

The Electrical Characteristics of Active Sodium Transport in the Toad Bladder

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ABSTRACT The mechanism responsible for active sodium transport in the urinary bladder of the toad appears to be located at the serosal boundary of the epithelial cell layer of the bladder. Studies of the potential step observed at the serosal boundary in the open-circuited state were undertaken in an attempt to define the factors responsible for its production. Glass micropipettes were used to measure the serosal potential step in bladders exposed on the serosal side to solutions of high potassium or of high potassium and low chloride concentration. Observed potentials exceed the maximum values which would have been expected if the serosal potential step were a potassium or chloride diffusion potential. Measurements of net cation flux exclude the possibility of a diffusion potential at this border due to the passive movement of any anionic species. The observed independence of transbladder potential and short-circuit current from the pH of the serosal medium over a wide range of pH makes it unlikely that the observed serosal potential step is a hydrogen ion diffusion potential. We conclude that the active sodium transport mechanism in toad bladder is "electrogenic."

INTRODUCTION

The active transport of sodium from the mucosal to the serosal side of the toad bladder gives rise to an electrical potential oriented so that the serosal surface is positive to the mucosal surface (1). This transbladder potential is made up of two potential steps of the same polarity as the transbladder potential, the first being located at or near the mucosal surface of the single layer of bladder epithelial cells, the second at or near the serosal surface of this same layer of cells (2). The electrical potential step at the mucosal surface appears to be a consequence of the passive movement of sodium ions down a concentration gradient from the mucosal medium, across a sodium-selective permeability barrier in the mucosal surface, into the cell interior (3). The present study is concerned with the origin of the electrical potential step at the serosal boundary of the layer of bladder epithelial cells.

In general, the operation of either of two types of mechanism at the serosal boundary could give rise to the observed electrical potential step. The sodium pump, which is located at this surface and acts to extrude sodium from the cell interior up an electrochemical gradient into the serosal bathing medium, could be of the "electrogenic" type, that is, it could accomplish the transfer of sodium ions alone from the cell to the serosal medium. The resulting electrical potential gradient would, of course, pull anions in the same direction so that the over-all process would be a net transbladder transport of neutral salt. However, the slight lag between the primary transfer of sodium ions and the resulting movement of anions could create the observed electrical potential step. It is in this sense that the adjective "electrogenic" is applied to the sodium pump. Alternatively, the serosal potential step could represent a diffusion potential generated by passive movements of ions down their concentration gradients. In this latter view, recently proposed by Koefoed-Johnsen and Ussing (4) to account for certain of the electrical characteristics of frog skin, the sodium pump is a neutral one, extruding a sodium ion into the serosal medium in exchange for a potassium ion which is transferred into the cell interior. There is no net charge transfer by the pump, hence no potential is generated by it. The concentration gradient for potassium, developed across the serosal membrane by the operation of the neutral pump, provides the driving force for diffusive movement of potassium across this potassium-selective barrier, giving rise to a diffusion potential.

The experiments reported here show that under certain conditions, the magnitude of the serosal potential step in toad bladder exceeds the maximum value predicted for a diffusion potential due to potassium or chloride ions. Further experimental evidence indicates that the serosal potential step is not due to diffusion of hydrogen ion or some anion other than chloride. We conclude that the serosal potential step probably results from an "electrogenic" sodium pump.

METHODS

A. Solutions

The composition of each of the experimental solutions is given in Table I.

Two test conditions were applied in the measurements of the electrical potential step at the serosal surface of the bladder epithelial cell. In the first, the effect of a high concentration of potassium in the serosal bathing medium was tested. Solution I, ordinary Ringer's solution, was used as the mucosal medium. Solution II, potassium Ringer's solution, which differed from ordinary Ringer's in the substitution of potassium for all of the sodium, was used as the serosal bathing medium.

