Active H$^+$ Transport in the Turtle Urinary Bladder

Coupling of Transport to Glucose Oxidation

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ABSTRACT The turtle urinary bladder acidifies the contents of its lumen by actively transporting protons. H$^+$ secretion by the isolated bladder was measured simultaneously with the rate of $^{14}CO_2$ evolution from [^{14}C]glucose.

The application of an adverse pH gradient resulted in a decline in the rate of H$^+$ secretion (J_H) and in the rate of glucose oxidation ($J^*_{CO_2}$). The changes in J_H and $J^*_{CO_2}$ were linear functions of the pH difference across the membrane. Hence, J_H and $J^*_{CO_2}$ were linearly related to each other. The slope, $\partial J_H/\partial J^*_{CO_2}$, was found to be similar in half-bladders from the same animal but was seen to vary widely in a population of turtles.

To investigate the effect of pH gradients on $\partial J_H/\partial J^*_{CO_2}$, two experiments were performed in each of 14 hemibladders. In one, J_H and $J^*_{CO_2}$ were altered by changing the luminal pH. In the other, they were altered by changing the ambient pCO$_2$ while the luminal pH was kept constant. The average slope, $\partial J_H/\partial J^*_{CO_2}$, in the presence of pH gradients was 14.45 eq·mol^{-1}. In the absence of gradients in the same hemibladders it was 14.72, $\Delta = 0.27 \pm 1.46$.

The results show that H$^+$ transport is organized in such a way that leaks to protons in parallel to the pump are negligible. Analysis of the transport system by use of the Essig-Caplan linear irreversible thermodynamic formalism shows that the system is tightly coupled. The degree of coupling, q, given by that analysis was measured and found to be at or very near the maximum theoretical value.

INTRODUCTION

Urinary acidification occurs throughout the renal tubules and is thought to be an active process (18). The finding that the turtle urinary bladder acidifies its contents (19) had led to a clarification of the cellular processes responsible for acid secretion. It has been shown that urinary acidification occurs against a gradient (29) and that the active step appears to be located at the luminal border of the epithelium (7, 27). The weight of evidence favors the view that the transported species are protons (21) (or OH$^-$ in the opposite direction) as opposed to HCO$_3^-$ absorption (19). H$^+$ secretion is stimulated by the presence of ambient CO$_2$ and is dependent on the activity of carbonic anhydrase in the epithelial cell (22, 23). It is inhibited by anoxia and by metabolic inhibitors (28) and is stimulated by the hormone aldosterone (3).

Active transport, as defined by Kedem (10), occurs when the flow of the transported species is coupled to the flow of metabolic reactions. On the basis of this formulation, Essig and Caplan developed a theoretical analysis of active ion transport in epithelia, using linear irreversible thermodynamics (6). Experimental use of this formalism has been hampered by the lack of methods that measure the flow of the relevant metabolic reaction coupled to transport. However, the rate of overall oxidative reactions was recently used as an index of the flow in the metabolic pathway, and the coupling between sodium transport and the simultaneously measured O_2 consumption (5, 33) or CO_2 production (2) was investigated in amphibian epithelia. In the present study we use a method that allows measurement of the flow of one subset of the metabolic pathways, namely the rate of glucose oxidation. We describe the relation of H^+ transport to total oxidative metabolism and to glucose oxidation. The results show that H^+ transport is tightly coupled to total respiration and to glucose oxidation. The H^+ transport system can be described as a pump-leak system where the leak is vanishingly small. As a result the rate of transport is effectively regulated by the development of transepithelial gradients. The efficiency of energy conversion is seen to be very high. Analysis of the coupling by irreversible thermodynamics shows that the system is tightly coupled and that the degree of coupling is at or very close to the theoretical maximum. Proton conductors such as 2,4-dinitrophenol effectively "uncouple" the system by increasing the parallel back-leak.

MATERIALS AND METHODS

Fresh-water turtles *Pseudemys scripta elegans* (Educational Products Div., The Mogul Corp., Oshkosh, Wis.), were kept in tap water at room temperature. Hemibladders from doubly pithed turtles 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 millimoles/liter, NaCl 113, KCl 3.5, Na_2HPO_4 2.0, $CaCl_2$ 1.0, $MgCl_2$ 0.5. The pH of the Ringer was adjusted to the desired value with 1 N HCl. After each experiment the exposed area of the membrane was cut, and the dry weight was determined after drying in a hot air oven at 90°C overnight (mean dry wt ± SE 22.1 mg ± 1.80). The spontaneous transepithelial potential difference was sensed by 3 M KCl-agar bridges and balanced calomel half-cells connected to a Keithley model 600B voltmeter (Keithley Instruments, Inc., Cleveland, Ohio). An automatic voltage clamp supplied enough current to nullify this spontaneous PD via Ag-AgCl electrodes and KCl-agar bridges. The short-circuit current was read from a Weston DC microammeter.

Measurement of the Rate of H^+ Secretion

In all experiments 0.5 mM ouabain was added to the serosal bathing medium. The short-circuit current rapidly declined and reversed in sign to reach a stable rate within 2 h. We measured the net rate of urinary acidification by the pH stat technique (30) simultaneously with the short-circuit current in 19 hemibladders. In the absence of exogenous CO_2 the slope of the short-circuit current against the pH stat rate was 0.998 ± 0.00 $n = 5$. With 1% CO_2 in air as the gassing mixture the slope was 1.04 ± 0.11 $n = 14$. These results include values obtained with the mucosal pH being varied from 5 to 7.4. The equivalence of those two methods of measurements confirms the earlier findings of Steinmetz (30).

Measurement of the Total CO₂

Total CO_2 production was measured by the method of Maffly and Steele (15) as previously described (2). Turtle bladders were mounted in chambers and bubbled with CO_2-free air. The gas effluent from the chamber, now containing CO_2 produced by the bladder, was passed into a glass coil to equilibrate with a moving stream of dilute NaOH. The conductivity of the alkali solution was measured before and after it equilibrated with the CO_2-containing gas, by using flow-through conductivity cells. The output of these cells was connected to a Wescan differential conductivity meter (Wescan Instruments, Santa Clara, Calif.). Appropriate calibrations were performed daily with known volumes of CO_2.

