J. Membrane Biol. 22, 91–105 (1975)

Coupling of Sodium Transport to Respiration in the Toad Bladder

Qais Al-Awqati*, Renaud Beauwens* and Alexander Leaf

Massachusetts General Hospital and Harvard Medical School,
Boston, Massachusetts 02114

Received 31 October 1974; revised 20 January 1975

Summary. Energy expenditure and transepithelial sodium transport were measured continuously and simultaneously from isolated urinary bladders of the Dominican toad, *Bufo marinus*. Sodium transport was measured as the short-circuit current and CO_2 produced by the bladder was measured conductometrically by the method of Maffly. The rates of sodium transport and CO_2 production were linearly related. The slope of the regression of sodium transport on CO_2 production, dJ_{Na}/dJ_{CO_2}, was found to be quite similar in paired half bladders but to differ significantly between bladders from different toads. Thus, in this preparation there appears to be no unique stoichiometric ratio characterizing sodium transport and metabolism and past efforts to arrive at such a value by averaging results obtained from different animals do not seem warranted. The CO_2 production by the isolated bladder which is unrelated to sodium transport was determined by two means: 1) extrapolating the regression of J_{Na} on J_{CO_2} to $J_{Na=0}$, and 2) measuring CO_2 production with sodium transport suppressed by removal of all sodium from the mucosal bathing medium. The two methods gave values which were in close agreement in each preparation. This suggests that metabolism which supports nontransport activities in this tissue cannot be recruited to support the energy requirement of sodium transport and vice versa.

The coupling between transepithelial sodium transport and metabolism has been investigated by measuring transport activity and oxygen consumption in several tissues [4, 7, 8, 11, 12, 17]. The techniques used for measuring transport and metabolism allowed only mean values of each to be used to estimate the energy cost of sodium transport. This yielded, of necessity, only average values for this "stoichiometry". Recently, Vieira, Caplan and Essig [16] using a method which allowed simultaneous and continuous examination of oxygen consumption and sodium transport demonstrated that individual frog skins have different "stoichiometric ratios".

* *Present Address:* Department of Internal Medicine, University of Iowa, Iowa City, Iowa 52242.

The present study reports initial observations using a method, devised by Maffly *et al.* [10], for making continuous measurements of CO_2 production simultaneously with sodium transport by toad bladders. The coupling of sodium transport to metabolism can thus be examined in detail in individual tissues under a variety of conditions.

It was found that while the sodium transport per CO_2 produced was constant in each bladder and similar in paired half bladders, no unique value characterized this relationship when the result from one animal was compared with that from others. The metabolism of the bladder unrelated to sodium transport was estimated by two methods and the two values were found in good agreement when both were determined in the same tissue.

Materials and Methods

Female specimens of *Bufo marinus*, obtained from the Dominican Republic (National Reagents, Bridgeport, Conn.), were kept at room temperature on moist pine shavings. Hemibladders from doubly pithed toads were mounted in lucite chambers which were constructed to give large exposed areas of the membrane (9.1 cm^2) and small volumes (3 ml on each side).

The membrane was bathed by a modified Ringer's solution which contained (in mmoles/liter): NaCl 113, KCl 3.5, Na_2HPO_4 2, $CaCl_2$ 1. The pH of the Ringer's was adjusted to 7.0 by titration with 1 N HCl. In some experiments the NaCl in this medium was substituted by choline chloride or magnesium chloride.

After each experiment the exposed area of the bladder was cut and its weight determined after drying in a hot air oven at 90 °C overnight (mean dry weight $\pm$ SD was 25.3 mg $\pm$ 8). The spontaneous transepithelial potential difference was sensed by 3 M KCl-agar bridges and balanced calomel half-cells connected to a Keithley model 600 B voltmeter. An automatic voltage clamp supplied enough current to nullify this spontaneous p.d. via Ag-$AgCl_2$ electrodes and KCl-agar bridges. The short-circuit current was read from a Weston d-c microammeter.

Continuous Measurement of Total CO_2

The total CO_2 produced by the toad bladder was measured according to the method of Maffly *et al.* [10] (Fig. 1). Compressed air, or 20% O_2 in N_2 was passed through two tall ($\sim$1 meter) columns of 3 M NaOH or KOH. The resulting CO_2-free air was introduced into both sides of the chamber at a rate of 80–100 ml/min. The effluent air was collected and passed into the CO_2 measuring apparatus. This consisted of a large $3\frac{1}{2}$ liter plastic beaker containing 1 mM NaOH or $Ba(OH)_2$ which was continuously stirred by a rotating magnet. On a platform, a long (5 meters) double spiral glass coil was placed. The bulk medium was aspirated by a Buchler polystaltic pump (model 2–6100, Buchler Instruments, Chicago, Illinois) through a flow-through conductivity cell (model CDC 114, Radiometer A/S, Copenhagen, Denmark) and then was pumped through one of two side arms of the glass coil. The air after leaving the toad bladder chamber was introduced into the other side arm. The ratio of fluid-to-air flow in the spiral was approximately 1:10. The fluid after circulating through the spiral was allowed to return to the bulk medium through a second conductivity cell. The two conductivity cells were

