The Effect of Mucosal and Serosal Solution Cations on Bioelectric Properties of the Isolated Toad Bladder

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ABSTRACT   The spontaneous transtissue potential and the DC conductance of the isolated toad bladder were measured when the tissue was exposed to sulfate Ringer's solutions of modified ionic composition. Na^+ was replaced to varying extents by $(C_2H_5)_3NH^+$, $(C_2H_5)_4N^+$, Li^+, Cs^+, K^+, or Rb^+. Reversible and irreversible changes were observed. The reversible changes were consistent with equations derived from the Nernst-Planck diffusion equation, and gave the following functional description of the bladder: (a) the potential measurements were compatible with two membranes in series; (b) the mucosal surface was more permeable to Na^+ than to other monovalent cations; (c) the serosal surface was permeable to both K^+ and Na^+ but preferentially to K^+; (d) the rate of Na^+ diffusion across the mucosal membrane appeared to approach a maximum but two alternative interpretations are discussed; (e) the conductance data were consistent with the assumption of a constant concentration gradient for the penetrating ions within the membrane (Henderson's assumption) provided suitable hypotheses are made concerning the Na^+ distribution between the membrane surfaces and the bulk phases of the adjacent solutions; (f) the conductance and spontaneous potential data suggested that the mucosal membranes of a small fraction of the epithelial cells were more permeable than the mucosal membranes of the majority of these cells. The irreversible changes were almost entirely associated with cation substitution in the serosal solution. However, Li^+ produced an irreversible fall in voltage when added to either side of the tissue.

INTRODUCTION

The model of the epithelial cell of the frog skin (Koefoed-Johnson and Ussing, 1) was based on measurements of the spontaneous transtissue potential. In a

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sulfate Ringer's solution the skin responded like a Na^+ electrode to changes in the Na^+ concentration of the outside solution. Changes in the K^+ concentration of the inside solution produced alterations of the transtissue potential consistent with the behavior of a K^+ electrode. Similar results have been obtained in a more extensive study by Lindley and Hoshiko (2).

The epithelial cell was described as an aqueous phase separated from the bathing media by the mucosal and serosal cell membranes. The transtissue potential was thought to be the sum of a Na^+ diffusion potential across a mucosal membrane selectively permeable to Na^+ and a K^+ diffusion potential across a serosal barrier selectively permeable to K^+. A non-electrogenic active sodium-potassium exchange system was also located at the serosal membrane.

Microelectrode penetrations of the frog skin had demonstrated potential steps of the proper polarity (3). Optical measurements of skin epithelial cell swelling confirmed the selective permeabilities of the mucosal and serosal membranes to Na^+ and K^+ and the relative impenetrability of the sulfate ion (4). The inhibition of salt transport by removal of K^+ from the bathing solution was cited by Ussing (5) as evidence for a stoichiometric sodium-potassium exchange "pump."

The pattern of solute and water transport of the isolated toad bladder was found by Leaf and coworkers (6, 7) to be similar to that of the frog skin. In addition, the omission of K^+ from the bathing media completely blocked net Na^+ transport (8). Two potential steps of increasing positivity were observed as microelectrodes were advanced from mucosa to serosa (9). The mucosal potential jump was shown to be qualitatively dependent on the Na^+ concentration in the mucosal solution.

The toad bladder has been described as a single continuous layer of mucosal epithelial cells separated from the serosa by connective tissue containing smooth muscle bundles and blood vessels (6). A significant DC resistance and spontaneous potential were not observed for serosa (peritoneum) removed from an area adjacent to the bladder (10). It was concluded that the epithelial layer was the site of the spontaneous transtissue potential.

Although the above functional and morphological observations support the extension of the frog skin cell model to the toad bladder, objections have been raised concerning the existence of a K^+ diffusion potential and coupled sodium-potassium exchange across the serosal membrane (10, 11). A serosal potential step was found when the tissue was bathed in a potassium chloride Ringer's solution and when the serosal surface was bathed in a potassium sulfate Ringer's solution containing the concentration of K^+ presumed to be present in the epithelial cells. The failure of Na^+ transport to occur in the absence of serosal K^+ was attributed to a reduction in passive Na^+ entry through the mucosal barrier rather than a decrease in the active exchange of

Na^+ for K^+. Finally, the ratio of K^{42} uptake from the serosal solution to net transtissue Na^+ transport was far less than one.

The following experiments were designed to determine the effects of the substitution of monovalent cations in the serosal and mucosal solutions on DC tissue conductance and the spontaneous transtissue potential of the isolated toad bladder. The interpretation of the data is similar to that implicit in the epithelial cell model proposed by Koefoed-Johnson and Ussing.

TABLE I
COMPOSITION AND PROPERTIES OF
EXPERIMENTAL SOLUTIONS

Concentration, mM	Cl Ringer's	SO₄ Ringer's
Composition		
NaCl	113	—
Na₂SO₄	—	56.5
NaHCO₃	0.5	0.5
KHCO₃	2.0	2.0
CaCl₂	1	—
Ca(gluconate)₂	—	1
Properties		
pH	8	8
Colligative	≈ 115 mM NaCl/liter	≈ 80 mM NaCl/liter

METHODS

A. *Experimental Animals*

Toads (*Bufo marinus*) of both sexes were obtained from the Lemberger Co. (Oshkosh, Wisconsin) and Tarpon Zoo (Tarpon Springs, Florida). The animals were stored in pens at room temperature and force-fed liver or earthworms weekly. By periodic immersion in water the toads maintained their hydration.

B. *Experimental Solutions*

Isolated bladders were exposed to the Ringer's solutions described in Table I. Additional sulfate Ringer's solutions were prepared by partially replacing Na₂SO₄ with Li, K, Rb, Cs, tetraethylammonium, or triethylammonium sulfate. All salts were reagent grade except Cs₂SO₄ "purified" (Fisher Scientific Co., Pittsburgh, Pennsylvania) and Rb₂SO₄ 99.9 per cent (A. D. Mackay, New York). Triethylammonium and tetraethylammonium sulfate were prepared by titrating a measured volume of 1.0 N H₂SO₄ to pH 8 with the base (Distillation Products Industries, Rochester, New York). The vapor pressure or freezing point of all solutions used in the transtissue potential-tissue conductance studies was within ± 4 mM NaCl/liter of the control (113.5 mEq Na⁺/liter) sulfate Ringer's solution.

C. Volume of Distribution Studies

The apparent volumes of distribution of C^{14}-inulin (New England Nuclear Corporation, Boston, Massachusetts) and $S^{35}O_4^=$ (Abbott Laboratories, Oak Ridge, Tennessee) in the bladder were determined by the same general procedure. Bladders of 40 to 200 mg wet weight were removed from doubly pithed animals and incubated in oxygenated Ringer solution for one-half hour. The tissues were transferred to vials containing the appropriate radioactive Ringer's solution at 21°C. Mixing and oxygenation were accomplished by slowly bubbling oxygen through the solution. At various time intervals all the tissues were removed from a group of vials, blotted on Whatman no. 1 filter paper moistened with a drop of radioactive solution, and placed in tared flasks. The flasks were weighed to the nearest 0.1 mg and placed in a drying oven at 95°C for 24 hours. These time and temperature conditions have been reported to yield consistent tissue water analyses (12). After the drying period the flasks were reweighed and the tissue water calculated from the difference between wet and dry weights.

