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Metabolic Evidence that Serosal Sodium Does Not Recycle Through the Active Transepithelial Transport Pathway of Toad Bladder

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Summary. The possibility that sodium from the serosal bathing medium "back-diffuses" into the active sodium transport pool within the mucosal epithelial cell of the isolated toad bladder was examined by determining the effect on the metabolism of the tissue of removing sodium from the serosal medium. It was expected that if recycling of serosal sodium did occur through the active transepithelial transport pathway of the isolated toad bladder, removal of sodium from the serosal medium would reduce the rate of CO_2 production by the tissue and enhance the stoichiometric ratio of sodium ions transported across the bladder per molecule of sodium transport dependent CO_2 produced simultaneously by the bladder (J_{Na}/J_{CO_2}). The data revealed no significant change in this ratio (17.19 with serosal sodium and 16.13 after replacing serosal sodium with choline). Further, when transepithelial sodium transport was inhibited (a) by adding amiloride to the mucosal medium, or (b) by removing sodium from the mucosal medium, subsequent removal of sodium from the serosal medium, or (c) addition of ouabain failed to depress the basal rate of CO_2 production by the bladder [(a) rate of basal, nontransport related, CO_2 production ($J^b_{CO_2}$) equals 1.54 ± 0.52 with serosal sodium and 1.54 ± 0.37 without serosal sodium; (b) $J^b_{CO_2}$ equals 2.18 ± 0.21 with serosal sodium and 2.09 ± 0.21 without serosal sodium; (c) 1.14 ± 0.26 without ouabain and 1.13 ± 0.25 with ouabain; unite of $J^b_{CO_2}$ are nmoles mg d.w.$^{-1}$ min^{-1}]. The results support the hypothesis that little, if any, recycling of serosal sodium occurs in the toad bladder.

The permeability of the basal-lateral plasma membranes of the epithelial cells of toad bladder to sodium from the serosal bathing medium has been difficult to assess. It is important in understanding the process of active sodium transport across this tissue to know if sodium from the serosal medium can gain access to the interior of the transporting layer of cells and co-mingle there with sodium from the mucosal medium which is in transit across these cells. Such recycling of serosal sodium would not contribute to the net reabsorption of sodium which takes place from mucosa to serosa. It would, however, increase the energy costs of sodium transport in proportion to the number of sodium ions recycled.

Equilibration studies reveal that some 80 percent of sodium within the noninulin space of mucosal epithelial cells scraped from toad bladders equilibrates with sodium from the serosal medium [10]. The possibility exists, therefore, that this large proportion of sodium within the noninulin space competes with sodium of mucosal origin for active extrusion out of the cell across their basolateral plasma membranes.

Kinetic wash-out or loading measurements have proven unsatisfactory as methods with which to measure the "active transport pool" of sodium or the permeability coefficients of the mucosal cells of the toad bladder because of the underlying sub-mucosal tissue [10]. We have, therefore, used metabolic measurements to assess back leakage of serosal sodium into the cells. If such leakage with recycling occurs, it will require the expenditure of energy to pump the sodium uphill against an electrochemical potential. Therefore, the metabolism of the bladder should be reduced if sodium is removed from the serosal medium making such recycling impossible. Measurements of the rate of CO_2 production by isolated toad bladders revealed no significant changes in the presence or absence of sodium in the serosal medium. From these findings it is concluded that no significant back leak with recycling of serosal sodium occurs in this tissue.

Materials and Methods

Preparation

Dominican female toads (*Bufo marinus*) were used (National Reagents, Bridgeport, Conn.). Hemibladders from doubly pithed large toads were mounted between the two halves of a lucite chamber which exposed 9.3 cm^2 of membrane to 3.5 ml of medium on each side. The half bladders were mounted, serosal side down, over a nylon mesh and with a teflon ring coated with Dow Corning High Vacuum Silicone Grease as sealant at the margins of the bladder. This means of reducing edge damage of bladders mounted in chambers is shown in Table 1. Note the reduction in passive conductance, K_p[1], the lower ratio of passive to total transepithelial conductance (K_p/K_t) and higher spontaneous open circuit potential ($\Delta\psi$) of half bladders sealed with the silicone grease as compared with the mixture of petrolatum and lanolin used previously as the sealant. The inverse relationship between K_p and spontaneous trans-epithelial potential is similar to that reported by Higgins *et al.* [6].