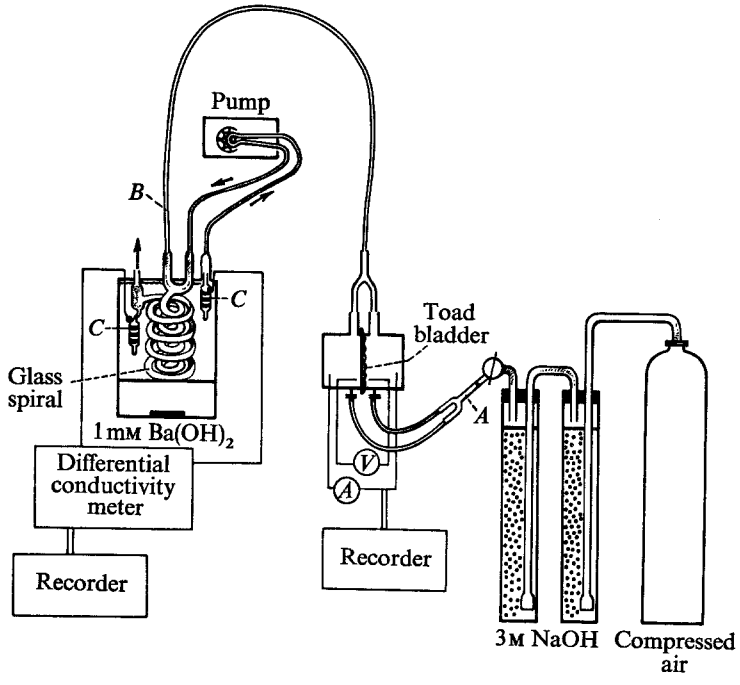

Fig. 1. Diagrammatic representation of the apparatus for the measurement of total CO_2 (*see text* for description). Points *A* and *B* were used to inject known amounts of CO_2. *C* refers to flow-through conductivity cell

connected to a differential conductivity meter (Wescan model 211, Wescan Instruments, Cupertino, California). The output of the conductivity meter was connected to a recorder (Varian model G22A, Varian Instruments, Palo Alto, California or Hewlett-Packard model 7130 A/B, Hewlett-Packard, San Diego, California).

It was found that the conductivity of $Ba(OH)_2$ and NaOH was a linear function of the concentration in the range 0.1 to 2 mM. The experiment was started by allowing CO_2-free air to circulate through the system at 80–100 ml/min. The recording of the difference in conductivity was checked for drift and the experiment was not started until at least an hour of recording showed no appreciable drift in this baseline. Calibration was done by injecting into the CO_2-free air known volumes of air from a reference cylinder, the CO_2 content of which was measured by the Scholander method [14]. It was also measured by allowing a solution of dilute $NaHCO_3$ of known concentration to equilibrate with the CO_2-containing air and calculating the CO_2 content from the equilibrium pH by the Henderson-Hasselbach equation. Both methods agreed to within 5%. Ten to 100 ml of this known standard CO_2 was injected slowly by a Harvard constant infusion pump (Harvard Apparatus Company Inc., Millis, Mass.) at rates of 3 ml/min. One to four such injections were performed in each experiment. At these rates of infusion the deflections recorded were similar in magnitude to those produced by the toad bladder. The different curves were integrated and plotted as volumes of CO_2 *vs.* mm^2, measured by planimetry. The relationship was invariably linear with an intercept not different from zero. An amount of CO_2 injected at different rates gave the same area with an accuracy of 2%.

To minimize bacterial growth, all media were first filtered through Millipore filters (# HAWP 04700, Millipore Corp., Bedford, Mass.). Three antibiotics were added to the medium, penicillin 0.1 mg/ml, gentamicin 10 μg/ml and colistin 5 μg/ml. The choice of antibiotics was based on disc sensitivity testing of the cultured microorganisms. The toad bladder was washed three times for at least 30 min with the antibiotic-containing solution before beginning each experiment.

Criteria for Successful Experiments

Before any experiment was included for analysis it had to be demonstrated that bacteria were not responsible for the CO_2 being measured. At the end of the experiment after removal of the bladder, CO_2-free air was bubbled through the medium and the experiment was discarded if CO_2 continued to be produced by the medium. This was performed in all experiments with substrate in the medium and in about half the experiments without substrate. At the end of each experiment CO_2-free air was passed through the system again to check for drift in the baseline. No experiment was accepted if the baseline has changed by more than 5% of the full scale. Because of the several connections present in the apparatus and the positive pressure inside, leakage of air was checked for by injecting into the CO_2-free air stream at point *A* in Fig. 1 a known volume of the standard CO_2-containing air. The deflection produced was compared to that obtained when the same volume was injected into the air stream close to the spiral (point *B*, Fig. 1) where no connections which might lead to leakage existed. No experiment was used unless there was agreement to within 5% of these areas measured.

Chemicals used for preparation of the media were reagent grade chemicals. Carbonic anhydrase was obtained from Sigma. All data are presented as means $\pm$ SD. Regression analysis was performed using the least-squares method and comparison of the slopes was performed using standard statistical analysis [15]. The sodium transport (J_{Na}) is expressed as nanoequivalents per mg dry weight per minute. The CO_2 production (J_{CO_2}) is expressed as nanomoles per mg dry weight per minute.

