J. Membrane Biol. 20, 387–401 (1975)

Some Effects of Ouabain on Cellular Ions and Water in Epithelial Cells of Toad Urinary Bladder

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Received 19 July 1974; revised 7 November 1974

Summary. Transepithelial sodium transport was virtually abolished when toad urinary hemibladders, mounted in chambers and short-circuited, were exposed on their serosal surface to ouabain, 10^{-2} M, for 60 minutes. Epithelial cells scraped from such hemibladders gained sodium and lost an equal quantity of potassium when compared with controls not exposed to cardiac glycoside. Their total cellular cation content, chloride content and water content were unchanged. Experiments in which ^{24}Na, amiloride, or sodium-free mucosal solutions were used, revealed that a large, though variable, percentage of the sodium gained by cells exposed to ouabain, came from the mucosal medium, a finding consistent with the model of passive sodium entry from the mucosal medium followed by active sodium extrusion to the serosa. The ouabain-insensitive maintenance of cellular volume which was observed did not depend upon transepithelial sodium transport which had been virtually completely inhibited by ouabain. Neither did the maintenance of a normal cellular potassium content depend upon transepithelial sodium transport, for cellular potassium was unaffected when the mucosal medium was sodium-free or when it contained sufficient amiloride, 10^{-3} M, to virtually abolish such transport.

Cardiac glycosides, such as ouabain, inhibit active sodium transport in a variety of epithelia such as frog skin (Koefoed-Johnsen, 1957), amphibian renal proximal tubule (Schatzmann, Windhager & Solomon, 1958), rabbit ileum (Schultz & Zalusky, 1964), toad bladder (Herrera, 1966) and gall-bladder (Martin & Diamond, 1966). This effect is thought to be related to inhibition of a specific enzyme system, (Na-K)-activated, Mg-dependent ATPase, located in the cellular membrane and intimately involved in energy-dependent movement of sodium across this membrane (Skou, 1965).

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Transepithelial sodium transport by the toad bladder has been extensively investigated by a number of workers and the bladder has served as a useful tissue for delineating properties of the transport mechanism. However, the study of one major area – the relationships of the intracellular composition of the transporting epithelial cells to transcellular sodium movement – has until recently been complicated by the fact that the only analyses reported were of total tissue composition which inevitably reflects a large contribution of the underlying connective tissue and smooth muscle as well as the contents of the epithelial cells themselves. Recently, several groups (Lipton & Edelman, 1971; Macknight, DiBona, Leaf & Civan, 1971; Handler, Preston & Orloff, 1972) have published results showing the effects of ouabain on the composition of epithelial cells isolated from toad urinary bladder and incubated *in vitro*. These studies serve to demonstrate the expected effect of oubain, a loss of cellular potassium in exchange for extracellular sodium. They have the major disadvantage, however, that they do not allow determination of the relative contribution of sodium of mucosal and serosal origin to this exchange. This question is of particular interest for it is often assumed that sodium entry to the epithelial cells from the mucosal medium is a passive process occurring as the result of facilitated diffusion whereas the energy-dependent step is the extrusion of sodium from the cells across the baso-lateral membrane to the serosal medium (Leaf, 1965). The specific inhibition by ouabain of the energy-dependent step in transepithelial transport might then be expected to be associated with an increase in cellular sodium of mucosal origin. However, Herrera (1968), from studies of the composition of whole bladders, concluded that almost all the sodium gained by the tissue after exposure to ouabain was of serosal origin.

Experiments were therefore performed using the techniques previously described (Macknight *et al.*, 1971; Macknight, Leaf & Civan, 1971; Macknight, Civan & Leaf, 1975) to examine the effects of ouabain on epithelial cells scraped from hemibladders which had been mounted in chambers. The results show that a large percentage of the sodium gained by toad bladder epithelial cells exposed to ouabain is of mucosal origin. They also provide some new information about relationships between transepithelial sodium transport, cellular potassium accumulation and the maintenance of cellular volume.

