observed decrease in resistance nor the frequency of blistered profiles was significantly different using choline or sodium as the principal cation in the Ringer's solution.

---

Substituting Eq. (a) into the above expression for the fractional change in resistance:

$$\frac{\left(\frac{R_0}{R_e}-\frac{R_0}{R_i}\right)_{\text{exp}}}{\left(\frac{R_0}{R_e}-\frac{R_0}{R}\right)_{\text{control}}}=\frac{\left(\frac{\bar{g}_e m}{\bar{g}_0 m}-\frac{\bar{g}_i m}{\bar{g}_0 m}\right)_{\text{exp}}}{\left(\frac{\bar{g}_e m}{\bar{g}_0 m}-\frac{\bar{g}_i m}{\bar{g}_0 m}\right)_{\text{control}}}. \tag{b}$$

If the experimental and control tissues do, in fact, have the same transport properties at the onset of the experiment,

$$(\bar{g}_0)_{\text{exp}}=(\bar{g}_0)_{\text{control}}. \tag{c}$$

Therefore,

$$\frac{\left(\frac{R_0}{R_e}-\frac{R_0}{R_i}\right)_{\text{exp}}}{\left(\frac{R_0}{R_e}-\frac{R_0}{R_i}\right)_{\text{control}}}=\frac{(\bar{g}_e-\bar{g}_i)_{\text{exp}}}{(\bar{g}_e-\bar{g}_i)_{\text{control}}}. \tag{d}$$

Thus, the fractional change in conductance, as defined above, is equal to the ratio of the increment in conductance/transporting tissue for the experimental quarter-bladder to that for the control.

Table 1. Summary of relative changes in resistance defined as $\frac{(R_e/R_i)\text{ experimental}}{(R_e/R_i)\text{ control}}$ (*see* Materials and Methods) and of percentage blistering under a variety of experimental conditions[a]

| Exp. | Solutions | | Relative change in resistance | % Blistering | | |
|---|---|---|---|---|---|---|
| | Experimental | Control | | Experimental | Control | Experimental-Control |
| 1. | *M*: Hypertonic (KCl to ChR)<br>*S*: Isotonic (ChR) | *M*: Isotonic (ChR)<br>*S*: Isotonic (ChR) | $0.37 \pm 0.052$<br>$P < 0.01$ | $90.2 \pm 0.93$ | $0 \pm 0$ | $90.2 \pm 0.93$ (3)<br>$P < 0.001$ |
| 2. | *M*: Hypertonic (KCl to NaR; 385–406 mOsm/kg water)<br>*S*: Isotonic (NaR) | *M*: Hypertonic (Mannitol to NaR; 450–467 mOsm/kg water)<br>*S*: Isotonic (NaR) | $0.58 \pm 0.021$<br>$P < 0.005$ | $94.0 \pm 3.0$ | $89.2 \pm 2.8$ | $4.8 \pm 5.7$ (3)<br>$P > 0.4$ |
| 3. | *M*: Hypertonic (Raffinose to NaR; 645–672 mOsm/kg water)<br>*S*: Isotonic (NaR) | *M*: Hypotonic (Mannitol to NaR; 450–467 mOsm/kg water)<br>*S*: Isotonic (NaR) | $1.54 \pm 0.30$<br>$P > 0.2$ | $18.3 \pm 3.9$ | $91.6 \pm 1.8$ | $-73.2 \pm 2.4$ (3)<br>$P < 0.005$ |
| 4. | *M*: Isotonic (NaR)<br>*S*: Hypotonic (water to NaR; 134–146 mOsm/kg water) | *M*: Hypertonic (water to NaR; 134–146 mOsm/kg water)<br>*S*: Hypotonic (water to NaR; 134–146 mOsm/kg water) | $0.44 \pm 0.032$<br>$P < 0.005$ | $89.6 \pm 2.0$ | $0 \pm 0$ | $89.6 \pm 2.0$ (3)<br>$P < 0.001$ |
| 5. | *M*: Hypertonic (NaCl to NaR)<br>*S*: Isotonic (NaR) | *M*: Hypertonic (NaCl to NaR)<br>*S*: Hypertonic (NaCl to NaR) | $0.75 \pm 0.085$<br>$P > 0.05$ | $91.2 \pm 0.48$ | $0 \pm 0$ | $91.2 \pm 0.48$ (4)<br>$P < 0.001$ |

[a] *M* and *S* refer to the mucosal and serosal bathing media, respectively. NaR and ChR refer to the sodium Ringer's and choline Ringer's solutions described in Materials and Methods. The differences between the experimental and control solutions are underlined. Numbers in parentheses represent the number of paired quarter-bladders studied during the course of each series of experiments. *P* represents the probability of the null hypothesis, and has been calculated on the basis of Student's t-test.

