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The use of amiloride to uncouple branchial sodium and proton fluxes in the brown trout, *Salmo trutta*

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Abstract Resting proton, ammonium and sodium fluxes in *Salmo trutta* were 492.6 ± 19.5 (n = 29); 122.9 ± 34.2 (n = 28) and 277.1 ± 18.5 (n = 50) $\mu mol \cdot kg^{-1} \cdot h^{-1}$, respectively. The resting transepithelial potential was found to be composed of three successive potentials, the outermost averaging −7.36 ± 0.19mV, the second, −14.3 ± 1.4 mV and the third −37 ± 1.7 mV. Amiloride inhibits the proton, ammonium and sodium fluxes in a dose-dependent manner at concentrations of 0.5 $mmol \cdot l^{-1}$ and 0.1 $mmol \cdot l^{-1}$, but at 0.01 $mmol \cdot l^{-1}$, proton and ammonium fluxes remained at control levels whilst the sodium was reduced to 70.59 ± 7.29 $\mu mol \cdot kg^{-1} \cdot h^{-1}$. The trans-epithelial potential was effected in a bi-phasic manner by 0.5 $mmol \cdot l^{-1}$ amiloride. An initial hyperpolarisation of ca. 6 mV was followed by a sustained depolarisation of ca. 14 mV (towards zero) which persisted until the amiloride was washed off the gill. The initial hyperpolarisation was thought to reflect a rapid inhibition of a positive inward sodium current and the subsequent depolarisation was due to the inhibition of a positive outward current (proton) which would abolish the transepithelial potential. However, at 0.01 $mmol \cdot l^{-1}$ only the hyperpolarisation was seen, due to the inhibition of only the inward sodium current. Acetazolamide (0.1 $mmol \cdot l^{-1}$) was found to have no significant effect on the proton, ammonium and sodium fluxes. These results indicate that the proton and sodium fluxes across the gill of the freshwater trout are not tightly linked. While this suggests that the trout gill resembles the model of Ehrenburg et al. (1985) of sodium uptake in frog skin, the apical potentials measured in the pavement epithelial cell(s) are too low to account for sodium uptake unless the activity of the sodium in the cells is very low.

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Abbreviations *MRC* mitochondrial-rich cell(s) *fw* freshwater · *TEP* transepithelial potential · *I* net H^+ proton net flux · *PEC* pavement epithelial cell(s)

Introduction

The mechanisms by which protons (H^+), sodium (Na^+) and ammonium (NH_4^+) ions move across the gill epithelia in FW teleosts and the site of these exchanges are controversial. Krogh (1938) first proposed that Na^+ was exchanged for NH_4^+ but later evidence showed that the situation was more complicated. Molecular ammonia (NH_3) is much more permeant than the larger hydrated NH_4^+. In FW fish most of the ammonia is lost in the molecular form but NH_3 combines with H^+ in the external medium to produce NH_4^+. Any proton flux will therefore appear partly as NH_4^+. In fish, Na^+ exchange has been reported to be coupled both to H^+ excretion and also to NH_4^+ excretion (Kerstetter and Keeler 1976; Payan 1978 a, b) or to both (Evans 1985; Wright and Wood 1985) However, opinion at this time is that in fish Na^+ influx is more closely linked to H^+ excretion than to NH_4^+ excretion; whether H^+ and Na^+ fluxes are tightly linked or follow the model of Ehrenfeld et al. (1985) or not is uncertain. Ehrenfeld et al. (1985) have shown that in frog skin an active (H^+) pump creates an electrical potential across the apical membrane which is negative on the cytoplasmic side of the membrane. This gradient passively drives the concomitant influx of Na^+ through channels which are amiloride sensitive (Avella and Bornancin 1989; Wright and Wood 1985; Evans and Cameron 1986; Wood 1991). The favoured model for acid/base/anion/cation exchange is Cl^-/HCO_3^- and Na^+/H^+. However, the model is based upon a very limited number of species, and it is clear that there is considerable inter-specific variation.

