J. Membrane Biol. 34, 289–312 (1977)

Interrelationships of Sodium Transport and Carbon Dioxide Production by the Toad Bladder: Response to Changes in Mucosal Sodium Concentration, to Vasopressin and to Availability of Metabolic Substrate*

Norman S. Coplon, Roderic E. Steele** and Roy H. Maffly

Veterans Administration Hospital and the Department of Medicine, Stanford University School of Medicine, Palo Alto, California 94304

Received 9 April 1976; revised 30 August 1976

Summary. Active sodium transport and CO_2 production were measured simultaneously in toad bladders mounted in membrane chambers. The rate of sodium transport was varied by changing the concentration of sodium in the mucosal bath (substitution with choline), by adding vasopressin, by adding metabolic substrates and by adding malonate, and the ratio of the change of sodium transport and CO_2 production was determined Mean values for $\Delta Na/\Delta CO_2$ (equiv/mole) were: Na⇄choline 18.3 ± 1.1; vasopressin 15.5 ± 2.8; and pyruvate (corrected for the increment in "nontransport" CO_2) 15.4 ± 3.5. Based on previously determined values for the respiratory quotient (R.Q.), calculated mean values for $\Delta Na/\Delta O_2$ ranged between 15.5 and 18.5 equiv/mole. It appears that basal metabolism does not contribute to metabolism supporting sodium transport when the rate of sodium transport is varied. "Transport" metabolism appears much more responsive to changes in the availability of endogenous and exogenous substrates than does "nontransport" metabolism. We conclude that "transport" and "nontransport" metabolism are functionally separated in the toad bladder.

How the chemical energy of cellular metabolism is converted to the electrochemical energy of active ion transport remains an important biologic challenge. One experimental approach to this problem has been the comparison of the effects of various factors on rate of ion transport to their effects on rate of respiration. A number of studies have been

* These results were presented in part at the Annual Meeting of the American Society of Nephrology, November 1973.

** *Present Address:* Laboratory of Technical Development, National Heart and Lung Institute, Bethesda, Maryland 20014.

performed correlating effects on active sodium transport and oxygen consumption. When changes of sodium transport have been induced in a variety of epithelial tissues, oxygen consumption has been found to change in parallel, at a ratio of 10 to 30 equivalents of sodium per mole of oxygen (*see* Ref. [24]), and from these figures conclusions have been drawn about the relationship between sodium transport and cellular metabolism. These interpretations depend, however, on the assumption that as the rate of metabolism supporting sodium transport varies, the rate of basal, nontransport metabolism remains constant. Instead, if with the stimulation of sodium transport the required supporting metabolism comes in part from basal metabolism, then metabolism supporting transport will be underestimated and the calculated ratio of sodium ions transported to oxygen consumed will be erroneously high.

Measurement of active sodium transport can be easily performed with certain epithelial membranes, including the urinary bladder of the toad, by the short-circuit current technique [23]. Because simultaneous measurement of oxygen consumption has proven difficult, we began several years ago the simultaneous measurement of sodium transport and carbon dioxide production by the toad bladder. With the volumetric method originally employed, CO_2 effluxing from the *in vitro* bladder was collected for 1-h periods and measured [2, 18]. Subsequently, a conductometric method was developed which permitted the measurement of micromolar quantities of CO_2 [17], and with it we have carried out studies of the simultaneous effect of a number of variables on the short-circuit current and CO_2 production measured during successive 4-min periods. These studies have permitted a more critical evaluation of the quantitative relationship between sodium transport and tissue metabolism and of the problem of possible changes in basal metabolism when the rate of sodium transport is varied. It has also been possible to make a quantitative comparison of the metabolism supporting sodium transport and that supporting nontransport processes.

Materials and Methods

Toads (*Bufo marinus*) were obtained from Tarpon Zoo, Tarpon Springs, Florida and originated from Colombia, South America. They were kept on moist dirt or sawdust, were not fed, and were sacrificed several days to several weeks after arrival in the laboratory. After pithing the toad, a hemibladder was mounted as a diaphragm between two halves of a glass chamber specially designed for the simultaneous measurement of short-circuit current and CO_2 efflux [2]. The exposed area of the membrane was 5.7 cm^2. The tissue

was held in the midline by nylon hair netting or stocking on both surfaces. 5 ml of bathing solution was added to each side.

A small amount of an antifoaming agent (Antifoam A Spray, Dow Corning Corp., Midland, Mich.) was applied to each fluid surface. A stream of CO_2-free air was then introduced into the lower portion of each hemi-chamber and bubbled through the solution; gas effluxed from the chamber at a measured flow rate of 80 ml/min (Flowmeter #F1100, Roger Gilmont Instruments, Great Neck, New York). The gas was carried into a glass spiral containing 16 ml of 0.002 N NaOH; the conductivity of the NaOH, which was continuously monitored, declined as CO_2 was absorbed. This conductometric method has been previously described and characterized [17]. Successive 4-min periods of measurement were employed.

The short-circuit current was continuously measured with an automatic voltage clamp [18]. The outputs from the conductivity meter and voltage clamp were recorded on a dual-channel recorder (Model G2000, Varian Associates, Palo Alto, Calif.). In the "paired" experiments, a duplicate set-up was employed so that short-circuit current and CO_2 efflux could be measured simultaneously with two hemibladders. Room temperature was held at 25 ± 0.5 °C during the experiments.

Bacterial growth in the chamber and tubing, which if not minimized led to significant production of CO_2, was avoided by washing the chamber thoroughly before use and using fresh or clean tubing; antibiotics (penicillin 0.1 mg/ml, streptomycin 0.1 mg/ml and polymixin B sulfate 0.01 mg/ml) were added fresh to all Ringer's solution used. Diffusion of atmospheric CO_2 into the chamber or gas stream was avoided by employing glass tubing where possible; otherwise we used flexible polyvinyl chloride tubing (Tygon, Norton Plastics and Synthetics Division, Akron, Ohio), the permeability of which is low to CO_2 compared to most other flexible tubing. Just before the hemibladder was mounted in the chamber a blank run was made with the chamber plus 10 ml of Ringer's solution. Any small background of CO_2 "production" was measured and was subtracted from the measurements subsequently made with the bladder in place; in 32 consecutive experiments over a one-year period, a mean blank value of 1.9 ± 0.3 µl per hr was measured. In addition, at the conclusion of 19 of these experiments (up to 6 hr after mounting) the bathing solution was collected in a syringe, the chamber was taken down, the tissue was cut away and removed, the chamber was set up again, and the original Ringer's solution was returned to the chamber; after an equilibration period of 8 to 20 min, CO_2 "production" was again measured: it proved consistently to be similar to the blank value (difference $= +1.3 \pm 0.7$ µl per hr) and was a small fraction of the CO_2 measured with the tissue in place.

Dry weight of the experimental tissue was obtained by cutting out the exposed tissue at the conclusion of the experiment and placing it in an oven at 100 °C overnight.

The usual bathing solution was a phosphate-buffered (HCO_3^--free) frog Ringer's solution (Na 111.0, K 4.0, Ca 1.8, Cl 113.0, HPO_4 2.0 and H_2PO_4 2.0 mEquiv per liter; pH 6.4; 220 mOsm/kg H_2O). Where specified, a sodium-free choline Ringer's solution (choline chloride substituted for sodium chloride) was employed as the mucosal bath. Test substances were made up in solutions of pH and osmolality corresponding to the Ringer's solution; these solutions were bubbled with CO_2-free air for 10–20 min prior to their addition to the serosal bathing medium via syringe and needle through a rubber serum cap; 30 sec later an equal amount of bathing solution was removed so as to maintain a volume of 5 ml in each hemi-chamber.