Some Problems Relating to the Measurement of Fast Changes in CO_2 Production

CO_2 produced by the toad bladder diffuses out into the bulk medium where a large part escapes as free CO_2 directly into the gas phase. Another part of the produced CO_2, however, is hydrated in the bulk solutions and the bicarbonate pool so formed will influence the kinetics of CO_2 evolution. For a given CO_2 flux the size of the bicarbonate pool depends on the volume of the bulk medium and on its pH. Furthermore, the kinetics of CO_2 evolution from this pool will be influenced by the rate of dehydroxylation of bicarbonate.

To minimize the size of the bicarbonate pool the volume of the chamber was decreased to 6–7 ml and the pH of the medium was reduced from the usual pH of 7.8 to 7.0. Fig. 2 shows that at pH 7.0 the short-circuit current was about 80% of the value at pH 7.8.

To simulate the washout of the bicarbonate pool, a small volume of Ringer's solution at pH 7.0 previously equilibrated with 5% CO_2 was injected directly into the chamber containing phosphate buffered Ringer's solution at pH 7.0 in absence of a toad bladder. Curve *B* in Fig. 3 shows that this washout had a half time of 160 sec. When the experiment was repeated with 0.2 mg/ml carbonic anhydrase in the medium this washout was enhanced; curve *C*, Fig. 3 $t_{\frac{1}{2}} = 67$ sec. These curves were compared with the one representing the washout of a CO_2 pulse. CO_2 was injected at point *A* in Fig. 1, into the stream of CO_2-free air flowing through the chamber: curve *A* in Fig. 3. Since the rate of flow of air was fast (80 ml/min) it would be expected that little CO_2 would be absorbed by the fluid;

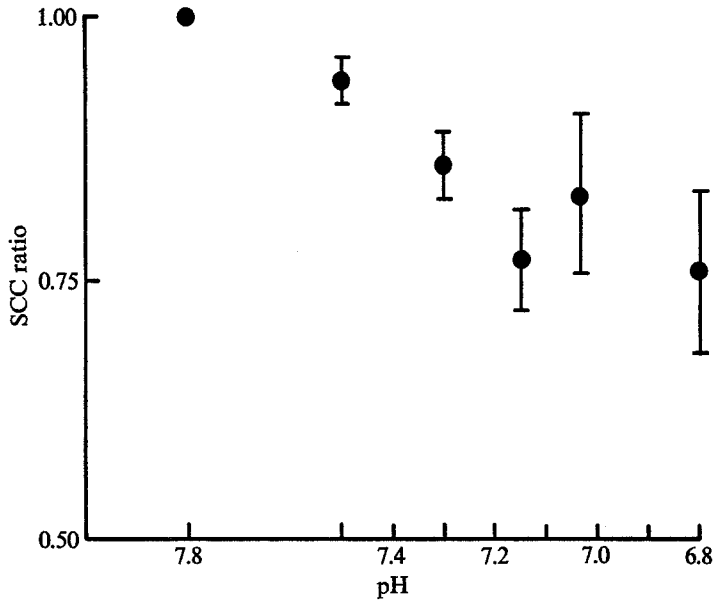

Fig. 2. Effect of changing medium pH on the short-circuit current (SCC). Bladders were bathed with phosphate-buffered Ringer's solution at pH 7.8. After stabilization of the SCC the medium was changed to a Ringer's solution with a different pH. The SCC was observed until it stabilized (approx. 20 min); the solution was then changed to pH 7.8. The SCC ratio mentioned in the figure was calculated as the SCC observed during the experimental period divided by half the sum of the SCC in the periods preceeding and following the experimental period

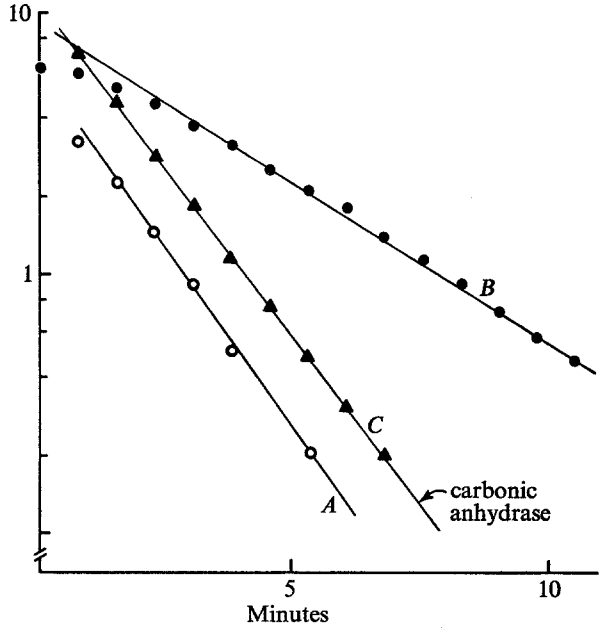

Fig. 3. Semilog plot of kinetics of washout of CO_2 from the chamber. *See text* for details

consequently this curve characterizes the dead space of the system. It can be seen that curves *A* and *C* are almost identical. Since curve *A* represents the fastest possible washout under these specified conditions the equality of curves *A* and *C* suggests that in the presence of carbonic anhydrase the dehydration reaction is no longer rate-limiting.

