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The cultured branchial epithelium of the rainbow trout as a model for diffusive fluxes of ammonia across the fish gill

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Summary

A novel branchial epithelial preparation grown in L-15 medium in culture was used as a model system for understanding the diffusion of ammonia across the gills of the rainbow trout *Oncorhynchus mykiss*. The epithelium is known to contain both respiratory and mitochondria-rich cells in the approximate proportion in which they occur *in vivo* and to exhibit diffusive fluxes of Na^+ and Cl^- similar to *in vivo* values, but does not exhibit active apical-to-basolateral transport of Na^+. Transepithelial resistance and paracellular permeability are also known to increase when the apical medium is changed from L-15 medium (symmetrical conditions) to fresh water (asymmetrical conditions). In the present study, net basolateral-to-apical ammonia fluxes increased as basolateral total ammonia concentration, basolateral-to-apical pH gradients and basolateral-to-apical P_{NH_3} gradients were experimentally increased and were greater under asymmetrical than under symmetrical conditions. The slope of the relationship between ammonia flux and P_{NH_3} gradient (i.e. NH_3 permeability) was the same under both conditions and similar to values for other epithelia. The higher fluxes under asymmetrical conditions were explained by an apparent diffusive flux of NH_4^+ that was linearly correlated with transepithelial conductance and was probably explained by the higher electrochemical gradient and higher paracellular permeability when fresh water was present on the apical surface. In this situation, NH_4^+ diffusion was greater than NH_3 diffusion under conditions representative of *in vivo* values, but overall fluxes amounted to only approximately 20 % of those *in vivo*. These results suggest that branchial ammonia excretion in the intact animal is unlikely to be explained by diffusion alone and, therefore, that carrier-mediated transport may play an important role.

Key words: *Oncorhynchus mykiss*, rainbow trout, gill, cultured epithelium, ammonia diffusion, transepithelial resistance, transepithelial conductance, P_{NH_3} gradient, NH_4^+ electrochemical gradient, pH gradient, paracellular pathway.

Introduction

Ammonia excretion across the gills of fish was first studied by some of the fathers of comparative physiology more than half a century ago (Smith, 1929; Krogh, 1939), but the mechanisms involved still remain clouded in controversy. There is evidence for NH_3 diffusion along P_{NH_3} gradients from blood plasma to water (in some cases facilitated through diffusion-trapping of NH_3 by boundary layer acidification), for NH_4^+ diffusion along electrochemical gradients from blood plasma to water, for apical membrane Na^+/NH_4^+ exchange, for apical membrane Na^+/H^+ exchange, for basolateral membrane transport by substitution for K^+ on the Na^+/K^+-ATPase and/or substitution for K^+ on the $Na^+/K^+/2Cl^-$ cotransporter, for direct basolateral membrane transport by Na^+/NH_4^+-ATPase and for various combinations of these passive and active mechanisms (Maetz and Garcia-Romeu, 1964; Maetz, 1972, 1973; Payan, 1978; Kormanik and Cameron, 1981; Cameron and Heisler, 1983, 1985; Wright and Wood, 1985; Cameron, 1986; Evans and Cameron, 1986; Evans and More, 1988; McDonald and Prior, 1988; McDonald and Milligan, 1988; Balm et al., 1988; Heisler, 1990; Wilson and Taylor, 1992; Wilson et al., 1994; Salama et al., 1999). Wilkie (1997) provides a recent critical review.

Even in situations in which simple diffusion appears to predominate, it has not been possible to determine whether diffusion is transcellular, paracellular or both. Differences amongst species, salinities and in the ammonia, ionic and acid–base status of the animals in different studies have undoubtedly contributed to this uncertainty. However, the complex geometry of the intact gills with respect to both blood and water flow has also been an important factor. In particular, it has not been possible to determine the exact P_{NH_3}, NH_4^+ and pH levels on the two sides of the branchial epithelium because of unknown boundary layer and transit time effects, considerable differences in the composition of blood and water entering and leaving the gills and the possible heterogeneity of different parts of the gill surface.

An alternative approach is to develop simple model systems in which some of these factors can be better controlled. Recently, our laboratory has developed a

cultured branchial epithelial preparation ('double-seeded preparation') that incorporates the two major cell types of the freshwater gill, respiratory cells ('pavement cells', approximately 85 %) and mitochondria-rich cells ('chloride cells', approximately 15 %) in the approximate proportions in which they occur *in vivo* (Fletcher et al., 2000; Kelly et al., 2000). The freshwater rainbow trout *Oncorhynchus mykiss*, the species that has been studied most for ammonia excretion, is the source of the cells, and the preparation is an extension of an earlier 'single-seeded' trout gill epithelium that contained only respiratory cells (Wood and Pärt, 1997; Wood et al., 1998; Gilmour et al., 1998). These preparations are flat and thus amenable to manipulation and sampling of the medium on either side for measurements of flux, pH and electrical characteristics.

The cultured epithelia are grown with culture medium (a blood-plasma-like substance) on both surfaces ('symmetrical conditions'), but are tolerant of subsequent exposure to fresh water on the apical surface ('asymmetrical conditions'), as *in vivo*. This exposure causes an overall increase in epithelial resistance. Simultaneous flux measurements with the paracellular marker polyethlene glycol-4000 (PEG-4000) have shown that the phenomenon is due to a decrease in transcellular permeability since paracellular permeability actually increases at this time (Wood et al., 1998; Gilmour et al., 1998; Fletcher et al., 2000), although it remains far lower than if sea water is placed on the apical surface (Fletcher, 1997). While the 'double-seeded' preparation does not exhibit active Na^+ uptake (i.e. transport from the apical to the basolateral surface), it appears faithfully to duplicate the passive electrical and diffusive properties of the intact gill for Na^+ and Cl^- movements (Fletcher et al., 2000). It may, therefore, be particularly suitable for analysing passive ammonia movements across the gill. In the present study, we evaluate this preparation for the general study of ammonia excretion and use it to examine the roles of pH and electrical gradients and of paracellular *versus* transcellular pathways and the importance of NH_3 *versus* NH_4^+ diffusion.

Materials and methods

Preparation of cultured branchial epithelia

Rainbow trout [*Oncorhynchus mykiss* (Walbaum)] weighing 90–150 g were obtained from a local supplier and held in dechlorinated running Hamilton tapwater (composition: [Na^+], 0.55; [Cl^-], 0.70; [Ca^{2+}], 1.00; [Mg^{2+}], 0.15; [K^+], 0.05 mmol l^{-1}; pH 7.8–8.0). Photoperiod and temperature (15–19 °C) varied seasonally.

