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A NEW ANALYSIS OF AMMONIA AND SODIUM TRANSPORT THROUGH THE GILLS OF THE FRESHWATER RAINBOW TROUT (*SALMO GAIRDNERI*)

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Summary

The mechanism of ammonia excretion and sodium absorption was re-examined in trout using the isolated-perfused head preparation. Preliminary experiments in which ammonia concentration was increased on the blood side (internal) showed that ammonia and sodium transport was uncoupled.

For ammonia excretion, our results showed that gill tissue endogenously produces ammonia. A correlation was demonstrated between ammonia excretion and the internal–external ammonia gradient. We conclude that diffusion in the form of NH_3 was responsible for ammonia efflux and we were therefore able to estimate its diffusion coefficient ($D_{NH_3} = 1{\cdot}55 \times 10^{-6}\,cm^2\,s^{-1}$) and permeability coefficient ($6 \times 10^{-3}\,cm\,s^{-1}$). This ammonia diffusion was shown to be modified according to the external proton availability.

For sodium absorption, significant changes were caused by indirect modifications of intracellular pH brought about by addition of acetazolamide inside or ammonia outside or by acidification of the internal or external medium. The relationship between sodium and proton transport was further confirmed by the action of the drug amiloride and the measurement of H^+ excretion.

A possible model representing sodium, proton and ammonia transport through the gill epithelium is proposed.

Introduction

Freshwater teleosts compensate for the constant diffusional loss of ions by active branchial absorption of sodium and chloride from the hypo-osmotic environment. It has been shown that the mechanisms of Na^+ and Cl^- uptake are independent, and Krogh (1938) proposed that Na^+ is exchanged for NH_4^+, which at the same time facilitates excretion of nitrogenous waste. According to Smith (1929), Cameron & Wood (1978) and Evans (1982), loss of ammonia occurs mainly through the gills. Further investigations have provided more precise information for determination of the physicochemical parameters such as the pK and solubility

Key words: fresh water, trout, isolated head, ammonia, sodium, protons.

coefficient for ammonia in water and plasma at different temperatures (Kormanik & Cameron, 1981; Cameron & Heisler, 1983).

However, some controversy exists over the endogenous ion exchange in Na^+ uptake (Maetz *et al.* 1976; Evans, 1985). Thus, in rainbow trout, *Salmo gairdneri*, Na^+ uptake has been coupled with NH_4^+ excretion (Kerstetter & Keeler, 1976; Payan, 1978*a*,*b*), H^+ excretion (Kerstetter *et al.* 1970; Cameron & Heisler, 1983; Heisler, 1984) or with both H^+ and NH_4^+ excretion (Evans, 1985; Wright & Wood, 1985). Moreover, recent studies have led to a new appraisal of the relative NH_3 and NH_4^+ gradients and Na^+ transport across fish gills (Wright & Wood, 1985; Evans & Cameron, 1986).

In the light of this recent work, the present investigation employed the isolated-perfused head of rainbow trout, a technique allowing constant control of both the internal and external components, to study Na^+ absorption coupled with H^+ or NH_4^+ excretion.

Materials and methods

Biological material and isolated-perfused head technique

Rainbow trout (*Salmo gairdneri*) of either sex and weighing about 200 g were obtained from a local source (St Jeannet, Alpes-Maritimes, France). The fish were housed in large circular glass fibre tanks supplied with aerated city tap water ($NaCl \approx 160\,\mu mol\,l^{-1}$; $Ca^{2+} \approx 1{\cdot}6\,mmol\,l^{-1}$; $K^+ \approx 80\,\mu mol\,l^{-1}$; $pH \approx 8{\cdot}0$) at constant temperature (15 °C); they were kept unfed for 1 week before the experiments.

The isolated-perfused head preparation was prepared as described by Payan & Matty (1975) and Girard & Payan (1977). This technique involved the setting up of a circulation in the isolated head allowing measurements of branchial water and ionic permeability. Before experimentation, a blood sample was taken from the caudal vein for chemical analysis.

The head of a previously heparinized trout was sectioned 1 cm behind the pectoral fins, and the gills were then irrigated by a current of fresh water and temporarily perfused under constant pressure. The perfusion liquid had physico-chemical parameters similar to those of the trout blood (Bornancin *et al.* 1985). The perfusion was performed at a constant flow rate ($150\,ml\,h^{-1}\,100\,g^{-1}$) chosen to avoid any excess pressure in the lamellae. The input pressure was constantly controlled by a recorder.

These conditions have been proved to prevent any oedema formation: this was regularly controlled by morphological examination of the gills at the end of the preparation. During experimentation, the head was perfused for a 10-min period before sample collection. Arterial, venous and external samples were collected at regular intervals. The experiments never exceeded 1 h.

Measurement and calculation of Na^+ transport

^{22}Na (3 μCi, CEA, Saclay) was added to the external medium (170 ml), and its

appearance in the efferent arterial and venous fluids determined. Secondary lamellar sodium influx [J_{in}^{Na}(SL), expressed as μmol Na^+ h^{-1} 100 g^{-1}] was calculated from:

$$J_{in}^{Na}(SL) = \frac{Qa \times F_{SL} \times [Na^+]_e}{W \times Qe} \times 60 \times 100,$$

where Qa and Qe are the arterial and external radioactivities (disints min^{-1} ml^{-1}), respectively, determined on a Packard gamma counter; F_{SL} represents total efferent flow rate (ml min^{-1}); $[Na^+]_e$ is the external sodium concentration (μmol ml^{-1}) determined by Eppendorf flame photometer; and W is the fish mass (g).

Primary lamellar sodium influx values were calculated but not stated because no significant changes appeared during our experiments, and most Na^+ absorption in fresh water (80 %) has been shown to occur through the secondary lamellae (Gardaire *et al.* 1985; Avella *et al.* 1987).

Measurement and calculation of ammonia excretion

The word 'ammonia' is used to refer to the total ammonia (T_{Amm}) in solution; particular chemical forms are referred to as NH_3 or NH_4^+.

According to the experimental design, one of the following techniques was chosen.

(1) In a closed external circuit, ammonia excretion ($J_{net}^{T_{Amm}}$ expressed in μmol h^{-1} 100 g^{-1}) was measured by the external appearance of ammonia as follows:

$$J_{net}^{T_{Amm}} = \frac{\Delta[T_{Amm}]_e \times V_e}{W \times \Delta t} \times 60 \times 100,$$

where $\Delta[T_{Amm}]_e$ represents the difference in external ammonia concentrations (μmol l^{-1}) during the time (Δt, expressed in min) between two sample collections; V_e is the volume of the external medium ($\approx$0·170 l); and W is the fish mass (g).

External ammonia concentration was determined by the manual method of Koroleff (1969).

