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The Sodium Transport Pool in Toad Urinary Bladder Epithelial Cells

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Summary. The sodium which equilibrates with ^{24}Na in epithelial cells of toad urinary bladders has been determined. With sodium Ringer's bathing both mucosal and serosal surfaces, ^{24}Na in the mucosal medium equilibrated with about 35 mmoles cellular sodium/kg cellular dry weight, representing about 20% of the total cellular sodium determined flame photometrically; ^{24}Na in the serosal medium equilibrated with 120 mmoles cellular sodium/kg cellular dry weight, about 80% of the total cellular sodium. With ^{24}Na in both media all cellular sodium was labeled within 30 min. In the absence of serosal sodium, total cellular sodium and that sodium which equilibrated with mucosal ^{24}Na in sodium Ringer's were both similar to the cellular sodium of mucosal origin which had been determined in epithelial cells exposed on both surfaces to sodium Ringer's. Sodium-free mucosal medium, and sodium Ringer's containing amiloride 10^{-4} or 10^{-3} M in the mucosal medium, both virtually completely inhibited transepithelial sodium transport. But, whereas the cellular sodium of mucosal origin fell to only 2 mmoles/kg cellular dry weight with sodium-free mucosal medium, an appreciable labeling of cellular sodium was found whether amiloride was present before, or only after, exposure of tissue to mucosal ^{24}Na. Rapid washing of the mucosal surface of hemibladders just before removal of epithelial cells for analysis removed most of this sodium labeled in the presence of amiloride, suggesting that the cellular sodium of mucosal origin consists of at least two fractions with only about two-thirds truly intracellular. The sodium transport pool measured directly in these experiments is appreciably smaller than any previous estimates of pool size all of which have been obtained by indirect techniques involving use of whole hemibladders rather than epithelial cells alone.

The urinary bladder of the toad *Bufo marinus* has proved a useful tissue in which to examine transepithelial transport of various solutes and water as well as the modifications of transport produced by two important mammalian hormones, vasopressin and aldosterone (Leaf, 1965; Sharp & Leaf,

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1966). To delineate the steps involved in the active transepithelial transport of sodium, it is important to know the quantity of sodium in the epithelial cells which is awaiting transport from the cells to the serosal fluid – the so-called active transport pool of sodium. Unfortunately, the size of this pool remains controversial. With the layer of transporting epithelial cells supported on a submucosa containing connective tissue, bundles of smooth muscle, capillaries and a serosal layer, analyses of whole tissue composition must include the contents of all the supporting tissue as well as the epithelial cells themselves. Similarly, the interpretation of experiments designed to determine the size of the epithelial cell active transport pool through the use of radioactive isotopes of sodium is complicated by the presence of the submucosal tissues (Leaf, 1966), whether tissue analyses or kinetic "wash-out" measurements are employed.

It has recently proved possible to develop a method by which epithelial cells can be removed from hemibladders and subsequently analyzed for their water and ion contents, after the bladders have been mounted in chambers and short-circuited (Macknight, DiBona, Leaf & Civan, 1971). The present studies examine relationships between total intracellular sodium and intracellular sodium of mucosal origin in an attempt to characterize more precisely the sodium transport pool in toad bladder epithelial cells.

Materials and Methods

The sodium Ringer's solution contained (mM): 117 Na^+, 3.5 K^+, 1.0 Ca^{2+}, 117 Cl^-, and 10 glucose, buffered at pH 7.8 by 2 mM HPO^{2-}. Sodium-free choline Ringer's solution contained (mM): 117 choline, 4.0 K^+, 1.0 Ca^{2+}, 117 Cl^-, and 10 glucose, buffered at pH 7.8 by 2 mM HPO^{2-}.

Inulin-carboxyl-^{14}C and inulin-methoxy-^{3}H were obtained from New England Nuclear Corporation, and ^{24}Na from the Australian Atomic Energy Commission, Lucas Heights, New South Wales. Amiloride was the gift of Merk, Sharp and Dohme.

Toads of the species *Bufo marinus*, obtained from the Dominican Republic (National Reagents, Bridgeport, Conn.) were doubly pithed and their hearts immediately perfused with sodium Ringer's to deblood the bladders.

Paired hemibladders were mounted in chambers of area 8.04 cm^2. Initially, both mucosal and serosal surfaces were bathed with sodium Ringer's and the open-circuit potential difference recorded. They were then continuously short-circuited. Air was bubbled through both mucosal and serosal solutions throughout the experiments.

Once the short-circuit current (SCC) had stabilized, the chambers were drained and refilled with the appropriate solutions as indicated. Whenever it was desired to bathe the hemibladders with sodium-free solutions, the appropriate chambers were drained, then filled and drained five times using fresh choline Ringer's before filling with choline Ringer's. This procedure allowed washout of sodium from the tissue and prevented any appreciable concentration of sodium in the final solution during the course of the experiment. The sodium contents of these solutions were checked by flame photometry

at the end of each experiment. Both the mucosal and serosal solutions contained radioactive inulin for at least 60 min before the end of each experiment. Since there is no measurable flux of inulin across the bladder, the use of ^{3}H-inulin in one solution and ^{14}C inulin in the other allowed separate correction of tissue composition for any mucosal and serosal extracellular fluid in the sample.

At the conclusion of each experiment, the chambers were drained, the portion of the hemibladder exposed in the chambers removed, and blotted several times on Whatman filter paper no. 542 until no visible moisture was transferred to the paper. It was then placed mucosal surface up on a Pyrex petri dish and the epithelial cells were removed by scraping with a glass slide. It has been shown that this procedure removes over 90% of the epithelial cells from the underlying tissue without contamination by other tissue elements (Macknight *et al.*, 1971). The epithelial cell scrapings were then transferred to tared Pyrex tubes.

Scrapings were weighed, dried to constant weight at 105 °C in a hot air oven, cooled at room temperature for 40 min, and reweighed. The water content of the scraped tissue was taken to be equal to the loss of weight upon drying. In experiments with ^{24}Na, epithelial cell scrapings were first weighed, then tissue and samples of medium were counted in a Packard Gamma counter. Scrapings were then dried as above.