Continuous Measurement of ¹⁴CO₂ from Labeled Substrates

This method developed by Tolbert (32) utilizes an ionization chamber. ¹⁴C-Labeled substrates (uniformly labeled [¹⁴C]glucose [New England Nuclear, Boston, Mass.] was used in the present series) were placed in the serosal chamber in the appropriate specific activity. The gas effluent from the chamber was passed through a 10-ml drying chamber containing silica gel (Tel-Tale, grade 44, mesh size 3–8, Davison Chemical, Baltimore, Md.). The gas was then passed through a Cary-Tolbert ionization chamber of 275 ml volume (Cary Instruments no., 3595600, Varian Instrument Division, Cary Products, Monrovia, Calif.). The ionization chamber was connected to two 45-V Burgess batteries in series so that 90 V were present between the central rod of the chamber and the outer shell. The output of the chamber was connected to a Cary vibrating reed electrometer (Cary Instruments, model 401). The instrument was placed in the current mode with the 10^{12} Ω resistor used. The vibrating reed electrometer was connected to a recorder.

Initial calibration of the ionization chamber was done with ¹⁴CO₂ generated from $Ba^{14}CO_3$ of accurately known specific activity (New England Nuclear, no. NES 002). The calibration curve was linear and gave a value of 630.4 dpm/mV. It was found that this calibration constant did not vary over periods as long as 2 yr. Furthermore, the calibration constant was the same whether there was continuous air flow in the ionization chamber or not. Care had to be taken to insure that the ionization chamber remained dry throughout the experiment.

Calculation of the rate of ¹⁴CO₂ production was performed according to the following equation:

$$J^*_{CO_2} = \frac{(C)(A)(6)(R)}{(SA)(V)(DW)},$$

where C is the calibration constant in dpm/mV, A is the airflow through the chamber in ml/min, R is the electrometer reading in mV, SA is the specific activity of [U-¹⁴C]glucose in dpm/nmol of total glucose in the medium, DW is the dry weight of the bladder in mg, V is the volume of the ionization chamber = 275 ml, and the factor 6 comes from the fact that oxidation of 1 mol of glucose liberates 6 mol of CO_2. The final glucose concentration was 10 mM. In preliminary experiments it was found that the rate of glucose utilization was a curvilinear function of glucose concentration up to 20 mM. At 10 mM the glucose utilization rate did not result in a change in the bulk glucose concentration by more than a few percent for each 4-h experiment.

In all CO_2 experiments the media contained 0.5 mg/ml carbonic anhydrase, 0.1 mg/ml penicillin, 40 μg/ml gentamicin, and 0.5 μg/ml colistin. All media were then filtered through Millipore filters (no. HAWP 04700, Millipore Corp., Bedford, Mass.). The bladder was washed three times in these media for a total of 30 min, and then fresh

solutions were placed and the experiment started. The pH of the serosal medium was kept near 7.0. The luminal pH was changed by addition of dilute HCl to the desired level during the experiment. At the end of the experiment the media were bubbled with air and checked for CO_2 production as an index of bacterial growth. Cultures showed that in spite of the presence of antibiotics, bacteria and fungi continue to grow, albeit at low rates. The $^{14}CO_2$ production measured by bubbling the medium at the end of the experiments was usually about twice the amount of the background radioactivity of the system measured at the beginning of each experiment. It was assumed that this increase in "background" during the experiment occurred in a linear fashion and was subtracted from the total $^{14}CO_2$ signal of the bladder.

The change in background due to bacteria was a fraction of the signal produced by the epithelium. The $^{14}CO_2$ production during each experiment varied with the rate of H^+ transport. The lowest signal, observed at zero net transport, was at least three times the total background (background radioactivity plus $^{14}CO_2$ production due to microorganisms). Frequently, it was much higher; hence, although microorganisms grow logarithmically, their rate of $^{14}CO_2$ production was sufficiently low with respect to the signal observed that the difference between a linear increase and a logarithmic one at these rates is probably small and would not contribute a significant error into the calculation of the $^{14}CO_2$ production by the membrane.

All of the results of the rate of H^+ transport and CO_2 production were normalized to the dry weight of the exposed area of the bladder. In each experiment the J_H and J_{CO_2} were plotted against the pH gradient across the membrane and against each other, and the slopes and intercepts were calculated by linear regression analysis. Statistical analysis was performed using Student's t-test for paired populations in all of the studies. The confidence intervals for evaluation of q were performed according to the one-tailed distribution (26) where 95% limit = $\bar{x} - 1.645(SE)$ and 99% limit = $\bar{x} - 2.326(SE)$.

RESULTS

Relation of the Rate of Transport of CO_2 Production

Most of the short-circuit current of turtle bladders is due to sodium transport. Simultaneous measurement of CO_2 production (conductometrically) and Na transport showed that J_{Na} and J_{CO_2} are linearly related. This is similar to what we found in the toad bladder (2). In three experiments the initial J_{Na} averaged 8.32 neq·min^{-1}·mg dw^{-1}; and the initial J_{CO_2} was 1.870 nmol·min^{-1}·mg dw^{-1}. The J_{Na} slowly declined with time; the J_{CO_2} showed a parallel change. The slope of J_{Na} against J_{CO_2} gave a value of 12.42 eq·mol^{-1} (average of 20.1, 10.5, and 6.67). When J_{Na} was nullified by adding 0.5 mM ouabain, J_{CO_2} declined to 1.014 nanomol·min^{-1}·mg dw^{-1}. Hence, it appears that in these experiments 46% of the total CO_2 produced is related to sodium transport. Schwartz and Steinmetz (23) found that ouabain (0.1 mM) inhibited both the short-circuit current and the J_{CO_2} (measured conductometrically by a different method); with this dose of ouabain 28% of the CO_2 production was related to Na transport. The $\partial J_{Na}/\partial J_{CO_2}$ in their experiments averaged 7.78 eq·mol^{-1}.

Ouabain induced a reversal in the sign of the short-circuit current. As was pointed out in Methods, this reversed current can be completely accounted for by H^+ transport into the luminal medium. The rate of H^+ secretion (J_H) averaged 0.355 neq·min^{-1}·mg dw^{-1}. Addition of HCl in quantities sufficient to

reduce the pH of the luminal medium resulted in a decline in J_H (29). When J_H was nullified, the J_{CO_2} was 0.909 nmol·min^{-1}·mg dw^{-1}. The slope of J_H on J_{CO_2} in these three experiments averaged 3.3 eq·mol^{-1} (average of 4.19, 2.87, and 2.84).

With turtle bladders mounted in Ussing chambers, addition of uniformly labeled [^{14}C]glucose (final glucose concentration 10 mM) results in the production of $^{14}CO_2$ which reaches a steady state after about 2 h. The J_H is measured as the short-circuit current after the addition of 0.5 mM ouabain to the serosal side in all of the experiments reported in this paper. Provided that the luminal pH is kept constant, the rate of J_H is remarkably constant for several hours.

