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Metabolic Cost of Sodium Transport in Toad Urinary Bladder

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Summary. The metabolic cost of active sodium transport was determined in toad bladder at different gradients of transepithelial potential, $\Delta\psi$, by continuous and simultaneous measurements of CO_2 production and of transepithelial electric current. Amiloride was used to block active sodium transport in order to assess the nontransport-linked, basal, production of CO_2 and the passive permeability of the tissue. From these determinations active sodium transport, J_{Na}, and suprabasal CO_2 production, $J^{sb}_{CO_2}$, were calculated. Since large transients in J_{Na} and $J^{sb}_{CO_2}$ frequently accompanied any abrupt change in $\Delta\psi$, steady state conditions were carefully defined.

Some 20 to 40 min were required after a change in $\Delta\psi$ before steady state of transport activity and of CO_2 production were achieved. The metabolic cost of sodium transport proved to be the same whether the bladder expended energy moving sodium against a transepithelial electrical potential grandient of +50 mV or whether sodium was being pulled through "the active transport pathway" by an electrical gradient of −50 mV. In both cases the value of the ratio $J_{Na}/J^{sb}_{CO_2}$ averaged some 20 sodium ions transported per molecule of CO_2 produced.

When the Na pump was blocked by 10^{-2} M ouabain, the perturbations of the transepithelial electrical potential did not elicit changes of J_{Na} nor, consequently, of $J^{sb}_{CO_2}$.

The independence of the ratio $J_{Na}/J^{sb}_{CO_2}$ from $\Delta\psi$ over the range ± 50 mV indicates a high degree of coupling between active sodium transport and metabolism.

All animal cells engage in active transport, the movement of ions or uncharged solutes against an electro-chemical ptoential gradient. Such activity maintains cell volume and excitability. In transporting tissues such as intestinal or renal tubular epithelia, frog skin, and toad bladder, active transport of solutes has been studied and assessment of the metabolic costs of transport attempted. That a coupling of energy metabolism to active transport occurs has been repeatedly shown and, in fact, is a thermodynamic requirement. Until recently, however, methods have been inadequate to permit quantitative assessment of the constancy of such coupling.

In this study an attempt is made to assess the constancy of such coupling in toad bladder by measuring simultaneously the rate of CO_2 production by the tissue which is dependent on sodium transport and the rate of sodium transport. The transepithelial electrical potential was varied from +50 mV to −50 mV; sodium movement through the "active transport pathway" was measured together with the suprabasal rate of CO_2 production (the basal rate of CO_2 production is that produced by the bladder in the absence of transepithelial active sodium transport).

It was found that large abrupt changes in the transepithelial electrical potential triggered time-dependent changes in ion transport and in CO_2 production lasting 20 to 40 min. When a steady state of transport activity and CO_2 production were finally achieved, however, the metabolic cost of sodium transport proved to be the same whether the bladder was expending energy in moving the sodium against an electrical gradient of +50 mV or whether sodium was being pulled by a negative electrical potential gradient through the "active transport pathway" of the bladder. In both cases the number of sodium ions traversing the active transport pathway, J_{Na}, and the simultaneous suprabasal rate of CO_2 production $J^{sb}_{CO_2}$ were such that the ratios $J_{Na}/J^{sb}_{CO_2}$, were the same.

Materials and Methods

Preparation

Female toads (*Bufo marinus*) from the Dominican Republic were used (National Reagents, Inc., Bridgeport, Conn.). Hemibladders from doubly pithed toads were mounted between the two halves of a lucite chamber which exposed 9.3 cm^2 of bladder 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 edges of the bladder which greatly decreased the conductance increase from edge damage to the bladder.

After mounting, the half bladders were rinsed twice with Ringer's solution and CO_2-free air was bubbled continuously through the chamber. Experiments were started after an hour of equilibration in open circuit. The medium in all experiments was a bicarbonate-free phosphate Ringer's solution with a pH of 7.2 containing carbonic anhydrase. Antibiotics were added as described [4] to the Ringer's solution to suppress bacterial growth. No substrates were added to the medium. Bladders with initial open circuit transmural potentials less than +50 mV (serosa positive) were excluded.

Methods

For the electrical measurements an automatic voltage clamp was used to set the transepithelial electrical potential, $\Delta\psi$, in the range of ±100 mV. Ag/AgCl electrodes measured the transepithelial current and carefully balanced calomel electrodes monitored $\Delta\psi$.

Table 1. The effect of $\Delta\psi$ on the basal CO_2 production, $J^b_{CO_2}$, by toad bladder

$\Delta\psi$ (mV)	0	+50	−50
$\bar{x} \pm$ SEM	0.85±0.11	0.84±0.11	0.84±0.11
n=17			

Amiloride (0.1 mM) was added to the mucosal side when the voltage was clamped at $\Delta\psi = 0$. J_{CO_2} was recorded for at least 30 min before $\Delta\psi$ was sequentially varied for 15-min periods. Mean values are nmoles per mg dry wt per min.

A +10 mV pulse (serosa positive) of one sec duration was applied every 3 min to monitor the transepithelial conductance. A Keithley model 200 B voltmeter was used to measure $\Delta\psi$.

CO_2 production by the half bladders was measured continuously and simultaneously with the transepithelial transport as described by Al-Awqati, Beauwens and Leaf [1], and Canessa, Labarca and Leaf [4] based on a conductometric method developed by Maffly [8].

The current and CO_2 measurements used for the calculation of the sodium flux, J_{Na}, and rate of CO_2 production, J_{CO_2} were made during an interval of 2, 10 or 60 min in each experimental condition by integrating the area under the respective tracings. Correction was made for the lag in the arrival of CO_2 from the chamber to the conductometric cell. For every hemibladder the actual delay in the CO_2 recording was determined by injecting a pulse of CO_2 into the chamber and noting the interval until it was recorded. The lag was found to be 1 to 2 min.