In additional experiments, sodium was retained in the bathing media, but active sodium transport was reduced by means of inhibitors. In six experiments, ouabain was added to the serosal medium of the experimental side to a final concentration of $1 \times 10^{-3}$ M. After 36 to 88 min, the short-circuit current had fallen to $12 \pm 2.0\%$ and the resistance had risen to $131 \pm 11\%$ of their initial values. At that point, KCl was added to the mucosal solutions of both control and experimental sides of three hemibladders, while the same amount of NaCl was added to the mucosal solutions of the remaining three hemibladders. The tissues were fixed 4.5 to 9.0 min later. The fractional change in conductance (as defined above) produced by KCl in the presence of ouabain with respect to that in its absence ranged from 0.79 to 3.52 (mean $\pm$ SEM $= 1.96 \pm 0.81$). The fractional change in conductance produced by NaCl in the presence of ouabain to that in its absence ranged from 0.70 to 1.49 (mean $\pm$ SEM $= 1.14 \pm 0.23$). Combining all six experiments, the mean $\pm$ SEM was $1.55 \pm 0.42$. For the same six experiments the frequency of blistered junction profiles was $92 \pm 2\%$ and $93 \pm 2\%$ on the experimental and control sides, respectively (Table 2). Ouabain, there-

Table 2. Summary of fractional changes in conductance defined as $\left(\frac{R_0}{R_e} - \frac{R_0}{R_i}\right)_{\text{exp}} \Big/ \left(\frac{R_0}{R_e} - \frac{R_0}{R_i}\right)_{\text{control}}$ (*see* Results) and of percentage blistering in the presence or absence of inhibition of active sodium transport[a]

| Exp. | Solutions | | Fractional change in conductance | % Blistering | | |
|---|---|---|---|---|---|---|
| | Experimental | Control | | Experimental | Control | Experimental-Control |
| 1. | *M*: Hypertonic (KCl to ChR)<br>*S*: Isotonic (ChR) | *M*: Hypertonic (KCl to NaR)<br>*S*: Isotonic (NaR) | $0.78 \pm 0.24$<br>$P > 0.5$ | $91.6 \pm 2.6$ | $91.6 \pm 1.0$ | $0.03 \pm 1.6$ (3)<br>$P > 0.98$ |
| 2. | *M*: Hypertonic (KCl/NaCl to NaR)<br>*S*: Isotonic (NaR)<br>Ouabain | *M*: Hypertonic (KCl/NaCl to NaR)<br>*S*: Isotonic (NaR)<br>No ouabain | $1.55 \pm 0.42$<br>$P > 0.2$ | $91.7 \pm 1.8$ | $92.6 \pm 2.1$ | $-0.9 \pm 3.7$ (6)<br>$P > 0.8$ |
| 3. | *M*: Hypertonic (KCl to NaR)<br>Amiloride<br>*S*: Isotonic (NaR) | *M*: Hypertonic (KCl to NaR)<br>No amiloride<br>*S*: Isotonic (NaR) | $0.56 \pm 0.12$<br>$P > 0.05$ | $89.0 \pm 1.1$ | $94.8 \pm 2.0$ | $-5.8 \pm 1.2$ (3)<br>$P < 0.05$ |

[a] *See* Table 1 legend for definition of symbols.

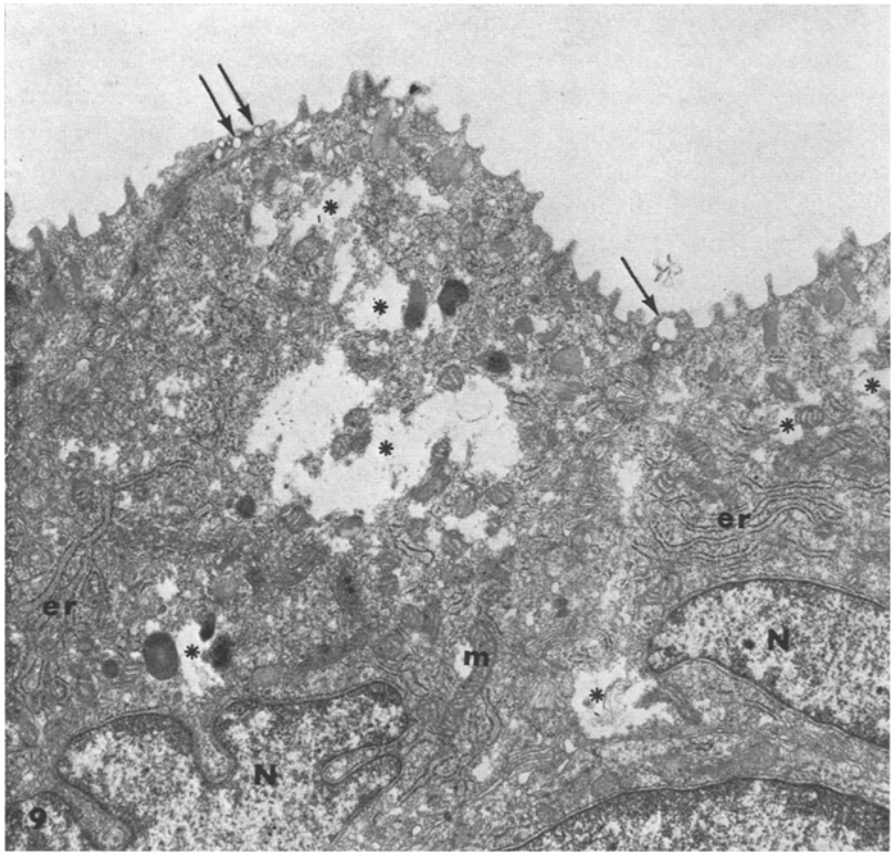


Fig. 9. Appearance of bladder epithelium after bathing with choline Ringer's solution. Clear zones (*) appear within the cytoplasm which are not membrane bound and show no definite substructure. These regions are found whether or not the mucosal bath is made hypertonic. In this case mucosal tonicity was raised to 385 mOsm/kg water with KCl; note blistered junctions (arrows). Mitochondria *m*, rough endoplasmic reticulum *er*, nuclei *N*, and other cell organelles are not noticeably affected. × 5,000

fore, significantly affected neither the resistance change nor the blistering induced by mucosal hypertonicity.

