J Comp Physiol B (1989) 158:627–635

Journal of Comparative Physiology B Biochemical, Systemic, and Environmental Physiology

Fish gill water boundary layer: a site of linkage between carbon dioxide and ammonia excretion

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Accepted August 30, 1988

Summary. Carbon dioxide excreted across fish gills is hydrated catalytically to form HCO_3^- and H^+ ions in water near the gill surface. We tested the possibility that CO_2 excretion is functionally linked to ammonia excretion through chemical reactions in the gill-water boundary layer. A blood-perfused trout head preparation was utilized in which the convective and diffusive components of branchial gas transfer were controlled. Pre-incubation of blood perfusate with the carbonic anhydrase inhibitor, acetazolamide, reduced both carbon dioxide and ammonia excretion in the blood-perfused preparation. Increasing the buffering capacity of inspired ventilatory water significantly reduced ammonia excretion, but carbon dioxide excretion was unaffected. Each of these experimental treatments significantly reduced the acidification of ventilatory water flowing over the gills. It is proposed that the catalysed conversion of excreted CO_2 to form HCO_3^- and H^+ ions provides a continual supply of H^+ ions need for the removal of NH_3 as NH_4^+. We suggest, therefore, that acidification of boundary layer water by CO_2 enhances blood-to-water NH_3 diffusion gradients and facilitates ammonia excretion.

Introduction

Molecular CO_2 excreted across fish gills hydrates to form HCO_3^- and H^+ ions in the expired water (Wright et al. 1986). Recent advances indicate that carbonic anhydrase (CA), the enzyme that catalyses the CO_2 hydration reaction, is present on the external surface of the gill (Rahim et al. 1988). Wright et al. (1986) concluded that mucus adjacent to the gill epithelial surface contains CA activity because (1) water downstream from the gill is more acidic than inspired water but in pH equilibrium and (2) epidermal mucus, which is chemically similar to gill mucus, contains CA activity.

A possible role of external gill CA is to maintain an acidic boundary layer next to the gill surface to facilitate ammonia excretion (the term ammonia or Tamm will be used to indicate the total ammonia concentration, while NH_4^+ and NH_3 will refer to ammonium ion and non-ionic ammonia, respectively). In freshwater fish, ammonia is excreted across the gills by diffusion of non-ionic NH_3 and via the Na^+/NH_4^+ ion exchange mechanism (Maetz and Garcia Romeu 1964; Maetz 1973; Kirschner et al. 1973; Evans 1977; Payan 1978; Cameron and Heisler 1983; Wright and Wood 1985). Theoretically, sustained NH_3 diffusion is dependent upon NH_3 removal from the boundary layer because the accumulation of excreted NH_3 in the boundary layer will reduce the blood to water NH_3 partial pressure (P_{NH_3}) diffusion gradient. NH_3 can be physically removed from the boundary layer by diffusion into the bulk water flowing past the gills, and/or removed chemically by combining with a H^+ ion to form NH_4^+ in the boundary layer. The maintenance of P_{NH_3} gradients across the gill will therefore depend, in part, upon the availability of H^+ ions in the boundary layer to facilitate interconversion of NH_3 to NH_4^+. A possible role of external gill CA may be to catalyse the conversion of excreted CO_2 to HCO_3^- and H^+ ions in the boundary layer to supply H^+ ions for the reaction $NH_3 \rightleftharpoons NH_4^+$ (Fig. 1).

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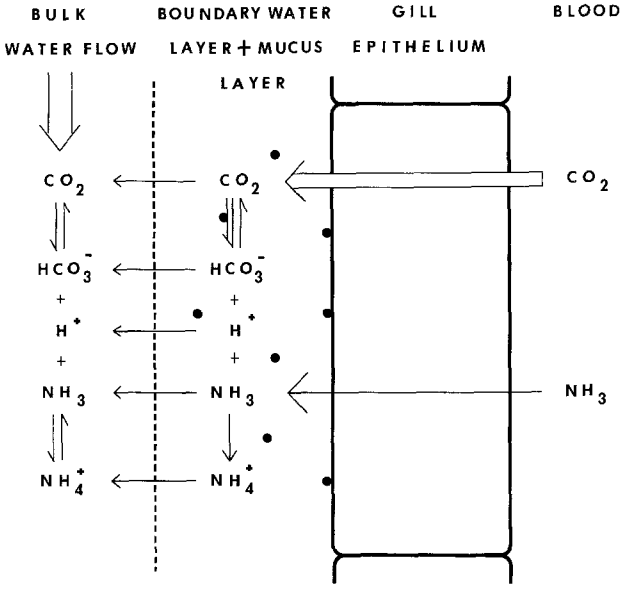