In all experiments, tissue was extracted for 7–10 days in 10 ml of 0.1 M nitric acid. Sodium and potassium were measured with an EEL flame photometer and chloride with a Cotlove titrator.

Inulin-^{14}C and inulin-^{3}H were determined by adding 15 ml scintillation solution to 2 ml tissue extract neutralized with 0.2 ml of 1 M sodium hydroxide. The samples were then counted in a three-channel Packard Tri-Carb Liquid Scintillation counter. Samples of medium, 0.1 ml, were diluted by the addition of 3 ml 0.1 M nitric acid and 2 ml of this was then neutralized and counted in the same way. Samples from experiments with ^{24}Na were counted after that isotope had completely decayed.

The tissue values of water and ions were corrected for contamination with extracellular fluid using the assumption that isotopically labeled inulin equilibrated in the extracellular space and that the ions in this space were at the same concentration as in the bulk of the medium. Recent work (McIver & Macknight, 1974) confirms that inulin is a suitable extracellular marker for toad bladder epithelial cells under these experimental conditions.

The intracellular water content is expressed as kg H_2O/kg dry weight. The ion contents of the cells are shown in mmoles/kg dry weight. Their concentrations in mmoles/kg intracellular water can be derived by dividing the ion content by the cellular water content.

The intracellular Na content determined by ^{24}Na was calculated in the following way:

$$\text{I.C. Na content} = \frac{(\text{Intracellular counts})(Na_m) \times 10^3}{(\text{Medium counts})(\text{Tissue dry wt})}$$

where I.C. Na content = mmoles of sodium/kg tissue dry weight from ^{24}Na, Na_m = μmoles of sodium/ml of mucosal medium, intracellular counts = total tissue counts/minute corrected for ^{24}Na in inulin space of the mucosal and serosal surfaces, medium counts = counts/min/ml mucosal medium, and tissue dry wt = tissue dry weight in mg.

Values presented in text and Tables represent the mean ± SEM where appropriate, of the number of observations shown. Means of paired analyses are presented with their difference and its SE. Significances of differences between means have been evaluated using Student's *t* test.

Results

Comparison of Labeling of Intracellular Sodium from Mucosal and Serosal Media

The mean sodium content of the scraped mucosal cells was found to be 161 ± 4 mmoles ($n = 64$) per kg dry solids with a concentration of 48 ± 1 mmoles/liter of intracellular water. The question arises as to how much of this sodium exchanges with radioactive sodium placed on the mucosal and serosal surfaces, respectively. To examine this question, paired hemibladders were mounted in chambers and bathed on both their surfaces by sodium Ringer's. One hemibladder was exposed on its mucosal surface to ^{24}Na, the other hemibladder had ^{24}Na in the serosal medium. After 60 min bladders were removed, blotted and the epithelial cells scraped for analysis.

Table 1 shows the results of these measurements. The equality of water, potassium, chloride and sodium contents of the paired tissues have been previously documented (Macknight *et al.*, 1971). This allows comparison of the isotopic labeling of the sodium content from mucosal medium in one set of observations with the isotopic labeling from the serosal side in the paired tissue. Radioactive sodium placed in the mucosal medium labeled $23.2 \pm 1.5\%$ of the intracellular sodium content while from the serosal side $78 \pm 4.3\%$ was labeled. The sum of the two indicates that all intracellular sodium exchanges with sodium in the bathing media after 1 hr of incubation under these conditions.

To determine if a steady-state distribution of sodium occurs in less than 60 min paired tissues were immersed in sodium Ringer's solution containing

Table 1. Comparison of total cellular sodium with amount derived from mucosal and serosal media

Medium with ^{24}Na	Cellular sodium		
	Total (mmoles/kg dry wt)	Isotopic	%
Mucosal	162 ± 7	37 ± 3	23 ± 1.5
Serosal	155 ± 13	122 ± 15	78 ± 4.3
$n = 8$			

Paired hemibladders were intially incubated with sodium Ringer's on both mucosal and serosal surfaces. Once SCC stabilized, chambers were drained. One hemibladder was bathed on its mucosal surface with sodium Ringer's + ^{24}Na + ^{14}C-inulin and on its serosal surface with sodium Ringer's + ^{3}H-inulin. The paired hemibladder was bathed on its mucosal surface with sodium Ringer's + ^{3}H-inulin and on its serosal surface with sodium Ringer's + ^{24}Na + ^{14}C-inulin. After a further 60 min both hemibladders were removed from the chambers, blotted, and the epithelial cells scraped off and collected for analysis.

^{24}Na and ^{14}C-inulin. One hemibladder was removed at 30 min and another at 60 min. The mucosal cells were scraped off and analyzed chemically and isotopically for their sodium content. At 30 min $98 \pm 2\%$ of total intracellular sodium had a specific activity equal to that of the medium and at 60 min the corresponding value was $102 \pm 2\%$. Thus a steady state of tissue labeling with ^{24}Na occurs within 30 min of immersion of the tissue in the sodium Ringer's solution.

Effect of Sodium-Free Mucosal Medium on Cellular Sodium

If the cellular sodium which has equilibrated with ^{24}Na from the mucosal medium provides a meaningful estimate of the size of the sodium transport pool, then removal of sodium from the mucosal medium should cause not only a loss of isotopically-labeled sodium from the cells but also a comparable loss of total intracellular sodium as determined flame photometrically, together with chloride and, if these ions are osmotically active in free solution within the epithelial cells, water. Results of experiments designed to examine this hypothesis are presented in Table 2, together with a summary of the protocol. Table 2A shows that, as predicted, when sodium is removed from the mucosal medium the cells lose equivalent quantities of isotopically labeled sodium, and sodium measured flame photometrically, together with chloride. That this loss is not the consequence only of diffusion of sodium from the cells to the sodium-free mucosal medium is suggested by the fact (Table 2B) that the presence of amiloride, 10^{-4} M, in the sodium-free mucosal medium did not modify the results though it might be expected to markedly inhibit sodium movements across the mucosal cell membrane in both directions as it does in frog skin (Morel & Leblanc, 1973). (At a concentration of 10^{-4} M, amiloride in the mucosal medium with sodium Ringer's virtually abolishes SCC within 5 min of its addition.) Table 2C presents the pooled data from both experiments. Clearly the cells have lost the same amounts of isotopically labeled and flame photometrically measured sodium, with a comparable quantity of chloride. The very small residual sodium content equilibrated with ^{24}Na probably reflects the fact that the mean mucosal sodium concentration in the mucosal medium at the end of the experiment was 0.71 ± 0.03 mM, not zero. Though both the absence of sodium from the mucosal solution (Table 2A) and the presence, in addition, of 10^{-4} M amiloride (Table 2B) were associated with the same small loss of water, in neither experiment was this loss statistically significant. Nor was it significant when the data were combined. However it is somewhat less than the loss of water of 0.29 kg H_2O/kg dry wt which would