In a further series of three experiments, active sodium transport was inhibited by the diuretic amiloride. Ten to 30 min after introducing amiloride into the mucosal medium of the experimental side to a final concentration of $10^{-4}$ M, the short-circuit current had fallen to $14 \pm 2.5\%$ (mean ± SEM) and the resistance had risen to $128 \pm 9.2\%$ of their initial values, respectively. KCl was then added to the mucosal medium of both experimental and control sides. The fractional change in conductance (as defined above for the studies with choline) in the presence of amiloride with respect to that in its absence ranged from 0.34 to 0.77; the mean ± SEM was $0.56 \pm 0.12$, con-

siderably different from 1, but not quite significant at the 0.05 probability level (Table 2). Subsequent electron-micrographs demonstrated blistering of $89 \pm 1\%$ of the junctions from the experimental tissue, and $95 \pm 2\%$ of those from the control; this relatively small difference in frequency of blistering was just significant at the 0.05 probability level. Therefore, while mucosal hypertonicity produced both resistance changes and blisters of comparable degree in the presence or absence of amiloride, marginally significant changes in both effects were noted in the presence of the diuretic.

*Effect of Solute Size*

To examine more precisely the effect of the molecular size of the solute used on the osmotically-induced changes, the following studies were carried out. Six hemibladders were mounted and bathed with sodium Ringer's solution. Mannitol was subsequently added to the mucosal medium of each of the control quarter-bladders, to a final tonicity of 450 to 467 mOsm/kg water. KCl was added to the mucosal medium of three experimental quarter-bladders to a final tonicity of 385 to 406 mOsm/kg water; The relative change in resistance following KCl to that following mannitol was 0.54 to 0.61 (mean $\pm$ SEM $= 0.58 \pm 0.021$). Raffinose was added to the mucosal medium of the remaining three experimental quarter-bladders to a final concentration of 645 to 672 mOsm/kg water; the relative change in resistance following raffinose to that following mannitol was 1.10 to 2.36 (mean $\pm$ SEM $= 1.54 \pm 0.30$). Therefore, KCl produced a significantly larger fall in tissue resistance than did mannitol in a higher concentration. Similarly, mannitol in this concentration produced a larger fall, albeit not statistically significant at the 0.50 probability level, than did a still higher concentration of raffinose (Table 1).

The results of electron-microscopy were particularly informative with these samples. While KCl had produced almost twice as large a fall in resistance as did mannitol, the frequency of blistered junction profiles in each case was greater than 89% (Table 1). To better evaluate the morphologic differences between the samples, observed blisters were measured with a centimeter scale engraved on the fluorescent screen of the microscope. Without prior knowledge of pretreatment, samples from each of the six individual quarter-bladders were examined and every junction encountered was classified either as closed or blistered. The number of discernible deformations at a magnification of 30,000 diameters was noted for each; the largest blister in each profile was measured and classified on the basis of its largest diameter. A histogram (Fig. 10) presenting these results as the percentage of blisters in each range of size demonstrates that the degree of