Fig. 1. A simplified cross-section through the gill epithelium showing the bulk water flow, the boundary water layer and associated mucus layer containing carbonic anhydrase molecules represented by ●. We have not shown the series of chemical reactions involving CO_2 and H_2O, but instead have represented the catalysed reaction as a summary mass transfer statement. The catalysed hydration of CO_2 in the boundary layer to $HCO_3^- + H^+$ facilitates ammonia excretion by promoting the interconversion of NH_3 to NH_4^+, thereby preventing reductions in the transepithelial NH_3 diffusion gradient due to accumulation of NH_3. The thickness of the arrows denotes the magnitude of the particular process illustrated (not precisely drawn to scale)

To investigate the possible linkage of CO_2 and ammonia excretion at the gill it was imperative to employ a perfused gill preparation in which the independent variables (e.g. ventilation and perfusion) could be precisely controlled. In preliminary in vivo studies (unpublished data), the independent variables fluctuated during experimental manipulations and the results were therefore equivocal. In contrast, the blood-perfused trout head preparation allows the investigators to control the convective components of gas transfer (blood flow rate through the gills and water flow rate over the gills), as well as manipulate the chemical composition of the blood perfusate and ventilatory water.

Materials and methods

Experimental animals. Rainbow trout (*Salmo gairdneri*) of both sexes were obtained from Thistle Springs Trout Farm (Ashton, Ontario) and transported to the University of Ottawa. Fish were held indoors in rectangular fiberglass tanks supplied with flowing, aerated and dechlorinated tapwater (pH = 7.5–8.0, $[Na^+] = 0.10$; $[Cl^-] = 0.15$; $[Ca^{2+}] = 0.35$; $[K^+] = 0.03$ mmol·l^{-1}, temperature = 6–9 °C). Fish were fed a daily diet of commercial trout pellets.

Blood collection and preparation. Isolated trout heads were perfused with whole blood, collected from fish with dorsal aortic cannulae (Soivio et al. 1972), implanted 24 h earlier. Blood was collected immediately prior to each experiment, as described by Perry et al. (1985a). The haematocrit (hct) of the pooled blood was measured (mean hct = 17.1% ± 0.5; mean ± SEM) and adjusted to 13–15% (mean = 14.4% ± 0.3) with Cortland saline (Wolf 1963) containing 2.2% Bovine Serum Albumin (BSA, Sigma). In pilot experiments the hct of the pooled blood was adjusted to 10%; however, we observed that gas transfer across the gills was considerably less than at a hct of 13–15%, thus the higher hct was adopted. The extent of dilution depended on the hct of the pooled donor blood, but plasma osmotic pressure was probably constant in all experiments since 2.2% Bovine Serum Albumin (BSA, Sigma) was present in the saline. In preliminary experiments we also found that the addition of the colloid osmotic filler, polyvinylpyrrolidone, PVP (see Perry et al. 1984a), to the saline in place of BSA resulted in blood leakage at the gills, incomplete perfusion of gill arches, and extremely high perfusion (afferent) pressure values (> 120 cm H_2O) in the isolated head preparation. Thus, saline which was used to dilute the blood as well as perfuse the head on the operating table (see below) contained only BSA. Diluted blood was added in equal portions to two reservoirs and stirred gently for 90 min in an ice bath. The blood was equilibrated with 0.4% CO_2 and 4% O_2, balanced with N_2 (P_{CO_2} 3 Torr, P_{O_2} 30 Torr), for 90 min to simulate venous blood gas tensions.