Changing the serosal pH over a range of 5.6–7.3 had no effect on J_H or $J^*_{CO_2}$ as shown in Fig. 1. In contrast, small changes in mucosal pH had large effects on both the rate of transport and of glucose oxidation. A typical experiment is shown in Fig. 2 where the pH of the luminal bathing solution was changed by adding HCl or NaOH. In the bottom panel of Fig. 2, it is seen that after
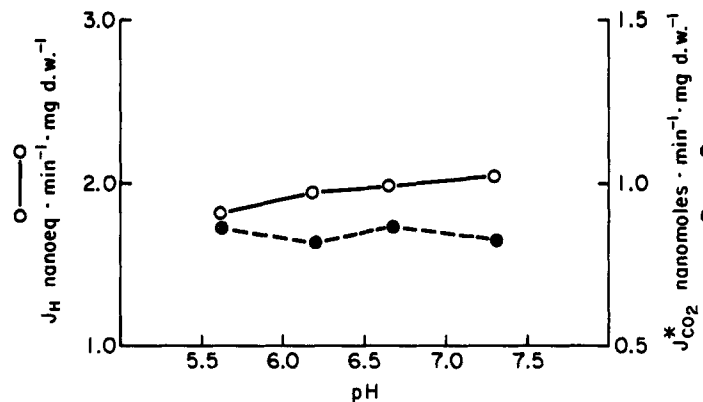

FIGURE 1.   The effect of change of serosal pH on the rate of H^+ secretion (J_H) and the rate of $^{14}CO_2$ production ($J^*_{CO_2}$). Mucosal pH was kept constant at 7.2.

alterations in the mucosal pH, J_H changed almost instantaneously, reaching a new steady state within 1 min. J_H increased as the luminal pH increased. The changes in J_H were accompanied by slower changes in $J^*_{CO_2}$ in the same direction, reaching a new steady state within 10–15 min; Fig. 2, top panel.

A series of experiments was performed where the pH of the luminal medium was changed in a random fashion at constant serosal pH and the steady-state J_H, $J^*_{CO_2}$, and transepithelial pH difference were recorded. All the experiments were performed in the short-circuited state. The relation of J_H and $J^*_{CO_2}$ to the pH difference and to each other was analyzed by linear regression. In each bladder, applying an adverse pH gradient resulted in a decline in J_H and $J^*_{CO_2}$. The relation of J_H and $J^*_{CO_2}$ to the pH was highly linear, as shown in Fig. 3. Table I shows the results of this series of experiments where five to seven measurements were performed in each bladder. The regression coefficient, r, is seen to be high for all of these experiments, exceeding 0.89 in all experiments. Fig. 3 shows a representative experiment. Since J_H and $J^*_{CO_2}$ were linear in pH, it is to be

expected that they would be linearly related to each other, as shown in Table I and Fig. 3, bottom panel.

Constancy of the "Basal Metabolism"

When J_H was brought to zero, a significant amount of $J^*_{CO_2}$ remained, as shown in Fig. 3, bottom panel. In analyzing the results, it is assumed that this "basal metabolism" remains constant during the period of the experiment. This assumption is validated by two observations. The impressive linearity obtained in the experiments shown in Table I and Fig. 3 would have been highly unlikely if the basal metabolism was changing. This is particularly so since the periods obtained in these experiments included changes in J_H produced in random order. In addition, five experiments were conducted in which J_H was brought to zero by the application of a pH gradient, and the $(J^*_{CO_2})_{J_H=0}$ was followed for 2 h. 1 h after the abolition of J_H, the average $J^*_{CO_2}$ was 0.557 nmol·min^{-1}·mg dw^{-1}, and the value after 2 h was 0.507, $\Delta \pm SE = 0.05 \pm 0.03$.
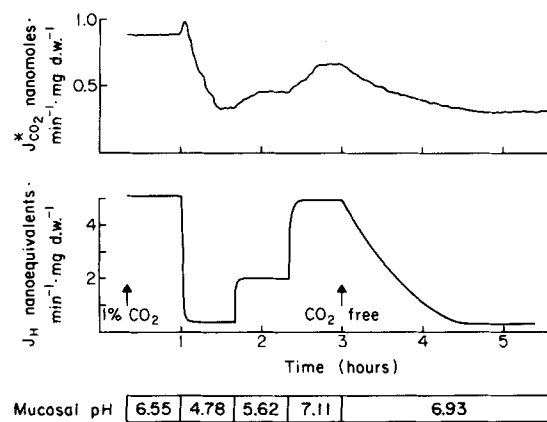

Figure 2. Effect of changing mucosal pH on the rate of H$^+$ secretion (J_H) and the rate of $^{14}CO_2$ production ($J^*_{CO_2}$) with constant serosal pH. The gassing mixture was 1% CO_2 in air which was later changed to CO_2-free air at the arrow marked CO_2-free.

The Effect of Acetazolamide

A set of four experiments was performed to investigate the effect of this agent on basal metabolism. The J_H was nullified by luminal acidification, and the $J^*_{CO_2}$ was followed until it reached a steady state. Acetazolamide 1 mM was added to the serosal side, and the CO_2 production was followed for 1 h more. In these experiments the $J^*_{CO_2}$ before acetazolamide, 0.230 nmol·min^{-1}·mg dw^{-1}, was not different from that after, 0.231, $\Delta \pm SE = 0.001 \pm 0.008$, $n = 4$. This suggests that when the luminal bath pH is lowered to the level where net H$^+$ transport is zero, the rate of the H$^+$ pump at that point is also zero. Had there been any active transport going on, it would be expected that acetazolamide at this high dose would have inhibited it, thus reducing $J^*_{CO_2}$. This point will receive further attention in the Discussion.
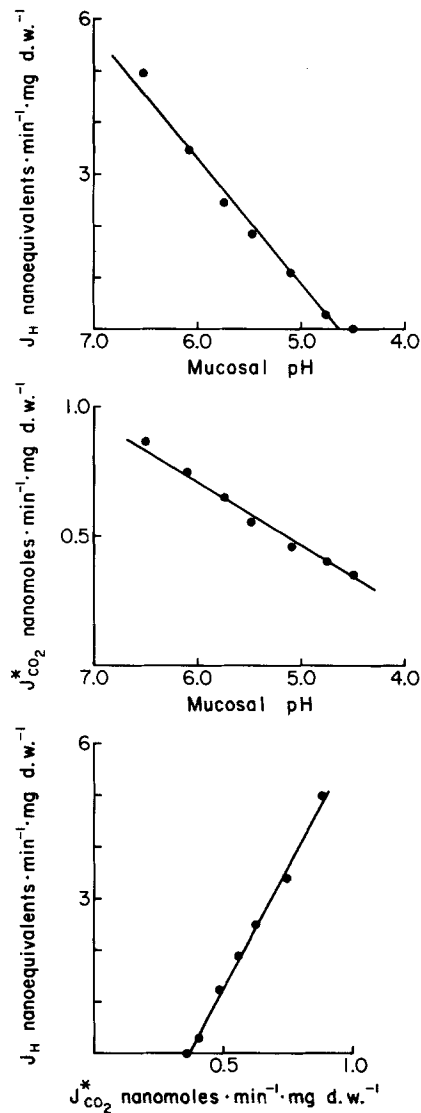
FIGURE 3. The relation of the rate of H⁺ secretion (J_H) and the rate of $^{14}CO_2$ production ($J^*_{CO_2}$) to the mucosal pH and to each other at constant serosal pH.