The exact site of Na^+ exchange has also been the subject of debate. The MRCs have been favoured as the site of Na^+ and Cl^- uptake on the basis of morphology, mitochondrial content and correlation between cell

number and ion flux (Pisam and Rambourg 1991; Laurent et al. 1985; Avella et al. 1987; Perry and Laurent (1989). More recently it has been shown by Goss et al. (1992a) in the rainbow trout, *Onchorhynchus mykiss*, that MRCs are more involved with Cl^- than Na^+ uptake. During experimental hypercapnia, a decrease in Cl^- uptake was accompanied by a decrease in the area of MRCs exposed to the medium suggesting that these are the site of Cl^- uptake. Also, Morgan et al. (1994) measured intracellular ion concentrations by X-ray microprobe analysis and demonstrated that the PECs are more probably the site of Na^+ uptake and that the MRC would seem to be the site of Cl^- uptake. It is shown below that it is possible to uncouple the Na^+ and H^+ fluxes through the use of pharmacological agents and that these agents have an effect on the electrophysiology of the PECs.

Materials and methods

Brown trout, *Salmo trutta*, weighing 150–250 g were obtained from Dunsop Bridge Trout Farm, Dunsop Bridge, Lancs., UK. The fish were stored in a circular tank in aerated, running fresh water supplied from Thirlmere reservoir in the Lake District. A water analysis is shown in Table 1.

Sodium flux measurements

Live fish were placed in a Perspex flux box of 5 l volume at 10 °C. The fish was left in flowing water overnight to acclimatise and the experiment was started by closing the flow circuit and adding 2 μCi (74 kBq) $^{22}NaCl$ to the medium. After 5 min to allow mixing, duplicate 5-ml water samples were taken and counted for 1 h in a Canberra Packard 1600 Liquid Scintillation Counter. From the mean count of the two samples C_{10} was calculated (see below). Subsequent duplicate samples were taken at 1 intervals and counted (C_{IT}) (count at time T). Flux constants were calculated using the formula:

$$K_1 + K_2 = \frac{1}{T} \ln \frac{C_{1_{Tn}} - C_{1_\infty}}{C_{1_{Tn+1}} - C_{1_\infty}} \quad (1)$$

where

$$C_{1_\infty} = \frac{Na_1}{Na_1 + Na_2} \times C_{10} \quad (2)$$

and

$$K_1 Na_1 = K_2 Na_2 \quad (3)$$

Na_1 = sodium concentration in the fish (37.2 mmol·kg^{-1})
Na_2 = sodium concentration in the media (0.32 mmol·l^{-1})
K_1 = rate constant of sodium in the water
K_2 = rate constant of sodium in the fish
C_{10} = counts in the media at time T = 0
C_{1Tn} = counts in the media at time $T = n$ h
From this the sodium influx in μmol·kg^{-1}·h^{-1} was calculated.

A typical experiment lasted for 7 h. In control experiments, the rate of influx remained essentially constant.

For experiments involving the use of the drugs, a control period of 4 h was allowed to establish the resting flux rate. After 4 h the drug was added to the medium and the measurement of fluxes continued to assess the effect of the drug upon the fish.

Table 1 Composition of aquarium water

Ion	Concentration
Sodium	0.32 mmol·l^{-1}
Chloride	0.29 mmol·l^{-1}
Calcium	0.20 mmol·l^{-1}
Potassium	0.02 mmol·l^{-1}
Magnesium	0.07 mmol·l^{-1}
Bicarbonate	< 0.001 mmol·l^{-1}
Sulphate	< 0.001 mmol·l^{-1}

Measurement of H^+ and ammonium fluxes

The H^+ flux was measured by a method based on that of Wood (1991). A fish was placed in a closed system and 200-ml water samples were taken every hour. The samples were weighed to 0.001 g for accuracy and buffered with 200 mmol·l^{-1} imidazole, diluted to a final concentration of 2 mmol·l^{-1} and aerated overnight to remove respiratory CO_2. The sample was then divided into four 50-ml aliquots (weighed for accuracy), three of which were titrated using a Mettler DL21 autotitrator to pH 8 using dilute NaOH. The titre obtained was termed the titratable acidity. The titrations were carried out in triplicate and the mean of the three results calculated. The fourth aliquot of buffered water sample was used to determine the ammonium concentration of the water. This was obtained using an ABB Kent-Taylor ammonium-sensitive electrode, calibrated using NH_4Cl solution and coupled to a standard extended-scale pH meter.

