J. Membrane Biol. 38, 359–386 (1978)

Pathways for Movement of Ions and Water Across Toad Urinary Bladder

III. Physiologic Significance of the Paracellular Pathway

Mortimer M. Civan and Donald R. DiBona

Departments of Physiology and Medicine, University of Pennsylvania School of Medicine, Philadelphia, Pa. 19174, and
Laboratory of Renal Biophysics, Massachusetts General Hospital, Boston, Massachusetts 02114

Received 6 April 1977; revised 22 August 1977

Summary. Hypertonicity of the mucosal bathing medium increases the electrical conductance of toad urinary bladder by osmotic distension of the epithelial "tight" or limiting junctions. However, toad urine is not normally hypertonic to plasma. In this study, the transmural osmotic gradient was varied strictly within the physiologic range; initially hypotonic mucosal bathing media were made isotonic by addition of a variety of solutes. Mucosal NaCl increased tissue conductance substantially. This phenomenon could not have reflected solely an altered conductance of the transcellular active transport pathway since mucosal KCl also increased tissue conductance, whether or not Na^+ was present in the bathing media. The effect of mucosal NaCl could not have been mediated solely by a parallel transepithelial pathway formed by damaged tissue since mucosal addition of certain nonelectrolytes also increased tissue conductance. Finally, the osmotically-induced increase in conductance could not have occurred solely in transcellular transepithelial channels in parallel with the active pathway for Na^+, since the permeability to ^{22}Na from serosa to mucosa (*s* to *m*) was also increased by mucosal addition of NaCl; a number of lines of evidence suggest that *s*-to-*m* movement of Na^+ proceeds largely through paracellular transepithelial pathways. The results thus establish that the permeability of the limiting junctions is physiologically dependent on the magnitude of the transmural osmotic gradient. A major role is proposed for this mechanism, serving to conserve the body stores of NaCl from excessive urinary excretion.

Since Ussing and his colleagues initiated the modern era of transport physiology (Ussing, 1949) our concepts of transepithelial NaCl transport have been formulated almost exclusively in terms of salt movement through the transporting cells, i.e., through transcellular transepithelial pathways. Early morphologic results seemed to support the concept that only the transcellular pathways were of physiologic importance in regulating transepithelial salt movement. In their classic studies, Farquhar

0022-2631/78/0038-0359 $5.60

and Palade (1963, 1965) reported that the most apical portion of the intercellular junctional complex was the limiting barrier to transepithelial passage of large molecules, such as hemoglobin. They called this element the *zonula occludens* or tight junction, a term which has suggested to physiologists and morphologists that the junction effectively blocks transepithelial passage of all solutes and water between the transporting cells, i.e., through the paracellular channels. Subsequently these elements have also been called limiting junctions (DiBona, 1972; DiBona & Civan, 1973), a convention we shall follow here.

The presumption that the paracellular pathway was insignificant persisted over succeeding years, despite evidence that hypertonicity of the outer medium bathing frog skin can either produce new parallel transepithelial shunt pathways or increase the magnitude of existing parallel channels (Ussing, 1963; Ussing & Windhager, 1964), and despite indirect evidence that such shunts are present in toad urinary bladder under physiologic conditions (Civan, Kedem & Leaf, 1966). More recently, both morphologic and electrophysiologic measurements have suggested that, at least in more "leaky" epithelia such as proximal renal tubule (Boulpaep, 1972; Grandchamp & Boulpaep, 1974; Rawlins et al., 1975), rabbit gallbladder (Frömter & Diamond, 1972), and rabbit ileum (Frizzell & Schultz, 1972), the paracellular pathway through the "tight" junctions cannot be neglected.

Recent studies have further demonstrated that establishment of reversed osmotic gradients across "tight" epithelia such as toad urinary bladder, frog skin and *Xenopus* skin, either by reducing the serosal osmolality or increasing the mucosal osmolality, causes bullous deformations of the apical limiting junctions (DiBona, 1972; DiBona & Civan, 1973; Wade, Revel & DiScala, 1973). The osmotically-induced widening of the space between the two apposed plasma membranes comprising the limiting junction permits ions to cross the paracellular channels more readily, resulting in an increased tissue conductance (DiBona & Civan, 1973; Civan & DiBona, 1974). Although of investigational interest, such results were of limited physiologic relevance. Frogs and *Xenopus* are normally exposed to hypotonic saline solutions, while toad urine is never hypertonic to toad plasma (Sawyer, 1956; Leaf, Anderson & Page, 1958).

In the present study, we have examined the effects of varying the osmolality of the mucosal medium bathing toad urinary bladder within the physiologic range, hypotonic or isotonic to toad plasma. On the basis of the results presented, we propose that the osmolality of the mucosal medium plays a physiologic role in regulating salt movement through the

paracellular transepithelial pathway. We further propose that this osmotic regulation may serve an important function, limiting excessive urinary excretion of NaCl.

Certain of the current findings have been presented in preliminary form elsewhere (DiBona & Civan, 1972*b*).

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 or sphagnum moss. Paired experimental and control tissues from the same toad were provided, either by mounting the hemibladder in Lucite double-chambers of 2.5 cm^2 cross-sectional area, or by mounting the paired hemibladders in separate Lucite chambers of 5.9 cm^2 cross-sectional area. In each case, the serosal surface of the tissues was supported by nylon mesh. The volume of the mucosal medium always exceeded that of the serosal medium to maintain a slight gradient of hydrostatic pressure ($\sim$1 cm) across the preparation.

The isotonic sodium Ringer's solution consisted of (mM): Na^+, 115.1; K^+, 3.3; Ca^{2+}, 0.8; Cl^-, 113.9; HCO_3^-, 2.2; HPO_4^{2-}, 1.8; $H_2PO_4^-$, 0.3; the pH was 7.7–8.1 and tonicity 218–226 mOsm/kg water. The hypotonic sodium Ringer's solution consisted of (mM): Na^+, 14.0; K^+, 0.3; Ca^{2+}, 0.07; Cl^-, 10.4; HCO_3^-, 0.3; HPO_4^{2-}, 1.8; $H_2PO_4^-$, 0.3; the pH was 7.8 and tonicity 26–27 mOsm/kg water. Isotonic and hypotonic choline Ringer's solutions had the same composition as the corresponding sodium Ringer's solutions, except for the equimolar replacement of Na^+ by choline ions.

Phloretin was obtained from K & K Laboratories, Inc. (Plainview, N.Y.), xylitol from Pfanstiehl Laboratories, Inc. (Waukega, Ill.), and adonitol both from Pfanstiehl Laboratories, Inc., and from Calbiochem (La Jolla, Calif.). For many of the experiments, the adonitol was recrystallized 1–3 times from cold ethanol, without measurably altering the results. ^{22}Na was obtained in the form of neutral $^{22}NaCl$ from the Amersham/Searle Corp. (Arlington Heights, Ill.); the radioactive samples were counted with a model # 1185 Automatic Gamma System (Searle Analytic, Inc., Arlington Heights, Ill.).

As previously described (DiBona & Civan, 1973), the experimental procedure was to clamp the transepithelial potential at 0 mV, except for 9-sec intervals every 30 sec when the transepithelial potential was increased to 10 or to 12 mV (serosa positive to mucosa) by means of chlorided silver electrodes in series with 3 M KCl agar bridges. Potentials were monitored by means of calomel electrodes in series with similar salt bridges. Transepithelial electrical current was continuously monitored and displayed on a dual pen recorder.

The initial electrical measurements were performed using the electrical circuit previously described in detail (DiBona & Civan, 1973). By introduction of a suitable feedback loop to an otherwise conventional voltage-clamping arrangement, it is possible to correct automatically for the resistance of the bathing media and to control the difference in electrical potential across the tissue, itself. This feedback loop is particularly useful under conditions where the electrical resistance of the bathing solutions is an appreciable fraction of the total resistance of the preparation, and where the solution resistance is changed during the course of the experiment.

In many experiments, however, nonelectrolytes were added to the mucosal media, changing the solution resistance very little. In addition, the radioactive flux measurements were conducted in the larger chamber equipped with silicone rubber gaskets

treated with a silicone rubber grease (High Vacuum Grease, Dow Corning Corp., Midland, Mich.), a technique used effectively in other preparations (Higgins et al., 1975; Lewis & Diamond, 1976) to minimize edge damage; under these circumstances, the solutions made only a smaller contribution to the total resistance of the preparation. In both the latter cases, it proved unnecessary to introduce the feedback loops, and the more conventional circuit was used. Here, however, the solution resistance was subtracted from the resistance of the total preparation; all values reported in *Results* reflect specifically the tissue conductance.

Essentially a single experimental design was used throughout the study. Paired tissues were initially bathed with "baseline solutions", consisting of isotonic Ringer's solution on their serosal surfaces, and with hypotonic Ringer's solution on their mucosal surfaces. The osmolality of the mucosal solution bathing the experimental tissue was then raised to isotonicity by adding a small volume of the same hypotonic Ringer's solution containing a high concentration of the experimental solute. In this way, the concentration of each of the components of the mucosal medium was unchanged by the mucosal addition, except that of the experimental solute. An identical volume of hypotonic Ringer's solution was always added to the mucosal medium of the control tissue.

