J. Membrane Biol. 59, 27–34 (1981)

The Journal of
Membrane Biology

Coupling between H^+ Transport and Anaerobic Glycolysis in Turtle Urinary Bladder: Effects of Inhibitors of H^+ ATPase

Philip R. Steinmetz, Russell F. Husted, Allan Mueller, and Renaud Beauwens

Laboratory of Epithelial Transport and Kidney Physiology, Departments of Medicine and Physiology, University of Iowa College of Medicine, Iowa City, Iowa

Summary. The coupling between H^+ transport (J_H) and anaerobic glycolysis was examined *in vitro* in an anaerobic preparation of turtle urinary bladder. J_H was measured as the short-circuit current after Na^+ transport was abolished with ouabain and by pH stat titration. The media were gassed with N_2 and 1% CO_2 ($PO_2 < 0.5$ mm Hg) and contained 10 mM glucose. Under these conditions, J_H was not inhibited by 3 mM serosal (*S*) cyanide or by 0.1 mM mucosal (*M*) dinitrophenol. Control anaerobic lactate production (J_{lac}) of 47 bladders was plotted as a function of simultaneously measured J_H. The slope of J_{lac} on J_H was 0.58 ± 0.12 with an intercept for J_{lac} at $J_H = 0$ of 0.55 μmol/hr. Values for $\delta J_{lac}/\delta J_H$ were determined in groups of individual bladders when J_H was inhibited by an opposing pH gradient (0.55 ± 0.16), by acetazolamide (0.58 ± 0.19) and by dicyclohexylcarbodiimide, DCCD (0.58 ± 0.14). The constancy of $\delta J_{lac}/\delta J_H$ indicates a high degree of coupling between J_H and J_{lac}. Since the anaerobic metabolism of glucose produces one ATP for each lactate formed, the $\delta J_{lac}/\delta J_H$ values can be used to estimate the stoichiometry of H^+ translocation. The movement of slightly less than 2 H^+ ions is coupled to the hydrolysis of one ATP. During anaerobiosis (absence of mitochondrial ATPase function) the acidification pump was not inhibited by *M* addition of oligomycin but was inhibited by *M* addition of DCCD and Dio-9, inhibitors of H^+ flow in the proteolipid portion of H^+-translocating ATPases. DCCD inhibited anaerobic J_H without change in $\delta J_{lac}/\delta J_H$ or basal J_{lac} and, therefore, acted primarily on the H^+ pump. *S* addition of vanadate also inhibited J_H, but the inhibition was associated with an increase in J_{lac}. The site of this apparent uncoupling remains to be defined. The acidification pump of the luminal cell membrane of the turtle bladder has H^+-ATPase characteristics that differ from mitochondrial ATPase in that H^+ transport is oligomycin-resistant and vanadate-sensitive. As judged from the flows of H^+ and lactate, the H^+/ATP stoichiometry of the pump is about 2.

The fresh water turtle is able to withstand O_2 deprivation for prolonged periods during diving or N_2 inhalation (Dodge & Folk, 1963; Robin et al., 1964). The isolated urinary bladder of the turtle shares this ability since it can maintain appreciable rates of Na^+ absorption and H^+ secretion *in vitro* during deoxygenation with N_2 (Klahr & Bricker, 1964; Schwartz & Steinmetz, 1977). The H^+ pump of the luminal cell membrane is especially well equipped to operate anaerobically. In the presence of glucose and exogenous CO_2 it maintains steady transport rates for several hours. Under anaerobic conditions the metabolic pathways which provide energy to the H^+ pump are greatly simplified. The flow of metabolic energy can be monitored as the rate of lactate production from glucose and can be related to the simultaneous rate of H^+ transport (J_H). Furthermore, since mitochondrial ATPase function is excluded, it becomes possible to examine the acidification pump of the luminal membrane by means of ATPase inhibitors which otherwise might affect H^+ secretion indirectly by inhibiting mitochondrial respiration.

In the present study we examined the coupling between active H^+ transport and anaerobic glycolysis and explored how the two flows are affected by ATPase inhibitors. The studies suggest that the proton pump of the turtle bladder is a H^+-translocating ATPase with characteristics that differ from mitochondrial ATPase in that the proton pump is oligomycin-resistant and vanadate-sensitive. Anaerobic J_H is closely coupled to lactate production when J_H is inhibited by a variety of maneuvers. Slightly less than 2 H^+ ions are translocated per lactate (or ATP)

0022-2631/81/0059-0027 $01.60

formed from glucose. Some of the experiments for this paper have been reported in preliminary form (Steinmetz, Mueller & Beauwens, 1979; Steinmetz, Husted & Mueller, 1980).

Materials and Methods

Urinary bladders of adult fresh water turtles, *Pseudemys scripta*, (Lemberger Co., Germantown, Wisc.) were mounted in Lucite chambers with an exposed tissue area of 8 cm^2. H^+ secretion (J_H) was measured as the short-circuit current in bladders in which Na^+ transport was abolished by 0.5 mM ouabain (Schwartz, 1976). In a few groups of experiments J_H was measured also by pH stat titration as indicated in the text. All rates were expressed in μmol/hr per 8 cm^2 membrane area. The mean dry wt of this bladder area has ranged from 13.8 ± 0.9 to 14.7 ± 0.6 mg in previous studies from our laboratory (Steinmetz, 1974).

The bathing solution contained in mmol per liter: Na, 118; K, 3.5; Ca, 1.8; Mg, 0.5; Cl, 122; HPO_4, 2; and dextrose, 10. The mucosal solution was gassed with 100% N_2 and the serosal solution with 1% CO_2 and 99% N_2. The pH of the solutions was kept at 7.4. The chambers were covered with parafilm to exclude ambient oxygen. Samples of the fluid adjacent to the bladder were taken with a long needle and a gas-tight syringe. PO_2 measurements were made with a model 113 blood gas analyzer (Instrumentation Laboratory, Inc.).