The H^+ flux was calculated using the following formula:

$$J_{net}\ H^+(\mu mol \cdot h^{-1} \cdot kg^{-1}) = \Delta \text{Titratable acidity } (\mu mol \cdot h^{-1} \cdot kg^{-1}) + \text{Ammonium flux } (\mu mol \cdot h^{-1} \cdot kg^{-1})$$

Microelectrode experiments

The apical and transepithelial potentials were measured by glass microelectrodes. Fish were killed by a blow to the head and the gills were immediately removed and placed in chilled, aerated Cortland saline. Single filaments were dissected from the gill arches and fixed onto glass microscope slides using cover slips, cut to size using a diamond pen. The cover slips were anchored over the filaments using molten wax applied to the slide away from the gill filament so as not to damage the filament (Fig. 1).

A perfusion chamber was then fixed to the slide, enclosing the preparation using Baysilone high-viscosity paste and fw was perfused over the filament from base to apex. Glass microelectrodes

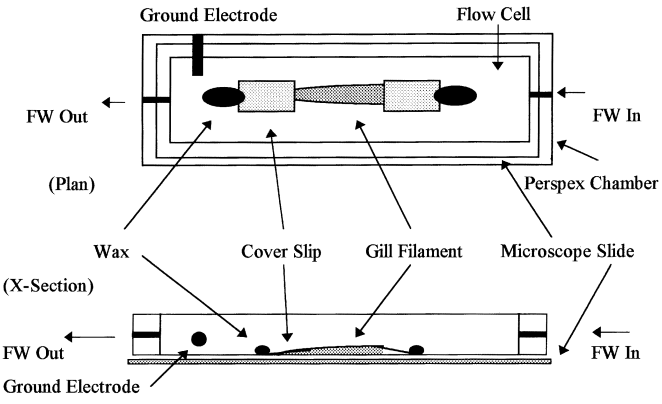

Fig. 1 Diagram of perfusion chamber used in microelectrode experiments

were pulled to an impedance of 50–80 mΩ (tip diam. ~ 0.5 μm) using an LKB vertical microelectrode puller. The preparation was viewed using a WPI inverted microscope in the magnification range 60–400×. The electrodes were held in WPI electrode holders and manipulated using WPI micro-manipulators attached to fine vernier controls. Impalements were made towards the tips of the filaments. The MRCs are found mainly towards the base of the lamellae and on the filaments, while the outer portions of the lamellae are covered almost entirely with pavement cells. While it was not possible to distinguish between the two kinds of cells under the light microscope we believe that the cells impaled were pavement cells. The electrode output was routed to the high-impedance probe of an HSE microelectrode/voltage clamp amplifier and from there to the main amplifier and onto a Grass Model 79D Polygraph recorder and Tektronics storage oscilloscope. Measurements (± 0.1 mV) of the recorded potentials were made using a digital multimeter.

Ussing chamber experiments

In order to confirm results obtained using the microelectrodes, a second method of measuring the TEP was employed. A whole gill arch was removed from a fish and the gill filaments removed from half of the arch. One cut end, with filaments still attached, was sealed with Vaseline which was injected into the open ends of the blood vessels and the other end was pulled through a rubber membrane in which was a small hole. This was then placed in an Ussing chamber and the potential difference between each side was measured by the use of IVM Ag/AgCl flat-tip probes. Drugs were applied to the end with intact filaments and any changes recorded on a Grass Model 79D polygraph chart recorder. Measurements of TEP (± 0.1 mV) were made using a digital multi-meter.