Basal CO_2 Production

The basal CO_2 production rate, $J^b_{CO_2}$, was obtained at the end of each experiment by adding 0.1 mM amiloride to the mucosal medium. This concentration of amiloride promptly abolishes the active transepithelial transport of sodium and its associate CO_2 production, $J^{sb}_{CO_2}$. Previous studies had shown that the basal CO_2 production is very constant with time, decaying only 1 to 2% per hr. In 4 of the 17 experiments from which Table 1 was prepared, the three periods shown were followed by an additional 30 min with $\Delta\psi = 0$. The decline in $J^b_{CO_2}$ averaged 1.4% per hr over the 90 min of measurement. To determine the possible effect of $\Delta\psi$ on basal CO_2 production, 17 half bladders were short-circuited for one half hr after which 0.1 mM amiloride was added to the mucosal medium to abolish active sodium transport, $J_{Na} = 0$, and its associated CO_2 production, $J^{sb}_{CO_2} = 0$. Once a steady state of $J^b_{CO_2}$ was obtained at 0 mV, the transepithelial potential was clamped at ± 50 mV during 15 min periods. As shown in Table 1, no effect of $\Delta\psi$ on $J^b_{CO_2}$ was observed. Furthermore, in 7 paired half bladders with one half bladder maintained at +50 mV and the other at −50 mV for one hr, the subsequent mean values for $J^b_{CO_2}$ averaged 0.62±0.44 and 0.62±0.28 nmoles per (mg dw) per (min), respectively. Thus, the actual value of basal CO_2 production is not affected by the conditions of the experiments and, therefore, the value of sodium transport dependent CO_2 production (or suprabasal CO_2 production, $J^{sb}_{CO_2}$) is $J^{sb}_{CO_2} = J^{Total}_{CO_2} - J^b_{CO_2}$ for $-50\text{ mV} < \Delta\psi < +50\text{ mV}$.

Active Sodium Transport at Different $\Delta\psi$

The total electric current flowing across the toad bladder, I_T, at any $\Delta\psi$ is $I_T = J_{Na}F - K_p\Delta\psi$ where $J_{Na}F$ is the current due to sodium transport through the active pathway,

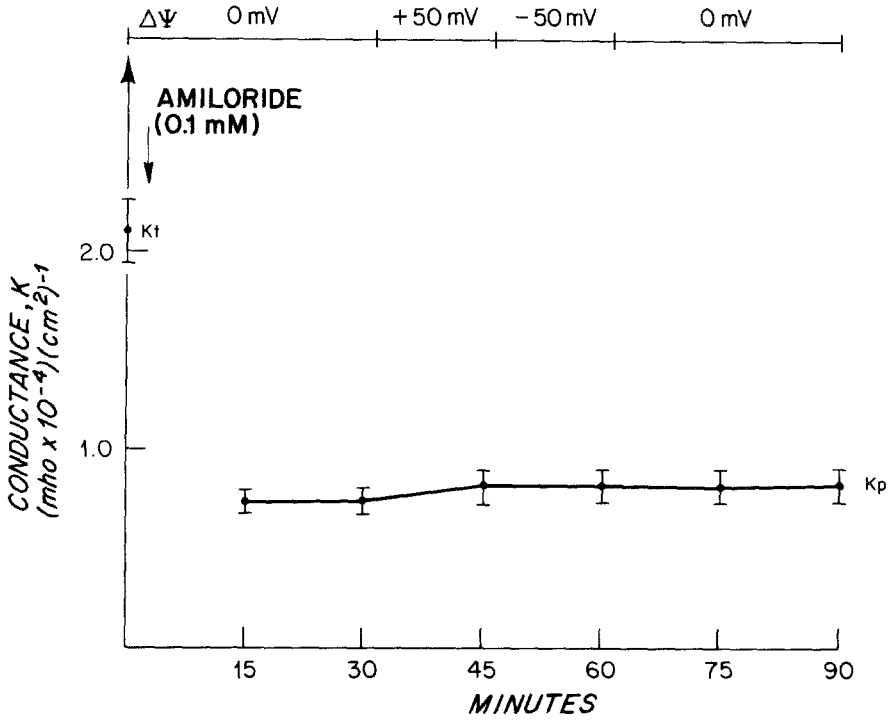

Fig. 1. The effect of transepithelial potential changes on the passive conductance, K_P, across the toad bladder. The mean total transepithelial conductance, K_T, is shown on the ordinate. At zero time amiloride (0.1 mM) was added to the mucosal medium to block sodium transport thus reducing K_T to K_P, the passive conductance. K_P was constant and unaffected by $\Delta\psi$ over the subsequent 90 min of observation. (n=19)

since only sodium is transported actively by this tissue [6], F is the faraday, K_p is the conductance through the passive pathways and $\Delta\psi$ is the transepithelial electrical gradient, defined as positive when the serosal side of bladder is positive to the mucosal surface. Then $J_{Na}F=I_T+K_p\Delta\psi$. Since K_p was measured only at the end of each experiment when J_{Na} was abolished by amiloride, any change in K_p with time will introduce error in the estimate of J_{Na} and will be larger if the ratio of K_p/K_T is high at the start of an experiment. The following precautions were, therefore, taken in order to avoid experimental error introduced by change (generally increase) in K_p:

1. Silicone grease, as described, was used as sealant to minimize edge damage. Previous studies [4] showed that this decreases the K_p/K_T ratio.

2. Bladders with K_p/K_T ratios greater than 0.5 at the end of an experiment were eliminated.

With these precautions a further set of control observations were made on 19 hemibladders which were mounted carefully in the chambers, washed twice with Ringer's solution, left one hr open-circuited while aerated with CO_2-free air, and then short-circuited during 30 min. Amiloride 0.1 mM was then added to the mucosal medium to block sodium transport. After a further 30 min, the transepithelial potential was clamped at +50 and −50 mV sequentially for 15 min each and finally returned for 30 min again to $\Delta\psi=0$. Throughout these observations transepithelial conductance was determined as described. The results

Table 2. The effect of changing $\Delta\psi$ at 10-min intervals on the ratio $J_{Na}/J^{sb}_{CO_2}$ in toad bladder

Δ (mV)	0	+50 mV	−50 mV	0
$J_{Na}/J^{sb}_{CO_2} \pm$SEM	41.6±7.8	21.7±3.1	39.2±8.2	22.4±4.3
$\Delta \pm$SE		19.8±4.8	17.4±5.6	16.8±3.9
p		<0.005	<0.025	<0.005
(n=8)				

The hemibladders were kept in open-circuit prior to the changes of $\Delta\psi$ which were applied in the sequence $\Delta\psi$=0, +50, −50, 0 mV. J_{Na} and $J^{sb}_{CO_2}$ were calculated by integration of the last 2 min of the 10 min periods of recording.

are shown in Fig. 1, in which it can be seen that only a small increase of some 7.3% in K_p occurred during the 90 min of observation. In these 19 control studies the K_A/K_T ratio averaged 0.64.