The radioactivity in each bladder was extracted with 0.1 N or 1.0 N HNO_3 and continuous shaking for 48 hours. A similar tissue extraction has been described by Page and Solomon (13) and Frazier et al. (14). Duplicate samples of the extract which contained S^{35} were counted with a Geiger tube and scaler. C^{14} was estimated using a thin window gas flow detector and scaler. The standard error of each count was less than 3 per cent. Agreement between duplicate sample counts averaged 5 per cent.

The C^{14}-inulin space associated with the mucosal surface was obtained from tissues clamped in the apparatus used for the transtissue potential measurements. The mucosal surface of the bladder was exposed to sulfate Ringer's solution containing C^{14}-inulin, while inulin-free sulfate Ringer's bathed the serosal side. The solutions on both sides were changed every 40 minutes. After 3 hours of incubation a piece of tissue was removed from the apparatus and blotted on the mucosal side with filter paper moistened with radioactive Ringer's solution. The serosal surface was blotted with filter paper moistened with a drop of non-radioactive Ringer's solution. Tissue water and radioactivity were determined by the procedures previously outlined. In all volume of distribution studies 50 microliter samples of the appropriate radioactive Ringer's solution were carried through the drying, extraction, and counting procedures.

D. Transtissue Potential—Tissue Conductance Studies

The transtissue potential and DC tissue conductance were measured at 21°C in an apparatus similar to the one first described by Ussing and Zerahn (15). A tissue surface area of 3.14 cm² was exposed to the 8 ml of Ringer's solution bathing each side of the bladder.

Two syringes and a three-way stopcock attached to each lucite compartment— bubble pump assembly made it possible to change the solutions on each side of the bladder simultaneously. About 0.3 ml remained in each compartment after the solutions were removed.

The transtissue potential was monitored by agar–KCl bridges connected through

calomel half-cells to a recording DC voltmeter (Yellow Springs Instrument Co., Yellow Springs, Ohio) of 1 megohm impedance and ± 1 mv accuracy. The leak of KCl from a bridge into the bathing media approximated 8 micromoles per hour.

The DC tissue conductance was determined by impressing an external EMF across the bladder and measuring the transtissue potential and current flow through the external circuit. Carbon electrodes connected the external battery to the bathing solution. The external current was measured with a DC microammeter (50 μa full scale, 2 per cent accuracy). The tissue followed Ohm's law for two 20 μa steps on
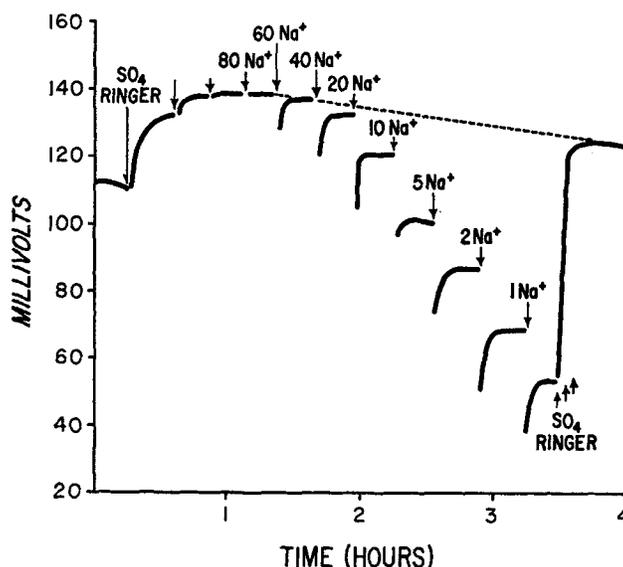
FIGURE 1. The effect of the Na^+ concentration in the mucosal solution on the transtissue potential (see text). The response of a single bladder in sulfate Ringer's solution. Mucosal Na^+ was replaced by K^+. The numbers preceding Na^+ are the mucosal concentrations in milliequivalents per liter.

either side of the open circuit (Fig. 5). The DC tissue conductance was calculated as the reciprocal of the slope of the voltage-current plot.

The time course of transtissue potential depicted in Fig. 1 illustrates the protocol followed in the transtissue potential–tissue conductance studies. After mounting the tissue in the apparatus, control sulfate Ringer's solution was placed on both sides of the bladder. All tissues were incubated with control sulfate Ringer's for at least 45 minutes. During this period the solutions were changed at least twice (three times in Fig. 1). Often, when the solutions were changed, the potential dropped and then returned to a steady-state after 5 to 15 minutes. A steady-state potential was defined arbitrarily as a potential which remained constant for at least 6 minutes. The potential drop could be prevented by supporting the tissue with thin silk cloth. However, the transtissue potential and resistance of supported bladders declined rapidly after 90 minutes, presumably because of friction between the cloth and tissue.

At the fourth arrow a solution with 33.5 mEq/liter of the Na^+ replaced by K^+

(final Na^+ concentration—80 mEq/liter) was added to the mucosal compartment, while control sulfate Ringer's was placed in the serosal chamber. This procedure was repeated as the mucosal Na^+ concentration was reduced to 1 mEq/liter. Conductance measurements were made after the steady-state potentials had been established. At the twelfth arrow the solutions bathing both sides of the bladder were replaced three times with control sulfate Ringer's. After the potential had reached a steady-state, a final conductance measurement was made. In an alternative protocol the mucosal surface was washed three times with 1 mEq Na^+/liter. Then the mucosal Na^+ concentration was increased. Both types of procedure gave the same results. Similar potential changes were observed when the sequence of test solutions was randomized.

Preliminary experiments had shown that control steady-state potentials greater than 60 mv were maintained ($\pm$ 10 mv) for at least 4 hours. Therefore a test series was not started unless the transtissue potential in control Ringer's exceeded 60 mv. Since the transtissue potentials of bladder lobes from the same animal sometimes differed by 10 mv or more, each tissue was used as its own control. The potential changes in the presence of test solutions were corrected for shifts in control potential on the assumption that the shift was a linear function of time. This relationship is illustrated by the dashed line in Fig. 1. Data from "reversible" experiments were not accepted if the final control voltage was less than 70 per cent of the control potential before the start of the test sequence. These restrictions eliminated about 10 per cent of the experiments.

In an attempt to detect intracellular changes in Na^+ and K^+ concentration, a procedure similar to the transtissue potential studies was adopted. After a control steady-state potential had been established, the mucosal surface was washed three times with a K_2SO_4-substituted solution containing 1 mEq Na^+/liter. A new steady-state potential was recorded and a portion of the tissue was removed from the apparatus. The mucosal surface was blotted on filter paper moistened with the test solution, while the serosal surface was blotted with filter paper moistened with control sulfate Ringer's solution. The same procedure was followed for control tissues except that both sides of the bladder were exposed to control sulfate Ringer's solution (113.5 mEq Na^+/liter). Tissue water was determined by the method used in the volume of distribution studies. After extracting the tissue with 0.1 N HNO_3 for 48 hours, the extract Na^+ and K^+ concentrations were determined by flame photometry (Zeiss spectrophotometer PMQ II with flame attachment).

RESULTS

The interpretation of the electrical changes accompanying alterations in the cation composition of the bathing solution depends upon the assumption that the sulfate ion does not pass across the bladder and does not penetrate into the epithelial cells. Permeability coefficients listed by Leaf and Hays (16) indicate that the sulfate flux is small compared to that of Na^+. Preliminary studies in this laboratory showed that the sulfate flux across the short-circuited bladder exposed to sulfate Ringer's solution was only 3 per cent of the

active Na^+ transport. These flux studies demonstrated that there is at least one barrier to sulfate movement across the tissue.