All procedures for gill cell isolation were conducted in a laminar flow hood using sterile techniques. Methods for initial gill cell isolation were based on those originally developed by Pärt et al. (1993), with modifications described by Wood and Pärt (1997), and methods for epithelial preparation and culture were based on those outlined by Fletcher et al. (2000). Briefly, gill cells were initially obtained from excised gill filaments by two consecutive cycles of tryptic digestion (Gibco BRL Life Technologies, 0.05 % trypsin in phosphate-buffered saline, PBS, with 5.5 mmol l^{-1} EDTA) and resuspended in culture medium (Leibovitz's L-15 supplemented with 2 mmol l^{-1} glutamine, 5 % foetal bovine serum, 100 i.u. ml^{-1} penicillin, 100 μg ml^{-1} streptomycin and 200 μg ml^{-1} gentamycin). The cells were then directly seeded onto permeable Falcon cell culture inserts (Cyclopore polyethylene terephthalate 'filters'; Becton Dickinson, Franklin Lakes, New Jersey, USA; pore density 1.6×10^6 pores cm^{-2}, pore size 0.45 μm, growth surface 0.9 cm^2) at a density of 2×10^6–2.5×10^6 viable cells cm^{-2}. Initially, 0.8 ml and 1.0 ml of medium plus antibiotics (see above) were added to the apical (insert) and basolateral (companion wells) sides of the preparations respectively. One day after seeding, inserts were rinsed with 0.4 ml of PBS (pH 7.7) to remove non-adherent cells and mucus, and new cells freshly isolated from a second fish were then seeded at the same density onto the adherent layer of cells established in the inserts. After 24 h, non-adherent cells and mucus were again rinsed off the inserts, and 1.5 ml and 2.0 ml of antibiotic-free medium (Leibovitz's L-15 supplemented with 2 mmol l^{-1} glutamine and 5 % foetal bovine serum) were added to the apical and basolateral sides of the preparations respectively. Media were changed every 48 h thereafter and remained antibiotic-free throughout the culture period. Epithelial preparations were incubated at 18 °C in an air atmosphere. Full details of the procedures for the preparation and culture of rainbow trout epithelia can be found in Kelly et al. (2000).

All experiments were conducted on epithelia 6–7 days after initial seeding, a time when transepithelial resistance was close to maximum plateau values (Fletcher et al., 2000). When fresh water was used to replace the apical medium (asymmetrical or simulated *in vivo* conditions), it (sterilized, chemical composition same as original holding water) was added to the apical side of the insert after several rinses to ensure removal of any residual medium.

Electrophysiological measurements

Transepithelial resistance (TER) was monitored using STX-2 'chopstick' electrodes connected to an EVOM epithelial voltohmeter, custom-modified by the manufacturer (World Precision Instruments, Sarasota, Florida, USA) to measure resistances up to 100 000 Ω. To correct for background resistance, the TER of an identical vacant culture insert was determined and subtracted from the TER of the culture inserts containing cultured epithelia. The corrected value in ohms (Ω) was then multiplied by the effective growth area (0.9 cm^2) to yield the final value for TER (in Ω cm^2). Conductance was calculated as the inverse of TER. In a parallel series of experiments, we determined strong linear regression relationships between measured transepithelial potential (TEP; expressed with respect to the apical surface as 0 mV) and measured TER under both symmetrical and asymmetrical conditions, as reported in fig. 6 of Fletcher et al. (2000); these relationships were used to predict TEP for the purposes of calculation in the present experiments.

Experimental series

Three experimental series were performed, each with the

same basic sampling and flux measurement procedures. Two were essentially preliminary, to evaluate the suitability of the preparation for the study of ammonia excretion, and the third comprised a detailed examination in which pH and chemical and electrical gradients were carefully measured and the effects of symmetrical *versus* asymmetrical conditions and the directionality of diffusion gradients were assessed. Because of seasonal variation in TER of epithelia grown from fish at different times of the year, discussed by Wood et al. (1998) and Fletcher et al. (2000), each series was performed using cells from a discrete batch of fish, at one time. This eliminated potential confounding effects between epithelia prepared from different batches in which large natural variations in epithelial 'tightness' have previously been observed to occur. Selection of inserts for experimental manipulation within each series was conducted according to matching criteria based on TER measurements at day 6–7 under unmanipulated conditions (i.e. final plateau TER measurements).

Series 1

Selected epithelia with almost identical TERs under symmetrical culture conditions (L-15 apical/L-15 basolateral) were exposed to asymmetrical conditions (fresh water apical/L-15 basolateral) after adding varying levels of NH_4Cl (BDH, Toronto, Ontario, Canada) to the basolateral medium (N=10 for each group). The added nominal concentrations of NH_4Cl were 0, 250 and 1000 μmol l^{-1}, but since the L-15 medium contains 100–150 μmol l^{-1} of endogenous ammonia, final measured total ammonia concentrations (T_{Amm}) on the basolateral side were 123±2, 400±5 and 1055±10 μmol l^{-1} respectively. After a 3 h flux period, apical fresh water was collected for the analysis of total ammonia concentration (T_{Amm}). TER across the epithelia was recorded prior to experimental manipulation and at 0 and 3 h after the addition of fresh water. On the basis of our observations in this experiment, a final T_{Amm} concentration of approximately 650 μmol l^{-1} was selected for all subsequent experiments, achieved by adding 500 μmol l^{-1} NH_4Cl.

Series 2

To determine whether basolateral pH influenced ammonia flux rates (J_{Amm}) from the basolateral to the apical compartment of cultured epithelia under asymmetrical conditions, basolateral medium pH (pH_{Bl}) was manipulated by adding appropriate amounts of either 140 mmol l^{-1} HCl or 140 mmol l^{-1} NaOH, and T_{Amm} was set to 650 μmol l^{-1}. Availability of inserts allowed for six groups (N=3 for each group) with pH_{Bl} values of 7.0, 7.4, 7.6, 7.8, 8.2 and 8.6 (±0.05 pH units). Apical water samples were collected before and after a 3 h flux period for analysis of T_{Amm}. TER measurements were recorded immediately before and after the introduction of asymmetrical conditions and just prior to the collection of apical water at the end of the 3 h flux period.

Series 3

This most detailed series examined the effects of basolateral pH manipulations, of symmetrical *versus* asymmetrical conditions and of directionality of gradients (i.e. basolateral-to-apical *versus* apical-to-basolateral gradients) under symmetrical conditions. The pH of the basolateral medium (set to 650 μmol l^{-1} T_{Amm}) was manipulated as described previously (see above), and a range of values from 7.0 to 8.6 was assessed. Separate preparations were used for apical freshwater treatments (asymmetrical; N=3 at each pH_{Bl}) and apical L-15 (symmetrical; N=6 at each pH_{Bl}) treatments at each pH. Apical water and basolateral L-15 samples were taken at 0, 3 and 6 h for analysis of T_{Amm}, apical pH (pH_{Ap}), pH_{Bl} (and adjustment of pH if necessary), together with TER measurements.