An estimate of the size of the bicarbonate pool in the presence of carbonic anhydrase was obtained by allowing a toad bladder to reach a steady state of CO_2 production. A small volume of 1 N hydrochloric acid was added to the medium in quantities sufficient to reduce the pH to 2.0. The evolved CO_2 was taken as the measure of the total pool in the chamber. In six experiments the pool measured 136 ± 35 nequiv. The ratio of the flux of CO_2 (in nequiv/min) to the total CO_2 pool in these experiments was 0.1 ± 0.04 min^{-1}. This rate constant, however, does not reflect the overall kinetics of the system but rather provides an upper boundary, since a large part of the CO_2 escapes into the gas phase without being hydrated. The overall rate constant can be estimated by examining the approach to a new steady state from a steady rate of CO_2 production. A potential was imposed on the toad bladder of sufficient magnitude to alter the rate of net sodium transport. The time taken for the CO_2 production to reach a new steady state was 10 min in the presence of carbonic anhydrase. In comparing the rate of CO_2 production to the short-circuit current, a 4-min delay time was used.

Another factor that could influence the washout kinetics is the rate of equilibration of CO_2 in the bulk medium with the CO_2-free air that passes through the chamber. The primary determinant of that is the surface area available for diffusion of CO_2 from the liquid to the gas phase. A bubble-lift was used as a stirring device and care was taken to insure that the bubbles were small enough to have large surface-to-volume ratios.

Results

CO_2 Efflux from Mucosal and Serosal Border

The efflux of CO_2 across both borders of the epithelium was measured in four experiments with carbonic anhydrase in the medium. It was found that the efflux was slightly higher from the mucosal border. The average ratio of mucosal/serosal rates was 1.15 ± 0.06.

Correlation of CO_2 Production with Sodium Transport

Toad bladders were mounted in chambers and the simultaneous rates of sodium transport and CO_2 production were measured continuously. The short-circuit current was used as the measure of net sodium transport across the membrane [5]. In most of the experiments no exogenous substrate was present. The short-circuit current was allowed to decline with time. These changes were relatively slow. It was noted (Fig. 4) that the decline in sodium transport was accompanied by a similar decline in CO_2 production. When the sodium transport was plotted as a function of CO_2 production it was found that the relation was highly linear (Fig. 5). The degree of linearity was estimated from the correlation coefficient (r) of the least-squares fit of the regression analysis. In 50 experiments the minimum value of r was 0.864, the mean r was 0.974 ± 0.03 (SD).

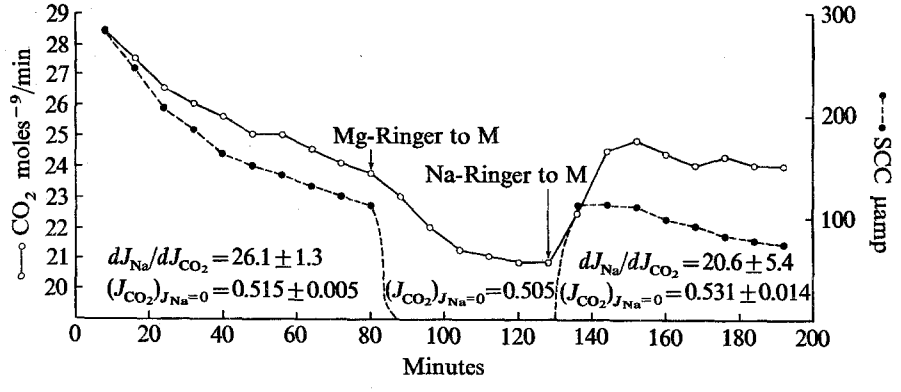

Fig. 4. Representative experiment of simultaneous and continuous measurement of CO_2 and short-circuit current. The SCC was allowed to decline spontaneously. At the first arrow the mucosal medium was changed to a Na-free magnesium Ringer's solution. At the second arrow Na-containing Ringer's solution was replaced on the mucosal side. The numbers given, normalized to the dry weight of the bladder (41 mg), were obtained from a plot of CO_2 production against Na transport by least-squares analysis

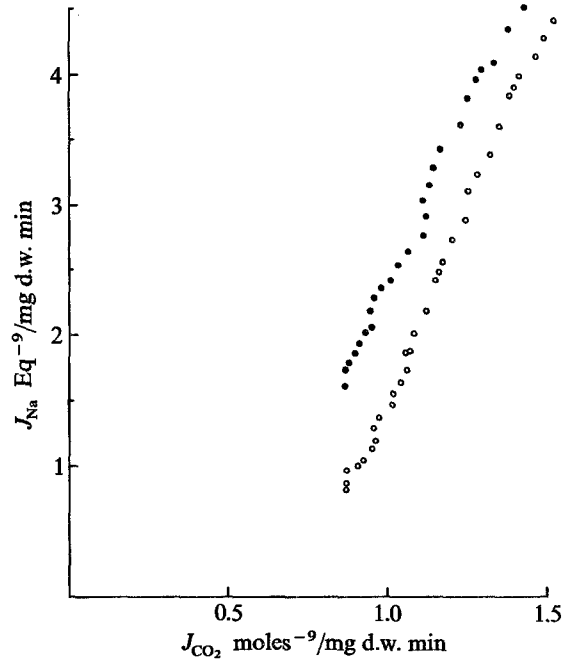

Fig. 5. Representative paired experiment. Simultaneous measurement of CO_2 and Na in paired hemibladders from the same toad. The values of dJ_{Na}/dJ_{CO_2} were 5.8 ± 0.07 (open circles) and 5.2 ± 0.12 (closed circles)