Moreover, comparison of the $S^{35}O_4$ and C^{14}-inulin spaces in the bladder indicated that sulfate was distributed almost exclusively in the extracellular space. The C^{14}-inulin space (Fig. 2) was 50 per cent of the tissue water in sulfate or chloride Ringer's solution. Inulin spaces of 47 per cent (12) and 51 per cent (14) have been reported previously for the bladder in chloride Ringer's solution. The sulfate space (Fig. 2) was larger, amounting to 65 per
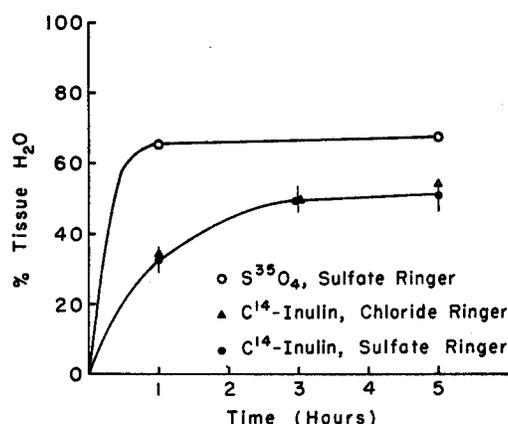
FIGURE 2. Apparent volumes of distribution of $S^{35}O_4$ and C^{14}-inulin in the isolated toad bladder. For the distribution of $S^{35}O_4$ in sulfate Ringer's, each point represents the mean of at least fifteen tissues. The vertical lines of the C^{14}-inulin-chloride Ringer's points are the standard errors calculated from at least eight tissues and are representative of the other points. Each of the C^{14}-inulin-sulfate Ringer's points was obtained from twelve tissues.

cent of the tissue water. This may be because sulfate ions accumulate in the epithelial mucous cells as reported by Choi (17).

The interpretation of the results also depends upon the assumption of constant intracellular concentrations of Na^+ and K^+. When the cation composition of a tissue is modified by changing the composition of a solution to which it is exposed, the tissue cells most apt to be involved are those in immediate contact with the modified solution. Similarly, if the cation alteration is limited to extracellular rather than intracellular fluid, the extracellular compartment which is most readily modified is the one adjacent to the altered external solution. Table II describes changes in tissue Na^+ and K^+ when the mucosal solution composition was changed from 113.5 mEq Na^+/liter and 2 mEq K^+/liter, to 1 mEq Na^+/liter and 114.5 mEq K^+/liter. The tissue concentration changes are reconcilable with the assumption that, without entering the cells, Na^+ and K^+ ions equilibrate with an extracellular tissue

volume of less than 10 per cent of the tissue water (last column, Table II). This volume compares favorably to the mucosal inulin space of 6.1 per cent (± 1.5 SE) determined for six tissues in sulfate Ringer's solution. A mucosal inulin space of 7 per cent has been reported by Maffly et al. (12) for the bladder in chloride Ringer's solution. It seems reasonable to conclude that changes in the cation composition of the mucosal solutions were not accompanied by changes in the intracellular cation composition.[1]

The lack of change in cation composition adds further support to the conclusion that sulfate does not penetrate into the epithelial cells responsible for the electrical properties of the tissue because sulfate accumulation would be expected to induce alterations in intracellular cation composition.

TABLE II

THE ESTIMATION OF THE MUCOSAL SPACE FROM
CHANGES IN TISSUE Na+ AND K+ CONTENT

	n	Mucosal fluid composition		Tissue concentration		Δ Concentration	Volume of distribution*
		Control	Low Na+	Control mean (±SE)	Low Na+		
				mEq/kg tissue H₂O		mEq/kg tissue H₂O	per cent tissue H₂O
Na+	10	113.5	1	74.2 (±4.4)	67.2 (±3.4)	7.0	6
K+	10	2	114.5	42.8 (±1.8)	53.2 (±3.2)	10.4	9

* Calculation assumes constant cellular [Na+] and [K+]; constant cell, serosal and mucosal volumes.

Studies on the Mucosal Surface

The partial replacement of Na+ in the mucosal solution by K+, Cs+, or Rb+ resulted in a reversible decrease in potential. The cumulative volt-

[1] The measurements listed in Table II do not eliminate the possibility of major changes in the intracellular cation composition of the epithelial cells if the contribution of these cells to the tissue content is small. Similarly, small changes in cation composition would be difficult to detect even if all the tissue content was present in the epithelial cells. It is unlikely, however, that the deviation from the response of an ideal sodium electrode depicted in Fig. 3 can be explained by a change in intracellular sodium, if the epithelial cells contain a reasonable fraction of the tissue cation. For example, if the tissue is represented as the two epithelial cell membranes in series, transtissue potentials or intracellular cation concentrations can be calculated with the aid of equation (5) of the Appendix. If the sum of the cell Na+ and K+ concentrations remains constant, and if reasonable control values are assumed for these concentrations and K_s, then the Na+ electrode hypothesis requires a loss of about 30 mEq Na+/kg of cell water to account for the decrease in transtissue potential which accompanied the mucosal solution change from control to low Na+ Ringer. This loss of intracellular Na+, in addition to the alteration in mucosal extracellular composition, predicts a change in tissue Na+ approximately twice that reported in Table II if the epithelial cells occupy 50 per cent of the non-inulin space.

age change was a linear function of the log of the Na^+ concentration in the mucosal solution (Fig. 3). The slopes of the lines were approximately half the 58.4 mv per tenfold concentration change predicted for an ideal Na^+ electrode. Linear regression analysis of Fig. 3 revealed that the slopes for Cs^+, K^+, and Rb^+ substitution were respectively 27.5, 30, and 25 mv.

For Na^+ concentrations below 60 mEq/liter voltage responses similar to those in Fig. 3 were observed when tri- and tetraethylammonium ions were
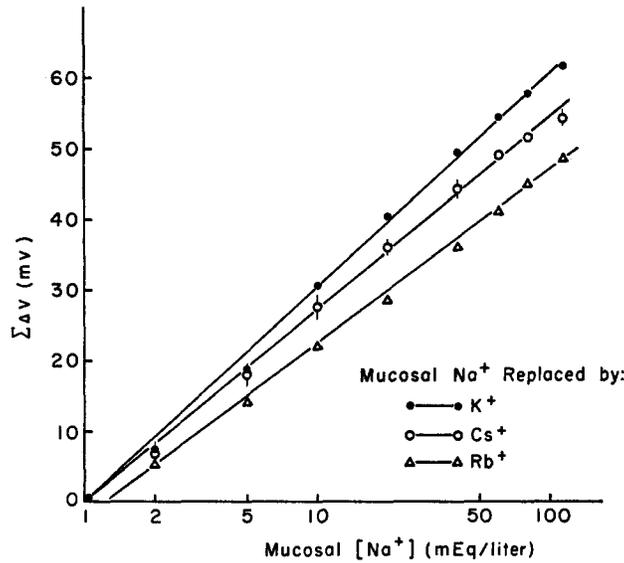
FIGURE 3.   The increment in transtissue potential with increasing Na^+ concentration in the mucosal solution. The data were obtained from tissues in sulfate Ringer's solution. For the replacement of mucosal Na^+ by Cs^+, Rb^+, and K^+, each point depicts respectively the mean of six, two, and at least eleven tissues. The vertical lines of the Cs^+ points are the standard errors and are also representative of the K^+ data. The slopes of lines indicate 27.5, 25, and 30 mv changes per tenfold Na^+ concentration change for Cs^+, Rb^+, and K^+ replacement respectively.

used (Fig. 4). At high Na^+ concentrations the points deviated from the log-linear response. When the Na^+ concentration of the mucosal solution was lowered from 113.5 to 80 mEq/liter the transtissue potential actually increased by 3.8 mv for tetraethylammonium and by 5.5 mv for triethylammonium substitution.

