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EVIDENCE FOR THE PRESENCE OF AN ELECTROGENIC PROTON PUMP ON THE TROUT GILL EPITHELIUM

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Summary

Ion transport inhibitors, amiloride, SITS, vanadate and acetazolamide, were added to the water to determine the effect of ion transfer mechanisms on the acidification of water passing over the gills. In neutral water, proton excretion causes a marked reduction in gill water pH. If water pH is 2.5 units lower than blood pH, however, then this proton excretion is inhibited and all water pH changes can be accounted for by CO_2 hydration and ammonia protonation. Proton excretion across the gills is insensitive to 0.1 mmol l^{-1} amiloride and SITS but sensitive to vanadate, acetazolamide and water pH; thus, we conclude that proton excretion is mediated by an active proton pump on the apical membrane of the gill epithelium similar to that reported for the frog skin. Higher concentrations of amiloride (0.5 and 1 mmol l^{-1}) reduced both ammonia and acid excretion, presumably because of inhibition of Na^+/K^+-ATPase on the basolateral border of the gill epithelium.

Introduction

Carbon dioxide and proton excretion acidifies water as it passes over the gills, whereas NH_3 excretion raises pH. The effect of carbon dioxide and proton excretion dominates at water pH above 5.3. Below pH 5.3, the effect of ammonia excretion dominates and the pH of water increases as it passes over the gills (Lin and Randall, 1990).

Rainbow trout gills constitute a tight epithelium, permeable to NH_3 and CO_2. Carbon dioxide is excreted from the blood as CO_2, the gill epithelium being impermeable to HCO_3^- (Perry *et al.* 1982). It has been suggested that a portion of the carbon dioxide entering the gill epithelium is hydrated, forming bicarbonate, which is then exchanged for chloride across the apical membrane. The addition of 4-acetamido-4′-isothiocyanatostilbene-2-2′-disulphonic acid (SITS), known to block Cl^-/HCO_3^- exchange in red blood cells, to the water results in a rise in blood pH in trout (Perry *et al.* 1981) and a reduction in Cl^- uptake (Perry and Randall, 1981).

Key words: acidification, carbon dioxide, ammonia, H^+-ATPase, SITS, amiloride, vanadate, acetazolamide, gill, rainbow trout, *Oncorhynchus mykiss*.

The trout gill NH_3 permeability coefficient of 6×10^{-3} cm s^{-1} (Avella and Bornancin, 1989) is intermediate between values reported for the toad bladder and mammalian kidney tubule. It has been suggested that ammonia excretion, although dominated by NH_3 diffusion (Hillaby and Randall, 1979; Cameron and Heisler, 1983), is also mediated by Na^+/NH_4^+ exchange on the apical surface (Payan, 1978; Wright and Wood, 1985). The stimulatory effect of NH_4^+ on sodium flux, however, could be explained in terms of a pH effect of the ammonia addition (Cameron and Kormanik, 1982), and Avella and Bornancin (1989) concluded that the balance of evidence was against the presence of Na^+/NH_4^+ exchange across the apical surface of trout gills. They considered the trout gill to be similar to other tight epithelia, such as frog skin and toad bladder, in that passive sodium uptake from water is indirectly coupled to an active electrogenic proton transport system.

The object of this study was to determine the relative contributions of these various components to the change in pH of water as it passes over the gills.

Materials and methods

Animals and preparation

Rainbow trout *Oncorhynchus mykiss* (Walbaum) weighing 202–592 g were maintained in outdoor fibreglass tanks supplied with flowing dechlorinated Vancouver tapwater. Fish were fed daily with commercial trout pellets and feeding was suspended for at least 48 h prior to experimentation.

Surgery was performed on each fish under general anaesthesia (1:10 000 MS222 solution, pH adjusted to 7.5 with $NaHCO_3$) to fix an opercular cannula for sampling expired water. Fish were then confined, but not physically restrained, in a black chamber to recover for at least 24 h. This black chamber was supplied with aerated dechlorinated tapwater during the recovery period. Three hours prior to the experiment, the water supply was switched to the aerated test solution (40 mmol l^{-1} NaCl and 0.5 mmol l^{-1} $CaCl_2$ in dechlorinated tapwater, from Wright *et al.* 1986) with a buffering capacity (β) of 81 μequiv l^{-1} pH unit^{-1}. The test solution had the same ionic strength as the buffer solution used to calibrate the pH electrodes. By using this test solution, we reduced the response time of the pH electrode, increased its stability and thus obtained more precise water pH measurements. Temperature was regulated to that of tapwater with a cooling coil.

Experimental protocols

Amiloride, SITS and pH treatment

Three sets of experiments were carried out using a recirculating system connected to the black chamber. One was a control experiment with the test solution only. The other two were amiloride and SITS treatments, with either amiloride or SITS added to the test solution to give a final concentration of 0.1 mmol l^{-1}. In each set of experiments the fish was exposed for 30 min to four environmental pH levels – pH 7, pH 6, pH 5 and pH 4. The volume of the

recirculating system was 6 l and it was aerated and controlled at temperatures between 6.5 and 8.5 °C (winter experiments). A magnetic stirring bar was used in the reservoir to ensure complete mixing. The pH of the test solution was adjusted to 7 by adding 0.1 mol l^{-1} NaOH or 0.1 mol l^{-1} HCl and then quickly lowered to 6, 5 and 4 by adding 0.1 mol l^{-1} HCl at the beginning of each different environmental pH exposure. The amount of acid added was measured precisely and pH was recorded to construct buffer curves. The water pH of the recirculating system changed slightly (<0.3 unit) over the 30-min experimental period, and no attempt was made to stabilize pH during this period. Inspired and expired water samples (approximately 5 ml each) were withdrawn from the outlets of the glass electrode chambers at the end of each 30-min exposure. Between each exposure there was a 15-min exchange time during which the water was mixed and pH changed to a new level. The results of pH 7 and pH 6 treatments were pooled for data analysis because there was no significant difference between them.