Table 2. Effects of sodium-free mucosal media on epithelial cell composition

	H_2O (kg/kg dry wt)	Na (mmoles/kg dry wt)	^{24}Na	K	Cl
A. Choline Ringer's mucosa; sodium Ringer's serosa					
Control	3.04	174	38	403	219
Sodium-free mucosa	2.91	140	2	413	195
Δ	-0.13 ± 0.18	-34 ± 11	-36 ± 3	$+10 \pm 9$	-24 ± 11
p	>0.40	<0.02	<0.001	>0.20	<0.05
n = 8					
B. Choline Ringer's + amiloride, 10^{-4} M, mucosa; sodium Ringer's serosa					
Control	3.09	172	36	477	226
Sodium-free + amiloride mucosa	2.85	140	2	463	194
Δ	-0.24 ± 0.12	-32 ± 11	-34 ± 4	-14 ± 15	-32 ± 3
p	>0.10	<0.05	<0.001	>0.30	<0.001
n = 6					
C. Pooled results: sodium-free mucosa; sodium Ringer's serosa					
Control	3.06	173	37	431	222
Sodium-free mucosa	2.88	140	2	432	195
Δ	-0.18 ± 0.11	-33 ± 7	-35 ± 2	$+1 \pm 9$	-27 ± 7
p	>0.10	<0.001	<0.001	>0.90	<0.005
n = 14					

Paired hemibladders were initially incubated with sodium Ringer's on both mucosal and serosal surfaces. Once SCC stabilized, chambers were drained and refilled with sodium Ringer's + ^{24}Na + ^{14}C-inulin mucosa, sodium Ringer's serosa. After 40 min all chambers except the mucosal chamber of the control hemibladder were drained. The serosal chamber of the control hemibladder was refilled with sodium Ringer's + ^{3}H-inulin. The mucosal and serosal chambers of the experimental hemibladder were washed five times by rapidly filling and immediately draining, repeating this procedure five times. The mucosal washing solution was either choline Ringer's (part A) or choline Ringer's + amiloride, 10^{-4} M (part B); the serosal washing solution was sodium Ringer's. The chambers were then refilled with either choline Ringer's + ^{14}C-inulin mucosa, sodium Ringer's + ^{3}H inulin serosa (part A) or choline Ringer's + amiloride, 10^{-4} M + ^{14}C-inulin mucosa, sodium Ringer's + ^{3}H-inulin serosa. After a further 60 min both hemibladders were removed from the chambers, blotted, and the epithelial cells scraped off and collected for analysis.

be predicted given a sodium loss of 34 mmoles/kg dry wt when sodium-free solution bathes the mucosal surface, and the assumption that such a loss was of osmotically active intracellular sodium and chloride. Too much should not be made of this discrepancy, since the difference between a mean loss of 0.18 kg H_2O/kg dry wt and the theoretically predicted 0.29 kg H_2O/kg dry wt probably lies within the errors inherent in the calculations of intracellular water. However, evidence will be provided later which sug-

Table 3. Epithelial cell sodium equilibrated with serosal ^{24}Na in the absence of mucosal sodium

	H_2O (kg/kg dry wt)	Na (mmoles/kg dry wt)	^{24}Na	K	Cl
After 60 min	3.40	138	141	490	255
After 120 min	3.33	118	124	480	251
Δ	-0.07 ± 0.19	-20 ± 16	-17 ± 21	-10 ± 36	-4 ± 11
p	>0.70	>0.20	>0.40	>0.70	>0.70

Paired hemibladders were initially incubated with sodium Ringer's both on mucosal and serosal surfaces. Once SCC stabilized, chambers were drained. The mucosal surfaces of both hemibladders were rapidly washed five times with choline Ringer's. They were then filled with choline Ringer's + ^{3}H-inulin mucosa, sodium Ringer's + ^{24}Na + ^{14}C-inulin serosa. After a further 60 min, one hemibladder was taken for scraping and analysis of the epithelial cells; the other hemibladder was removed after 120 min.

gests that perhaps 10–15 mmoles of the sodium within the cellular transport pool may not be truly intracellular. Thus only perhaps 20 mmoles Na/kg dry wt is intracellular and contributing to cellular osmotic pressure and this would represent an isoosmotic solution with 0.18 kg H_2O/kg dry wt.

Table 1 shows that when sodium was available to the epithelial cells from both mucosal and serosal solutions about 78% of the total cellular sodium had equilibrated with the serosal sodium. In the absence of mucosal sodium one would predict that all the remaining cellular sodium should have equilibrated with serosal sodium. Table 3 shows that within 60 min of removal of mucosal sodium all the cellular sodium measured flame photometrically had equilibrated with ^{24}Na in the serosal medium and that no significant changes in cellular composition occurred over a further 60-min incubation.

Effects of Amiloride on Cellular Sodium

Amiloride blocks sodium entry to the cells from the mucosal medium (Bentley, 1968), and, at a concentration of 10^{-4} M, produces a rapid (less than 5 min) inhibition of more than 99% of the active transepithelial sodium transport as judged by the fall in SCC. At this concentration, therefore, the presence of amiloride in sodium Ringer's bathing the mucosal surface of the cells should mimic the effects on cellular composition of removal of sodium from the mucosal medium. (In some experiments amiloride was also used at a concentration of 10^{-3} M. This 10-fold increase in concentration had no detectable effect on any of the results.)