Materials and Methods

The methods used were those presented in the previous paper (Macknight *et al.*, 1975). Ouabain, obtained from Sigma or Calbiochem, was dissolved in the appropriate

media at a final concentration of 5×10^{-3} or 10^{-2} M. The experimental protocols are outlined with the appropriate Tables, which present means of paired analyses with the difference and its SE. Significances of differences between means have been evaluated using Student's *t* test.

Results

In the experiments to be described, relatively large concentrations of ouabain (5×10^{-3} or 10^{-2} M) were dissolved only in the serosal medium. Preliminary experiments failed to reveal any effects on either transepithelial sodium transport or intracellular composition when ouabain was present only in the mucosal medium. Toad bladder epithelial cells are relatively insensitive to this cardiac glycoside and it was important to achieve virtually complete inhibition of transepithelial transport to examine the relationship between such transport and the changes in intracellular composition produced by ouabain. Lower concentrations of ouabain do however produce smaller changes in composition which are qualitatively similar. Unless otherwise indicated, the hemibladders were removed from chambers and the epithelial cells scraped off and taken for analysis 60 min after exposure to ouabain, by which time the SCC had stabilized at virtually zero, evidence of complete inhibition by ouabain of active transepithelial sodium transport.

Table 1 shows the effects of ouabain in one set of experiments, on the composition of epithelial cells scraped from paired hemibladders exposed on both their mucosal and serosal surfaces to sodium Ringer's, the mucosal medium containing ^{24}Na. The detailed protocol is provided in the Table.

Table 1. Effect of ouabain, 10^{-2} M, on epithelial cell composition with sodium Ringer's bathing both surfaces

	H_2O (kg/kg dry wt)	Na (mmoles/kg dry wt)	^{24}Na	K	Cl
Control	2.91	164	30	414	212
Ouabain	2.86	253	83	312	208
Δ	-0.05 ± 0.15	$+89 \pm 38$	$+52 \pm 4$	-102 ± 14	-4 ± 23
p	>0.70	<0.05	<0.001	<0.001	>0.80
n = 8					

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, serosal chambers were drained and the serosal chamber of the control refilled with sodium Ringer's $+^{3}H$-inulin + mannitol, 10^{-2} M, while the serosal chamber of the experimental hemibladder was refilled with sodium Ringer's $+^{3}H$-inulin + ouabain, 10^{-2} M. After a further 60 min both hemibladders were removed from the chambers, blotted and the epithelial cells scraped off and collected for analysis.

Table 2. Effect of ouabain, 10^{-2} M, on epithelial cell composition with sodium available from either both media or serosal medium only

	H_2O (kg/kg dry wt)	Na (mmoles/kg dry wt)	K	Cl
Serosal Na	2.78	182	365	202
Na, both media	2.86	310	259	219
Δ	$+0.08 \pm 0.11$	$+128 \pm 12$	-106 ± 8	$+17 \pm 8$
p	>0.40	<0.001	<0.001	>0.05
n = 7				

Paired hemibladders were initially incubated with sodium Ringer's on both mucosal and serosal surfaces. Once SCC stabilized chambers were drained. The mucosal surface of one hemibladder was washed by filling the appropriate chamber with choline Ringer's, immediately draining, repeating this process five times. This chamber was then filled with choline Ringer's. The other chambers were refilled with sodium Ringer's. After 20 min all chambers were drained. The mucosal chamber bathed with choline Ringer's was washed once with choline Ringer's, then refilled with choline Ringer's and ^{14}C-inulin. The other mucosal chamber was refilled with sodium Ringer's + ^{14}C-inulin. Both serosal chambers were filled with sodium Ringer's + ^{3}H-inulin + ouabain, 10^{-2} M. After a further 60 min both hemibladders were removed from the chambers, blotted, and the epithelial cells scraped off and collected for analysis.

Clearly, the epithelial cells lost potassium and gained a comparable amount of sodium, without a change in total cation, chloride or cellular water content. Furthermore, a large fraction of the total gain in cellular sodium was provided by sodium of mucosal origin, as judged by cellular sodium equilibrated with ^{24}Na in the mucosal medium.