(2) In an open external circuit, ammonia excretion through the secondary lamellae [$J_{out}^{T_{Amm}}$(SL) expressed in μmol h^{-1} 100 g^{-1}] was calculated by the rate of clearance from the internal medium as follows:

$$J_{out}^{T_{Amm}} = \frac{[C_p^{T_{Amm}} - C_{ae}^{T_{Amm}}] \times F_{SL}}{W} \times 60 \times 100,$$

where $C_p^{T_{Amm}}$ is the ammonia concentration in the perfusion fluid (μmol ml^{-1}) and $C_{ae}^{T_{Amm}}$ is the ammonia concentration in the arterial effluent (μmol ml^{-1}).

Internal ammonia concentration was determined by automatic analyser (Technicon) with a dialysis membrane according to Logsdon (1960). Concentrated NH_4Cl solution was the source for all experiments involving ammonia addition, and actual ammonia measurements were undertaken immediately after experimentation.

Measurement and calculation of H^+ excretion

Net hydrogen flux (J^H_{net} in $\mu equiv\,h^{-1}\,100\,g^{-1}$) was determined from the variations of H^+ concentration in the external medium as a function of time. Assuming that NH_3 was equilibrated with NH_4^+ at the expense of H^+ in the external medium, the sum of the titratable acidity measured plus ammonia excretion was termed the net apparent H^+ efflux (Kirschner *et al.* 1973; Cameron, 1986; Vermette & Perry, 1987).

The H^+ concentration of the external solution (buffered with $2\,mmol\,l^{-1}$ imidazole) was determined by titration with $2\,mmol\,l^{-1}$ NaOH and calculated from the quantity of base added to reach the starting pH. The samples were first aerated overnight, to eliminate respiratory CO_2 produced by the head, and measurements were then made on 2-ml samples with a radiometer autotitrator in conjunction with an autoburette unit and a titrigraph recorder.

Statistical analysis

Data shown in figures are means ± S.E. Student's unpaired *t*-tests were used to compare most of the sample means. However, when appropriate, Student's paired *t*-tests were used and are mentioned in the text.

Results

Relationship between sodium influx and ammonia excretion (Na^+/NH_4^+ hypothesis)

A low external Na^+ concentration of $200\,\mu mol\,l^{-1}$ [approximate K_m of sodium absorption (Avella *et al.* 1987) and see Fig. 12] was employed so that the rate of Na^+ influx could be readily enhanced by increasing the internal ammonia concentration, if a Na^+/NH_4^+ exchange existed. When the internal ammonia concentration was gradually increased from 0 to $1\,mmol\,l^{-1}$ at constant pH (Fig. 1), no significant change in sodium absorption was observed. However, ammonia excretion increased as a function of internal ammonia concentration, showing a complete uncoupling between sodium and ammonia transport.

Ammonia excretion

Endogenous ammonia production

Basal ammonia excretion in the external medium was measured in the absence of ammonia in the perfusion fluid in an external closed circuit and was equal to $5{\cdot}7 \pm 0{\cdot}7\,\mu mol\,h^{-1}\,100\,g^{-1}$ ($N = 4$). Ammonia was produced by the branchial epithelium as a result of cellular metabolism of irrigated tissues. Glutamine, which is rich in NH_2 residues, would stimulate endogenous ammonia production, if added to the perfusate. Branchial cell uptake of glutamine is followed by an enzymatic deamination producing the nitrogenous waste, ammonia (Goldstein & Forster, 1961). After glutamine addition, basal ammonia excretion to the external medium was stimulated by 80% within 6 min (Fig. 2). Because of the closed

external circuit, external ammonia accumulated and the flux progressively decreased (see below). Excretion of ammonia in the efferent arterial fluid (basal excretion) was also observed.

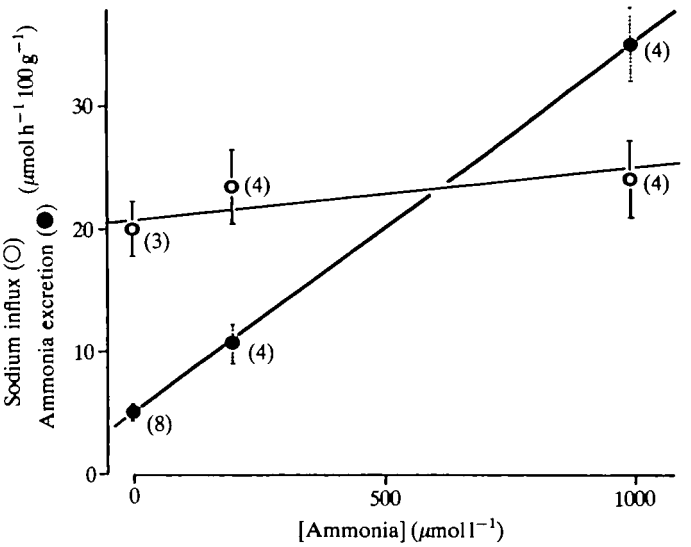

Fig. 1. Sodium influx (○) and ammonia excretion (●) as a function of internal ammonia concentration. The external medium was closed and consisted of fresh water ($[Na^+]_e < 160\,\mu mol\,l^{-1}$). Mean values ± S.E. The numbers of experiments are given in parentheses.

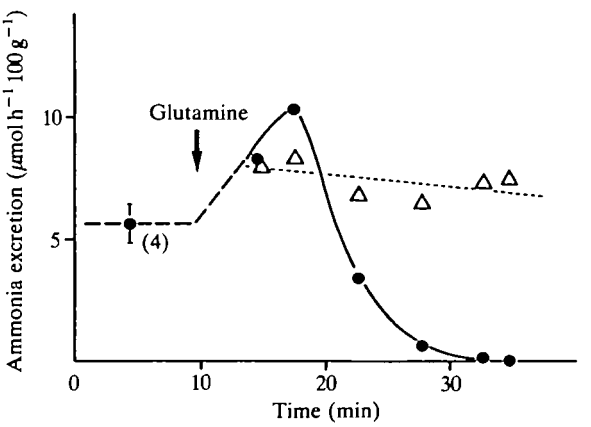

Fig. 2. Secondary lamellar ammonia excretion: effect of glutamine addition to the internal medium. After a control period (10 min), glutamine was added (arrow) to the perfusion fluid. The external medium was closed and contained 0·5 mmol l^{-1} NaCl; ammonia was absent from the internal medium. Ammonia excretion was measured, either by external appearance (●) or by internal appearance (= diffusion through the basal membrane: △). Control value is mean ± S.E. The number of experiments is given in parentheses. Lines fitted by eye.