Results

It became apparent early in these studies that with large, abrupt changes in transepithelial electrical potential, $\Delta\psi$, transient changes occured in both electrical current and CO_2 production before steady state conditions were achieved. Undetected, these transients would have seriously altered the conclusions of this study. Table 2 shows the results, which we now regard as spurious, obtained before steady state conditions were established. In these experiments bladders were maintained in the open circuited state until the voltage clamp set the values of $\Delta\psi$ for 10 min each sequentially at 0 mV, +50 mV, −50 mV, and back to 0 mV. Integration of the area under the current and CO_2 tracings for the last 2 min or each 10-min period yielded the results presented in Table 2. Note the high stoichiometric ratios obtained, the decline in the ratio when sodium transport was opposed by +50 mV, and increase when transport was favored by −50 mV. When short-circuit current conditions were reestablished the value for $J_{Na}/J^{sb}_{CO_2}$ had fallen to approximately half its initial value. Examination of Fig. 2 shows the transient changes in J_{Na} and J_{CO_2} that occurred with each change of potential. Without the capability of measuring J_{Na} and J_{CO_2} simultaneously and continuously the nonsteady state nature of such measurements might easily be overlooked.

Similar experiments were repeated in another set of bladders except that the periods of voltage clamping were prolonged to 15 min rather than only 10 min. Values were obtained during the last 2 min of each 15 min period. Table 3 shows the results in these 8 experiments. Now

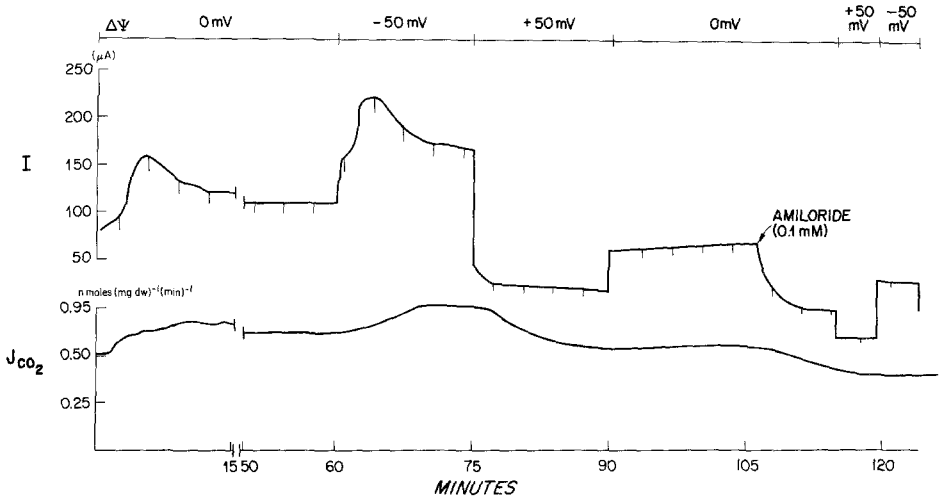

Fig. 2. The effect of transepithelial potential changes, $\Delta\psi$, on transport, I, and metabolism, J_{CO_2}, of toad bladder. This is a representative tracing of simultaneous transport and metabolic activity of toad bladder. Note that changes in J_{CO_2} commenced promptly with changes in $\Delta\psi$ but were slow to reach stable values. Changes in I, however, reached stable values either slowly as initially at 0 mV and -50 mV, or promptly as seen with $+50$ mV and on return to 0 mV. The small deflections of I at 3-min intervals are the current responses to the one-sec pulses of $+10$ mV. From these pulse responses transepithelial conductance was calculated. The addition of amiloride blocks active sodium transport thus reducing I to zero and J_{CO_2} to $J^b_{CO_2}$. The application of ± 50 mV across the bladder after amiloride allows determination of that portion of I occurring via passive pathways

Table 3. The effect of changing $\Delta\psi$ at 15 min intervals on J_{Na}, $J^{sb}_{CO_2}$, and $J_{Na}/J^{sb}_{CO_2}$ in toad bladder

	0	+50 mV	−50 mV	0
J_{Na}	8.1 ± 0.9	3.9 ± 0.47	9.1 ± 0.97	4.2 ± 0.44
$J^{sb}_{CO_2}$	0.4 ± 0.05	0.24 ± 0.03	0.37 ± 0.04	0.22 ± 0.03
$J_{Na}/J^{sb}_{CO_2}$	20.6 ± 2.1	18.4 ± 2.2	24.4 ± 2.4	20.2 ± 1.6
Δ (n=8)	2.2 ± 1.6	6.0 ± 3.2	4.2 ± 1.9	

The numbers are mean values ± SEM
J_{Na} and $J^{sb}_{CO_2}$ are in nmoles per mg dry wt per min.

the ratio $J_{Na}/J^{sb}_{CO_2}$ has the same value during short circuiting at the start and end of the observations and the values at $\Delta\psi = +50$ or -50 mV do not differ significantly from each other or from the ratios obtained during short-circuiting. This apparent constancy of the ratio, despite