Amiloride treatments

This study employed the recirculating system described above. Three concentrations of amiloride were utilized and their effects on CO_2, ammonia and net proton excretion determined. Each experiment started with a 1-h control period, with the fish resting in the recirculating system containing the test solution alone. Inspired and expired water samples were taken at 30 min and 60 min. The system was flushed with fresh test solution and amiloride was added to the system to give a final concentration of 0.1, 0.5 or 1 mmol l^{-1}. Recirculation was restored and the amiloride treatment lasted for another hour. Water samples were taken at 30 min and 60 min. The buffer capacities of inspired and expired water were measured later by titrating the stored water samples using 0.1 mol l^{-1} NaOH and 0.1 mol l^{-1} HCl. The results of the 30-min and 60-min sampling were pooled for data analysis since there was no significant difference between them. Experiments were performed during the summer at temperatures between 13 and 17 °C.

Vanadate and acetazolamide treatment

Using the same recirculating system, 0.1 mmol l^{-1} vanadate or 0.1 mmol l^{-1} acetazolamide was added to the external water to examine their effect on CO_2, ammonia and proton excretions. Each experiment started with a 1-h control period followed by a 1-h treatment period. Since the ammonia accumulation in the system was very low (less than 100 μmol l^{-1} after 2 h), flushing the system with fresh test solution at the beginning of the treatment period was considered unnecessary. For the vanadate treatment, the pH of the test solution was adjusted to between 7 and 8 and, 30 min later, to between 8 and 9 by titrating with 0.1 mol l^{-1} NaOH, in order to investigate proton excretion in the pH range 7–9. Freshly made 3 mmol l^{-1} sodium orthovanadate (Na_3VO_4) solution was boiled and the cooled solution was neutralized with 0.1 mol l^{-1} HCl. After the control period, 200 ml of the 3 mmol l^{-1} vanadate solution was added to the 6-l recirculating system to obtain a final concentration of 0.1 mmol l^{-1}. External water pH was

adjusted to the control value during the two 30-min treatment periods. For the acetazolamide treatment, water pH was adjusted to around 7 and acetazolamide was added to the system to give a final concentration of 0.1 mmol l^{-1} after the control period. The same set of measurements as described in 'amiloride treatment' was performed every 30 min in both control and treatment periods. Temperature in these experiments was 7–9 °C (spring experiments).

Analytical techniques and calculations

Inspired and expired water pH were monitored during the whole experimental period with combination glass pH electrodes housed in two water-jacketed glass chambers (Wright *et al.* 1986). Inspired pH (pH_{in}) and expired pH (pH_{ex}) values were recorded at each sampling.

Total carbon dioxide contents of inspired water $[CO_2]_{in}$ and expired water $[CO_2]_{ex}$ were measured immediately with a Carle gas chromatograph (model III) containing a CO_2 discriminating column (porapak Q) (Boutilier *et al.* 1985; Lenfant and Aucutt, 1966).

Total ammonia contents of inspired water $[Amm]_{in}$ and expired water $[Amm]_{ex}$ were measured by a micro-modification of the salicylate–hypochlorite assay with frozen water samples (Verdouw *et al.* 1978). To ensure that there was no ammonia loss from water in the recirculating system, two experiments were carried out in which known amounts of NH_4Cl were added to the system without fish. No loss from the system occurred.

The ammonia excretion rate of the fish was calculated as:

$$\text{Ammonia excretion rate} = \frac{([Amm]_{in,f} - [Amm]_{in,i})V}{tW},$$

where i and f refer to the initial and final ammonia concentrations in inspired water in μmol l^{-1}, V is the volume of the system (6 l in our study), t is the elapsed time in hours and W is the mass of the fish in kilograms.

Bicarbonate concentrations in inspired water $[HCO_3^-]_{in}$ and expired water $[HCO_3^-]_{ex}$ were calculated from $[CO_2]_{in}$, pH_{in} and $[CO_2]_{ex}$, pH_{ex}, respectively, by the Henderson–Hasselbalch equation, using the pK_{CO_2} and α_{CO_2} values from Boutilier *et al.* (1985). The difference in $[HCO_3^-]$ between the expired and inspired water, $[HCO_3^-]_{ex} - [HCO_3^-]_{in}$, was equivalent to the calculated proton addition due to CO_2 excretion by the fish (assuming no HCO_3^- excretion). Carbonate formation is negligible over the pH range.

Ammonium ion concentrations in inspired water $[NH_4^+]_{in}$ and expired water $[NH_4^+]_{ex}$ were calculated from $[Amm]_{in}$, pH_{in} and $[Amm]_{ex}$, pH_{ex}, respectively, by the Henderson–Hasselbalch equation, using the pK_{Amm} value from Cameron and Heisler (1983). The difference in $[NH_4^+]$ between the expired and inspired water, $[NH_4^+]_{ex} - [NH_4^+]_{in}$, was equivalent to the calculated proton consumption due to NH_3 excretion by the fish (assuming no NH_4^+ excretion).

The calculated proton concentration increase in expired water, $[H^+]_{cal}$, was

obtained by subtracting the calculated proton consumption from the calculated proton addition:

$$[H^+]_{cal} = ([HCO_3^-]_{ex} - [HCO_3^-]_{in}) - ([NH_4^+]_{ex} - [NH_4^+]_{in}).$$

The measured proton concentration increase in expired water, $[H^+]_{meas}$, which was equivalent to the amount of protons actually added to the water, was calculated from the appropriate buffer curve and pH_{in} and pH_{ex}.

Net proton excretion was obtained by subtracting the calculated proton concentration increase from the measured proton concentration increase in expired water:

$$\text{Net proton excretion} = [H^+]_{meas} - [H^+]_{cal}.$$

Data are presented as means±standard error. Student's two-tailed *t*-test and analysis of variance (ANOVA) were used to test for significant differences between means. Tests of significance were conducted at the 5 % level of rejection. Regression analyses were used to describe relationships between variables.

Results

The differences in total CO_2 content between inspired water and expired water with different treatments are presented in Table 1. $[CO_2]_{ex}-[CO_2]_{in}$ represents the CO_2 excretion rate if we assume that ventilation rate is constant. There was no significant difference in $[CO_2]_{ex}-[CO_2]_{in}$ between control and drug-treated animals except in the case of acetazolamide. CO_2 excretion increased when fish were exposed to 0.1 mmol l^{-1} acetazolamide in water.