Drugs were made up to the required concentration in fw. A typical experiment consisted of a control period of approximately 10 min during which fw was perfused over the gill, followed by an experimental period of 10 min during which the drug was applied and a final 10-min wash in clean fw to allow recovery.

Amiloride and acetazolamide were obtained from Sigma and ^{22}Na was obtained from DuPont.

In vivo measurement of transepithelial potential

The TEP was measured by indwelling peritoneal catheter according to the method of Potts and Eddy (1973).

Results

Na^+, H^+ and NH_4^+ fluxes

The mean Na^+ flux under control conditions was 277.1 ± 18.5 $\mu mol \cdot kg^{-1} \cdot h^{-1}$ (n = 50), the mean H^+ flux was 492.6 ± 19.5 $\mu mol \cdot kg^{-1} \cdot h^{-1}$ (n = 29) and the mean NH_4^+ flux was 122.9 ± 34.2 (n = 28) (Fig. 2).

Trans-epithelial potential measurements

Three successive potentials could be measured upon application of microelectrodes to the gill filament. The first was measured as the tip of the electrode reached the tissue. This averaged −7.36 ± 0.19 mV. On examination at 400× it could be seen that this potential was achieved as the tip of the electrode pierced the cell membrane; however, the potential was short lived and declined after

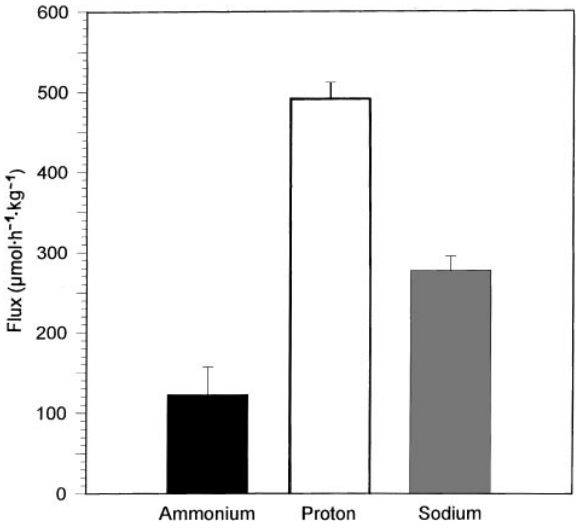

Fig. 2 Mean rates of ion efflux from *S. trutta*. All points are mean ± SEM

the electrode entered the cell. Thus, it was impossible to make steady recordings of the potential. The second potential developed when the electrode penetrated further into the tissue of the gill. This potential averaged −14.3 ± 1.4 mV (n = 13 filaments, 34 impalements). The third potential was encountered upon further advance into the tissue and averaged −37.0 mV ± 1.7 (n = 13 filaments, 33 impalements). These results are summarised in Fig. 3 and polygraph traces showing the entry of the electrodes into the filament is shown in Fig. 4.

To confirm that the third potential was the TEP, experiments were undertaken using an Ussing chamber. Potentials measured in the Ussing chamber ranged from −18 to −30 mV but the results were more erratic than the overall impalement potentials. However, this method served to confirm that the third potential was the TEP of the gill. In vivo experiments averaged close to 30 mV, again confirming the third potential was the TEP. As the

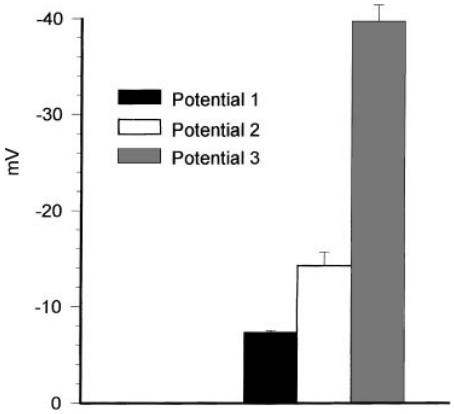

Fig. 3 Mean values of potentials measures using microelectrodes in isolated gill filaments of *S. trutta*. All points are mean ± SEM