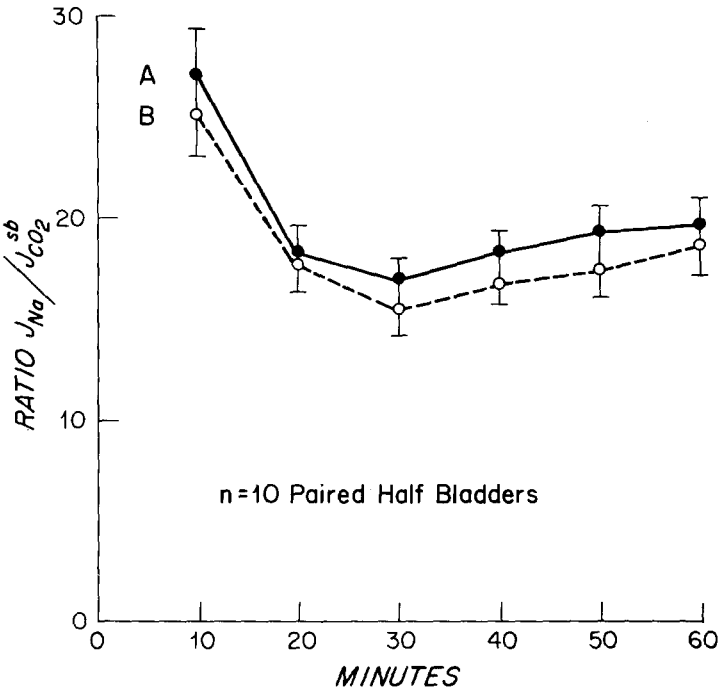

Fig. 3. The ratios of $J_{Na}/J^{sb}_{CO_2}$ during one hr of short-circuiting ($\Delta\psi = 0$) in paired hemibladders, *A* and *B*. Note the significant drop in the ratio that occurs after 10 min. The ratios measured between 20 and 60 min showed no significant differences

the range of $\Delta\psi$, was achieved in the face of significant changes in sodium transport and CO_2 production, shown also in Table 3.

The effect of time on the ratio $J_{Na}/J^{sb}_{CO_2}$ was examined further in 7 paired half bladders in which measurements were made (after one hr with bladders short-circuited) first for 10 min and then for 15 min at $\Delta\psi = \pm 50$. The sequence of potential change was reversed in each pair. Again the results showed significant changes in the ratio $J_{Na}/J^{sb}_{CO_2}$ with the applied potentials at 10 min, whereas no significant differences were observed at 15 min—as seen in initial experiments in Table 3. Furthermore, the order in which the plus or minus 50 mV was applied did not affect the results.

With such clear evidence that time-dependent perturbations were affecting our results, the changes in $J_{Na}/J^{sb}_{CO_2}$ with time were examined.

Fig. 3 shows the time course of this ratio in paired, short-circuited half bladders during a 60-min period. The results were very similar in the paired bladders, and, although lowest values were obtained at 30 min, no statistically significant changes occurred from 20 to 60 min. In a

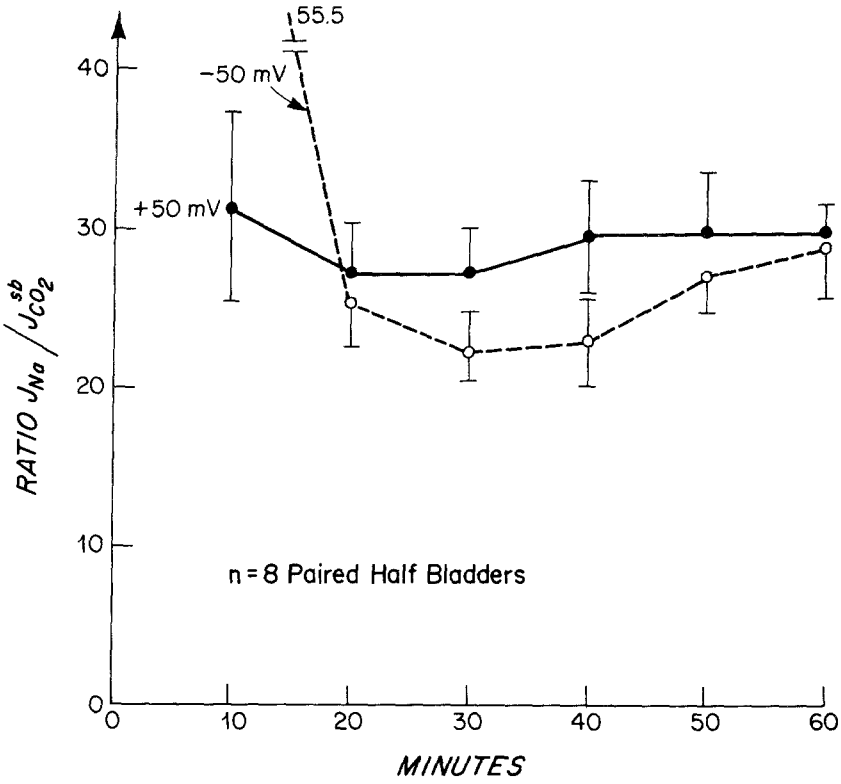

Fig. 4. The ratios of $J_{Na}/J_{CO_2}^{sb}$ during one hr in paired half bladders with tranepithelial potential clamped at +50 mV in one half and −50 mV in the other. Note that only after 20 min did the ratio approach constant values

few experiments the period of short-circuiting was extended to 135 min and the mean value for the ratio $J_{Na}/J_{CO_2}^{sb}$ showed no further significant change (averaging 20.0 ± 1.2, 21.1 ± 1.6, and 22.5 ± 1.3 at 60, 90, and 135 min, respectively).

With this knowledge regarding the similarity of response of paired half bladders, the time course of $J_{Na}/J_{CO_2}^{sb}$ was examined. One half bladder was maintained for 60 min with $\Delta\psi = +50$ mV while its paired half was kept for the same time at $\Delta\psi = -50$ mV. The results are shown in Fig. 4. It can be seen that after the first 20 min the ratio of $J_{Na}/J_{CO_2}^{sb}$ was statistically the same whether the half bladder had to do work in moving sodium against an electrical potential of +50 mV or whether sodium moved through the active transport pathway down an electrical gradient of −50 mV.