Table 4. Effects of amiloride, 10^{-4} M, on epithelial cell composition

	H_2O (kg/kg dry wt)	Na (mmoles/kg dry wt)	^{24}Na	K	Cl
A. Cells equilibrated with ^{24}Na before addition of amiloride					
Control	3.54	142	38	480	203
+Amiloride	3.51	110	16	489	172
Δ	-0.03 ± 0.11	-33 ± 14	-22 ± 6	9 ± 9	-31 ± 9
p	>0.70	<0.06	<0.02	>0.30	<0.02
n=7					
B. Cells exposed to ^{24}Na after addition of amiloride					
	3.80 ± 0.12	156 ± 11	18 ± 2	462 ± 6	208 ± 8
n=8					

Paired hemibladders were initially incubated with sodium Ringer's on both mucosal and serosal surfaces. Once SCC stabilized, chambers were drained. Results shown in Table 4A were obtained after hemibladders were first exposed to sodium Ringer's + ^{24}Na + ^{14}C-inulin mucosa, sodium Ringer's serosa. After 40 min chambers were drained. The chambers of the control hemibladder were refilled with sodium Ringer's + ^{24}Na + ^{14}C-inulin mucosa, sodium Ringer's + ^{3}H-inulin serosa, the chambers of the experimental hemibladder with sodium Ringer's + ^{24}Na + amiloride, 10^{-4} M, + ^{14}C-inulin mucosa, sodium Ringer's + ^{3}H-inulin serosa. After a further 60 min both hemibladders were removed from the chambers, blotted, and the epithelial cells scraped off and collected for analysis. In separate experiments summarized in Table 4B, hemibladders were first exposed to sodium Ringer's + amiloride, 10^{-4} M mucosa, sodium Ringer's serosa. After 40 min, chambers were drained and refilled with sodium Ringer's + ^{24}Na + amiloride, 10^{-4} M + ^{14}C-inulin mucosa, sodium Ringer's + ^{3}H-inulin serosa. After a further 60 min hemibladders were removed from the chambers, blotted, and the epithelial cells scraped off and collected for analysis.

Table 4A shows the results of a series of experiments in which, after a 40-min equilibration with ^{24}Na in the mucosal sodium Ringer's, transepithelial transport was inhibited by 10^{-4} M amiloride. After a 60-min exposure to amiloride the epithelial cells had lost a significant amount of the sodium equilibrated with ^{24}Na but, unlike cells exposed to a sodium-free mucosal solution, they still retained an appreciable quantity of such sodium. A similar quantity of sodium equilibrated with ^{24}Na was also found in tissues exposed on their mucosal surfaces to ^{24}Na and amiloride only after initial incubation with 10^{-4} M amiloride (Table 4B) which was expected to prevent equilibration of any ^{24}Na with cellular sodium.

These findings appeared to conflict with the results obtained when sodium was removed from the mucosal medium. Both the absence of mucosal sodium and the presence of amiloride, 10^{-4} M, in mucosal sodium Ringer's produced virtually complete inhibition of transepithelial sodium transport. This, together with the finding that the same fraction of sodium equilibrated

Table 5. Effects of washing of mucosal surface of hemibladders exposed to mucosal ^{24}Na, just before analysis of cellular ^{24}Na

	^{24}Na content (mmoles/kg dry wt)
Sodium Ringer's (n = 22)	35 ± 2
Sodium Ringer's washed (n = 22)	20 ± 2
Sodium Ringer's + amiloride (n = 22)	13 ± 1
Sodium Ringer's + amiloride, washed (n = 12)	3 ± 0.2

Experiments with unwashed hemibladders were performed following the standard protocol. Washed hemibladders were also incubated in the normal way. However, just before their removal from the chambers the serosal chambers were drained and remained unfilled. The mucosal chambers were drained, filled rapidly with nonradioactive solution, immediately drained, and this process was repeated five times before the hemibladders were removed from the chambers, blotted, and the epithelial cells scraped off and collected for analysis. Washing solutions were either choline Ringer's, choline Ringer's + amiloride, 10^{-4} or 10^{-3} M, or sodium Ringer's + amiloride 10^{-3} M.

with ^{24}Na whether amiloride was present before or only after exposure of the mucosal surface to ^{24}Na, suggested that the sodium which had equilibrated with ^{24}Na in the presence of amiloride was not in the intracellular sodium transport pool.

This possibility was investigated in experiments in which hemibladders exposed on their mucosal surfaces to sodium Ringer's containing ^{24}Na were rapidly washed (by draining both mucosal and serosal medium from the chambers and then filling the mucosal chamber rapidly with isotope-free solution, immediately draining, and repeating this procedure five times over not more than 60 sec) immediately before the hemibladders were removed, blotted, and the epithelial cells scraped off and collected for analysis. Washings were usually performed with solutions of choline Ringer's containing amiloride, 10^{-3} M. In one set of experiments in which ^{24}Na had equilibrated with cellular sodium the effects of washing with choline Ringer's with or without amiloride, 10^{-3} M were compared. Cells washed with choline Ringer's containing amiloride did lose slightly less of their labeled sodium but the difference, 4 ± 4 was not statistically significant ($p > 0.30$). Washing with sodium Ringer's containing amiloride, 10^{-3} M, had an effect no different from the effect of choline Ringer's with amiloride. In an additional series of experiments washing with sucrose + amiloride had similar effects.

The pooled results of these experiments are given in Table 5. The cells incubated with sodium Ringer's and ^{24}Na and those incubated with amiloride in addition, both lost comparable quantities of labeled sodium after rapid washing of the mucosal surface. But whereas the cells without amiloride

during incubation still retained 20 ± 2 mmoles of labeled sodium/kg dry wt, those incubated with amiloride had only 3.0 ± 0.2 mmoles labeled sodium/kg dry wt after washing. Thus, washing had removed almost all of the labeled sodium from the cells incubated with amiloride.

The problem remains to decide the origin of the sodium readily washed from the mucosal surface of the epithelial cells. Since amiloride at the concentrations used here rapidly inhibits transepithelial sodium transport by more than 99% it is unlikely that the sodium readily washed from the mucosal surface of cells which had been incubated with amiloride had come from an intracellular sodium transport pool. Nor is it likely that a large fraction of the sodium washed from the mucosal surface of cells incubated without amiloride came from the intracellular sodium transport pool, for the presence of amiloride in the washing media should have prevented such loss.