Under these conditions the bladders tolerated the anaerobiosis well for periods ranging from 4 to 7 hr as judged from steady H^+ secretion rates and preserved electrical resistances. This tolerance is in contrast to the tissue injury observed previously under different anaerobic conditions by Oliver, Himmelstein and Steinmetz (1975) when the serosal solution contained 20 mM HCO_3^- and was deoxygenated with N_2 without CO_2. In this previous study the electrical resistance often fell sharply presumably because of the alkalinity imposed on the epithelial cells by the serosal HCO_3^- in the absence of metabolic or exogenous CO_2.

After a 2-hr period of deoxygenation the effects on anaerobic J_H of the following agents were studied. 2,4-dinitrophenol (DNP) was added to a final concentration of 0.1 mM, dicyclohexylcarbodiimide (DCCD) was added in concentrations ranging from 0.1–1.0 mM, oligomycin (Sigma Chemical Co., St. Louis, Mo.) in a concentration of 100 μg/ml and Dio-9 (Gist-Brocades NV, Delft, Netherlands) in concentrations from 50–500 μg/ml; these agents were added to the mucosal solution. Sodium orthovanadate was added either to the mucosal or serosal solution as indicated in a final concentration of 1 mM; at pH 7.4 the $H_2VO_4^-$ concentration of the media was 0.1 mM (Pope & Dale, 1968). Sodium cyanide (NaCN) and acetazolamide (American Cyanamid Co., Pearl River, N.Y.) were added to the serosal solution in final concentrations of 3 and 0.05 mM, respectively. DCCD, Dio-9, DNP, and oligomycin were dissolved in ethanol, the other agents in water.

The accumulation of lactate was measured in the mucosal and serosal solutions by removing samples of 200 μl at half-hour periods with a long needled Hamilton syringe. Lactate was determined by an enzymatic (LDH) method (Diagnostic Kits, Sigma Chemical Co.) in which NAD is converted to NADH in an alkaline medium. NADH was determined in a model 25 spectrophotometer (Beckman) at an ultra-violet wavelength of 340 nm. More than 95% of the produced lactate appeared in the serosal fluid. All lactate production rates given are total rates (J_{lac}).

An attempt was made to strip the serosal loose tissue layer with its network of muscle fibers from the mucosal cell layer. In nine experiments it was possible to mount an 8-cm^2 area of stripped mucosa on the Lucite chambers. These stripped tissue behaved similarly to the other tissues. As indicated, J_{lac} in stripped tissues was only slightly lower than in unstripped tissue. The relationship of lactate production to H^+ secretion was studied in a variety of ways. In a group of nine stripped bladders and 38 nonstripped bladders the spontaneous rates of lactate production and H^+ secretion were compared under control conditions. In groups of experiments the change in J_{lac} in individual bladders was followed after inhibition of H^+ secretion by acetazolamide, DCCD, or vanadate. J_{lac} was also measured after the rate of H^+ secretion was either decreased or increased by changing mucosal pH. After an experimental maneuver, J_{lac} was measured for three half-hour periods. Values are given as mean values with the standard error of the mean. The slopes and intercepts of the relationship between lactate production and hydrogen secretion were determined by analysis of covariance for the group data and by linear regression analysis for individual experiments.

Results

1. H^+ Transport and Lactate Production by the Anaerobic Turtle Bladder

During deoxygenation under our experimental conditions, O_2 was not detectable (< 0.5 mm Hg) in the solutions adjacent to the bladder ($n = 17$). The results of Table 1 demonstrate that the preparation was indeed anaerobic. Serosal addition of 3 mM sodium cyanide had no effect on the rate of H^+ secretion (J_H). Anaerobic J_H remained relatively constant in both the control and experimental halves of the bladders. The same concentration of NaCN inhibits J_H by about 40% in the presence of O_2 and 1% CO_2 (Schwartz & Steinmetz, 1977). In a group of five other experiments anaerobic J_H was unchanged by 0.1 mM 2,4-dinitrophenol (DNP); control J_H was 14.2 ± 1.6 μA and J_H 1 hr after DNP was 14.4 ± 1.4 μA. The failure of cyanide and DNP to decrease J_H indicates that the H^+ pump received all its metabolic energy from anaerobic glycolysis.

J_H was dependent on the presence of glucose. In four experiments in which glucose was removed from the media J_H fell from 27 ± 7 μA to zero; readdition of glucose restored the rate to 17 ± 4 μA.

Table 1. Failure of sodium cyanide to affect H^+ secretion during anaerobiosis

Time (min)	H^+ Secretion (μA)	
0	Deoxygenation with 99% N_2–1% CO_2	
	Experimental	Control
60	22 ± 4	24 ± 7
90	21 ± 4	23 ± 7
120	21 ± 4	23 ± 7
121	NaCN	Control
150	20 ± 4	22 ± 6
180	20 ± 4	21 ± 6

Values for mean and SE are given for five pairs of hemibladders. NaCN was added to the serosal solution in a final concentration of 3 mM at 121 min to the experimental halves.