1 Glossary of Symbols: J_{CO_2} = rate of total CO_2 production (nmoles per mg d.w. per min; $J^{sb}_{CO_2}$ = rate of suprabasal CO_2 production or sodium transport related CO_2 production; $J^{b}_{CO_2}$ = rate of basal, nontransport related, CO_2 production; F = Faraday; J^{0}_{Na} = short circuit current (μamp); $\Delta\psi$ = transepithelial electrical potential difference (mV); K_T = total transepithelial electrical conductance (10^{-4} mho/cm^2); K_p = "passive" transepithelial electrical conductance; K_a = transepithelial conductance dependent on active sodium transport; J_{Na}/J_{CO_2} = stoichiometric ratio of sodium ions transported across the bladder per molecule of sodium transport dependent CO_2 produced simultaneously by the bladder.

Table 1. Total (K_T), passive (K_p), active (K_a) conductances and electrical potential gradient ($\Delta\psi$) in toad bladders mounted with and without silicone sealant

	K_T	K_P	K_a	K_p/K_t	$\Delta\psi$
	(10^{-4} mho/cm^2)				(mV)
A. Without silicone ($n=10$)	2.75±0.50	1.21±0.15	1.53±0.44	0.50±0.08	60.8±7.7
B. With silicone ($n=9$)	2.59±0.34	0.87±0.01	1.72±0.30	0.35±0.04	79.6±7.3

Total transepithelial conductance, K_T, was determined from the deflections in the current caused by the 10 mV potential difference imposed across the bladder wall for one sec every three min. K_p, the passive conductance, was similarly determined after all transepithelial sodium transport was blocked by amiloride. The difference between K_T and K_p is considered to be the conductance through the active sodium transport pathway.

After mounting, the half bladders were rinsed with 3 to 5 washes of the appropriate Ringer's solution over a period of at least one hr before experiments were commenced, but the spontaneous open-circuit electrical potential was monitored continuously. CO_2-free air was bubbled through the chamber. The CO_2-free air was prepared by bubbling room air through two one meter tall columns in series each containing 2 to 3 M sodium hydroxide to absorb CO_2. To deliver a constant pressure of CO_2-free air, the incoming air was vented through two similar columns of 2 M sodium chloride solution. Teflon tubing was used to join parts of the system with rubber connections as necessary to allow flexibility, but reduced to minimal length to avoid ingress of CO_2 into the CO_2-free system.

Methods

Sodium transport was measured as the short circuit current by the method of Ussing and Zerahn [15]. CO_2 production by the half bladders was measured simultaneously, essentially as described by Al Awqati [1] based on the method of Maffly [12] with the following modifications to increase sensitivity and stability of the CO_2 determination:

1. The tank serving as the reservoir for the 1.0 mM sodium hydroxide was closed to the atmosphere by a teflon ring upon which the lid rested.

2. The magnetic stirrer in the sodium hydroxide solution was rotated by an air driven rotor to reduce the electrical noise and the heating effects of the conventional electrical rotor.

3. A high flow rate of CO_2-free air was bubbled through each chamber giving a rate of 30 bubbles per min through the glass coil of the differential conductometric system. This gave a more stable tracing of CO_2 production at recorder speeds of 0.07 to 1.0 inch per min.

4. The differential conductometric apparatus was calibrated by injecting a known volume of gas of known CO_2 concentration from a gas-tight syringe directly into the conductometric apparatus at a slow rate – 2.5 to 10.0 ml of gas mixture in 2 to 3 min. The area under the curve of the CO_2 tracing, representing thus a known quantity of CO_2, was used to calculate the CO_2 produced by the bladder.