Chemicals employed, all of reagent grade, were glucose (J.T. Baker, Philadelphia, Penn.) and pyruvic acid and malonic acid (Sigma Chemical Company, St. Louis, Missouri). Vasopressin was used as Pitressin (Parke, Davis & Co., Detroit, Mich.).

Statistical significance was determined with Student's *t*-test. Results are ordinarily expressed as mean $\pm$ SE of n experiments.

Results

Baseline Values

Measurements were made of the short-circuit current (SCC) and of CO_2 production prior to the addition of any agents in hemibladders from 58 toads, 1/2 to 3 hr after the institution of the short-circuited state. The mean value of the SCC was 278 ± 17 μA and of CO_2 production was 40.3 ± 1.8 μl (STP) per hour expressed per 5.7 cm^2 of exposed membrane. Where dry weights of the exposed tissue were available the mean values ($n=48$) were 9.2 ± 0.7 μA/mg dry wt and 1.34 ± 0.06 μl per hr/mg dry wt. The mean of the ratios of SCC to CO_2 production (hereafter termed "total SCC/total CO_2") was 6.98 ± 0.30 μA/μl per hour ($n=58$); this corresponds to 5.79 ± 0.25 equivalents of Na per mole of CO_2.[1]

Although in most experiments variables were introduced which subsequently altered SCC and CO_2 production, in some instances none were introduced and the spontaneous changes in sodium transport and respiration were observed. During the period of 2 to 5 hr after beginning the short-circuited state, the SCC declined by an average of $19.4 \pm 3{,}5\%$ per hr and CO_2 production declined by an average of $9.5 \pm 1.6\%$ per hr; the mean of the ratios of the absolute decrements was 12.2 ± 2.3 equivalents Na/mole CO_2 ($n=12$). Total SCC/total CO_2 declined from 4.86 to 4.37 equivalents/mole (-0.49 ± 0.20, $p<0.05$).

Changes Induced by Varying the Sodium Concentration in the Mucosal Bathing Medium

(a) Na→choline. Mucosal concentration of sodium was reduced by replacing the mucosal bathing medium with choline Ringer's solution on 10 occasions, in the absence of exogenous substrate. (An example of the effects of mucosal Na→choline is included in Fig. 1.) The mean results of the 10 experiments are depicted in Fig. 2. The SCC dropped quickly to 7% of the baseline value and remained there or lower for the one hour of the experiment. CO_2 efflux declined more gradually, plateauing at 60% of baseline by 20 min.

The decrement of SCC was divided by the decrement of CO_2 efflux to obtain a decremental ratio (Δ equivalents Na/Δ moles CO_2), and

1 This conversion is made by multiplying by the factor

$$0.83 \frac{\text{equiv}/\mu\text{A}}{\text{moles}/\mu\text{l per hr}}$$

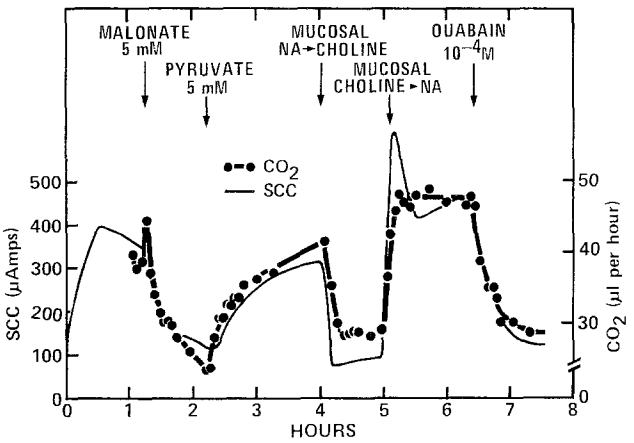

Fig. 1. Example of an experiment demonstrating the effects of several variables on short-circuit current (SCC) and CO_2 efflux

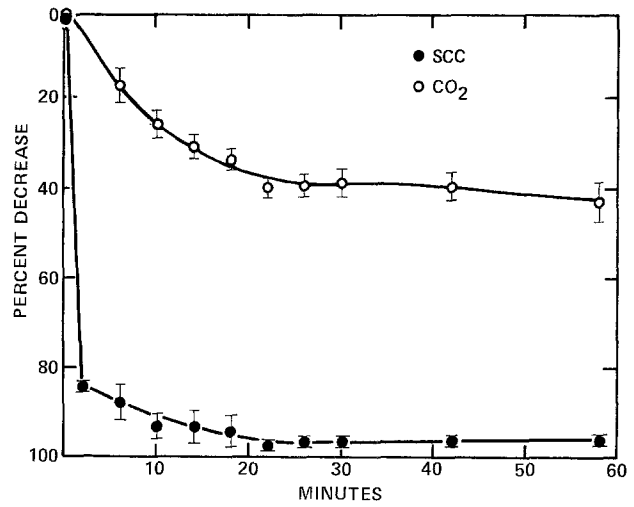

Fig. 2. Effects of substituting mucosal sodium with choline. At time zero minutes the mucosal bathing solution was changed from sodium Ringer's solution to choline Ringer's solution; the serosal medium was sodium Ringer's throughout. Plotted are the percentage decreases in short-circuit current and CO_2 efflux (mean $\pm$ standard error, $n=10$)

the mean values of this ratio are plotted in Fig. 3. Since the SCC dropped more rapidly than the CO_2 *efflux*, the initial ratios were high. This does not mean that the ratios of Δ sodium transport to ΔCO_2 *production* were equally as high. Rather, the initial high ratios occurred in large part if not entirely because the CO_2 *efflux* rate did not decrease as rapidly as the CO_2 *production* ratio: With decrease in the latter, the

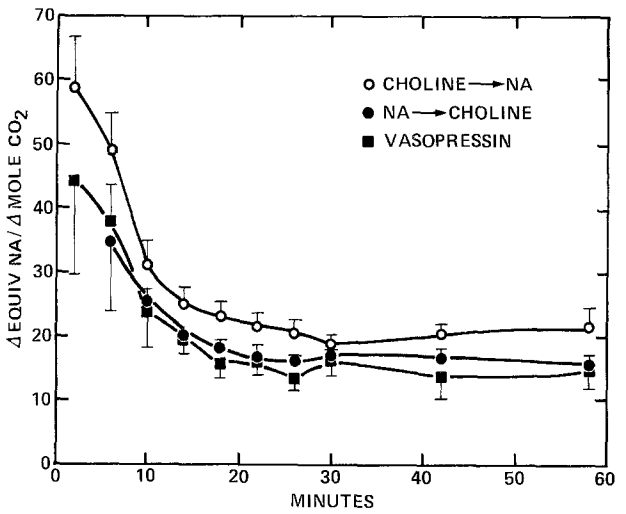

Fig. 3. Ratios of simultaneous changes of sodium transport and CO_2 efflux. Experimental variables were introduced at time zero minutes. The values plotted are the means and standard errors of the ratios of the simultaneous increments (or decrements) of sodium transport and CO_2 efflux, expressed as equivalents of sodium per mole of CO_2 ($n=7$ for choline→Na, 10 for Na→choline and 5 for vasopressin)

mean partial pressure of CO_2 in the tissue decreased, and thereafter there occurred a relatively slow loss of CO_2 from spaces in the bladder and the Ringer's solution (the loss from the bladder was found to occur with a time constant of approximately 4 min and loss from the Ringer's solution with a time constant of approximately $2^1/_2$ min. The data presented as a function of time show that stability in the ratio was achieved by 30 min. Hence the steady-state values during the second half hour (30, 42 and 58 min) were averaged for each experiment and the mean value obtained was 16.5 ± 1.2 equiv/mole ($n=10$).