An alternative explanation might be that the sodium washed from the epithelial cells lay in an extracellular compartment which does not equilibrate with inulin. In our experiments the inulin space on the mucosal side normally lies between 2 and 6% of the total tissue water. It has been calculated that if all the ^{24}Na washed from the mucosal surface had been of extracellular origin then the extracellular space on the mucosal side would have to represent 15–20% of the tissue water. It is most unlikely that inulin could underestimate the size of the mucosal extracellular space to this extent for experiments in which this space was measured simultaneously with ^{3}H-mannitol and ^{14}C-inulin showed that both markers occupied virtually the same fraction of the tissue wet weight after 60 min of incubation (6.1 and 5.3% tissue wet wt, respectively, $\Delta\ 0.8 \pm 0.1$, $p < 0.02$) in spite of the large difference in molecular size.

It therefore seems unlikely that the sodium removed by washing was in an extracellular compartment inaccessible to inulin. One possibility which remains to be explored is that it was in some way associated with the outer, mucosal surface of the cell membrane, perhaps even related to the postulated carriers thought to be involved in sodium entry to the cells from the mucosal medium. Such a speculative interpretation would imply that amiloride inhibits transepithelial sodium transport by toad bladder epithelial cells not by blocking the initial association of sodium and carrier but at a subsequent step in the penetration to the cell interior.

Effects of Sodium-Free Serosal Medium on Cellular Sodium

The results presented show that the cellular sodium transport pool normally contains about 35–40 mmoles/kg dry wt of sodium of mucosal

Table 6. Epithelial cell sodium equilibrated with mucosal ^{24}Na in the absence of serosal sodium

	H_2O (kg/kg dry wt)	Na (mmoles/kg dry wt)	^{24}Na	K	Cl
After 45 min	3.23	43	35	382	253
After 120 min	3.00	41	35	373	229
Δ	-0.23 ± 0.12	-2 ± 5	0 ± 4	-9 ± 11	-24 ± 11
p	>0.10	>0.60	>0.90	>0.40	>0.05
n = 7					

Percentage change in SCC after choline Ringer's serosa
After 45 min: $-2.9 \pm 8.7\%$
After 120 min: $-27.6 \pm 5.5\%$

Final medium Na concentration (mM/liter)
Hemibladder removed at 45 min: 1.06 ± 0.12
Hemibladder removed at 120 min: 0.77 ± 0.07

Paired hemibladders were initially incubated with sodium Ringer's on both mucosal and serosal surfaces. Once SCC stabilized chambers were drained. The mucosal chambers were refilled with sodium Ringer's + ^{24}Na + ^{14}C-inulin. The serosal chambers were washed five times with choline Ringer's. They were then filled with either choline Ringer's + ^{3}H-inulin (hemibladder to be removed after 45-min incubation), or choline Ringer's. After 15 min both serosal chambers were drained, washed once with choline Ringer's, and refilled with either the choline Ringer's + ^{3}H-inulin, or choline Ringer's as before. After 45 min one hemibladder was taken for scraping and analysis of the epithelial cells. After 60 min the serosal chamber of the other hemibladder was drained, washed once with choline Ringer's, and refilled with choline Ringer's + ^{3}H-inulin. After a further 30 min the serosal chamber was drained, washed once with choline Ringer's, and refilled with choline Ringer's + ^{3}H-inulin. After 120 min this hemibladder was removed from the chamber, blotted, and the epithelial cells scraped off and collected for analysis. The washing ensured that no appreciable sodium or ^{24}Na accumulated in the serosal medium in these experiments in which there was a gradient for net sodium diffusion from the mucosal to the serosal medium.

origin under steady-state conditions with sodium Ringer's bathing both the mucosal and serosal surfaces. This represents about 20% of the total cellular sodium. One would predict that, in the absence of serosal sodium, the pool would remain of similar size, and experiments were therefore performed to examine this. Table 6 summarizes the results obtained when paired hemibladders were incubated for 45 or 120 min with sodium Ringer's + ^{24}Na bathing the mucosal surface and choline Ringer's the serosal surface, before the epithelial cells were removed for analysis.

The important point to emerge from these experiments was that there was a dramatic fall in cellular sodium so that, after 45-min incubation of hemibladders in an isoosmotic sodium-free serosal medium, the cellular

sodium content, measured flame photometrically was similar to that labeled by ^{24}Na in the mucosal medium. Both were comparable to the sodium of mucosal origin when sodium Ringer's bathed both surfaces (Tables 1 and 2). This sodium content was maintained over a further 75-min incubation. These results are therefore totally consistent with the conclusion that sodium of mucosal origin normally constitutes about 40 mmoles/kg dry wt.

Two other points deserve brief comment. First, since both cellular water and chloride contents were comparable to those found in hemibladders bathed on both surfaces by sodium Ringer's, choline must have entered the cells from the serosal medium to replace cellular sodium. Second, in the absence of serosal sodium there was probably some loss of cellular potassium, for the potassium contents found after 45 and 120 min of incubation, though similar to each other, were somewhat lower than those usually found when sodium Ringer's bathes the mucosal and serosal surfaces of hemibladders (control values in Tables 2 and 4).

Discussion

Before discussing the significance of the results, it is important to examine the validity of the technique used for the determination of the sodium transport pool in relation to total cellular sodium.