Using preparations derived from the same batch of fish, a concurrent experiment was performed to determine whether the cultured epithelia exhibited any signs of rectification. This was done under symmetrical culture conditions in which the apical medium of another set of epithelia, matched to approximately the same TER, was pH-manipulated and loaded to 650 μmol l^{-1} T_{Amm} (N=5–6 at each pH_{Ap}). Both apical and basolateral L-15 samples were collected for the analysis of T_{Amm}, pH_{Ap}, pH_{Bl} (and adjustment of pH if necessary) and TER measurements at 0, 3 and 6 h.

Analytical techniques and calculations

In water, T_{Amm} was determined by the salicylate/hypochlorite method (Verdouw et al., 1978). Components of the L-15 medium interfere with colour development in this assay so, in medium, T_{Amm} was determined using the enzymatic method of Mondzac et al. (1965) employing Sigma ammonia kit no. 171-UV according to the manufacturer's instructions for blood plasma samples. The two assays were cross-validated to produce the same results using both saline- and water-based standards, although the precision of the colorimetric assay was greater than that of the enzymatic method. Both water and medium pH were measured with a Radiometer E5021 microelectrode system, thermostatically controlled to the experimental temperature (18 °C).

The concentrations of NH_3 and NH_4^+ and the partial pressure of ammonia (P_{NH_3}) in both water and medium samples were calculated from the respective pH and T_{Amm} measurements in the two solutions using the Henderson–Hasselbalch equation and appropriate values of pK and ammonia solubility (α_{NH_3}) in plasma and fresh water from Cameron and Heisler (1983), as detailed in Wright and Wood (1985). As L-15 medium is very similar in ionic composition to trout plasma (Wood and Pärt, 1997), plasma values were employed for L-15. The difference (ΔP_{NH_3}) between simultaneous determinations of P_{NH_3} in apical water or medium and basolateral medium was calculated as a measure of the NH_3 diffusion gradient. The electrochemical gradient for NH_4^+ diffusion (e.g. basolateral-to-apical) was calculated as the driving force, i.e.

$$\mathrm{TEP} + \frac{\boldsymbol{R}T}{zF} \ln \frac{[NH_4^+]_{Bl}}{[NH_4^+]_{Ap}}, \qquad (1)$$

where concentrations are in molar units, TEP is in volts, and

R, *T*, *z* and *F* in the Nernst component have their usual thermodynamic values. P_{NH_3}, pH and electrochemical gradients were averages from measurements made at the start and end of the associated ammonia flux measurements.

Ammonia flux rates (J_{Amm}, nmol cm^{-2} h^{-1}), from either apical-to-basolateral or basolateral-to-apical compartments of the cultured epithelia, were calculated in the standard fashion from the appearance of T_{Amm} (nmol ml^{-1}) in the unmanipulated compartment (the one with a lower T_{Amm}), multiplied by volume (ml) and factored by time (h) and surface area (cm^2). In the experiments of series 3, we checked whether disappearance from the basolateral compartment (L-15, higher concentration) corresponded to appearance in the apical compartment (fresh water, lower concentration). There were never any significant differences between the two measurements of flux, but the lower precision of the enzymatic assay, coupled with the problem of detecting small changes against high background levels in the basolateral compartment, resulted in higher variability in the disappearance measurement, so only appearance measurements are reported. We also ran tests with vacant filter inserts to check whether the ammonia diffusion resistance of the filter was significant relative to that of the cultured epithelium. The results demonstrated that the ammonia permeability of the filter alone was at least 10-fold greater than the permeability of the epithelium plus filter under symmetrical conditions and at least fivefold greater under asymmetrical conditions, indicating that this was not an important source of error.

Data have been expressed as means ± 1 S.E.M. Statistical comparisons were made by Student's paired or unpaired *t*-test (two-tailed) or by one-factor or two-factor analysis of variance (ANOVA) followed by a Student–Newman–Keuls test to detect specific differences, as appropriate (Nemenyi et al., 1977). Regression relationships were fitted by the method of least squares, and the significance of Pearson's correlation coefficient was assessed. A fiducial limit of $P \leqslant 0.05$ was used throughout.

Results

Cultured epithelia

After 6–7 days in culture, the preparations exhibited a high transepithelial resistance (TER) that was consistent with the formation of an electrically 'tight' epithelium. Prior to experimental manipulation, the mean TER of epithelia used in the current studies was 3491±329 Ω cm^2 (N=112) under symmetrical conditions.

Series 1

The 30 preparations used in this experiment were selected for initial uniform TER under symmetrical conditions (3000–3500 Ω cm^2), and the addition of various levels of T_{Amm} as NH_4Cl to the basolateral medium did not affect TER (Fig. 1A). However, when the apical medium was changed to fresh water, TER increased significantly, approximately threefold, and became more variable in all groups (Fig. 1A).

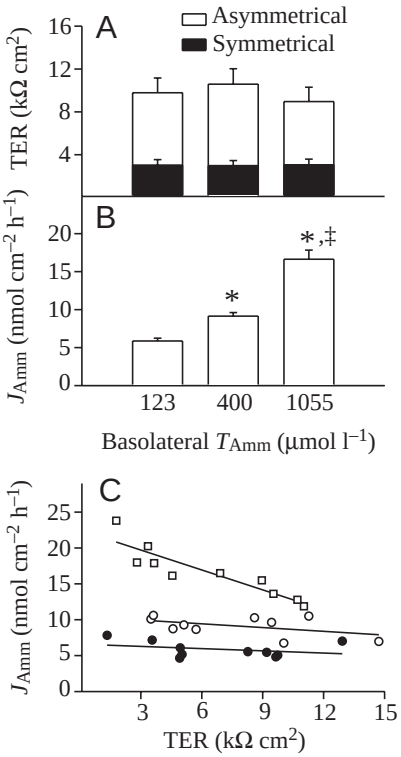

Fig. 1. (A) Transepithelial resistance (TER) of cultured epithelia before (symmetrical conditions, L-15 apical/L-15 basolateral) and after (asymmetrical conditions, fresh water apical/L-15 basolateral) the addition of apical fresh water at three different basolateral concentrations of total ammonia (T_{Amm}) in series 1. Values are means +1 S.E.M. (N=10 per group). All increases in TER were highly significant (P<0.001), but there were no significant differences (P>0.05) associated with different T_{Amm} levels. (B) Ammonia flux rates (J_{Amm}) from basolateral-to-apical compartments under asymmetrical conditions over a 3 h flux period at the three different basolateral T_{Amm} levels in the same preparations. An asterisk denotes a statistically significant difference (P<0.05) from the 123 μmol l^{-1} group and a double dagger denotes a statistically significant difference (P<0.05) from both other groups. (C) Relationships between J_{Amm} and TER in individual cultured epithelia under asymmetrical conditions in each of the three groups in B. The regression equations are as follows. At 123 μmol l^{-1}, filled circles, J_{Amm}=–0.103TER+6.593, r=0.33, N=10, P>0.05; at 400 μmol l^{-1}, open circles, J_{Amm}=–0.176TER+10.495, r=0.49, N=10, P>0.05; at 1055 μmol l^{-1}, open squares, J_{Amm}=–0.924TER+22.483, r=0.90, N=10, P<0.0001.