When water pH was approximately neutral, $[HCO_3^-]_{in}$ was always greater than $[HCO_3^-]_{ex}$ (Fig. 1), indicating that the water was acidified as it passed over the gills by something other than CO_2 addition. When proton excretion was inhibited

Table 1. *Total CO_2 excretion by fish (μmol l^{-1} kg^{-1})*

	$[CO_2]_{ex}-[CO_2]_{in}$	
	Control	Treatment
0.1 mmol l^{-1} amiloride N=6 paired	39.73±6.57	32.75±5.30
0.5 mmol l^{-1} amiloride N=6 paired	71.00±8.04	71.48±10.43
1 mmol l^{-1} amiloride N=6 paired	62.46±8.11	57.77±7.49
0.1 mmol l^{-1} SITS N=6 unpaired	66.73±13.13	59.49±9.69
0.1 mmol l^{-1} vanadate N=10 paired	76.94±4.41	63.68±5.60
0.1 mmol l^{-1} acetazolamide N=4 paired	55.11±9.91	82.90±10.32*

* indicates a significant difference from the control value.

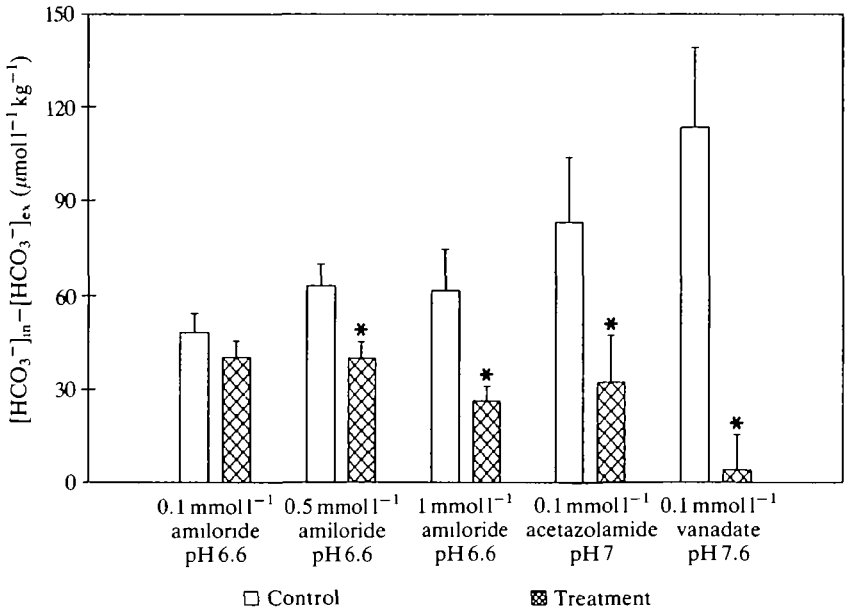

Fig. 1. Bicarbonate concentration differences between inspired and expired water of rainbow trout under control, amiloride, acetazolamide and vanadate treatments. * indicates a significant difference between the control and treatment values ($P<0.05$); bars show standard errors; $N=6$.

by 0.1 mmol l^{-1} vanadate, 0.1 mmol l^{-1} acetazolamide or 0.5 or 1 mmol l^{-1} amiloride (see Figs 5, 6), the extent of bicarbonate dehydration decreased and $[HCO_3^-]_{ex}$ increased. Therefore, $[HCO_3^-]_{in}-[HCO_3^-]_{ex}$ was significantly lower than the control value. In addition, the control $[HCO_3^-]_{in}-[HCO_3^-]_{ex}$ value at pH 7.6 was greater than that at pH 6.6, indicating that the excretion of acid equivalents was higher at pH 7.6.

As external water pH decreased, the ammonia excretion rate was not statistically different from that at neutral pH within control, 0.1 mmol l^{-1} amiloride and 0.1 mmol l^{-1} SITS treatments (Fig. 2). The difference between control and SITS treatments, tested by ANOVA, was not significant, but there was a significant difference between control and amiloride treatments (notice that control, amiloride and SITS treatments were performed on different group of animals). Ammonia excretion of the same animals was not significantly inhibited by 0.1 mmol l^{-1} amiloride (Fig. 3). However, higher concentrations of amiloride induced a reduction in ammonia excretion, by 58 % with 0.5 mmol l^{-1} amiloride and by 87 % with 1 mmol l^{-1} amiloride (Fig. 3). Vanadate and acetazolamide had no significant effect on ammonia excretion.

In external water below pH 6, net proton excretion was approximately zero (Fig. 4), and the addition of either 0.1 mmol l^{-1} amiloride or 0.1 mmol l^{-1} SITS had no effect on net proton excretion (Fig. 4). At neutral pH, however, there was

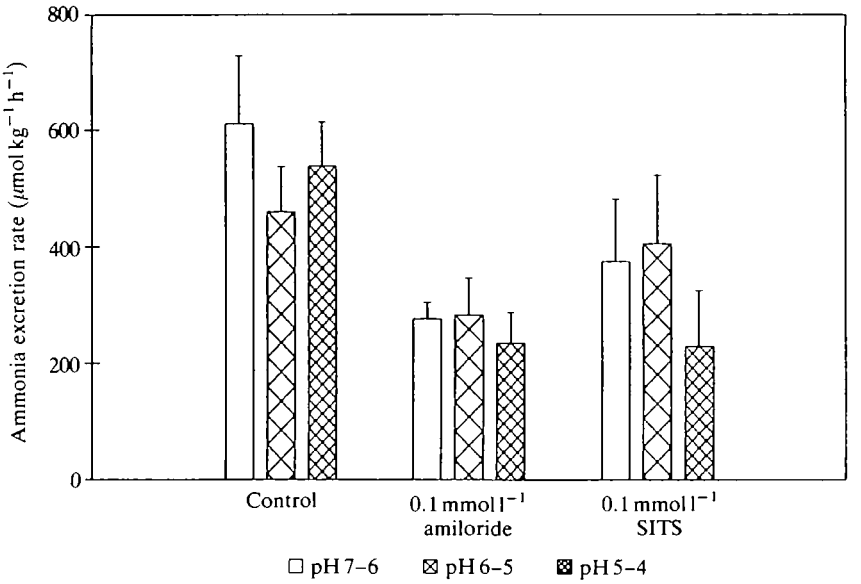

Fig. 2. Ammonia excretion rates of rainbow trout under control, amiloride and SITS treatments. External water pH was lowered from 6 to 5 to 4 within each treatment. Control and treatment groups were carried out on different fish. Under one-way analysis of variance, there was no significant difference within each treatment as pH decreased or between the control and SITS treatments, but there were significant differences between the control and amiloride treatments. Bars show standard errors; N=6.