Data reduction was carried out by measuring g_o (the electrical conductance of the tissue just before adding mucosal solute) and g_e (the tissue conductance during the experimental period). Unless otherwise specified, analysis was performed by calculating the ratio γ_g, defined as (g_e/g_o), both for the experimental (exp) and control (con) preparations. Finally, $\Delta\gamma_g$ was calculated as $(\gamma_g)_{exp}-(\gamma_g)_{con}$. A value of $\Delta\gamma$ of greater than, less than, or equal to zero, indicates that the conductance of the experimental side increased more than, less than, or equally to that of the control tissue, respectively.

The analogous parameters $(\gamma_I)_{exp}$, $(\gamma_I)_{con}$ and $\Delta\gamma_I$ were calculated for the current (I) necessary to reduce the transepithelial potential to zero. For convenience, we shall refer to I as the "short circuit current", whether or not the preparations were bathed with identical solutions on their two surfaces. Similar parameters were calculated for the Na^+ permeability P_{Na}, and for the various components of the total tissue conductance.

Unless otherwise stated, all values reported in *Results* are presented as means $\pm$SEM. All probabilities (P) of the null hypothesis have been calculated on the basis of the Student's t-test. Such probabilities are meaningful only if the sample populations do not deviate significantly from t-distributions. For this reason, all of the data presented in the tables were subjected to the Kolmogorov-Smirnov test, as previously described (Siegel & Civan, 1976). In each case, the probability was $>20\,\%$ that the data presented are characterized by a t-distribution, providing a rational basis for the application of the Student's t-test.

When tissue fixation was performed, suitable volumes of 50 % (wt/vol) glutaraldehyde (Fisher Scientific Co., Pittsburgh, Pa.) were added simultaneously to the mucosal and serosal solutions to provide a final glutaraldehyde concentration of 1 % (DiBona & Civan, 1972*a*). After standing 15 to 30 min, rectangles of tissue were excised and immersed in a 1 % solution of glutaraldehyde in phosphate buffer. Tissue samples were post-fixed in osmium tetroxide, dehydrated and embedded in epoxy as described earlier (DiBona, Civan & Leaf, 1969*a*); one-half of each of the samples was also stained *en bloc* with uranyl acetate (Farquhar & Palade, 1963) to facilitate observations of junction membranes. Sections were cut with a Reichert OmU2 ultramicrotome (G. Reichert Werke, A.G., Vienna, Austria) or with an LKB-Ultratome III (LKB Produkter, Bromma, Sweden). 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

Representative results obtained with the protocol for the first series of experiments are illustrated in Fig. 1. Hemibladders were bathed with baseline solutions in the smaller double chambers. Under these conditions, the average tissue conductance was 0.28 ± 0.018 mmho $\cdot$ cm^{-2}. At

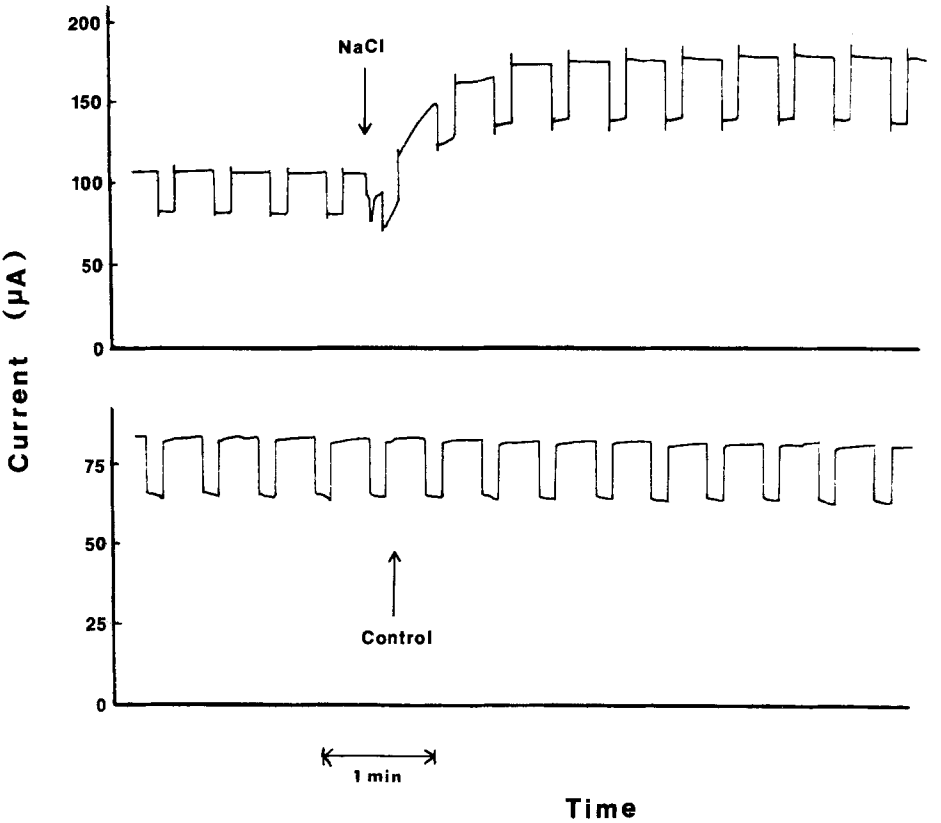

Fig. 1. Conductance changes induced by adding NaCl to the mucosal medium, increasing its osmolality to isotonicity. The upper curve was obtained from the experimental side, and the lower curve from the control side, of adjoining quarter-bladders. The abscissa is time. The ordinate is the current necessary to reduce the transepithelial potential to zero (upper envelope of each curve) or to (+) 12 mV (lower envelope). The tissue conductance is thus given by the magnitude of the current deflection, divided by 12 mV. Within 2 min after the mucosal additions, the tissue conductance (g_{exp}) and short circuit current (I_{exp}) of the experimental side increased to new steady-state values. At 4 min after adding NaCl, g_{exp} had risen from 0.83 mmhos $\cdot$ cm^{-2} to 1.26 mmhos $\cdot$ cm^{-2}, and I_{exp} had reached 174 μA from an initial value of 105 μA. On the control side, little change was noted during the same period. The experiment was conducted with an electrical circuit incorporating a feedback loop which automatically corrected for the solution resistance. Prior to adding the NaCl to the experimental tissue, it was necessary to reduce the correction setting. The distortion of the upper current at the vertical arrow is an artifact reflecting this resetting of the feedback loop

the vertical arrow marked "NaCl", a sufficient volume of 2.4 osM NaCl solution was added to the mucosal medium of the experimental tissue to render it isotonic to the serosal medium; an identical volume of hypotonic sodium Ringer's solution was added to the mucosal medium of the control tissue. The short-circuit current and tissue conductance of the experimental quarter-bladder increased within seconds and stabilized at a new steady state 1.5–2 min after adding the NaCl solution.

In a series of 8 experiments, $\Delta\gamma_g$ was 0.8 ± 0.14 ($P < 0.001$) and $\Delta\gamma_I$ was 1.3 ± 0.39 ($P < 0.02$). These positive values, significantly different from zero, indicate that rendering the mucosal medium isotonic by addition of NaCl increases the electrical conductance as well as the short circuit current across the isolated toad bladder.

The rise in short circuit current might reflect an increase in conductance of the transcellular active transport pathway; however, several lines of evidence suggest that the addition of NaCl increased the conductance of one or more passive transepithelial pathways as well. The first series of experiments supporting this conclusion is represented by the data of Fig. 2, which were generated from the same tissue as that of Fig. 1, but with the experimental and control roles reversed. The serosal surfaces were washed several times with fresh isotonic sodium Ringer's solution, and the mucosal surfaces with fresh hypotonic sodium Ringer's solution. After a steady state was attained, the mucosal medium of the experimental tissue was rendered isotonic by addition of concentrated KCl solution; an identical volume of hypotonic sodium Ringer's solution was added to the mucosal medium of the adjoining quarter-bladder. As with NaCl, increasing the tonicity of the mucosal medium increased the tissue conductance but, in this case, the short circuit was reduced. For the entire series of 8 experiments, $\Delta\gamma_g$ and $\Delta\gamma_I$ were measured to be 0.4 ± 0.13 ($P < 0.02$) and -0.16 ± 0.072 ($P < 0.01$), respectively. The presence of K^+, *per se*, in the mucosal medium has been reported to have little effect on the short circuit current (Robinson & Macknight, 1976*a–c*) or on the unidirectional flux of Na^+ from mucosa to serosa (Frazier & Leaf, 1964). Thus, the present result indicates that increasing the osmolality of the mucosal medium to isotonicity induces an increased conductance of pathways functioning in parallel with the transepithelial active Na^+ transport channel.