In addition to examining the effects of $\Delta\psi$ on transport and metabolism of paired half bladders with one half exposed to +50 mV and

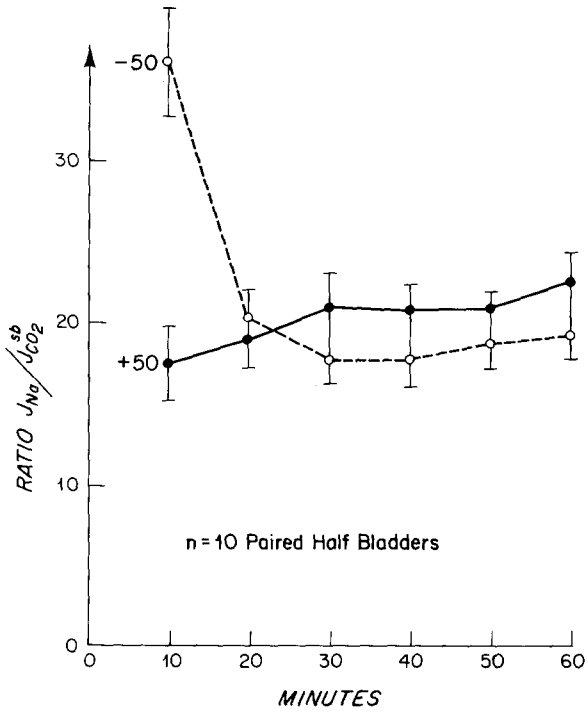

Fig. 5. The ratios of $J_{Na}/J_{CO_2}^{sb}$ during two periods of one hr in paired half bladders with each bladder exposed first to +50 mV and then to −50 mV while the transepithelial potentials were applied in reverse order to the paired half bladder. Note that again steady values of the ratio were attained by 20 min

its paired half to −50 mV for 60 min as shown in Fig. 4, a further series of experiments was performed in which one half bladder was exposed to 60 min at +50 mV followed by a second 60 min at −50 mV. The paired half bladder was exposed similarly to two 60 min periods but with the order of the potentials reversed. The results of this series of 10 paired half bladders are shown in Fig. 5. Although the plus and minus 50 mV potentials were applied sequentially in each half bladder the data are presented in this figure in the same manner as that shown in Fig. 4. Note the striking similarity in the time course of these separate measurements. The values for the calculated ratio $J_{Na}/J_{CO_2}^{sb}$ in the first set of experiments of Fig. 4 are higher on the average than that seen in any of the other studies. Aside from the fact that this series used a single shipment of toads, we have no definite explanation for the high values obtained in this set of experiments.

Examination of Figs. 4 and 5 suggests that the initial high ratios at $\Delta\psi = -50$ mV are compensated later by ratios which tend to be lower

Table 4. Comparison of $J_{Na}/J^{sb}_{CO_2}$ calculated for the total 60-min period and for the last 10 min of the hr

	60 min	last 10 min	Δ
A. At $\Delta\psi = 0$ mV			
$\bar{x} \pm$ SEM (n= 20)	19.2 ± 0.9	19.6 ± 0.9	0.32 ± 0.79
B. At $\Delta\psi = -50$ mV			
$\bar{x} \pm$ SEM (n= 8)	28.5 ± 1.8	28.5 ± 2.1	0.75 ± 0.88
At $\Delta\psi = +50$ mV			
$\bar{x} \pm$ SEM (n= 8)	29.8 ± 3.2	29.9 ± 3.5	0.05 ± 1.5
C. At $\Delta\psi = -50$ mV			
$\bar{x} \pm$ SEM (n= 10)	21.7 ± 1.8	19.8 ± 1.6	1.87 ± 0.65
At $\Delta\psi = +50$ mV			
$\bar{x} \pm$ SEM (n= 10)	19.8 ± 1.6	22.5 ± 1.8	2.99 ± 1.5

than for the values obtained at $\Delta\psi = +50$ mV. This possibility was examined by integrating the area under the curves for J_{Na} and for $J^{sb}_{CO_2}$ for the full 60 min and comparing the resulting ratio with that obtained by integrating the area under the curves for just the last 10 min of each hr. The results of these comparisons are shown in Table 4. Clearly from examination of Fig. 3, one would expect similar values for the entire hour and the last 10 min of each study. However, very good agreement was found as well for Fig. 4 as shown in Part B of Table 4 in spite of the unusually high values, as noted, for the ratios in these experiments. Similarly for Fig. 5, as seen in Table 4, Part C, the agreement is satisfactory. This does suggest that the transients in sodium transport and CO_2 production are real and indicates that the last 10 min of the hour period represent steady state conditions. Perhaps even more striking and significant for the present study is the excellent agreement in Parts B and C of the ratios $J_{Na}/J^{sb}_{CO_2}$ calculated for the entire 60-min periods at $\Delta\psi = -50$ mV with the value in the paired or same tissue for the entire period at $\Delta\psi = +50$ mV. The values are 28.5 and 29.8 for Part B and 21.7 and 19.8 for Part C, respectively. This is further assurance that the metabolic cost of sodium transport is the same whether sodium is transported by the bladder against an electrical potential of +50 mV

Table 5. Effect of changing $\Delta\psi$ on J_{Na} and $J^{sb}_{CO_2}$ in toad bladder

$\Delta\psi$	+50 mV		−50 mV	
Expt. No.	J_{Na}	$J^{sb}_{CO_2}$	J_{Na}	$J^{sb}_{CO_2}$
1	123.6	6.1	389.9	12.4
2	65.0	3.9	208.4	12.7
3	77.4	3.5	99.7	5.0
4	94.6	5.6	446.8	17.4
5	334.3	18.0	319.5	19.2
6	201.6	13.5	275.2	14.0
7	54.2	4.3	176.9	13.6
8	212.7	9.6	360.5	14.1
9	161.3	6.3	275.4	10.5
10	60.9	2.6	154.6	7.1
$\bar{x} \pm$ SEM	139 ± 28.4	7.3 ± 1.6	270 ± 35.1	12.6 ± 1.4
$\bar{\Delta}J_{Na} = 132 \pm 34$			$\bar{\Delta}J^{sb}_{CO_2} = 5.3 \pm 1.2$	

$\Delta\psi$ was maintained at either +50 or −50 mV for 60 min and then changed to −50 or +50 mV, respectively, for the second hr. Values for J_{Na} and $J^{sb}_{CO_2}$ are all expressed as nmoles per mg dry wt per hr.

or whether the transepithelial potential gradient of −50 mV assists in drawing sodium through the transport channels of the bladder.