a net proton excretion across the gill epithelium. This excretion was also unaffected by the addition of either 0.1 mmol l^{-1} SITS or 0.1 mmol l^{-1} amiloride. Increasing amiloride concentration in the external medium induced a reduction in proton excretion (Fig. 5), but more than 50 % of the net proton excretion was still sustained even in the presence of 1 mmol l^{-1} amiloride. 0.1 mmol l^{-1} vanadate treatment resulted in reductions of net proton excretion by 58 % and 67 % when fish were exposed to neutral and moderately alkaline water, respectively (Fig. 6). Acetazolamide treatment also caused a 48 % reduction in net proton excretion (Fig. 6).

The relationship between net proton excretion and the pH difference across the gill epithelium is illustrated in Fig. 7. When water pH is below blood pH by more than 2.5 units, there is no net proton excretion; that is, any change in water pH can be accounted for by CO_2 hydration/HCO_3^- dehydration and/or NH_3 protonation. Net proton excretion was completely inhibited at low external pH. When the pH difference was more than −2.5 units, net proton excretion increased with inspired water pH. Acid excretion was maximal when inspired water pH was equal to or higher than blood pH.

Fig. 8 shows the relationship between net proton excretion and expired water

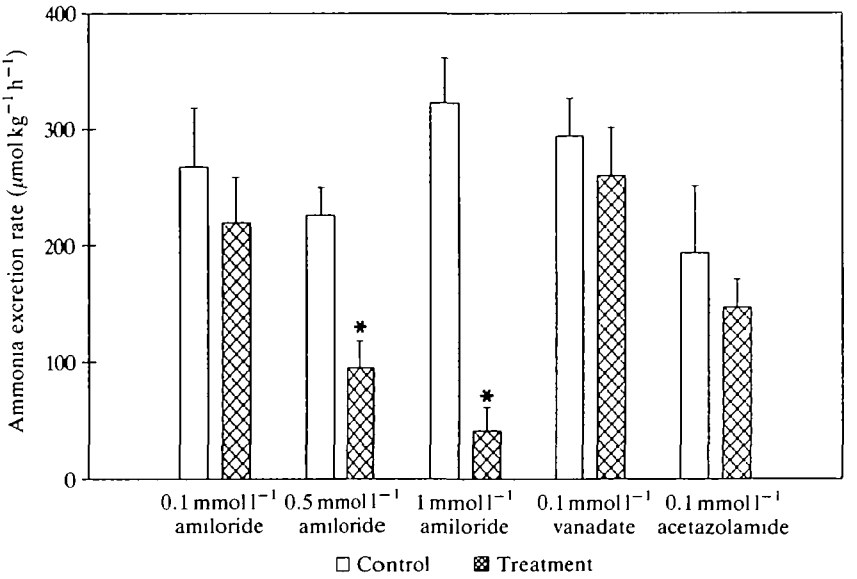

Fig. 3. Ammonia excretion rates of rainbow trout under control, amiloride, acetazolamide and vanadate treatments. * indicates a significant difference between control and treatment values ($P<0.05$); bars show standard errors; $N=6$.

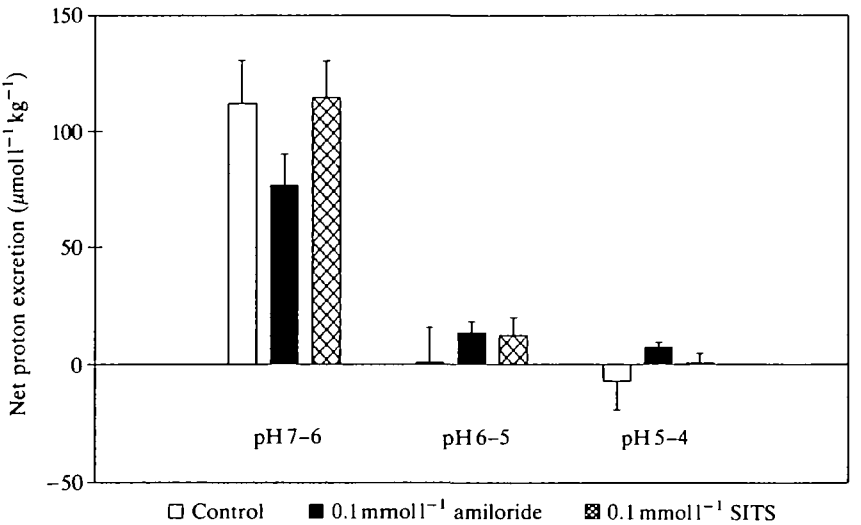

Fig. 4. Net proton excretion across the gill epithelium of rainbow trout exposed to different external water pH values under control, amiloride and SITS treatments. Bars show standard errors; $N=6$.