In each experiment the slope of the line dJ_{Na}/dJ_{CO_2} and the intercept $(J_{CO_2})_{J_{Na}=0}$ were calculated by the least-squaress method. In seven experiments the slope of the regression line dJ_{Na}/dJ_{CO_2} was compared to the value

of the total sodium transport divided by the supra basal CO_2 production $\Delta J_{Na}/\Delta J_{CO_2}$. The latter ratio was obtained by integrating the short-circuit current curve over 1 hr and dividing it by the CO_2 produced in the same period minus that produced in the absence of sodium transport. The two methods of calculation were in good agreement, dJ_{Na}/dJ_{CO_2} 10.0, $\Delta J_{Na}/\Delta J_{CO_2}$ 9.1, $\Delta = 0.9 \pm 2.3$. This validates the use of the regression slope, dJ_{Na}/dJ_{CO_2} as the measure of the relation between Na transport and CO_2 production.

Paired Experiments

To evaluate the validity of the method, experiments were carried out on the two hemibladders from individual toads. The experiments were performed in two ways. A group of five experiments were done in which the CO_2 production and sodium transport were measured simultaneously. A second group of three experiments were performed in which the two hemibladders were mounted and examined "in series". No difference was seen between the two methods used.

The results of these experiments were plotted and the slope dJ_{Na}/dJ_{CO_2} as well as $(J_{CO_2})_{J_{Na}=0}$, i.e., the zero intercept, were compared. These results are shown in Table 1 and representative results from paired hemibladders are shown in Fig. 5. There was good agreement between the slopes calculated from both hemibladders. The mean difference between members of each pair amounted to 17% of the mean value. In four of the eight pairs the difference was less than 10% of the mean of the pair. In no experiment was the difference greater than 48% of the mean of the pair. Comparison of the results of the zero intercept $(J_{CO_2})_{J_{Na}=0}$ shows similar agreement; members of a pair differed by an average of 21% of the mean value of the pair.

Whether these differences represent real differences between paired hemibladders or simply reflect the experimental error of the measurements cannot be resolved at present. Of importance is the finding that values of dJ_{Na}/dJ_{CO_2} vary much more between bladders from different animals than between paired hemibladders from the same toad. This establishes that the large differences between animals in this ratio are real. Fig. 6 shows the value for dJ_{Na}/dJ_{CO_2} from 28 unpaired experiments measured over a period of 11 months. The average value was 7.9 but the spread was 1 to 27 which is much greater than the average difference between members of a pair which was $\pm$ 17% of the mean value for a pair. It is evident that no single value of this ratio is representative of all the experimental values.

The range of sodium transport over which these experiments were performed averaged 1.65 ± 1 nequiv/min mg dry wt. The initial rates of sodium

Table 1. The relation of CO_2 production to sodium transport in paired hemibladders

dJ_{Na}/dJ_{CO_2} (Equiv/mole)	Δ	$(J_{CO_2})_{J_{Na}=0}$ (nmoles/mg dry wt min)	Δ
11.3 ± 0.4		0.61 ± 0.004	
8.6 ± 0.4	2.7 ± 0.5 [a]	0.54 ± 0.008	0.07 ± 0.009 [a]
4.1 ± 0.2		0.66 ± 0.016	
4.4 ± 0.1	0.3 ± 0.2	0.60 ± 0.007	0.06 ± 0.017 [a]
5.8 ± 0.1		0.74 ± 0.006	
5.2 ± 0.1	0.6 ± 0.1 [a]	0.54 ± 0.012	0.2 ± 0.013 [a]
3.8 ± 0.2		0.22 ± 0.013	
4.0 ± 0.5	0.2 ± 0.5	0.28 ± 0.027	0.06 ± 0.030
3.0 ± 0.3		1.82 ± 0.03	
2.6 ± 0.5	0.4 ± 0.6	1.54 ± 0.109	0.28 ± 0.113
5.6 ± 0.6		0.27 ± 0.022	
3.5 ± 0.07	2.1 ± 0.9	0.44 ± 0.034	0.17 ± 0.040 [a]
4.0 ± 0.2		0.33 ± 0.053	
3.4 ± 0.2	0.6 ± 0.3	0.35 ± 0.01	0.02 ± 0.054
7.7 ± 0.3		0.33 ± 0.03	
8.2 ± 0.6	0.44 ± 0.7	0.25 ± 0.03	0.07 ± 0.043
Mean difference ± SD	0.92 ± 0.94		0.117 ± 0.09

CO_2 production (J_{CO_2}) and sodium transport (J_{Na}) was examined in hemibladders from individual toads. The least-squares fit of the slope dJ_{Na}/dJ_{CO_2} and the zero intercept $(J_{CO_2})_{J_{Na}=0}$ are given ± SD.
[a] $p < 0.05$.

transport averaged 3.8 nequiv/min mg dry wt (range 0.6 to 9.7). There was no significant correlation between the dJ_{Na}/dJ_{CO_2} and the initial short-circuit current ($r = 0.101 \pm 0.14$, $n = 50$) nor between the dJ_{Na}/dJ_{CO_2} and the total resistance ($r = 0.086 \pm 0.23$, $n = 19$).