In the second series of experiments, the effect on the serosal potential of altering the cell to serosa gradients for both potassium and chloride was examined. Solution

III, potassium sulfate Ringer's solution, was used on both sides of the bladder. Because no active sodium transport and no spontaneous transbladder potential appear in the absence of sodium in the mucosal medium (2, 3), sodium was included at a concentration of 50 meq per liter. This concentration has been shown previously to permit maximum active sodium transport by the bladder (3). The potassium and chloride concentrations were chosen to minimize or reverse the usual concentration gradients of these ions across the serosal boundary of the bladder epithelial cell, while maintaining the potassium chloride product of normal Ringer's solution. Bicarbonate ion was included to stabilize the pH at 8.1 to 8.2 when the solution was equilibrated with air at room temperature. Osmotic restrictions dictated the choice of a divalent anion for the remainder of the salts. The solution was saturated with calcium sulfate to bring the concentration of ionized calcium to adequate levels.

TABLE I
COMPOSITION OF THE EXPERIMENTAL SOLUTIONS
Concentrations are given in millimoles per liter.

Salt	Designation of solution			
	I	II	III	IV
NaCl	114			114
NaHCO ₃	3			3
CaCl ₂	0.9	0.9		0.9
KCl	3	114	3	
KHCO ₃		3	3	
K ₂ SO ₄			55	
Na ₂ SO ₄			25	
CaSO ₄			Saturated	

For the net cation flux experiments all that was desired was to have sodium but no potassium in the mucosal bathing medium, solution IV, and potassium but no sodium in the serosal medium, solution II.

B. Measurements of the Electrical Potential Profile

The methods have been discussed in detail elsewhere (2) and, except as they have been altered for the present experiments, will be mentioned only in outline here. Isolated urinary bladders from male and female specimens of the toad, *Bufo marinus*, were soaked in the serosal test solution for at least 15 minutes and mounted on a cylinder of the test solution containing 2 per cent agar. The assembly was suspended, mucosal side out, in a bath of the same or a different test solution at room temperature and the measurement of the electrical potential profile was carried out by means of Ling-Gerard micropipettes of borosilicate glass. The method of determining the position within the bladder of a given potential measurement involved intermittent short-circuiting of the bladder during impalement, with measurement of the relative d.c. resistance of that portion of the bladder between the micropipette tip and the reference electrode on the serosal surface of the bladder. The potential difference

between the micropipette tip and the reference electrode was recorded on a model G-22 recorder (Varian Associates, Palo Alto, California). Intermittent short-circuiting was carried out at a frequency of 9 cycles per minute. At this switching rate the recorder showed 100 per cent of the response to the same potential step at a frequency of zero. Because some of the test solutions contained very low concentrations of chloride ion, the membrane was brought to the short-circuited condition by the passage of current from an external circuit through salt bridges of the test solution, rather than by the use of silver-silver chloride electrodes located in the bathing media on the two sides of the membrane.

C. Serosal Limiting Membrane

The body cavity of the toad is lined by a layer of peritoneum which is reflected over the surfaces of the viscera, including most of the urinary bladder. Sheets of this membrane could not be obtained in undamaged form from the bladder itself but were taken from the portion which lines the posterior wall of the body cavity, where the peritoneum is unattached to visceral or parietal structures. Portions of the membrane were soaked for at least 15 minutes in 30 ml of potassium sulfate Ringer's solution, solution III, and then mounted between the two halves of a lucite chamber 0.5 cm² in area and 0.1 ml in total volume. One side of the chamber was connected to a reservoir containing 5 ml of solution III with tracer amounts of K⁴² which circulated continuously through one half of the chamber. The other side of the chamber was connected to a source of inactive solution of otherwise identical composition. This solution flowed through the other half of the chamber at 4 to 6 ml per minute and was collected in separate, 1 minute samples for counting. The samples were adjusted to equal volume before counting in an Auto-gamma well counter (Packard Instrument Company, La Grange, Illinois). Appropriate decay corrections were applied. The rate of appearance of K⁴² on the sink side of the membrane reached a steady value in less than 1 minute in each of 5 membranes. From this steady rate of appearance of K⁴² a permeability coefficient was calculated.

D. Net Cation Flux

The experiments were performed in lucite chambers of the type described by Ussing and Zerahn (5). Na²²-labeled solution IV bathed the mucosal side of the bladder, and K⁴²-labeled solution II was present on the serosal side. The radioactivity was added, 15 to 30 minutes were allowed for mixing, and the isotopic fluxes were measured by standard techniques. Individual periods of flux measurement lasted 30 to 90 minutes. The bladder was in the open-circuited or spontaneously active condition throughout, and the experiment was continued until the spontaneous transbladder potential fell to less than 20 mv. In no case was the duration of the experiment sufficiently long so that back flux of isotope from sink to source side constituted a significant fraction of estimated sampling and counting errors.