At a constant cation composition of the mucosal and serosal solutions, the voltage across the tissue was linearly related to the applied current. Fig. 5 depicts a typical experiment in which mucosal solution Na^+ was replaced by K^+. The slope of the lines is the reciprocal of the DC tissue conductance and a function of the cation composition of the mucosal solution. The DC tissue

conductance decreased with partial replacement of mucosal Na$^+$ by K$^+$, Cs$^+$, Rb$^+$ and (C$_2$H$_5$)$_4$N$^+$. The conductance ratio (Cr/Co) has been plotted as a function of the Na$^+$ concentration of the mucosal solution (Fig. 6) according to equation (10) of the Appendix. The ratio was obtained by dividing the tissue conductance (Cr) in the presence of the mucosal test solution by the control conductance (Co) before the start of the test sequence. This normalization of the data was necessary because control absolute conductances ranged
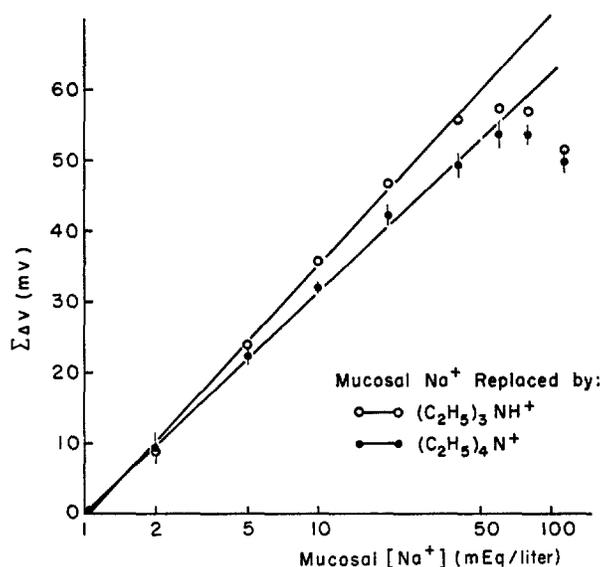
Figure 4. The cumulative voltage response with the replacement of Na$^+$ in the mucosal solution by tri- and tetraethylammonium. The data were obtained from tissues in sulfate Ringer's solution. Each point is the mean of three and five tissues for tri- and tetraethylammonium substitution. The vertical lines of the tetraethylammonium points represent the standard errors.

from 2.0×10^{-4} to 7.6×10^{-4} mho/cm^2. Cs$^+$, K$^+$, and Rb$^+$ gave a linear graph (Fig. 6) in conformity with equation (10). (C$_2$H$_5$)$_4$N$^+$ deviated from linearity at mucosal Na$^+$ concentrations above 60 mEq/liter. Conductance measurements were not made for (C$_2$H$_5$)$_3$NH$^+$ substitution. Comparison of the conductance in control Ringer's solution after the test period with the conductance observed initially indicated that the effects of K$^+$, Cs$^+$, Rb$^+$, and (C$_2$H$_5$)$_4$N$^+$ were reversible.

It was mentioned under D in the section on Methods that the potential of the tissue in control sulfate Ringer's solution shifted during the time course of an experiment. Although the voltage after the test period was sometimes greater than the potential recorded before the addition of the first test solution, the control potential of the majority of the tissues declined with time.

For the experiments which have been considered to be reversible, the mean decline in potential ranged from 4 (± 4 SE) mv for the replacement of mucosal Na^+ by $(C_2H_5)_4N^+$ to 20 mv for the two Rb^+ experiments. However, the drop in transtissue potential observed with the partial replacement of mucosal Na^+ with Li^+ was not reversed when Li^+ was washed from the mucosal com-
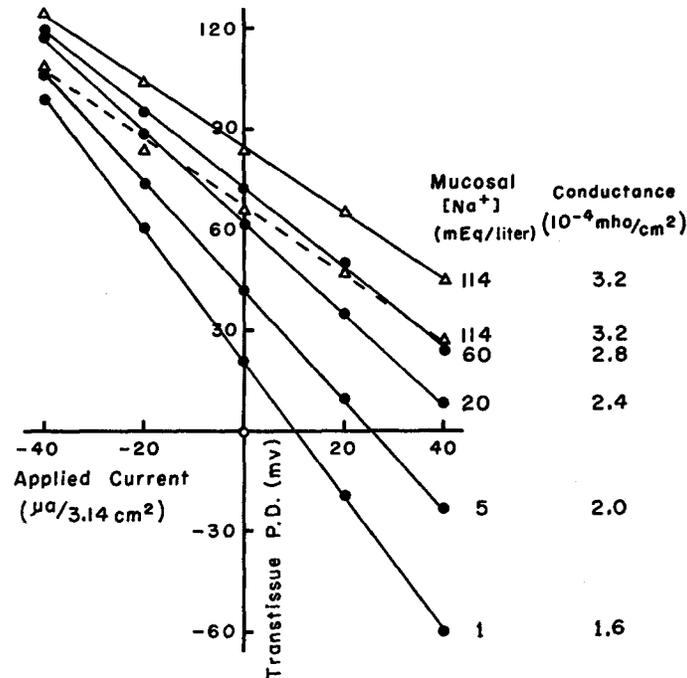
FIGURE 5. The effect of the Na^+ concentration in the mucosal solution on tissue DC conductance. The figure represents a typical response of a bladder in sulfate Ringer's solution. Currents which made the mucosa more negative have been given negative signs. Mucosal solution Na^+ has been replaced by K^+. The triangles represent points obtained in control solution. The points of the dashed line were obtained after the last experimental solution, 200 minutes later than the first control response (solid line). Each of the other lines represents the response of the tissue in the presence of the mucosal solution Na^+ concentration listed to the right of the 40 μa points.

partment. The mean irreversible change in potential for six tissues was 38 (± 4 SE) mv.