Gas tanks containing the CO_2 for calibration of the apparatus were obtained from Medical Technical Gases, Medford, Massachusetts. The CO_2 concentration of this standard gas mixture was checked by three different methods: (a) pH measurements were made of a 0.100 mM solution of $NaHCO_3$ equilibrated with the standard gas. This method gave non-reproducible results and consumed large volumes of the standard gas mixture. (b) The CO_2

concentration was determined by mass spectrometry (courtesy of Dr. Stanley Rosenthal, Harvard-MIT Biomaterials Research Project). (c) A primary gas standard of known CO_2 concentration was prepared by mixing in a special 1500 ml gas-tight syringe (Hamilton Laboratories, Reno, Nevada) highly purified 100 % nitrogen with 3 vols. percent of CO_2 calibrated by mass spectrometry. Different volumes of this gas mixture were then injected through the conductometric apparatus and a calibration curve prepared from the integrated area and the known CO_2 content of the gas. The CO_2 concentration of the standard gas mixture from the tank was then measured from this standard curve and subsequently served as a secondary standard for daily use in calibrating the CO_2 measurements. The last two methods were in good agreement and gave reproducible results. The secondary standards in the two tanks used in these studies had concentrations of CO_2 of 0.022 and 0.028 vols. percent, respectively.

The CO_2 produced by the toad bladder was calculated by determining the area under the tracing of the recorder with a planimeter and comparing it with the area under the tracing produced by a given volume of the standard gas mixture with known CO_2 concentration. The actual calculation then is:

$$J_{CO_2}=\frac{(A_x)(V_s)(C_s)(273)(P-p)}{(A_s)(22.4)(T)(760)(t)(\text{d.w})}$$

J_{CO_2} = CO_2 produced – moles per (mg d.w per min); A_x = area under tracing of CO_2 produced during experiment; A_s = area under tracing produced by standard gas mixture; V_s = liters of standard gas mixture used for calibration; C_s = liters of CO_2 per liter of standard gas mixture; T = absolute temperature; t = time – minutes; d.w = dry weight of bladder exposed in chamber – in mg; $P-p$ = barometric pressure minus water vapor pressure; A_x was determined over periods long enough to minimize errors due to lag in CO_2 recordings.

Electrical Measurements. For short-circuit current measurements, an automatic voltage clamp kept the transepithelial potential, $\Delta\psi$, at zero. Before commencing the CO_2 recording, the bladder was kept open-circuited and $\Delta\psi$ was recorded; a few bladders with $\Delta\psi < 50$ mV were discarded. CO_2-free air was used and antibiotics and carbonic anhydrase were present during this equilibration period of two hours [1]. Measurements of short circuit current and CO_2 were made from the recorder tracings only after a five min period elapsed following setting the voltage clamp at $\Delta\psi = 0$.

When gas containing CO_2 is introduced into the Ringer's solution in the chambers the transit time until the pulse of CO_2 is detected and recordered was 1 to 2 min. Thus, measurements of J_{CO_2} and J^0_{Na} were commenced from recorder tracings only after five min when both values had become stable. An example of the data as recorded is shown in Fig. 1.

Solutions. The sodium Ringer's solution was prepared without bicarbonate and contained (mM): Na, 119; K, 3.5; Ca, 0.9; Cl, 118; HPO_4, 2.4; and H_2PO_4, 0.57. This solution had a pH of 7.2 and a solute concentration of 220 mOsm. Sodium-free choline Ringer's solution contained (mM): choline, 113; K, 4.7; Ca, 0.9; Cl, 115; HPO_4, 2; and H_2PO_4, 0.16. The pH of both sodium-free solutions was 7.2 and their solute concentrations 220 mOsm. The sodium concentration was confirmed by flame photometric measurements. Sodium concentrations of the sodium-free solutions were determined by flame photometry at the start and completion of experiments.

Just prior to use 0.1 mg of K penicillin, 0.01 mg of gentamicin, and 0.05 mg of colistin[2] were added per ml to each solution and the medium was filtered through an 0.8 μ Millipore filter to minimize bacterial growth during the experiments. 0.2 mg of carbonic anhydrase were added per ml of solution to accelerate equilibration of CO_2.