If the changes in transepithelial potential difference were having little effect on J_{Na}, then equality of the ratios of $J_{Na}/J^{sb}_{CO_2}$ at plus and minus 50 mV might be expected. Table 5 shows, however, that for the experiments illustrated in Fig. 5 the changes in $\Delta\psi$ significantly affected both J_{Na} and $J^{sb}_{CO_2}$. The ratio of average J_{Na} at +50 mV to that at −50 mV is 0.51, while the ratio for the corresponding values of the average $J^{sb}_{CO_2}$ were 0.58, respectively. Changes of similar proportions occurred in the results of experiments shown in Fig. 4 in which $\Delta\psi$ was +50 mV for 60 min in one half bladder and −50 mV for the same time in the paired half bladder. The ratio of J_{Na} was 0.67 and of $J^{sb}_{CO_2}$ 0.66 in that series of experiments. Thus the changes in $\Delta\psi$ had the large expected effects on the magnitude of J_{Na} but $J^{sb}_{CO_2}$ was similarly affected to maintain a constant ratio of $J_{Na}/J^{sb}_{CO_2}$.

Since movement of sodium through the "active transport" pathway of the mucosal cells exacts its toll of metabolic energy irrespective of whether sodium is moved "up hill" or "down hill" electrically, it was of interest to see if sodium could still move "down hill" through the active transport pathway if metabolic energy were excluded from the

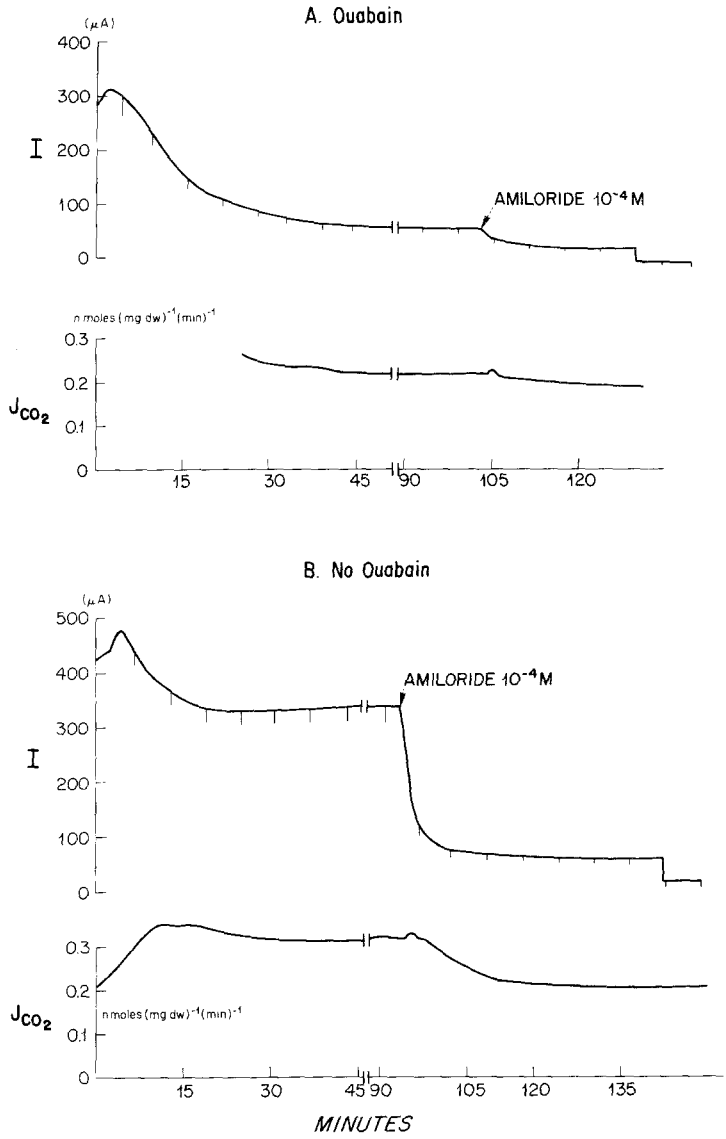

Fig. 6. The effect of a transepithelial potential of -50 mV on paired half bladders in the presence (*A*) or absence (*B*) of ouabain (10^{-2} M). Note that ouabain markedly inhibited both I and J_{CO_2}. In fact the reductions occurred proportionately so that the ratio $J_{Na}/J^{sb}_{CO_2}$ remained constant. In the ouabain treated half bladder the ratio calculated for the interval just before addition of amiloride was 29.7. In the absence of ouabain the ratio determined for the comparable time interval was 31.2. In spite of the transepithelial potential favoring sodium movement across the bladder, therefore, the action of ouabain blocks movement of sodium through the active transport pathway

sodium pump. Ouabain was used to block the sodium, potassium adenosine triphosphatase of the bladder and thus to exclude ATP energy utilization by the pump. In the presence of 10^{-2} M ouabain in the serosal medium and with $\Delta\psi = -50$ mV, J_{Na} fell to zero or to a very small value. Subsequent addition of amiloride (0.1 M) had no effect on J_{Na} or J_{CO_2} in two experiments and produced a small reduction in each in a third study. This last experiment is shown in Fig. 6. This indicates that inhibition of ATP hydrolysis at the sodium pump closes the active pathway to the movement of sodium even when a negative electrical gradient favors sodium movement across the epithelium.

Discussion

The development by Maffly [8] of a method for continuous measurement of CO_2 production by the isolated toad urinary bladder has allowed assessment of metabolism over periods of several hours and its relation to the transport activity of the tissue. Previous studies have indicated that the basal rate of CO_2 production, $J^b_{CO_2}$, changes very little with time with the bladder short-circuited [1, 4]. It is further demonstrated in Table 1 that $J^b_{CO_2}$ obtained by blocking sodium transport by amiloride is unaffected by changes in transepithelial electrical potential, $\Delta\psi$, imposed on the tissue in these studies. Thus $J^{sb}_{CO_2}$, the rate of CO_2 production related to ion transport, can be obtained in each experiment by subtracting the value of $J^b_{CO_2}$ determined at the end of each experiment from the total rate of CO_2 production during an experiment.