Measurements of the conductance of four tissues exposed to mucosal Li^+ were inconclusive. The conductance of two tissues was unchanged during the test solution sequence and return to control Ringer's. The conductance of the other tissues increased irreversibly during the test period to twice the control value.
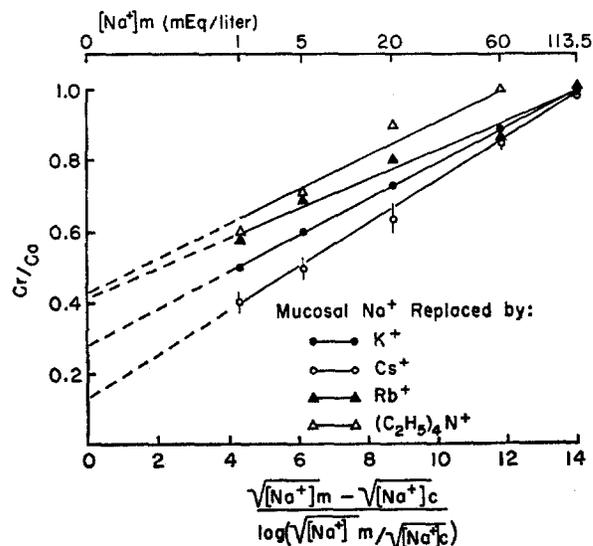
FIGURE 6.   The effect of the mucosal Na^+ concentration on the conductance ratio (see text). Each point for K^+, Cs^+, Rb^+, and tetraethylammonium substitution is the mean of observations on five, six, two, and five tissues respectively. The vertical lines of the Cs^+ points are the standard errors and are representative of the K^+ and tetraethylammonium points. Although a cell sodium concentration ($[Na^+]_c$) of 10 mEq/liter has been assumed, any reasonable constant value may be used without affecting the linearity or intercept of the relationship.
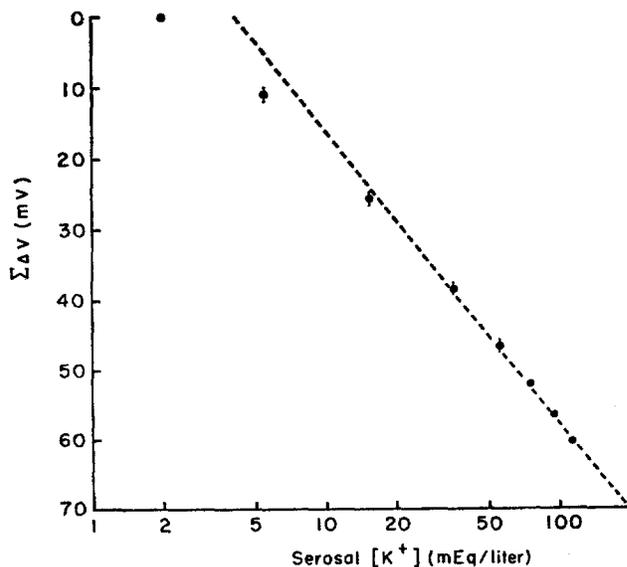
FIGURE 7.   The decrement in transtissue potential with increasing serosal K^+ concentration. Serosal Na^+ was replaced by K^+. Each point is the mean of observations on at least ten tissues. The vertical lines of any point represent the standard errors.

Studies of the Serosal Surface

A reversible reduction in the transtissue potential was associated with the replacement of serosal solution Na^+ by K^+. Fig. 7 illustrates the cumulative decrease in voltage plotted as a log function of the serosal K^+ concentration.

Six of the eight observations fell on a straight line with a gradient of 42 mv per tenfold concentration change. The voltage response deviated from the

$$\frac{([K^+]_s + \sqrt{[Na^+]_s}) - ([K^+]_c + \sqrt{[Na^+]_c})}{\log([K^+]_s + \sqrt{[Na^+]_s}) - \log([K^+]_c + \sqrt{[Na^+]_c})}$$
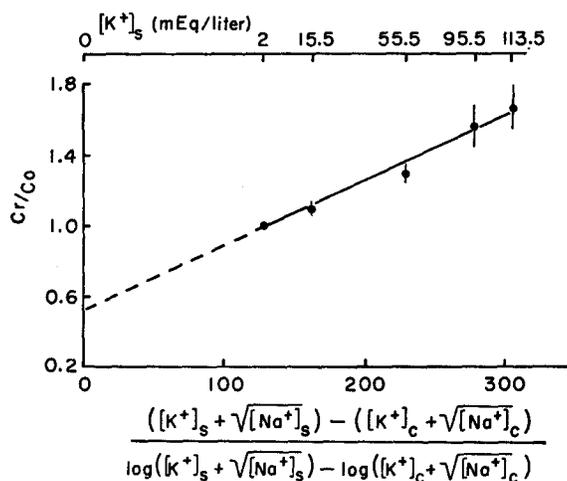
FIGURE 8. The effect of the serosal K^+ and Na^+ on the conductance ratio (see text). Serosal Na^+ was replaced by K^+. Each point is the mean of observations on eight tissues, with the exception of the point at the highest K^+ concentration which was obtained from five bladders. The vertical lines of any point represent the standard errors. A value of 150 has been assumed for the quantity containing the cell K^+ and Na^+ concentrations ($[K^+]_c + \sqrt{[Na^+]_c}$). As in Fig. 6 any reasonable constant value may be used.

line with serosal K^+ concentrations below 16 mEq/liter. Changes in the DC conductance ratio associated with serosal K^+ substitution are plotted according to equation (10) on the assumption that Na^+ and K^+ have equal mobilities and that the Na^+ concentration in the membrane is proportional to the square root of its concentration in the serosal solution (Fig. 8).

The irreversible decrement in transtissue potential which followed the replacement of serosal Na^+ by monovalent cations has been recorded in Table III. All the ions substituted for serosal Na^+, except K^+ and $(C_2H_5)_4N^+$, produced large reductions in the control transtissue potential. If the potential changes associated with the replacement of Na^+ by $(C_2H_5)_4N^+$ are corrected for the irreversible voltage drop, a mean reversible decrease of 27 (±7 SE) mv was obtained for the change from 113.5 to 2 mEq Na^+/liter. This decrease was considerably less than the total reversible voltage decrement of 61 mv observed with replacement by K^+.

Serosal Li+, Cs+, and tetraethylammonium decreased the DC tissue conductance to about half the initial control conductance in the majority of the tissues tested. The decrease was not a function of the concentration of substituted cation in the sense that the maximum decrease was sometimes observed with concentrations as small as 13.5 mEq/liter. Rb+ and triethylammonium substitution appeared to have little effect on tissue conductance. However, when triethylammonium was washed from the serosal compartment, the conductance of the bladder increased twofold. The ratio of the final control conductance (C_f) to the pretest conductance (C_o) for each serosal cation series has also been reported in Table III. The effects on tissue conductance appeared to be reversible with all cations except triethylammonium.

TABLE III
THE EFFECT OF SEROSAL CATIONS
ON THE CONTROL TRANSTISSUE POTENTIAL
AND TISSUE CONDUCTANCE

Cation replacing Na^+	n	Irreversible potential decrement mean $(\pm SE)$	n	Conductance C_f*/C_o mean $(\pm SE)$
		mv		
K^+	15	7 (± 3)	8	1.22 (± 0.14)
Li^+	6	56 (± 7)	6	0.92 (± 0.17)
Rb^+	6	58 (± 7)	5	1.06 (± 0.16)
Cs^+	6	83 (± 7)	6	0.82 (± 0.16)
$(C_2H_5)_3NH^+$	5	55 (± 5)	5	2.44 (± 0.63)
$(C_2H_5)_4N^+$	6	20 (± 4)	6	0.85 (± 0.09)

* C_f is the conductance of the tissue in control (113.5 mEq Na^+/liter) sulfate Ringer's following the test solution sequence.

DISCUSSION

The physiological properties of this preparation were similar to those reported for other preparations of toad bladder; namely a similar total extracellular space of 50 per cent, a mucosal inulin space of less than 10 per cent, and a marked impermeability to the sulfate ion. The tissue was able to maintain constant intracellular concentrations of Na^+ and K^+ in the face of large changes in the concentrations of these ions in the mucosal solution (Table II). As further evidence of the viability of this preparation, intracellular K^+ concentrations were maintained at a much higher level than intracellular Na^+. For example, the tissue concentrations quoted in Table II give the following intracellular concentrations if a total extracellular space of 50 per cent and a mucosal space of 10 per cent are assumed. When bathed with the control Ringer's, intracellular Na^+ and K^+ were respectively 35 and 84 mEq/kg tissue H_2O; with the mucosal solution composition changed to 1 mEq Na^+/

liter and 114.5 mEq K^+/liter, the corresponding intracellular values were 43 and 82 mEq/kg tissue H_2O.