(b) Choline → Na. Mucosal sodium concentration was abruptly raised in seven experiments by changing a mucosal choline Ringer's solution to sodium Ringer's in the absence of exogenous substrate (Fig. 1 contains an example of such a change). In Fig. 4 the results are depicted as approach to the 1-hr value, in per cent. The SCC rose sharply to a peak within 10 min, then fell in a curvilinear fashion to the 1-hr value. CO_2 efflux rose more gradually to a maximum between 20 and 30 min and then declined. In Fig. 3 are plotted the ratios of the increments of SCC and CO_2 efflux. As with the Na→choline experiments the initial ratios were high and dropped to fairly stable values by 30 min, averaging 20.3 ± 1.7

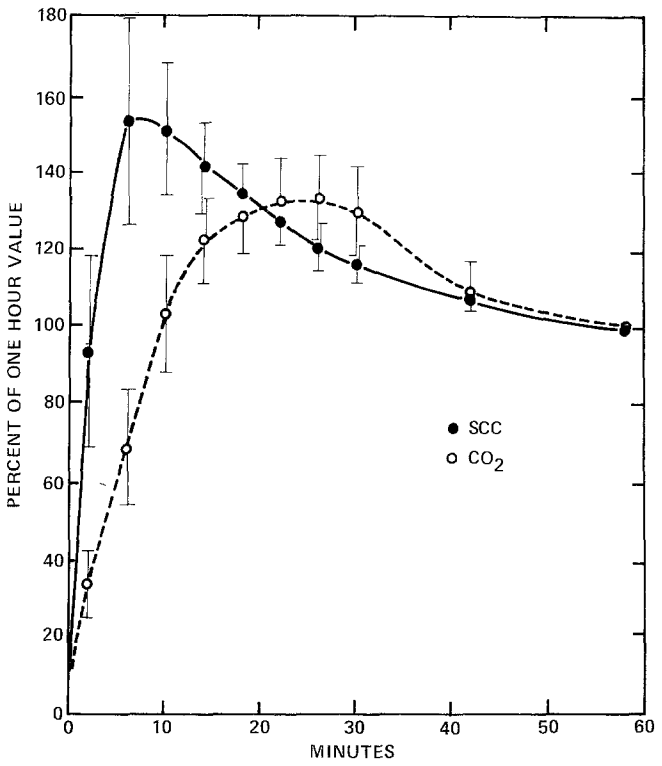

Fig. 4. Effects of replacing mucosal choline with sodium. At time zero minutes the mucosal bathing solution was changed from choline Ringer's solution to sodium Ringer's solution; the serosal medium was sodium Ringer's throughout. Plotted are the percentage increases in SCC and CO_2 efflux, taking the values at 1 hr to be 100% ($n=7$)

equiv/mole ($n=7$) during the second half hour. This value is higher than that found in the Na→choline experiments, but the difference between them (3.8 ± 2.1) is not statistically significant.

Any spontaneous decline in CO_2 production which occurred over the period of observation would exaggerate the CO_2 decrement in the Na→choline experiments—hence decrease the decremental ratio—and would diminish the CO_2 increment in the choline→Na experiments—hence increase the incremental ratio. In the absence of sodium transport, CO_2 production continued at a substantial rate—approximately 60% of that occurring in the presence of sodium transport. This "basal" [20, 24] or "resting" [10] respiration, which is associated with processes other than active sodium transport, we have termed "nontransport" respiration. Evidence that nontransport CO_2 declined spontaneously with time is provided by the ratios previously noted for the simultaneous

spontaneous declines of SCC and total CO_2 production: The value of 12.2 is lower than the 16.5 and 20.3 found in the Na⇄choline experiments, indicating that CO_2 production declined to a greater extent than expected from decline of SCC alone. If 18:1 is taken to be the expected ratio of decline of equivalents Na to moles CO_2 (*see below*), then based on the observed spontaneous decline of sodium transport, CO_2 declined by an average rate of $6.1 \pm 2.3\%$ per hour in the 12 experiments. It seemed possible that the decline in nontransport CO_2 was due to reduction in available endogenous substrate and could be prevented or minimized by providing substrate exogenously. Hence five experiments were performed in which Na→choline and choline→Na mucosal substitution were carried out in succession in the same hemibladder, in the presence of 5 mM pyruvate serosally. The mean value for Na→choline, 18.8 equiv/mole, was the same as that for choline→Na, 18.6 (difference $= 0.2 \pm 1.3$), consistent with prevention of decline of nontransport respiration.

It seems likely that spontaneous decline of nontransport CO_2 does account for the difference observed between the Na→choline and choline→Na experiments. We therefore consider the best estimate of the ratio of change of sodium transport and respiration in the absence of exogenous substrate to be the average of the mean ratios for the 10 Na→choline and the 7 choline→Na experiments, 18.3 ± 1.1 equivalents of Na per mole of CO_2.

Changes Induced by Vasopressin

Vasopressin stimulates sodium transport and respiration in the toad bladder [12]. The simultaneous effects of vasopressin (as Pitressin) 40 mU/ml on SCC and CO_2 efflux are plotted in Fig. 5 ($n = 5$). The curve for the incremental ratio (Fig. 3) could be almost superimposed on the curve for Na→choline and was of the same shape but lower than the curve for choline→Na. The mean ratio for the second half hour was 15.5 ± 2.8 equivalents Na/mole CO_2, which was somewhat lower than the mean of the Na⇄choline experiments but not significantly so.

Changes Induced by Provision of Metabolic Substrates

(a) Glucose. The addition to the serosal bathing medium of the toad bladder of certain metabolic substrates, including glucose and pyruvate, can stimulate the short-circuit current [19]. In the present studies glucose,

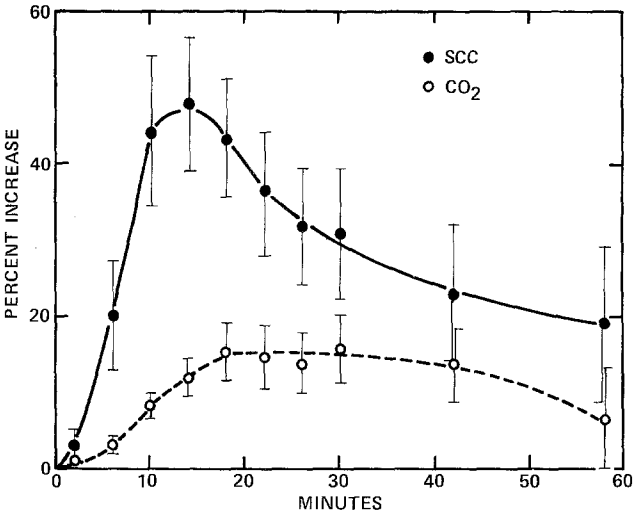

Fig. 5. Effects of vasopressin. At time zero minutes vasopressin (as Pitressin) 40 mU/ml was added to the serosal bathing medium ($n=5$). The SCC rose by a maximum of $47.8 \pm 8.4\%$ at 15 min and was $+19.0 \pm 9.8\%$ at 1 hr. CO_2 efflux plateaued at $+15.4 \pm 3.4\%$ at 20 min and was $+6.5 \pm 6.2\%$ at 1 hr. Total SCC/total CO_2 rose from 5.76 to 6.39 equiv/mole ($+0.63 \pm 0.16$, $p<0.02$)

5 or 10 mM, was added on 13 occasions; substantial stimulation of the SCC (increase of 94 to 250 μA in 1 hr) occurred in six experiments, minimal effect (increase of 8 to 35 μA) occurred in three, and no discernible effect occurred in four. In the six experiments with substantial stimulation of the SCC, CO_2 efflux rose fairly linearly. The ratio of total SCC/total CO_2 rose significantly (preglucose 3.59, 1 hr post-glucose 5.61 equiv/mole; $\Delta = +2.02 \pm 0.75$, $p<0.05$). The incremental ratio rose to a value of 13.6 ± 2.3 equiv/mole at 60 min. In one of the experiments neither the SCC nor CO_2 efflux changed until 20 min after addition; when recalculation was performed with addition of glucose considered to have been made at time 20 min, mean percentage rises of the SCC and CO_2 efflux (Fig. 6) were changed slightly, and the incremental ratio at 60 min (Fig. 7) became 15.8 ± 3.2 equiv/mole.