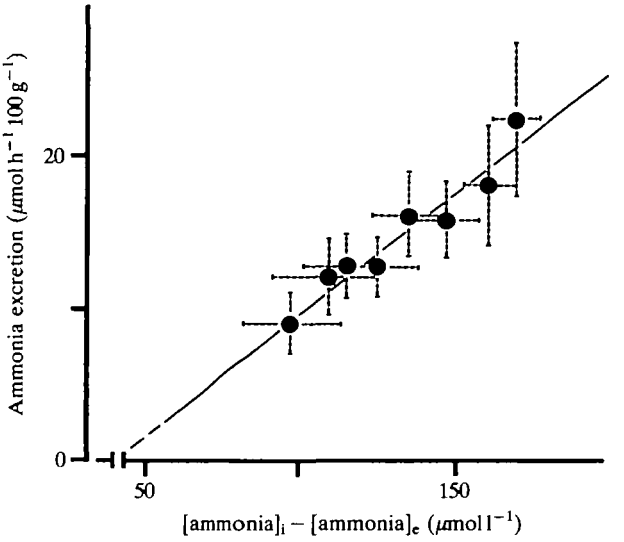

Fig. 3. Relationship between ammonia excretion and the internal – external ammonia gradient. The external medium was closed and consisted of fresh water ($[Na^+] < 160\,\mu mol\,l^{-1}$). The perfusion fluid contained $200\,\mu mol\,l^{-1}$ ammonia. $y = (0{\cdot}156 \pm 0{\cdot}018)x - (5{\cdot}9 \pm 2{\cdot}5)$, $N = 8$, $r = 0{\cdot}96$. Mean values ± S.E. ($N = 4$).

Mechanism and modulation of ammonia excretion

Experiments with a closed external circuit (see Wright & Wood, 1985) showed that the decrease in ammonia excretion was proportional to the internal–external ammonia gradient (Fig. 3).

When the external medium was open (to prevent any ammonia accumulation which could artificially reduce the efflux), ammonia branchial clearance was measured under conditions of increasing internal [T_{Amm}]. Ammonia excretion was directly proportional to the internal ammonia concentration within the range studied ($100–900\,\mu mol\,l^{-1}$, Fig. 4). The limits chosen were physiological values observed *in vivo* ($231{\cdot}2 \pm 33{\cdot}6\,\mu mol\,l^{-1}$; $N = 5$). Addition of sodium ($1\,mmol\,l^{-1}$) to the external medium stimulated ammonia excretion.

The buffer (Tris–H_2SO_4, pH 8·0; $50\,mmol\,l^{-1}$) was used to alter the environmental ability to donate protons. Tris buffer, with a pK of about 8·0, is a good proton donor to the NH_3/NH_4^+ system, which has a pK of around 9·3. After a control period of 20 min, buffer was added to the external medium, causing an immediate response which resulted in a 50 % increase in ammonia excretion (Fig. 5).

Sodium influx

Internal addition of acetazolamide

Acetazolamide, the carbonic anhydrase inhibitor which reduces intracellular proton production and causes an alkaline shift in the intracellular pH (Hersey &

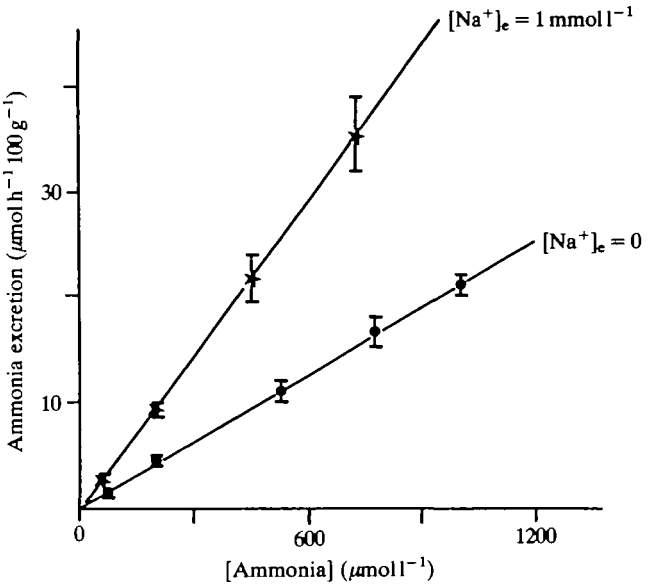

Fig. 4. Ammonia excretion as a function of internal ammonia concentration; effect of addition of sodium to the external medium. The external circuit was open: (●) $[Na^+]_e = 0$, $y = 0{\cdot}048x - 0{\cdot}26$, $r = 0{\cdot}999$; (✱) $[Na^+]_e = 1\ mmol\ l^{-1}$, $y = 0{\cdot}021x + 0{\cdot}053$, $r = 0{\cdot}999$. Mean values ± S.E. ($N = 5$).

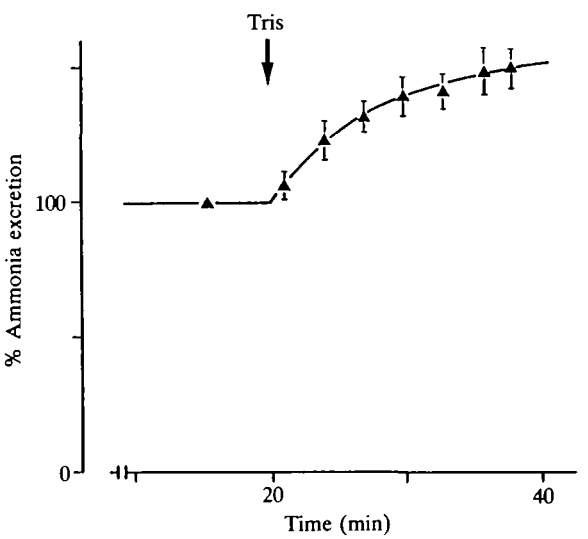

Fig. 5. Secondary lamellar ammonia excretion: effect of buffer (Tris) addition to the external medium. The external medium was open. After a control period (20 min) Tris buffer (50 mmol l^{-1}; H_2SO_4; pH 8·0) was added to the external medium ($[Na^+]_e \approx 0{\cdot}5$ mmol l^{-1}; pH 8·0). Mean values ± S.E. ($N = 4$).

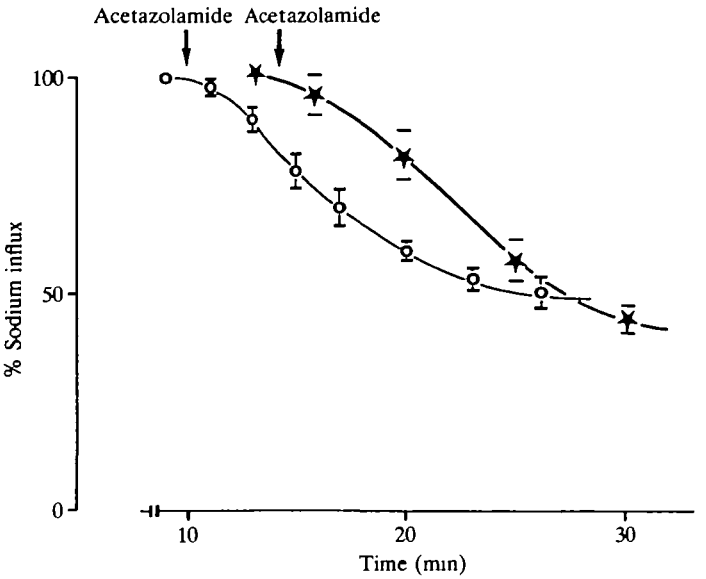

Fig. 6. Acetazolamide addition to the internal medium: effect on ammonia excretion in the presence (160 μmol l^{-1}) (○) or absence (●) of internal ammonia. After a control period (13–15 min) acetazolamide (10^{-4} mol l^{-1}) was added (arrows) to the perfusion fluid. The external medium was closed and contained 1 mmol l^{-1} NaCl. Mean values ± S.E. ($N = 4$).