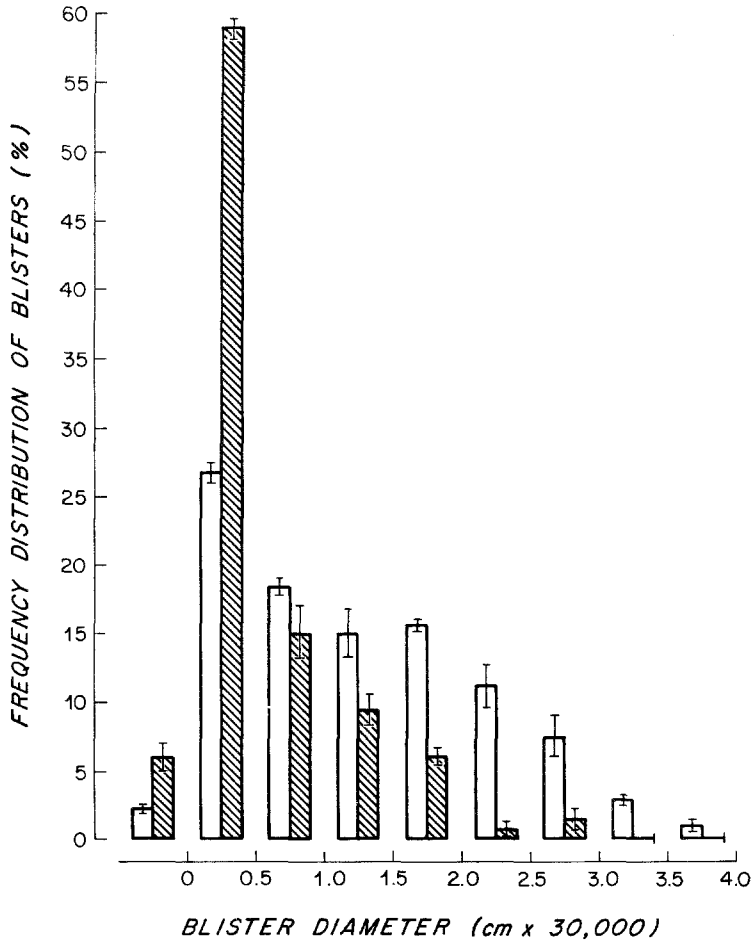


Fig. 10. Comparative size distribution of blisters elicited by KCl (clear bars) or mannitol (shaded bars). KCl was added to the isotonic mucosal solution bathing one quarter-bladder of three preparations, raising the tonicity to 385 to 406 mOsm/kg water; mannitol was added to the mucosal solution of each adjoining quarter-bladder, raising the tonicity to 450 to 467 mOsm/kg water. The tissues were subsequently examined at a magnification of 30,000 diameters; 111 to 145 apical junctions from each sample were studied, and the maximum diameter of the blisters observed in each junction was measured. Data were normalized by dividing the number of blisters observed in each size range by the total number of junctions observed. Values to the left of zero on the abscissa represent unblistered junction profiles. The height of each bar is the mean $\pm$ SEM of the percentage observed in each size range indicated on the abscissa, obtained by averaging the data from the three preparations. It is clear that a lower concentration of KCl produced larger blisters (mean = 1.5 cm) than did a higher concentration of mannitol (mean = 0.8 cm)

junction disruption was clearly greater with KCl than with mannitol. The KCl-treated preparation also revealed that 18% of the observed junction profiles contained more than a single blister while, following mannitol,

only 6% showed multiple deformations. Comparison between mannitol- and raffinose-treated samples demonstrated a marked difference in the frequency of blistered junctions; $92 \pm 2\%$ of the junctions were blistered by mannitol, as opposed to $18 \pm 4\%$ by raffinose. It was of interest to note the frequency distribution of cell types adjoining the junctions deformed by raffinose. Only 15 of the 181 junctions observed to be bounded by two granular cells were blistered, whereas 27 of the 28 junctions bounded by one granular cell and either a mitochondria-rich or goblet cell were deformed. Since some 80% of the surface epithelial cells are granular cells [6, 22], the characteristics of the apical junctions appear to depend upon the properties of the contiguous cells forming the junction.

An effort was made to determine whether morphologic changes other than blistering could be correlated with the decrease in resistance produced by mucosal hypertonicity. Although some small change in epithelial cell volume is likely to have occurred, cell shrinkage could be only rarely appreciated from the electron-micrographs. This observation is in agreement with previous studies of epithelial structure following dilution of the mucosal medium [5, 13, 28]. In the absence of vasopressin, the permeability of the apical plasma membranes to water is clearly very low.

Vacuolation of the granular epithelial cells was occasionally noted, but seems to have been unrelated to the osmotically-induced resistance changes. The vacuolation was most evident in tissues treated with the larger solutes (mannitol, sucrose, raffinose) which were less effective in reducing tissue resistance. Specifically, in the paired studies of KCl and mannitol, KCl produced a substantially larger fall in resistance and a greater junctional deformation, inducing cell vacuolation in 0.0 to 4.7% (mean $\pm$ SEM $= 2.8 \pm 1.4\%$), whereas mannitol produced a smaller resistance change, inducing vacuolation in 18.5 to 29.0% (mean $\pm$ SEM $= 23.5 \pm 3.0\%$) of the granular cells observed. Similarly, comparison of mannitol- and raffinose-treated samples indicated that mannitol produced a larger fall in resistance but induced far less vacuolation (5.0 to 22.5%; mean $\pm$ SEM $= 15.4 \pm 3.9\%$) of the observed granular cells than did raffinose (45.6 to 61.5%; mean $\pm$ SEM $= 53.9 \pm 3.4\%$).

### *Effect of Other Changes in the Tonicity of the Bathing Media*

Although mucosal hypertonicity was regularly associated with both the appearance of blisters and a fall in resistance, it was unclear whether these effects were determined by the tonicity of the mucosal medium *per se* or by the osmotic gradient established across the preparation. The following studies were, therefore, performed. In a series of three experiments, isotonic