Surgical procedure for preparing isolated perfused head. Trout (216–353 g, mean weight 269 ± 6 g, $n = 31$) were removed from holding tanks and immediately injected via the caudal vein/artery with 1 ml of saline containing 2500 units·ml^{-1} ammonium heparin. After 10 min, fish were removed from the water and quickly decapitated and the head (mean weight 74 ± 1 g) was immediately transferred to an operating table. Details of the surgical procedures have been previously described (Perry et al. 1985a), with the following exceptions. After the cardiovascular surgery was complete, opercular cavity cannulae were affixed to the isolated head in order to sample expired water downstream from the gill. Polyethylene cannulae (PE 90) were stitched in position under, and midway along, the opercular openings (Wright et al. 1986). A small tube (Tygon, 0.5 cm diameter) was stitched to the floor of the buccal cavity and the tongue just at the opening of the mouth to ensure water flow over the gills during the subsequent experiment. The mouth was sealed around this tube by two large stitches between the upper and lower jaw, on either side. The opercular openings were loosely closed by two stitches along the ventral openings to prevent the opercular valves from flaring open and resulting in poor ventilatory flow through the gill arches. A variety of methods of gill irrigation were tried in pilot experiments; for example, an inflow tube was simply placed into the mouth without sealing the mouth or restraining the opercular valves. This method has been widely used in other isolated head studies (e.g. Perry et al. 1984c; Perry and Wood 1985; Bornancin et al. 1985). We noticed, however, that water tended to flow back out of the mouth, and the water passing through the opercular cavities did not flow in a uniform pattern.

Finally, a rubber membrane (condom) was fitted around the head by first making a groove on the external surface with a heavy piece of silk (size 0) tied around the fish, posterior to the pectoral fins. Surgery was completed within approximately 15 min. Preparations were discarded in cases where blood clearance from the gills was incomplete (approximately 5%).

Experimental apparatus. The isolated head was transferred to the plastic chamber (inset Fig. 2) and the condom was fitted