In the four experiments with no response of the SCC upon the addition of glucose, there was also no increase in CO_2 efflux (mean change in 1 hr $= -7.1 \pm 3.9\%$, not significantly different from the spontaneous decline found previously in the absence of substrate). Thus, the added glucose either did not enter the cells of the bladder or upon entering was not metabolized to CO_2. Metabolic stimulation was nonetheless

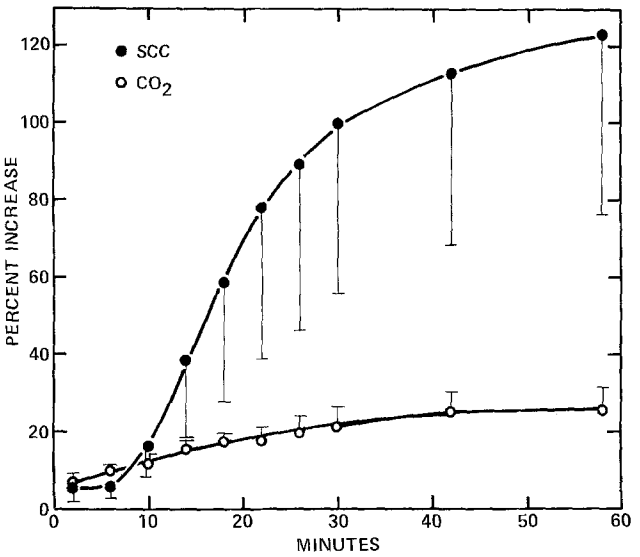

Fig. 6. Stimulation by glucose. At time zero minutes 5 mM glucose was added to the serosal bathing medium. The results are plotted as the percentage increases in six experiments in which stimulation of the SCC was substantial (>75 μA)

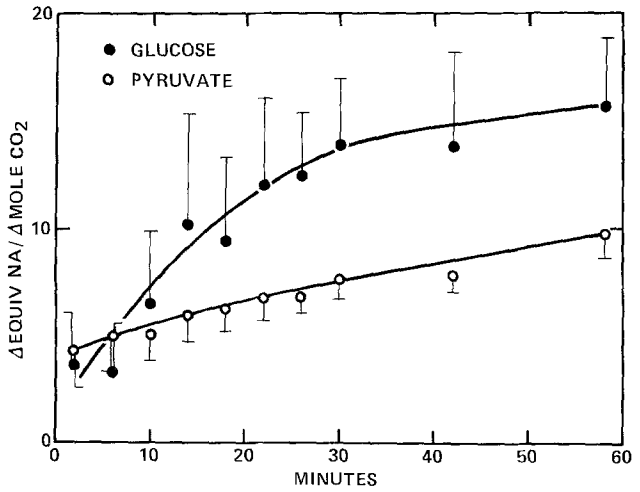

Fig. 7. Ratios of simultaneous increments of sodium transport and CO_2 efflux after addition of glucose and pyruvate. The values were obtained from the experiments included in Figs. 6 and 8 ($n=6$ for glucose and 16 for pyruvate)

possible in such hemibladders: When 5 mM pyruvate was subsequently added to three of these tissues, the SCC and CO_2 production were both promptly stimulated in two and slightly stimulated in the third.

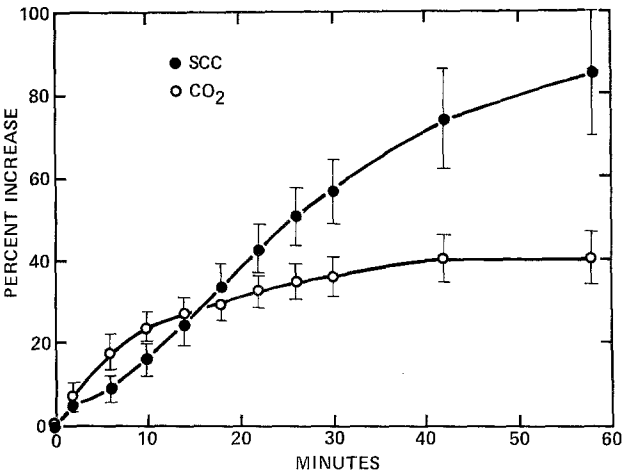

Fig. 8. Stimulation by pyruvate. At time zero minutes 5 mM pyruvate was added to the serosal bathing medium. The results are plotted as the percentage increases in 16 experiments in which stimulation of the SCC was substantial (>75 μA)

(b) Pyruvate. Pyruvate (5 mM) was added on 19 occasions. In three instances significant stimulation of the SCC did not occur (maximum increase at 1 hr was 13 μA), although CO_2 efflux rose moderately (5 to 8 μl per hr—an increase of 14 to 22%) over the ensuing 60 min. In the other 16 additions, the SCC rose by 80 to 261 μA in 1 hr. (Fig. 8; the result of an individual addition is included in Fig. 1.) Total SCC/total CO_2 rose significantly (4.70→5.75 equiv/mole, $\Delta = +1.05 \pm 0.24$, $p < 0.001$). The incremental ratio is plotted in Fig. 7. There was a fairly linear rise from 4.3 ± 1.8 equiv/mole in the first 4-min period to 9.9 ± 1.2 equiv/mole at 1 hr. The latter was significantly less than the ratio for both choline→Na (difference $= 11.7 \pm 3.1$, $p < 0.01$) and Na→choline (difference $= 5.9 \pm 1.8$, $p < 0.01$).

We assumed that the lower incremental ratio for the pyruvate experiments was due to nontransport CO_2 produced from the added pyruvate. To confirm this, 11 experiments were carried out in which 5 mM pyruvate was added to hemibladders bathed with sodium Ringer's solution serosally but choline Ringer's solution mucosally. With sodium transport thus minimized (SCC $= 9 \pm 4$ μA), the effect of pyruvate on non-transport CO_2 was observed. CO_2 efflux rose in curvilinear fashion to an approximate plateau at 20 min (Fig. 9). The small SCC rose by a mean of 11 ± 4 μA; each experiment was corrected for any rise in SCC by subtracting transport CO_2 at a ratio of 18:1 (the SCC increment was so small