2 Since colistin contains sodium it was not used when sodium-free media were employed.

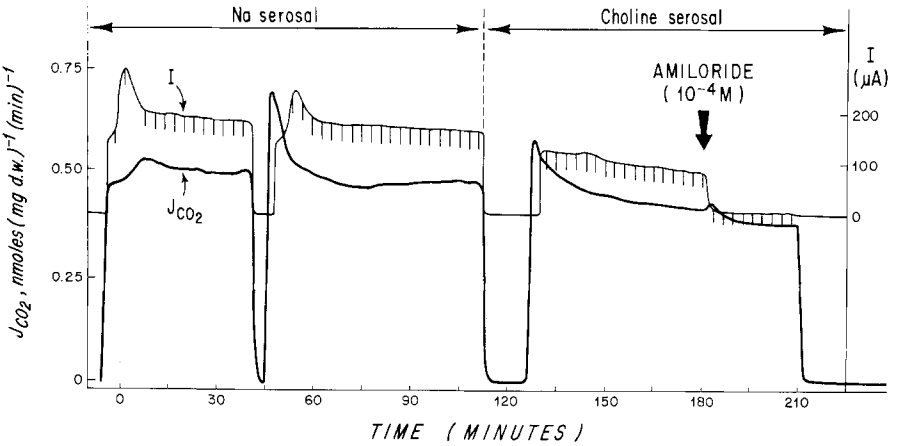

Fig. 1. A representative record of sodium transport measured as the short-circuit current, I, in microamperes, μA, and of the simultaneous rate of CO_2 production, J_{CO_2} by the toad bladder. Note the transient over-shoot of current and CO_2 production with changes in applied transmembrane potentials or of bathing solutions. The dips in the CO_2 measurements at 43 to 45 and at 112 to 127 min were caused when the zero calibrations for the measurements of J_{CO_2} were obtained by passing the CO_2-free gas directly into the conductometric apparatus, thus by-passing the Ussing chamber. The short-circuit current at the same time was turned off to obtain the calibration for zero current. The basal rate of CO_2 production was obtained by blocking all transepithelial sodium transport with amiloride at the end of the study. The terminal fall in the CO_2 measurement represents a final calibration of the zero with CO_2-free air. The short current circuit measurement was simultaneously turned off and no further fall in I occurred indicating total blockade of transepithelial transport by the amiloride. The small vertical deflections from the current tracing result from pulses of potential of 10 mV applied for one sec every three min across the bladder with serosa made positive to mucosa. From these deflections the transepithelial conductance was calculated

To determine the basal rate of CO_2 production in the absence of sodium transport, two methods were employed. The mucosal medium was changed to a sodium-free choline or magnesium Ringer's solution or Amiloride was added to the mucosal medium to a final concentration of 10^{-4} M to block all sodium transport [2]. The latter procedure was preferred as it obviated the tissue damage and decrease of electrical resistance that often accompanied changes of medium, it minimized the time for reequilibriation with the CO_2-free air, and it obviated also the small residual current from sodium which failed to be removed from the mucosal medium in spite of rinses with the sodium-free Ringer's solutions or from sodium which diffused from serosal to mucosal media during an experiment.

Dry weight of tissue was determined for the area of bladder exposed in the chamber to the bathing solutions. Tissues were dried in a tared vessel at 100 °C for 24 hr.

Results

The possibility that sodium from the serosal medium recycles through active transport pathways and thus contributes to CO_2 production, J_{CO_2}, was tested by three different experimental protocols:

Table 2. The effect of Na-free serosal medium on the $J_{Na}/J^{sb}_{CO_2}$ in toad bladders; mucosal medium is sodium Ringer's solution in all experiments