Another series of six experiments was performed where the rates of J_H and $J^*_{CO_2}$ were measured, and then the luminal pH was decreased in three to four steps until J_H was abolished. Acetazolamide, final concentration 20 μM, was added to the serosal side, and the procedure was repeated. The rate of H⁺ transport in the absence of electrochemical gradients was 3.342 neqv·min⁻¹·mg dw⁻¹, after acetazolamide J_H declined, to reach a steady level (in the absence of gradients) of 1.407, Δ = 2.03 ± 0.42, P < 0.01. The $J^*_{CO_2}$ in the absence of electrochemical gradients decreased from 0.457 to 0.295 nmol·min⁻¹·mg dw⁻¹, Δ = 0.153 ± 0.04, P < 0.01.

When J_H was abolished by luminal acidification, $J^*_{CO_2}$ in the control period was 0.236 nmol·min^{-1}·mg dw^{-1}; in the acetazolamide period that value was 0.227 nmol·min^{-1}·mg dw^{-1}, Δ = 0.009 ± 0.010. Likewise, acetazolamide had no effect on the $\partial J_H/\partial J^*_{CO_2}$, control 15.42 eq·mol^{-1}, acetazolamide 18.62, Δ = 3.2 ± 2.8.

Replicability in Paired Experiments

Experiments were performed on paired hemibladders from the same animal in order to validate the method and to estimate the error involved in these measurements. The J_H was varied by altering the mucosal pH and $J^*_{CO_2}$ was simultaneously measured. The lines for $\partial J_H/\partial J^*_{CO_2}$, $\partial J^*_{CO_2}/\partial pH_m$, and $\partial J_H/\partial pH_m$ were obtained, and the intercept $(J^*_{CO_2})_{J_H=0}$ was calculated by the method of least squares. Some of these results are presented in Table II. It is seen that there is satisfactory agreement between the members of a pair. The average difference in the slope of $\partial J_H/\partial J^*_{CO_2}$ was 22% of the mean value, and for $(J^*_{CO_2})_{J_H=0}$ it was 19%.

TABLE I

RELATION OF J_H AND $J^*_{CO_2}$ TO THE APPLIED GRADIENT AND TO EACH OTHER

n	$\partial J_H/\partial J^*_{CO_2}$	r	$\partial J_H/\partial \Delta_{pH}$	r	$\partial J^*_{CO_2}/\partial \Delta_{pH}$	r
7	9.45±0.37	0.9962	2.44±0.06	0.9908	0.258±0.01	0.9954
6-5	27.23±5.01	0.9385	1.402±0.19	0.9734	0.039±0.015	0.8375
5	32.85±2.62	0.9906	0.807±0.065	0.9936	0.024±0.001	0.9979
7	15.68±1.20	0.9857	0.781±0.022	0.9980	0.048±0.0049	0.9748
5	13.31±2.72	0.9427	0.209±0.049	0.9271	0.014±0.0041	0.8935
6	9.94±1.48	0.9583	0.363±0.017	0.9956	0.034±0.004	0.9726
6	18.51±1.73	0.9829	0.707±0.028	0.9968	0.037±0.0026	0.9901
		0.9707		0.9822		0.9517
		±0.009		±0.0097		±0.0234

The linear regression slopes are given ± SD for individual hemibladders. n refers to the number of experimental periods in each hemibladder, and r is the regression coefficient.

"Stoichiometric Ratio"

The relation between respiration and transport has been the subject of numerous investigations (2, 5, 10, 12, 13, 15, 16, 33, 36). Particular attention has been paid recently to the lack of a unique "stoichiometric ratio" between transport and metabolism (2, 33). In the present experiments the rate of metabolism has been taken as the rate of glucose oxidation $(J^*_{CO_2})$, and the slope of J_H on $J^*_{CO_2}$ is a measure of the coupling between H$^+$ transport and glucose oxidation. The values of these slopes are similar in hemibladders from the same animal—Table II. However, they vary between 4.4 ± 1.2 and 38.2 ± 3.5 in the population of 40 bladders presented in Tables I-V.

Since the fraction of total respiration contributed by glucose oxidation is probably variable in different animals, a series of experiments was performed by using a method for measurement of the total CO_2 (2). In six experiments the rate of J_H was varied by changing the pH in the mucosal medium, and the simultaneous rate of total CO_2 production (J_{CO_2}) was measured. In these experiments the medium was bubbled with CO_2-free air; hence, the values of J_H were much lower

than the experiments where it was possible to use high ambient pCO_2 levels. The relation of J_H to J_{CO_2} was highly linear over the ranges of H^+ secretion seen. The average correlation coefficient was 0.86, but only two of the six experiments had correlation coefficients below 0.87. In these two experiments the rate of transport was the lowest in this series. In Table III the results are shown. It is seen that the "stoichiometric ratio," $\partial J_H/\partial J_{CO_2}$, varies from 1.41 ± 0.26 to 11.23 ± 1.17. The average $\partial J_H/\partial J_{CO_2}$ was 4.8 ± 1.42. This is to be compared with an average $\partial J_H/\partial J^*_{O_2}$ of 15 ± 3, $n = 40$. This suggests that one-third of the CO_2 production in the active H^+ transport pathway comes from exogenous glucose oxidation.