If indeed sodium of mucosal origin makes an important contribution to the total cellular gain of sodium when hemibladders are exposed to ouabain, absence of mucosal sodium should be associated with a smaller exchange of cellular potassium for extracellular sodium. Table 2 shows the results of experiments in which paired hemibladders were exposed on their mucosal surfaces to either sodium Ringer's or sodium-free choline Ringer's while their serosal surfaces were bathed by sodium Ringer's containing ouabain, 10^{-2} M. The availability to the cells of sodium from both media was associated with a marked increase in cellular sodium and a substantially greater loss of cellular potassium after 60-min incubation. As was expected from our previous results (Table 2 of the preceding paper, Macknight *et al.*, 1975), the absence of mucosal sodium was associated with a small fall in cellular water and chloride contents and there was a difference between the potassium lost and sodium gained by the cells of 22 mmoles/kg dry wt reflecting the small loss of sodium which would occur from the cells exposed to sodium-free mucosal medium.

Table 3. Effect of ouabain, 10^{-2} M, on epithelial cell composition with sodium Ringer's only on the serosal surface

	H_2O (kg/kg dry wt)	Na (mmoles/kg dry wt)	K	Cl
Control	2.93	129	450	247
Ouabain	2.93	177	398	239
Δ	0.00 ± 0.13	$+48 \pm 6$	-52 ± 15	-9 ± 12
p	>0.95	<0.001	<0.02	>0.50
n = 7				

Paired hemibladders were initially incubated with sodium Ringer's on both mucosal and serosal surfaces. Once SCC stabilized, chambers were drained. The mucosal surfaces of both hemibladders were washed by filling the chambers with choline Ringer's, immediately draining, repeating this process five times. The mucosal chambers were then filled with choline Ringer's + ^{14}C-inulin, the serosal chamber of the control was filled with sodium Ringer's + ^{3}H-inulin + mannitol, 10^{-2} M, the experimental with sodium Ringer's + ^{3}H-inulin + ouabain, 10^{-2} M. After a further 60 min both hemibladders were removed from the chambers, blotted, and the epithelial cells scraped off and collected for analysis.

Table 4. Effect of ouabain 10^{-2} M, and amiloride, 10^{-3} M, on epithelial cell composition with sodium Ringer's bathing both surfaces

	H_2O (kg/kg dry wt)	Na (mmoles/kg dry wt)	^{24}Na	K	Cl
Amiloride	2.72	173	12.7	430	173
Amiloride + Ouabain	2.88	223	11.3	387	192
Δ	$+0.16 \pm 0.09$	$+50 \pm 11$	-1.4 ± 2.5	-43 ± 11	$+19 \pm 4$
p	>0.10	<0.005	>0.50	<0.01	<0.005
n = 8					

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 + amiloride, 10^{-3} M, mucosa, sodium Ringer's serosa. Once SCC had fallen and stabilized, chambers were drained, and refilled with sodium Ringer's + ^{14}C-inulin + amiloride, 10^{-3} M, mucosa, and either sodium Ringer's + ^{3}H-inulin, or sodium Ringer's + ^{3}H-inulin + ouabain, 10^{-2} M, serosa. After a further 60 min both hemibladders were removed from the chambers, blotted, and the epithelial cells scraped off and collected for analysis.

The results presented in Tables 3 and 4 provide strong support for the conclusion that when sodium is unavailable to the cells from the mucosal medium, ouabain causes smaller changes in cellular sodium and potassium. Table 3 shows the results of experiments in which paired hemibladders were exposed on their mucosal sides to sodium-free choline Ringer's for 60 min

Table 5. Effect of ouabain, 5×10^{-3} M, on epithelial cell composition with sodium Ringer's only on the mucosal surface

	H_2O (kg/kg dry wt)	Na (mmoles/kg dry wt)	K	Cl
Control	3.12	61	389	186
Ouabain	2.98	134	268	172
Δ	-0.14 ± 0.07	$+74 \pm 13$	-121 ± 19	-14 ± 10
p	>0.05	<0.001	<0.001	>0.10
n = 8				