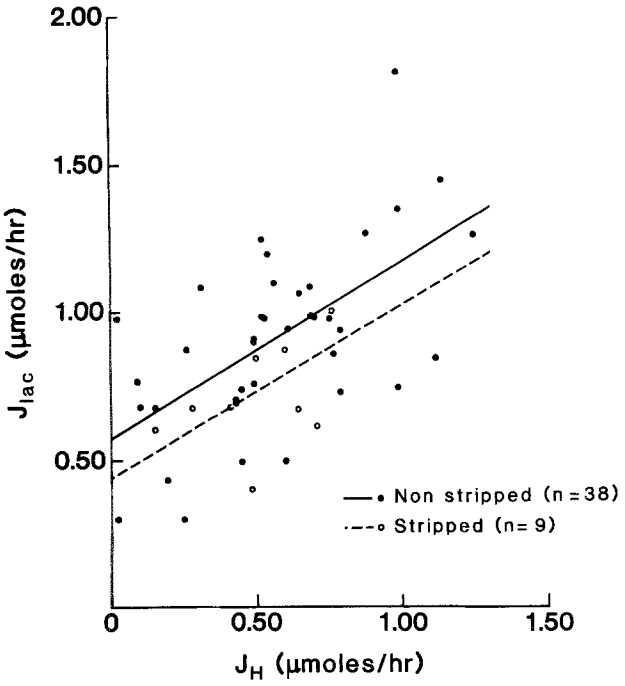

Fig. 1. Relationship of lactate production (J_{lac}) to H^+ secretion (J_H) in 47 bladders. The slope of the combined regression is 0.58 ± 0.12 and the intercept is 0.55 ± 0.08. The lines shown in the figure have the same slope but have intercepts calculated separately for the stripped and nonstripped bladders (*see* Table 2)

In Fig. 1 the rates of lactate production (J_{lac}) are plotted against anaerobic J_H for a group of 38 bladders (solid dots) as well as for a group of 9 bladders in which the serosal loose tissue layer with its network of muscle bundles had been stripped off (open circles). J_{lac} varied as a function of J_H in these groups of bladders. Mean J_{lac} was 0.91 ± 0.05 μmol/hr in the nonstripped bladders and 0.73 ± 0.06 in the stripped bladders. Mean anaerobic J_H was similar in the two groups, 0.57 μmol/hr (15 μA) for the nonstripped group and 0.51 μmol/hr (14 μA) for the stripped group. The observed rates of lactate production were at least twice as high as those reported previously by Klahr and Bricker (1965) and Schwartz and Steinmetz (1977). The high rates observed in the present study may be explained by the more prolonged and, perhaps, more complete anaerobiosis and also by the higher glucose concentrations used. In the study of Klahr and Bricker glucose was absent from the Ringer's solution, and in the experiments of Schwartz and Steinmetz the glucose concentration was only 2 mM.

In Table 2 the slope and the intercept of the data of Fig. 1 are shown. Analysis of covariance indicated that neither the slope nor the intercept were significantly different between groups of stripped and nonstripped bladders. The slope for the combined groups

Table 2. Coupling between anaerobic H^+ secretion (J_H) and lactate production (J_{lac})

	n	$\delta J_{lac}/\delta J_H$	J_{lac} at $J_H=0$ μmol/hr
Group of Fig. 1	47	0.58 ± 0.12	0.55 ± 0.08
Nonstripped bladders	38		0.58 ± 0.09
Stripped bladders	9		0.44 ± 0.18
Nonstripped bladders			
ΔpH method	7	0.55 ± 0.16	0.59 ± 0.10
Acetazolamide (0.05 mM)	6	0.58 ± 0.19	0.61 ± 0.05
Stripped bladders			
DCCD (0.5 mM)	5	0.58 ± 0.14	0.45 ± 0.07

Mean ±SE are given for groups of experiments as indicated. For the bladders of Fig. 1 the slope and intercept are for the group data. For the other three groups of bladders, the reported values are the averages of the slopes and intercepts calculated for individual experiments.

was 0.58 ± 0.12. For comparison with subsequent studies in individual bladders, the intercepts for stripped (0.44 ± 0.18 μmol/hr) and nonstripped bladders (0.58 ± 0.09 μmol/hr) are also shown. The intercepts represent basal metabolism unrelated to H^+ secretion. The difference between stripped and nonstripped bladders presumably represented lactate production by muscle and other cells of the serosal loose tissue layer.

These results suggest that the rate of anaerobic glycolysis is coupled to J_H. To examine this coupling further J_H was varied experimentally in individual bladders by different maneuvers and the ensuing change in J_{lac} was measured.

2. Coupling between H^+ Transport and Anaerobic Glycolysis

Active H^+ transport can be increased or decreased promptly by changing the pH gradient against which transport occurs. Figure 2 shows an individual experiment in which J_H was first decreased in two steps and then increased by changing luminal pH. J_{lac} decreased as J_H was reduced and both rates increased as the luminal pH was returned to 7.4. This experiment is shown in Fig. 3 by the solid dots and line. Basal J_{lac} was 0.64 μmol/hr and the slope, $\delta J_{lac}/\delta J_H$ was 0.50. Figure 3 also shows two other experiments in which J_H could be varied over a wide enough range to make serial measurements of J_{lac} at several levels of J_H. The intercepts and slopes of these experiments were similar. The average $\delta J_{lac}/\delta J_H$ for the group of seven experiments in which J_H was varied by the ΔpH method was 0.55 ± 0.16 while basal J_{lac} was 0.59 ± 0.10 μmol/hr (*see* Table 2). These values obtained in