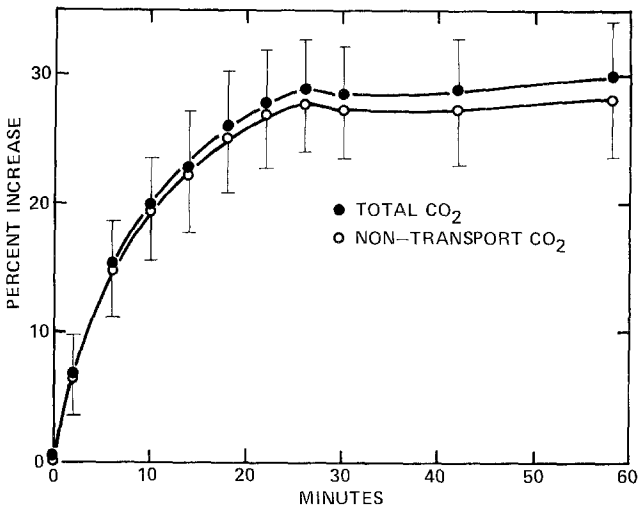

Fig. 9. Effect of pyruvate on CO_2 efflux when the mucosal medium was choline Ringer's solution. At time zero minutes 5 mM pyruvate was added to the serosal (sodium Ringer's) bathing medium ($n=11$). The actual CO_2 efflux observed is identified as "total CO_2". Upon subtracting CO_2 calculated to be associated with the small amount of sodium transport present, the "nontransport CO_2" curve was obtained

that the exact value employed for correcting CO_2 production was not critical) and a nontransport CO_2 curve was obtained (Fig. 9). This rise in CO_2 production confirmed that the low incremental ratio for the pyruvate experiments carried out in sodium Ringer's solution was at least in part attributable to increases in nontransport CO_2.

Approaches were then sought which would permit an estimate of the quantitative relationship between the increment in transport CO_2 (rather than the total increment in CO_2 depicted in Fig. 8) and the increment in sodium transport which occurred upon addition of pyruvate. Three approaches were employed:

1) Five Na⇄choline experiments (previously referred to) were carried out in the presence of 5 mM pyruvate. For the second half hours the decremental and incremental ratios averaged 18.7 ± 1.7 equiv/mole. Since the addition of pyruvate had induced a doubling of the SCC by the time the Na→choline substitution was performed—a mean increase of $+103.2 \pm 21.7\%$,—it appeared that at this time endogenous and exogenous substrates were contributing approximately equally to metabolism supporting sodium transport. If this balance persisted during the subsequent mucosal substitution with choline (an assumption of unknown

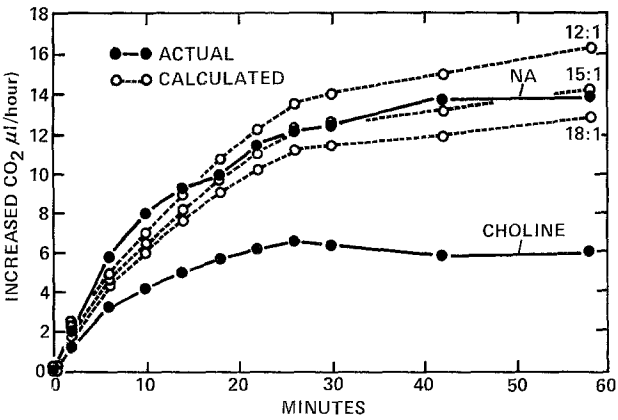

Fig. 10. Calculated versus actual CO_2 curves following addition of pyruvate. The two curves labeled Na and Choline are the mean experimental curves obtained for the increase in CO_2 efflux upon addition of pyruvate; the mucosal surfaces of the hemibladders were bathed in sodium Ringer's solution ($n=16$) and choline Ringer's solution ($n=11$), respectively. (The curves are similar to the CO_2 curves in Figs. 8 and 9, respectively, except that the CO_2 increments are expressed in microliters per hour rather than in percent.) The three dotted curves are calculated curves, based on the assumption that sodium transport and CO_2 production are associated with ratios of 12:1, 15:1 or 18:1 equivalents per mole; each represents the *sum* of *a*) the (actual) Choline curve and *b*) a (calculated) curve obtained by dividing the mean SCC incremental curve, resulting from addition of pyruvate in the presence of sodium Ringer's solution, by 12, 15 or 18 equivalents per mole. During the second half hour after addition of pyruvate, the actual curve is fit best by the calculated curve of 15 equivalents per mole

validity) the $\Delta Na/\Delta CO_2$ for pyruvate must have been close to that previously found for endogenously supported transport -18 equiv/mole.

2) The curve of the increment in total CO_2 following addition of pyruvate to bladders bathed in sodium Ringer's solution was assumed to be the summation of two curves, one for transport CO_2 and the other for nontransport CO_2. Transport CO_2 curves were then calculated from the mean SCC curve (i.e. the SCC curve in Fig. 8), using the assumption that after addition of pyruvate the incremental change in SCC occurred at a fixed ratio to CO_2. For the purposes of this calculation, the mean curves for absolute, rather than per cent, increase of total CO_2 were used. Ratios of 12:1, 15:1 and 18:1 equiv/mole were assumed, three respective transport CO_2 curves were calculated, and each was added to the nontransport CO_2 curve obtained in the choline-pyruvate experiments to obtain three predicted curves for total incremental CO_2. These curves are depicted in Fig. 10, where they are compared to the total incremental CO_2 curve actually obtained in sodium Ringer's solu-

Table 1. Effect of 5 mM pyruvate

Paired Hemibladders, Mucosal Sodium *vs.* Choline
($n=8$)

	ΔSCC (μA)			ΔCO_2(μl per hr)			Ratio of differences	
	Na	Choline	Difference	Na	Choline	Difference	μA/μl per hr	equiv/mole
	−1	0	−1	+4.7	+5.4	−0.7		
	+13	+4	+9	+5.0	+6.3	−1.3		
	+77	+22	+55	+6.4	+5.8	+0.6		
	+80	0	+80	+10.6	+3.3	+7.3	11.0	9.1
	+176	+44	+132	+23.9	+10.2	+13.7	9.6	8.0
	+156	0	+156	+13.3	+8.0	+5.3	29.4	24.4
	+216	0	+216	+18.8	+4.5	+14.3	15.1	12.5
	+261	+33	+228	+19.6	+11.4	+8.2	27.8	23.1
Mean	+122	+13	+109	+12.8	+6.9	+5.9	18.6	15.4
SE	33	6	31	2.6	1.0	2.2	4.2	3.5

tion upon adding pyruvate. By 20 min after addition of pyruvate the latter curve falls very close to the 15:1 curve.

3) In eight instances the addition of pyruvate was made to paired hemibladders, one with a mucosal bath of sodium Ringer's solution, the other with a mucosal bath of choline Ringer's solution. (The individual results were included in the grouped sodium and choline data presented previously.) The results are presented in Table 1, where the experiments are listed in order of increasing differential stimulation of the SCC. Over a period of 1 hr, the SCC rose by a mean of 122 μA in the sodium hemibladders and 13 μA in their choline pairs, a difference of 109 ± 31 μA ($p < 0.01$); CO_2 efflux rose 12.8 and 6.9 μl per hour, respectively, a difference of 5.9 ± 2.2 μl per hour ($p < 0.05$). The ratio of these mean differences (18.5 μA/μl per hr) is 15.4 equivalents Na/mole CO_2. In five of the eight experiments, SCC and CO_2 differences were large enough to permit calculation of meaningful paired incremental ratios, and a mean value of 15.4 ± 3.5 equiv/mole was obtained. When the 60-min integrals of the sodium-minus-choline increments for SCC and CO_2 were employed, the mean for the five experiments was 14.4 ± 2.5 equiv/mole. Thus, each of the three analyses of the paired experiments gave estimates for the pyruvate incremental ratio of SCC to transport CO_2 which were close to 15 equivalents Na/mole CO_2.