This conclusion was further examined under conditions where active transport of Na^+ was specifically prevented. Hemibladders were mounted in double-chambers, and bathed with baseline choline Ringer's solutions. When the mucosal medium of the experimental tissue was

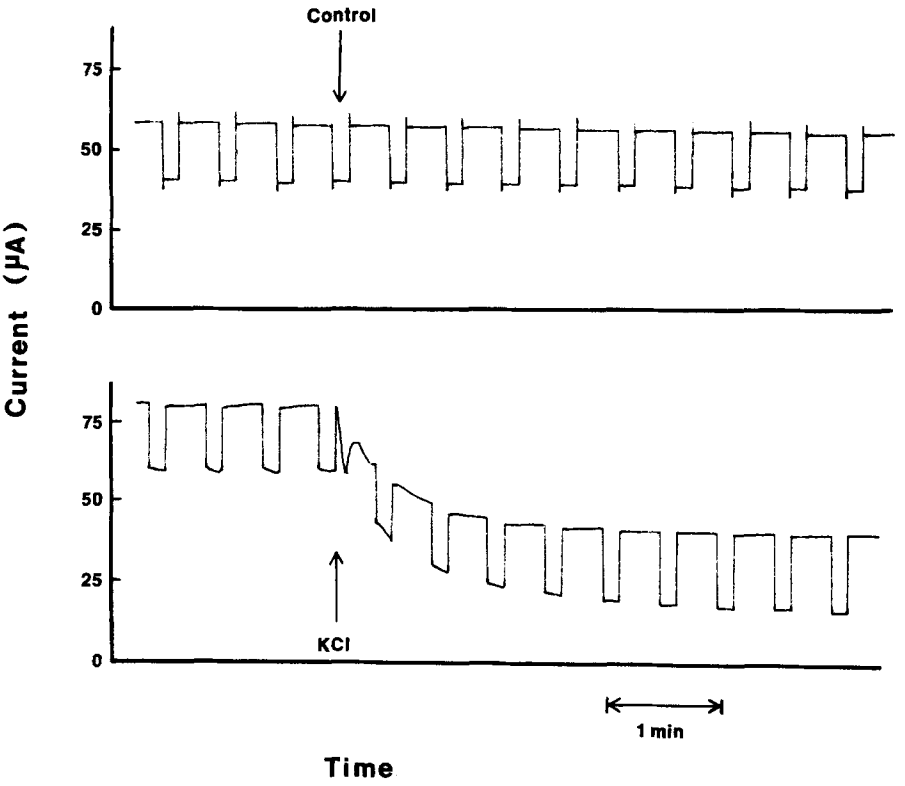

Fig. 2. Conductance changes induced by adding KCl to the mucosal medium, increasing its osmolality to isotonicity. By 4 min after the mucosal additions, the conductance of the experimental tissue had increased from approximately 0.68 to 0.82 mmhos·cm^{-2}, while the short circuit current had fallen from 81 to 41 μA. The conductance and short-circuit current of the control changed very little

made isotonic by addition of concentrated KCl solution, the tissue conductance increased with a time course similar to that noted above in the presence of Na^+. In a series of 10 experiments, $\Delta\gamma_g$ was found to be 1.9 $\pm$0.15, 5 min after KCl addition, supporting the concept that the tonicity of the mucosal medium regulates, at least in part, the conductance of transepithelial channels in parallel with the active transport pathways for Na^+.

The results of these first experiments do not establish the anatomic identity of the osmotically sensitive pathways. However, from the known effects of mucosal hypertonicity, it seemed reasonable that the junctions would also be responsive over the physiologic range of tonicities. Therefore, experiments were performed as above, but control and experimental quarter-bladders were fixed 4–16 min after addition of solute to the

mucosal medium of the experimental preparation. As expected from previously published findings (Peachey & Rasmussen, 1961; DiBona, Civan & Leaf, 1969*b*; DiBona & Civan, 1973), no morphological changes in the cells were evident after changing the tonicity of the mucosal bath. In addition, no changes were apparent in the geometry of the lateral intercellular spaces. In several experiments, the width of the spaces between the points of contact within the limiting junction appeared increased, as anticipated from our previous studies of reversed osmotic gradients. However, this structural modification was not observed in all of the preparations studied. We conclude, therefore, that junctional changes could not be rigorously documented with the present techniques.

Given the evident limitations of direct morphologic approaches to this problem, additional experiments were designed to further specify the site of this gradient-sensitive transepithelial channel. Possibilities to be considered were: (i) a paracellular pathway, through the limiting junction and the lateral intercellular spaces, (ii) a transcellular route in parallel with the transcellular active transport pathway, and (iii) a conducting pathway across damaged tissue within the area of contact between the Lucite chamber and the epithelium ("edge-damage").

Edge-damage in a chamber-mounted epithelium can contribute significantly to the measured transmural conductance (Dobson & Kidder, 1968; Walser, 1970; Helman & Miller, 1971). Increasing the ionic concentrations of the mucosal medium and thus the average ionic concentrations of the transepithelial pathway through the damaged tissue would enhance the total tissue conductance. Changes in conductance of any edge-damage-pathway are not to be expected if the ionic strength of the bathing solutions is maintained constant while the mucosal tonicity is elevated by addition of nonelectrolytes. This reasoning must be qualified, however. The conductance of any osmotically sensitive pathway might not be as responsive to the addition of nonelectrolytes as to NaCl or KCl; therefore, a small effect by nonelectrolytes is not necessarily convincing evidence for passive transport predominantly by edge-damage pathways. Indeed, studies of hypertonic mucosal solutions revealed that the magnitudes of the changes in structure and conductance induced by the solute tested were inversely dependent on the solute size.

The effects of three nonelectrolytes (xylitol, adonitol and urea) were studied. Addition of xylitol to the mucosal medium to isotonicity produced no significant change in tissue conductance. In 6 experiments, $\Delta\gamma_g$ was 0.04 ± 0.032. On the other hand, making the mucosal medium isotonic by adding adonitol resulted in a small but significant increase in

Table 1. Fractional conductance changes following addition of adonitol or KCl to mucosal medium

	γ_g	
	Adonitol	KCl
Exp	1.15 ± 0.023	2.39 ± 0.33
Con	1.03 ± 0.019	1.01 ± 0.014
Δ	0.12 ± 0.021	1.38 ± 0.33
n	11	10
P	<0.001	<0.005

In 10 of the 11 experiments summarized, the initially hypotonic mucosal medium of the experimental quarter-bladder was made isotonic several times sequentially. After each part of the experiment the serosal and mucosal media were replaced several times with fresh isotonic and hypotonic sodium Ringer's solution, respectively. In the same 10 experiments, KCl was the solute added in the second or third mucosal addition. Adonitol was the solute added in all other mucosal additions. In each experiment, $(\gamma_g)_{exp}$, $(\gamma_g)_{con}$ and $\Delta(\gamma_g)$ were calculated as the means of the series of determinations obtained following addition of adonitol.

tissue conductance. Adonitol also produced a small increase in short circuit current ($\Delta\gamma_I = 0.13 \pm 0.025$), usually peaking at 8–16 min after mucosal addition. Measured at the time of the peak current effects, $\Delta\gamma_g$ was found in a series of 11 experiments to be 0.12 ± 0.021 (Table 1).

Urea might be expected to be more effective than adonitol since it is a smaller molecule and was nearly as effective as NaCl or KCl in studies of mucosal hypertonicity (DiBona & Civan, 1973). Yet, in three preliminary experiments, addition of urea to mucosal isotonicity produced no detectable changes in tissue conductance. This may reflect the entry of urea both into the epithelial cells and the limiting junctions, preventing the establishment of the osmotic gradients necessary to alter the paracellular pathway (Civan & DiBona, 1974).

This possibility was tested by repeating these experiments in the presence of phloretin, which inhibits the facilitated diffusion of urea into the cells (Levine, Franki & Hays, 1973). Phloretin was added to adjoining experimental and control quarter-bladders bathed with baseline solutions to a final mucosal concentration of 5 mM. After a steady state was attained, the mucosal medium of the experimental tissue was rendered isotonic by addition of concentrated urea solution, while an identical volume of hypotonic sodium Ringer's solution was added to the mucosal medium of the control. In each experiment, the addition of urea increased the absolute value of the conductance. In 10 experiments

Table 2. Fractional conductance changes following addition of urea or KCl to mucosal medium

	γ_g	
	Urea	KCl
Exp	1.15 ± 0.023	2.21 ± 0.11
Con	1.04 ± 0.016	1.01 ± 0.013
Δ	0.11 ± 0.012	1.19 ± 0.11
n	10	10
P	< 0.001	< 0.001

Initially, urea was added to render the mucosal medium isotonic; an identical volume of hypotonic Ringer's solution was added to the mucosal medium of the adjoining control quarter-bladder. Phloretin was present in the mucosal medium and in the mucosal addition for the experimental and control tissues. Subsequently, the mucosal medium was replaced three times with fresh baseline solutions. Here (γ_g) is defined as the ratio of the tissue conductance measured after adding urea to the average conductance measured in hypotonic mucosal medium before and after the experimental period.

In the second half of each experiment, the roles of the experimental and control quarter-bladders were switched. KCl was now added to the mucosal medium of the previously control tissue rendering it isotonic; this side had until now been bathed only with hypotonic Ringer's solution. The identical volume of hypotonic Ringer's solution was added to the mucosal medium of the adjoining control tissue, which had served previously as the experimental side. At the conclusion of the experiment, fresh baseline solutions were replaced three times. The values of (γ_g) were calculated as for the first half of the experiment.

(Table 2) $\Delta\gamma_g$ was found to be 0.11 ± 0.012, establishing that urea does significantly increase the tissue conductance when its entry into the epithelial cells is inhibited. Therefore, apart from any contribution of edge-damage, increasing the tonicity of the mucosal medium over the physiologic range increases tissue conductance.

It should be noted that the effects induced by adonitol and urea are clearly much smaller than that induced by comparable milliosmoles of KCl. In the same series of experiments, the serosal and mucosal media were replaced several times with fresh baseline Ringer's solutions. After a new steady state was attained, KCl was added to the mucosal medium of the experimental quarter-bladder, and an identical volume of hypotonic sodium Ringer's solution was added to that of the adjoining control tissue. In each case, the KCl induced a roughly ten-fold greater increase in conductance than had the adonitol (Table 1) or urea (Table 2).