The Measurement of Serosal Extracellular Space

Some controversy has surrounded the appropriate choice of an extracellular marker for experiments with toad bladder epithelial cells. The finding that isotopes of sucrose occupied a larger fraction of the tissue water than did inulin (Al-Awqati, Leaf, Macknight & Civan, 1972) raised the possibility that sucrose was a more appropriate extracellular marker than inulin for this tissue. A fuller investigation of this problem, however, has resolved this diffuculty (McIver & Macknight, 1974). It was found (*i*) that sucrose was metabolized by toad bladder; it must therefore have entered cells as it does in mammalian kidney slices; (*ii*) that both ^{14}C- and ^{3}H-inulin occupied the same space; (*iii*) that ^{3}H-PEG 1000, though it entered cells in slices of mammalian liver and kidney, occupied the same space as inulin in preparations of toad bladder epithelial cells. It therefore appears that inulin provides as good an estimate as any of the size of the extracellular space in toad bladder epithelial cell preparations. The fact that horseradish peroxidase (mol wt 40,000) enters the lateral intercellular spaces in toad bladders (Masur, Holtzman, Schwartz & Walter, 1971) together with the fact that

the distribution of inulin (mol wt not greater than 6,000) in toad bladders reaches a steady state within 20 min (Finn, Handler & Orloff, 1966) suggest that inulin should distribute itself through all the accessible extracellular water, including that within the lateral intercellular spaces, by 60 min, the shortest time that hemibladders were exposed to inulin in these experiments.

Nevertheless, the finding that about 80% of the sodium in the noninulin water did not equilibrate with ^{24}Na in the mucosal medium might suggest that the contribution of extracellular sodium to the total sodium in the epithelial scrapings has been underestimated. In particular, could all of the sodium in the cells be equilibrated with the sodium of mucosal origin, the remaining sodium being not intracellular, but extracellular? It is possible, using representative values, to calculate the size that the extracellular space would have to be if all the sodium of serosal origin attributed to the cells were instead extracellular. The serosal space would have to be almost twice as large as that measured by inulin, before the calculated cellular sodium was solely sodium equilibrated with mucosal sodium. Even sucrose, which apparently enters cellular water, only measured a space about 20% greater than that occupied by inulin (McIver & Macknight, 1974). Indeed, the serosal extracellular space could not be so large or else the concentration of cellular potassium (derived by dividing the much smaller intracellular water content which is a consequence of the assumption of a greatly increased extracellular space, into the cellular potassium content which, because of the very low potassium concentration in the medium, is almost unaffected by any variation in extracellular space) would be about 230 mM/liter, much higher than that found in any other amphibian or mammalian cells. Using the noninulin water, the calculated concentration is approximately 140 mM/liter which is very similar to the concentration found in other amphibian and mammalian cells.

An alternative possibility, that sodium of serosal origin in the epithelial cell preparation is sodium bound in the extracellular space, cannot be excluded, though there is no evidence at present to support such a proposal.

Though it is possible that inulin does not faithfully reflect the extracellular space on the serosal side, it is clearly impossible that the space could be great enough to invalidate our conclusion that ^{24}Na in the mucosal medium does not equilibrate with all the cellular sodium.

The Concentration of Sodium in the Serosal Extracellular Space

A second criticism may be raised, that, although the volume of the serosal extracellular space is reasonably accurate, the concentration of

sodium in that space, which is used in correcting the total measured tissue sodium for extracellular sodium, is larger than that in the medium bathing the serosal surface of the hemibladders. It has been shown that tissues transporting isotonic extracellular fluid may indeed develop gradients of sodium concentration in their lateral intercellular spaces (Kaye, Wheeler, Whitlock & Lane, 1966; Diamond, 1971). It is possible that such gradients exist within lateral intercellular spaces in the toad bladder epithelial cells though they are unlikely to be as great as the 10 mM above the bathing solution concentration estimated by Machen and Diamond (1969) in the rabbit gallbladder, for the rate of sodium transport in this tissue (13 μmoles/cm^2 hr; Diamond, 1968) is much greater than that in the toad bladder (1.6 μmoles/cm^2 hr; Leaf, Anderson & Page, 1958). But, even if restricted regions of increased sodium concentration do exist, they cannot contribute any significant error to the cellular sodium content, for it can be calculated that the sodium concentration throughout all of the serosal extracellular water removed from the hemibladders with the epithelial cells themselves, and not just in the lateral intercellular spaces, would have to be 80 mM/liter greater than the concentration of 117 mM/liter in the bulk of the serosal extracellular fluid, for all the cellular sodium unlabeled by mucosal ^{24}Na to lie outside of the cells. This is obviously impossible.

The Effects of Accumulation of Mucosal ^{24}Na in the Serosa on the Labeling of Cellular Sodium

In experiments with ^{24}Na in the mucosal medium, when sodium transport was not inhibited, some ^{24}Na accumulated in the serosal medium. During the calculation of cellular sodium labeled with ^{24}Na the total tissue counts were corrected for the ^{24}Na present in the medium using the serosal inulin space and the assumption that ^{24}Na was at the same concentration in the serosal extracellular fluid as it was in the bulk of the serosal medium. This correction reduced the value by only about 5%. (Though the mucosal extracellular space is very much smaller than the serosal, 2–6% compared with 30–45%, the correction of the total tissue cpm for mucosal contamination, which was always done, has a very great effect on the calculated cellular sodium labeled with ^{24}Na, for the specific activity of ^{24}Na in this medium is of course very much greater.) The fact that the cpm in the serosal medium, even with ^{24}Na movement from the mucosa to the serosa, are much lower than in the mucosal medium (in two typical experiments the serosal counts at the end of 60 min averaged 0.51 ± 0.08% of the mucosal counts, $n = 16$) means that, though this serosal ^{24}Na may be labeling the cellular sodium

which equilibrates with the serosal sodium and which amounts to about 80% of cellular sodium, the contribution of this labeling to the cellular sodium counts corrected for extracellular ^{24}Na can be no more than 2 to 4%. Therefore, such labeling would lead to an error of no more than 5% in the calculated sodium pool – an error within the SEM of the values shown in the Tables.

Is the Sodium Labeled from the Mucosal Medium in the Cells?

One major criticism of experiments in which tissue ^{24}Na labeling in whole tissue has been used to estimate the size of the sodium transport pool is that much of the sodium actually counted may represent radioactive sodium which has already been transported by the epithelial cells (Leaf, 1966; Zehran, 1969), for in such experiments it has often been difficult to detect effects on pool size of procedures known to affect transepithelial sodium transport. This does not seem to represent a major problem in the present experiments where only the epithelial cells themselves, together with related extracellular fluid, are analyzed. Three lines of evidence support this conclusion: first, the excellent agreement between the losses of isotopically labeled and flame photometrically determined sodium when sodium-free medium bathed the mucosal surface of the cells (Table 2); second, the fact that it has proved possible in previous work using these techniques to dissociate changes in intracellular sodium, measured flame photometrically, from changes in transepithelial sodium transport (Macknight *et al.*, 1971); and third, the finding, reported in the following paper (Macknight, Civan & Leaf, 1975), that the presence of ouabain, 10^{-2} M, which virtually abolishes transepithelial transport, is associated with a marked increase in the size of the sodium pool equilibrated with mucosal ^{24}Na.