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, the serosal chambers with choline Ringer's, after washing by filling the chambers with choline Ringer's, immediately draining and repeating this process five times. After 25 min chambers were again drained. The mucosal chambers were refilled with sodium Ringer's + ^{14}C-inulin, the serosal chambers with choline Ringer's + ^{3}H-inulin and either mannitol, 10^{-2} M (control), or ouabain, 10^{-2} M. After a further 60 min both hemibladders were removed from the chambers, blotted, and the epithelial cells scraped off and collected for analysis. (Choline Ringer's was prepared just before use by diluting a twice isotonic solution of choline chloride with diluent containing appropriate concentrations of K, Ca and glucose. One portion of the concentrated choline chloride solution contained ouabain, 10^{-2} M, thus the final medium concentration of ouabain was 5×10^{-3} M. Since 1×10^{-3} M ouabain appears to produce a profound inhibition of transepithelial sodium transport in toad bladder, the use of 5×10^{-3} M rather than 10^{-2} M ouabain in this experiment should not affect any conclusions.)

while their serosal surfaces were bathed with sodium Ringer's with or without ouabain. The changes in potassium and sodium in the presence of ouabain were only about half of those previously observed with sodium Ringer's in both media. As in the results shown in Table 1, total cation, chloride and water content remained constant. In the experiments summarized in Table 4, amiloride was used to inhibit mucosal entry of sodium. As in Table 3, the cells lost some potassium in exchange for sodium but the changes were again only about one-half of those observed when sodium was freely available to the cells from both the mucosal and serosal media. Once more, cellular volume was unaffected by the presence of ouabain.

The conclusion that epithelial cells gain more sodium during 60 min of incubation with ouabain when sodium is available to the cells from the mucosal medium as well as the serosal medium, is also supported by experiments in which paired hemibladders were incubated with sodium Ringer's bathing their mucosal surfaces while sodium-free choline Ringer's replaced sodium Ringer's as the serosal medium. As shown in Table 5, these cells,

Table 6. Effect of ouabain, 10^{-2} M, on epithelial composition after one hour or four hours of incubation with sodium Ringer's only on the serosal surface

	H_2O (kg/kg dry wt)	Na (mmoles/kg dry wt)	K	Cl
Ouabain, 1 hr	2.62	151	363	170
Ouabain, 4 hr	2.50	288	277	159
Δ	-0.12 ± 0.07	$+77 \pm 12$	-86 ± 10	-11 ± 9
p	>0.10	<0.001	<0.001	>0.20
n=8				

Paired hemibladders were initially incubated with sodium Ringer's on both mucosal and serosal surfaces. Once SCC stabilized, chambers were drained. The mucosal surfaces of both hemibladders were washed by filling the chambers with choline Ringer's, immediately draining, repeating this process five times. The mucosal chambers were then filled with choline Ringer's + ^{14}C-inulin, the serosal chamber of the hemibladder to be removed after 1-hr incubation was filled with sodium Ringer's + ^{3}H-inulin + ouabain, 10^{-2} M. However, to ensure that there was no possibility of distortion of the results by any penetration of inulin into the cellular water during prolonged incubation, the serosal chamber of the hemibladder to be removed at 4 hr was filled with sodium Ringer's + ouabain, 10^{-2} M. After 3 hr this serosal chamber was drained and refilled with sodium Ringer's + ^{3}H-inulin + ouabain, 10^{-2} M. Therefore, both hemibladders were only exposed on their serosal surfaces to inulin for the 60 min prior to their removal from the chambers. Hemibladders were removed from the chambers at 1 or 4 hr, blotted, and the epithelial cells scraped off and collected for analysis.

exposed to 5 mM ouabain, lost 121 mmoles potassium/kg dry wt. They gained 74 mmoles/kg dry wt of sodium which could only be of mucosal origin. Since neither cellular water nor chloride content changed significantly the difference between cellular potassium loss and sodium gain of 47 mmoles/kg dry wt must represent a cellular gain of choline of serosal origin. (It is clear both from results presented in Table 5 and in our previous paper (Macknight *et al.*, 1975) that choline in the serosal medium replaces cellular sodium in cells bathed on their mucosal surfaces with sodium Ringer's and on their serosal surfaces with choline Ringer's.)