High, 1971), was introduced into the perfusion fluid, causing an inhibition of sodium influx (50%) within 15 min in the presence of 160 μmol l^{-1} internal ammonia (Fig. 6). The same phenomenon was apparent when ammonia was absent from the internal medium. Because sodium entry decreased both with or without ammonia in the perfusion fluid, the action of acetazolamide cannot be interpreted simply as an effect on Na^+/NH_4^+ exchange.

External addition of ammonia

According to Boron & De Weer (1976), addition of ammonia to the external medium is followed by a rapid cellular diffusion of NH_3, which binds H^+, causing intracellular alkalinization. Ammonia removal, however, is immediately followed by intracellular acidification. This treatment was applied to the isolated-perfused head preparation (Fig. 7): 20 mmol l^{-1} ammonia was added to the external medium for a 20-min period (phase *b*) and then quickly removed (phase *c*).

During ammonia exposure, sodium entry was inhibited by 40% (phase *b*). In this condition, a rapid (within 30 s) alkalinization of the efferent arterial fluid was observed for each preparation exposed to external ammonia. The efferent arterial pH increased significantly from 7·50 ± 0·04 to 7·80 ± 0·05 ($N = 6$) (Student's paired *t*-test: $\Delta pH = 0{\cdot}300 \pm 0{\cdot}016$, $P = 0{\cdot}0001$). As the perfusion fluid contained a coloured indicator, acidification was noticed immediately, and the pH measurements of the arterial effluents were performed with an electrode without delay on

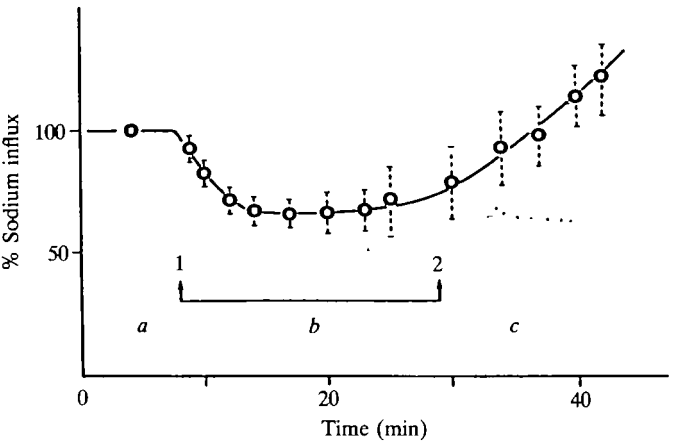

Fig. 7. Ammonia addition to the external medium: effect on secondary lamellar sodium influx. The external medium was closed and contained 1 mmol l^{-1} NaCl. Three successive phases (*a*, *b* and *c*) were observed. Control period (9 min) – $[T_{Amm}]_i$ = 200 μmol l^{-1}; $[T_{Amm}]_e = 0$. Arrow 1: ammonia addition (20 mmol l^{-1}) to the external medium (20 min) – $[T_{Amm}]_i$ = 200 μmol l^{-1}; $[T_{Amm}]_e$ = 20 mmol l^{-1}. Arrow 2: ammonia removal from the external and internal media – $[T_{Amm}]_i = 0$; $[T_{Amm}]_e = 0$. Mean values ± S.E. ($N = 4$).

efferent samples. This result provides evidence for NH_3 diffusion through the branchial epithelium (cellular and/or paracellular pathways) and could argue for an intracellular alkalinization responsible for sodium influx inhibition.

Conversely, the removal of both external and internal ammonia, which could result in an intracellular acidosis, led to a stimulation of Na^+ absorption (phase *c*).

Internal and external acidification

The decrease of internal pH by 1 unit stimulated sodium uptake by 40 % (Fig. 8). Moreover, in the same conditions, ammonia excretion was significantly inhibited by 30 % and fell from 51 ± 3 μmol h^{-1} 100 g^{-1} at pH 8·0 to 35 ± 5 μmol h^{-1} 100 g^{-1} at pH 7·0 ($N = 3$) (Student's paired *t*-test: difference in rates of ammonia excretion = 15·6 ± 5·0 μmol h^{-1} 100 g^{-1}, $P = 0{\cdot}004$).

During external acidification, sodium and ammonia excretion were measured simultaneously (Fig. 9). To maintain the imposed pH, the external milieu was buffered, and to prevent ammonia accumulation the latter was open and continuously renewed. When external pH was decreased from 8·0 to 6·0, a marked stimulation (100 %) of ammonia excretion was observed and sodium absorption was inhibited by 80 %.

Sodium and ammonia transport had become completely 'uncoupled'.

External addition of amiloride

The diuretic amiloride is known to inhibit sodium entry and/or Na^+/H^+ exchange, according to the experimental concentration employed (Benos, 1982).

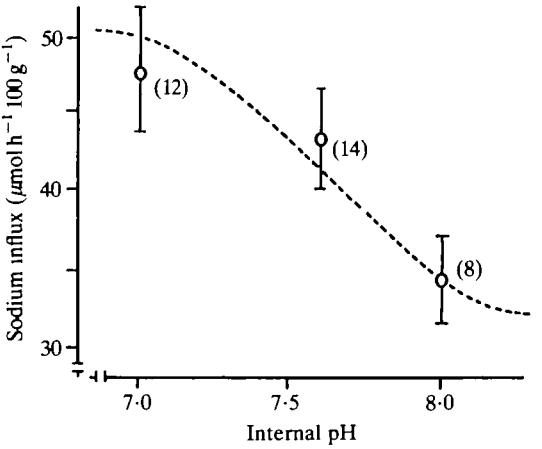

Fig. 8. Secondary lamellar sodium influx as a function of internal pH. The external medium was closed and contained 1 mmol l^{-1} NaCl. The perfusion fluid contained 1 mmol l^{-1} ammonia. After a period where the perfusion fluid was maintained at a certain pH, changes of pH (increase and/or decrease) were performed at random on the same fish (to prevent possible time-dependent effects). Acidification were carried out with H_2SO_4, and alkalinization with KOH. Mean values ± S.E. The numbers of experiments are given in parentheses.