"*Basal Metabolism*"

Removal of sodium from the mucosal medium results in a rapid fall of the short-circuit current to levels at or near zero. The CO_2 production also falls though its approach to a steady value is less rapid than that of sodium transport (Fig. 4)[1]. The steady-state value for CO_2 production in the absence

1 CO_2 evolution reached a new steady state within 45 min; a longer period than that observed in experiments in which the rate of sodium transport was changed by maneuvers that did not involve replacement of the bathing media. One reason for this delay could be that the new solutions were CO_2-free and would have to trap CO_2 and bicarbonate before steady-state conditions are again established. Washout of unstirred layers could further complicate the kinetics of CO_2 evolution during this period. Following replacement of the bathing media 1 hr was therefore allowed for attainment of the steady state.

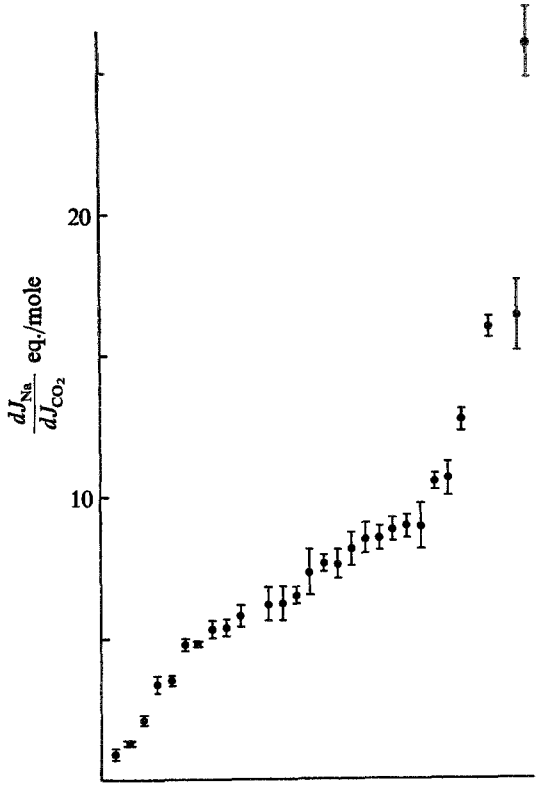

Fig. 6. A plot of the slope of sodium transport on CO_2 production (dJ_{Na}/dJ_{CO_2}) in 28 individual toad bladders showing the values in ranking order $\pm$ one SD

Table 2. $(J_{CO_2})_{J_{Na}=0}$ (nmoles/min mg dry wt)

Extrapolated	Observed	Δ
0.52 ± 0.007	0.49	-0.03
0.60 ± 0.004	0.45	-0.15
0.72 ± 0.043	0.72	0.00
0.62 ± 0.015	0.64	$+0.02$
0.52 ± 0.015	0.50	-0.02
0.75 ± 0.039	0.72	-0.03
0.52 ± 0.031	0.52	0.00
Mean $\pm$ SD		
0.61 ± 0.1	0.58 ± 0.12	-0.03 ± 0.06

The CO_2 production (J_{CO_2}) and sodium transport (J_{Na}) were measured in individual bladders. After a period where the spontaneous decline was followed the mucosal solution was changed to a Na-free solution and the CO_2 production measured. The first part was plotted as J_{CO_2} *vs.* J_{Na} and the zero intercept $(J_{CO_2})_{J_{Na}=0}$ was calculated by the least-squares method and compared to the value of the J_{CO_2} observed in the absence of mucosal Na.

Table 3. Constancy of CO_2 production in the absence of sodium transport J_{CO_2} in nmoles/min mg dry wt

60 min	120 min	Δ
0.49	0.51	+0.02
0.46	0.40	−0.06
0.72	0.65	−0.06
0.43	0.40	−0.03
0.89	0.90	+0.01
0.64	0.57	−0.07
0.55	0.53	−0.02
Mean ± SD		
0.60 ± 0.16	0.57 ± 0.17	−0.03 ± 0.04

The CO_2 production was measured in the absence of mucosal sodium. The value of the J_{CO_2}, 60 min after removal of the mucosal sodium was compared to that 120 min after the substitution.

of mucosal sodium, $(J_{CO_2})_{J_{Na}=0}$, was measured in seven experiments and the results are shown in Table 2. In these experiments the short-circuit current was allowed to decline for a period then the mucosal solution was changed to choline Ringer's or magnesium Ringer's. The slope of the line dJ_{Na}/dJ_{CO_2} and its zero intercept of $(J_{CO_2})_{J_{Na}=0}$ were calculated as before. Comparison between the zero intercept and the CO_2 production in the absence of mucosal sodium is presented in Table 2. It can be seen that in most of the instances the differences were minor and inconstant in direction. The mean difference was 0.03 ± 0.06 and was not a significant difference.