Basolateral pH was not controlled in this series but was approximately 7.4–7.6. Under these asymmetrical conditions, increasing basolateral T_{Amm} from 123 to 400 and 1055 μmol l^{-1} resulted in significant increases in ammonia flux rates (J_{Amm}) from basolateral to apical surfaces across cultured epithelia (Fig. 1B), although there was again no effect on TER. There was no significant relationship between J_{Amm} and TER at the two lower T_{Amm} levels, but a significant negative relationship at a basolateral T_{Amm} of 1055 μmol l^{-1} (Fig. 1C).

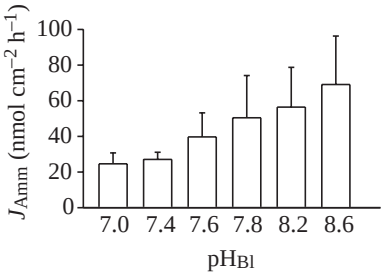

Fig. 2. The effects of varying basolateral pH (pH_{Bl}) at total ammonia (T_{Amm})=650 μmol l^{-1} on ammonia flux rates (J_{Amm}, over 3 h) across cultured branchial epithelia under asymmetrical (fresh water apical/L-15 basolateral) conditions in series 2. Values are means +1 S.E.M. (N=3 per group). The overall effect of pH_{Bl} was significant (P<0.05), although none of the individual differences was significant.

Series 2

In this series, basolateral T_{Amm} was set to 650 μmol l^{-1} and pH_{Bl} was manipulated experimentally. No statistical differences were found between the initial TER measured (under symmetrical culture conditions) across groups designated for different pH_{Bl} treatments. The mean TER for all preparations was 1823±180 Ω cm^2 (N=18), and this increased significantly to 4206±387 Ω cm^2 (N=18) when the apical medium was changed to fresh water. Similarly, after a 3 h flux period under asymmetrical culture conditions, no significant differences between TER measurements for groups treated with varying pH_{Bl} were detected. Overall, there was a clear significant effect of pH_{Bl} on J_{Amm} (one-way ANOVA) in these 3 h experiments. Basolateral-to-apical J_{Amm} increased from approximately 25 to approximately 70 nmol cm^{-2} h^{-1} in pH_{Bl} treatments of 7.0 and 8.6 respectively (Fig. 2), although none of the individual differences was significant by itself. Apical freshwater pH was not controlled in these experiments, but generally fell from initial values of approximately 8.0 to 7.0–7.4, with larger changes occurring at lower pH_{Bl}.

Using a gill surface area of 2500 cm^2 kg^{-1} for freshwater trout (Wood, 1974), J_{Amm} values of around 55 nmol cm^{-2} h^{-1} at pH_{Bl} 7.8 (Fig. 2) yield predicted *in vivo* rates of 138 μmol kg^{-1} h^{-1}. This is approximately 25–50 % of typical reported *in vivo* rates, even though a basolateral T_{Amm} of 650 μmol l^{-1} is somewhat higher than *in vivo* values for non-feeding fish. For example, at 15 °C, Cameron and Heisler (1983) reported J_{Amm} for resting trout to be approximately 330 μmol kg^{-1} h^{-1} when water pH was 7.0, blood plasma pH was 7.75 and T_{Amm} was 320 μmol l^{-1}. Overall, series 1 and 2 indicated that the cultured epithelium provided rates of J_{Amm} that were low but not completely unrealistic relative to *in vivo* values and that responded to basolateral T_{Amm} and pH_{Bl} manipulations in a consistent fashion. They therefore suggested that this cultured epithelial preparation was a suitable model for examining diffusive fluxes of ammonia in detail in series 3.

Series 3

Basolateral T_{Amm} was again set to 650 μmol l^{-1}, pH_{Bl} was experimentally manipulated and pH_{Ap} was closely monitored

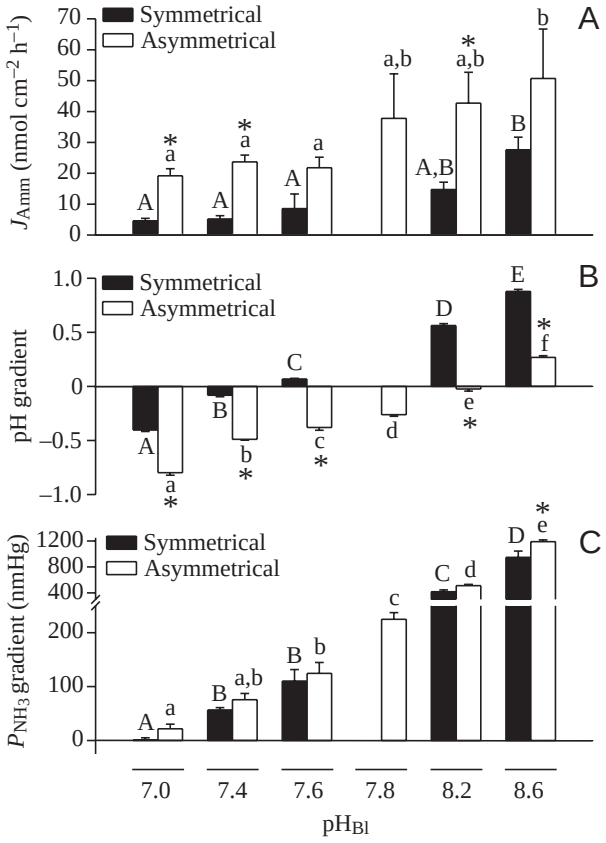

Fig. 3. (A) A comparison of ammonia flux rates (J_{Amm}, over 6 h) between epithelia exposed to either symmetrical (L-15 apical/L-15 basolateral; filled columns) or asymmetrical (fresh water apical/L-15 basolateral; open columns) conditions at varying basolateral pH (pH_{Bl}), total ammonia (T_{Amm})=650 μmol l^{-1}, in series 3. The overall effects of pH and of symmetrical *versus* asymmetrical conditions were significant (P<0.05). (B) Mean pH gradients and (C) mean P_{NH_3} gradients associated with these treatments. Values are means +1 S.E.M. (N=6 for all symmetrical groups and N=3 for all asymmetrical groups). Significant differences between specific groups within a treatment (pH_{Bl} effect) are denoted by different letters; significant differences between specific groups held under either symmetrical or asymmetrical conditions are denoted by an asterisk. 1 nmHg=0.133 Pa.

to keep it close to starting values (pH 7.5 for apical L-15, pH 8.0 for apical fresh water) in preparations under either symmetrical (apical L-15/basolateral L-15) or asymmetrical (apical fresh water/basolateral L-15) conditions. Flux periods of 6 h (average of two 3 h periods, with pH adjustment at 3 h) were employed to control pH levels better. In this series, there were no significant differences in TER associated with apical freshwater exposure, and no significant influence of pH_{Bl} on TER, which averaged 4443±548 Ω cm^2 (N=45).