Changes Induced by Addition of Malonate

Malonate (5 mM) when added in the absence of substrate reduced both the SCC and the CO_2 efflux (Fig. 11; an example of an individual addition is included in Fig. 1). At 60 min SCC had declined by $60.5 \pm 8.0\%$ and CO_2 efflux by $39.2 \pm 4.2\%$ ($n=5$). Total SCC/total CO_2 decreased significantly ($7.71 \rightarrow 4.81$, $\Delta = -2.90 \pm 0.64$, $p < 0.02$). The curve for the decremental ratio dropped gradually to a value of 11.7 ± 0.6 equiv/mole at 60 min. This is significantly less than that calculated in the Na $\rightleftarrows$ choline experiments and not far from that found for pyruvate.

In the presence of glucose, malonate inhibited the SCC (by $43.5 \pm 6.8\%$ at 60 min) and CO_2 production (by 13.8 ± 3.5, $n=8$) whereas in the presence of pyruvate there was minimal if any effect. The decremental ratios in the presence of glucose proved to be variable because of the relatively small values for ΔCO_2 in the denominators; at 60 min the values averaged 25.1 ± 6.4 equiv/mole and for the second half hour were 23.1 ± 4.4.

Changes Induced by Addition of Ouabain

Results with ouabain have been reported [3]. Fig. 1 includes an example of the effect of this agent. The majority of the experiments were carried out with pyruvate present; in those experiments the mean decremental ratio at 60 min was 15 equiv/mole.

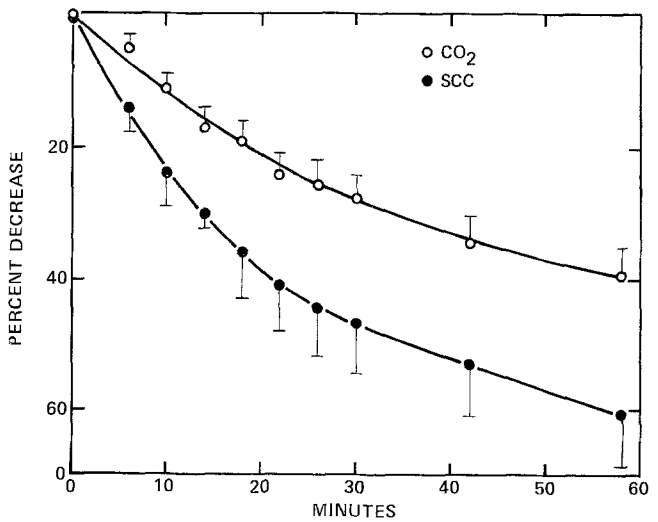

Fig. 11. Effects of malonate in the absence of exogenous substrate. At time zero minutes 5 mM malonate was added to the serosal bathing medium ($n=5$)

Discussion

Relationship of Rate of Sodium Transport to Rate of Respiration

The approach employed in the present study offered several advantages over previous studies of sodium transport and respiration in anuran membranes: The sensitivity of the conductometric method for measuring CO_2 permitted measurement of respiration at intervals of 4 min rather than intervals of up to 1 hr used previously, including our earlier study with CO_2 [18]. And measurement of the simultaneous responses of respiration and ion transport to the addition of metabolic substrates permitted an approach to the problem of possible contributions of basal energy metabolism to ion transport upon stimulating the latter.

The QCO_2 obtained from toad bladder supported by endogenous substrate was 1.34 µl per hr/mg dry wt within 3 hr of instituting the short-circuit state. With the respiratory quotient (R.Q.) of 0.92 previously measured [22] the expected QO_2 would be 1.46. This value is similar to the values found in Warburg experiments (1.38–1.57) in which toads from both Colombia and the Dominican Republic were employed, and in which Ringer's solutions of more alkaline pH and containing HCO_3^- were in some cases employed [7, 8, 13, 15].

The rate of active sodium transport was varied by three methods in these studies: *a)* by change in the concentration of sodium in the mucosal bathing medium, *b)* by addition of vasopressin, and *c)* by affecting the availability of metabolic substrates.

(a) Changes in mucosal sodium concentration. Upon varying the sodium concentration in the mucosal medium, both sodium transport and the efflux of CO_2 changed in the same direction. The steady-state ratio between these variables averaged 18 equivalents of sodium transported per mole of CO_2 produced. Employing an R.Q. of 0.9 [22], we obtain a figure of 16 1/2 equivalents of sodium transported per mole of oxygen consumed, comparable to the mean values for anuran members found by others [1, 13, 14, 21, 24, 26).

Leaf and co-workers [6], employing toads from the Dominican Republic showed that the SCC continues to be an accurate measure of active sodium transport when the mucosal bath has been partially replaced, and the serosal bath completely replaced, with choline Ringer's solution. Recently Ludens and Fanestil [16] have demonstrated in bladders from Colombian toads that hydrogen ion transport can occur concomitantly with, but opposite in direction to, sodium transport. Under this circumstance the measured SCC will underestimate the rate of net sodium

flux. However, this phenomenon correlated with the presence of HCO_3^- and CO_2 in the incubation medium and was small (about 4% of sodium transport) when phosphate-buffered Ringer's solution and low P_{CO_2} were employed, as was the case in our studies. Furthermore the rate of hydrogen ion transport was shown to be the same whether sodium transport was simultaneously present or absent (inhibited by mucosal choline substitution, amiloride or ouabain). As a consequence, in our Na⇄choline experiments any small discrepancy between the SCC and net sodium transport due to hydrogen ion transport was presumably present both before and after mucosal substitution with choline, and therefore the measured changes in SCC were very likely equal to the changes in net sodium transport.

In our previous studies [18] we obtained a somewhat lower ratio, 14 equivalents Na/mole CO_2. The results were based on 1-hr collections beginning 20–30 min after change of the mucosal solution. The discrepant results are most likely due to the different time responses of the SCC and the CO_2 efflux: As seen in particular in Fig. 4, the SCC response leads the change in CO_2 efflux; by beginning a 1-hr period of measurement 20 min after mucosal substitution, an excessive amount of CO_2 would be collected during the first portion of the period, hence the ratio of change of SCC to CO_2 would be unduly low.

(b) Addition of vasopressin. The response to posterior pituitary products has been employed previously to obtain Na/O_2 ratios in frog skin [14] and toad bladder [12]. The values obtained, 18 to 19, were similar to those obtained with removal of sodium from the external or mucosal bathing medium. In the present studies, employing the R.Q. of 1.0 observed previously for vasopressin-induced sodium transport [22], the calculated ratio for equivalents Na/mole O_2 is 15 1/2, in reasonable agreement with the previous studies.

(c) Addition of metabolic substrates. Upon adding metabolic substrates, glucose and pyruvate, both CO_2 efflux and sodium transport increased. For glucose an incremental ratio in the range of 14 to 16 equivalents per mole was obtained. With pyruvate a ratio of 10 equiv/mole was obtained 1 hr after addition, which was distinctly less than that obtained in Na⇄choline experiments. This low ratio was due at least in part to the increased production of nontransport CO_2 upon adding pyruvate, as evidenced by observing increased CO_2 production upon adding pyruvate in the absence of sodium transport. Efforts were made to obtain the best estimate of the ratio of sodium transported to transport CO_2.