TABLE II

PAIRED EXPERIMENTS

$\partial J_H/\partial J^*_{O_2}$	Δ	$(J^*_{O_2})_{J_H=0}$	Δ
28.21		0.059	
25.18	3.03	0.032	0.027
18.42		0.112	
20.18	1.76	0.127	0.015
4.39		0.495	
7.28	2.89	0.556	0.061
5.37		0.428	
8.47	3.1	0.411	0.017
28.02		0.266	
21.32	6.7	0.323	0.057
16.17		0.185	
21.91	5.74	0.200	0.015
22.12		0.104	
14.01	8.11	0.147	0.043
9.29		0.266	
10.18	0.89	0.199	0.067
7.73		0.284	
7.86	0.13	0.329	0.045
10.01		0.161	
9.19	0.82	0.289	0.128

Mean	14.76±1.74		0.249±0.032
Mean difference	3.317±0.85		0.048±0.011

$\partial J_H/\partial J_{CO_2}$ was obtained in paired hemibladders from the same animal using three to four periods in each hemibladder. The values presented are obtained from linear regression analysis. The difference is given regardless of sign.

$\partial J_H/\partial J^*_{O_2}$ in the Presence or Absence of H^+ Gradients

As will be shown in the Discussion, the relation of J_H to $J^*_{CO_2}$ obtained in the presence of a pH gradient need not be the same as the ratio obtained in the absence of a gradient. Since the quantitative relationship between these two "stoichiometric ratios" is important, it is necessary to measure them in the same hemibladder. Because of bacterial and fungal growth in the medium, the experiments could not be carried out over prolonged periods.

To obtain the relation of J_H to $J^*_{O_2}$ in the presence of a gradient, the rate of H^+ transport was changed by altering the luminal pH keeping the serosal pH

constant. This method is similar to the experiments presented so far and was shown graphically in Fig. 2. In order to measure the $\partial J_H/\partial J^*_{CO_2}$ in the absence of a pH gradient, the luminal and serosal pHs were titrated to 7.0. The J_H and $J^*_{CO_2}$ were recorded, and then the gassing mixture was changed from the standard 1% CO_2 in air to CO_2-free air. It is well known that J_H is exquisitely sensitive to the ambient pCO_2 (23). On decreasing the ambient pCO_2, J_H and $J^*_{CO_2}$ fell (Fig. 2). The luminal and serosal pHs were titrated back to 7.0 and the steady-state J_H and $J^*_{CO_2}$ were used to construct a curve of $\partial J_H/\partial J^*_{CO_2}$. The values of these two slopes obtained in the presence and absence of electrochemical gradients are presented in Table IV. In 4 out of the 14 experiments the order of the procedure was changed, but there was no difference in the mean of these experiments from the mean of the 10 others. $\partial J_H/\partial J^*_{CO_2}$ in the presence of a gradient averaged 14.72 eq·mole^{-1}, and in its absence it was 14.45, $\Delta \pm SE = 0.27 \pm 1.46$.

TABLE III

COUPLING BETWEEN H^+ TRANSPORT (J_H) AND TOTAL CO_2 PRODUCTION

(J_{CO_2})

	$(J_H)_{\Delta pH=0}$	$(J_{CO_2})_{J_H=0}$	$\partial J_H/\partial J_{CO_2}$
	$nmol·min^{-1}·mg\ dw^{-1}$	$nmol·min^{-1}·mg\ dw^{-1}$	$eq·mol^{-1}$
	1.587	0.952	11.23 ± 1.17
	0.687	0.558	5.87 ± 0.70
	0.279	0.332	3.19 ± 0.70
	0.817	0.708	1.41 ± 0.26
	0.656	0.879	4.10 ± 0.63
	0.421	0.589	3.01 ± 0.92
Mean$\pm$SE	0.741 ± 0.187	0.670 ± 0.093	4.80 ± 1.42

These experiments were performed with the conductometric method for total CO_2 measurement. The gassing mixture was CO_2-free air.

It is to be expected that the error in the slope $\partial J_H/\partial J^*_{CO_2}$ at constant gradients will be larger than that at varying gradients since in the former only two periods were available, while in the latter three to four readings were obtained. In the Discussion it will be shown that the ratio of the two slopes is equal to the square of the degree of coupling.

The Effects of 2,4-Dinitrophenol

Dinitrophenol (DNP) increases the proton conductance in artificial and natural membranes leading to uncoupling of oxidative phosphorylation. We used low doses of DNP applied to the luminal solution in an effort to induce a leak for H^+ in parallel with the H^+ "pump." 1 μM DNP, when added to the luminal side, had minor effects on J_H or $J^*_{CO_2}$ in the absence of electrochemical gradients (Table V).[1] This suggests that in this dose DNP had not uncoupled mitochondrial oxidative phosphorylation, since that would have led to an increase in $J^*_{CO_2}$ when $\Delta pH = 0$.

[1] Although $(J_H)_{\Delta pH=0}$ is significantly lower after DNP, the change is quite small, amounting to only 8%. This may have been due to the spontaneous decline since the experiment with DNP was done 1-2 h after the control period.

Higher doses of DNP (10 μM added to the serosal bath) reduced $(J_H)_{\Delta pH=0}$ by 50% and increased $J^*_{CO_2}$, suggesting that DNP has uncoupled oxidative phosphorylation leading to a reduction in ATP levels and, hence, to the rate of H^+ transport (Fig. 4).

When an opposing H^+ gradient is imposed on the system by acidifying the luminal bath in the presence of DNP, J_H declines. The relation of J_H to the luminal pH remains linear. However, $\partial J_H/\partial \Delta pH$ is significantly increased from 0.89 neq·pH U^{-1}·min^{-1}·mg dw^{-1} to 0.98 neq·pH U^{-1}·min^{-1}·mg dw^{-1} after 1 μM DNP, $\Delta = 0.09 \pm 0.04$, $P < 0.05$. The relation of J_H to the pH difference across the membrane is a measure of the conductance of protons through the system. An increase in this conductance supports the view that DNP acts as a proton conductor, since $\partial J_H/\partial \Delta pH$ in the control period reflects primarily the

TABLE IV

$\partial J_H/\partial J^*_{CO_2}$ IN THE PRESENCE AND ABSENCE OF A pH GRADIENT

	$\left[\dfrac{\partial J_H}{\partial J^*_{CO_2}}\right]_{\Delta\bar{\mu}_H=0}$	$\left[\dfrac{\partial J_H}{\partial J^*_{CO_2}}\right]_{\Delta\bar{\mu}_H\neq0}$	q
	6.41	9.87	0.806
	38.24	28.21	1.164
	25.56	25.18	1.008
	20.78	19.56	1.031
	8.9	18.42	0.695
	11.24	20.18	0.746
	18.46	14.34	1.135
	3.08	4.39	0.838
	4.98	7.28	0.827
	5.82	5.37	1.041
	8.60	8.47	1.008
	19.83	24.77	0.895
	15.82	10.84	1.208
	14.57	9.19	1.259
$\bar{x}$	14.45	14.72	0.97 ± 0.05
		$\Delta = 0.27\pm1.46$	