Samples were pipetted into a liquid scintillation solution (6) and K⁴² activity was determined in a Tri-Carb liquid scintillation spectrometer (Packard Instrument Company, LaGrange, Illinois) at settings which reduced the Na²² counting rate in the sample to approximately 5×10^{-5} of its optimal counting rate. After a 2 week

period for decay of K^{42} , the samples were recounted for Na^{22} activity. Decay corrections were applied to the measured K^{42} counting rates; activities were kept sufficiently low so that dead time corrections were not necessary.

K^{42} -chloride was obtained from Iso/Serve, Inc., of Boston, from material irradiated in the reactor at the Massachusetts Institute of Technology, Cambridge. The Na^{22} -chloride was obtained from Nuclear Science and Engineering Corporation, Pittsburgh.

E. Dependence of Transbladder Potential and Short-Circuit Current on pH of the Serosal Medium

The bladder preparation was mounted between the two halves of a lucite chamber similar to that originally described by Ussing and Zerahn (5). The transbladder potential was monitored by a model 200B d.c. vacuum tube voltmeter (Keithley Instruments, Inc., Cleveland). Short-circuiting of the bladder was carried out by means of a modification of the voltage clamp apparatus described by Menninger, Snell, and Spangler (7), and the short-circuit current recorded on a model G-11A recorder (Varian Associates, Palo Alto, California). Both surfaces of the bladder were exposed to ordinary Ringer's solution. The pH of the serosal medium was adjusted initially with 0.1 N NaOH and then acidified by the addition of small volumes of 0.1 N HCl. A model H2 glass electrode pH meter (Beckman Instruments, Inc., Fullerton, California) was used to measure the pH of the serosal solution.

RESULTS

A. The Serosal Limiting Membrane

One of the experimental tests of the origin of the potential step across the serosal surface of the bladder epithelial cell involved exposing the serosal surface of the bladder to solutions containing high concentrations of potassium. The serosal surface of the bladder epithelial cell is shielded from the serosal bathing medium by a loose, thin layer of connective tissue and the peritoneal or serosal limiting membrane. In order to estimate the concentration of potassium in the connective tissue layer in apposition to the serosal surface of the bladder epithelium it was necessary to determine the potassium permeability of the serosal limiting membrane.

In a series of fifty 1 minute periods in 5 peritoneal membrane preparations, the average permeability coefficients for each membrane ranged from 0.45 to 2.3×10^{-4} cm sec⁻¹, with a mean for the series of 1.3×10^{-4} cm sec⁻¹.

Assuming that the connective tissue layer between the serosal limiting membrane and the serosal surface of the bladder epithelium is 40 μ thick, a generous estimate, and that anion or other cation movement is not rate limiting, the half-time for equilibration of this space with potassium in the serosal bathing medium is 21 seconds. In the absence of vasopressin there is at best a negligible net transfer of water across the bladder (8), hence there

is no alteration in the rate of distribution of potassium due to the bulk flow of solvent. Thus it seems probable that the extracellular environment of the serosal surface of the bladder epithelial cells has a potassium concentration indistinguishable from that of the bulk serosal bathing medium.

The serosal limiting membrane does not generate a spontaneous membrane potential which might distort observations of the potential profile of the bladder epithelial cell (2).

B. *The Electrical Potential Profile*

The electrical potential profile of the bladder was measured under two different sets of conditions.

In the first, the effect of reducing the potassium ion gradient across the serosal surface of the bladder epithelial cell was tested by exposing the serosal surface of the bladder to 2 per cent agar in potassium Ringer's solution, solution II. Ordinary Ringer's solution bathed the mucosal surface. In five technically acceptable measurements in 2 bladders two steps were observed, the second or serosal potential step ranging from 17 to 28.5 mv with the bladder in the open-circuited state. Spontaneous transbladder potentials in these preparations ranged from 25 to 42 mv. The values for the serosal potential step, plotted against the relative d.c. resistance between the micro-pipette tip and the serosal reference electrode, are shown as open circles in Fig. 1.