Ringer's solution was initially applied to the surfaces of experimental and control quarter-bladders. All solutions were subsequently drained, and isotonic Ringer's solution restored to the mucosal side of the experimental tissue. Ringer's solution diluted with equal volumes of distilled water (R/2) of tonicity 134 to 146 mOsm/kg water, was added to the serosal medium of the experimental side. Since the volume of the epithelial cells increases following dilution of the serosal medium [13, 28], and since cell volume is thought to be one determinant of the transepithelial resistance [32] the dilute Ringer's solution (R/2) was added simultaneously to the serosal and mucosal surfaces of the control preparation, avoiding the establishment of an osmotic gradient. Following the change in solutions, the short-circuit current of the quarter-bladders transiently decreased but at the time of fixation, some 22 to 27.5 min later, the currents were very little changed in comparison to the pretreatment values ($+4 \pm 17\%$ on the experimental side, and $-3 \pm 13\%$ on the control side). At that time, the resistance of the control tissues had increased by 3, 16 and 33%, while that of the experimental tissue had fallen by 41, 50 and 49%, respectively. The mean $\pm$SEM for the relative change in resistance (as defined in Materials and Methods) was therefore calculated to be $0.44 \pm 0.032$, significantly different from 1 at the 0.005 probability level (Table 1).

Upon microscopic examination of the tissue, the epithelial cells of the experimental and control tissues appeared swollen to comparable degrees. No evidence of vacuolation was found. Blisters were observed in 90% of the apical junctions observed from the experimental tissue, and in none of those from the control tissue (Table 1). Fig. 11 illustrates the results.

It was also of interest to compare the effect of mucosal hypertonicity with the effect of hypertonicity of both serosal and mucosal solutions, in the absence of a transepithelial osmotic gradient. Small volumes of concentrated NaCl solution were added to the serosal and mucosal media bathing the control tissue, and to the mucosal medium bathing the experimental tissue, to a final tonicity of 390 to 410 mOsm/kg water; a similar volume of isotonic Ringer's solution was added to the serosal medium

Fig. 11. Comparative views of bladder epithelium exposed to an osmotic gradient by different means. (*a*) Hypotonic serosa (134 mOsm/kg water), isotonic mucosa; (*b*) Isotonic serosa, hypertonic mucosa (410 mOsm/kg water tonicity elevated with NaCl). In each case the apical junction is prominently blistered; the most apical point of remaining contact is indicated by an arrow. The intercellular space between granular cells $C_1$ and $C_2$ and between these and the underlying basal cell *BC* is extremely narrow. In (*a*), however, the cells are swollen; mitochondria *m* are swollen and distorted compared to those in (*b*) and the cytoplasm is less condensed. In (*b*), where the glycocalyx *gc* or "fuzz" is well preserved, it is clear that this is not present within the blister itself which originates therefore, as presumed, from within the junction. Each $\times$ 16,000