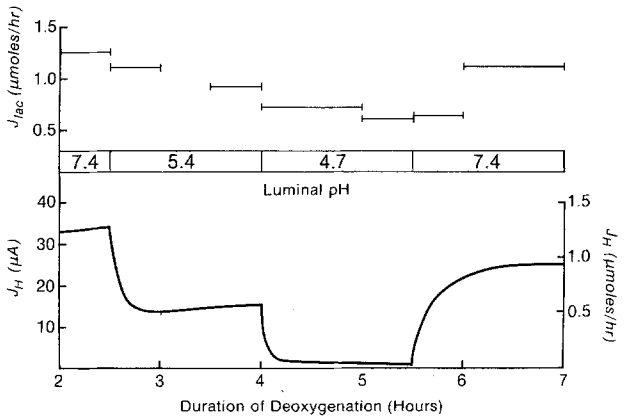

Fig. 2. ΔpH-induced changes in lactate production (J_{lac}) and H^+ secretion (J_H) in a representative experiment. Luminal pH was changed by addition of 0.1 N HCl or 0.1 N NaOH

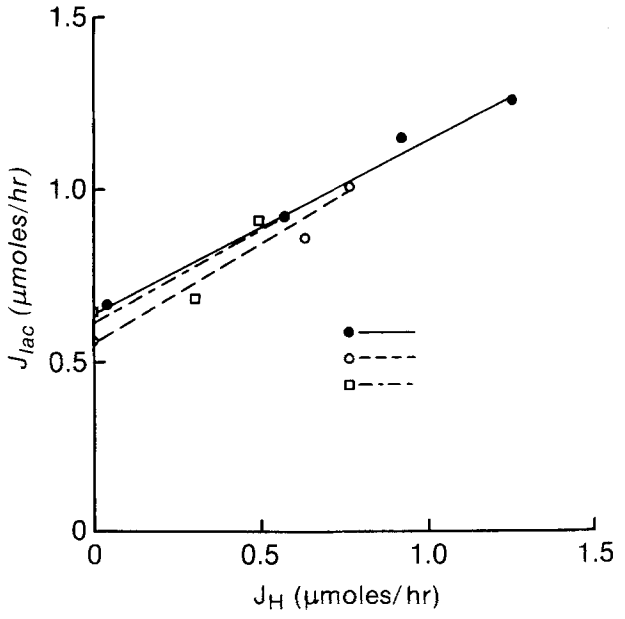

Fig. 3. Relationship between lactate production (J_{lac}) and hydrogen secretion (J_H) during ΔpH-induced changes in J_H in 3 bladders. Slopes and intercepts of the lines are 0.50 and 0.64 (●), 0.55 and 0.55 (○), and 0.50 and 0.61 (□)

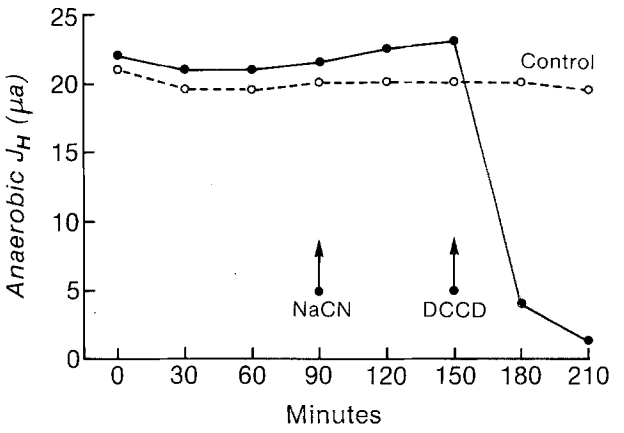

Fig. 4. Anaerobic H^+ secretion (J_H) in two halves of the same bladder. In the control half (dashed line) J_H was constant for the 3½ hr of observation. Serosal addition of 3 mM sodium cyanide had no effect on anaerobic J_H. In contrast, the luminal addition of 0.5 mM DCCD caused a rapid and marked inhibition of J_H

individual bladders are very close to the values observed when control J_{lac} was compared with the spontaneous anaerobic secretion rate for H^+ in the group of different bladders of Fig. 1. Average $\delta J_{lac}/\delta J_H$ was 0.58 ± 0.19 for a group of experiments in which J_H was reduced by acetazolamide and 0.58 ± 0.14 for a group of experiments in which J_H was inhibited by addition of 0.5 mM dicyclohexylcarbodiimide (DCCD) to the luminal solution (Table 2). DCCD did not alter basal J_{lac} as judged from control J_{lac} at $J_H = 0$ in stripped bladders and the inhibition of J_H by DCCD reduced J_{lac} with the same average $\delta J_{lac}/\delta J_H$ as was obtained with the ΔpH method or with acetazolamide. The average slopes ranged between 0.55 and 0.58 among the different groups of experiments. The fact that DCCD did not alter $\delta J_{lac}/\delta J_H$ or basal J_{lac} suggests that DCCD acted primarily on the H^+ pump.

3. Inhibitor Characteristics of Anaerobic H^+ Transport

Dicyclohexylcarbodiimide (DCCD) is thought to bind to the proteolipid or channel component of most H^+-ATPases in mitochondria, chloroplasts, bacterial membranes, and certain animal plasma membranes and, thereby, to inhibit the translocation of protons across the membrane (Racker, 1976; Kagawa et al., 1979). As shown in the experiment of Fig. 4, mucosal addition of 0.5 mM DCCD caused a marked inhibition of anaerobic J_H in a bladder in which NaCN had no effect on the anaerobic rate. Anaerobic J_H in the control half of the same bladder remained constant. In Fig. 5, the concentration dependence of the inhibition of anaerobic J_H is shown. Fifty percent inhibition was reached after about 30 min exposure to 0.1 mM DCCD and after about 15 min of exposure to 0.5 or 1.0 mM DCCD. A similar time course and degree of inhibition occurred if the luminal membrane was exposed to DCCD for only 5 min and then washed. Since DCCD had no effect (*see* above) on basal J_{lac} or the number of protons translocated per lactate formed (Table 2), the inhibition of J_H appears to represent a primary action on the proton pump of the luminal cell membrane of the bladder.