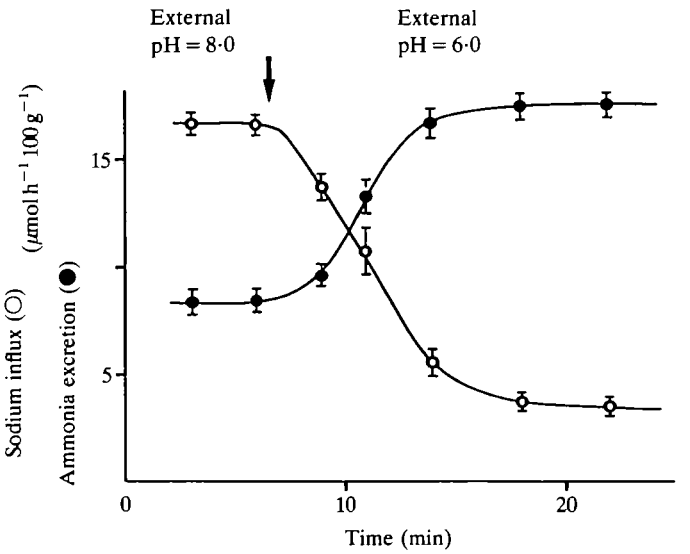

Fig. 9. External acidification: effect on secondary lamellar sodium influx (○) ($N = 3$) and ammonia excretion (●) ($N = 4$). The external medium was open, buffered (2 mmol l^{-1} imidazole) and consisted of fresh water ($[Na^+] < 160$ μmol l^{-1}). At the arrow, pH was changed from 8·0 to 6·0. The external alkalinization (pH 8·0) was performed with KOH, and acidification (pH 6·0) with H_2SO_4. The perfusion fluid contained 200 μmol l^{-1} ammonia. Mean values ± S.E.

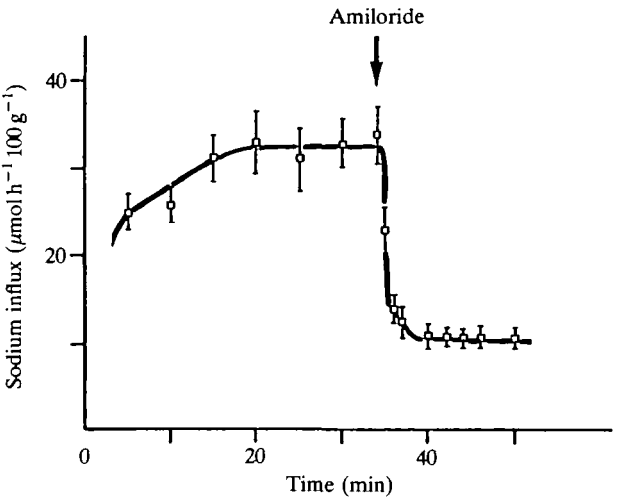

Fig. 10. Amiloride addition to the external medium: effect on secondary lamellar sodium influx. After a control period (35 min), amiloride (10^{-4} mol l^{-1}) was added to the external medium, which contained 1 mmol l^{-1} NaCl. The internal medium contained 1 mmol l^{-1} ammonia. Mean values ± S.E. ($N = 6$).

It was added to the external medium to exert effects on the mucosal side of the epithelium. Amiloride (Merk Sharp & Dohme, Chibret) was used initially at 10^{-4} mol l^{-1} to modify sodium and ammonia fluxes. After a control period, when sodium influx was stable, an immediate fall in sodium absorption was observed (Fig. 10). Sodium entry quickly stabilized and the percentage inhibition was calculated. In these conditions, ammonia excretion was stable: 20·0 ± 1·9 μmol h^{-1} 100 g^{-1} for controls and 16·9 ± 2·6 μmol h^{-1} 100 g^{-1} ($N = 4$) during amiloride exposure (no significant difference: Student's paired t-test: difference between rates of ammonia excretion = 3·0 ± 4·3 μmol h^{-1} 100 g^{-1}, $P = 0{\cdot}26$).

Different concentrations of the diuretic (range 10^{-6}–10^{-3} mol l^{-1}) were also tested, producing an inhibition of sodium influx which was calculated as a percentage of the control flux (Fig. 11). The concentration required for 50% inhibition (IC_{50}) was 10^{-5} mol l^{-1}.

Further, sodium influx was measured as a function of the external sodium concentration (Fig. 12), in the presence (curve C) or absence (curve A) of external amiloride (10^{-4} mol l^{-1}). In contrast to the previous experiments, amiloride was present from the beginning of the experiment. Under these conditions, the remaining sodium influx increased linearly as a function of the external sodium concentration. This regression line (curve C) could be taken to represent the diffusional fraction of the total sodium absorption. When diffusional fluxes (curve C) were subtracted from total fluxes (curve A) a saturation curve was obtained (curve A') and the kinetic parameters could be calculated: $V_{max} = 42{\cdot}8 \pm 1{\cdot}9$ μmol h^{-1} 100 g^{-1}; $K_m = 305 \pm 45$ μmol l^{-1} ($N = 6$).

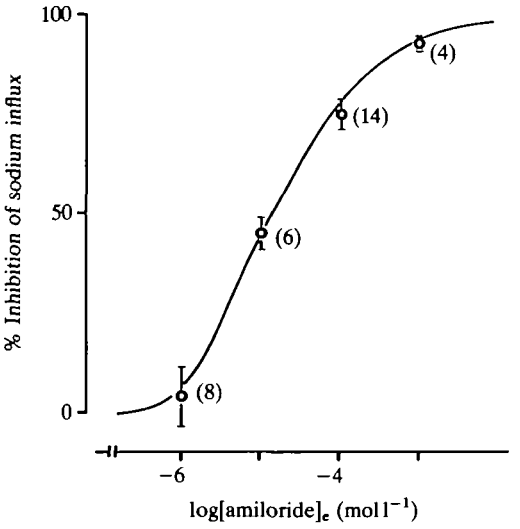

Fig. 11. Percentage inhibition of secondary lamellar sodium influx as a function of external amiloride concentration (amiloride dose–response curve). The external medium was closed and contained 1 mmol l^{-1} NaCl. The perfusion fluid contained 1 mmol l^{-1} ammonia. Each value was obtained according to the protocol described in Fig. 10. Mean values ± S.E. The numbers of experiments are given in parentheses.

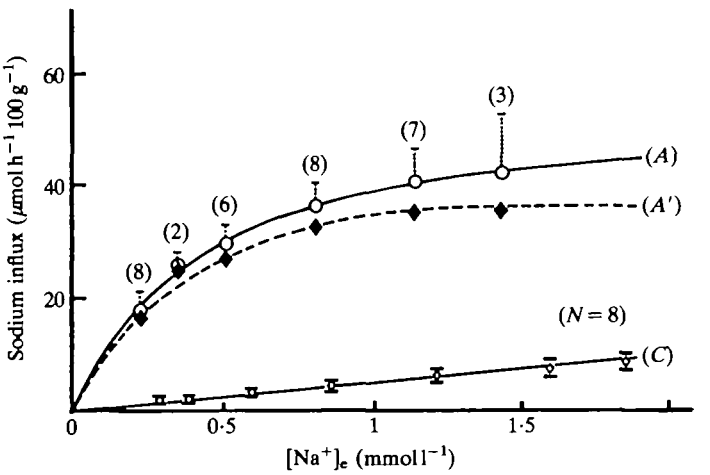

Fig. 12. Secondary lamellar sodium influx as a function of external sodium concentration: effect of amiloride. (*A*) Controls in fresh water, (*C*) amiloride addition (10^{-4} mol l^{-1}) to the external medium from the beginning of the experiment, $y = (3{\cdot}9 \pm 0{\cdot}2)x$, $N = 7$, $r = 0{\cdot}99$; $A' = A - C$. Mean values ± S.E. The numbers of experiments are given in parentheses.