As in series 2, basolateral-to-apical J_{Amm} increased significantly with increasing pH_{Bl} (one-way ANOVA), and this overall effect was seen under both asymmetrical and symmetrical test conditions (Fig. 3A). Furthermore, the rates at pH_{Bl} 8.6 were significantly greater than those at all other

pH_{Bl} levels below 7.8 under both conditions. (Because of the limited number of matched preparations available, pH_{Bl} 7.8 was not tested under symmetrical conditions.) When a two-way ANOVA was applied, there was a significant overall effect of asymmetrical *versus* symmetrical conditions, with consistently greater J_{Amm} values when the apical medium was fresh water. Individual differences were significant at pH_{Bl} values of 7.0, 7.4 and 8.2 (Fig. 3). However, since L-15 contains an endogenous level of T_{Amm} whereas the starting fresh water did not, and since the pH_{Ap} values were different in the two media, the pH, P_{NH_3} and NH_4^+ gradients were not necessarily the same under the two conditions. Indeed, the basolateral-to-apical pH gradients were substantially lower or more negative at every pH_{Bl} value when the apical medium was fresh water (Fig. 3B), but the P_{NH_3} gradients were very similar in the two treatments (Fig. 3C). Very clearly, the ammonia fluxes (Fig. 3A) tended to track the P_{NH_3} gradient (Fig. 3C) in each treatment. Relationships between J_{Amm} and the respective P_{NH_3} and NH_4^+ gradients are examined in greater detail in Fig. 5 and Fig. 6 (see below).

When NH_4Cl was added to the apical medium (T_{Amm}) rather than the basolateral medium under symmetrical conditions, and pH_{Ap} rather than pH_{Bl} was manipulated experimentally, ammonia flux now occurred in the apical-to-basolateral direction in all pH treatments (Fig. 4A). Again, there was a significant overall effect of pH on J_{Amm}. Interestingly, there was also a significant effect of direction (by two-way ANOVA), with consistently higher apical-to-basolateral flux rates than in the 'normal' direction. None of the individual differences was significant but, at every pH, the average J_{Amm} values in the basolateral-to-apical direction were 40–60 % lower. These differences occurred despite the facts that there were no significant differences in TER (overall mean $4723\pm579\,\Omega\,cm^2$, N=58) and that we were successful in closely matching the pH gradients (Fig. 4B) and P_{NH_3} gradients (Fig. 4C) under the two test conditions.

Fig. 3 and Fig. 4 both suggest that J_{Amm} was correlated with the relevant P_{NH_3} gradient, but not in a directly proportional fashion. Fig. 5 provides a closer examination of possible relationships between P_{NH_3} gradient and J_{Amm} and Fig. 6 of possible relationships between the electrochemical gradient for NH_4^+ and J_{Amm} using all individual data points from the experiments of series 3. For each of the three overall conditions (asymmetrical with basolateral-to-apical gradients, symmetrical with basolateral-to-apical gradients and symmetrical with apical-to-basolateral gradients), J_{Amm} was positively correlated with the P_{NH_3} gradient, suggesting that NH_3 diffusion plays an important role in ammonia flux across this epithelium. These relationships were significant for the first two of the conditions (Fig. 5A,B) but not for apical-to-basolateral gradients (Fig. 5C). A comparison of Fig. 5A and Fig. 5B indicates that the same range of P_{NH_3} gradients was achieved in these two conditions, despite the difficulties with different starting T_{Amm} levels in the apical medium of the two treatments. Furthermore, at a comparable P_{NH_3} gradient, J_{Amm} was approximately twofold higher under asymmetrical

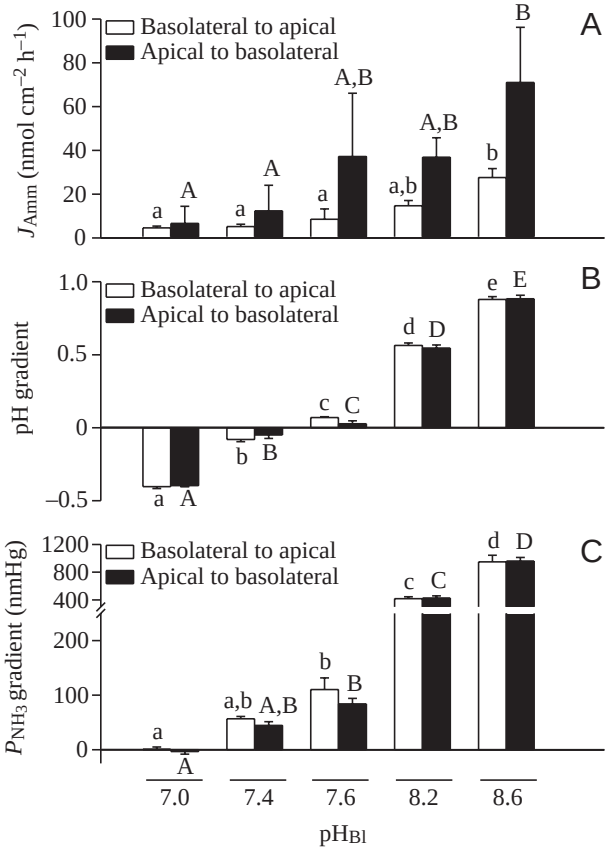

Fig. 4. (A) A comparison of ammonia flux rates (J_{Amm}, over 6 h) between epithelia exposed under symmetrical conditions (L-15 apical/L-15 basolateral) to either basolateral manipulations (basolateral-to-apical fluxes; open columns, data from Fig. 3) or apical manipulations (apical-to-basolateral fluxes; filled columns) of pH at total ammonia (T_{Amm})=650 μmol l^{-1} in series 3. The overall effects of pH and of direction were significant (P<0.05), although there were no significant differences with respect to direction at the same pH values. (B) Mean pH gradients and (C) mean P_{NH_3} gradients associated with these treatments. In B and C, there were no significant differences (P>0.05) attributable to direction, although the overall effects of pH were highly significant (P<0.0001). Values are means +1 S.E.M. (N=6 per group for all basolateral manipulations and N=5–6 per group for all apical manipulations). Significant differences between specific groups within a treatment (pH_{Bl} effect) are denoted by different letters. 1 nmHg=0.133 Pa.

conditions (Fig. 5A) than under symmetrical conditions, confirming that apical fresh water really does increase J_{Amm} independently of P_{NH_3} gradient. The intercepts of the two regression relationships were significantly different (P<0.05), whereas the slopes were not.