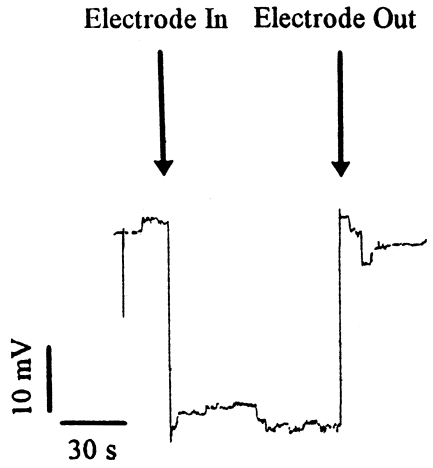

Fig. 4 Polygraph traces showing entry of microelectrode into cell and subsequent stable recording of membrane potential

third potential was the most stable, experiments were confined to this potential.

Effect of amiloride

Amiloride was added to the water at three concentrations, 0.5, 0.1 and 0.01 mmol·l^{-1}. At 0.5 and 0.1 mmol·l^{-1}, a dose-dependent reduction in all three fluxes was seen; however, at 0.01 mmol·l^{-1} it was found that the fluxes were uncoupled, only the Na^+ flux being reduced whilst the H^+ and NH_4^+ fluxes were left unchanged (Table 2, Fig. 5).

When amiloride (0.5 mmol·l^{-1}) was applied to isolated filaments which had been impaled by a microelectrode, the effects on the potential were as follows: at 0.01 mmol·l^{-1}, a hyperpolarisation of 5.07 ± 1.26 mV which persisted until the amiloride was removed. At 0.5 mmol·l^{-1} a hyperpolarisation of 4.8 ± 2.8 mV was seen but this was followed by a depolarisation of 14.1mV ± 2.5 mV which again persisted until washout. Occasionally, after washout, the tissue continued to depolarise. Figure 6 shows the effects of amiloride on the potential.

Effect of acetazolamide.

Acetazolamide at a concentration of 0.1 mmol·l^{-1} was found to have no significant effect on any of the three fluxes measured. (Table 3). Acetazolamide has previously been shown to be effective in rainbow trout *Onchorhyneus mykiss* by Avella and Bornancin (1989) and Lin and Randall (1991).

Table 2 Effect of amiloride on ion fluxes in *S. trutta*

Flux of	Control	0.01 mmol·l^{-1} Amiloride	0.01 mmol·l^{-1} Amiloride	0.5 mmol·l^{-1} Amiloride
Proton	100	96.32	43.6	1.34
Ammonium	100	92.41	54.2	35.8
Sodium	100	31.7	31.38	11.73

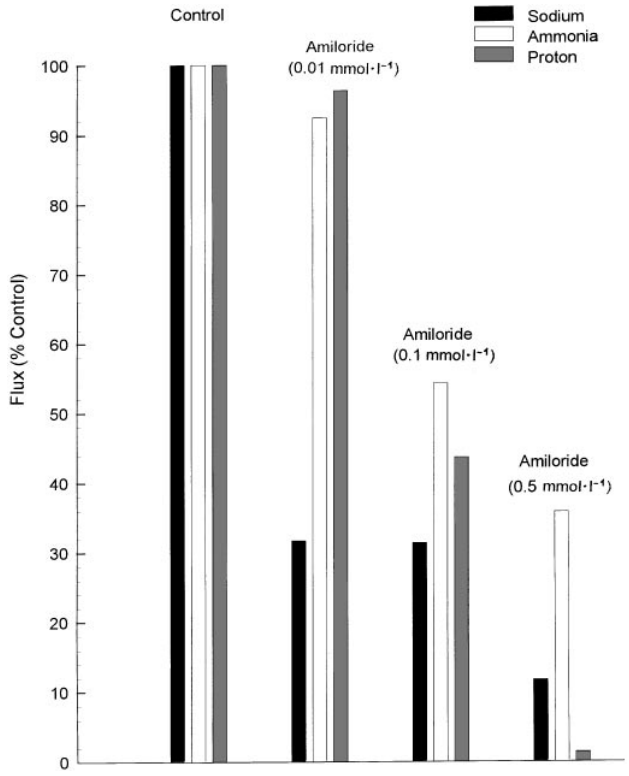

Fig. 5 Graph showing effect of three concentrations of amiloride on ion fluxes in *S. trutta*. All points are % control values