Table 2. Relationship between active sodium transport and respiration

	n	μA	Equivalents Na	R.Q. assumed	Equivalents Na
		μl/hr	Mole CO_2		Mole O_2
I. ΔNa transport/Δ total respiration					
Spontaneous	12	14.7±2.8	12.2±2.3	0.9	11
Na⇄choline	17	22.1±1.3	18.3±1.1	0.9	$16^1/_2$
Vasopressin	5	18.7±3.4	15.5±2.8	1.0	$15^1/_2$
Glucose	6	19.0±3.9	15.8±3.2	1.0	16
Pyruvate	16	11.9±1.4	9.9±1.2	1.2	12
Malonate	5	14.1±0.7	11.7±0.6	0.9	$10^1/_2$
II. ΔNa transport/Δ "transport" respiration					
Pyruvate					
Na⇄choline	5	~22	~18	1.2	~22
Na *vs.* choline, unpaired	16, 11	18	15	1.2	18
Na *vs.* choline, paired	5				
Values at 1 hr		18.6±4.2	15.4±3.5	1.2	$18^1/_2$
Integrals for 1 hr		17.3±3.0	14.4±2.5	1.2	17

By an initial method, a ratio of 18 equiv/mole was calculated. Two other methods of analysis, considered to be more accurate, provided ratios of 15 equivalents Na/mole CO_2. Therefore, with a respiratory quotient for pyruvate of 1.2, we take the most likely ratio to be 18 equivalents Na/mole of oxygen. This would be consistent with 3 Na^+ transported per molecule of ATP consumed.

These results, which are summarized in Table 2, appear to shed new light on the problem of basal respiration. A number of authors have pointed out that incremental or decremental ion/oxygen ratios obtained by primarily stimulating or inhibiting ion transport may be erroneously low because basal (nontransport) respiration might not remain constant as transport changes. Rather, upon stimulating transport, the resting respiration might be "dipped into" for use in sodium transport [4, 9, 10, 20, 26]. This has been a difficult concern with which to deal, and little evidence either for or against dipping into basal respiration is available. In favor of its possible occurrence, Hokin and Hokin [9] noted that pancreatic secretion can be stimulated with no increase in respiration; and Whalen [25] concluded that the resting respiration of skeletal muscle is dipped into during isotonic contraction. In counter-argument,

Martin and Diamond [20] emphasized that the ratio is approximately the same in several epithelia with sodium pumps, although these pumps account for quite different percentages of the total oxygen uptake by these tissues.

We consider that our approach with pyruvate provides persuasive evidence that resting respiration is not dipped into to support sodium transport. There is no compelling reason why basal oxygen consumption should contribute to the energy of sodium transport when sodium transport is stimulated by the provision of exogenous substrate. To the contrary, obtaining a similar ratio for ions transported per molecule of oxygen utilized whether employing Na⇄choline substitution or adding pyruvate strongly suggests that basal metabolism is not significantly changed when rate of sodium transport is stimulated primarily. Hence we conclude that in the toad bladder a mean ratio of 16 to 18 sodium ions transported per molecule of oxygen consumed can be accepted with some confidence.

Vieira, Caplan and Essig [24] have suggested that in the frog skin the number of sodium ions transported per molecule of O_2 utilized is not fixed, but rather varies from tissue to tissue. Al-Awqati, Beauwens and Leaf [1] have drawn the same conclusion for the toad bladder, based on CO_2 studies. From our results we can neither confirm nor deny such a possibility; our interpretations are based simply on the average behavior of the tissues studied whether or not the values measured represent a homogeneous or a heterogeneous population. However, it must be kept in mind that Na/O_2 and Na/CO_2 ratios based on observations of spontaneous decline, as was the case in the studies cited [1, 24], are subject to the complications introduced by possible variations in the decay rate of nontransport metabolism. In our studies we obtained substantial evidence that nontransport CO_2 declined in experiments where endogenous metabolism was providing the only energy:

1. Spontaneous decline of sodium transport and CO_2 production occurred at a significantly lower ratio than did changes induced by Na⇄choline substitution (12.2 *vs.* 18.3 equiv/mole, difference $= 6.1 \pm 2.6$, $p < 0.05$), but the ratio was not significantly different from that found by Vieira *et al.* (14.6) [24] or by Al-Awqati *et al.* (10.0) [1]. Based on our figures the nontransport CO_2 declined by 6% per hr.

2. Choline→Na gave higher ratios than Na→choline, consistent with a decline of nontransport CO_2.

3. When exogenous substrate (pyruvate) was present, the differences between choline→Na and Na→choline were not observed.

In addition, in our experiments the range of values measured for $\Delta Na/\Delta CO_2$, when considered in relation to the experimental error, was not so excessive that we are inclined to invoke any explanation save experimental reproducibility. The coefficient of variation for our ratios was 21% ($n=29$ different toads) whereas we calculate that the value of Vieira *et al.* was 44% ($n=10$) and of Al-Awqati *et al.* was 65% ($n=28$).

Separation of Transport and Basal Respiration

The demonstration that transport metabolism does not dip into basal metabolism indicates that the metabolism supporting active transepithelial sodium transport is functionally, if not spatially, separate from metabolism supporting other tissue processes in the toad bladder. Such a separation was proposed previously on the basis of tentative evidence only [19] and was supported by the demonstration that the R.Q. of metabolism supporting sodium transport (0.86) was different from that of nontransport metabolism (0.93) [22]. Further evidence that transport and nontransport metabolism behave differently is provided by analyzing their relative responses to experimental variables:

If the sodium: CO_2 ratio is known, the ratio of total SCC to total CO_2 production can be employed to determine the fraction of the total respiration of the tissue which is providing energy for sodium transport at that time. That is, total SCC/total $CO_2 \div \Delta SCC/\Delta CO_2 =$ transport CO_2/total CO_2. During the first 3 hr after mounting, the mean ratio of total SCC to total CO_2 production was approximately 6 equiv/mole; and in the absence of exogenous substrate, 18 equivalents of sodium transported entails on the average 1 mole of CO_2 production. Under the baseline *in vitro* conditions of our experiments, therefore, in freshly removed tissue 1/3 of the respiration was devoted on the average to the support of active sodium transport.

Based on studies of the supporting layer scraped free of epithelial cells, the supporting layer contributes 15–20% of the total respiration of the toad bladder.[2] Thus, half of the total tissue respiration must be nontransport respiration generated by the epithelial cells. Although at the present time we can provide no information on the relative re-

2 The supporting layer has been estimated to make up approximately 80% of the mass of the toad bladder [7, 15]. When epithelial cells have been scraped from it, the supporting tissue has had a QO_2 averaging 0.3 μl/mg dry weight [7, 8, 15]. Thus, the supporting tissue contributes 0.24 μl of the 1.40–1.50 μl produced per mg dry weight of intact, sodium-transporting tissue, or 16–17%.

sponses of the two nontransport CO_2 sources (supporting layer and epithelial layer) to our experimental variables (e.g. the passage of time, exogenous substrates, metabolic inhibitors), transport metabolism appears to be much more sensitive and responsive than nontransport metabolism considered as a whole. This can be inferred from evaluation of the effects of variables on the ratios of total SCC to total CO_2 production and on the incremental and decremental ratios:

(a) Substrate stimulation. Total SCC/total CO_2 rose significantly after adding either glucose or pyruvate. If transport and nontransport respiration were each stimulated by pyruvate in proportion to their pre-pyruvate magnitudes, the ratio would drop because the R.Q. of pyruvate (1.2) is greater than the R.Q. of toad bladders respiring in the absence of added substrate (0.9) [22]. The observed increase thus is attributable to a greater proportionate increase in transport CO_2 than in nontransport CO_2. By the calculation presented in Table 3, the difference was six fold.