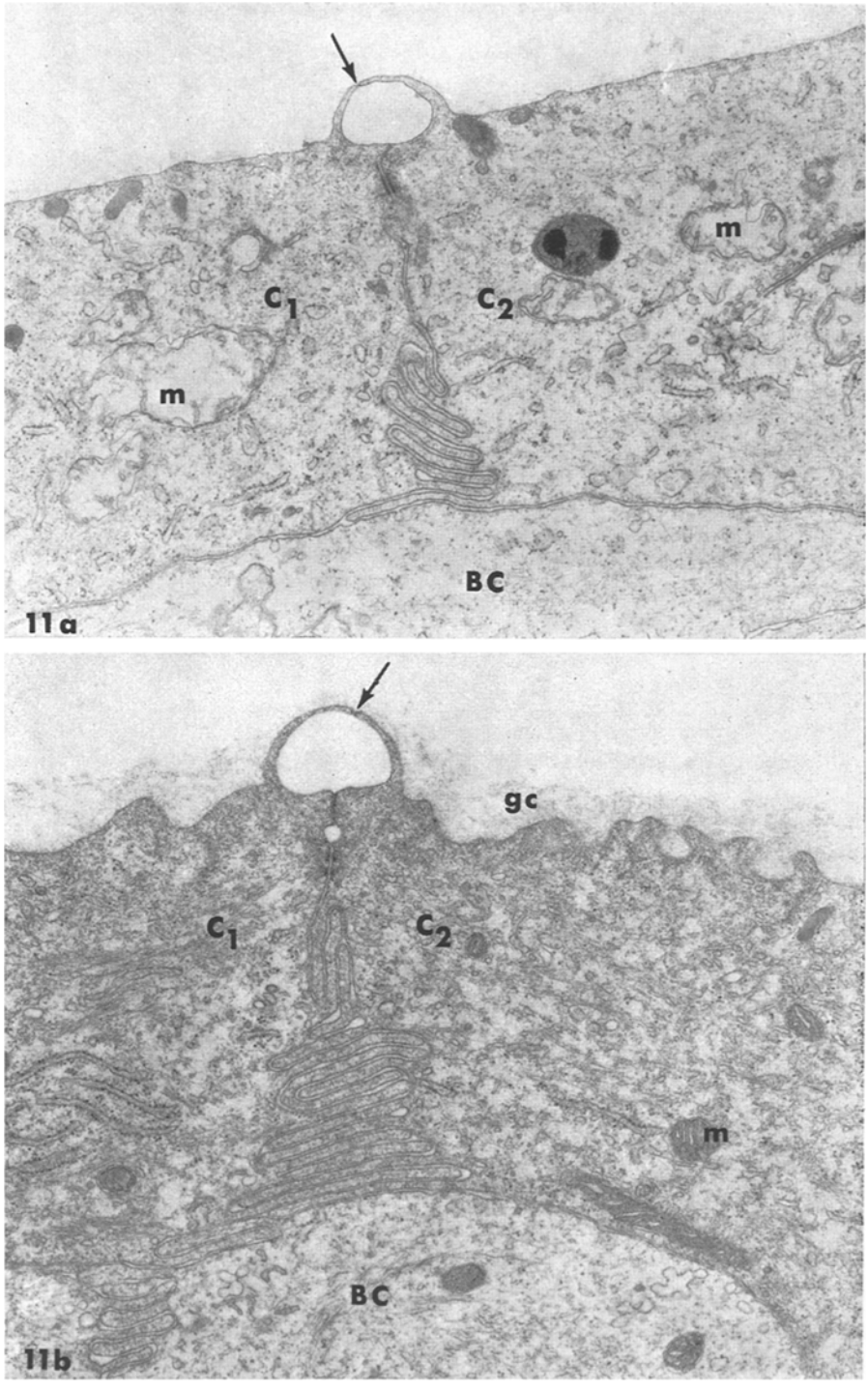


Fig. 11a and b

bathing the experimental tissue. Following the substitution of solutions, the short-circuit current of the control side transiently increased slightly, but then fell to $52 \pm 7.4\%$ of the pretreatment value. The short-circuit current of the experimental side also underwent a slight and transient increase. This slight increase was sustained in one preparation, but fell to values not very different from the initial short-circuit current in the remaining three experiments; averaging the results for the four experiments, mucosal hypertonicity increased the short-circuit current on the experimental side by $5 \pm 3.8\%$ (mean $\pm$ SEM). The resistance of the control tissue fell by $32 \pm 2.6\%$ (mean $\pm$ SEM), but in each case, the resistance of the experimental tissue fell even more during the same period of time (3.5 to 10.5 min); the mean value for the relative change in resistance was $0.75 \pm 0.085$. Electron-microscopy of the tissue exposed to a transepithelial osmotic gradient showed a deformation of 91% of the apical junctions examined, while no blistering whatsoever was noted in the control tissue (Table 1).

### *Presence of Diffusion Potential*

In the present studies, the changes induced in resistance by adding solutes to the mucosal medium were entirely unrelated to the changes induced in the short-circuit current. Addition of NaCl to the mucosal sodium Ringer's solution characteristically caused at least a transient increase in short-circuit current, whereas addition of KCl, mannitol, sucrose and raffinose all caused decreases in short-circuit current when added to the mucosal medium; addition of urea produced comparatively little change in short-circuit current. In all cases, however, addition of the solute in sufficiently high concentration reduced the tissue resistance.

When added to bladders with markedly inhibited active sodium transport (choline substitution, or addition of ouabain or amiloride), KCl invariably increased the "short-circuit current" while reducing the electrical resistance. Although KCl might conceivably have activated a previously low level of active sodium transport, such an interpretation is most unlikely since KCl added to a mucosal medium of sodium Ringer's solution significantly reduces short-circuit current. It is far more likely that the addition of large amounts of KCl provided a diffusion potential across the tissue, which was particularly prominent when sodium transport was inhibited. Since the ratio of the permeability coefficients of potassium to chloride across toad bladder is some 1.36 (T. Saito, P. D. Lief & A. Essig, *unpublished*), and the ratio of the mucosal to serosal concentrations was 29.0 and 1.8 for potassium and chloride, respectively, the potential produced by the mucosal KCl was in the direction predicted on the basis of a diffusion potential.

## Discussion

Addition, in sufficient quantity, of a variety of solutes to an initially isotonic mucosal medium reduces the electrical resistance across the urinary bladder of the toad. Mucosal hypertonicity also induces bullous deformation (blisters) of the most apical junctions (generally termed tight junctions) of the mucosal epithelium. The results of the present study demonstrate that all of the solutes examined (NaCl, KCl, urea, mannitol, sucrose and raffinose) which reduced tissue resistance also produced blistering in the same preparations. Furthermore, study of the effect of the addition and removal of mannitol indicated that both phenomena, the osmotically-induced resistance change and the junctional deformations, are reversible, following the same time course.

The simplest hypothesis accommodating these data is that musocal hypertonicity reduces tissue resistance specifically by enlarging the space between cells at the level of the apical junction. If so, and if active transport of sodium proceeds only through the cells and not through the junctional complexes [24, 25], the osmotically-induced resistance change should be independent of active transepithelial sodium transport. This proposition was examined by inhibiting sodium transport by three different techniques: (a) amiloride was added to the mucosal medium to inhibit sodium entry from the mucosal medium into the tissue [1]; (b) ouabain was added to the serosal medium to inhibit sodium extrusion from the cell into the serosal medium [21]; and (c) sodium was quantitatively replaced by choline. In each of these three experimental conditions, the magnitudes of the osmotically-induced resistance changes were comparable in the presence or absence of inhibition of sodium transport. The data do not preclude the possibility that mucosal hypertonicity may alter the resistance of the pathways through which sodium is actively transported. However, most of the osmotically-induced resistance change is independent of active sodium transport and must be mediated through passive parallel pathways.