The concentration of potassium in solution II, 117 meq per liter, approaches that of potassium in the cell water of the bladder epithelial cell, which averages 110 meq per liter of tissue water in the non-inulin space of the bladder (8, 17). The magnitudes of the observed serosal potentials are too large to be compatible with the hypothesis that they originate in the diffusion of potassium from the bladder epithelial cell across the serosal boundary into the extracellular space.

This experiment does not rule out the alternative possibility of chloride diffusion down its concentration gradient from the serosal extracellular space across the serosal boundary into the bladder epithelial cell, with the development of a potential difference of the observed polarity. To test this hypothesis, as well as to confirm the previous findings with respect to potassium, the bladder was bathed in potassium sulfate Ringer's solution for 15 to 30 minutes and mounted as described, with potassium sulfate Ringer's solution, solution III, as both mucosal and serosal medium, and serial determinations of the potential profile were carried out for periods as long as 6 hours on a single preparation. Thirty-four technically acceptable measurements were obtained from 6 bladders. A representative impalement is shown in Fig. 2. The potential profile in the open-circuited state, the lower envelope of the curve, is qualitatively the same as that found in experiments with ordinary sodium

Ringer's solution on the two sides of the bladder (2), or with potassium Ringer's solution as the serosal medium. It consists of two positive-going potential steps on crossing the bladder from the mucosal to the serosal side.

The values for the second of these potential changes, the potential step at the serosal boundary of the bladder epithelial cell, are shown as solid circles in Fig. 1. The observed potentials are plotted against the relative d.c. re-

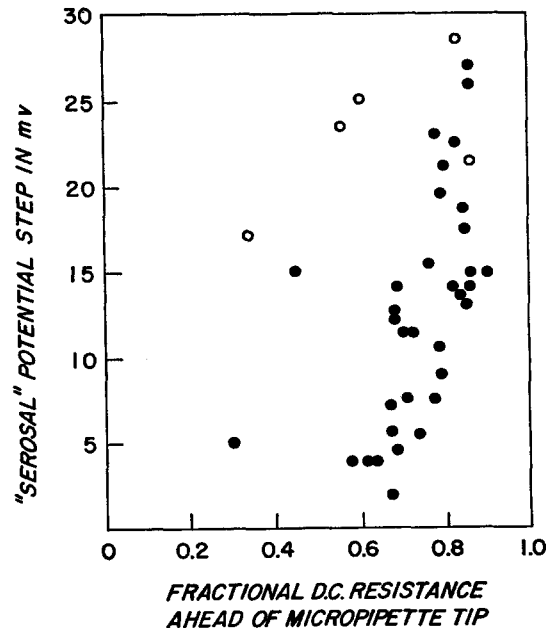
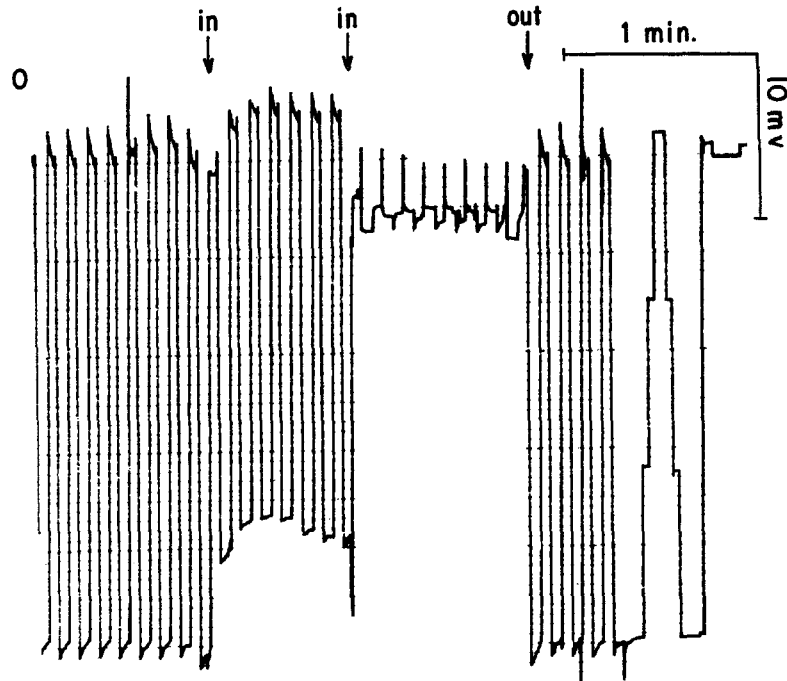