$(\partial J_H/\partial J^*_{CO_2})_{\Delta\bar{\mu}_H=0}$ was obtained by varying the ambient pCO_2. $(\partial J_H/\partial J^*_{CO_2})_{\Delta\bar{\mu}_H\neq0}$ was performed by changing the luminal pH at constant ambient pCO_2. The gassing mixture in the latter case was 1% CO_2 in air. Both slopes were obtained in the same bladder.

conductance in the active pathway while after DNP this slope is equal to the active conductance plus the leak induced by DNP. Surprisingly, the relation of J_H to pH remains linear after DNP. This suggests that the leak pathway is linear in pH. We expected it to be linear in the concentration of H^+. However, the mechanism of action of DNP is quite complex and includes contributions from partitioning in the membrane, its pK, the surface charge of the membrane, and its diffusion through unstirred layers. At least to the extent that we have measured it in three to four periods in each experiment, there do not appear to be any systematic deviations from linearity.

In contrast to the control period, the decrease in J_H induced by a pH gradient in the DNP period is accompanied by an *increase* in $J^*_{CO_2}$, as shown in Fig. 4. If the

luminal pH was then elevated, J_H increased but $J^*_{CO_2}$ *decreased*. Hence, the sign of $\partial J_H / \partial J^*_{CO_2}$ was reversed, as shown in Table V. Despite this change, the relation of J_H to $J^*_{CO_2}$ remained linear. In the experiments with 10 μM DNP the average correlation coefficient before DNP was 0.98 which was not significantly different from that of 0.89 in the periods after the addition of DNP. The reversal of the sign of $\partial J_H / \partial J^*_{CO_2}$ was seen when the low as well as the high dose of DNP was used.

We point out that J_H in the presence of DNP now includes a "leak" pathway induced by the uncoupler and does not reflect mostly the active pathway as it did in the control state. We suggest that the increase of $J^*_{CO_2}$ when the luminal bath is acidified is due to an increased flux of H^+ into the cell, leading to stimulation of the H^+ pump. This "recycling" of protons through the active pathway is the cause of the increased CO_2 production in the presence of H^+ gradients.

TABLE V

EFFECT OF 2,4-DINITROPHENOL (DNP) ON NET H^+ TRANSPORT (J_H) AND GLUCOSE OXIDATION (J_{CO_2})

	$(J_H)_{\Delta pH=0}$	$(J^*_{CO_2})_{\Delta pH=0}$	$\partial J_H / \partial J^*_{CO_2}$
	$nmol \cdot min^{-1} \cdot mg^{-1}$	$nmol \cdot min^{-1} \cdot mg^{-1}$	$eq \cdot mol^{-1}$
Control	2.10	0.98	12.12
1 μM DNP to M	1.94	0.81	−4.47
$\Delta \pm SE$	−0.16±0.04*	−0.16±0.06*	16.58±0.36*
Control	4.97	0.53	13.57
10 μM DNP to S	2.71	0.78	−5.14
$\Delta \pm SE$	−2.26±0.47*	0.25±0.11*	18.71±2.3*

DNP was added to the luminal bath only, final concentration 1 μM, n = 4 (top set). In another series of five experiments, DNP was added to the serosal medium, final concentration 10 μM (bottom set). Before addition of DNP a pH gradient was imposed on the turtle bladder, and three periods were performed. After addition of DNP the J_H and $J^*_{CO_2}$ were allowed to reach a new steady state, and the luminal pH was lowered for two to three periods. A representative experiment is shown in Fig. 4.
* P < 0.05.

DISCUSSION

The results presented show that the rate of net H^+ transport from the serosal side into the luminal medium (J_H) is exquisitely sensitive to alteration in the H^+ activity in the mucosal medium. At constant serosal pH, then, the J_H is proportional to the pH difference across the epithelium. However, the system appears to be asymmetrical since large changes in the serosal pH fail to affect the transport rate. The electrophysiological studies of Hirschhorn and Frazier (7) and intracellular pH measurements (using DMO distribution) of Steinmetz (27) suggest that the active process of acidification is located at the luminal border of the epithelium. Hence, the electrochemical gradient of interest is that across the luminal border. Alterations in the serosal pH probably do not result in significant changes in the cell pH. Large changes of ambient pH at constant pCO_2 result in much smaller changes in the intracellular pH of mammalian renal tubular epithelial cells (31) and muscle (1). In the turtle urinary bladder the apparent cell pH determined by the DMO distribution method changed from 7.56 to 7.42

when the ambient pCO_2 was increased from near zero to 40 mm Hg (27). Other maneuvers, e.g., deoxygenation or acetazolamide, failed to change the cell pH by more than 0.25 U. The serosal pH in the experiments reported in the present communication was kept between 7.0 and 7.4, and no attempt was made to rigorously maintain it there. The pH difference across the relevant barrier was considered to be the difference between the luminal pH and the assumed cell pH of 7.4.

The rate of glucose oxidation ($J^*_{\text{CO}_2}$), used as an index of the rate of oxidative metabolic reactions, was found to vary with the rate of net H^+ transport. It was also found to vary with the applied pH gradient across the membrane. The system of H^+ transport, then, appears to be coupled to the flow of glucose oxidation. This coupling between transport and metabolism is the hallmark of active transport as suggested by Kedem (10). In the range of transport activity
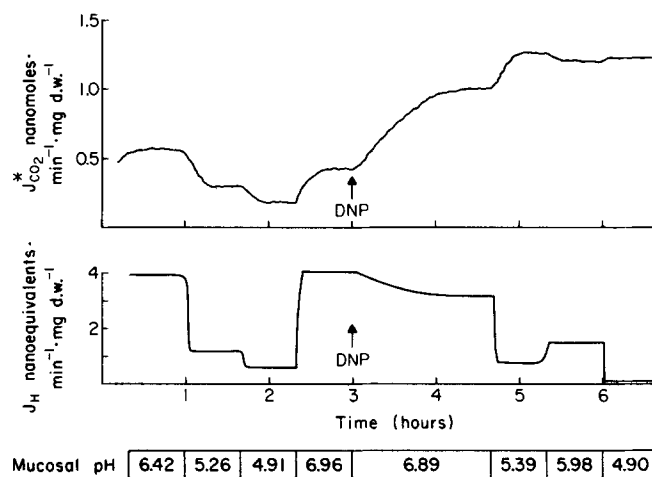

Mucosal pH

6.42	5.26	4.91	6.96	6.89	5.39	5.98	4.90

FIGURE 4. The effect of 10^{-5} M 2,4-dinitrophenol on the rate of H^+ secretion (J_H) and $^{14}CO_2$ production ($J^*_{\text{CO}_2}$) as they are altered by changes in mucosal pH.

and applied gradients examined, the system exhibits impressive linearity. In all the experiments performed, the correlation coefficient of the linear regression analysis of the data showed very high values which were relatively independent of the number of experimental periods of observation. The linearity of this system facilitates its formal thermodynamic analysis. Recently, it has been shown in amphibian skin (5, 33, 34) and urinary bladder (2) that the sodium transport system can be described by linear phenomenologic equations.