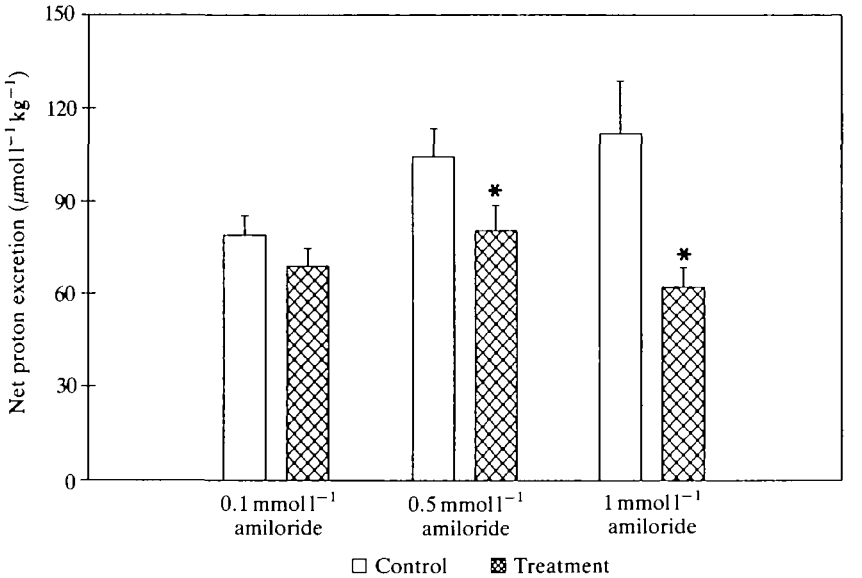

Fig. 5. Net proton excretion across the gill epithelium of rainbow trout exposed to different concentrations of amiloride. * indicates a significant difference between the control and treatment values ($P<0.05$); bars show standard errors; $N=6$.

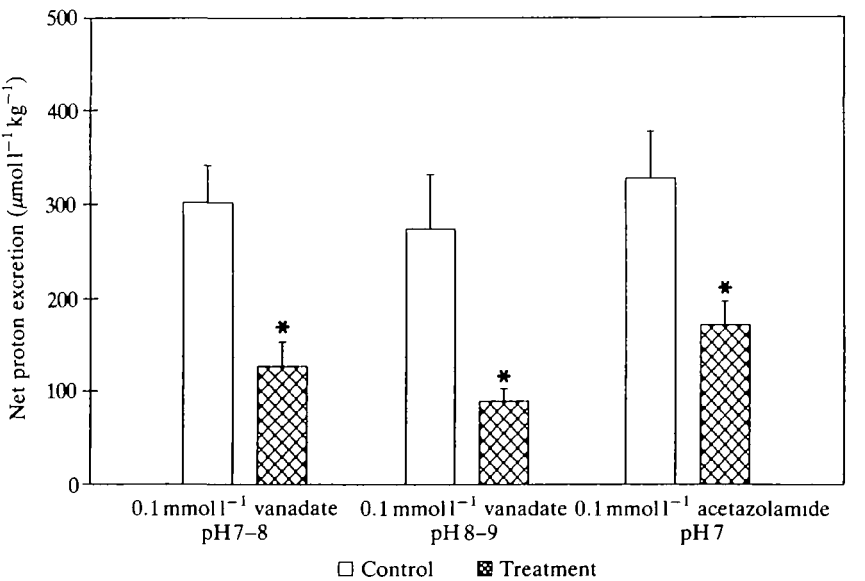

Fig. 6. Net proton excretion across the gill epithelium of rainbow trout exposed to 0.1 mmol l^{-1} vanadate or 0.1 mmol l^{-1} acetazolamide. * indicates a significant difference between the control and treatment values ($P<0.05$); bars show standard errors; $N=6$.

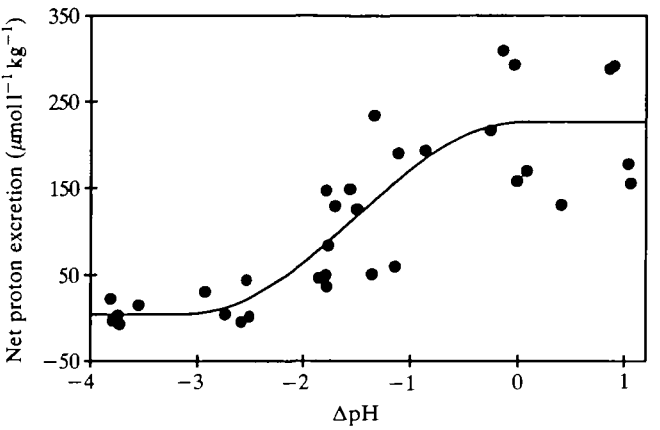

Fig. 7. The relationship between the net proton excretion and the pH difference across the gill epithelium ($\Delta pH=pH_{in}-pH_{blood}$) was expressed by a five-degree regression curve ($r^2=0.853$). Blood pH values were taken from Lin and Randall (1990).

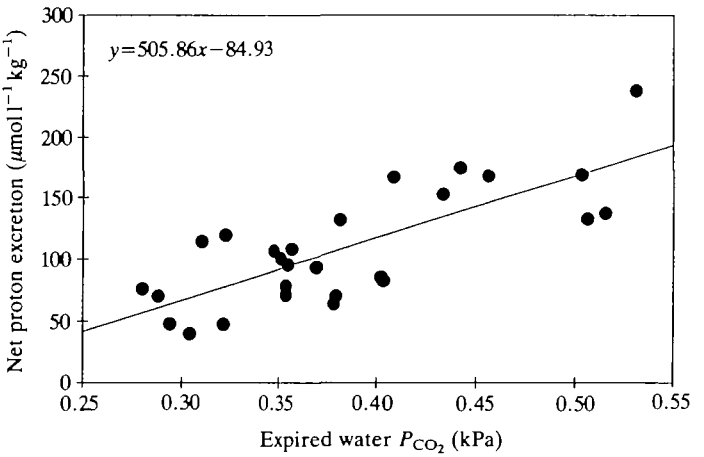

Fig. 8. The relationship between net proton excretion and expired water carbon dioxide levels. The linear regression line has an r^2 value of 0.77. The slope of the regression line is significantly different from zero ($P<0.05$).

carbon dioxide levels in a neutral environment. There is a general tendency for increased P_{CO_2} in expired water to lead to an increase in net proton excretion.