Serosal medium	$J_{Na}/J^{sb}_{CO_2}$		
A	Na	Choline	Na
	26.66	34.02	46.10
	17.80	21.87	24.20
	24.60	28.06	27.70
	17.00	24.04	21.20
	26.23	24.85	26.00
	12.04	9.61	7.90
$\bar{x} \pm$ SEM	20.74±2.7	23.74±3.6	25.54±5.5
B	Na	Na	Choline
	10.95	14.68	16.68
	13.56	17.79	25.00
	22.59	25.40	28.50
	14.62	26.42	19.79
	6.03	9.79	11.73
	6.07	7.51	10.86
	6.69	7.02	5.96
$\bar{x} \pm$ SEM	11.50±2.5	15.51±3.3	16.86±3.3
C	Choline	Choline	Na
	7.00	12.40	9.91
	5.46	8.06	5.00
	5.62	7.38	16.90
	15.80	11.60	8.90
	5.90	10.40	8.40
	4.60	6.80	4.70
$x \pm$ SEM	7.40±1.7	9.40±0.9	8.90±1.8

1. The effect of removing sodium from the serosal medium on the ratio J_{Na}/J_{CO_2} was determined. With sodium Ringer's solution bathing both surfaces of the bladder, J_{CO_2}, total CO_2 production, was measured simultaneously with the short-circuit current. The serosal medium was then replaced by a sodium-free choline Ringer's solution with the bladder short-circuited and the effect of removing sodium from the serosal medium on J_{CO_2} was examined. J^0_{Na} was determined simultaneously as the short circuit current since the latter still measures net transepithelial sodium transport under these conditions [4].

The effect on the stoichiometric ratio, J_{Na}/J_{CO_2}, of removing sodium from the serosal bathing medium was determined. As shown in Table 2,

this ratio was unaffected by removal of serosal sodium. However, there usually occurred an increase in the ratio with each change of medium and the washings entailed. By randomizing the sequence of solutions bathing the serosal medium, the effect of repeated changes was cancelled out – Parts *B* and *C*, Table 2. The mean differences were not significantly different from zero. Thus, the presence or absence of sodium in the serosal medium was found not to affect the ratio J_{Na}/J_{CO_2}, consistent with no recycling of serosal sodium through the active transport pathway, but the disturbing effect of changing the bathing medium might have obscured a small effect. The lower mean values for $J_{Na}/J^{sb}_{CO_2}$ in Parts *B* and *C* as compared with Part *A* of Table 2 reflect differences obtained with different batches of toads and seasonal variability, as previously reported [1].

2. The effect of removing sodium from the serosal medium on the rate of basal CO_2 production, $J^b_{CO_2}$, was determined. In these experiments active sodium transport was first inhibited with amiloride, which prevents sodium transport by blocking sodium of the mucosal medium from entering the transporting cells of the bladder [10]. The rate of basal CO_2 production, $J^b_{CO_2}$, was determined. The serosal medium was then replaced by a sodium-free choline or magnesium Ringer's solution and the $J^b_{CO_2}$ was again determined. Table 3 shows that, though there was a progressive small reduction in basal CO_2 production with time, on the average there was no change when the serosal medium was changed from a sodium-containing to a sodium-free choline Ringer's solution. Table 4 shows comparable data for sodium-free magnesium Ringer's solution replacing

Table 3. The effect of replacing serosal medium by choline on the basal CO_2 production, $J^b_{CO_2}$, at $J_{Na}=0$ in toad bladder

Experiment		$J^b_{CO_2}$ nmoles (mg d.w.)$^{-1}$ (min)$^{-1}$		
		(Period 1)	(Period 2)	(Period 3)
	Serosal medium	Na	Choline	Na
1		1.54	1.33	1.12
2		1.30	1.50	1.38
3		0.57	0.63	0.60
4		1.34	0.93	0.79
5		2.26	1.90	1.67
6		2.94	2.98	2.89
	$\bar{x}$	1.66	1.54	1.41
	±SEM	0.37	0.37	0.37

Amiloride, 0.1 mM in mucosal medium, $J_{Na}=0$.