The antibiotic Dio-9, a potent inhibitor of H^+-ATPase in chloroplasts and mitochondria (Carmeli, Lifshitz & Gepshtein, 1975; Racker, 1976), caused

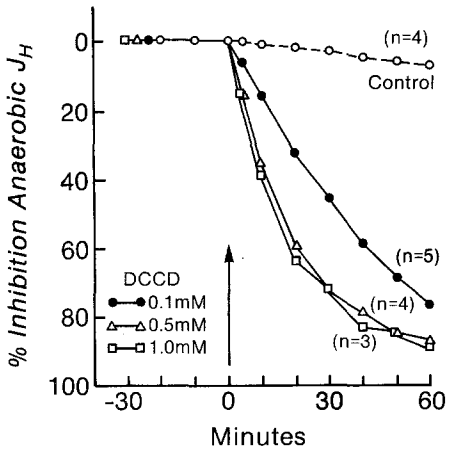

Fig. 5. Inhibition of anaerobic J_H by dicyclohexylcarbodiimide (DCCD). DCCD was added to the luminal solution in the concentrations indicated. Control anaerobic J_H was 15 ± 3 μA

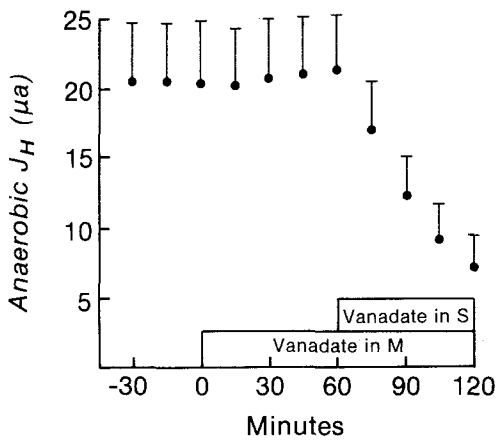

Fig. 6. Effects of mucosal and serosal vanadate on anaerobic H^+ secretion. Mucosal addition of 1 mM Na_3VO_4 did not affect J_H. Serosal vanadate caused $67 \pm 6\%$ inhibition at 1 hr

an almost instantaneous inhibition of anaerobic J_H. In five experiments the luminal addition of 500 μg/ml Dio-9 reduced J_H by more than 50% within 1 min.

To ensure that the inhibition of the short-circuit current in ouabain-treated bladders represented inhibition of H^+ secretion, we made simultaneous measurements of J_H by pH stat titration in five bladders exposed to DCCD and three exposed to Dio-9. Similar large reductions in anaerobic J_H were measured by this method.

In contrast to the inhibition of DCCD and Dio-9, anaerobic J_H was not inhibited by oligomycin added to the luminal solution in a final concentration of 100 μg/ml. In six experiments mean control J_H was 18 ± 2 μA at 120 and 150 min of anaerobiosis. Thirty and 60 min after oligomycin, J_H was 17 ± 2 μA. Under aerobic conditions luminal addition of as little as 20 μg/ml is sufficient to cause 40% inhibition of J_H. There is little doubt, therefore, that the oligomycin concentrations were adequate. The failure of oligomycin to inhibit anaerobic J_H is interesting since in the yeast *Schizosaccharomyces pombe* (Delhez et al., 1977) and in *Neurospora crassa* (Bowman et al., 1978) the plasma membrane contains a H^+ translocating ATPase that differs from mitochondrial ATPase in that it is oligomycin insensitive. Another difference between the plasma membrane and the mitochondrial ATPase in these fungi is that the plasma membrane but not the mitochondrial H^+-ATPase is inhibited by vanadate. Figure 6 shows the effects of luminal and serosal addition of vanadate on anaerobic J_H in six bladders. Luminal addition of vanadate had no effect on anaerobic J_H, whereas serosal addition caused a reduction in J_H from 21 ± 4 to 7 ± 2 μA within 1 hr. The $H_2VO_4^-$ anion is thought to be the major active form of vanadate and to act on the cytoplasmic side of the ATPase (Simons, 1979). The effective concentration of $H_2VO_4^-$ at the pH of the media was about 0.1 mM (Pope & Dale, 1968). The failure of vanadate to inhibit J_H from the luminal side is probably due to the inability of the anions to cross the luminal cell membrane. The serosal cell membrane has a high anion permeability (Cohen, Mueller & Steinmetz, 1978) and would be expected to allow entry of vanadate anions. Beauwens, Crabbé and Rentmeesters (1980) also observed that in toad bladder the inhibitory effect of vanadate on Na transport is exerted from the serosal side. The inhibition was reduced by pretreatment of the serosal surface with a disulfonic stilbene, a result which suggests that vanadate enters the cell through specific anion transport sites.

4. *Uncoupling of J_H and J_{lac} by Vanadate*

In Table 2 we showed that in the anaerobic preparation J_{lac} is related to J_H. It should be mentioned parenthetically that H^+ secretion was the principal transport process since Na^+ transport was abolished by ouabain. The relationship $\delta J_{lac}/\delta J_H$ was remarkably constant when J_H was varied by different experimental maneuvers.

In contrast to these results of Table 2, the serosal addition of vanadate caused an apparent uncoupling of J_H from anaerobic glycolysis. As shown in Table 3 the inhibition of J_H by vanadate was associated with increased rather than decreased lactate production. To determine whether this increased J_{lac} resulted from uncoupling at the catalytic site of the H^+ pump or from increased glycolysis unrelated to H^+ transport, we measured J_{lac} when J_H was reduced to zero either by an opposing pH gradient or by 0.5 mM DCCD.