The results presented in Tables 1 through 5 taken together establish that, when sodium is available in both the media, epithelial cells exposed on their serosal surfaces to ouabain show a greater loss of potassium and gain of sodium, partly of mucosal origin, than do cells which can obtain sodium only from the serosal medium. Indeed, over 60 min, loss of cellular potassium for sodium of serosal origin seemed to represent a quite constant value of about 50 mmoles/kg dry wt of cells. But, as the results presented in Table 6 establish, this does not represent a steady-state situation, for hemibladders incubated for 240 min with sodium-free choline Ringer's in

the mucosal medium and sodium Ringer's with 10 mM ouabain in the serosal medium, lost more potassium in exchange for sodium than did paired hemibladders similarly incubated but for only 60 min. The longer period of incubation did not however produce any change in cellular water content, total cellular cation content or chloride content.

Discussion

The Source of the Sodium Gained by Toad Bladder Epithelial Cells Exposed to Ouabain

The results presented establish that a large percentage of the sodium gained in exchange for potassium lost by epithelial cells scraped from hemibladders which have been mounted in chambers, bathed on both mucosal and serosal surfaces by sodium Ringer's, short-circuited and exposed to ouabain, is of mucosal origin. This finding refutes the suggestion, made from analysis of whole toad bladder rather than from analysis of epithelial cells alone, that the source of the sodium gained is almost entirely the serosal medium (Herrera, 1968). As pointed out in the previous paper (Macknight *et al.*, 1975), discrepancies between results obtained when epithelial cells alone are analyzed and when attempts are made to deduce epithelial cell composition from analysis of whole bladder, are not unexpected, for in a series of 15 observations the epithelial cells, with their extracellular fluid, scraped from the hemibladders represented only 10% of the total tissue dry weight and 17% of the total tissue water content. These epithelial cell scrapings contained only 16% of the total tissue sodium, 19% of the total tissue potassium and 15% of the total tissue chloride. Thus, changes which ouabain produces in the subepithelial tissues, and which have been reported by Herrera (1968), may well mask the relatively small, but, in absolute terms highly significant, changes occurring in the epithelial cells themselves.

The results are totally consistent with the model which places the ouabain-inhibited active step in transepithelial transport at the basolateral boundary of the epithelial cell and ascribes sodium entry, from the mucosal medium to the cells, to a passive process of facilitated diffusion (Leaf, 1965). Such a model would predict that the cells would gain sodium of mucosal origin when exposed to ouabain. It appears, however, that the actual gain by epithelial cells of sodium from the mucosal medium, and concomitant loss of cellular potassium presumably into the serosal medium, when both surfaces are bathed with sodium Ringer's may be quite variable from hemibladder to hemibladder.

Table 1 shows the results from one such set of experiments. In a second set the cellular gains in total sodium, sodium equilibrated with mucosal ^{24}Na and losses of potassium were somewhat greater. Taking the two sets together ($n = 16$) the mean cellular loss of potassium was 142 ± 17 mmoles/kg dry wt ($p < 0.001$) while the mean increase in cellular sodium was 133 ± 23 mmoles/kg dry wt ($p < 0.001$) of which 70 ± 6 mmoles were provided by sodium of mucosal origin ($p < 0.001$). In addition, other experiments have been performed in which the effects of ouabain, 10^{-2} M, on the cellular composition of hemibladders bathed on both surfaces with sodium Ringer's have been observed, though without the paired hemibladders serving as appropriate controls. In a total of 37 hemibladders exposed to ouabain under these conditions, the mean cellular sodium content equilibrated with ^{24}Na in the mucosal medium was 119 mmoles/kg dry wt with a standard deviation of ± 48, a standard error ± 8, and a range from 65 to 269 mmoles/kg dry wt. The reasons for this variability in cellular gain of mucosal sodium after ouabain remain to be defined. However, one possibility is that it is related to the variable sodium transporting capacities of the cells scraped from different hemibladders. The sodium transport can be expressed as SCC per mg cellular dry weight. There are marked differences in this value from toad to toad but the paired hemibladders from each toad show very similar transport rates when expressed in this way. For example, in 45 separate experiments the difference in SCC per mg cellular dry wt between paired hemibladders bathed on both surfaces with sodium Ringer's was 3 ± 3 ($p > 0.30$) yet the range between bladders from different toads was from 14 to 193, with a mean of 58 μA per mg cellular dry wt.