Results with nonionic solutes established, then, that under physiologic conditions, the mucosal tonicity regulates the conductance of

transepithelial channels which are in parallel to the active transport pathway for Na^+; these pathways are distinct from any nonspecific channels resulting from damage to the tissue. In order to distinguish whether these channels were primarily paracellular or transcellular, measurements were made of the unidirectional flux of ^{22}Na from serosa to mucosa. The flux of Na^+ in this direction is a reliable index of the permeability of the paracellular pathway since several lines of evidence considered in the *Discussion* indicate that Na^+ enters the transporting cells from the serosal medium very slowly.

The fluxes were measured across paired hemibladders mounted in the larger Lucite chambers. When bathed with baseline sodium Ringer's solutions, the tissue conductance was 0.16 ± 0.023 mmhos $\cdot$ cm^{-2}. After a steady state was attained, ^{22}Na was added to the serosal media of the paired experimental and control hemibladders. Samples of solution were taken every 30 min during a baseline period of 2 hr. A small volume of concentrated NaCl solution was then added to bring the mucosal medium of the experimental hemibladder to isotonicity; an identical volume of hypotonic sodium Ringer's solution was added to the mucosal medium of the control preparation. Samples of solution were taken once again 5 min after the mucosal additions (by which point the tissue conductances were in a new steady state), and at 30-min intervals for another 2-hr period.

An average permeability, P^*_{Na} was calculated in cm $\cdot$ sec^{-1} from the relationship:

$$P^*_{Na} = \frac{\Delta n^*}{[Na^*]_s \cdot \Delta t \cdot S} \tag{1}$$

where $[Na^*]_s$ is the serosal concentration of ^{22}Na (in moles $\cdot$ cm^{-3}), and where Δn^* is the absolute number of moles of ^{22}Na transferred from serosa to mucosa during the time period Δt (in sec), across the total surface area (S) of exposed tissue (5.9 cm^2). An average tissue conductance for the same time period was calculated as the mean of the three values measured: just before the onset, at the mid-point, and just before the end of the sample period.

P^*_{Na} measured during the first 30-min period following mucosal addition was compared with that measured during the final 30-min baseline period before adding NaCl. In each case that mucosal isotonicity produced an increase in g_T, P^*_{Na} also underwent an absolute increase. As noted in Table 3, the fractional change in P^*_{Na} (calculated as $\Delta \gamma_p$) was

Table 3. Changes in conductance and Na^+ permeability induced by adding NaCl to mucosal medium

A. Changes in total conductance and Na^+ permeability

	g_o	g_e	γ_g	$(P^*_{Na})_o$	$(P^*_{Na})_e$	γ_P
	(mmhos · cm^{-2})			(10^{-8} cm · sec^{-1})		
Exp	0.13 ± 0.016	0.16 ± 0.020	1.25 ± 0.055	2.8 ± 0.57	4.1 ± 0.86	1.5 ± 0.14
Con	0.16 ± 0.046	0.14 ± 0.042	0.85 ± 0.028	3.6 ± 0.59	3.7 ± 0.68	1.02 ± 0.068
Δ			0.40 ± 0.046			0.49 ± 0.088
n			10			10
P			< 0.001			< 0.001

B. Changes in calculated conductance of paracellular pathway

	$(g_L)_o$	$(g_L)_e$	$\left[\frac{(g_L)_e-(g_L)_o}{g_o}\right]$
	(mmhos · cm^{-2})		
Exp	0.010 ± 0.0021	0.37 ± 0.0077	0.21 ± 0.032
Con	0.013 ± 0.0021	0.013 ± 0.0025	0.002 ± 0.0085
Δ			0.21 ± 0.032
n			10
P			< 0.001

$(g_L)_e$ and $(g_L)_o$ are theoretical estimates of the conductance of the paracellular pathway calculated from the measurements of $(P_{Na})_e$ and $(P_{Na})_o$, using Eqs. (A3) and (A4) of the Appendix. The results suggest that roughly half of the osmotically-induced change in tissue conductance, $\Delta(\gamma_g)$, can be ascribed to changes in conductance of the paracellular pathway,

$$\Delta\left[\frac{(g_L)_e-(g_L)_o}{g_o}\right].$$

substantial. $\Delta\gamma_p$ was 0.49 ± 0.088, slightly but not significantly exceeding the fractional increase ($\Delta\gamma_g$) in total tissue conductance of 0.40 ± 0.046.

It was of interest to estimate from the data of Table 3 how much of the total increase in g_T could be ascribed to changes induced exclusively in the paracellular pathways. Taking an approach similar to that of Saito, Lief & Essig (1974), we may estimate the conductance through the paracellular pathway (g_L) from measurements of P^*_{Na} in the serosal-to-mucosal direction using Eqs. (A3) and (A4) of the *Appendix*. We wish to compare the absolute change in paracellular conductance $[(g_L)_e-(g_L)_o]$ with the change in total conductance (g_e-g_o). In order to relate these changes to those spontaneously occurring in the paired control and in other experimental and control hemibladders, these differences are normalized to the total conductance (g_o) measured just before the experi-

mental period. The normalized change in total conductance, corrected for the spontaneous changes noted in the control, is then calculated as the difference

$$\left[\frac{(g_e - g_o)}{g_o}\right]_{\text{exp}} - \left[\frac{(g_e - g_o)}{g_o}\right]_{\text{con}}.$$

This normalized corrected difference is simply equal to the parameter $\Delta\gamma_g$ we have been using throughout *Results*. On the other hand, the comparable parameter for g_L,

$$\left[\frac{(g_L)_e - (g_L)_o}{g_o}\right]_{\text{exp}} - \left[\frac{(g_L)_e - (g_L)_o}{g_o}\right]_{\text{con}}, \quad \text{is a new index.}$$

All of the relevant parameters are presented in Table 3.

From the results of Table 3, we estimate that half of the osmotically-induced increase in total conductance can be ascribed to the paracellular pathway, *per se*. The remainder of the change must have been mediated through transcellular pathways and through damaged tissue. It is likely that the active transport pathway contributed significantly to the observed increase in total tissue conductance, since mucosal NaCl addition consistently increased short circuit current; for the 10 experiments of Table 3, $\Delta\gamma_I$ was 0.45 ± 0.045. However, the contribution of edge damage must have been very small. Averaging the measurements of P^*_{Na} obtained under isotonic conditions for the 10 experiments, the mean ($\pm$SEM) may be calculated to be 4.1 $(\pm 0.86) \times 10^{-8}$ cm $\cdot$ sec^{-1}. This value compares favorably with previous measurements obtained with bladders mounted either in chambers ($10.4 \pm 0.7 \times 10^{-8}$ cm $\cdot$ sec^{-1}; Saito et al., 1974) or as sacs ($4.6 \pm 0.5 \times 10^{-8}$ cm $\cdot$ sec^{-1}; Walser, 1970), where care was taken to minimize the effects of edge damage.

For the purposes of later discussion, it is helpful to obtain an estimate of g^a_{Na}, the conductance of the active transport pathway for Na^+. This value was determined for tissues bathed with isotonic solutions by subtracting the paracellular conductance $(g_L)_e$ from the total conductance for each of the experimental tissues of Table 3. Calculated this way, $g^a_{\text{Na}} = 0.12 \pm 0.017$ mmhos $\cdot$ cm^{-2}. For the same tissues, the fractional tissue conductance provided by the active transport pathways (g^a_{Na}/g_e) may be calculated to be 0.77 ± 0.036. Again, this value compares favorably with other published values obtained with a similar technique (0.60–0.68; Saito et al., 1974) or by electrophysiologic methods (0.44–0.48, calculated from the data of Reuss & Finn, 1975).

Finally, it will be useful to estimate g^p_{Na}, that part of the total paracellular conductance specifically reflecting movement of Na^+

through the paracellular channel. From Eq. (A1) of the *Appendix*, and Tables 3 and 4, g^p_{Na} is calculated to be 0.018 ± 0.0038 mmhos $\cdot$ cm^{-2} when the bladder is bathed with isotonic mucosal and serosal Ringer's solutions.

Discussion

The establishment of reversed osmotic gradients, by applying either mucosal hypertonicity or serosal hypotonicity, markedly increases the electrical conductance of toad urinary bladder. This phenomenon has been the central concern of the present series of mansuscript. In the first paper, we established that the application of these nonphysiologic reversed osmotic gradients increases tissue conductance specifically through the paracellular pathway by altering the structure of the intercellular limiting or tight intercellular junctions (DiBona & Civan, 1973). In the second paper of the series, we examined interactions between the paracellular and transcellular transepithelial pathways (Civan & DiBona, 1974).

The data presented in those reports suggested the following mechanism of action of reversed osmotic gradients. The presence of a relative excess of solute molecules in the mucosal medium establishes a concentration gradient, favoring solute entry both into the cytoplasm of the surface cells and into the interstitial fluid contained within the intercellular tight junctions. If the plasma membranes are relatively impermeable to the solute added, the diffusion of solute into the limiting junctions produces an osmotic gradient favoring water entry into the junctions from the basal intercellular spaces and from the cytoplasm of the adjoining epithelial cells. The resulting bullous deformation of the apical junctions entails a greater separation of the contiguous plasma membranes increasing the conductance of the paracellular pathway across the toad bladder.