The mechanism by which mucosal hypertonicity induces blisters is suggested by the results of two further series of experiments. First, an osmotic gradient was established across tissues by adding distilled water to the serosal medium rather than by adding solute to the mucosal medium. Serosal hypotonicity was found to decrease the transepithelial resistance and to produce blisters indistinguishable from those induced by mucosal hypertonicity. It seems clear, therefore, that blisters are induced by the osmotic gradient established across the epithelium and, therefore, across the junction, and not simply by the tonicity of the mucosal medium alone. The direction of the gradient is, of course, critical. Dilution of the mucosal medium [11]

or addition of solute to the serosal medium [9] does not induce blisters and increases, rather than decreases, the tissue resistance. Furthermore, the results suggest that the osmotically-induced resistance change is not primarily determined by changes in epithelial cell volume. Application of an osmotic gradient across the preparation reduces resistance and causes blisters whether the osmotic forces tend to swell the cells (serosal hypotonicity) or to shrink the cells (mucosal hypertonicity).

Second, the size of the solutes studied was related to their relative effectiveness in producing blisters and reducing resistance. Addition of mannitol and KCl to the mucosal media of adjoining quarter-bladders demonstrated that potassium (of crystal radius 1.33 Å) and chloride (of crystal radius 1.81 Å) were clearly more effective than mannitol (of radius 4.2 Å) [29]. A similar comparison of mannitol and raffinose suggested that mannitol was more effective than raffinose (of radius 6.0 Å) [29] in inducing both effects.

These data suggest that although larger molecules such as peroxidase [27] are restricted from entering the apical junction, water and smaller solutes can penetrate these junctions. When added to the mucosal medium, molecules as large as raffinose appear to diffuse into the junctional complexes. An increase in the tonicity of the fluid within the apical junction then induces movement of water directly from the lateral intercellular spaces and/or from the adjacent cell cytoplasm into the junctions; in the latter event, water will enter the cells in accordance with the gradient in chemical activity across the lateral plasma membranes, indirectly resulting in the observed closure of the lateral intercellular spaces. With increase in size of the blister within the junction, further solute may diffuse into the junctional complex from the mucosal medium stimulating transfer of more water, and further deformation of the junctions. With increased separation of the lateral plasma membranes of the adjoining cells, the resistance to passage of ions across the entire preparation is markedly reduced, lowering the total electrical resistance measured across the entire preparation. Similarly, the presence of such blisters will enhance the permeability to water flow through the intercellular spaces; an increase in tissue permeability to water is, in fact, produced by mucosal hypertonicity [30].

The results suggest, therefore, that the apical junctions present a limited, and perhaps adjustable, resistance to the passage of solutes and water across the urinary bladder of the toad, a conclusion consistent with recent results obtained with a number of other epithelia [3, 19, 20, 26]. Movement of ions across the junctional complexes and through the lateral intercellular spaces is, therefore, likely to constitute at least one of the parallel shunt pathways previously hypothesized on the basis of purely electrophysiologic evidence

[8, 35]. For this reason, the terms "tight junction" and "*zonula occludens*", [15] based on anatomic considerations and perhaps falsely evoking the concept of an infinite resistance to the passage of all solutes and water, should best be replaced by the term "limiting junction" [9] which describes the probable physiologic function of this structure.

The technical assistance of Miss Bonnie Lord is gratefully acknowledged. The authors wish to thank Drs. Qais Al-Awqati, Alvin Essig and Alexander Leaf for critical review of the manuscript.

This work was supported in part by Grant No. HE-06664 from the National Heart and Lung Institute and by Grant-In-Aid No. 71-847 from the American Heart Association. Dr. Civan is an Established Investigator (70-148) of the American Heart Association.

## References

1. Bentley, P. J. 1968. Amiloride: A potent inhibitor of sodium transport across toad bladder. *J. Physiol. (London)* **195**:317.
2. Biber, T. U. L., Curran, P. F. 1968. Coupled solute fluxes in toad skin. *J. Gen. Physiol.* **51**:606.
3. Boulpaep, E. L. 1967. Ion permeability of the peritubular and luminal membrane of the renal tubular cell. *In*: Symposium über Transport und Funktion Intracellularer Elektrolyte. F. Kruck, editor. pp. 98–107. Urban and Schwarzenberg, Munich.
4. Brightman, M. W., Reese, T. S. 1969. Junctions between intimately apposed cell membranes in the vertebrate brain. *J. Cell Biol.* **40**:648.
5. Carasso, N., Favard, P., Valérien, J. 1962. Variations des ultrastructures dans les cellules epithéliales de la vessie du crapaud après stimulation par l'hormone neurohypophysaire. *J. Microscopie* **1**:143.
6. Choi, J. K. 1963. The fine structure of the urinary bladder of the toad, *Bufo marinus*. *J. Cell Biol.* **16**:53.
7. Civan, M. M. 1970. Effects of active sodium transport on current-voltage relationship of toad bladder. *Amer. J. Physiol.* **219**:234.
8. Civan, M. M., Kedem, O., Leaf, A. 1966. Effect of vasopressin on toad bladder under conditions of zero net sodium transport. *Amer. J. Physiol.* **211**:569.
9. DiBona, D. R. 1972. Passive pathways in amphibian epithelia: Morphologic evidence for an intercellular route. *Nature, New Biol.* **238**:179.
10. DiBona, D. R., Civan, M. M. 1972. Anatomic site of osmotically-induced conductance changes in toad urinary bladder. *Fed. Proc.* **31**:279 Abs.
11. DiBona, D. R., Civan, M. M. 1972. Osmotically-induced conductance changes in toad bladder under physiologic conditions. *Int. Union of Pure and Applied Biophysics IVth Congress. (In press).*
12. DiBona, D. R., Civan, M. M., Leaf, A. 1969. The anatomic site of the transepithelial permeability barriers of toad bladder. *J. Cell Biol.* **40**:1.
13. DiBona, D. R., Civan, M. M., Leaf, A. 1969. The cellular specificity of the effect of vasopressin on toad urinary bladder. *J. Membrane Biol.* **1**:79.
14. Farquhar, M., Palade, G. E. 1963. Junctional complexes in various epithelia. *J. Cell Biol.* **17**:375.
15. Farquhar, M., Palade, G. E. 1965. Cell junctions in amphibian skin. *J. Cell Biol.* **26**:263.
16. Finkelstein, A. 1964. Electrical excitability of isolated frog skin and toad bladder. *J. Gen. Physiol.* **41**:545.