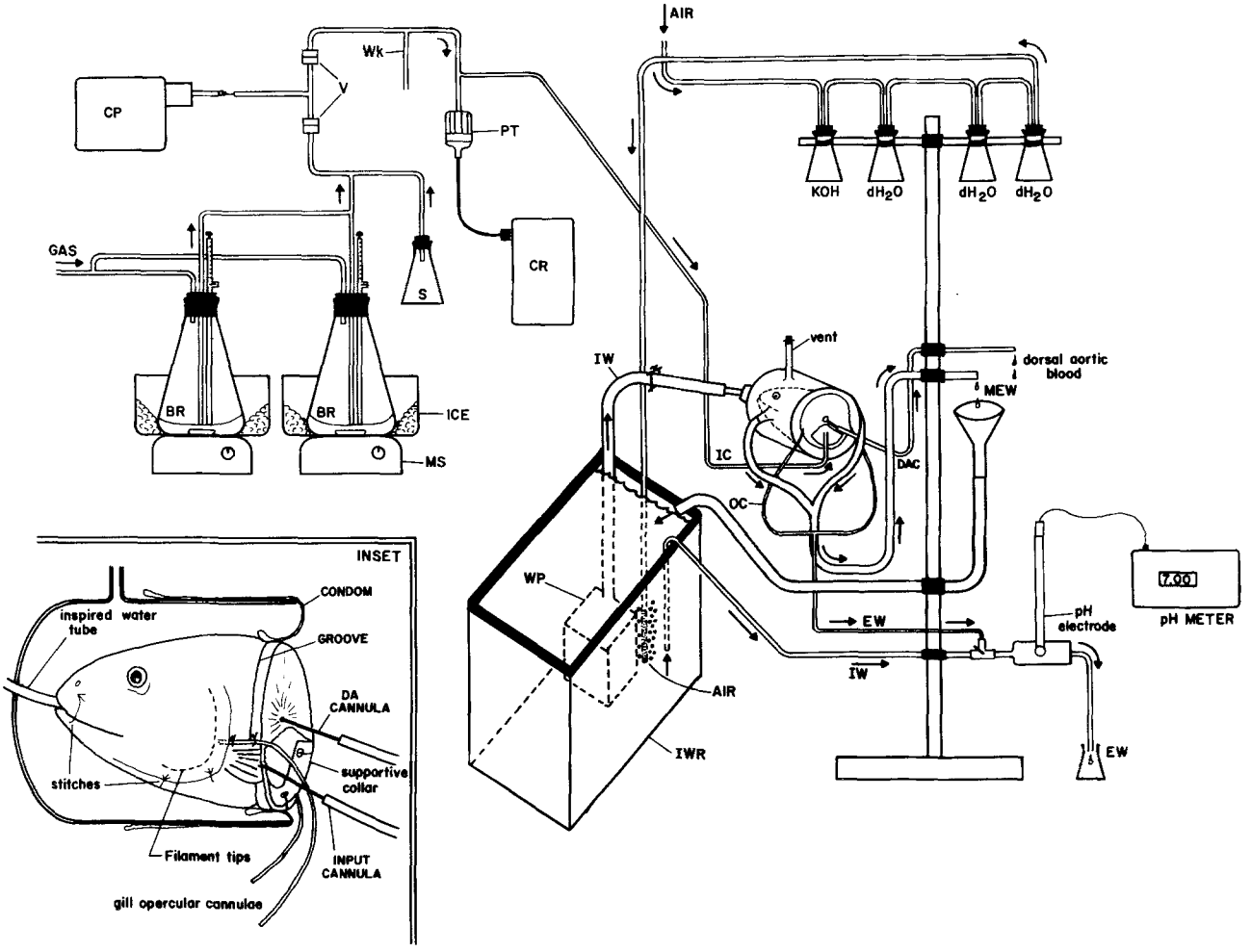

Fig. 2. Experimental apparatus for the isolated head preparation. The inset illustrates a close-up of the head which was placed in the plastic experimental chamber after surgery and sealed with a condom. The placement of the dorsal aortic (DA), venous (input), and opercular cannulae is shown. *BR* blood reservoir; *MS* magnetic stirrers; *CP* cardiac pump; *S* saline; *V* one-way valves; *Wk* Wind-kessel; *PT* pressure transducer; *CR* chart recorder; *IC* input cannulae; *DAC* dorsal aortic cannula; *OC* opercular cannulae; *EW* expired water; *MEW* mixed expired water; *IW* inspired water; *WP* water pump

around the outside of the chamber to make a tight seal. Water flow ($\dot{V}w$) over the gills was set to approx. 840 $ml \cdot min^{-1} \cdot kg^{-1}$ (water temp. 8 °C). Tapwater was air-equilibrated to ensure that water CO_2 levels were in equilibrium with air. The gills were perfused at constant pulsatile flow (blood flow rate $\dot{V}b = 11\ ml \cdot min^{-1} \cdot kg^{-1}$) with gas-equilibrated (see above) whole venous blood (diluted to 13–15% hct) from one of the two blood reservoirs. For details of the modified cardiac pump refer to Davie and Daxboeck (1983). Input (afferent) pressure (*Pi*) was monitored with a pressure transducer via a T-junction in the input catheter (Bell and Howell) and displayed on a Harvard chart recorder. Pulse pressure was kept constant at 10 cm H_2O by adjusting the size of a gas space at the top of a wide-bore side-arm (Windkessel) in the perfusion line. The pressure drop across the input catheter was measured after each experiment with ligatures still in place. Catheter resistance ($Rs = 13$ cm $H_2O \pm 1$ ($n = 18$)) was subtracted from measured total input pressure ($Pt = 81$ cm $H_2O \pm 3$ ($n = 18$)) for each preparation to determine corrected input pressure ($Pi = 69$ cm $H_2O \pm 4$ ($n = 18$)). Dorsal aortic pressure (Pda) was maintained between 10 and 15 cm above the level of the dorsal aorta (see Fig. 2; Perry et al. 1985a).

To test whether water flow was distributed evenly between both gills, a non-toxic dye (food colour) was added to the inflow water, and the pattern of flow from each opercular opening was assessed visually at the onset and termination of each experiment. Preparations were discarded if the water flow pattern was not approximately matched on both sides. The head was removed from the chamber at the end of the experiment, and the gills were examined once again to ensure that blood perfusion had been complete. The placement of the mouth tube was also checked, along with the position of the opercular catheters.