FIGURE 1. The relation between the magnitude of the second or serosal potential step and the fractional d.c. resistance between the micropipette tip and the serosal reference electrode at the point where the serosal potential step is measured. The results indicated by open circles are from bladders with sodium Ringer's solution, solution I, on the mucosal side and potassium Ringer's solution, solution II, on the serosal side. The remaining points (solid circles) are from experiments in which both sides of the bladder were bathed by high potassium, low chloride, sulfate Ringer's solution, solution III. The magnitudes of the observed potentials are too great to be accounted for by a potassium or chloride diffusion potential in most instances.

sistance between the micropipette tip at the point where the potential was recorded and the serosal reference electrode. The values for d.c. resistance fall between 0.3 and 0.9 of the total d.c. resistance of the bladder, indicating that the micropipette tip was within the bladder epithelial cell layer at the point where the potential was recorded (2).

The important feature of the results is the magnitude of the observed serosal potential step. Exposure of the serosal surface of the bladder to the high potassium, low chloride, sulfate Ringer's solution suppresses, and perhaps



neg.

FIGURE 2. Record of the impalement of an intermittently short-circuited bladder with high potassium, low chloride, sulfate Ringer's solution bathing both sides of the bladder. The lower envelope of the curve represents the potential profile of the bladder in the open-circuited condition. As the micropipette tip is advanced from the mucosal medium there is a positive potential step at the mucosal boundary of the bladder epithelial cell layer. A second advance of the micropipette is associated with a second positive potential step at the serosal boundary of the epithelial cell layer.

The upper envelope of the curve is the potential profile of the bladder in the short-circuited condition. On crossing the mucosal border of bladders with ordinary Ringer's solution on both sides of the bladder or ordinary Ringer's solution on the mucosal side and potassium Ringer's solution on the serosal side, a zero potential difference or a small negative cellular potential is found. The substitution of sulfate ion for chloride ion on the serosal side is usually associated with the presence of a small potential, averaging 6.0 mv, with the interior of the bladder epithelial cell positive to the serosal reference point.

The height of the vertical excursion is proportional to the relative d.c. resistance of that portion of the bladder between the micropipette tip and the serosal reference electrode. The record is run continuously during the course of the impalement and withdrawal. Voltage calibration appears on the right.

reverses, the normal concentration gradients for both ions at this surface. Using the Nernst equation, it can be calculated that even if a gradient of 1.3 to 1 in the usual direction still persisted, the expected potential step would be only 6.6 mv. Twenty-six of the thirty-four observations lie above this value

and half are above 12.5 mv, a potential which is equivalent, from the Nernst equation, to a concentration gradient of 1.6 to 1. Hence it seems quite unlikely that the serosal potential step represents a potassium or chloride diffusion potential.

C. Net Cation Flux

Although the magnitude of the observed serosal potential step in solutions of low chloride concentration appears to rule out a chloride diffusion potential, it is possible to test for an anionic diffusion potential by a more general method. We might expect that with the bladder in the open-circuited condition the operation of the sodium pump would result in net cation movement from the mucosal to the serosal medium. With the bladder open-

TABLE II
NET CATION FLUX IN THE OPEN-CIRCUITED TOAD
BLADDER

Experiments	Periods		Flux in 10^{-12} mole sec^{-1} cm^{-2}	
	Number	Duration	Sodium M to S*	Potassium S to M†
8	28	30 to 90 min.	140.3	84.6

Mean difference in cation fluxes = 55.7×10^{-12} mole sec^{-1} cm^{-2} .

S.E. of mean difference = $\pm 6.9 \times 10^{-12}$ mole sec^{-1} cm^{-2} .

$P < 0.001$.

* M to S = mucosal to serosal flux, measured isotopically with Na^{22} in solution IV.

† S to M = serosal to mucosal flux, measured isotopically with K^{42} in solution II.

circuited there can be no current flow, so that a net movement of cation from mucosa to serosa obligates an equivalent movement of anion in the same direction. If the bladder is in a steady state with respect to volume, the movement of cation and anion occurs across both borders of the epithelial cell layer. But if anion movement across the serosal boundary of the bladder epithelial cell is passive, net anion movement in the direction cell interior to serosal medium is incompatible with a serosal potential step of the observed polarity due to anionic diffusion from serosal medium back into cell interior. The experimental problem, then, is to measure the direction and magnitude of the cationic fluxes across the open-circuited toad bladder.