In the absence of mucosal sodium the basal metabolism was constant. Table 3 shows the rate of change in basal metabolism. There was no significant difference 2 hr after removal of sodium as compared to 1 hr.

Discussion

To be able to measure relatively rapid changes in CO_2 production and correlate them with the simultaneously occurring changes in transport the bicarbonate pool in the chamber must be reduced to a minimum. One approach is to decrease the pH. The problem, as shown in Fig. 2, is that the short-circuit current falls gradually as ambient pH falls. To avoid too marked a reduction in ambient pH we elected to use pH 7.0. At this pH, however, the pool of bicarbonate is still significant. Small volume chambers and carbonic anhydrase were used. Carbonic anhydrase accelerates the dehydration reaction of bicarbonate to CO_2 and OH^-, and Fig. 3 shows

that following the addition of carbonic anhydrase the kinetics of CO_2 evolution approach or equal those dictated by the geometry of the apparatus. Although the ambient bicarbonate pool is small, measuring 136 nequiv per chamber, some slowly exchanging bicarbonate must remain, located probably in the cell water and the unstirred layers. This latter pool will become important if sudden large changes in CO_2 production are being measured; however, for the purposes of the experiments reported here it could be ignored.

The major advantage of this method is that it can yield information on the production of CO_2 continuously and simultaneously with the measured rate of sodium transport in the same bladder. The urinary bladder of the toad *Bufo marinus* transports sodium from the mucosal side to the serosal side actively. This transport activity can be monitored continuously by the short-circuit current method. It has been demonstrated under a variety of conditions that the short-circuit current is an excellent measure of net sodium transport in one subspecies of *Bufo marinus*, that obtained from the Dominican Republic [5]. Another subspecies, Colombian toads, have been shown to secrete hydrogen ions from serosal to mucosal side 'electrogenically' [3, 9, 13] and to absorb chloride ions [2]. No hydrogen ion secretion has been demonstrated in the Dominican toad [7, 13].

It was noted that the CO_2 production varied with sodium transport. This variation appeared linear over a large range of sodium transport activity. When sodium transport was plotted as a function of CO_2 production over the range of values seen in individual bladders it was seen that the slope, dJ_{Na}/dJ_{CO_2}, for each bladder varied significantly among different toads. To test the validity of these measurements, studies were performed on paired hemibladders from the same toad. The assumption was that the slope of both hemibladders would be identical. Table 1 shows the results in eight pairs. The difference in half of them was below 10% of the mean value and none differed by more than 50%; the mean difference was 17%.

In a large number of such experiments the values of the slope of J_{Na} on J_{CO_2} varied over a wide range. Fig. 6 shows all these values arranged in ranking order. It is apparent that no unique value characterizes the relation between transepithelial sodium transport and respiration in this tissue. This confirms the observations of Vieira *et al.* [16] on the coupling between sodium transport and oxygen consumption in the frog skin.

The range of values of dJ_{Na}/dJ_{CO_2} in the toad bladder was as large as that observed in the frog skin [8, 16, 17]. In the present experiments, the small differences seen with paired hemibladders from the same toad indicates clearly that the much larger differences obtained with hemibladders from

different animals are real and cannot be attributed to experimental error. Furthermore, the CO_2 production unrelated to sodium transport was measured directly and found to be constant over the period of observation. The isolated urinary bladder of the toad seems quite capable of transporting sodium at distinctly different energy costs during prolonged periods of observation under near steady-state conditions. Hence, conclusions regarding stoichiometry based on data obtained by averaging ratios from different animals [4, 8, 11, 12, 17] should be interpreted with caution. It should be emphasized, however, that for each tissue the ratio of transport to metabolism remained constant over the period of observation.

What, then, could account for these observed differences in the "stoichiometric ratios"? At the basolateral borders of the transporting layer of mucosal cells where active transport of sodium is presumed to occur, the molecular relations between splitting of ATP and sodium transport may indeed occur with fixed stoichiometry. The differences observed in the whole tissue might then be attributable to some aspect of the complex organization of the transport activity within the whole tissue. Possibilities include: 1) variability in the metabolism supporting the nontransport activities of the tissue (rendered unlikely by the constancy observed in the direct measurement of this moiety of metabolism); 2) variable contribution of non-CO_2 producing energy metabolism to sodium transport, e.g. glycolysis. Under conditions when glycolysis is the principal energy-yielding reaction as during anaerobiosis, sodium transport falls to levels that are only 30% of the aerobic level [6]. In the presence of oxygen, lactate production is only one-seventh that during anaerobiosis [7]. Thus, although glycolysis can support sodium transport it is unlikely that during aerobic conditions it can significantly contribute to the total energy production enough to account for the observed differences in the sodium to CO_2 ratios. 3) A back leak of sodium from the serosal medium into the cell would lead to a "recycling" of sodium transport. Even with a constant metabolic requirement for each sodium ion transported actively from cell interior to serosal medium, the ratio of net transepithelial sodium transport (which is what the short-circuit current measures) to CO_2 production would vary as the ratio of sodium entering the cell from the mucosal medium versus that entering via the serosal back leak. On the basis of a close study of the current-voltage relationship in the toad bladder, Civan [1] came to the conclusion that sodium traverses the active pathway largely if not entirely in one direction. However, that study does not rule out the possibility of exchange diffusion. 4) Differences from one bladder to the next in the efficiency of synthesis of ATP or other high energy intermediates which drive sodium transport would also affect the