The data presented in the first two papers of the series provided no information concerning whether the junctions are responsive to osmotic gradients over the physiologic range (mucosal medium hypotonic or isotonic to plasma). In addition, the physiologic significance of the osmotic regulation of these junctions has been unclear. The present study has been addressed to these two questions.

The first of the current observations was that addition of NaCl to the mucosal medium increases the tissue conductance (Fig. 1). In principle, the change could be mediated by changes in: (i) the transcellular pathways for active Na^+ transport, (ii) a factitious pathway produced by

crushing tissue during the mounting procedure, (iii) a transcellular pathway parallel to the active transport channel, and (iv) the physiologic paracellular pathway through the apical limiting junction and lateral intercellular space.

Two of the other data presented here indicate that the increase in conductance induced by adding NaCl cannot be exclusively mediated by changes in the active transport pathway. Addition of equimolar concentrations of KCl to the hypotonic mucosal media increased the tissue conductance (Fig. 2), but could not have stimulated active Na^+ transport (Frazier & Leaf, 1964; Robinson & Macknight, 1976*a–c*). The introduction of KCl into the mucosal medium increases the tissue conductance even when active transport has been inhibited by replacing Na^+ with choline ions.

It is also clear for at least two reasons that edge damage *per se* could not account for the increased conductance induced by mucosal NaCl. First, the nonelectrolytes, adonitol and urea, also induced an increase in tissue conductance; the effect of urea was not demonstrable until its facilitated diffusion into the cell was inhibited with phloretin. Second, the addition of mucosal NaCl increased the permeability of Na^+ from serosa to mucosa. Such an effect could not arise if the sole effect of adding mucosal electrolyte were to increase the conductance of crushed tissue at the chamber-bladder interface unless the added electrolyte induced a substantial change in the configuration of this interface.

Finally, the results of the radioactive Na^+ fluxes also indicate that the effect of mucosal tonicity is not mediated solely through a putative transcellular pathway at least partly in parallel with the active transport channel. Several lines of indirect evidence have suggested that the unidirectional serosal-to-mucosal flux of Na^+ proceeds through the physiologic paracellular pathway, and that very little of the intracellular Na^+ of the surface transporting cells is of serosal origin. First, measurements of the current-voltage relationship at large hyperpolarizations have suggested rectification of Na^+ through the active transport transcellular channels (Civan, 1970). Second, amiloride blocks active sodium transport by inhibiting Na^+ entry into the cell from the mucosal medium (Bentley, 1968; Macknight, Civan & Leaf, 1975), but has no effect on the serosal-to-mucosal flux of Na^+ (Hong & Essig, 1976). Third, the rates of CO_2 production (Coplon & Maffly, 1972; Canessa, Labarca & Leaf, 1976) and of pyruvate utilization (Sharp et al., 1966) appear dependent on the rate of active Na^+ transport from mucosa-to-serosa, but independent of the presence or absence of serosal Na^+. These considerations

do not entirely preclude the possibility of a passive transcellular Na^+ pathway. However, such a pathway would necessarily be insensitive to amiloride and contain Na^+ immiscible with the active transport pools. In the absence of direct support for such a model, it seems likely that almost all of the serosal-to-mucosal transmural Na^+ movement proceeds through the paracellular pathway.

Because of the above considerations, we conclude that the osmotically-induced increase in serosal-to-mucosal flux of Na^+ reflected a decreased resistance to movement of ions through the paracellular pathway. The apical limiting junctions constitute the rate limiting barrier in this pathway, and are therefore the likely site of action. Electron microscopic examinations did not demonstrate a consistent widening of the intercellular space within the junctions. However, the direct morphologic approach is relatively insensitive to the subtle structural changes expected under the physiologic conditions studied.

In summary, the results of the present study support the concept that, over the physiologic range of tonicities, the mucosal osmolality regulates the resistance to ionic movement through the paracellular pathway across toad bladder. This regulation may possibly provide the toad with a physiologic mechanism for conserving its body stores of NaCl (Fig. 3). Reuss and Finn (1975) have reached a similar conclusion. However, using intracellular micropipets, they were unable to demonstrate an effect of osmolality (as opposed to Na^+ concentration) over the hypotonic-to-isotonic range of mucosal osmolalities. The current data now indicate that a more general osmotic mechanism is at work over this range, as well.

This concept can be developed quantitatively within the framework of the equivalent circuit of Fig. 4. The analysis excludes the possible effects of solvent drag (Andersen & Ussing, 1957) from consideration, insofar as solvent drag has never been rigorously demonstrated in toad bladder. Data which formerly had been thought to reflect solvent drag (Leaf & Hays, 1962) are now thought to have reflected unstirred water layers (Hays, 1972). The considerations guiding the choice of the circuit elements are discussed in the Appendix. The expression relating net NaCl transport (J_{NaCl}) to these circuit elements is given by Eq. (A13) of the Appendix:

$$J_{NaCl} = (1/F)\left[\frac{E-(e_{Cl})\left(\frac{2g^p_{Na}}{g^a_{Na}}+1\right)}{\frac{k}{g^p_{Na}}+\frac{1}{g^a_{Na}}(1+k)}\right] \tag{A13}$$

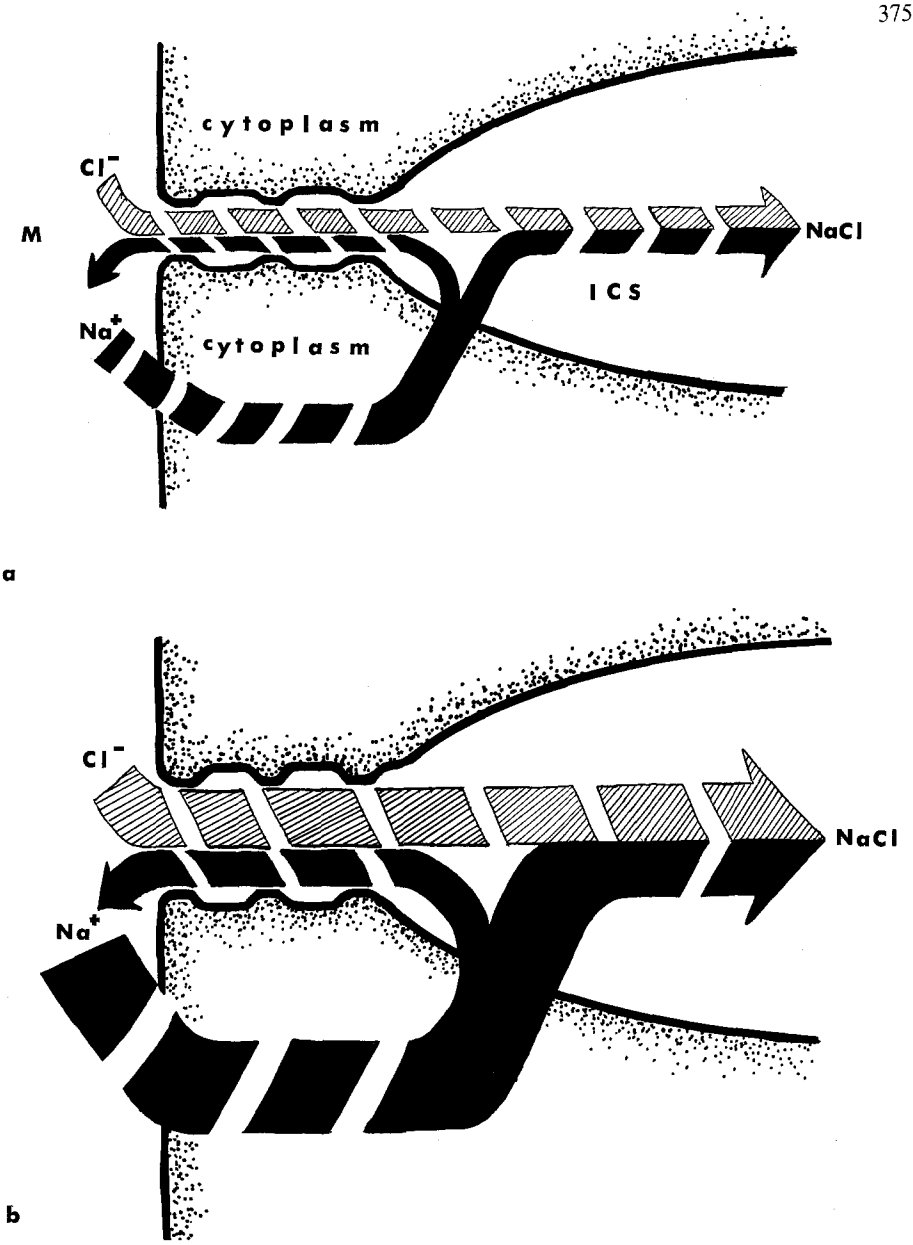

Fig. 3. Proposed control of transepithelial NaCl transport by osmotic regulation of the apical intercellular junctions. Na^+ is actively transported through a transcellular pathway, entering the surface cells by facilitated diffusion from the mucosal medium and leaving the cell by active transport into the serosal medium. In vivo, in the absence of net current movement, the transcellular movement of Na^+ is balanced by paracellular movement of Cl^- from mucosa to serosa and of paracellular movement by Na^+ from serosa to mucosa; the less abundant ionic species are considered to play negligible roles. (*a*): In the presence of hypotonic mucosal media, the apical junctions are relatively closed, limiting transepithelial movement of Cl^-, and thus of NaCl. (*b*): When bathed with isotonic mucosal media, the apical junctions are relatively open, enhancing net NaCl reabsorption from the mucosal medium