Table 4. The effect of replacing serosal medium by magnesium on basal CO_2 production, $J^b_{CO_2}$, at $J_{Na}=0$ by toad bladder

Experiment		$J^b_{CO_2}$ nmoles (mg d.w.)$^{-1}$ (min)$^{-1}$		
		(Period 1)	(Period 2)	(Period 3)
	Serosal medium	Na	Mg	Na
1		1.24	0.92	0.68
2		0.96	0.81	0.91
3		3.38	2.73	2.83
4		1.57	1.23	0.82
5		2.08	2.04	1.86
6		0.47	0.44	0.40
7		2.42	1.80	1.94
	$\bar{x}$	1.73	1.42	1.35
	$\pm$SEM	0.40	0.33	0.36

Amiloride 0.1 mM in mucosal medium, $J_{Na}=0$.

the standard sodium Ringer's solution as the serosal bathing medium. In both Tables 3 and 4 the CO_2 production was determined after total inhibition of sodium transport by amiloride and with the sodium-free measurements bracketed between prior and subsequent measurements with standard sodium Ringer's solution bathing the serosal surface of the bladder. The absence of any detectable, significant reduction in basal CO_2 production when sodium was removed from the serosal bathing medium supports the hypothesis that recycling of sodium through the active transport pathway in toad bladder does not occur.

A further series of observations were made in which basal CO_2 production, $J^b_{CO_2}$, was determined with a sodium-free choline Ringer's solution bathing the mucosal surface to stop transepithelial sodium transport. The serosal sodium Ringer's solution was then replaced also by sodium-free choline Ringer's solution and amiloride was added to the mucosal medium. As shown in Table 5 in this series, removal of sodium also from the serosal medium again failed to affect the basal CO_2 production by the bladder.

3. Finally, the effect of ouabain on basal CO_2 production was determined. With sodium Ringer's solution bathing both surfaces but with sodium transport blocked by amiloride, the effect of ouabain 10^{-3} M added to the serosal medium on basal CO_2 production was determined. Ouabain at this concentration blocks sodium transport by inhibiting the Na, K-dependent ATPase at the baso-lateral borders which seems instrumental in extruding sodium actively from cell interior to serosal

Table 5. The effect of replacing Na from the serosal medium by choline on the basal CO_2 production, $J^b_{CO_2}$ by toad bladder at $J_{Na}=0$

Experiment		$J^b_{CO_2}$ nmoles (mg d.w.)$^{-1}$ (min)$^{-1}$		
		(Period 1)	(Period 2)	(Period 3)
	Mucosal media	Choline	Choline	Choline + Amiloride
	Serosal media	Na	Choline	Choline
1		1.63	1.66	1.49
2		2.35	2.35	1.88
3		2.42	2.50	2.35
4		2.31	2.28	2.17
	$\bar{x}$	2.18	2.20	1.97
	±SEM	0.21	0.21	0.22

Amiloride 0.1 mM in mucosal media.

Table 6. The effect of ouabain on basal CO_2 production, $J^b_{CO_2}$, at $J_{Na}=0$ by toad bladder

	$J^b_{CO_2}$ nmoles (mg d.w.)$^{-1}$ (min)$^{-1}$		
	Amiloride (10^{-4} M)	Ouabain (10^{-3} M)	Δ
A. Serosal sodium	0.62	0.57	−0.05
	0.72	0.70	−0.02
	0.57	0.54	−0.03
	0.66	0.63	−0.03
	2.61	2.53	−0.07
	1.88	1.94	+0.06
	1.12	1.24	+0.12
	2.77	2.72	−0.05
	0.88	0.83	−0.05
	0.29	0.31	+0.02
	0.51	0.51	0.00
$\bar{x}$ ± SEM	1.14±0.26	1.13±0.25	−0.01±0.06
B. Serosal choline	2.53	2.37	
	0.78	0.76	

Amiloride 0.1 mM in mucosal medium: $J_{Na}=0$.

medium. As seen in Table 6, the addition of ouabain had no detectable effect on basal CO_2 production, confirming an absence of significant recycling of serosal sodium through the active transport pathway. Ouabain did not affect $J^b_{CO_2}$ when choline replaced sodium in the serosal medium, as shown in the last two observations of Table 6.

Discussion

The present observations confirm other recent reports which have noted a considerable variability in the sodium ions transported per molecule of supra-basal oxygen consumed [14, 16] or CO_2 produced [1].