Discussion

Valuable information can be obtained by the application of microelectrodes to isolated filaments. Discussion will be confined to the total TEP which was stable and reproducible in all the filaments examined. The potentials recorded depended on the depth of impalement. The initial potential recorded may correspond to the potential inside the PECs located on the surface of the gill, the apical membrane potential. However, this potential would seem to be too low for the Ehrenfeld model of sodium uptake. The second potential may be due to some of the numerous cells of unknown function, lying between the PEC and blood space, recorded as the tip of

Table 3 Effect of acetazolamide on ion fluxes in *S. trutta*

Flux of	Control (μmol·kg^{-1}·h^{-1})	0.1 mmol·l^{-1} Acetazolamide (μmol·kg^{-1}·h^{-1})
Sodium	249.17 ± 5.48	252.53 ± 42.71
Proton	354.7 ± 38.69	304.79 ± 36.01
Ammonium	212.84 ± 23.91	206.54 ± 7.81

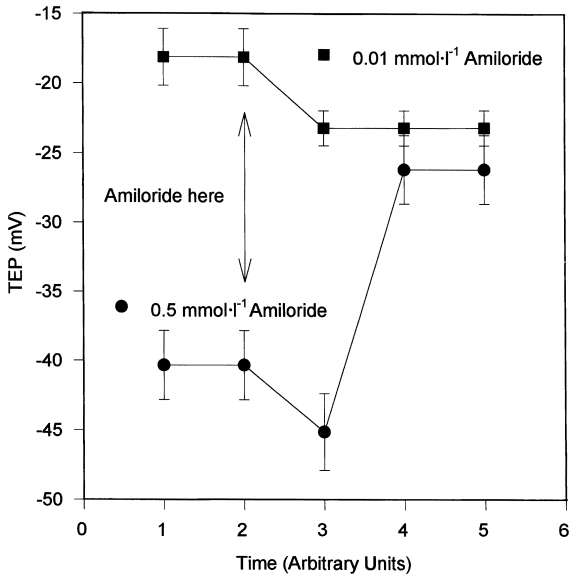

Fig. 6 Graph showing effect of two concentrations of amiloride on the TEP of isolated gill filaments of *S. trutta*. All points are mean ± SEM

the electrode is driven through the filament. The final, largest potential would seem to be the total TEP recorded when the electrode enters the blood space of the gill. This potential was similar in magnitude to that recorded in the Ussing chamber using whole filaments and to the *in vivo* potential of the whole fish, measured with an indwelling peritoneal catheter. Of the three potentials which could be measured, the third, deepest potential gave the most stable and reproducible results, and it was this potential which was examined in greatest detail. Thus, the potentials discussed below are believed to be the TEP of the gill.

The H^+ fluxes reported here may appear large and possibly reflect those of a fish which has been stressed. Every effort was made to reduce stress and to allow the fish to acclimate to the experimental conditions, but it was necessary to confine the trout, an active fish, in a small volume in order to obtain significant results and this could have stressed the fish. However, the measurements taken were consistent throughout the project.

At concentrations of 0.1 and 0.5 $mmol \cdot l^{-1}$ amiloride the Na^+, H^+ and ammonium fluxes were all reduced by an amount dependent upon the concentration of the drug. However, at the lowest concentration of 0.01 $mmol \cdot l^{-1}$ the fluxes were uncoupled; Na^+ being reduced by approx. 70% with H^+ and ammonium remaining close to control values. These results are not consistent with the presence of a $Na^+ \backslash H^+$ exchange system but are consistent with a potential driven Na^+ influx through amiloride-sensitive Na^+ channels. However, the initial potential measured seems too low to maintain a net Na^+ influx unless the intracellular Na^+ activity is very low.