The rate of net acidification in the absence of transepithelial electrochemical gradients can be taken to equal the rate of transport in the active pathway. A realistic model for H^+ transport should include a passive "leak" pathway in parallel with the active pathway. In the presence of an opposing gradient the decline in the rate of acidification could reflect a decrease in the active rate or an increase in flow through the leak pathway or both. Obviously, the rate of CO_2 production is linked only to the active rate. Hence, a decline in $J^*_{\text{CO}_2}$ seen on

application of an opposing gradient is evidence that at least part of the decline in the net transport is due to a reduction in the active rate. This indicates that the H^+ pump rate is sensitive to the transepithelial H^+ gradient.

The net rate of H^+ transport can be nullified by increasing the H^+ concentration in the luminal medium. If the leak rate is significant, then at the point of net zero H^+ secretion a substantial pump rate will remain, balanced by the opposing leak flux. Hence, the CO_2 production would include a moiety which is due to the activity of the H^+ pump. The magnitude of the pump rate and its CO_2 production under conditions of zero net flux will vary directly with that of the leak pathway. It is to be expected that inhibition of the pump will result in a further decline in $J^*_{CO_2}$. We used 1 mM acetazolamide, a dose known to abolish H^+ transport (25). At zero net H^+ transport acetazolamide did not induce a decline in $J^*_{CO_2}$, suggesting that the pump rate was nullified, implying that the net rate and the pump rate are identical. We conclude that within the error of the methods used a back leak of protons is undetectable.

To examine this point further a series of experiments was performed where the $\partial J_H/\partial J^*_{CO_2}$ was measured under the two sets of conditions. It was reasoned that in the absence of H^+ gradients the $\partial J_H/\partial J^*_{CO_2}$ would be independent of the presence of a parallel leak of H^+. However, if such leaks are significant, then the $\partial J_H/\partial J^*_{CO_2}$ in the presence of gradients would be lower than in their absence, since J_H is a measure of the net H^+ transport rate and not the active H^+ rate. These experiments were performed in the same hemibladders, and it was found that the $\partial J_H/\partial J^*_{CO_2}$ was the same in the presence and the absence of gradients. These results further support the contention that the passive proton conductance is very low.

It appears that the proton permeability of the epithelium as a whole is quite low, implying that not only the cell membrane but also the intercellular pathways have a low conductance for H^+. The proton conductance of bacteria, mitochondria, and chloroplasts is very low. This has evolved as a central issue in the chemiosmotic hypothesis of energy transduction where the low proton permeability allows the development of gradients (the protonmotive force) that can be dissipated through selective sites for ATP synthesis. Uncouplers of oxidative phosphorylation, e.g., dinitrophenol (DNP), are seen to exert their effect by increasing the proton conductance, thus collapsing the gradients (17, 25). Artificial membranes have a negligible H^+ permeability, but the addition of uncouplers such as dinitrophenol specifically increases the proton permeability (8).

We added DNP to the luminal surface of the turtle bladder in very low doses (1 μM) and found that when the luminal pH was 7.4 there was little change in J_H or $J^*_{CO_2}$. This suggests that DNP had not entered the cell. Had it done so, it would be expected to uncouple mitochondrial oxidative phosphorylation, resulting in large increases in CO_2 production and a decline in J_H as seen with the larger dose (10 μM), Fig. 4, Table V.

The lack of effect of DNP at luminal pH of 7.4 supports our assumption of a cell pH near 7.4, since no net flow in the DNP-induced pathway is expected when both media have the same pH and since in the short-circuited state the potential difference across the luminal membrane is quite small (7). The relation

of J_H to the pH difference across the membrane is a measure of the proton conductance of the system. DNP increased $\partial J_H/\partial \Delta pH$, confirming our expectation that this agent induces a proton shunt in the membrane.

In the presence of DNP, decreasing the luminal pH caused a fall in J_H; however, in contrast to the control state, $J^*_{CO_2}$ *increased*. The change in $J^*_{CO_2}$ was reversible as shown in Fig. 4. We interpret these results as follows: DNP partitions into the luminal membrane and acts as a shunt in parallel with the H^+ pump. When the luminal solution is acidified, a driving force favoring H^+ flow into the cell will be created. The entry of H^+ leads to an increase in availability of protons to the pump. Provided that the pump is not "saturated," this increase in availability will result in a stimulation of the active transport rate. This situation is analogous to the mechanism of stimulation of active H^+ transport by increasing the ambient pCO_2 (23, 27, 28). The higher the flow through the shunt pathway, the more the H^+ pump will be stimulated and the greater will $J^*_{CO_2}$ be. As the luminal pH is elevated, the shunt flow will decline and the process described above will be reversed, leading to a decrease in $J^*_{CO_2}$. We refer to this process as recycling of H^+ through the pump. Note that when the pH of the luminal medium is lowered, $J^*_{CO_2}$ will increase, but J_H will decrease since J_H is now the "pump rate" *minus* the flow through the shunt. Hence, the relation of J_H to $J^*_{CO_2}$ will have a reversed sign from that under control conditions.