17. Franz, T. J., Van Bruggen, J. T. 1967. Hyperosmolality and the net transport of nonelectrolytes in frog skin. *J. Gen. Physiol.* **50**:933.
18. Franz, T. J., Galey, W. R., Van Bruggen, J. T. 1968. Further observations on asymmetrical solute movement across membranes. *J. Gen. Physiol.* **51**:1.
19. Frizzell, R. A., Schultz, S. G. 1972. Ionic conductances of extracellular shunt pathway in rabbit ileum. Influence of shunt on transmural sodium transport and electrical potential differences. *J. Gen. Physiol.* **59**:218.
20. Frömter, E., Diamond, J. 1972. Route of passive ion permeation in epithelia. *Nature* **235**:9.
21. Herrera, F. C. 1966. Action of ouabain on sodium transport in toad urinary bladder. *Amer. J. Physiol.* **210**:980.
22. Keller, A. R. 1963. A histochemical study of the toad urinary bladder. *Anat. Rec.* **147**:367.
23. Lindley, B., Hoshiko, T., Leb, D. E. 1964. Effects of $D_2O$ and osmotic gradients on potential and resistance of the isolated frog skin. *J. Gen. Physiol.* **47**:773.
24. Macknight, A. D. C., DiBona, D. R., Leaf, A., Civan, M. M. 1971. Measurement of the composition of epithelial cells from the toad urinary bladder. *J. Membrane Biol.* **6**:108.
25. Macknight, A. D. C., Leaf, A., Civan, M. M. 1971. Effects of vasopressin on the water and ionic composition of toad bladder epithelial cells. *J. Membrane Biol.* **6**:127.
26. Mandel, L., Curran, P. F. 1972. Response of the frog skin to steady-state voltage clamping: I. The shunt pathway. *J. Gen. Physiol.* **59**:503.
27. Masur, S. K., Holtzman, E., Schwartz, I. L., Walter, R. 1971. Correlation between pinocytosis and hydroosmosis induced by neurohypophyseal hormones and mediated by adenosine 3′,5′-cyclic monophosphate. *J. Cell Biol.* **49**:582.
28. Peachey, L. D., Rasmussen, H. 1961. Structure of the toad's urinary bladder as related to its physiology. *J. Biophys. Biochem. Cytol.* **10**:529.
29. Schultz, S. G., Solomon, A. K. 1961. Determination of the effective hydrodynamic radii of small molecules by viscometry. *J. Gen. Physiol.* **44**:1189.
30. Urakabe, S., Handler, J. S., Orloff, J. 1970. Effect of hypertonicity on permeability properties on the toad bladder. *Amer. J. Physiol.* **218**:1179.
31. Ussing, H. H. 1963. Effects of hypertonicity produced by urea on active transport and passive diffusion through the isolated frog skin. *Acta Physiol. Scand.* **59** (Suppl. 213):155.
32. Ussing, H. H. 1965. Relationship between osmotic reactions and active sodium transport in the frog skin epithelium. *Acta Physiol. Scand.* **63**:141.
33. Ussing, H. H. 1966. Anomalous transport of electrolytes and sucrose through the isolated frog skin induced by hypertonicity of the outside bathing solution. *Ann. N. Y. Acad. Sci.* **137**:543.
34. Ussing, H. H. 1969. The interpretation of tracer fluxes in terms of membrane structure. *Quart. Rev. Biophys.* **1**:365.
35. Ussing, H. H., Windhager, E. E. 1964. Nature of shunt path and active sodium transport path through frog skin epithelium. *Acta Physiol. Scand.* **61**:484.
36. Wade, J. B., DiScala, V. A. 1971. The effect of osmotic gradients on the ultrastructure of the zonulae occludentes in toad bladder epithelia. *11th Annual Meeting, Amer. Soc. Cell Biol.*, Abstr. 622.