Analysis of the relationship between the SCC in μA per mg cellular dry wt, calculated from the SCC measured just prior to the addition of ouabain, which should provide an estimate of the tissue's capacity for sodium transport, and the gain of sodium of mucosal origin after 60-min exposure to the cardiac glycoside, revealed a positive correlation between these two variables. The greater the SCC per mg dry wt, the greater the entry of sodium from the mucosal surface after ouabain, with a correlation coefficient $r = 0.7614$, $n = 16$. Since both variables showed a normal distribution, $p < 0.001$. The significance of this observation is supported by an analysis of the relationship between the SCC per mg cellular dry wt immediately before incubation with ouabain and the size of the sodium pool equilibrated with mucosal sodium, using all 37 observations obtained with sodium Ringer's bathing both surfaces and ouabain on the serosal side. (Since not all hemibladders so treated had as their control a hemibladder bathed with Na Ringer's on both surfaces, only the relationship between

total cellular sodium of mucosal origin and not the gain in such sodium after ouabain can be used in the analysis. The two values of course both provide a measure of the sodium entry from the mucosal surface after ouabain.) Again, there is a positive correlation, the greater the SCC per mg dry wt the greater the total quantity of sodium of mucosal origin in the cells after ouabain (correlation coefficient $r = 0.4818$, $n = 37$, $p < 0.005$). This positive correlation between the sodium of mucosal origin gained by the cells after ouabain and the sodium transporting capacity of the cells as assessed by the SCC in μA per mg dry wt, would be consistent with the model which places the rate-limiting step for transepithelial transport of sodium at the mucosal surface of the cell (Leaf, 1965). Thus, the greater the SCC per mg dry wt under control conditions with sodium Ringer's on both surfaces, the greater the rate of sodium entry to the cells under control conditions and therefore the greater the gain in sodium of mucosal origin when sodium extrusion is inhibited by ouabain.

In contrast to the variability in the gain of sodium of mucosal origin after ouabain, cells which were able only to exchange potassium for sodium of serosal origin showed a much more predictable gain in sodium and loss of potassium over 60 min (Tables 3 and 4), the change in each case being about 50 mmoles cation/kg dry wt. Unlike the gain in sodium of mucosal origin, there was no significant relationship from a total of 21 experiments between SCC, μA per mg cellular dry wt measured initially with sodium Ringer's bathing both surfaces, and serosal sodium gained when sodium was unavailable from the mucosal medium ($r = -0.2245$, $n = 21$, $p > 0.30$).

Significance of the Results in Relation to the Cellular Sodium Transport Pool

In the previous paper (Macknight *et al.*, 1975), we provided evidence that the cellular sodium which equilibrated with ^{24}Na in the mucosal medium, represented about 20% of the cellular sodium determined flame photometrically. The clear demonstration in the present paper that the sodium equilibrated with mucosal ^{24}Na increased markedly when transepithelial sodium transport was completely inhibited by ouabain lends strong support to our claim that we are indeed measuring with this technique cellular sodium and not sodium which has already been transported by the cells from the mucosal to the serosal extracellular fluid. If this had been the case, inhibition of sodium transport by ouabain would have been associated with a decrease in the size of the measured cellular sodium and, additionally, there should not have been the excellent agreement between the increase in cellular sodium equilibrated with ^{24}Na and the loss of cellular potassium in excess of the gain in sodium of serosal origin. The fact that

amiloride prevented any gain of mucosal sodium by cells exposed to ouabain provides evidence that mucosal sodium gained after ouabain had entered the cells by the same pathway as it would have under normal conditions.