Discussion

The observation that SITS treatment did not result in a reduction of carbon dioxide excretion across the gills indicates that Cl^-/HCO_3^- exchange is playing

only a minor role in carbon dioxide excretion. If 10 % of the total CO_2 excretion is in the form of HCO_3^- in exchange for Cl^-, then blockage of this exchange pathway may result in an elevation of P_{CO_2} in the fish gill, which, in turn, will enhance the molecular CO_2 diffusion and re-establish normal CO_2 excretion rates. The extent of conversion of CO_2 to HCO_3^- in expired water is unimportant for CO_2 excretion, because water pH has no effect on carbon dioxide excretion (Lin and Randall, 1990), and is consistent with the view that the rate-limiting step in carbon dioxide excretion is Cl^-/HCO_3^- exchange across the red blood cell membrane (Perry *et al.* 1982). SITS had no measurable effect on acid excretion across the trout gill in this study, but Perry *et al.* (1981) reported that SITS reduced chloride influx and caused an alkalosis in trout after 6 h of exposure. This indicates that the presence of a Cl^-/HCO_3^- exchange mechanism on the fish gill has only a minor effect on acid–base balance of the fish, below that detectable in this study.

It has long been hypothesized that $Na^+/H^+(NH_4^+)$ electroneutral exchange is the principal mechanism of acid–base regulation in the gill epithelium of fish (Wright and Wood, 1985). This antiport exchange process is blocked by $0.1\,mmol\,l^{-1}$ amiloride, a very potent and relatively specific inhibitor of sodium transport in a wide variety of cellular and epithelial transport systems (Benos, 1982). 84 % and 94 % reductions of the Na^+ uptake by the gills of intact freshwater rainbow trout exposed to $0.1\,mmol\,l^{-1}$ amiloride in the external medium were reported by Perry and Randall (1981) and Wright and Wood (1985), respectively. In our studies, this concentration of amiloride had no effect on either net proton or ammonia excretion when compared with control values from the same animals (Figs 3 and 5). Our experimental conditions are similar to those of Perry and Wright. This indicates that sodium influx and either proton or ammonium ion efflux are not directly coupled (see also Avella and Bornancin, 1989).

It is well documented that proton transport in mammalian kidney (Steinmetz, 1985), amphibian urinary bladder (Al-Awqati, 1978; Steinmetz, 1986) and frog skin (Ehrenfeld *et al.* 1985) is mediated by an electrogenic proton pump. The gill epithelium in freshwater fish is considered to be 'tight' (Sardet, 1980) and resembles frog skin and turtle bladder epithelia functionally and morphologically. Our studies demonstrated that proton excretion in trout was unaffected by low concentrations of amiloride but was inhibited by vanadate, acetazolamide and low water pH, which is associated with a reversal of gill transepithelial potential (McWilliams and Potts, 1978; Ye *et al.* 1991). In addition, elevated water P_{CO_2} stimulated proton excretion in fish gills. All these phenomena have been reported for frog skin (Ehrenfeld *et al.* 1985; Ehrenfeld and Garcia-Romeu, 1977) and turtle bladder (Al-Awqati, 1978; Steinmetz, 1986). Considering all of these lines of evidence together, we agree with Avella and Bornancin (1989) and conclude that the fish gill has an electrogenic proton pump in the mucosal membrane, similar to that reported for frog skin and toad bladder, rather than a Na^+/H^+ exchange mechanism.

The electrogenic proton pump, or H^+-translocating ATPase, on the apical membrane removes protons from the cell and generates a negative potential on

the inner side of the apical membrane (Fig. 9). Sodium influx, driven by the negative potential, occurs *via* a sodium channel that is highly sensitive to amiloride. A $Na^+/K^+(NH_4^+)$-ATPase in the basolateral membrane pumps sodium out of the cell into the blood. Thus, proton excretion and sodium uptake are intimately, but indirectly, linked. Since Avella *et al.* (1987) showed that branchial sodium uptake was proportional to the number of chloride cells in the gills and since proton pumps consume energy and chloride cells are rich in mitochondria and can supply the energy demand, we conclude that the electrogenic proton pump is located in the chloride cell (Fig. 9).

Ammonium ions can replace potassium on the Na^+/K^+-ATPase and thus enter the cell and form NH_3 and protons (Evans *et al.* 1989). The deprotonation of NH_4^+ could supply the proton pump and NH_3 could diffuse passively across the apical membrane into the water. Although much less sensitive than the Na^+ channel, the Na^+/K^+-ATPase in the basolateral membrane can be inhibited by amiloride that has entered the cell when applied in high concentrations to the mucosal side (Knauf *et al.* 1976; Kleyman and Cragoe, 1988). Thus, the reduced proton and ammonia excretion in 0.5 and 0.1 mmol l^{-1} amiloride treatments could be accounted for by the inhibitory effect of amiloride on $Na^+/K^+(NH_4^+)$-ATPase in the basolateral membrane. In support of this contention, Evans *et al.* (1989)

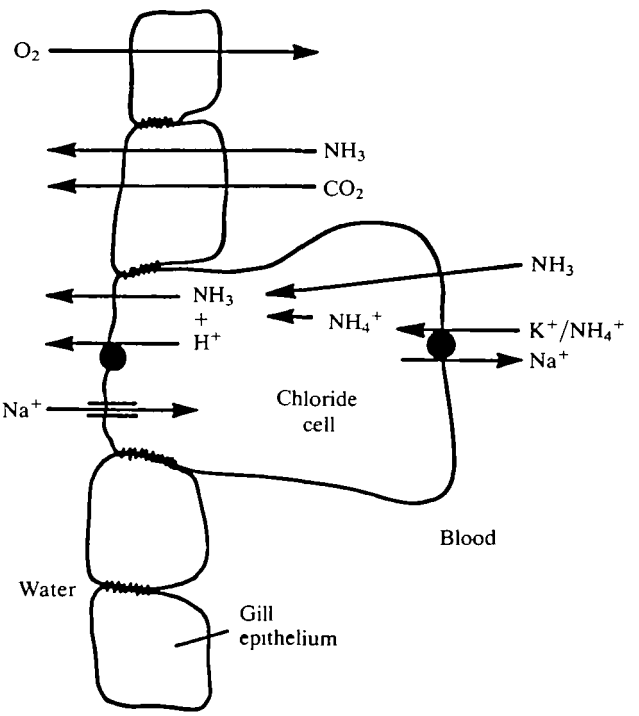

Fig. 9. Schematic representation of gas and ion transport across the gill epithelium of rainbow trout. ATP-driven pumps are denoted by filled circles, passive diffusion by arrows. See text for details.