The electrical data may be divided for purposes of discussion into the reversible and irreversible. The reversible data were obtained under steady-state conditions and can therefore be interpreted in terms of the Nernst-Planck diffusion equation as discussed in the Appendix.

Reversible Electrical Effects

The reversible potential changes resulting from changes in the composition of the bathing solutions will be interpreted in terms of potential changes across either the mucosal- or serosal-facing membrane of the bladder epithelial cell. Specifically, if the compositions of the serosal solution and of the intracellular solution remain constant, transtissue voltage changes resulting from alterations in the cation concentration of the mucosal solution reflect equal changes in the voltage across the mucosal-facing membrane of the epithelial cell.

The voltage changes induced by mucosal substitution (Figs. 3 and 4) are similar in magnitude and direction irrespective of the nature of the cation replacing Na^+ except that $(C_2H_5)_3NH^+$ and $(C_2H_5)_4N^+$ are atypical for the solution changes between 60 and 113.5 mEq Na^+/liter. The simplest interpretation is that the mucosal surface is selectively permeable to Na^+ and responds to concentration changes like an ideal Na^+ electrode according to equation (2) of the Appendix. The semilog plots of Figs. 3 and 4 indicate a linear relation as predicted by this equation, but the slope of the lines, instead of the theoretical 58.4 mv, is approximately half this value.

Two obvious mechanisms which might lead to a voltage response less than the theoretical are (a) Na^+ migration that is accompanied by net movements of solution anions and (b) a net movement of solution Na^+ into the cells that is associated with an equivalent loss of cell K^+. Evidence presented in the Results indicated that movement of the major solution anion, sulfate, into the epithelial cells did not occur. Furthermore, the apparent constancy of the intracellular K^+ and Na^+ concentrations in the presence of mucosal solutions of widely varied cation composition rules out the exchange of intracellular K^+ for solution Na^+.

If the observed voltage changes were due entirely to an Na^+ diffusion potential, a gradient of 50 per cent of the theoretical might be due to one of two causes, according to equation (2) in the Appendix. The sodium ion might have an effective charge of two. Flux ratio measurements of K^+ diffusion across the membrane of the squid axon were compatible with this ion having an effective charge $z = 2.5$, and Hodgkin and Keynes (18) suggested that such a phenomenon might result from the diffusion of ions through long pores. A second possible cause is a concentration of Na^+ at the surface of the

membrane that is proportional to the square root of the Na^+ concentration in the bathing solution. A square root relationship may originate in different ways. Na^+ may be physically adsorbed on the mucosal surface of the bladder according to the classical adsorption isotherm:

$$x/m = KC^{1/n}$$

where x = the weight adsorbed, m the amount of adsorbing material, and C the concentration in the solution. For example, n is reported to vary between 2 and 4 for the adsorption of various substances from aqueous solution by charcoal.

Alternatively, the square root relationship might result from the combination of Na^+ with divalent "carrier" or "receptor" located at each surface of the epithelial membrane. For example, the mucosal cell membrane might consist of three diffusion barriers: an outer barrier at each surface of the membrane (the mucosal solution–facing and cytoplasm-facing surfaces) which is permeable to the uncharged disodium carrier complex, and an inner barrier which is impermeable to the carrier and limits the rate of Na^+ diffusion across the membrane. If it is assumed that the first Na^+ is bound strongly to the carrier, so that all carrier in the outer surface contains at least one Na^+, then the attachment of a second Na^+ to the carrier will determine the rate of transport of the uncharged complex across the outer barrier. Thus, the rate of transport of the uncharged complex across the mucosal solution–facing barrier will be proportional to the product of the concentration of Na^+ in the solution and the concentration of singly charged sodium-carrier complex. The complex, on passing the outer barrier, rapidly dissociates making two Na^+ available for diffusion across the inner rate-determining barrier.

This reversible sequence of reactions may be represented by the steady-state equation:

$$k_{in} [\text{Na-carrier}^-]_m [\text{Na}^+]_m = k_{out} [\text{Na}^+]_i^2 [\text{carrier}^=]_i$$

where k_{in} and k_{out} are each the product of an association (or dissociation) rate constant and an influx (or outflux) constant for the movement of the uncharged complex across the barrier; the subscripts m and i refer to concentrations at the mucosal border of the barrier and on the inner side of the barrier. Rearranging the above equation we obtain:

$$[\text{Na}^+]_i = \left(\frac{[\text{Na}^+]_m k_{in} [\text{Na-carrier}^-]_m}{k_{out} [\text{carrier}^=]_i} \right)^{1/2}$$

To the extent that the ratio of the concentration of the singly charged carrier to the concentration of the divalent carrier stays constant, it follows that the concentration of Na^+ inside the barrier (and therefore at the rate-determining

diffusion barrier) is approximately proportional to the square root of the concentration at the mucosal surface. An obvious candidate for the divalent carrier is the sulfate ion. However, preliminary experiments in this laboratory indicate that similar voltage responses are obtained with the monovalent impermeant anion, methylsulfate.

In the case of replacement of Na^+ in the serosal solution of K^+, the semilog plot gives a linear relationship only for serosal K^+ concentrations greater than 16 mEq/liter (Fig. 7). The gradient of the linear portion of 42 mv per tenfold concentration change deviates from the theoretical value of 58.4 for a membrane selectively permeable to K^+ ions. A linear semilog plot having the 58.4 mv gradient is obtained if the data are plotted according to equation (5) of the Appendix, which assumes that the membrane is equally permeable to both K^+ and Na^+ and that Na^+ follows the square root relation as discussed for the mucosal membrane. Thus the serosal membrane potential would be described by the equation:

$$E_2 - E_1 = \frac{RT}{F} \ln\left([K^+]_s + \sqrt{[Na^+]_s}/[K^+]_c + \sqrt{[Na^+]_c}\right)$$

where subscripts s and c refer to the concentration of ions in the serosal solution and inside the epithelial cell respectively.

Equation (5) in the Appendix may supply an alternative interpretation of the potential changes produced by mucosal and serosal replacement of Na^+. The potential changes due to mucosal solution substitution correctly fit equation (5) if it is assumed that the relative permeability coefficient K changes with the ionic composition of the mucosal solution. Its mean value over any given potential change may be computed from equation (5) by substituting the cation concentrations between which the voltage change was observed. The results of these calculations are plotted in Fig. 9 as a function of the log of the Na^+ concentration at the midpoint of the solution change. The relative permeability coefficient increased from 0.02 at 1 mEq Na^+/liter to at least 0.5 for the solution change from 80 to 113.5 mEq/liter. The values of K calculated for substitution of tri- and tetraethylammonium ions follow the same general curve as Fig. 9, starting at 0.02 at 1 mEq Na^+/liter but increasing to values greater than unity for the 80 to 113.5 mEq Na^+/liter change. A variable K may be interpreted as a change in the mobility of Na^+ (u_{Na}) or of the substituent cation (u_r) or both. However, since Na^+ was the ion common to all five sets of experiments, it is simpler to assume that u_{Na} decreased as the mucosal Na^+ concentration increased. The direction of the change in u_{Na} would indicate that the penetration of Na^+ into the epithelial cells would approach a maximum rate as the mucosal Na^+ concentration is increased. This interpretation supports the conclusions of Frazier, Dempsey, and Leaf (14), who found that the Na^+ concentration in a "tissue sodium pool" reached a maximum as

the mucosal Na+ concentration was increased, and more recently Frazier and Hammer's (19) observation of a rate-limited efflux of Na+ across the mucosal-facing membrane of the epithelial cell.