We also suggested in the previous paper (Macknight *et al.*, 1975) that a part of the cellular sodium equilibrated with mucosal ^{24}Na, though it lay outside the mucosal extracellular space accessible to markers such as inulin and mannitol, was nevertheless not intracellular since it could readily be removed by a rapid washing of the mucosal surface of the hemibladders in the chambers just before the epithelial cells were obtained for analysis. This interpretation was supported by the fact that though, in the presence of amiloride the cellular sodium labeled from the mucosal medium was still about one-third of normal in spite of the virtual abolition of transepithelial transport, almost all of this sodium was removed from epithelial cells scraped from hemibladders which had been washed on their mucosal surfaces. An alternative, though unconvincing explanation for this observation might have been that washing removed from epithelial cells sodium of mucosal origin which had entered them in spite of the presence of amiloride in the mucosal medium. The results presented in Table 4 help refute this argument for if mucosal sodium continued to enter epithelial cells exposed to 10^{-3} M amiloride on their mucosal surface, those cells which were incubated with amiloride and ouabain should have shown an increase in cellular sodium of mucosal origin when compared to the controls incubated only with amiloride. This was not the case.

Though the present results demonstrate an entry into the epithelial cells of a variable amount of sodium from the mucosal medium with concomitant loss of cellular potassium after incubation of hemibladders with ouabain there is also an exchange of sodium of serosal origin for cellular potassium. This indicates that some part of the cellular potassium may be related to the cellular sodium which readily exchanges with the serosal medium (Macknight *et al.*, 1975). For example, it seems very likely that the basal cells, exposed as they must be only to serosal sodium, make a contribution to this serosal sodium-cellular potassium exchange.

Relationships Between Transepithelial Sodium Transport, Cellular Potassium and the Regulation of Cellular Volume

There is at present much uncertainty as to the relationships between transepithelial sodium transport, cellular accumulation of potassium and the regulation of cellular volume. In recent years considerable evidence has accumulated which suggests that the concept of a ouabain-sensitive sodium-potassium exchange pump situated in the basolateral membranes of the cells

and responsible for transepithelial sodium transport, cellular potassium accumulation and cellular volume regulation is not correct. Thus, workers using a variety of tissues, not all of them composed of epithelial cells (rat diaphragm, Kleinzeller & Knotkova, 1964*b*; kidney, Kleinzeller & Knotkova, 1964*a*; Macknight, 1968; Whittembury, 1968; Maude, 1969; uterine smooth muscle, Daniel & Robinson, 1971; erythrocytes, Kregenow, 1973; Parker, 1973; liver, Macknight, Pilgrim & Robinson, 1974) have provided evidence that cellular volume regulation occurs by an ouabain-insensitive, potassium-independent, energy-dependent mechanism. This mechanism excludes sodium, chloride and water from the cells as demanded by the hypothesis, first proposed independently by Wilson (1954) and Leaf (1956) that sodium exclusion opposes the colloid osmotic force generated by the intracellular macromolecules to which the cellular membranes are impermeable. However, the actual nature of this mechanism remains controversial. One model (Whittembury, 1968; Giebisch, Boulpaep & Whittembury, 1971) suggests that the epithelial cells lining the renal proximal tubules possess two sodium pumps, one a ouabain-sensitive, potassium-dependent pump largely concerned in basolateral sodium-potassium exchange, the other a ouabain-insensitive potassium-independent pump regulating cellular volume. Both pumps may be concerned in transepithelial sodium transport. Part of the evidence for this second pump is, however, based on the results of experiments with the diuretic ethacrynic acid, the interpretation of which remains controversial, for some workers (Macknight, 1969; Poat, Poat & Munday, 1970; Epstein, 1972) claim that the effects of ethacrynic acid reflect a nonspecific interference with cellular metabolism rather than a specific direct inhibition of a sodium pump.