In order to permit the quantification of net cation flux, solution IV, containing sodium as cation, and solution II, containing potassium as cation, were placed on the mucosal and serosal sides of the bladder, respectively. Sodium movement in the direction mucosa to serosa and potassium movement in the reverse direction were determined by labeling with Na^{22} and K^{42}

as described in Methods. The results of the experiments are shown in Table II. There is a substantial and statistically significant preponderance of cation movement in the direction from mucosa to serosa. There was no significant change in the ratio of dry to wet weight in these preparations, the experimental tissues having 18.2 ± 0.5 (S.E.M.) per cent solids while freshly excised control bladders showed 19.1 ± 0.4 ($P > 0.15$). In the absence of significant tissue swelling, the net movement of cation from mucosal to serosal media

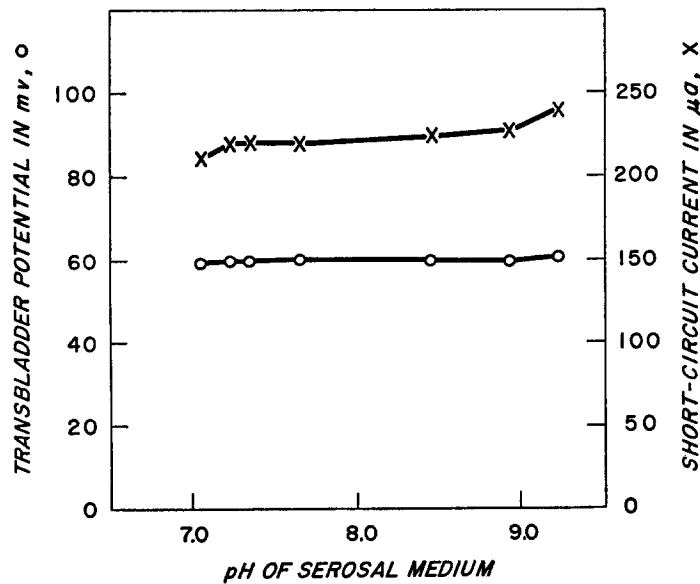


FIGURE 3. The independence of the electrical activity of the toad bladder and the pH of the serosal medium. The bladder is bathed on both sides by ordinary Ringer's solution. The serosal pH is adjusted initially to 9.23 by the addition of 0.1 N sodium hydroxide and the bladder is permitted to come to a new steady state. Small increments of 0.1 N hydrochloric acid are then added to the serosal medium at 5 to 10 minute intervals. The transbladder potential, short-circuit current, and serosal pH are measured immediately before the addition of each increment of acid.

must have obligated an equivalent movement of anion in the same direction. This result is incompatible with a net movement of anion from serosal medium to cell interior, and hence with an anionic diffusion potential at the serosal boundary of the bladder epithelial cell, if anion movement is passive in both directions at this site.

D. Active Sodium Transport and Transbladder Electrical Potential as a Function of Serosal pH

In many types of animal cells, the cytoplasm is acid to the surrounding fluid. In the case of the toad bladder in a bicarbonate-buffered Ringer's solution

this difference amounts, on the average, to 0.15 pH units (9). Although this gradient is not sufficient to account for the magnitude of the majority of the observed serosal potential steps, it is possible that the cytoplasmic pH was substantially lower than the average value in at least some of the bladder epithelial cells. Hydrogen ion cannot be excluded from consideration on the basis of its very low concentration relative to that of other ion species present, since Eigen (10) has shown that proton mobilities in aqueous systems may be orders of magnitude greater than would be expected on the basis of the behavior of the alkali metal ions.

For these reasons the influence of the pH of the serosal medium on the active sodium transport and transbladder potential was examined. One of three such experiments, all of which gave similar results, is shown in Fig. 3. As can be seen from the figure, alteration of the hydrogen ion concentration in the serosal medium by a factor of 150 caused no significant change in either the transbladder potential or the rate of active sodium transport. Thus it seems unlikely that hydrogen ion uptake across the serosal boundary of the epithelial cell is coupled with the active extrusion of sodium at the same site, or that the serosal potential step is a hydrogen ion diffusion potential resulting from the proton gradient across the serosal boundary.