The results presented therefore cannot have been importantly distorted by problems with the technique. The experiments thus show that in the steady state, with sodium Ringer's bathing both the mucosal and serosal surfaces of toad bladder epithelial cells, it is possible to measure the quantity of cellular sodium of mucosal origin involved in transepithelial sodium transport. It seems likely that this pool, when it is derived by correcting the total tissue ^{24}Na counts in the scraped cells for ^{24}Na in the inulin space on the mucosal surface, is made up of two fractions, a smaller fraction which has not yet entered the epithelial cells, though it lies outside of the inulin space, and which can readily be washed from the cells, and a larger fraction, which is not found in cells exposed on their mucosal surface to amiloride and which is truly intracellular. Together these two fractions make up about 20% of the total cellular sodium. The size of the sodium pool measured

in this way varied as would be predicted when transepithelial transport of sodium was altered. Thus removal of sodium from the mucosal medium, or inhibition by amiloride of sodium entry to the cells both caused a very substantial loss of sodium from the transport pool. Alternatively as shown in the following paper (Macknight *et al.*, 1975), inhibition of sodium extrusion from the cells by ouabain was associated with a significant increase in the size of the sodium pool. It has also been established that the pool measured in this way increases significantly when transepithelial sodium transport is stimulated by vasopression (Macknight, Leaf & Civan, 1971) a finding which provides direct evidence that such stimulation is the result of an increased entry of sodium to the epithelial cells from the mucosal medium.

It is of considerable interest to compare the size of the sodium pool measured directly on the epithelial cells with some of the estimates of sodium pool size which have been made previously. In general, two techniques have been used to make such determinations. In one technique, the pool has been determined by loading hemibladders, or portions thereof, with radioactive sodium in the mucosal medium, under a variety of experimental conditions, and then counting whole tissue radioactivity with or without correction for any extracellular radioactivity. A knowledge of whole tissue radioactivity and of the specific activity of sodium in the mucosal medium then allows a calculation of the sodium in the tissue which has equilibrated with the medium sodium. This technique is in principle that used in these experiments, but with the crucial difference that the present results are derived from analysis of only the epithelial cells themselves (together with adherent extracellular fluid for which correction is made), and not from the analysis of the radioactivity in the whole tissue. Workers who have employed this type of approach with toad bladder include Frazier, Dempsey and Leaf (1962), Herrera (1966, 1968), Crabbé and de Weer (1969), and Gatzy (1971).

An alternative approach has been to load the tissues with radioactive sodium from the mucosal medium and then, once a steady state has been established with respect to transepithelial movement of isotope, to determine the rates at which radioactivity is lost from the tissues into both mucosal and serosal medium. This technique has recently been used by Finn and Rockoff (1971) who have developed methods for calculating from their washout data, not only the sodium transport pool but also the rate constants for, and unidirectional fluxes of, sodium across the mucosal and serosal membranes of the cell.

It is of importance to compare the results presented in this paper with those reported by others. To do this we have recalculated our results to obtain values in terms of total tissue in the following way.

Table 7. The relative contributions of epithelial cell scrapings and remaining tissue to the composition of toad hemibladders

	Epithelial scrapings	Remaining tissue	% Contribution of epithelial scrapings to total composition
Wet wt (mg)	13.7	73.3	15.8
Dry wt (mg)	2.28	19.1	10.7
Tissue H_2O (mg)	11.4	54.2	17.4
Extracellular H_2O (mg)	5.61	24.9	18.4
Intracellular H_2O (mg)	5.83	29.3	16.6
Tissue sodium (μmoles)	0.97	4.97	16.3
Tissue potassium (μmoles)	0.96	4.07	19.2
Tissue chloride (μmoles)	1.04	5.83	15.1
Cellular sodium (μmoles)	0.30	1.98	13.2
Cellular potassium (μmoles)	0.94	3.98	19.1
Cellular chloride (μmoles)	0.37	2.84	11.5

Values shown represent the means obtained from the analyses of 15 consecutive epithelial cell scrapings together with their remaining tissue.

To determine their relative contributions to whole tissue composition, both the epithelial scrapings and the remaining subepithelial tissue were analyzed. The means of 15 analyses are shown in Table 7. Since we have previously shown (Macknight *et al.*, 1971) that scraping removes about 95% of the epithelial cells and virtually none of the subepithelial components (deblooding by perfusion of the heart with sodium Ringer's before removal of the hemibladders ensures that the scrapings are not contaminated by erythrocytes which would be squeezed from the submucosal blood vessels), the values in the Table should reflect quite accurately the true relationship between epithelial cell composition and the composition of the total tissue. It is obvious from Table 7 that analysis of whole tissue may fail to reveal even quite large alterations in epithelial cell composition and that, alternatively, alterations in the composition of the subepithelial components may mask changes in the composition of the epithelial cells, for the epithelial cells provide only a small fraction of the total water and ions in the tissue. Having obtained the values shown in Table 7 it is now possible to calculate how much sodium of epithelial cell origin would be present per unit weight of whole tissue water or whole tissue dry weight and therefore to compare our results with those presented in the literature. Table 8 provides a summary of these. Results from whole tissue counting are not always strictly comparable. For example, Herrera (1968) presents results obtained after the mucosal