There has been great interest in the magnitude of the "sodium transport pool" within toad bladder epithelial cells. This is the quantity of sodium within transporting epithelial cells which is destined for extrusion across their basolateral plasma membranes and which is miscible with, but not necessarily limited to, that sodium which enters the epithelial cells from the mucosal medium. The indeterminate variable has been the accessibility of this transport pool to sodium in the serosal medium. If only sodium entering the cells through their apical surfaces is destined for active extrusion at the serosal surface, then transepithelial transport of sodium will be maximally efficient in that every sodium ion extruded from cell to serosal medium will be a contribution to transepithelial sodium transport. To the extent that sodium from the serosal medium can enter this active transport pool, there will have to be more than a single sodium ion actively transported outward across the basolateral cell membranes for each unit charge contributing to the short circuit current or net transepithelial transport of sodium.

The present study tested the possibility of recycling of serosal sodium by three experimental protocols. The basis of each, however, was to measure the effect of the presence or absence of sodium in the serosal medium on tissue metabolism. Without sodium in the serosal medium there is no possibility of recycling, and CO_2 production by the tissue should be reduced. Since some 80% of the sodium in the noninulin space of scraped epithelial cells equilibrates with sodium in the serosal medium [10], removal of sodium from the serosal medium should significantly reduce CO_2 production by the bladder if this sodium were entering the active sodium transport pool and being recycled.

The most direct experimental approach would be to determine J_{Na}/J_{CO_2} with a sodium Ringer's solution bathing both surfaces of the bladder and then to replace the serosal medium with a sodium-free Ringer's solution. Unfortunately, changing the serosal medium was associated with some changes in J_{Na}/J_{CO_2}. The mean values showed no effect from removing serosal sodium on this stoichiometric ratio when the sequence of changes of the serosal medium were varied. Other experimental approaches, however, were tried.

With sodium removed from the mucosal medium or its entry into the

mucosal cells blocked with amiloride, transepithelial sodium transport stops. Recycling of serosal sodium could still occur and its energy requirement would contribute to the rate of basal CO_2 production, $J^b_{CO_2}$. However, removal of serosal sodium had no effect upon $J^b_{CO_2}$ as shown in Tables 3, 4, and 5. Finally, ouabain at concentrations which inhibit all transepithelial sodium transport when added to the serosal medium failed to depress $J^b_{CO_2}$ in the presence of serosal sodium. At the concentrations of ouabain used, 10^{-3} M, all net transepithelial sodium transport stops. Since sodium from the serosal medium which could enter the active transport pool would be indistinguishable from sodium entering this pool from the mucosal medium, ouabain would block recycling of serosal sodium through the sodium pump. The absence of an effect of ouabain on $J^b_{CO_2}$ thus further supports the hypothesis that no recycling, in fact, occurs. This finding also indicates that ouabain-sensitive sodium transport, which must be occurring in the connective tissue, epithelial and smooth muscle cells of the submucosal structures, contribute little to $J^b_{CO_2}$. These experiments, however, provide no evidence for or against the possibility that sodium from the serosal medium may enter some compartment in the epithelial cells but not be miscible with the active sodium transport pool.

The argument developed here assumes that transepithelial sodium transport involves entry of the transported sodium into some or all of the mucosal epithelial cells of the bladder. That the pathway of sodium transport is through mucosal cells seems established. (1) These cells have adenosinetriphosphatase lining their basolateral but not their apical margins [13]. (2) Stimulation of transepithelial sodium transport by vasopressin and aldosterone is associated with an increased sodium content of the epithelial cell layer [5, 8, 11]. (3) Amiloride which blocks active sodium transport has no effect on the passive ionic permeability of toad bladder as indicated by a lack of effect on the transepithelial fluxes of Cl, K in either direction, or in Na flux from serosa to mucosa [7]. Morphological evidence supports the view that the pathway for passive permeation is via the intercellular route [3]. (4) Inhibition of transepithelial sodium transport by ouabain results in a reciprocal loss of potassium and gain of sodium in the epithelial cell layer [9].