Table 1. *Comparisons between sodium influx and ammonia and/or proton excretion*

Internal [ammonia] ($mmol\,l^{-1}$)	Sodium influx	Ammonia excretion	Titratable acidity	Proton excretion*
1	43·0 ± 4·0 (6)	35·0 ± 3·0 (4)	10·0 ± 2·0 (6)	45·0
0	34·0 ± 4·4 (6)	5·9 ± 0·6 (6)	27·9 ± 3·9 (6)	33·8

* Obtained by calculation as described in the text.

The external medium was closed and contained deionized water, supplemented with 1·5 $mmol\,l^{-1}$ $CaSO_4$, 2 $mmol\,l^{-1}$ imidazole and 0·5 $mmol\,l^{-1}$ Na_2SO_4.

The numbers of experiments are given in parentheses.

All values are in $\mu equiv\,h^{-1}\,100\,g^{-1}$.

Correlation between proton excretion and sodium influx

Although experimental conditions were complex (there is an acidic mucus layer around the head and respiratory CO_2 is produced in the external medium), proton excretion was measured (Table 1). With ammonia (1 $mmol\,l^{-1}$) in the perfusion fluid, titratable acidity was very low, but in the absence of internal ammonia it increased. Thus, as defined in Materials and methods, total proton excretion can be considered as the sum of titratable acidity and ammonia excretion. These results (Table 1) confirm the presence of a substantial proton excretion, which could be correlated quantitatively with Na^+ influx.

Discussion

Mechanism of ammonia excretion

In our experimental preparation, the external compartment has a limited volume and thus the difference in total ammonia concentration between the internal and external compartments tends to decrease as $NH_3 + NH_4^+$ builds up externally. Indeed, ammonia does not leave the external compartment since the rate of diffusion of NH_3 to the atmosphere is very slow. NH_3 diffusion to the atmosphere is a function of the ratio of the capacitances:

$$\frac{\beta_{atm}^{NH_3}}{\beta_w^{NH_3}} = 1{\cdot}2 \times 10^{-3} \text{ (at } 15\,^{\circ}C),$$

where the capacitance (β_w) of NH_3 in water is 47·53 $mmol\,l^{-1}\,mmHg^{-1}$ (Cameron & Heisler, 1983) and the capacitance (β_{atm}) of ammonia in air is $55{\cdot}68 \times 10^{-3}$ $mmol\,l^{-1}\,mmHg^{-1}$ (Dejours, 1975) (1 mmHg = 133·3 Pa).

Total ammonia excretion would stop under our experimental conditions (of temperature and pH) if the difference in concentration between the internal and external compartment decreased to 50 $\mu mol\,l^{-1}$ (see possible extrapolation on Fig. 3).

Table 2. *Calculation of variations in total ammonia concentration in the external medium, in experimental conditions where NH_3 diffusion would stop*

	pH	pK	T_{Amm} ($mmol\,l^{-1}$)	$[NH_4^+]$ ($mmol\,l^{-1}$)	$[NH_3]$ ($mmol\,l^{-1}$)	pNH_3 (nmHg)
Internal medium	7·60	9·54	0·200	0·197	0·0023	46
External medium						
(a)	7·89 ± 0·04 (6)	9·47	0·087	0·085	0·0020	46
(b)	7·63 ± 0·03 (6)	9·47	0·140	0·138	0·0020	46

Experimental conditions
(a) measurements performed at the beginning of the experiment;
(b) measurements performed at the end of the experiment.
The external medium was closed.

Details of the calculation
Internal and external pH were respectively imposed and measured.
pK values were estimated from Cameron & Heisler (1983).

In the internal medium:
$[T_{Amm}]$: ammonia concentration was imposed and maintained constant at $200\,\mu mol\,l^{-1}$ in the perfusion fluid.
Respective $[NH_4^+]$ and $[NH_3]$ were determined according to the equations:

$$[NH_3] + [NH_4^+] = [T_{Amm}] \quad (1)$$

$$pK = pH - \log[NH_3]/[NH_4^+] \quad (2)$$

pNH_3 was calculated from:

$$[NH_3] = \alpha pNH_3, \quad (3)$$

where the solubility coefficient was estimated as the α value in plasma and at 15°C according to Cameron & Heisler (1983): $\alpha = 49{\cdot}15\,mmol\,mmHg^{-1}$.

In the external medium:
pNH_3: NH_3 diffusion would stop if there were no difference of pNH_3 between the internal and external medium: internal pNH_3 = external pNH_3 = 46 nmHg.
$[NH_3]$ was calculated according to equation 3 using the α value in water = $47{\cdot}53\,mmol\,mmHg^{-1}$.
$[NH_4^+]$ was determined from equation 2.
$[T_{Amm}]$ was estimated from equation 1.

Based on the solubility of ammonia in various media (fresh water, plasma, etc.) and on the characteristics (i.e. pK) of the $NH_3 - NH_4^+$ equilibria (Cameron & Heisler, 1983), the corresponding pNH_3, $[NH_3]$ and $[NH_4^+]$ values were calculated in another experimental situation in conditions where ammonia diffusion would stop (Table 2). These values indicate that excretion as a diffusion process should be totally inhibited when there is no difference in pNH_3 between internal and external media (internal pNH_3 = external pNH_3 = 46 nmHg). In the present case, the calculated difference between the internal and external total ammonia concentrations decreased to $60\,\mu mol\,l^{-1}$ ($0{\cdot}200\,mmol\,l^{-1} - 0{\cdot}140\,mmol\,l^{-1}$) by the

end of the experiment, when the external pH fell to 7·63. The predicted experimental value (observed in Fig. 3: 50 μmol l^{-1}) and the calculated value (60 μmol l^{-1}) are remarkably close, which would argue for a diffusion of NH_3 as a function of the ΔpNH_3 between the internal and external media.

Although some authors have shown that gills are permeable to NH_4^+ (Maetz, 1973; Goldstein *et al.* 1982; Evans, 1985), the uncharged form NH_3 is generally considered to be highly lipid-soluble and to diffuse rapidly through biological membranes. Non-ionic diffusion of free base NH_3 was first postulated as the mechanism of renal excretion of ammonia by Pitts (1948). Manning (1964) proposed that NH_3 penetrates cell membranes much more rapidly than does the ionic form. Many authors since then have studied NH_3 diffusion through the gill epithelium (Kirschner *et al.* 1973; Cameron & Heisler, 1983; Wright & Wood, 1985). Maetz (1973) reported that NH_3 diffusion occurred through the gill epithelium of goldfish. More recently, Evans (1984) showed the epithelium of elasmobranchs to be much more permeable to NH_3 than to NH_4^+, the former diffusing 300 times faster than the latter.