ratio of sodium transported per CO_2 produced. It does not seem likely that uncoupling between ATP synthesis and oxygen utilization (and hence CO_2 production) could exist to a sufficient degree to account for the large variations in dJ_{Na}/dJ_{CO_2} observed. Whether the latter two possibilities could account quantitatively for the apparent lack of stoichiometry remains to be determined. Other possibilities undoubtedly exist but the evidence on which to distinguish between them is not yet available.

Over the period of observation, sodium transport declined continuously and slowly while the CO_2 production unrelated to sodium transport (basal metabolism) remained constant. To the extent that the decline in sodium transport reflects depletion of a metabolic pool the constancy of the basal metabolism suggests that the two pools are separate and noncommunicating. The basal metabolism was measured by two independent methods: 1) extrapolating the regression line of J_{Na} on J_{CO2} to the point of $(J_{CO_2})_{J_{Na}=0}$ and 2) measuring the CO_2 production with sodium removed from the mucosal bathing medium. Both methods gave values in close agreement in individual bladders. This provides additional evidence that the transport-linked metabolism is separate from the basal metabolism.

We are grateful to Drs. R. H. Maffly and R. Steele of Stanford University for making available to us the technique of measurement of CO_2 production prior to its full publication. We thank Drs. P. R. Steinmetz and M. M. Civan for a critical reading of the manuscript. This work appeared in abstract form in Abstracts of the American Society of Nephrology, 1973, p. 2. Dr. R. Beauwens was a fellow of the Belgian American Education Foundation. This work was supported by a USPHS grant 2P01-HL 06664.

References

1. Civan, M. M. 1970. Effects of active sodium transport on current voltage relationship of toad bladder. *Amer. J. Physiol.* **219**:234
2. Finn, A. L., Handler, J. S., Orloff, J. 1967. Active chloride transport in the isolated toad bladder. *Amer. J. Physiol.* **213**:179
3. Frazier, L. W., Vanatta, J. C. 1971. Excretion of H^+ and NH_4^+ by the urinary bladder of the acidotic toad and the effect of short-circuit current on the excretion. *Biochim. Biophys. Acta* **241**:20
4. Kramer, K., Deetjen, P. 1964. Oxygen consumption and sodium reabsorption in the mammalian kidney. *In:* Oxygen Consumption in the Animal Organism. F. Dickens and E. Neil, editors. p. 411. MacMillan Company, New York
5. Leaf, A. 1965. Transepithelial transport and its hormonal control in toad bladder. *Ergebn. Physiol.* **56**:215
6. Leaf, A., Anderson, J., Page, L. 1958. Active sodium transport by the isolated toad bladder. *J. Gen. Physiol.* **41**:657
7. Leaf, A., Page, L. B., Anderson, J. 1959. Respiration and active sodium transport of isolated toad bladder. *J. Biol. Chem.* **234**:1625

8. Leaf, A., Renshaw, A. 1957. Ion transport and respiration of isolated frog skin. *Biochem. J.* **65**:82
9. Ludens, J. H., Fanestil, D. D. 1972. Acidification of urine by the isolated urinary bladder of the toad. *Amer. J. Physiol.* **223**:1338
10. Maffly, R. H., Steele, R. E., Walker, W. E., Coplon, N. S. 1973. Respiration and active sodium transport. *Abstr. Amer. Soc. Nephrol.* p. 72
11. Martin, D. W., Diamond, J. M. 1966. Energetics of coupled active transport of sodium and chloride. *J. Gen. Physiol.* **50**:295
12. Nellans, H. N., Finn, A. L. 1974. Oxygen consumption and sodium transport in the toad urinary bladder. *Amer. J. Physiol.* **227**:670
13. Rosen, S., Oliver, J. A., Steinmetz, P. R. 1974. Urinary acidification and carbonic anhydrase distribution in bladders of Dominican and Colombian toads. *J. Membrane Biol.* **15**:193
14. Scholander, P. F. 1942. Volumetric microrespirometers. *Rev. Sci. Instr.* **13**:32
15. Snedecor, G. W., Cochran, W. G. 1967. Statistical Methods, 6th Edition. Iowa State University Press, Ames, Iowa
16. Vieira, F. L., Caplan, S. R., Essig, A. 1972. Energetics of sodium transport in frog skin. I. Oxygen consumption in the short-circuited state. *J. Gen. Physiol.* **59**:60
17. Zerahn, K. 1956. Oxygen consumption and active sodium transport in the isolated and short-circuited frog skin. *Acta Physiol. Scand.* **36**:300