The potential traces recorded using micro-electrodes showed that the higher concentrations of amiloride induced an initial hyperpolarisation followed by a long sustained depolarisation. With the lowest concentration of amiloride, only the initial hyperpolarisation was seen. If the H^+ pump contributes to the TEP of the gill, then any influx of a positive ion would serve to reduce the polarity of the membrane and reduce the TEP by an amount dependent upon the magnitude of the positive influx. If the Na^+ influx were blocked then the depolarisation would be reduced and the polarity of the membrane would increase. Thus, the initial hyperpolarisation when amiloride is added is probably due to an inhibition of the Na^+ flux. Amiloride is well known to block Na^+ channels (Bentley 1968; Benos 1982), however, at high concentrations of amiloride the hyperpolarisation is followed by a large sustained depolarisation (Fig. 6). In Fig. 6 it should be noted that the initial potentials shown by the two traces differ by approximately 20 mV. The potentials varied from fish to fish and also depended on the time of year. The lower potential in Fig. 6 was measured in winter, the higher in spring. The difference in resting potentials may reflect differing metabolic rates induced by differing water temperatures. Although the experimental conditions were kept constant at 10 °C the temperature of the incoming water effected the temperature of the tanks in which the fish were stored.

Amiloride has been found to reduce the proton flux in other species (Lin and Randall 1991). Clarke (pers. comm.) has also found that amiloride will reduce the proton flux in *Platichthys flesus* in a concentration-dependent manner. In *Salmo trutta* at higher concentrations the amiloride directly inhibits the H^+ pump, as well as blocking the Na^+ channels and reducing the TEP. However, at lower concentrations, the later depolarisation does not take place and whole-fish studies have shown that the H^+ flux is not effected. It may be concluded that the depolarisations at concentrations of 0.1 and .0.5 $mmol \cdot l^{-1}$ amiloride are due to reductions in the H^+ efflux, which are not apparent at a concentration of 0.01 $mmol \cdot l^{-1}$, although the Na^+ flux is still reduced at this concentration. Consequently, the H^+ and Na^+ fluxes must have been uncoupled, and it may be concluded that the H^+ flux is independent of the Na^+ flux, although the latter may still be dependent of the H^+ flux.

The ammonium efflux is reduced when the H^+ flux is reduced by amiloride but not to the same extent as the Na^+ influx. At first sight this might indicate a linkage between the Na^+ influx and ammonium efflux but these results are also consistent with the theory that most of the ammonia is lost by diffusion in the molecular form. In that case the external H^+ would trap the ammonia, converting it into NH_4^+, maintaining a favorable gradient for the outward diffusion of ammonia. When the H^+ pump is reduced by high levels of amiloride, the ammonia gradient would be reduced.

Acetazolamide at a concentration of 0.1 $mmol \cdot l^{-1}$ has no significant effect on the fluxes in *S. trutta*. Acetazolamide is a carbonic anhydrase inhibitor which should reduce the availability of H^+ (Lin and Randall 1991; Avella and Bornancin 1989). If this were the case,

as the efflux of H^+ declines the Na^+ influx should also decline if the Na^+ influx were dependent on H^+ efflux. However, in this case sufficient H^+ appear to be available from another source to allow this system to continue. It is possible that a higher concentration of acetazolamide is required to induce this effect. Alternatively, another inhibitor of the H^+-ATpase, e.g. orthovanadate, might be used to demonstrate the dependence of Na^+ influx on H^+ efflux.

The teleosts are a large, very diverse group and they may not all have identical Na^+ uptake mechanisms, but it would seem that the Ehrenfeld model is applicable to the trout. However, it must be borne in mind that the system is very dynamic with many interlocking processes. Thus, it is difficult to use pharmacological agents to affect only one flux without effecting more than the areas of interest under study. However, the Ehrenfeld model seems to apply and this very elegant system of ion exchange, initially found in the frog skin, is more widely distributed, though whether it has evolved more than once or it is a common inheritance remains to be determined.

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