Experimental protocol and measurements. Two sets of paired arterial and venous blood samples were taken from each head preparation. The first samples (control) were collected 7 min after blood flow was initiated in the head preparation. At 8 min (elapsed time), the blood flow was drawn from the second blood reservoir and, depending on the experiment, the chemical composition of the inflow water was altered. The second samples (experimental), were collected 7 min later or after 15 min (elapsed time). The measurement times were chosen to allow adequate time for stabilization of the head preparation (based on perfusion pressure measurements) after the initiation of

Table 1. Differences in arterial-venous blood CO_2 (Ca_{CO_2}–Cv_{CO_2}), ammonia ($Tamm_a$-$Tamm_v$), and O_2 (Ca_{O_2}–Cv_{O_2}) content and blood flow rates ($\dot{V}b$) in the perfused head preparation. Means ± 1 SEM. Number of samples n in parentheses

	Ca_{CO_2}–Cv_{CO_2} (mmol·l^{-1})	$Tamm_a$–$Tamm_v$ (mmol·l^{-1})	Ca_{O_2}–Cv_{O_2} (mmol·l^{-1})
1. Control ($\dot{V}b^1 = 11.10 \pm 0.39$)			
Control	1.20 ± 0.13 (10)	0.40 ± 0.05 (10)	0.51 ± 0.06 (10)
Experimental	1.19 ± 0.16 (10)	0.40 ± 0.06 (10)	0.45 ± 0.06 (10)
2. Amiloride control ($\dot{V}b^1 = 11.48 \pm 0.63$)			
Control	2.20 ± 0.17 (8)	0.29 ± 0.04 (8)	0.44 ± 0.05 (8)
Experimental	1.85 ± 0.15 (8)	0.29 ± 0.04 (8)	0.41 ± 0.06 (8)
3. Acetazolamide ($\dot{V}b^1 = 11.46 \pm 0.34$)			
Control	1.70 ± 0.21 (8)	0.23 ± 0.04 (9)	0.35 ± 0.04 (5)
Experimental	0.66 ± 0.13* (8)	0.14 ± 0.04* (9)	0.18 ± 0.06* (5)
4. Tris buffer ($\dot{V}b^1 = 12.29 \pm 0.70$)			
Control	1.56 ± 0.06 (4)	0.26 ± 0.03 (4)	0.53 ± 0.11 (4)
Experimental	1.43 ± 0.19 (4)	0.19 ± 0.05* (4)	0.47 ± 0.05 (4)

[1] $\dot{V}b$ (ml·min^{-1}·kg^{-1}) did not vary between control and experimental periods, therefore values were averaged for each experiment

* Significantly different from control values, $P < 0.05$

blood flow and after a change in the composition of blood or water. The preparation was stable in terms of gas transfer for at least 30 min (Perry et al. 1985a). We were unable to assess the long-term stability of the preparation because the duration of perfusion was limited by the volume of blood in the reservoir. At the same time that blood samples were taken, inspired water pH (pHI) and mixed expired water pH (pHE) were recorded, along with $\dot{V}w$ (see below).

Four experimental treatments were performed:

1. In this treatment the chemical composition of the blood and water was not altered (control) to provide baseline excretion rates, and to determine the stability of the head preparation over the duration of the experiment.

2. In subsequent experiments designed to examine the linkage between branchial NH_3 diffusion and CO_2 excretion, it was important to isolate NH_3 from NH_4^+ excretion. In the second experiment, referred to as amiloride control, the Na^+ uptake blocker, amiloride (10^{-4} M) was added to the water in both the control and experimental periods to inhibit the excretion of NH_4^+, via the Na^+/NH_4^+ (H^+) ion exchange mechanism (e.g. Kirschner et al. 1973; Wright and Wood 1985). In freshwater fish, passive NH_4^+ flux across the gill is of minor quantitative importance (Kormanik and Cameron 1981a; Wright and Wood 1985). The chemical composition of the blood was not altered.