Under all three conditions, there was a positive electrochemical driving force for NH_4^+ in the direction of J_{Amm}, principally due to the addition of NH_4Cl to the 'driving side'. In themselves, these analyses provided no evidence for diffusion of NH_4^+ along its electrochemical gradient as a major pathway of ammonia flux because, under all three conditions, the relationship between J_{Amm} and electrochemical driving

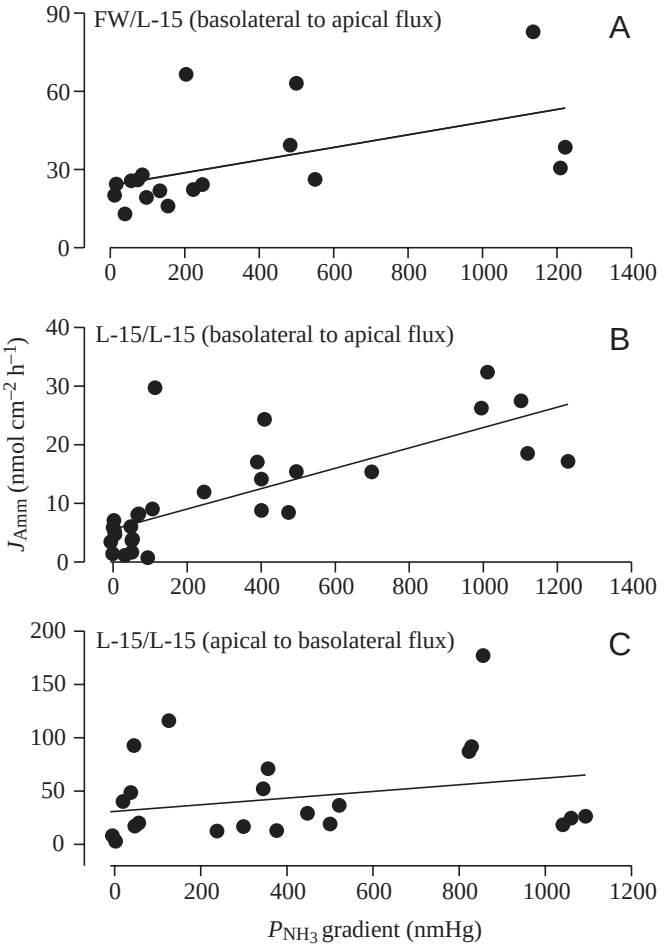

Fig. 5. Relationships between ammonia flux rates (J_{Amm}, over 6 h) and average P_{NH_3} gradients in individual epithelial preparations of series 3 under (A) asymmetrical conditions with basolateral-to-apical fluxes, (B) symmetrical conditions with basolateral-to-apical fluxes and (C) symmetrical conditions with apical-to-basolateral fluxes. The regression equations are as follows: (A) J_{Amm}=+0.0242P_{NH_3}+23.94, r=0.53, N=18, P<0.05; (B) J_{Amm}=+0.0174P_{NH_3}+5.52, r=0.74, N=31, P<0.05; (C) J_{Amm}=+0.0313P_{NH_3}+30.95, r=0.28, N=27, P>0.05. 1 nmHg=0.133 Pa. FW, fresh water; L-15, L15 medium.

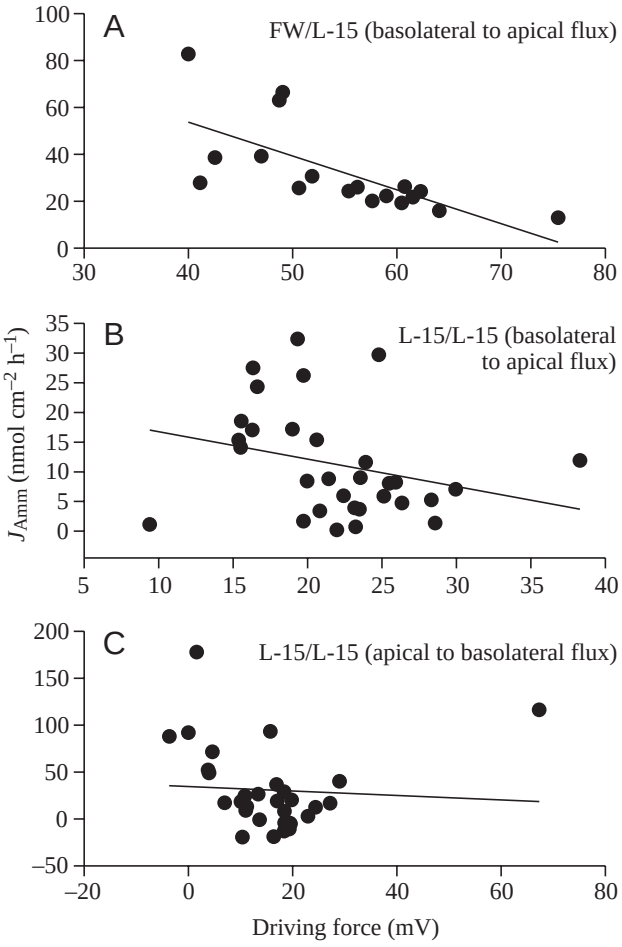

Fig. 6. Relationships between ammonia flux rates (J_{Amm}, over 6 h) and average net driving force for NH_4^+ (DF) in individual epithelial preparations of series 3 under (A) asymmetrical conditions with basolateral-to-apical fluxes, (B) symmetrical conditions with basolateral-to-apical fluxes and (C) symmetrical conditions with apical-to-basolateral fluxes. The regression equations are as follows: (A) J_{Amm}=–1.4415DF+111.40, r=–0.69, N=18, P<0.05; (B) J_{Amm}=–0.4616DF+21.37, r=–0.28, N=33, P>0.05; (C) J_{Amm}=–0.2370DF+34.40, r=–0.03, N=29, P>0.05. FW, fresh water; L-15, L15 medium.

force was actually negative (Fig. 6), although this was significant only under asymmetrical conditions (Fig. 6A). The negative relationships reflect the fact that J_{Amm} was greater at high pH and P_{NH_3} gradients, resulting in greater NH_4^+ accumulation on the recipient side and, therefore, lower electrochemical gradients when averaged over 6 h.