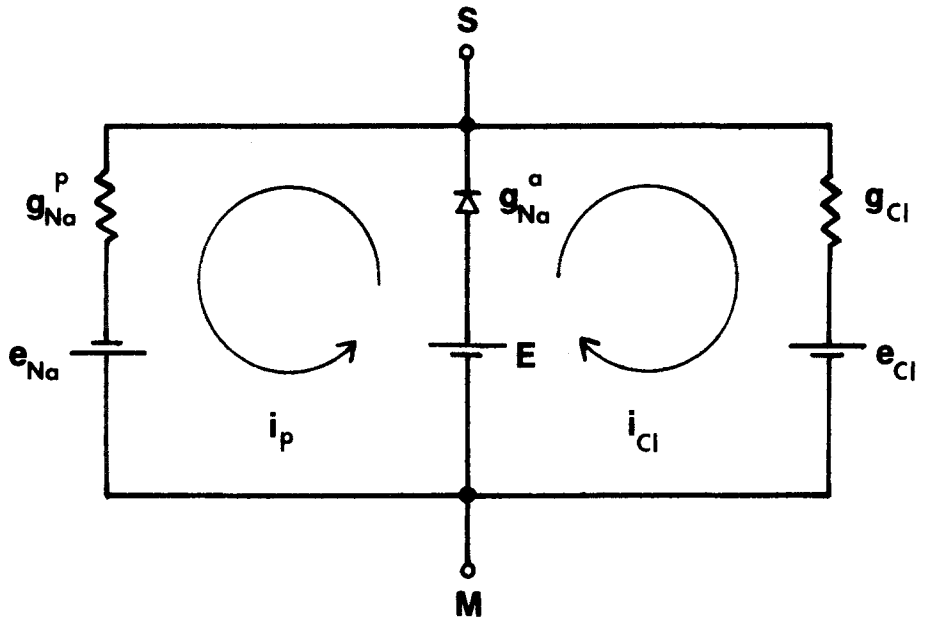

Fig. 4. Equivalent circuit of transepithelial transport of NaCl

where F is the Faraday constant, E and g^a_{Na} are the electromotive driving force and electrical conductivity, respectively, for the sodium ion in the active transport channels, k is the relative ionic conductance of Na^+ to that of Cl^- through the paracellular pathways, g^p_{Na} is the specific ionic conductivity for Na^+ through the paracellular channels, and e_{Cl} is the equivalent concentration driving force on the chloride ion in those channels, given by the Nernst equation [Eq. (A9), *Appendix*].

Under conditions of marked hypotonicity of the mucosal medium, the Cl^- concentration may fall sufficiently to reduce the net driving force on the NaCl to zero (Civan et al., 1966), or even to reverse its sign, favoring loss of NaCl from the body into the urine; e_{Cl} may become very large in vivo since the Cl^- concentration of toad urine has been observed to be as low as 3.3 mM (Leaf et al., 1958). For example, if the numerator of the right hand side of Eq. (A13) were to become negative, a fall in g^p_{Na} would make that term less negative; a fall in g^p_{Na} would also increase the magnitude of the denominator, further reducing the rate of loss of NaCl. Thus, salt conservation would be favored by reducing g^p_{Na} by increasing the resistance to ionic movement through the apical tight junctions, as seems to be the case.

On the other hand, when the urine produced by the kidneys is isotonic, with concentrations of Na^+ and Cl^- approximately equal to those of the interstitial fluid bathing the basolateral surfaces of the transporting cells, e_{Cl} will fall to approximately zero, and Eq. (A13) will reduce to:

$$J_{NaCl} = (1/F)\left[\frac{E}{(k/g^p_{Na}) + (1/g^a_{Na})(k+1)}\right]. \quad (2)$$

Under these conditions, NaCl reabsorption will be increased by increasing g^p_{Na}, as was observed.

We have tacitly made the conservative assumption that k is unchanged by rendering the mucosal medium isotonic. If anything, isotonicity is likely to reduce k, further enhancing J_{NaCl} in this system [Eq. (2)]. As discussed above, we expect isotonicity to lead to a widening of the intercellular space within the limiting junctions, which should reduce the significance of ionic interactions with the pathway walls. Under these conditions, k should approach more closely the permeability ratio (P_{Na}/P_{Cl}) given by the ratio of the limiting ionic conductances ($\lambda_{Na}/\lambda_{Cl}$) in bulk aqueous medium [Eq. (A2) of the Appendix]. Thus, we would anticipate that, in the case of mucosal hypotonicity, k is even larger than the value of 0.98 reported by Saito et al. (1974) and used in the present calculations.

On the other hand, in other epithelial systems, k need not change or might even increase following application of isotonicity. For example, Cl^- might cross an epithelium both through a transcellular route (characterized by a low value of k) and through a paracellular route (characterized by a high value of k). In the presence of mucosal isotonicity, the contribution of the paracellular pathway to the total conductance could become greater, increasing the value of k characteristic of the entire system. This mechanism might be the basis for the reduced J_{NaCl}, reported for *Necturus* proximal tubule following saline loading, despite an increase in the paracellular shunt conductance (Boulpaep, 1972). Such a consideration, based on possible heterogeneity of transepithelial Cl^- pathways, does not appear applicable to toad bladder; little, if any, Cl^- enters the mucosal cells from the mucosal medium (Macknight, 1977), indicating that transepithelial Cl^- movement proceeds exclusively through the paracellular pathway.

The quantitative importance of the osmotically-induced changes in the paracellular pathway can be estimated from Eq. (2), the data sum-

marized in *Results*, and the calculations of the *Appendix*. Taking $k=0.98$ (Saito et al., 1974), we have estimated that $g^a_{Na}=0.13\pm0.017$ mmhos $\cdot$ cm^{-2}, and that $g^p_{Na}=0.018\pm0.0038$ mmhos $\cdot$ cm^{-2} under isotonic conditions. From Table 3, we conclude that g^p_{Na} was at least $21\pm3.2\%$ smaller in the presence of mucosal hypotonicity; here, $g^p_{Na}=0.018/1.21=0.015$ mmhos $\cdot$ cm^{-2}. By using Eq. (2) we conclude that the osmotically-induced increase in g^p_{Na} from 0.015 to 0.018 mmhos $\cdot$ cm^{-2} would increase transepithelial NaCl transport in vivo by some 16%. This degree of transport regulation might initially be considered quantitatively insignificant. However, it could actually be of enormous importance in the day-to-day regulation of salt balance; e.g., aldosterone is necessary for human survival, and yet alters the reabsorption of filtered Na^+ by only 1–2% (Ganong & Mulrow, 1958). It will also be appreciated that the value of 16% represents only a conservative estimate. To the extent that edge damage was present, the true contribution of osmotic regulation would be higher.

Using a similar approach, it may be shown that an increase in the conductance through the paracellular channels caused by increased concentrations of KCl in the urine could favor conservation of KCl within the body. On the other hand, changes in the tonicity of the urine brought about by changes in the concentrations of nonelectrolytes would be expected to have little effect on the freedom of passage of ions through the paracellular pathways. Indeed, it was only with considerable effort that any effect of mucosal nonelectrolytes could be demonstrated on these channels (Tables 1 and 2).

Considerable recent evidence has suggested a physiologic role for the apical tight intercellular junctions of leaky epithelia. For example, the hydrostatic pressure gradient established across the apical junctions of proximal renal tubules appears to regulate the permeability of those junctions to NaCl (Boulpaep, 1972; Seely, 1973; Grandchamp & Boulpaep, 1974). This regulation is likely to provide the physiologic basis for glomerulotubular balance of NaCl. We now suggest that the osmotic pressure gradient established across the apical intercellular junctions of toad bladder also regulates the permeability of those junctions to NaCl, serving as a physiologic mechanism for urinary salt conservation.

We thank Dr. Arnðst Kleinzeller for his kind help in recrystallizing the adonitol. We also thank Miss Kim Peterson, Mrs. Bernice Richardson, Mr. Harry Chao and Miss Bonnie Lord for their skillful technical assistance during various stages of the work. Finally, we thank Dr. Alvin Essig for helpful discussions and Drs. Theodore J. Century, Alexander Leaf, Anthony D.C. Macknight and Lawrence G. Palmer and Mr. Joel D. DeLong for critically reviewing the manuscript.

This study was supported, in part, by research grants from the National Institutes of Health (AM 16586, AM 17372 and HL 06664) and the National Science Foundation (PCM: 77-15682). During part of the research period, Dr. Civan was an Established Investigator of the American Heart Association.

Appendix

The aim of this section is to outline briefly the development of the equations used in the *Results* and *Discussion.*

Relationship of Na^+ Tracer Permeability (P^*_{Na}) to Total Conductance (g_L) through the Paracellular Pathway

The relationship can be derived in at least two ways, each involving different assumptions.

A. Development by integration of Nernst-Planck equation. We take the regions of tight membrane apposition within the apical limiting membranes as the permeability barrier primarily restricting ionic movement through the paracellular channels. As a first approximation, we consider ions to move through this pathway by free diffusion. Actually, ions appear to interact with the surrounding walls of the channel (Walser, 1972; Saito et al., 1974), but agreement is incomplete concerning the quantitative significance of the interaction.