The results provided in this paper show that toad bladder epithelial cells scraped from hemibladders incubated with ouabain have the same water contents as control tissue incubated without ouabain. A similar result has been reported previously from experiments with isolated toad bladder epithelial cells incubated in vitro (Lipton & Edelman, 1971; Macknight *et al.*, 1971; Handler, Preston & Orloff, 1972), and is consistent with the hypothesis that cellular volume may be regulated by an ouabain-insensitive mechanism independent of ouabain-sensitive sodium-potassium exchange. The present results however provide some additional information about the relationships between transepithelial sodium transport, cellular potassium accumulation and cellular volume regulation.

First, the presence of ouabain in the serosal medium at a concentration of 10^{-2} M virtually completely inhibited transepithelial sodium transport during incubation for 60 min, without cellular swelling. Therefore, at least

in toad bladder epithelial cells, the mechanism regulating cellular volume can function independently of the mechanism responsible for transepithelial sodium transport, a conclusion not consistent with the hypothesis that these two processes share a common pump.

Second, it is clear that transepithelial transport of sodium is not required for toad bladder epithelial cells to maintain a normal intracellular content of potassium. For example, the potassium contents of epithelial cells not exposed to ouabain in Table 3, whose transepithelial sodium transport was prevented by the substitution for sodium Ringer's of a sodium-free choline Ringer's in the mucosal medium, and in Table 4, where transepithelial sodium transport was inhibited by amiloride in the mucosal medium, are of the order seen in epithelial cells transporting sodium normally from the mucosal to the serosal solutions. A direct comparison between intracellular potassium contents in epithelial cells transporting sodium and epithelial cells in which transport is inhibited by absence of mucosal sodium is provided in Table 2 of the previous paper (Macknight *et al.*, 1975) and establishes this point uniquivocally.

This complete dissociation of cellular potassium content from transepithelial transport seems inconsistent with any hypothesis that there is necessarily specific tight coupling between cellular potassium accumulation and transepithelial sodium transport (for example, Finn & Nellans, 1972). Neither does it favor any dependence of potassium accumulation on electrogenic transepithelial sodium transport (Frazier & Leaf, 1963) for even in the short-circuited hemibladders abolition of such transport should have modified the driving force for potassium accumulation. It is of interest in this regard that comparison of paired hemibladders bathed on both surfaces with sodium Ringer's revealed no difference in cellular potassium content (429 and 425, difference $= 4 \pm 16$ mmoles/kg dry wt, $n = 8$, $p > 0.70$) or concentration (difference $= 3 \pm 5$ mmoles/kg intracellular H_2O, $p > 0.50$) when one hemibladder remained open-circuited while the other was continuously short-circuited for 90 min before the epithelial cells were removed for analysis (B. A. Robinson and A. D. C. Macknight, *unpublished observations*).

These results are consistent with a model which ascribes both transepithelial sodium transport and cellular sodium-potassium exchange to a ouabain-inhibitable, energy-dependent process, and the regulation of cellular volume to an independent process not sensitive to ouabin. Whether this is a second sodium pump in the conventional sense as suggested by Giebisch *et al.* (1971), or some other energy-dependent process which results in extrusion of sodium and chloride, and thereby water, from the cells, is still to be determined.

This work was begun in Boston and completed in Dunedin, New Zealand. It was supported in Boston in part by grants from the John A. Hartford Foundation, Inc., and by U.S. Public Health Service grants HE-06664 from the National Heart Institute and AM-04501 from the National Institute of Arthritis and Metabolic Diseases. The work was begun while A. D. C. M. was a Public Health Service International Post-doctoral Research Fellow (TW 01485-01). M. M. C. is an Established Investigator of the American Heart Association. The work in New Zealand was supported by the Medical Research Council of New Zealand. We are very grateful to Miss Bridget A. Robinson for her help with some of the experiments.

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