It is clear, therefore, that ammonia excretion could take place *via* the diffusion of the free base NH_3 into the external medium, where it would bind hydrogen ions to form relatively nondiffusible ammonium ions. NH_3 diffusion could be stimulated by further binding and NH_3 trapping, which would occur in the external medium: (1) if H^+ is excreted (addition of external Na^+ possibly enhancing a Na^+/H^+ exchange as in Fig. 4) or (2) with increasing proton reserves in the external medium (addition of a buffer as in Fig. 5; external acidification in presence of a buffer as in Fig. 9). In the internal medium or in the cells, NH_3 is regenerated through continuous dissociation of NH_4^+ to NH_3 and H^+. Excretion of total ammonia will continue as long as internal pNH_3 exceeds external pNH_3.

Gill diffusion and permeability coefficients for NH_3

Dejours (1975) stated that the calculation of the coefficient of diffusion of NH_3 (D_{NH_3}) is valid 'for the specific conditions we have stated, in the steady state and when there is the same medium on both sides of the diffusion barrier, i.e. when $\beta_1 NH_3$ and $\beta_2 NH_3$ are identical'. Under our experimental conditions, the media on both sides of the branchial epithelium can be assumed to have been well mixed and renewed, and their NH_3 concentrations to have been adequately estimated (i.e. $[NH_3]$ of the perfusion fluid is the value calculated according to the pH of the medium and the total ammonia concentration imposed in the medium; $[NH_3]$ in water is 0). Then their β coefficients (= capacitance coefficients, equivalent in some cases to solubility coefficients) are essentially identical (Cameron & Heisler, 1983) and the coefficient of diffusion of NH_3 (D_{NH_3}) can be calculated as:

$$D_{NH_3} = \dot{M}_{NH_3} \times \frac{1}{\Delta[NH_3]} \times \frac{1}{A} \times E,$$

where A is the surface area (200 cm^2 100 g^{-1}), E is the thickness (6×10^{-4} cm) of the branchial epithelium (see Cameron & Heisler, 1983) and $\dot{M}_{NH_3}$ and $\Delta[NH_3]$

were estimated from Fig. 4, in the case where external $[Na^+] = 0$. $\dot{M}_{NH_3}$ = ammonia production = 19 μmol h^{-1} 100 g^{-1}; perfusate pH = 7·60; $pK(NH_4^+ - NH_3)$ = 9·54 at our temperature and pH conditions; $\Delta[NH_3]$ = $[NH_3]$ perfusate − $[NH_3]$ water; $[NH_3]$ perfusate = $10^{7.60-9.54} \times [NH_4^+]$ perfusate; perfusate $[T_{Amm}]$ = 900 μmol l^{-1} = $[NH_3]$ perfusate + $[NH_4^+]$ perfusate; and $[NH_3]$ water = 0.

Thus:

$$D_{NH_3} = \frac{19{\cdot}0\ (\mu\text{mol h}^{-1}\,100\,\text{g}^{-1})}{3600\ (\text{s})} \times \frac{1}{10{\cdot}2\times10^{-3}\ (\mu\text{mol cm}^{-3})} \times \frac{6\times10^{-4}\ (\text{cm})}{200\ (\text{cm}^2\,100\,\text{g}^{-1})}.$$

The diffusion coefficient for NH_3, D_{NH_3}, is $1{\cdot}55\times10^{-6}$ $cm^2\,s^{-1}$.

Cameron & Heisler (1983) calculated a value of $1{\cdot}3\times10^{-5}$ cm^{-1} for D, which they called the 'gill permeability coefficient', and compared this value to the coefficients of diffusion for O_2 and CO_2. However, the values should only be compared with great caution, because NH_3 diffusion rate is determined not only by the membrane permeability to the free base but also by the pH on both sides of the membrane, and by the flow rate of the perfusion fluid, which could modify the instantaneous amount of NH_3 available to be excreted.

When Na^+ is added to the external medium (Fig. 4: $[Na^+] = 1$ mmol l^{-1}; $[T_{Amm}] = 900$ μmol l^{-1}; $\dot{M}_{NH_3} = 44$ μmol h^{-1} 100 g^{-1}), D_{NH_3} rises to $3{\cdot}6\times10^{-6}$ $cm^2\,s^{-1}$ which could indicate that NH_3 diffusion responds to H^+ external production and would be consistent with a sodium–proton correlation.

The permeability coefficient for NH_3, calculated as D_{NH_3}/E ($E = 6\times10^{-4}$ cm), is equal to 6×10^{-3} cm s^{-1} and is intermediate between the values reported for turtle urinary bladder ($3{\cdot}0\times10^{-4}$ cm s^{-1}; Schwartz & Tripolone, 1983) and renal tubules (in rat: $1{\cdot}0\times10^{-2}$ cm s^{-1}, Oelert *et al.* 1968; in rabbit: $1{\cdot}6\times10^{-2}$ cm s^{-1}, Garvin *et al.* 1987).

The possibility of NH_4^+ excretion through simple diffusion cannot be ruled out (Evans & Cameron, 1986). However, the diffusion of ammonium ions does not need to occur to account for total ammonia excretion in the gill. Moreover, in particular conditions, active excretion of ammonia (possibly through a Na^+/NH_4^+ exchange) may take place either in a restricted environment rich in ammonia, to get rid of ammoniacal metabolic byproducts (Cameron & Heisler, 1983) or, after exhaustive exercise leading to extracellular acidosis, to contribute with Na^+/H^+ exchange to the regulation of acid–base equilibrium (Wood, 1988).

Mechanism of sodium influx

Na^+/NH_4^+ exchange

In general, the results described above lead us to conclude that Na^+ influx and total ammonia excretion are not linked and the notion of Na^+/NH_4^+ exchange has been rejected.

This was clear when the pH of the perfusion fluid was decreased by 1 unit (Fig. 8 and text): since the ratio $[NH_3]/[NH_4^+]$ is equal to 10^{pH-pK}, the ratio must have decreased 10-fold. Total ammonia concentration being constant and equal to

1 mmol l^{-1} in the internal medium, $[NH_3]$ decreased from 28 to 3 μmol l^{-1} and $[NH_4^+]$ increased slightly (2·5%) from 972 to 997 μmol l^{-1}. This acidification phenomenon will similarly affect the branchial cell; although the existence of intracellular buffers will give some attenuation, it should be followed by a trend towards intracellular acidification affecting the $[NH_3]/[NH_4^+]$ ratio. From the Na^+/NH_4^+ exchange hypothesis, even a small decrease in pH should slightly stimulate the exchange. However, this is not consistent with our observations that ammonia excretion decreased (30%: see text) when sodium influx increased markedly (Fig. 8) during internal acidification.