The relation of ion transport to the metabolic activity of an epithelium has attracted the attention of many investigators (2, 5, 10, 12, 13, 15, 16, 33, 36). The ratio of the amount of ion transported to O_2 consumed or CO_2 produced has frequently been called the "stoichiometric ratio." This kind of analysis was based on averaging the results of transport rates and O_2 consumption (sometimes in different tissues). The existence of stoichiometry in the sense of a unique ratio has recently been challenged. Vieira et al. (33), using simultaneous measurements of sodium transport and O_2 consumption in the same frog skin over wide ranges of transport activity, found that in a population of skins no unique value of $\partial J_{Na}/\partial J_{O_2}$ appears to hold. Similar results were obtained for the coupling of sodium transport to CO_2 production in the toad urinary bladder (2). In the H^+ transport system, the $\partial J_H/\partial J^*_{CO_2}$ varied over a wide range from 4 ± 1.2 to 38 ± 3.5 in 40 bladders. It is to be emphasized that this variation is not produced by experimental error since the variation between the $\partial J_H/\partial J^*_{CO_2}$ of two hemibladders from the same turtle was quite small (Table II) compared to the variation in a population of turtles. The variable coupling between H^+ transport and glucose oxidation may reflect the extent to which glucose metabolism participates in the total energy-producing pathways. For example, it is well known that in several species the fraction of CO_2 production due to glucose oxidation may be quite variable depending on the nutritional status of the animal (35). In our experiments the nutritional status of the turtles was not controlled. However, to evaluate this point, we examined the rate of total CO_2 production measured conductometrically and followed its coupling to H^+ transport. Of necessity, these experiments have to be performed in the absence of exogenous CO_2; hence, the net rate of H^+ secretion was much lower than in the other experiments performed in the presence of CO_2. The results of $\partial J_H/\partial J_{CO_2}$ shown in

Table III show again that these values are distributed over a range from 1.41 ± 0.26 to 11.23 ± 1.17 similar in magnitude of variation to both $\partial J_H/\partial J^*_{O_2}$ and to $\partial J_{Na}/\partial J_{CO_2}$ reported previously (2, 33).

The biochemical mechanism of H^+ translocation in the turtle bladder is unknown. It appears that an ATPase is responsible for proton pumping in mitochondria (9, 17, 24) and bacteria (25). Of interest in this regard is the limiting stoichiometry of these ATPases which has been found to be two protons translocated per ATP hydrolyzed (or synthesized) (17, 24). Assuming that the majority of the CO_2 production comes from glucose oxidation and that complete oxidation of 1 mol of glucose yields 6 mol of CO_2 and 36 mol of ATP, then the stoichiometry of 2 H^+/ATP would equal 12 H^+/CO_2. The bladder with the highest $\partial J_H/\partial J_{CO_2}$ that we have observed is 11.23. It is tempting to speculate that epithelial H^+ transport occurs through a proton-translocating ATPase. Recently, it had been demonstrated that "HCO_3-stimulated" ATPases are present in the gastric mucosa (14) and in luminal membranes of the renal tubule (20) and turtle bladder (4). The mitochondrial and bacterial "HCO_3-stimulated" ATPase is the locus of H^+ transport (9, 17, 24, 25).

The analysis of the coupling of transport to metabolism has been facilitated by the use of irreversible thermodynamics. Using the analysis of Essig and Caplan (6), we have written the equation applicable to the H^+ transport system in the Appendix. The coupling between J_H and $J^*_{CO_2}$ can be described by two parameters: Z (Eq. A3), a stoichiometric ratio, and q (Eq. A4), the degree of coupling (11). The values of q range between 0 and ±1, where zero indicates that the system is uncoupled and 1 describes complete coupling.

The ratio of $\partial J_H/\partial J^*_{O_2}$ in the absence to that in the presence of electrochemical gradients is seen to equal q^2 (Eq. A7). In Table IV we show the values of q; the average is 0.97 ± 0.05 (SE). Since values of q above 1 are physically meaningless, we used a one-tailed analysis to evaluate the confidence intervals (26). The 95% lower confidence limit is 0.90, and the 99% lower confidence limit is 0.86. Thus, q on the average is not significantly different from 1 and in most of the cases is quite close to 1. The value is to be compared to that obtained in the toad skin by Danisi and Vieira of 0.94 (5). Their results were obtained by comparing the $\partial J_{Na}/\partial J_{O_2}$ in the presence of sodium concentration gradients to those obtained in its absence. However, these two groups of data were obtained in different animals. As pointed out earlier, the "stoichiometric ratios" are quite variable in different animals; hence, it is imperative that the ratios in the presence and absence of gradients be measured in the same tissue.

When $q = 1$, the system is completely coupled. The implications of this are several. First, the variation of the $\partial J_H/\partial J^*_{O_2}$ in different tissues cannot be ascribed to different degrees of coupling but is due to different values of Z in different tissues (Eq. A5 and A6). Hence, we can conclude that each tissue has a different but characteristic stoichiometric ratio. Second, the efficiency of energy conversion in this system appears to be high since Kedem and Caplan (11) have shown that the maximum efficiency, η_{max}, is given by:

$$\eta_{max} = \frac{q^2}{(1 + \sqrt{1 - q^2})^2} = 76\%.$$

Third, when $q = 1$, it follows that when $J_H = 0$, $J^*_{CO_2}$ in the *active transport pathway* vanishes, i.e. the CO_2 production at zero net H^+ secretion is unrelated to active H^+ transport. This moiety of metabolism is then the basal metabolism and is due to cellular activities other than transport. We emphasize that $(J_{CO_2})_{J_H=0}$ is equal to true basal metabolism only in the condition of complete coupling.

APPENDIX

The Essig-Caplan equations (6) written for the H^+ transport system are:

$$J_H = L_H X_H + L_{Hr} A, \tag{A1}$$

$$J_r = L_r A + L_{rH} X_H, \tag{A2}$$

where J_h and J_r are the flows of active H^+ transport and metabolism (glucose oxidation in this case), and L_H and L_r are "straight coefficients" that relate the flows to their conjugate driving forces. For H^+, the driving force is the electrochemical potential difference across the membrane (X_H) and the driving force of metabolism is the affinity of the reaction coupled to transport (A). The cross coefficients L_{rH} and L_{Hr} link the flow of one to the driving force of the other and, following Onsager, are assumed to be equal. Further, using the two parameters defined by Kedem and Caplan (11), the stoichiometric ratio, Z

$$Z = \left[\frac{L_H}{L_r}\right]^{1/2}, \tag{A3}$$

and the degree of coupling q

$$q = \frac{L_{Hr}}{(L_r L_H)^{1/2}}, \tag{A4}$$

we find that the relation of J_H to $J^*_{CO_2}$ in the presence of a finite X_H to be different from that in the absence of electrochemical gradients
when $X_H = 0$

$$\left[\frac{\partial J_H}{\partial J^*_{CO_2}}\right]_A = \frac{L_{Hr}}{L_r} = qZ, \tag{A5}$$

when $X_H \neq 0$

$$\left[\frac{\partial J_H}{\partial J^*_{CO_2}}\right]_A = \frac{L_H}{L_{rH}} = \frac{Z}{q}. \tag{A6}$$

Hence

$$q^2 = \frac{\partial J_H/\partial J^*_{CO_2}, \ X_H = 0}{\partial J_H/\partial J^*_{CO_2}, \ X_H \neq 0}. \tag{A7}$$

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