It was equally essential to have a means of continuously measuring ion transport by the bladder throughout the imposed changes in the transepithelial electrical potential, $\Delta\psi$. Double isotope experiments with ^{22}Na and ^{24}Na to measure the bidirectional fluxes of sodium across the tissue are the accepted method to determine net sodium transport, J_{Na}. However, this method is cumbersome, would not have permitted the prolonged periods of observation required here, would have necessitated sampling of bathing medium which would have upset the measurements of CO_2 production, and would not have afforded instantaneous and simultaneous comparisons to be made of J_{Na} with J_{CO_2}. Therefore, an electrical monitoring of transepithelial ion transport was utilized.

It is, of course, only with the bladder short-circuited ($\Delta\psi = 0$) that the electric current across the bladder just equals the net sodium transport [6]. However, the urinary bladder of *Bufo marinus* from the Dominican

Republic apparently transports only sodium actively [6] and this can be quickly, completely, and reversibly blocked by amiloride, as used in these studies [2]. It has been shown by Hong and Essig [5] that the transepithelial ion flux across the bladder in the presence of amiloride represents passive movement probably via paracellular channels. In the experiments reported here the residual conductance, K_p, in the presence of amiloride represented only 30 to 40% of the total conductance across the bladder wall. As shown in Fig. 1, furthermore, the value of K_p was not affected by the value of $\Delta\psi$. This and the absence of any affect of $\Delta\psi$ on J_{CO_2} in the presence of amiloride, Table 1, supports the completeness of blockage of J_{Na} and the absence of active transport of any other ion species under the conditions of this study.

By measuring the electrical current at the end of each experiment in the presence of amiloride at the values of $\Delta\psi$ used during the experiment, and subtracting this current from the total current obtained during the experiment, electrical current attributable to sodium moving through the active pathway was estimated. We have thus designated this electrical current, which is in excess of that obtained with amiloride, to be $J_{Na}F$. Wolff[1] has found that net sodium flux accounts for all the amiloride-inhibitable current over the range of $\Delta\psi$ from 0 to +50 mV.

Because of the possible, deleterious effects of imposing arbitrary values of $\Delta\psi$ across the bladder for prolonged periods, and to mimic conditions of studies used recently by others (12) who monitored oxygen consumption, initial experiments involved changes in $\Delta\psi$ at 10 min intervals. Results were somewhat erratic but did seem to show a higher metabolic cost when sodium transport was proceeding against an electrical potential gradient of +50 mV than when transport of sodium was "down hill" by −50 mV, as seen in Table 2. These differences, however, disappeared with time; they result from nonsteady state conditions which may occur after any large abrupt perturbation of $\Delta\psi$. Transient changes in J_{CO_2} seemed to occur invariably while transients in J_{Na} were less constant, as seen in Fig. 2. However, the likely occurance of such time dependant perturbations make invalid the assessment of the ratio of $J_{Na}/J^{sb}_{CO_2}$ over short periods following changes in $\Delta\psi$. In the present study the agreement found at the end of 60 min in $J_{Na}/J^{sb}_{CO_2}$ in the same or in paired half bladders with $\Delta\psi$ clamped at either +50 or −50 mV and the agreement in the ratio calculated for the entire 60 min with that calculated for the last 10 min of the hr, as seen in Table 4, all give assurance that steady state conditions were achieved in this study.

[1] D. Wolff, 1976. Isotope interaction in the Na active pathway of the toad bladder (*personal communication*).

Methods for measuring oxygen consumption, recently reported, have used short periods of observation [9, 12]. Furthermore, since measurement of oxygen consumption can only be made discontinuously over periods of time sufficiently long to allow measurement of the rate of disappearance of oxygen from the medium bathing the bladder, the occurrence of the metabolic transients would not have been observed. Vieira *et al.* [12] recognized the need for steady state conditions to exist in order to interpret their studies on the dependence of oxygen consumption and $\Delta\psi$. Since the early changes in metabolism may appear to be linear over short time intervals, as seen in Fig. 2, one may be misled by such apparent linearity into thinking steady state conditions have been achieved. However, by following CO_2 production and J_{Na} simultaneously and continuously from periods of 60 min or longer in our studies, it is evident that steady state conditions of transport and metabolism are achieved only some 20 to 40 min following large perturbation of $\Delta\psi$. This is true even in going from open circuit to short circuit conditions. The good agreement shown in Figs. 4 and 5 between $J_{Na}/J^{sb}_{CO_2}$ at the end of 60 min with $\Delta\psi$ changed at either $+50$ (a value close to the physiologic open circuited state) or -50 mV, indicates that the tissue is not damaged by prolonged voltage clamping.

In an earlier study [1] it was estimated that a lag of some 4 min might exist between a change in rate of CO_2 production in the bladder and its recording by the apparatus used. This time includes the one to two min instrumental lag determined for each experiment. The time-dependent perturbations after changes in $\Delta\psi$ are, therefore, not attributable to the method of measurement, but probably represent real metabolic perturbations. Whatever the basis of these early transients may be, the fact that such good agreement exists when the ratio $J_{Na}/J^{sb}_{CO_2}$ measured over the entire hr is compared with the ratio calculated from the measurements over just the last 10 min of the hr, indicates that the early transients are compensated for later. At least during the last 10 min of these hr periods, steady state conditions were achieved.

Zerahn [13] had measured oxygen consumption of frog skin during open-circuit and short-circuit and found the ratio of $J_{Na}/J^{sb}_{CO_2}$ to be the same under the two conditions. His early findings are thus similar to what we have found, but his results were determined over a more limited range of $\Delta\psi$. Nellans and Finn [9] measured oxygen consumption of toad bladder as a function of transepithelial gradients of electrical potential and sodium concentration. They found the ratio of J_{Na} to suprabasal oxygen consumption to increase with J_{Na} to maximal values of 20 to 25 sodium ions transported per molecule of oxygen consumed. The latter

values are similar to those reported here but the authors concluded that sodium transport is not tightly coupled to metabolism because of the low values of the ratio in some tissues with low rates of sodium transport. Such low ratios may result from the residual values of suprabasal oxygen consumption found with $J_{Na}=0$ in their regression of $J^{sb}_{O_2}$ on J_{Na}. Our experiments showed no such intercept, and therefore we regard their low values as artefactual; perhaps some measurements were not made under steady state conditions.