Earlier studies concluded that a Na^+/NH_4^+ exchange existed in trout (Kerstetter & Keeler, 1976; Payan, 1978*a*,*b*). Payan (1978*a*,*b*), however, hypothesized that part of the Na^+ influx was independent of internal $[NH_4^+]$. Even for a very low ammonia excretion rate (3·3 μmol h^{-1} 100 g^{-1}) he still observed a significant sodium influx (20·8 μmol h^{-1} 100 g^{-1}) and although amiloride inhibited Na^+ influx, he observed no such effect on NH_4^+ excretion. Hence, the stimulatory effect that an experimental increase in internal ammonia concentration had on sodium influx, as observed in trout (Kerstetter & Keeler, 1976; Payan, 1978*a*,*b*) and goldfish (Maetz & Garcia-Romeu, 1964), could instead be explained by the decrease in internal pH resulting from the internal addition of ammonia (Cameron & Kormanik, 1982; Cameron & Heisler, 1983).

Na^+/H^+ exchange

An alternative hypothesis is required to explain our results. Indirect modulation of epithelial cell pH (Figs 6, 7, 8) brought adjustments in sodium influx that are consistent with the proposed Na^+/H^+exchange.

In Fig. 9, the inhibition of sodium absorption during external acidification could be explained as a competition of external H^+ with external Na^+ on the sodium site of the Na^+/H^+ exchanger (Paris & Pouyssegur, 1983). Then amiloride, which could totally inhibit sodium influx, can be assumed either to inhibit directly the apical Na^+/H^+ exchange or to decrease sodium conductance in the apical region of the cells.

The effects of amiloride externally, and acetazolamide internally, which can only occur directly on the branchial cells – as no erythrocytes are present in the perfusion fluid used for the acetazolamide effect – indicate that the exchange seems to take place on the external face of the gill epithelium.

The correlation between the magnitude of sodium influx and that of proton excretion (Table 1) adds considerable weight in favour of this exchange. When ammonia is present in the internal medium, the sum of titratable acid and NH_4^+ excreted can be considered as the total efflux of protons, to counterbalance Na^+ influx. However, in the absence of ammonia in the perfusion fluid, the gill epithelium of trout will excrete enough protons to counterbalance the influx of positive charge, entering as sodium in that case.

It has been suggested that Na^+/H^+ exchange occurs in trout both in perfused head preparations (Perry *et al.* 1985) and *in vivo* (Kerstetter *et al.* 1970; Kerstetter

& Keeler, 1976; Cameron & Heisler, 1983). Results obtained in this study for H^+ excretion are similar to those obtained by these authors and are close to the values obtained by Wood *et al.* (1984) for a range of internal pH conditions similar to those prevailing in our experiments.

This experimental preparation allows a precise study of ionic fluxes, but the structural complexity (Pisam *et al.* 1987) of, and limited access to, the gill epithelium hinder a more detailed description of the exchange mechanism.

Potentially, two mechanisms could account for our results.

(1) A Na^+/H^+ exchange mediated by a carrier as is usually described in cells in contact with salt water, such as sea urchin eggs (Johnson *et al.* 1976), and for 'leaky' epithelia such as rat small intestine (Mürer *et al.* 1976), rabbit gall bladder (Cremashi *et al.* 1979) and renal proximal tubule (Kinsella & Aronson, 1980). Such carrier-mediated exchanges are electrically neutral, strictly coupled, and are not energy-consuming. Na^+ entry along its electrochemical potential triggers the sodium–proton countertransport.

(2) An indirect coupling of an active electrogenic proton transport and a passive sodium entry. Such a mechanism has been shown to operate in 'tight' epithelia in which the mucosal side is bathed by very dilute media (such as fresh water) and the serosal side by a liquid similar to plasma: urinary bladder of turtle and toad (Steinmetz *et al.* 1987; Ludens & Fanestil, 1972) and frog skin (Ehrenfeld & Garcia-Romeu, 1977; Ehrenfeld *et al.* 1985). In these epithelia, when the external

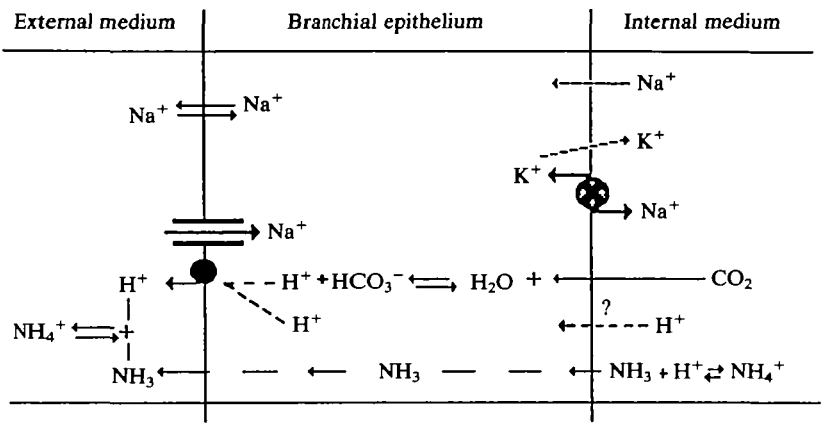

Fig. 13. Schematic representation of sodium and ammonia transport in the freshwater rainbow trout. Active pumps are denoted by circles; —> and —> are diffusion processes; ? indicates diffusion when electrochemical conditions are favourable. CO_2 enters the branchial cell, where it is hydrated by carbonic anhydrase to produce bicarbonate and protons. The latter are actively excreted electrogenically (together with internal and intracellular protons?) and could be responsible for the passive entry of Na^+ through an apical channel. Then, intracellular sodium is actively transported across the basal membrane by a Na^+/K^+-ATPase. Ammonia crosses the serosa and mucosa very quickly as the NH_3 form, the rate being a function of ΔpNH_3 between the internal and external media. In the external medium, it equilibrates with NH_4^+ according to the environmental pH and proton availability.

medium is diluted, H^+ excretion and Na^+ influx are correlated, but proton excretion can also occur in situations of low Na^+ influx (Ehrenfeld *et al.* 1985).

The gill epithelium of freshwater fish can be considered as being tight (Sardet, 1980; Pisam *et al.* 1987). In our experimental conditions, external sodium concentrations were very low (see freshwater composition in Materials and methods), always lower than or equal to $1\,mmol\,l^{-1}$. Thus, in terms of electrochemical potential, Na^+ could not be considered as a possible trigger of a strict Na^+/H^+ exchange. Moreover, for the passive proton excretion to trigger the exchange, cell pH would have to decrease far below 7, which is incompatible with intracellular metabolism and is not consistent with reported values for intracellular pH. Therefore, we propose that sodium influx in trout gills is electrically coupled to an intracellular negative potential resulting from the active excretion of protons. In other words, an electrogenic proton pump participates in the genesis of a favourable electrochemical driving force for apical sodium entry.

Fig. 13 summarizes the possible roles of sodium, protons and ammonia in the branchial epithelium. The site for those exchanges through the branchial epithelium has not been determined. However, according to Avella *et al.* (1987), chloride cells on the secondary lamellae have been shown to play a role in branchial sodium influx and, thus, are the likely site for this transport (they were well-developed in control conditions), but NH_3 diffusion probably takes place across the whole gill surface.

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