The evidence presented here based on metabolic requirements for active sodium transport supports other evidence summarized by Macknight, Civan and Leaf [10] that sodium from the mucosal medium which traverses epithelial cells in the course of trans-epithelial transport does not mix within the cells with sodium from the serosal medium. The data

support the view that the basal-lateral plasma membranes of cells engaged in active transepithelial transport effectively rectify in that they facilitate movement of sodium from cell interior to serosal medium; passive back leak or return of sodium through the pump mechanism does not occur to a significant extent in such cells.

The results do not provide evidence to determine whether transepithelial transport of sodium is a function of all mucosal epithelial cells including both the granular and mitochondrial-rich cell types or restricted to only one of these two possible candidates. The results also fail to locate the large fraction of sodium within the noninulin space of scraped epithelial cells which equilibrates with sodium of the serosal medium. If this sodium is within the epithelial cells it is neither miscible with sodium entering these cells from the mucosal medium nor is metabolic energy required to limit its accumulation; it must be in some compartment which equilibrates passively with serosal sodium.

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References

1. Al-Awqati, Q., Beauwens, R., Leaf, A. 1975. Coupling of Sodium transport to respiration in the toad bladder. *J. Membrane Biol.* **22**:91
2. Bentley, P.J. 1968. Amiloride: A potent inhibitor of Na transport across the toad bladder. *J. Physiol. (London)* **195**:317
3. DiBona, D.R., Civan, M.M. 1973. Pathways for movement of ions and water across toad urinary bladder. I. Anatomic site of transepithelial shunt pathways. *J. Membrane Biol.* **12**:101
4. Frazier, H.S., Dempsey, E., Leaf, A. 1962. Movement of sodium across the mucosal surface of the isolated toad bladder and its modification by vasopressin. *J. Gen. Physiol.* **45**:529
5. Handler, J.S., Preston, A.S., Orloff, J. 1972. Effect of ADH, aldosterone, ouabain and amiloride on toad bladder epithelial cells. *Am. J. Physiol.* **222**:1071
6. Higgins, J.T., Cesaro, L., Gebler, B., Fromter, E. 1975. Electrical properties of amphibian urinary bladder epithelia. Inverse relationship between potential difference and resistance in tightly mounted preparation. *Pfluegers Arch.* **358**:41
7. Hong, C.D., Essig, A. 1976. Effects of 2-deoxy-D-glucose, amiloride, vasopressin, and ouabain on active conductance and E_{Na} in the toad bladder. *J. Membrane Biol.* **28**:121
8. Leaf, A., Macknight, A.D.C. 1972. The site of the aldosterone induced stimulation of sodium transport. *J. Steroid Biochem.* **3**:237
9. Macknight, A.D.C., Civan, M.M., Leaf, A. 1975. Some effects of ouabain on cellular ions and water in epithelial cells of toad urinary bladder. *J. Membrane Biol.* **20**:387
10. Macknight, A.D.C., Civan, M.M., Leaf, A. 1975. The sodium transport pool in toad urinary bladder epithelial cells. *J. Membrane Biol.* **20**:365

11. Macknight, A. D. C., Leaf, A., Civan, M. M. 1971. Effects of vasopressin on the water and ionic composition of toad bladder epithelial cells. *J. Membrane Biol.* **6**:127
12. Maffly, R. H., Steele, R. E., Walker, W. E., Coplon, N. S. 1973. Respiration and active sodium transport. (Abstr.) *Am. Soc. Nephrol.* p. 72
13. Mills, J. W., Ernst, S. A. 1975. The localization of sodium pump sites in frog urinary bladder. *Biochim. Biophys. Acta* **375**:268
14. Nellans, H. N., Finn, A. 1972. Oxygen consumption and Na transport in the toad urinary bladder. *Am. J. Physiol.* **227**:670
15. Ussing, H. H., Zerahn, K. 1951. Active transport of sodium as the source of electric current in the short-circuited isolated frog skin. *Acta Physiol. Scand.* **23**:110
16. Vieira, F. L., Caplan, S. R., Essig, A. 1972. Energetic of sodium transport in frog skin. I. Oxygen consumption in the short-circuited state. *J. Gen. Physiol.* **59**:60