Table 2. *Comparison between the reduction in the rate of ammonia excretion and the reduction in the rate of net proton excretion under amiloride treatment*

Amiloride concentration ($mmol\,l^{-1}$)	Reduction in the rate of ammonia excretion ($\mu mol\,kg^{-1}\,h^{-1}$)	Reduction in the rate of net proton excretion ($\mu mol\,kg^{-1}\,h^{-1}$)
0.1	47.968±20.568*	60.806±36.178*
0.5	130.542±9.346	143.169±61.059
1	280.560±35.561	298.105±86.911

Net proton excretion was converted to $\mu mol\,kg^{-1}\,h^{-1}$ by assuming that the ventilation rate is $100\,ml\,min^{-1}$ (Lin and Randall, 1990).

* indicates a value not significantly different from zero.

showed that amiloride did not affect ammonia excretion if the perfused head of the toadfish was pretreated with ouabain, which blocks $Na^+/K^+(NH_4^+)$-ATPase. Proton excretion in frog skin was inhibited by $0.5\,mmol\,l^{-1}$ amiloride by 35 % but was not affected by $0.05\,mmol\,l^{-1}$ amiloride, whereas sodium uptake was completely abolished. If we assume that the ventilation rate of the fish was $100\,ml\,min^{-1}$ (Lin and Randall, 1990), we can compare the ammonia excretion rate with the net proton excretion rate under amiloride treatments (Table 2). The reduction in ammonia excretion was equivalent to that in proton excretion, indicating the possibility that NH_3 and protons were both originating from NH_4^+ transported into the epithelium *via* the $Na^+/K^+(NH_4^+)$-ATPase in the basolateral membrane (Fig. 9).

Ammonia elimination was not affected by vanadate or acetazolamide, indicating that proton and ammonia efflux from the gill epithelium are through different pathways. Thus, ammonium entry into the gill epithelium may affect proton excretion (Table 2), but variations in proton excretion do not appear to affect ammonia excretion. Ammonium cannot be the sole source of protons however, because proton excretion can be more than twice ammonia excretion in some instances. Elevated carbon dioxide levels appear to enhance proton excretion (Fig. 8), as observed in toad bladder (Al-Awqati, 1978), indicating that carbon dioxide is also a source of protons for the pump. Protons can be generated in the cellular compartment from CO_2 hydration catalysed by carbonic anhydrase. Acetazolamide, a traditional carbonic anhydrase inhibitor, inhibits proton excretion in fish gills (Fig. 6), as it does in frog skin and turtle bladder (Ehrenfeld and Garcia-Romeu, 1977; Steinmetz, 1986). While inhibiting proton excretion, acetazolamide also elevates carbon dioxide excretion across fish gills (Table 1). The reduction in intracellular CO_2 hydration will probably enhance the passive diffusion of CO_2. The amount of proton excretion in this study is of the same magnitude as that reported by Avella and Bornancin (1989).

Vanadate has a nonspecific inhibitory effect on ATPases and could be acting on Na^+/K^+-ATPase on the basolateral border of the fish gill. In our studies, more

than 50% of the net proton excretion across the gill epithelium was inhibited by 0.1 mmol l^{-1} vanadate applied to the mucosal membrane. De Sousa and Grosso (1979) showed that applying 1 mmol l^{-1} vanadate to the outer surface did not affect the Na^+/K^+-ATPase in the basolateral membrane of frog skin. Arruda *et al.* (1981) showed that vanadate had no effect on the backleak of proton or bicarbonate secretion but had a direct effect on H^+-translocating ATPase in turtle bladder. Thus, we conclude that the reduction in proton excretion observed in our studies was induced by the inhibitory effect of vanadate on the H^+-translocating ATPase in the apical membrane. The reason that the proton excretion was not completely abolished was, presumably, because of the difficulty of vanadate reaching the action site from the mucosal side (Arruda *et al.* 1981).

0.1 mmol l^{-1} amiloride had no effect on the putative fish gill proton pump in open-circuit conditions. Inhibition of sodium influx should have increased membrane potential and reduced proton excretion. This did not happen; therefore, if the proton pump does exist, there must be some other counter-ion that can replace sodium. Perry and Randall (1981) found that amiloride inhibited chloride influx in the fish gill. Inhibition of both chloride and sodium influx, when fish are exposed to amiloride, would tend to ameliorate any rise in potential across the apical membrane and, therefore, permit continued functioning of the proton pump.

The relationship between net proton excretion and ΔpH across the epithelial membrane shown in Fig. 7 is very typical of proton transport mediated by an electrogenic proton pump. At constant serosal pH, net proton secretion increased linearly with luminal pH over the physiological range of urine pH (4.4–7.4). Proton secretion was maximal at higher pH (Steinmetz, 1986). A linear relationship between proton excretion and mucosal pH over a limited range was also reported in frog skin by Ehrenfeld *et al.* (1985). This indicates that the electrochemical gradient for protons across the membrane is a fundamental regulator of the active proton transport, as discussed by Steinmetz (1986).

We found no inhibition of ammonia excretion at low pH, whereas Wright and Wood (1985) observed a marked depression of ammonia excretion in trout exposed to acid conditions. Low pH inhibits the proton pump, which will reduce epithelial pH, lower NH_3 levels in the epithelium and reduce ammonia excretion. Acid conditions in the water, however, will also lower NH_3 levels in the water and enhance ammonia excretion. The actual rate of ammonia excretion will vary initially, depending on the balance between the two effects, changing NH_3 levels and total ammonia stores in various compartments. Ultimately, excretion will match production and there is no reason to suppose that exposure to acid conditions has any effect on ammonium production.

In conclusion, our results provide evidence that the acidification of expired water in rainbow trout in neutral water is mainly caused by a net proton excretion mediated by an active proton pump on the apical membrane of gill lamellae. This proton pump is sensitive to vanadate and acetazolamide and is modulated by ambient CO_2 and pH. It resembles the electrogenic proton pump in frog skin and turtle bladder in many features.

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References

AL-AWQATI, Q. (1978). H^+ transport in urinary epithelia. *Am. J. Physiol.* **235**, F77–F88.