Table 8. Measurements of sodium transport pool in toad bladder epithelial cells

	Na transport pool	
	μmoles/g tissue H_2O	μmoles/g tissue dry wt
Calculated from whole tissue analysis		
A. From whole tissue counting		
Crabbé & de Weer, 1969	1–4	4–19
Frazier, Dempsey & Leaf, 1962	9.3	
Gatzy, 1971	6.6	
Herrera, 1966	12.5	
Herrera, 1968	7.4	
B. Computed from tissue washout		
Finn & Rockoff, 1971		105
C. Calculated from analysis of toad bladder epithelial cells, but expressed in terms of whole tissue [a]		
Total sodium in epithelial scrapings measured flame photometrically	14.8	45
Total cellular sodium measured flame photometrically but corrected for extracellular sodium	4.6	14
Cellular sodium equilibrated with mucosal ^{24}Na (assuming labeling of 20% cellular sodium)	1.0	2.8

[a] *See* text for methods of calculation.

surface was washed rapidly with sodium-free solution, Gatzy (1971) blotted the surface with moist filter paper before counting, Crabbé and de Weer (1969) corrected for mucosal contamination with inulin. However, in contrast to the normal concentration of sodium used by others, Crabbé and de Weer exposed tissue to only 23 mM sodium in the mucosal medium. Frazier, Dempsey and Leaf (1962) substituted choline Ringer's for the sodium Ringer's in the serosal medium. In spite of these variations in technique, however, all the results obtained with whole tissue analysis are reasonably comparable, and, when compared with the analysis of epithelial cells alone, actually measure as much or more sodium than the cells themselves could contain and appreciably more sodium than that which equilibrates with mucosal ^{24}Na. This analysis supports the contention that much of the sodium measured by this method has already been transported by the epithelial cells (Leaf, 1966).

Table 8 also contains values presented for the fast sodium pool by Finn and Rockoff (1971). (It is this pool which it is claimed is related to trans-

epithelial sodium transport.) This value, derived from a complex analysis of mucosal and serosal washout curves, is very much higher than that obtained by any direct method of analysis. It is 37.5 times higher than the actual sodium pool in the epithelial cells which has been directly measured in the present experiments, and, indeed, is actually more than twice as great as the total sodium per gram tissue dry wt measured flame photometrically in epithelial cell scrapings uncorrected for their extracellular sodium[1]. The size of the sodium transport pool thus computed can therefore not be correct and considerable caution should be exercised in accepting any conclusions based on this technique especially where such conclusions conflict with results obtained using more direct methods.

One major question which remains to be resolved is the relationship of the sodium of serosal origin to the transepithelial transport of sodium. The finding (Table 1) that about 80% of cellular sodium equilibrates with ^{24}Na in the serosal medium while about 20% equilibrates with ^{24}Na in the mucosal medium would be consistent either with all cellular sodium existing in a common pool, the rate of sodium entry to the pool being four times faster from the serosal medium, or, with the total cellular sodium existing in two relatively discrete pools. Three lines of evidence would be consistent with the second possibility. One is the observation that when sodium is absent from the serosal medium, as in the experiments summarized in Table 6, the short-circuit current does not rise, nor does the steady-state sodium pool decrease in size. If all cellular sodium occupied a single cellular pool with a much greater rate of entry of sodium from the serosal compartment to the pool, then removal of sodium should either increase the SCC as more transport sites became available to sodium of mucosal origin, or, alternatively, if the entry of sodium to the cells is the rate-limiting step in transepithelial sodium transport, the size of the sodium pool should decrease because of the availability of more transport sites within the cell. Second is the finding that ouabain inhibits metabolism of total hemibladders by a similar amount to the inhibition produced by removal of mucosal sodium (Coplon & Maffly, 1972). If four times more sodium were passing through the transport sites

1 An error in the estimate of the fraction of the epithelial cells scraped from the tissue could not explain the very large difference between the pool calculated by Finn and Rockoff (1971) and that measured here. Scraping would have to remove only one-eighth of the total epithelial cells to make even the total sodium measured flame photometrically equal in size to the pool reported by Finn and Rockoff (1971), but microscopy has shown that about 95% of the epithelial cells are removed by this technique (Macknight *et al.*, 1971). It is of interest that the mean total tissue sodium measured in the 15 experiments summarized in Table 7, 278 μmoles/g tissue dry wt, is comparable to the 350 μmoles/g tissue dry wt calculated from the data of Finn and Rockoff (1971).

per unit time from the serosal medium, inhibition of sodium transport by ouabain should have resulted in a much greater depression of metabolism than removal of mucosal sodium. Third is the relationships of pyruvate metabolism to sodium transport (Sharp, Coggins, Lichtenstein & Leaf, 1966), in tissues exposed to aldosterone. Removal of mucosal sodium decreased pyruvate metabolism to the same extent as it would were aldosterone not present. If a significant portion of the pump activity were involved in recirculation of serosal sodium, then removal of sodium from the mucosal medium alone should not so definitely suppress the metabolism of the tissue.

It is of interest that workers investigating the nature of the sodium transport pool in frog skin and using a variety of approaches to this problem have concluded that not all cellular sodium in the tissue participates in transepithelial sodium transport (Cereijido & Rotunno, 1967; Aceves & Erlij, 1971; Moreno, Reisin, Rodríguez Boulan, Rotunno & Cereijido, 1973; Cereijido, Rabito, Rodríguez Boulan & Rotunno, 1974).

The suggestion that there might be two discrete pools of sodium in toad bladder epithelial cells, one of mucosal, one of serosal origin, would raise the problem of the anatomical location of these pools. The statement that two cellular sodium pools exist would not necessarily mean that each cell contains two discrete pools. This might be true but an alternative interpretation would be that different cells might contain only sodium of mucosal or of serosal origin. Though entirely speculative, one possibility would be that under normal circumstances only the mitochondrial-rich cells contain the sodium of mucosal origin, the more plentiful granular cells together with the basal cells equilibrating with serosal sodium. The stimulation of transepithelial sodium transport by vasopression could then reflect an entry of mucosal sodium into the granular cells, which have been shown to be involved in the water movements which can be produced by this hormone (DiBona, Civan & Leaf, 1969). This fanciful interpretation of our results would provide one explanation for the recent claim (Mendoza, Handler & Orloff, 1970; Mendoza, 1972, 1973) that base-line sodium transport and vasopressin-stimulated sodium transport follow different pathways. Further work is clearly required to examine this problem of the relationships of cellular sodium of mucosal and serosal origin to transepithelial sodium transport by toad urinary bladder.

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