Nevertheless, the range of electrochemical driving forces was clearly higher under asymmetrical conditions (Fig. 6A) (generally +40 mV to +65 mV) than under symmetrical conditions (Fig. 6B,C) (generally +5 mV to +40 mV). This difference was reflected in mean values that were more than twice as high: +54.6±2.1 mV, N=18, under asymmetrical conditions *versus* +22.2±0.9 mV, N=33, in basolateral-to-apical trials and +16.9±2.3 mV, N=29, in apical-to-basolateral trials under symmetrical conditions. This difference was not due to the TEP component, which actually reduced the driving force under asymmetrical conditions (mean TEP –8.9±0.1 mV, N=18) and had a marginal influence under symmetrical conditions (mean TEP +2.4±0.3 mV, N=33, in basolateral-to-apical trials and +2.0±0.2 mV, N=29, in apical-to-basolateral trials). Rather, it was due to the higher starting T_{Amm} levels and NH_4^+ concentrations, and therefore lower Nernst component, when L-15 was used on the non-manipulated ('recipient') side in the symmetrical trials.

In theory, if NH_4^+ is diffusing across the epithelium, then its flux should be a linear function of conductance (the inverse of TER), all other factors being equal. The results of series 1 suggested that J_{Amm} decreased as TER increased in the expected fashion, at least at high basolateral concentrations of T_{Amm} when the apical medium was fresh water (Fig. 1). The results of the present series provide an opportunity to analyse

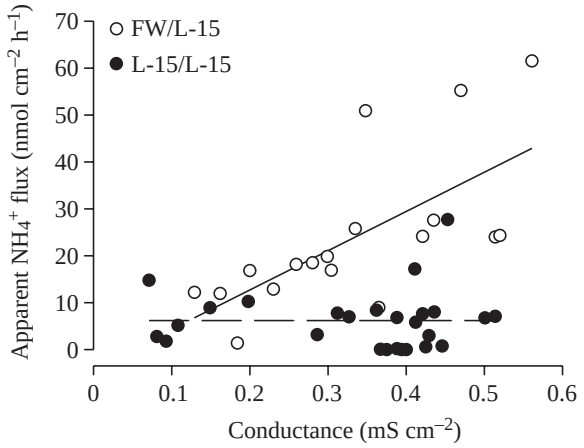

Fig. 7. Relationships between the apparent basolateral-to-apical flux rates of NH_4^+ (as calculated from ammonia flux rates, J_{Amm}, the relevant P_{NH_3} gradient and the regression slopes from Fig. 5A,B as described in the text) and the transepithelial conductance (*C*, inverse of transepithelial resistance) in individual cultured epithelial preparations of series 3 under symmetrical and asymmetrical conditions. The regression equations are as follows: asymmetrical conditions, NH_4^+ flux=85.05*C*–4.61, r=0.68, N=18, P<0.05; symmetrical conditions, NH_4^+ flux=0.03*C*+6.21, r=0.01, N=29, P>0.05. 1 nmHg=0.133 Pa. FW, fresh water; L-15, L15 medium.

possible conductance *versus* flux relationships in detail under conditions of both apical fresh water and apical L-15. To remove the influence of NH_3 flux, the product of the particular P_{NH_3} gradient and the slope of the relevant regression line (i.e. J_{Amm} per nmHg P_{NH_3} from Fig. 5; 1 nmHg=0.133 Pa) was subtracted from each value of total J_{Amm} to yield the apparent NH_4^+ flux (Fig. 7). Under asymmetrical conditions, this flux was strongly correlated with transepithelial conductance (r=0.68, N=18, P<0.05), whereas there was no relationship under symmetrical conditions (r=0.01, N=29, P>0.05). In neither case was the relationship improved by expressing J_{Amm} per millivolt driving force. This analysis suggests that diffusive NH_4^+ flux is important under asymmetrical conditions but not under symmetrical conditions.

Discussion

Overview

This 'double-seeded' cultured gill epithelium, which incorporates both respiratory and mitochondria-rich cells (Fletcher et al., 2000; Kelly et al., 2000), provides a useful *in vitro* model for understanding the diffusive fluxes of ammonia across the trout gill. One must always be cautious in extrapolating to the *in vivo* situation from an *in vitro* preparation, especially one that has actually been formed in culture rather than just maintained in culture. However, since the cultured gill epithelium closely mimics the intact gill in terms of diffusive Na^+, Cl^- and PEG-4000 permeability (Wood and Pärt, 1997; Wood et al., 1998; Fletcher et al., 2000), it is worthwhile to ask how well ammonia flux rates across this preparation correlate with those across the intact gill. A large discrepancy would suggest that additional (non-diffusive) mechanisms of ammonia transport may exist in the intact gill. As outlined in the subsequent section, the measured diffusive ammonia fluxes across the cultured epithelium do not appear to be large enough to account for the observed rates of ammonia excretion across the intact gill, thereby providing indirect support for the concept that carrier-mediated transport also plays an important role *in vivo*.

Leaving aside for the moment the curious influence of directionality (Fig. 4), several conclusions can be drawn about the passive movement of ammonia across the epithelium. The results confirm that the diffusion of NH_3 occurs across the epithelium and that this responds to variations in P_{NH_3} gradients achieved through pH manipulations (Fig. 3, Fig. 4, Fig. 5). Within any one condition, variations in NH_4^+ electrochemical gradients do not appear to be an important factor in variations in ammonia flux because there was either no relationship or a negative relationship between ammonia flux and net electrochemical driving force (Fig. 6). Nevertheless, the results indicate an important role for NH_4^+ diffusion under asymmetrical conditions. In this regard, the intercept of the regression of basolateral-to-apical ammonia flux on P_{NH_3} gradient was positive (i.e. some ammonia flux still occurred in the absence of a P_{NH_3} gradient) and much higher when fresh water was present on the apical surface, while the slopes (representing NH_3 permeability) were the same in the two treatments (Fig. 5A,B). A significant diffusive flux of NH_4^+ may, therefore, have contributed to the greater net ammonia fluxes seen when fresh water was present on the apical surface (Fig. 3).