Table 3. Uncoupling of anaerobic J_H and J_{lac} by vanadate

	J_H			J_{lac}		
	(μmol/hr)			(μmol/hr)		
	Control	$H_2VO_4^-$	ΔJ_H	Control	$H_2VO_4^-$	ΔJ_{lac}
1	0.69	0.18		0.98	1.58	
2	0.26	0.16		0.97	1.05	
3	0.60	0.32		0.50	1.34	
4	0.31	0.04		1.08	1.30	
5	0.30	0.07		0.72	1.24	
6	0.44	0.04		1.05	1.40	
Mean	0.44	0.14	−0.30	0.89	1.32	+0.43
±SE	0.07	0.04	0.06	0.09	0.07	0.11

Na_3VO_4 was added to the serosal solution to give a final concentration of $H_2VO_4^-$ of about 0.1 mM. This group of experiments was different from that of Fig. 4. The ΔJ_H and ΔJ_{lac} (paired analysis) are significant ($P<0.05$).

Vanadate failed to increase J_{lac} when the pump was stopped by a pH gradient, but did cause an increase in J_{lac} when the pump was stopped by DCCD. It is possible, therefore, that vanadate increased basal J_{lac} by stimulation of anaerobic glycolysis under some conditions. Vanadate may replace phosphate in glycolytic metabolism and promote rapid vanadolytic reactions (DeMaster & Mitchell, 1973). The apparent uncoupling between J_H and J_{lac} remains to be clarified. It is of interest that vanadate also causes a marked (about 80%) inhibition of J_H under aerobic conditions when several pathways for the supply of metabolic energy are available (Norby & Schwartz, 1978).

Discussion

These studies indicate that proton transport (J_H) by the isolated turtle bladder can be driven by the energy from anaerobic glycolysis. Under strictly anaerobic conditions, a portion of lactate production (J_{lac}) was clearly coupled to J_H. Such coupling was observed among bladders with different spontaneous rates of H^+ secretion as well as in individual bladders when J_H was altered experimentally by an opposing pH gradient or by certain inhibitors.

H^+ secretion accounts for most of the transport activity of the epithelium when sodium transport is abolished by ouabain[1]. Nevertheless, the rate of lactate production unrelated to H^+ secretion (J_{lac} at $J_H=0$) was appreciable: 0.58 μmol/hr in nonstripped bladders and 0.44 μmol/hr in stripped bladders. The small difference between stripped and nonstripped bladders indicate that the muscle bundles and other cells of the serosal loose tissue layer contribute relatively little to basal J_{lac}. Therefore, most of the basal J_{lac} must originate from the epithelial cells. Since H^+ secretion appears to be the function of only a population of carbonic anhydrase containing cells (Mueller et al., 1978; Husted et al., 1980), basal J_{lac} reflects the nontransport-related glycolysis of the H^+ secreting cells as well as the anaerobic metabolism of the large population of granular cells.

Of major interest in our anaerobic system is the consistent coupling observed between J_{lac} and J_H and the narrow range of $\delta J_{lac}/\delta J_H$ between 0.55 and 0.58 obtained when J_H was inhibited by the ΔpH method, acetazolamide or DCCD. This result indicates a high degree of coupling between J_H and the metabolic driving reaction and supports a similar conclusion reached by Beauwens and Al Awqati (1976) on the basis of studies carried out under aerobic conditions. Since H^+ secretion during prolonged anaerobiosis is critically dependent on the presence of glucose, we may assume that the transport-related lactate was derived from the breakdown of glucose. The anaerobic metabolism of one glucose results in the formation of two lactate and two ATP molecules. If ATP hydrolysis provides the free energy of H^+ translocation, the observed coupling would indicate that the H^+ pump transports slightly less than 2 protons per ATP hydrolyzed. This H^+/ATP stoichiometry is lower than the value of about 3 estimated by Dixon and Al Awqati (1980) from the average ATP/ADP·P_i ratios of all epithelial cells and the simultaneously measured maximal electrochemical potential gradient for protons ($\Delta\bar{\mu}_H$). Since it is as yet not feasible to obtain estimates of the free energy of ATP hydrolysis and the $\Delta\bar{\mu}_H$ of the H^+ secreting cell, the stoichiometry of 3 may prove to be too high. The H^+/ATP stoichiometry of just below 2 obtained in the present study depends on the ratio of the two flows J_H and J_{lac} rather than on the ratio of the two forces $\Delta G'_{ATP}$ and $\Delta\bar{\mu}_H$. J_H was measured by the current method which was in good agreement with the pH stat method. J_{lac} might underestimate ATP hydrolysis if O_2 entered the system or if glycogen rather than glucose was metabolized. These possibilities were unlikely and, furthermore, would change the stoichiometry in the wrong direction, i.e., to values less than 2. When the H^+/ATP stoichiometry is estimated from the ratio of the forces the assumption is made that there is a high degree of coupling between the two flows. As stated earlier our results support this assumption. At present there is no obvious explanation for the difference between the estimates made by the two experimental approaches. Perhaps the least certain

[1] Electroneutral Cl-HCO_3 exchange transport in turtle bladder is extremely O_2 dependent (Oliver et al., 1975) and would not be expected to operate under our conditions.

value is the $\Delta\bar{\mu}_H$ across the luminal cell membrane of the H^+ secreting cell.