We begin with the differential form of the conductance for each ion (j), obtained from the Nernst-Planck equation (Finkelstein & Mauro, 1963). This expression can be integrated across the limiting junctions, assuming: (i) a linear concentration profile for each ion, (ii) constancy of the cross-sectional area available for diffusion, and of the coefficient of diffusion, within the regions of tight membrane apposition, and (iii) the validity of the Nernst-Einstein relationship. Summing the ionic conductances, expressed in integrated form, the total conductance through the paracellular pathway becomes:

$$g_L = \frac{F^2 S}{RT} P^*_{Na} \sum_j z_j^2 (P_j/P^*_{Na}) [\tilde{j}]_{AJ} \tag{A1}$$

where F is the Faraday constant, S is the total bladder surface, R is the molar gas constant, T is the absolute temperature, and z_j, P_j and $[\tilde{j}]_{AJ}$ are the valence, permeability for net transport, and logarithmic mean of the

Table 4. Values of relative permeability and mean concentrations used to calculate the paracellular conductance

j	P_j/P_{Na}	$[\tilde{j}]_{Hypotonic}$ (mM)	$[\tilde{j}]_{Isotonic}$ (mM)
Na^+	1.00	48.0	117.1
K^+	1.39	1.3	1.3
Ca^{2+}	0.59	0.3	0.3
Cl^-	1.02	43.2	114.7
HCO_3^-	0.89	0.8	0.8
$H_2PO_4^-$	0.68	0.3	0.3
HPO_4^{2-}	0.68	1.8	1.8

concentration within the apical limiting junction, respectively, for the j-th ion.

In Eq. (A1), we have taken P_{Na}^* to be equal to P_{Na}, although in general they may differ because of isotope interaction and/or other coupled flows arising from a number of possible physical mechanisms (Chen & Walser, 1976). From the effect of transepithelial potential on the rate of radioactive Na^+ movement from mucosa to serosa, Saito et al. (1974) concluded that (P_{Na}/P_{Na}^*) is not significantly different from one for toad bladders mounted in chambers, as used in the present study. Smaller values have been reported for bladders mounted as sacs (Walser, 1970; Chen & Walser, 1976).

The values used for the relative permeabilities and ionic concentrations in Eq. (A1) are listed in Table 4. The values for (P_{Cl}/P_{Na}^*) have been taken from the data of Saito et al. (1974). The values for (P_{Ca}/P_{Na}^*) and (P_{HCO_3}/P_{Na}^*) have been calculated from the ratios (λ_j/λ_{Na}) of the limiting equivalent conductances (Robinson & Stokes, 1968):

$$(P_j/P_{Na}^*) = \left[\frac{\lambda_j/|z_j|}{\lambda_{Na}}\right]. \tag{A2}$$

The limiting ionic conductances of HPO_4^{2-} and $H_2PO_4^-$ are not readily available. Instead, the relative permeabilities of phosphate and Na^+ have been estimaed to be 0.68 from the results of Leaf and Hays (1962); their measurements probably reflected Na^+ and phosphate movement primarily through aqueous pathways in parallel with the transcellular channel.

Introducing the values of Table 4 into Eq. (A1), we may estimate g_L in mmhos$\cdot$cm^{-2} from P_{Na}^* in cm$\cdot$sec^{-1} under the currently used con-

ditions of mucosal hypotonicity $(g_L)_{hypo}$ and mucosal isotonicity $(g_L)_{iso}$:

$$(g_L)_{hypo} = 3.63 \times 10^5 P^*_{Na} \tag{A3}$$

$$(g_L)_{iso} = 8.97 \times 10^5 P^*_{Na}. \tag{A4}$$

The calculated proportionality factor of Eq. (A4) is close to that used by Saito et al. (1974). Under the conditions of the present study, the conductance of the paracellular pathway primarily reflects the ionic conductances of Na^+ and Cl^-; these ions provide 95 % of the conductance $(g_L)_{hypo}$ and 98 % of $(g_L)_{iso}$. The values calculated for g_L on the basis of Eqs. (A3)–(A4) have been entered in Table 3.

B. Development by macroscopic approach. We define a permeability (P_j) for net flux of ion j as the ratio of the net rate of transport ($\Delta n_j/\Delta t$) to the concentration ($[j]_s - [j]_m$) or equivalent electrical driving force, normalized to surface area (S). Thus, in the case of a concentration driving force,

$$P_j = \left(\frac{\Delta n_j}{\Delta t}\right)\left(\frac{1}{[j]_s - [j]_m}\right)\left(\frac{1}{S}\right) \tag{A5}$$

and for an electrical driving force,

$$P_j = \left(\frac{\Delta n_j}{\Delta t}\right)\left[\frac{RT}{z_j F(\psi_s - \psi_m)[j]_{AJ}}\right]\left(\frac{1}{S}\right). \tag{A6}$$

The ionic conductance g_j is the ratio of the electrical current (I_j) carried by ion (j) to the difference in electrical potential ($\psi_s - \psi_m$):

$$g_j = \frac{I_j}{\psi_s - \psi_m} = \frac{z_j F}{\psi_s - \psi_m}\left[\frac{\Delta n_j}{\Delta t}\right]. \tag{A7}$$

From Eqs. (A6)–(A7) and summing for all ions, we once again obtain Eq. (A1).

This approach avoids the need for simplifying assumptions in order to integrate the Nernst-Planck equation. However, the tacit assumption of the equivalence of electrical and concentration driving forces applied to the bulk bathing media need not necessarily be obeyed for several reasons. First, application of different driving forces between the two bulk phases may be distributed differently across the series permeabilities barriers within the tissue. Second, the application of concentration and electrical forces may change the state of the membrane system in different ways. Third, depending upon the nature and strength of in-

teractions between the ions and tissue, electrical and concentation driving forces need not be equivalent, even on a microscopic level.

Despite these caveats, we regard Eq. (A1) as a useful approximation of the relationship between g_L and P^*_{Na}, particularly since the same expression can be developed using two different sets of not unreasonable assumptions.

Relationship of Net NaCl Transport (J_{NaCl}) to the Ionic Conductances of the Paracellular Pathway

The equivalent circuit of Fig. 4 provides a useful framework. Na^+ is considered to be actively transported through transcellular channels of conductance g^a_{Na} by Na^+ pumps of driving force E. Both g^a_{Na} and E constitute lumped circuit elements which are dependent upon the driving forces and conductances characterizing both series plasma membranes of the transporting cells. The conductance element is symbolized as a diode because of the current-voltage relationship noted at large hyperpolarizations (Civan, 1970). On the other hand, Na^+ and Cl^- are thought to be free to move in either direction through the paracellular pathways. In these latter pathways, g^p_{Na} and e_{Na} symbolize the conductance and equivalent concentration driving force for net Na^+ movement, respectively, while g_{Cl} and e_{Cl} symbolize those parameters for net Cl^- movement. Under certain conditions, subspecies of *Bufo marinus* from Colombia can actively transport Cl^- (Finn, Handler & Orloff, 1967). However, under the current experimental conditions, net Cl^- transport is negligible (Davies, Martin & Sharp, 1968). Chemical analyses of such preparations indicates that little, if any, Cl^- enters the transporting cells from the mucosal medium (Macknight, 1977). Because of these considerations, we conclude that the paracellular pathway is the only physiologically important transepithelial channel for Cl^- movement, and is so indicated in Fig. 4.

Net movement of Na^+ through the active transport channels from mucosa (m) to serosa (s) is considered balanced by net movement through the paracellular channels of Cl (i_{Cl}) in the same direction and by net movement of Na^+ (i^p_{Na}) in the opposite direction. As a first approximation, net movement of the cations and anions in minor abundance is considered negligible.

e_{Na} and e_{Cl} are explicitly defined by the following expressions:

$$e_{Na} = \frac{RT}{F} \ln \frac{[Na^+]_s}{[Na^+]_m} \tag{A8}$$

$$e_{Cl}=\frac{RT}{F}\ln\frac{[Cl^-]_s}{[Cl^-]_m}. \tag{A9}$$

Under open-circuited conditions applying Kirchhoff's Rules to Fig. 4,

$$i_{Cl}=\frac{(E-e_{Cl})\left(\frac{1}{g^a_{Na}}+\frac{1}{g^p_{Na}}\right)-(E+e_{Na})\left(\frac{1}{g^a_{Na}}\right)}{\left(\frac{1}{g^p_{Na}}\right)\left(\frac{1}{g^a_{Na}}+\frac{1}{g_{Cl}}\right)+\left(\frac{1}{g^a_{Na}}\right)\left(\frac{1}{g_{Cl}}\right)}. \tag{A10}$$

In this approximate analysis, we treat the serosal and mucosal solutions as consisting primarily of NaCl solutions at two different concentrations, so that $e_{Cl}\doteq e_{Na}$, and $[Cl^-]_{AJ}\doteq[Na^+]_{AJ}$. Therefore.