It has been suggested that in leaky epithelia, such as intestinal mucosa [10] or renal proximal tubule [3], the ion which is actively transported may not actually "see" the $\Delta\psi$ which may be dominated by what occurs in the passive leak pathway. Thus imposed changes in $\Delta\psi$ may have little, if any, influence on the magnitude of the potential steps the ion must cross at the luminal and at the basolateral plasma membranes of the transporting cells. Such is not the case with a "tight" epithelium as the toad bladder, as indicated by the changes induced in the residual or amiloride-inhibited current, designated $J_{Na}F$. Tables 3 and 5 show that J_{Na} was significantly affected in the appropriate direction by the imposed values of $\Delta\psi$ and that $J^{sb}_{CO_2}$ was also proportionately affected. The constancy of $J_{Na}/J^{sb}_{CO_2}$ at different values of $\Delta\psi$ occurred with significant changes in ion transport by the bladder.

It has recently been demonstrated [4] that sodium from the serosal medium does not reflux back into the cells nor recycle through the active transport mechanism located in the basolateral plasma membranes of the mucosal epithelial cells. The present study indicates that sodium cannot move from mucosa to serosa through this layer of cells without a complete accompaniment of metabolic activity, even if the driving force for the movement of the sodium is, at least in part, provided by the imposed $\Delta\psi$. It had been our initial expectation that fewer sodiums would be transported per CO_2 produced with $\Delta\psi$ positive and more with $\Delta\psi$ negative.

It may be noted that in the previous study (4) $J_{Na}/J^{sb}_{CO_2}$ was not affected by removal of all Na from the serosal medium. Thus the metabolic cost of sodium transport across the toad bladder is not altered either by an electrical gradient or by a chemical gradient which favors the "down-hill" movement of sodium across this tissue.

Furthermore the dependence of sodium movement through this pathway on metabolic energy appears to be absolute. When the use of metabolic energy was excluded from the sodium pump no sodium could move through the active transport pathway even with external

electrical gradients favoring such movement. Ouabain blocks the Na, K-ATPase of toad bladder and J_{Na}; therefore ouabain was used to exclude ATP hydrolysis at the pump site. In the presence of ouabain J_{Na} and $J^{sb}_{CO_2}$ both fell to zero or near zero despite a $\Delta\psi$ of -50 mV indicating that, when the use of metabolic energy is impeded; sodium ions cannot be pulled through the active transport pathway by an external electrical gradient. The interpretation of these experiments must be qualified since the action of ouabain may be to block the pump and hence the splitting of ATP, rather than to block the supply of ATP to the pump.

Over the range studied, $+50$ to -50 mV, the ratio was found constant and thus the coupling of sodium transport to metabolism is obligatory and fixed. The constancy of this ratio in any single tissue indicates that a true stoichiometry exists between the energy cost for sodium transport irrespective of the work required in the transport process. At $J_{Na}/J^{sb}_{CO_2}$ of 18 to 20 some 3 sodium ions are transported per molecule of ATP hydrolyzed to ADP assuming a respiratory quotient for the tissue of 1.0.

In order to minimize the likelihood of bacterial growth in the bathing medium during a long experiment, no substrate was provided to the bladder. Thus the respiratory quotient could have varied from 0.7, if fat were the endogenous substrate, to 1.0, if glycogen were being utilized by the isolated tissue [11]. Thus a difference in the metabolic substrate being utilized could have affected $J^{sb}_{CO_2}$ by as much as 30%. This could have accounted for a significant portion of the differences observed in the ratio of $J_{Na}/J^{sb}_{CO_2}$ from bladder to bladder. Previously we had thought that the differences in the ratio $J_{Na}/J^{sb}_{CO_2}$ from paired half bladders was considerably less than that from the bladder of one toad to the next [1]. The large spread in the ratio observed from toad to toad we thought, therefore, to be real. In the present experiments (with improved technique) the range of values of the ratio in individual toads is considerably narrowed. Further, in examining by a partial analysis of variance differences between paired half bladders with those between bladders from different toads, it was found that though half bladders from the same animal had a somewhat smaller variance than that from bladders from different toads, the differences were not significant. This increases the likelihood that a single value of $J_{Na}/J^{sb}_{CO_2}$ prevails in this tissue and justifies, therefore, the calculation and presentation of average values. Perhaps providing exogenous substrate, so that respiratory quotients in different bladders are the same, might further reduce the range of experimental values. Our average figure, of 18 to 20 sodium ions transported

per molecule of CO_2 produced, is similar to that found earlier by Zerahn [13] and by Leaf and Renshaw [7] who measured sodium transport and oxygen consumption by frog skin.

The complete coupling of sodium transport to a supply of metabolic energy is demonstrated by the constancy of $J_{Na}/J^{sb}_{CO_2}$ over the range of $\Delta\psi$ studied. The need for an expenditure of metabolic energy when J_{Na} occurs against a $\Delta\psi$ of $+50$ mV is clear. The consumption of a similar amount of metabolic energy when sodium is pulled "down hill" through the transport pathway by the external electrical gradient is the inevitable consequence of the complete coupling of sodium transport to metabolism. ATP could determine the rate of sodium movement through the pump in the sense that the number of sodium ions that can move for each ATP hydrolyzed is fixed over the range of $\Delta\psi$ studied.

Although the ratio $J_{Na}/J^{sb}_{CO_2}$ was found to be the same over the range of $\Delta\psi$ tested, it is not proven that such will invariably be found. If net sodium transport can occur against much higher potentials than $+50$ mV, and spontaneous open circuited potentials of 130 mV have been observed, then the stoichiometric ratio may have to fall.

The determination of the degree of coupling between metabolism and active Na transport in toad bladder will be the subject of a future communication.

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