(b) Malonate inhibition. When added in the absence of exogenous substrate, malonate reduced sodium transport and CO_2 production with a decremental ratio significantly less than the ratio obtained in Na⇄choline experiments. This indicates that malonate inhibited both transport and nontransport metabolism. Since the ratio of total SCC to total CO_2 also dropped, transport metabolism was reduced to a greater extent than nontransport metabolism[3]; by calculation (Table 3) the reduction was threefold greater.

Malonate added in the presence of pyruvate had minimal effect on both transport and CO_2 production—consistent with the explanation that pyruvate leads to a concentration of succinate sufficient to overcome inhibition of succinic dehydrogenase [5, 11]. In the presence of glucose, however, malonate inhibited the SCC at a ratio of 23 ± 4 equiv/mole CO_2, suggesting that it inhibited only transport CO_2: Perhaps malonate penetrates mitochondria which provide energy for sodium transport more effectively than it does mitochondria supporting nontransport functions, or the concentration of succinate at the transport site may be lower than at nontransport sites.

(c) Spontaneous decline. The spontaneous declines in SCC and CO_2 production, observed 2 to 5 hr after mounting in substrate-free Ringer's solution and instituting the short-circuited state, occurred at a Na/CO_2

3 Assuming no major change in R.Q.

Table 3. Relative responses of transport and nontransport metabolism

Variable	SCC/CO_2	CO_2 Production rate				
	(Equiv/ mole)	Transport[a] / Total	Total[b] (μl per hr)	Transport[c] (μl per hr)	Non-transport (μl per hr)	Relative[d] response
Spontaneous						
Before	4.86	27%	40.7	11.0	29.7	
After	4.37	24%	36.9	8.9	28.0	
Change				−19%	−6%	3.2
Pyruvate						
Before	4.70	26%	37.6	9.8	27.8	
After	5.75	32%	51.6	16.3	35.3	
Change				+166%	+27%	6.2
Malonate						
Before	7.71	42%	41.0	17.2	23.8	
After	4.81	26%	25.0	6.5	18.5	
Change				−62%	−22%	2.8

Calculations are based on the changes induced by three experimental variables: the passage of time, addition of 5 mM pyruvate, and addition of 5 mM malonate. The *Before* values are the baseline values present at time 0. The *After* values are the values at time 60 min.

[a] Calculated in the *Before* situation as SCC/CO_2 ÷ 18.3 (*see* text). The *After* values for *spontaneous* and *malonate* experiments were calculated in this same way; for *pyruvate* the *after* value was obtained by taking *transport* CO_2 to equal *total* CO_2 minus the average CO_2 present in the 11 choline Ringer-pyruvate experiments.

[b] Mean experimental values obtained at time 0 and time 60 min.

[c] Calculated as the product of the two preceding columns.

[d] % change transport CO_2 ÷ % change nontransport CO_2.

ratio lower than that for Na ⇄ choline. Since total SCC/total CO_2 also dropped, transport metabolism decreased more than nontransport metabolism − 3 times greater by calculation (Table 3). The similarity of the ratios for spontaneous decline and pyruvate stimulation suggests that the spontaneous decline was due to a progressive decrease in available metabolic substrates which could be reversed by the addition of pyruvate.

We conclude that in the toad bladder metabolism supporting active transport of sodium is functionally separated from metabolism supporting other energy-requiring processes. Whether this separation is due to discrete delineation, as by membranes, or is due to some qualitative

difference between the metabolism supporting the different functions remains a subject of speculation and continued interest.

The authors are grateful for the technical assistance of Anne Chamberlain, Marjorie C. Smith and Helen Golbetz. This investigation was supported by a grant-in-aid from the American Heart Association, with funds contributed in part by the Santa Clara County Heart Association, by United States Public Health Service Grant AM-05678, and by the Medical Research Service of the Veterans Administration.

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. Coggins, C.H., Maffly, R.H. 1965. A method for measuring micromolar quantities of carbon dioxide and $C^{14}O_2$ produced by transporting epithelial membranes in vitro. *Anal. Biochem.* **10**:266
3. Coplon, N.S., Maffly, R.H. 1972. The effect of ouabain on sodium transport and metabolism of the toad bladder. *Biochim. Biophys. Acta* **282**:250
4. Davenport, H.W. 1952. Substrate and oxygen consumption during gastric secretion. *Fed. Proc.* **11**:715
5. Falchuk, M.Z., Sharp, G.W.G. 1968. On the role of the tricarboxylic acid cycle in the stimulation of sodium transport by aldosterone. *Biochim. Biophys. Acta* **153**:706
6. Frazier, H.S., Dempsey, E.F., 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
7. Gatzy, J.T., Berndt, W.O. 1968. Isolated epithelial cells of the toad bladder. Their preparation, oxygen consumption, and electrolyte content. *J. Gen. Physiol.* **51**:770
8. Hays, R.M., Singer, B., Malamed, S. 1965. The effect of calcium withdrawal on the structure and function of the toad bladder. *J. Cell Biol.* **25**:195
9. Hokin, L.E., Hokin, M.R. 1960. The role of phosphatidic acid and phosphoinositide in transmembrane transport elicited by acetylcholine and other humoral agents. *Int. Rev. Neurobiol.* **2**:99
10. Judah, J.D., Ahmed, K. 1964. The biochemistry of sodium transport. *Biol. Rev.* **39**:160
11. Krebs, H.A., Lowenstein, J.M. 1960. The tricarboxylic acid cycle. *In:* Metabolic Pathways. D.M. Greenberg, editor. Vol. 1, p. 129. Academic Press, New York
12. Leaf, A., Dempsey, E.F. 1960. Some effects of mammalian neurohypophyseal hormones on metabolism and active transport of sodium by the isolated toad bladder. *J. Biol. Chem.* **235**:2160
13. Leaf, A., Page, L.B., Anderson, J. 1959. Respiration and active sodium transport of isolated toad bladder. *J. Biol. Chem.* **234**:1625
14. Leaf, A., Renshaw, A. 1957. Ion transport and respiration of isolated frog skin. *Biochem. J.* **65**:82
15. Lipton, P., Edelman, I.S. 1971. Effects of aldosterone and vasopressin on electrolytes of toad bladder epithelial cells. *Am. J. Physiol.* **221**:733
16. Ludens, J.H., Fanestil, D.D. 1972. Acidification of urine by the isolated urinary bladder of the toad. *Am. J. Physiol.* **223**:1338
17. Maffly, R.H. 1968. A conductometric method for measuring micromolar quantities of carbon dioxide. *Anal. Biochem.* **23**:252

18. Maffly, R.H., Coggins, C.H. 1965. Carbon dioxide production and sodium transport by the toad bladder. *Nature (Lond.)* **206**:197
19. Maffly, R.H., Edelman, I.S. 1963. The coupling of the short-circuit current to metabolism in the urinary bladder of the toad. *J. Gen. Physiol.* **46**:733
20. Martin, D.W., Diamond, J.M. 1966. Energetics of coupled active transport of sodium and chloride. *J. Gen. Physiol.* **50**:295
21. Nellans, H.N., Finn, A.L. 1974. Oxygen consumption and sodium transport in the toad urinary bladder. *Am. J. Physiol.* **227**:670
22. Swenson, R.S., Maffly, R.H. 1968. Effect of sodium transport and vasopressin on the respiratory quotient of the toad bladder. *Nature (Lond.)* **218**:959
23. 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
24. 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
25. Whalen, W.J. 1965. Evidence for the Fenn effect in skeletal muscle exposed to low pO_2. *Nature (Lond.)* **205**:1224
26. Zerahn, K. 1956. Oxygen consumption and active sodium transport in the isolated and short-circuited frog skin. *Acta Physiol. Scand.* **36**:300