An important simplification that can be made during anaerobiosis is the exclusion of mitochondrial ATPase function. If the proton pump of the luminal cell membrane of turtle bladder is indeed an ATPase, it might share some of the characteristics of mitochondrial ATPase. Thus, in our studies DCCD and Dio-9, agents which block proton flow in the proteolipid portion of the H^+-ATPases of mitochondrial, chloroplast, and certain bacterial membranes (Carmeli et al., 1975; Racker, 1976) caused inhibition of anaerobic J_H. The fact that $\delta J_{lac}/\delta J_H$ was the same after inhibition of J_H by DCCD as by other methods indicates that DCCD acted primarily on the H^+ pump. In contrast to this similarity between the plasma membrane pump and mitochondrial ATPase, the plasma membrane pump was unaffected by concentrations of oligomycin which easily inhibit mitochondrial ATPase. Conversely, vanadate which does not inhibit mitochondrial ATPase (Bowman et al., 1978; Willsky, 1979) did inhibit J_H in turtle bladder. The acidification pump of the luminal cell membrane, therefore, differs from mitochondrial ATPase in several respects.

Unlike the (Na^+-K^+)-ATPase of the basolateral cell membrane, the luminal H^+ pump is not sensitive to ouabain (Solinger et al., 1968; Husted & Steinmetz, 1979). Both cell membrane pumps, however, are inhibited by vanadate. The vanadate sensitivity of anaerobic J_H in our study is of special interest since vanadate was the only inhibitor that uncoupled J_H from J_{lac}. J_{lac} increased significantly as J_H decreased. It appears that the active form of vanadate is probably the $H_2VO_4^-$ ion which resembles $H_2PO_4^-$ and may act on the phosphate release site of the ATPase (Simons, 1979; Grantham & Glynn, 1979). Vanadate inhibits the sodium pump from the inner side of the membrane. Similarly, in turtle bladder the inhibition of J_H occurred when the vanadate was added to the serosal side of the bladder. Since the serosal cell membrane has a much higher anion permeability than the luminal membrane (Cohen et al., 1978), it is likely that vanadate also acted on the cytoplasmic side of the proton pump.

The H^+ pump of the turtle bladder is electrogenic (Steinmetz, Omachi & Frazier, 1967) and closely controlled by the $\Delta\bar{\mu}_H$ across the transport pathway (Steinmetz, 1974; Beauwens & Al Awqati, 1976; Al Awqati, Mueller & Steinmetz, 1977). Of great interest is the recent study by Dixon and Al Awqati (1979), suggesting that when a sufficiently large $\Delta\bar{\mu}_H$ is imposed across the turtle bladder protons flow through the pump pathway in reverse direction and ATP is synthesized within the epithelial cell layer. They concluded that the H^+ pump of the luminal cell membrane is a reversible ATPase. In their study ATP synthesis was abolished by oligomycin and dinitrophenol and the plasma membrane pump closely resembled mitochondrial ATPase. Our anaerobic studies support the conclusion that the acidification pump is a proton-translocating ATPase but suggest that the characteristics of this ATPase may differ from those of mitochondrial ATPase.

The characteristics of the electrogenic proton pump of the turtle bladder are virtually identical to those of the H^+-ATPases of the plasma membranes of yeast (Delhez et al., 1977; Willsky, 1979) and the fungus *Neurospora crassa* (Bowman & Slayman, 1977; Bowman et al., 1978). In these organisms it has been possible to separate the H^+-ATPases of the plasma membrane from the mitochondrial ATPase. The plasma membrane ATPases translocate protons by an electrogenic mechanism and have a lower pH optimum for ATP hydrolysis and a simpler subunit structure than the mitochondrial enzyme. The plasma membrane ATPases are oligomycin resistant and vanadate sensitive (Bowman et al., 1978; Willsky, 1979).

In contrast to the well-characterized ATPases of yeast and fungi, relatively little is known about electrogenic proton pumps in epithelial membranes. Sachs, Spenney and Lewin (1978) have defined an apical membrane ATPase in gastric mucosa which is DCCD-sensitive and oligomycin-resistant. This gastric ATPase, however, appears to operate in a non-electrogenic mode by exchanging K^+ for H^+. Kinne-Saffran and Kinne (1979) have provided evidence for a bicarbonate-stimulated Mg^{++}-ATPase in brush border membranes isolated from rat kidney cortex. This ATPase is again oligomycin-resistant and differs in other respects from mitochondrial ATPase. The transport characteristics of this brushborder ATPase remain to be determined.

The proton pump of the turtle bladder has characteristics which are intermediate between those of mitochondrial ATPase and those of the metal cation ATPases. The stoichiometry of J_H and J_{lac} suggests that for each ATP hydrolyzed slightly less than two protons are translocated.

This work was supported by NIH grants AM 17568 and AM 24022.

References

Al-Awqati, Q., Mueller, A., Steinmetz, P.R. 1977. Transport of H^+ against electrochemical gradients in turtle urinary bladder. *Am. J. Physiol.* **233(6)**:F502

Beauwens, R., Al-Awqati, Q. 1976. Active H^+ transport in the turtle urinary bladder: Coupling of transport to glucose oxidation. *J. Gen. Physiol.* **68**:421

Beauwens, R., Crabbé, J., Rentmeesters, M. 1980. Effects of vanadate on the functional properties of the isolated toad bladder. *J. Physiol. (London) (in press)*

Bowman, B.J., Mainzer, S.E., Allen, K.E., Slayman, C.W. 1978. Effects of inhibitors on the plasma membrane and mitochondrial adenosine triphosphatases of *Neurospora crassa*. *Biochim. Biophys. Acta* **512**:13

Bowman, B.J., Slayman, C.W. 1977. Characterization of plasma membrane adenosine triphosphatase of *Neurospora crassa*. *J. Biol. Chem.* **252**:3357

Carmeli, C., Lifshitz, Y., Gepshtein, A. 1975. Control of proton translocation induced by ATPase activity in chloroplasts. *Biochim. Biophys. Acta* **376**:249

Cohen, L.H., Mueller, A., Steinmetz, P.R. 1978. Inhibition of the bicarbonate exit step in urinary acidification by a disulfonic stilbene. *J. Clin. Invest.* **61**:981