ARRUDA, J. A. L., SABATINI, S. AND WESTENFELDER, C. (1981). Vanadate inhibits urinary acidification by the turtle bladder. *Kidney International* **20**, 772–779.

AVELLA, M. AND BORNANCIN, M. (1989). A new analysis of ammonia and sodium transport through the gills of the freshwater rainbow trout (*Salmo gairdneri*). *J. exp. Biol.* **142**, 155–175.

AVELLA, M., MASONI, A., BORNANCIN, M. AND MAYER-GOSTAN, N. (1987). Gill morphology and sodium influx in the rainbow trout (*Salmo gairdneri*) acclimated to artificial freshwater environments. *J. exp. Zool.* **241**, 159–169.

BENOS, D. J. (1982). Amiloride: a molecular probe of sodium transport in tissues and cells. *Am. J. Physiol.* **242**, C131–C145.

BOUTILIER, R. G., IWAMA, G. K., HEMING, T. A. AND RANDALL, D. J. (1985). The apparent pK of carbonic acid in rainbow trout blood plasma between 5 and 15°C. *Respir. Physiol.* **61**, 237–254.

CAMERON, J. N. AND HEISLER, N. (1983). Studies of ammonia in the rainbow trout: physicochemical parameters, acid–base behaviour, and respiratory clearance. *J. exp. Biol.* **105**, 107–125.

CAMERON, J. N. AND KORMANIK, G. A. (1982). The acid–base responses of gills and kidneys to infused acid and base loads in the channel catfish, *Ictalurus punctatus*. *J. exp. Biol.* **99**, 143–160.

DE SOUSA, R. C. AND GROSSO, A. (1979). Vanadate blocks cyclic AMP-induced stimulation of sodium and water transport in amphibian epithelia. *Nature* **279**, 803–804.

EHRENFELD, J. AND GARCIA-ROMEU, F. (1977). Active hydrogen excretion and sodium absorption through isolated frog skin. *Am. J. Physiol.* **233**, F46–F54.

EHRENFELD, J., GARCIA-ROMEU, F. AND HARVEY, B. J. (1985). Electrogenic active proton pump in *Rana esculenta* skin and its role in sodium ion transport. *J. Physiol., Lond.* **359**, 331–355.

EVANS, D. H., MORE, K. J. AND ROBBINS, S. L. (1989). Modes of ammonia transport across the gill epithelium of the marine teleost fish *Opsanus beta*. *J. exp. Biol.* **144**, 339–356.

HILLABY, B. A. AND RANDALL, D. J. (1979). Acute ammonia toxicity and ammonia excretion in rainbow trout (*Salmo gairdneri*). *J. Fish. Res. Bd Can.* **36**, 621–629.

KLEYMAN, T. R. AND CRAGOE, E. J., JR (1988). Amiloride and its analogs as tools in the study of ion transport. *J. Membr. Biol.* **105**, 1–21.

KNAUF, H., SIMON, B. AND WAIS, U. (1976). Non-specific inhibition of membrane-ATPase by amiloride. *Naunyn-Schmiedeberg's Arch. Pharmac.* **292**, 189–192.

LENFANT, C. AND AUCUTT, C. (1966). Measurement of blood gases by gas chromatography. *Respir. Physiol.* **1**, 398–407.

LIN, H. AND RANDALL, D. J. (1990). The effect of varying water pH on the acidification of expired water in rainbow trout. *J. exp. Biol.* **149**, 149–160.

MCWILLIAMS, P. G. AND POTTS, W. T. W. (1978). The effects of pH and calcium concentrations on gill potentials in the brown trout, *Salmo trutta*. *J. comp. Physiol.* **126**, 277–286.

PAYAN, P. (1978). A studies of the Na^+/NH_4^+ exchange across the gill of the perfused head of the trout (*Salmo gairdneri*). *J. comp. Physiol.* **124**, 181–188.

PERRY, S. F., DAVIE, P. S., DAXBOECK, C. AND RANDALL, D. J. (1982). A comparison of CO_2 excretion in a spontaneously ventilating blood-perfused trout preparation and saline-perfused gill preparation: contribution of the branchial epithelium and red blood cell. *J. exp. Biol.* **101**, 47–60.

PERRY, S. F., HASWELL, M. S., RANDALL, D. J. AND FARRELL, A. P. (1981). Branchial ionic uptake and acid–base regulation in the rainbow trout, *Salmo gairdneri*. *J. exp. Biol.* **92**, 289–303.

PERRY, S. F. AND RANDALL, D. J. (1981). Effects of amiloride and SITS on branchial ion fluxes in rainbow trout, (*Salmo gairdneri*). *J. exp. Zool.* **215**, 225–228.

SARDET, C. (1980). Freeze fracture of the gill epithelium of euryhaline teleost fish. *Am. J. Physiol.* **238**, R207–212.

STEINMETZ, P. R. (1985). Epithelial hydrogen ion transport. In *The Kidney: Physiology and Pathophysiology* (ed. D. W. Seldin and G. Giebiach), pp. 1441–1458. New York: Raven.

STEINMETZ, P. R. (1986). Cellular organization of urinary acidification. *Am. J. Physiol.* **251**, F173–F187.

VERDOUW, H., VAN ECHTED, C. J. A. AND DEKKERS, E. M. J. (1978). Ammonia determination based on indophenol formation with sodium salicylate. *Water Res.* **12**, 399–402.

WRIGHT, P. A., HEMING, T. A. AND RANDALL, D. J. (1986). Downstream pH changes in water flowing over the gills of rainbow trout. *J. exp. Biol.* **126**, 499–512.

WRIGHT, P. A. AND WOOD, C. W. (1985). An analysis of branchial ammonia excretion in the freshwater rainbow trout: effects of environmental pH change and sodium uptake blockage. *J. exp. Biol.* **114**, 329–353.

YE, X., RANDALL, D. J. AND HE, X. (1991). The effect of acid water on oxygen consumption, circulating catecholamines and blood ionic and acid–base status in rainbow trout (*Salmo gairdneri*, Richardson). *Fish Physiol. Biochem.* **9**, 23–30.