A similar interpretation may be applied to the potential changes induced by substitution of serosal Na+ by K+. The calculated K values (Table IV) indicate that Na+ mobility decreases with increasing serosal Na+ concentrations.
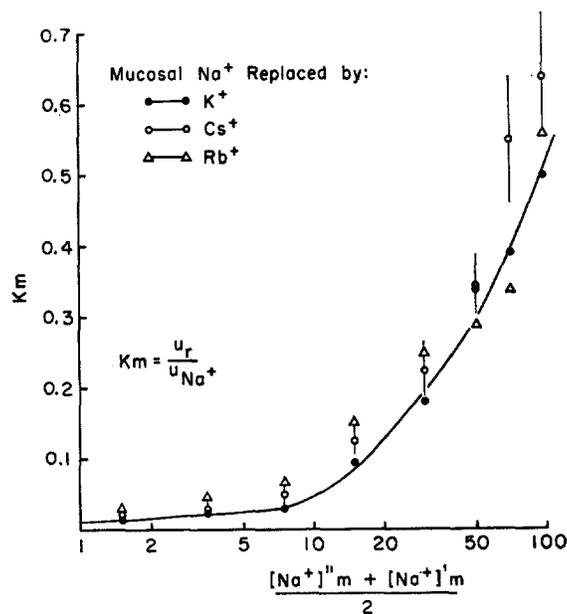
Figure 9. The effect of the mucosal Na+ concentration on the mucosal relative permeability coefficient (see text). The relative permeability coefficient has been calculated from the voltage changes of Fig. 3. A line has been drawn through the K+ points. The standard errors (vertical lines) of the Cs+ points are also representative of K+ and have been calculated from the variance computed from the expression:

$$\sigma^2_K = \left(\frac{\partial K}{\partial \Delta v}\right)^2 \sigma^2_{\Delta v}$$

Where Δv is the voltage change which accompanied the change in mucosal solution composition.

However, it is pertinent to note that the square root relationship will also approximate rate-limited diffusion kinetics. Thus the potential measurements in this report and evidence available in the literature are unable to distinguish between the two interpretations: the one based on equation (2) which postulates a unique permeability to Na+ (the square root or long pore effect), and the other based on equation (5) which proposes a permeability coefficient that

decreases at higher concentrations of Na^+. It was of interest to see whether the conductance data would distinguish between these interpretations.

Equations (2) and (3) in the Appendix were derived without any assumptions concerning ionic or electrical gradients within the membrane. To integrate the Nernst-Planck expression, it is necessary to know or assume a relationship between the concentration or electrical potential gradient and the thickness of the membrane. The Goldman assumption, *viz.*, a constant electrical gradient, has been applied to biological membranes (20). This approach

TABLE IV

THE SEROSAL RELATIVE PERMEABILITY COEFFICIENT

Solution K^+ (mEq/liter)Na^+		$K_s = \dfrac{u_{K^+}}{u_{Na^+}}$ mean ($\pm$SE*)
2	113.5	26 ($\pm$3)
5.5	110	17 ($\pm$2)
15.5	100	8.4 ($\pm$1.3)
35.5	80	8.6 ($\pm$2.4)
55.5	60	5.0 ($\pm$0.8)
75.5	40	6.0 ($\pm$2.4)
95.5	20	8.3 ($\pm$4.7)
113.5	2	

* The standard error of the mean was calculated from the variance computed from the equation:

$$\sigma^2_K = \left(\frac{\partial K}{\partial \Delta v}\right)^2 \sigma^2_{\Delta v}$$

where Δv is the voltage change which accompanied the change in serosal solution composition.

predicts that the DC conductance of a membrane, at constant ionic composition, is a function of the voltage across the membrane. However, the data in Fig. 5 indicate that over a wide voltage range the conductance is independent of voltage. On the other hand, the Henderson assumption, *viz.*, constant concentration gradient (equation (10) in the Appendix), predicts that conductance is independent of voltage at constant ionic composition. Furthermore, when the mucosal Na^+ concentration was changed, the conductance changes conformed to equation (10) if the square root of the Na^+ concentration was used (Fig. 6). The tri- and tetraethylammonium experiments deviated from the Henderson relation at Na^+ concentrations in excess of 60 mEq/liter.

Conductance changes which result from the replacement of serosal Na^+ by K^+ (Fig. 8) also conform to equation (10) if the same assumptions are

made as in the interpretations of the potential studies; *i.e.*, equal mobilities of Na^+ and K^+ and the square root relationship for Na^+.

Thus the interpretation of the potential data in terms of the square root assumption also provides an excellent description of the conductance data. The variable permeability coefficient approach does not provide an alternative interpretation of the conductance. When the K values of Fig. 9 were substituted in equation (10), the results showed no coherent relation to the observed conductance.

The Koefoed-Johnson and Ussing model of the epithelial cell (1) on which the present interpretation is based postulates two membranes in series; the mucosal- and serosal-facing membranes of the epithelial cell. Electrical leaks due to ion diffusion between the cells are regarded as negligible. Indeed, there is excellent electron microscopic evidence for close packing of the cells (21). The reversible potential changes are compatible with this model, but the conductance changes indicate the presence of at least two membranes in parallel. If the straight lines of Fig. 6 are extrapolated to zero mucosal Na^+ concentration (*i.e.*, zero on the abscissa), the intercept on the ordinate indicates the presence of residual conductance. Similarly, extrapolation of the line of Fig. 8 indicates a conductance even in the absence of Na^+ and K^+ in the serosal solution. Furthermore, the conductance data in complying with equation (10) indicate the absence of membranes in series. For example, the mucosal substitution data will obey equation (10) if the conductance of the serosal membrane (G_s) is very much greater than that of the mucosal-facing membrane (G_m).

For two membranes in series the total conductance (G_t) is given by:

$$G_t = \frac{G_m G_s}{G_m + G_s}$$

so that $G_t = G_m$ when $G_s \gg G_m$. Similarly, the serosal substitution data indicate that if a mucosal membrane is in series with the serosal barrier, its conductance must be very much greater. This apparently self-contradictory situation may be resolved in terms of the cell model depicted in Fig. 10.

The epithelial layer consists of two types of cells in parallel: type A has a mucosal membrane conductance (G_{mh}) which is very much greater than that of type $B(G_{ml})$ and also greater than the serosal conductance (G_s). The various conductances are related by the expression:

$$G_{mh} \gg G_s \gg G_{ml} \tag{a}$$

The total conductance (G_A) of cell A is determined by its serosal membrane conductance (G_s) and is independent of the mucosal Na^+ concentration. Similarly, the conductance (G_B) of cell B is determined by the conductance of

its mucosal membrane and is independent of the cation concentration of the serosal solution. G_B is effectively zero when the mucosal Na^+ concentration is zero. Thus an average intercept (Y_m) estimated from Fig. 6 is equal to G_A. If f is the fraction of cells of type A, it follows that:

$$Y_m = f\, G_s \qquad\qquad (b)$$

Similarly, the intercept for serosal substitution (Fig. 8) is related to G_{ml} as:

$$Y_s = (1 - f)\, G_{ml} \qquad\qquad (c)$$
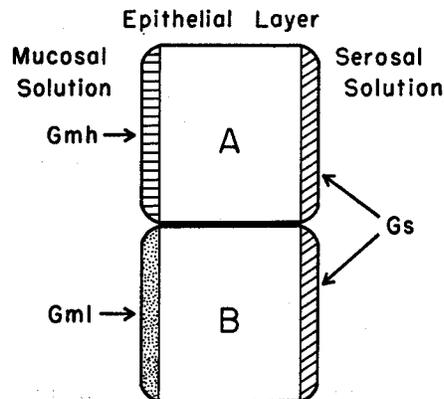
FIGURE 10. A model of the epithelial cell layer of the toad bladder (see text).