$$i_{Cl}=\frac{E-e_{Cl}\left[1+\frac{2g^p_{Na}}{g^a_{Na}}\right]}{\frac{k}{g^p_{Na}}+\frac{1}{g^a_{Na}}(1+k)} \tag{A11}$$

where

$$k\equiv\frac{g^p_{Na}}{g_{Cl}}=\frac{P_{Na}}{P_{Cl}}. \tag{A12}$$

In the absence of external current, the net flux of Cl^-, given by (i_{Cl}/F), is identical with J_{NaCl}:

$$J_{NaCl}=(1/F)\left[\frac{(E)-(e_{Cl})\left[\frac{2g^p_{Na}}{g^a_{Na}}+1\right]}{\frac{k}{g^p_{Na}}+\frac{1}{g^a_{Na}}(1+k)}\right]. \tag{A13}$$

References

Andersen, B., Ussing, H.H. 1957. Solvent drag on non-electrolytes during osmotic flow through isolated toad skin and its response to antidiuretic hormone. *Acta Physiol. Scand.* **39**:228

Bentley, P.J. 1968. Amiloride: A potent inhibitor of sodium transport across the toad bladder. *J. Physiol. (London)* **195**:317

Boulpaep, E.L. 1972. Permeability changes of the proximal tubule of *Necturus* during saline loading. *Am. J. Physiol.* **222**:517

Canessa, M., Labarca, P., Leaf, A. 1976. Metabolic evidence that serosal sodium does not recycle through the active transepithelial transport pathway of toad bladder. *J. Membrane Biol.* **30**:65

Chen, J.S., Walser, M. 1976. Effect of transepithelial concentration gradients on the passive fluxes of sodium across toad bladder. *J. Membrane Biol.* **37**:381

Civan, M.M. 1970. Effects of active sodium transport on the current-voltage relationship of toad bladder. *Am. J. Physiol.* **219**:234

Civan, M.M., Di Bona, D.R. 1974. Pathways for movement of ions and water across toad urinary bladder: II. Site and mode of action of vasopressin. *J. Membrane Biol.* **19**:195

Civan, M.M., Kedem, O., Leaf, A. 1966. Effect of vasopressin on toad bladder under conditions of zero net sodium transport. *Am. J. Physiol.* **211**:569

Coplon, N.S., Maffly, R.H. 1972. The effect of ouabain on sodium transport and metabolism of the toad bladder. *Biochim. Biophys. Acta* **282**:250

Davies, H.E.F., Martin, D.G., Sharp, G.W.G. 1968. Differences in the physiological characteristics of bladders from different geographical sources. *Biochim. Biophys. Acta* **150**:315

DiBona, D.R. 1972. Passive pathways in amphibian epithelia: Morphologic evidence for an intercellular route. *Nature New Biol.* **238**:179

DiBona, D.R., Civan, M.M. 1972*a*. Clarification of the intercellular space phenomenon in toad urinary bladder. *J. Membrane Biol.* **7**:267

DiBona, D.R., Civan, M.M. 1972*b*. Osmotically-induced conductance changes in toad urinary bladder under physiologic conditions. *Abstr. IXth Intern. Biophys. Cong.* Moscow, USSR

DiBona, D.R., Civan, M.M. 1973. Pathways for movement of ions and water across toad urinary bladder: I. Anatomic site of transepithelial shunt pathways. *J. Membrane Biol.* **12**:101

DiBona, D.R., Civan, M.M., Leaf, A. 1969*a*. The anatomic site of the transepithelial permeability barriers of toad bladder. *J. Cell Biol.* **40**:1

DiBona, D.R., Civan, M.M., Leaf, A. 1969*b*. The cellular specificity of the effect of vasopressin on toad urinary bladder. *J. Membrane Biol.* **1**:79

Dobson, J.G., Kidder, G.W., III. 1968. Edge damage effect in *in vitro* frog skin preparations. *Am. J. Physiol.* **214**:719

Farquhar, M., Palade, G.E. 1963. Junctional complexes in various epithelia. *J. Cell Biol.* **17**:375

Farquhar, M., Palade, G.E. 1965. Cell junctions in amphibian skin. *J. Cell Biol.* **26**:263

Finkelstein, A., Mauro, A. 1963. Equivalent circuits as related to ionic systems. *Biophys. J.* **3**:215

Finn, A.L., Handler, J.S., Orloff, J. 1967. Active chloride transport in the isolated toad bladder. *Am. J. Physiol.* **213**:179

Frazier, H.S., Leaf, A. 1964. Cellular mechanisms in the control of body fluids. *Medicine* **43**:281

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

Frömter, E., Diamond, J. 1972. Route of passive ion permeation in epithelia. *Nature (London)* **235**:9

Ganong, W.F., Mulrow, P.J. 1958. Rate of change in sodium and potassium excretion after injection of aldosterone into the aorta and renal artery of the dog. *Am. J. Physiol.* **195**:337

Grandchamp, A., Boulpaep, E.L. 1974. Pressure control of sodium reabsorption and intercellular backflux across proximal tubule. *J. Clin. Invest.* **54**:69

Hays, R.M. 1972. The movement of water across vasopressin-sensitive epithelia. *Current Topics in Membranes and Transport* **3**:339

Helman, S.I., Miller, D.A. 1971. In vitro techniques for avoiding edge damage in studies of frog skin. *Science* **173**:146

Higgins, J.T., Jr., Cesaro, L., Gebler, B., Frömter, E. 1975. Electrical properties of amphibian urinary bladder epithelia: I. Inverse relationship between potential difference and resistance in tightly mounted preparations. *Pfluegers Arch.* **358**:4

Hong, C.D., Essig, A. 1976. Effects of 2-deoxy-D-glucose, amiloride, vasopressin, and ouabain on active conductance and E_{Na} in the toad bladder. *J. Membrane Biol.* **28**:121

Leaf, A., Anderson, J., Page, L.B. 1958. Active sodium transport by the isolated toad bladder. *J. Gen. Physiol.* **41**:657

Leaf, A., Hays, R.M. 1962. Permeability of the isolated toad bladder to solutes and its modification by vasopressin. *J. Gen. Physiol.* **45**:921

Levine, S., Franki, N., Hays, R.M. 1973. Effect of phloretin on water and solute movement in the toad bladder. *J. Clin. Invest.* **52**:1435

Lewis, S.A., Diamond, J.M. 1976. Na^+ transport by rabbit urinary bladder, a tight epithelium. *J. Membrane Biol.* **28**:1

Macknight, A.D.C. 1977. The contribution of mucosal chloride to chloride in toad bladder epithelial cells. *J. Membrane Biol.* **36**:55

Macknight, A.D.C., Civan, M.M., Leaf, A. 1975. The sodium transport pool in toad urinary bladder epithelial cells. *J. Membrane Biol.* **20**:365

Peachey, L.D., Rasmussen, H. 1961. Structure of the toad's urinary bladder as related to its physiology. *J. Biophys. Biochem. Cytol.* **10**:529

Rawlins, F.A., González, E., Pérez-González, M., Whittembury, G. 1975. Effect of transtubular osmotic gradients on the paracellular pathway in toad proximal tubule: Electron microscopic observations. *Pfluegers Arch.* **353**:287

Reuss, L., Finn, A.L. 1975. Effects of changes in the composition of the mucosal solution on the electrical properties of the toad urinary bladder epithelium. *J. Membrane Biol.* **20**:191

Robinson, B.A., Macknight, A.D.C. 1976*a*. Relationships between serosal medium potassium concentration and sodium transport in toad urinary blader: I. Effects of different medium potassium concentrations on electrical parameters. *J. Membrane Biol.* **26**:217

Robinson, B.A., Macknight, A.D.C. 1976*b*. Relationships between serosal medium potassium concentration and sodium transport in toad urinary bladder: II. Effects of different medium potassium concentrations on epithelial cell composition. *J. Membrane Biol.* **26**:239

Robinson, B.A., Macknight, A.D.C. 1976*c*. Relationships between serosal medium potassium concentration and sodium transport in toad urinary bladder: III. Exchangeability of epithelial cellular potassium. *J. Membrane Biol.* **26**:269

Robinson, R.A., Stokes, R.H. 1968. Electrolyte Solutions (2nd Ed., revised). Butterworths, London

Saito, T., Lief, P.D., Essig, A. 1974. Conductivity of active and passive pathways in the toad bladder. *Am. J. Physiol.* **226**:1265

Sawyer, W.H. 1956. The antidiuretic action of neurohypophysial hormones in Amphibia. *Proc. Symp. Colston Res. Soc.* **8**:171

Seely, J.E. 1973. Effect of peritubular oncotic pressure on rat proximal tubule electrical resistance. *Kidney Int.* **4**:28

Sharp, G.W.G., Coggins, C.H., Lichtenstein, N.S., Leaf, A. 1966. Evidence for a mucosal effect of aldosterone on sodium transport in the toad bladder. *J. Clin. Invest.* **45**:1640

Siegel, B., Civan, M.M. 1976. Aldosterone and insulin effects on the driving force of the Na^+-pump in toad bladder. *Am. J. Physiol.* **230**:1603

Using, H.H. 1949. The distinction by means of tracers between active transport and diffusion. *Acta Physiol. Scand.* **19**:43

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

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

Wade, J.B., Revel, J.P., DiScala, V.A. 1973. Effect of osmotic gradients on intercellular junctions of the toad bladder. *Am. J. Physiol.* **224:** 407

Walser, M. 1970. Role of edge damage in sodium permeability of toad bladder and a means for avoiding it. *Am. J. Physiol.* **219:** 252

Walser, M. 1972. Components of sodium and chloride flux across toad bladder. *Biophys. J.* **12:** 351