Delhez, J., Dufour, J.P., Thines, D., Goffeau, A. 1977. Comparison of the properties of plasma membrane-bound and mitochondria-bound ATPases in the yeast *Schizosaccharomyces pombe*. *Eur. J. Biochem.* **79**:319

DeMaster, E.G., Mitchell, R.A. 1973. A comparison of arsenate and vanadate as inhibitors or uncouplers of mitochondrial and glycolytic energy metabolism. *Biochemistry* **12**:3610

Dixon, T.E., Al-Awqati, Q. 1979. Urinary acidification in turtle bladder is due to a reversible proton-translocating ATPase. *Proc. Nat. Acad. Sci. USA* **76**:3135

Dixon, T.E., Al-Awqati, Q. 1980. H^+/ATP stoichiometry of proton pump of turtle urinary bladder. *J. Biol. Chem.* **255**:3237

Dodge, C.H., Folk, G.E., Jr. 1963. Notes on comparative tolerance of some Iowa turtles to oxygen deficiency (hypoxia). *Proc. Iowa Acad. Sci.* **70**:438

Grantham, J.J., Glynn, I.M. 1979. Renal Na, K-ATPase: Determinants of inhibition of vanadium. *Am. J. Physiol.* **236**:F530

Husted, R.F., Mueller, A., Cohen, L.H., Steinmetz, P.R. 1980. Morphologic characteristics of H^+ secreting cells in turtle bladder: Studies with a disulfonic stilbene. *Fed. Proc.* **39**:739

Husted, R.F., Steinmetz, P.R. 1979. The effects of amiloride and ouabain on urinary acidification by turtle bladder. *J. Pharmacol. Exp. Ther.* **210**:264

Kagawa, Y., Sone, N., Hirata, H., Yoshida, M. 1979. Structure and function of H^+-ATPase. *J. Bionerg. Biomembr.* **11**:39

Kinne-Saffran, E., Kinne, R. 1979. Further evidence for the existence of an intrinsic bicarbonate-stimulated Mg^{++}-ATPase in brush border membranes isolated from rat kidney cortex. *J. Membrane Biol.* **49**:235

Klahr, S., Bricker, N.S. 1964. Na transport by isolated turtle bladder during anaerobiosis and exposure to KCN. *Am. J. Physiol.* **206**:1333

Klahr, S., Bricker, N.S. 1965. Energetics of anaerobic sodium transport by the fresh water turtle bladder. *J. Gen. Physiol.* **48**:571

Mueller, A.L., Kessell, R.G., Cohen, L.H., Husted, R.F., Steinmetz, P.R. 1978. Effect of a disulfonic stilbene (SITS) on surface morphology and urinary acidification in turtle bladder. *Fed. Proc.* **37**:511

Norby, L.H., Schwartz, J.H. 1978. Relationship between the rate of H^+ transport and pathways of glucose metabolism by turtle urinary bladder. *J. Clin. Invest.* **62**:532

Oliver, J.A., Himmelstein, S., Steinmetz, P.R. 1975. Energy dependence of urinary bicarbonate secretion in turtle bladder. *J. Clin. Invest.* **55**:1003

Pope, M.T., Dale, B.W. 1968. Isopoly-vandates, -niobates, and -tantalates. *Q. Rev. Chem. Soc.* **22**:527

Racker, E. 1976. A New Look at Mechanisms in Bioenergetics. Academic Press, New York

Robin, E.D., Vester, J.W., Murdaugh, H.V., Jr., Millen, J.E. 1964. Prolonged anaerobiosis in a vertebrate: Anaerobic metabolism in the fresh water turtle. *J. Cell. Comp. Physiol.* **63**:287

Sachs, G., Spenney, J.G., Lewin, M. 1978. H^+ transport: Regulation and mechanism in gastric mucosa and membrane vesicles. *Physiol. Rev.* **58**:106

Schwartz, J.H. 1976. H^+ current response to CO_2 and carbonic anhydrase inhibition in turtle bladder. *Am. J. Physiol.* **231**:565

Schwartz, J.H., Steinmetz, P.R. 1977. Metabolic energy and PCO_2 as determinants of H^+ secretion by turtle urinary bladder. *Am. J. Physiol.* **233(2)**:*F*145

Simons, T.J.B. 1979. Vanadate – a new tool for biologists. *Nature (London)* **281**:337

Solinger, R.E., Gonzalez, C.F., Shamoo, Y.E., Wyssbrod, H.R., Brodsky, W.A. 1968. Effect of ouabain on ion transport mechanisms in the isolated turtle bladder. *Am. J. Physiol.* **215**:249

Steinmetz, P.R. 1974. Cellular mechanisms of urinary acidification. *Physiol. Rev.* **54**:890

Steinmetz, P.R., Husted, R.F., Mueller, A. 1980. Coupling between H^+ transport and anaerobic glycolysis in turtle bladder. Vanadate sensitivity of H^+ pump. *Clin. Res.* **28(2)**: 560*A*

Steinmetz, P.R., Mueller, A., Beauwens, R. 1979. Effects of ATPase inhibitors on anaerobic H^+ transport by turtle urinary bladder. *Fed. Proc.* **38**:1121

Steinmetz, P.R., Omachi, R.S., Frazier, H.S. 1967. Independence of hydrogen ion secretion and transport of other electrolytes in turtle bladder. *J. Clin. Invest.* **46**:1541

Willsky, G.R. 1979. Characterization of the plasma membrane Mg^{++}-ATPase from the yeast, *Saccharomyces Cerevisiae*. *J. Biol. Chem.* **254**:3326

Received 23 June 1980; revised 24 October 1980