Since Y_s and Y_m are approximately equal, (b) and (c) can be equated to give:

$$\frac{1}{f} = \frac{G_s}{G_{ml}} + 1 \qquad\qquad (d)$$

and since $G_s \gg G_{ml}$, f must be very much less than one. In other words the fraction of cells of relatively high mucosal permeability is small. It is interesting to note that at least three morphologically distinct cell types have been observed in the bladder epithelial layer (17, 21). In addition, the serosal barrier has been reported to be more permeable than the mucosal barrier to lactate (22) and water (23). This model of toad bladder is also compatible with the series relationship derived from the potential studies providing that the mucosal membranes of cells A and B remain selectively permeable to Na^+.

Irreversible Changes

An adequate explanation of the irreversible drop in transtissue potential associated with mucosal Li^+ substitution and the replacement of serosal Na^+ by Li^+, Rb^+, Cs^+, or $(C_2H_5)_3NH^+$ awaits further study. The effect of Li^+ may be related to the inhibition of active Na^+ transported described by

Zerahn (24) for the isolated frog skin. The proximity of the active ion transport mechanism to the serosal membrane may be related to the intolerance of the tissue to the serosal substitution of all cations tested except K^+ and $(C_2H_5)_4N^+$.

Appendix

In the development of the equations which describe the electrical data obtained from the toad bladder, the tissue is regarded as a single continuous layer of epithelial cells. The solutions bathing each surface of the bladder are separated by two cell membranes in series; *viz.*, the mucosal- and serosal-facing membranes of the epithelial cell.

In general, the voltage across a biological membrane consists of three components; the electrical potential change due to Donnan equilibriums at the two surfaces of the membrane and an internal diffusion potential (25). The conditions in the experiments of this report were such that the internal composition of the cell remained constant and the ionic strength of the solutions bathing the mucosal and serosal surfaces of the bladder was unchanged. Thus the Donnan potentials cancel each other. Equations describing the internal diffusion potential were derived from the Nernst-Planck equation:

$$j_i = -z_i u_i RT \left[\frac{dC_i}{dx} + \frac{z_i FC_i}{RT} \frac{dE}{dx} \right] \tag{1}$$

where j_i is the current carried across the membrane by an ion i, and the remaining symbols have their usual significance. The activity coefficients are assumed to be constant. When a single mobile ion is present and there is no external current source, equation (1) may be integrated across the membrane to give:

$$E_2 - E_1 = \frac{RT}{z_i F} \ln (C_{i2}/C_{i1}) \tag{2}$$

where C_{i1} and C_{i2} are the respective concentrations of the ith ion at the two surfaces of the membrane. In the experiments reported here two mobile cations were present, Na^+ and a substituent cation r^+. Sulfate, the predominant anion, has practically zero mobility across the cell membrane. Thus the current flows j_{Na} and j_r are given by equation (1):

$$j_{Na} = -z_{Na} u_{Na} RT \left[\frac{dC_{Na}}{dx} + \frac{z_{Na} FC_{Na}}{RT} \frac{dE}{dx} \right] \tag{3}$$

$$j_r = -z_r u_r RT \left[\frac{dC_r}{dx} + \frac{z_r FC_r}{RT} \frac{dE}{dx} \right] \tag{4}$$

At zero applied current $j_{Na} + j_r = 0$, and if $z_{Na} = z_r = 1$, it follows from equations (3) and (4) that:

$$\frac{d}{dx} (u_{Na} C_{Na} + u_r C_r) + \frac{F}{RT} (u_{Na} C_{Na} + u_r C_r) \frac{dE}{dx} = 0$$

which may be rewritten as:

$$\frac{d \ln (u_{Na}C_{Na} + u_r C_r)}{dx} + \frac{F}{RT}\frac{dE}{dx} = 0$$

On integration across the membrane:

$$E_2 - E_1 = \frac{RT}{F}\ln\left[\frac{u_{Na}C_{Na2} + u_r C_{r2}}{u_{Na}C_{Na1} + u_r C_{r1}}\right]$$

or

$$E_2 - E_1 = \frac{RT}{F}\ln\left[\frac{C_{Na2} + KC_{r2}}{C_{Na1} + KC_{r1}}\right] \cdots \tag{5}$$

where $K = u_r/u_{Na}$. Usually it is assumed that the ion concentrations at the cell surface are proportional to the ion concentrations in the bathing solutions. However, as pointed out by Teorell (26) it is possible to introduce any ratio provided it can be defined or experimentally justified. In order to integrate the Nernst-Planck equation in the presence of an applied current, it is assumed that the ion concentration gradients in the membrane are linear, *i.e.*,

$$C_i = b_i x + C_{i1} \tag{6}$$

where C_i is the concentration of the ith ion at any point x in the membrane, b_i is a constant, and C_{i1} the ion concentration at surface 1. Differentiating equation (6) with respect to x and substituting in equation (1) we obtain:

$$j_i = -z_i u_i RT\left[b_i + \frac{z_i F}{RT}(b_i x + C_{i1})\frac{dE}{dx}\right] \cdots \tag{7}$$

The total applied current j_t is the sum of the individual current flows:

$$j_t = \Sigma j_i \tag{8}$$

For mobile cations of charge $z_i = 1$, we obtain from (7) and (8):

$$j_t = -RT\left[\Sigma u_i b_i + \frac{F}{RT}[\Sigma u_i(b_i x + C_{i1})]\frac{dE}{dx}\right]$$

which on rearrangement gives:

$$F\,dE + \frac{(RT\Sigma u_i b_i + j_t)dx}{\Sigma(u_i b_i x + u_i C_{i1})} = 0$$

Since

$$b_i = \frac{C_{i2} - C_{i1}}{a}$$

where a = the thickness of the membrane, then on integration across the membrane:

$$F(E_2 - E_1) + \frac{\left[RT\Sigma u_i\left(\dfrac{C_{i2} - C_{i1}}{a}\right) + j_t\right]}{\Sigma u_i\left(\dfrac{C_{i2} - C_{i1}}{a}\right)} \ln\left[\frac{\Sigma u_i C_{i2}}{\Sigma u_i C_{i1}}\right] = 0$$

or

$$j_t = -\left[F(E_2 - E_1)\Sigma u_i\left(\frac{C_{i2} - C_{i1}}{a}\right)\middle/ \ln\left[\frac{\Sigma u_i C_{i2}}{\Sigma u_i C_{i1}}\right]\right] - RT\Sigma u_i\left(\frac{C_{i2} - C_{i1}}{a}\right) \quad (9)$$

The conductance (G), defined as dj_t/dV where $V = E_2 - E_1$, is obtained by differentiation of equation (9):

$$G = \frac{dj_t}{dV} = \frac{F}{a}\,\Sigma u_i(C_{i2} - C_{i1})/\ln\left[\frac{\Sigma u_i C_{i2}}{\Sigma u_i C_{i1}}\right] \quad (10)$$

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