This possibility is supported by three other pieces of evidence. First, the mean basolateral-to-apical electrochemical driving force for NH_4^+ was more than twice as high (+55 mV *versus* +22 mV) when the apical medium was fresh water. Second, the apparent NH_4^+ flux was strongly correlated with transepithelial conductance in the expected fashion, but only under asymmetrical conditions (Fig. 7). Lastly, it is now well-documented that paracellular permeability (measured with PEG-4000) increases (generally by 50–100 %) when these preparations are exposed to apical fresh water (Wood et al., 1998; Gilmour et al., 1998; Fletcher et al., 2000). This increase in paracellular permeability is responsible for increased diffusive fluxes of ions such as Na^+, Cl^- and Ca^{2+} upon exposure of these cultured epithelia to fresh water, so it is not surprising that greater diffusion of NH_4^+ may also occur. However, it should be noted that this increase in paracellular permeability when the apical medium is changed from iso-osmotic L-15 medium (osmotically equivalent to approximately 30 % sea water) to fresh water is far smaller than when the apical medium is changed to full-strength sea water (Fletcher, 1997). Thus, the permeability characteristics of the cultured epithelium are in accord with the well-documented pattern that gill permeability *in vivo* is much lower in freshwater fish than in seawater fish (Evans, 1984).

The greater overall rates of J_{Amm} when the gradients were

reversed under symmetrical conditions was unexpected (Fig. 4). However, rectification of transport of many non-electrolytes across the fish gill is a well-documented phenomenon, with differences of up to 10-fold, although the phenomenon has not previously been reported for ammonia, and the mechanisms of rectification remain poorly understood (for a review, see Isaia, 1984). In the present study, there was no significant relationship between the apical-to-basolateral J_{Amm} and the P_{NH_3} gradient (Fig. 5C), the net driving force for NH_4^+ (Fig. 6C) or the transepithelial conductance (data not shown), so the mechanism is again unclear. *A priori*, it may seem counterintuitive for the gill to favour ammonia uptake (apical-to-basolateral flux) over ammonia excretion (basolateral-to-apical flux). However, we have recently presented evidence that rainbow trout may actually use low levels of ammonia in the environmental water to promote protein synthesis and growth (Linton et al., 1997; Morgan et al., 2001). Thus, a rectification mechanism favourable to ammonia uptake may be adaptive in some circumstances. Clearly, the phenomenon deserves further detailed study.

Comparison with in vivo *data*

Several studies of ammonia excretion in freshwater trout *in vivo* have estimated the 'diffusivity' of the gill to NH_3 as approximately 4–6 $\mu mol\,kg^{-1}\,h^{-1}\,nmHg^{-1}$ (Cameron and Heisler, 1983; Wright and Wood, 1985). These estimates were achieved by establishing conditions in which it was thought that only NH_3 diffusion was occurring and then dividing the measured J_{Amm} (approximately 300 $\mu mol\,kg^{-1}\,h^{-1}$) by the apparent P_{NH_3} gradient from blood to water (approximately 65 nmHg). Assuming a gill surface area of 2500 $cm^2\,kg^{-1}$ for freshwater trout (Wood, 1974), this yields an apparent gill NH_3 permeability of approximately 1.1×10^{-2} $cm\,s^{-1}$. Contrary to popular belief, NH_3 has low lipophilicity (Evans and Cameron, 1986), so this is a high permeability value for NH_3 relative to most other preparations (generally in the range 10^{-3} to 10^{-4} $cm\,s^{-1}$; see Cameron and Heisler, 1985; Evans and More, 1988) and comparable with the very high value seen in the rabbit proximal kidney tubule (Garvin et al., 1987). By way of comparison, at a basolateral-to-apical P_{NH_3} gradient of 65 nmHg, J_{Amm} across the cultured epithelium was approximately 25.5 $nmol\,cm^{-2}\,h^{-1}$ (from Fig. 3 or Fig. 5A), equivalent to only 64 $\mu mol\,kg^{-1}\,h^{-1}$, or approximately 20 % of the flux *in vivo*. However, on the basis of the slope of Fig. 5A (J_{Amm} per nmHg P_{NH_3} gradient), only approximately 6 % (4 $\mu mol\,kg^{-1}\,h^{-1}$) of this would be due to NH_3 diffusion, with the remainder presumably due to NH_4^+ diffusion. Thus, NH_3 permeability across the cultured epithelium is at most 2.3×10^{-3} $cm\,s^{-1}$ and could be as low as 1.5×10^{-4} $cm\,s^{-1}$, in general accord with values in the literature for other tissues and in accord with the predicted range (Isaia, 1984) for a substance whose oil:water partition coefficient is less than 0.1 (Evans and Cameron, 1986). NH_4^+ permeability across cultured epithelia under asymmetrical conditions (in the mid-range of conductance) would be approximately 10^{-5} $cm\,s^{-1}$, in accord with other analyses suggesting that the permeability ratio of NH_4^+ to NH_3 is somewhat higher in the tissues of ammoniotelic fish than in those of ureotelic higher vertebrates (for a review, see Wood, 1993). It is problematic whether significant NH_4^+ permeability occurs under symmetrical conditions; if so, it is small and hidden in the noise of the data (e.g. Fig. 7).

While these permeability estimates are only approximations, an inescapable conclusion from the present results is that passive fluxes of ammonia alone (as both NH_4^+ and NH_3) are insufficient to explain the rates of branchial ammonia excretion observed *in vivo*. This is especially striking since passive fluxes of Na^+ and Cl^- across this same preparation nicely match branchial Na^+ and Cl^- efflux rates recorded in intact freshwater rainbow trout (Fletcher et al., 2000). However, the conclusion is not surprising when one considers that concentrations of Na^+ and Cl^- in trout plasma are generally approximately 250–1000 times higher than those of T_{Amm} (e.g. 150 $mmol\,l^{-1}$ *versus* 150–600 $\mu mol\,l^{-1}$). Indeed, in hindsight, it is curious why so much emphasis has been placed on diffusion as the supposed dominant pathway for ammonia excretion across the gills of freshwater fish (Cameron and Heisler, 1983; Cameron and Heisler, 1985; Heisler, 1990; Wilson et al., 1994), an emphasis that has necessitated the implicit assumption of an unusually high permeability to NH_3. *In vivo*, there is abundant, though controversial, evidence for carrier-mediated NH_4^+ transport across the gills of freshwater fish (Maetz and Garcia-Romeu, 1964; Maetz, 1972; Maetz, 1973; Payan, 1978; Wright and Wood, 1985; Cameron, 1986; McDonald and Prior, 1988; McDonald and Milligan, 1988; Balm et al., 1988; Wilkie, 1997; Salama et al., 1999), generally associated directly or indirectly with active inward Na^+ transport. This active Na^+ transport does not occur in the cultured branchial epithelium (Fletcher et al., 2000), which probably explains the discrepancy.

In summary, the present results demonstrate that both NH_4^+ and NH_3 diffusion occur across the gill epithelium, that NH_4^+ diffusion, which probably proceeds *via* the paracellular pathway, is quantitatively the more important component, at least in apical fresh water, and that these fluxes are substantially less than rates of ammonia excretion *in vivo*. As such, they provide impetus to re-examine carrier-mediated ammonia flux in the intact gill and to investigate methods of promoting transepithelial active transport in the cultured branchial epithelium.

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