DISCUSSION

The observation that the inside surface of the frog skin behaved under certain conditions as a potassium electrode led Koefoed-Johnsen and Ussing to the hypothesis that a portion of the total skin potential was due to a potassium diffusion potential at this side. They further suggested that the active sodium transport mechanism located at this surface brought about an exchange of cellular sodium for extracellular potassium, that is, the operation of the sodium pump involved no net transport of charge (4). This hypothesis has aroused a great deal of interest, and was the stimulus for the study reported here. We have been unable to duplicate their results in the toad bladder, which possesses a sodium transport system similar to that of the frog skin. We have, therefore, sought by other approaches to test their hypothesis in the toad bladder.

Our measurements of the serosal potential step in toad bladder show it to be relatively unaffected by serosal media which are high in potassium and low in chloride. We conclude that the potential step at the serosal border of the bladder epithelial cell is not a diffusion potential due to the passive movement of potassium out of the cell or chloride into it.

The measurements of the potential profile of the bladder using the high potassium, low chloride solution III involved the substitution in the bathing fluid of an anion, sulfate, which is known to penetrate the bladder to a limited extent (11). Because it was present in higher concentration outside the cell

than in, it was possible that the observed potential step might be a sulfate diffusion potential. For this reason we felt obliged to test for the presence of an anionic diffusion potential across the serosal border of the epithelial cell, without regard to the identity of the anion. The net cation flux experiments in open-circuited bladders were designed to examine this possibility. They showed a substantial net cation flux in the direction mucosa to serosa. The existence of the equivalent net anion flux in this direction is incompatible with an anionic diffusion potential of the observed polarity at the serosal boundary, if anion movement is passive in both directions at this surface.

There is a mechanism which is consistent with anionic diffusion as the cause of the serosal potential step, and which retains the idea of a neutral sodium pump. In this model, the sodium which is actively transported from the cell interior to the serosal medium would be associated with the diffusible anion as an ion pair. In our experiments, the magnitude of the serosal potential step was not significantly affected by a substitution of sulfate for chloride in the serosal medium. This lack of dependence on the accompanying anion would seem to make such a neutral ion pair pump only a remote possibility.

Large changes in hydrogen ion concentration in the serosal medium failed to alter the transbladder potential or short-circuit current. This result effectively excludes the possibility of a hydrogen ion diffusion potential or coupling of hydrogen ion and sodium movement at the serosal boundary.

We have summarized the evidence for our belief that the serosal potential step is not a diffusion potential. The second feature of the hypothesis advanced by Koefoed-Johnsen and Ussing concerns the exchange of sodium for potassium in the operation of the sodium pump. The suggestion of this kind of coupling of sodium and potassium movement originates in the observation that active sodium extrusion from many cell types is suppressed in media containing little or no potassium. The evidence has been reviewed recently by Ussing (12). A similar relation obtains in the toad bladder when potassium is removed from the serosal bathing medium (13).

There are experimental preparations, however, in which it has been possible to dissociate sodium and potassium transport (14-16, for example). In this laboratory, attempts to demonstrate equality of active sodium transport and serosal uptake of potassium by the toad bladder have been unsuccessful (17). It is not our purpose to discuss at length the evidence for or against tight sodium-potassium coupling, with the neutral pump which this implies, in tissues in general. We would simply like to emphasize that in a variety of cell types the commonly accepted idea of active sodium-potassium exchange does not seem to apply.

In the toad bladder we have examined a consequence of the hypothesis of coupling between active sodium transport and the movement of other ions: a diffusion potential at the serosal boundary of the epithelial cell. We have

been unable to implicate potassium or hydrogen ions, chloride, or other uncharacterized anions in the generation of the observed serosal potential step. Accordingly, we are thrown back on the first possibility mentioned in the Introduction to this study, that the serosal potential step arises from the operation of an "electrogenic" sodium pump at this surface of the cell, the pump causing the transfer of a sodium ion from the cell interior to the serosal medium without a simultaneous and obligate transfer of an anion in the same direction or a different cation species in the opposite direction as an integral part of the active transport step.

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