3. In the third experiment, the head was perfused initially as in the amiloride control group but during the experimental period, the head was perfused with blood that had been incubated (90 min) with the carbonic anhydrase inhibitor, acetazolamide (10^{-4} M) (Maren 1977), in order to inhibit carbon dioxide excretion across the gills (Swenson and Maren 1987).

4. A final experiment was performed to test whether the possible link between CO_2 and ammonia excretion was due to chemical reactions that acidify the boundary layer. Tris buffer was added to the ventilatory water during the experimental period to raise the buffering capacity approximately 3-fold in order to impede acidification of the water boundary layer. A combination of 'Trizma base' ($C_{14}H_{11}NO_3$, Sigma) and 'Trizma hydrochloride' ($C_4H_{12}ClNO_3$, Sigma) was used to make a 4×10^{-4} M solution at pH = 8.0. We assume that given the flow rate of water over the gills and the relatively small volumes of the buccal and opercular chambers, that Tris reached the water boundary layer water relatively quickly.

Blood pH, oxygen tension (Po_2) and total O_2 content (Co_2), Cco_2, plasma Tamm, and hct were measured on both arterial and venous blood samples, along with $\dot{V}b$ and perfusion pressure. Plasma epinephrine levels were measured on venous samples. Blood pH and Po_2 were measured with a Radiometer Blood Microsystem (BMS3 Mk2) and associated acid-base analyser (PHM 71), maintained at the experimental temperature. Co_2 was determined by the Tucker method (Tucker 1967). Cco_2 was measured using a total CO_2 analyser (Corning). Plasma Tamm levels were determined enzymatically as described by Kun and Kearney (1971). Venous plasma samples were frozen in liquid N_2 and stored at −80 °C for later quantification of epinephrine levels (1.8×10^{-7} $M \pm 0.2 \times 10^{-7}$, $n = 18$) using high pressure liquid chromatograph and electrochemical detection (Woodward 1982). $\dot{V}b$ was measured at the beginning and the end of each experiment and the values averaged since there were only small differences (< 5%). The rates of oxygen uptake ($\dot{M}o_2$) and carbon dioxide ($\dot{M}co_2$) and ammonia ($\dot{M}$Amm) excretion were determined by the Fick principle, where arterial-venous differences were multiplied by $\dot{V}b$.

Measurements of pHI and pHE were made continuously with a Fischer Accumet pH meter (829MP) by allowing either inspired or expired water to flow through a sealed chamber containing the pH electrode (see Fig. 2). Ventilatory water flow

($\dot{V}w$) was determined by collecting mixed expired water leaving the head chamber in a given period of time and accounting for the flow (~ 30 ml·min^{-1}·kg^{-1}) through the opercular cannulae.

Data are presented as means ± SEM. The Student's paired t-test was used to determine significance between control and experimental values.

Results

The differences in arterial-venous blood CO_2, ammonia, and O_2 for each experiment are given in Table 1, along with blood flow rates ($\dot{V}b$). The calculated gas transfer rates in the control experiment, where the chemical compositions of the blood and water were not altered, are displayed in the first column of Figs. 3 and 4. The head preparation was apparently stable throughout the experiment since there was no significant difference between the values in the first (control) and second (experimental) measurement periods.

In the second treatment, where amiloride was present in the water throughout the experiment and blood chemistry was not altered (amiloride control), there were no changes in arterial-venous C_{CO_2}, Tamm, and C_{O_2} (Table 1), nor in $\dot{M}_{CO_2}$, $\dot{M}_{Amm}$, $\dot{M}_{O_2}$ (Fig. 3) and pHi–pHe (Fig. 4) between the control and experimental measurement periods. The difference between pHa and pHv (pHa–pHv) showed a small, but significant, decrease between the control and the experimental period (Fig. 3). In general, amiloride in the water reduced $\dot{M}_{Amm}$ by about 30% relative to the initial control experiment, and resulted in a stimulation of $\dot{M}_{CO_2}$ ($\sim 70\%$).

With amiloride in the water throughout and acetazolamide in the blood during the experimental period (experiment 3), arterial-venous C_{CO_2} and $\dot{M}_{CO_2}$ were both reduced by 60% and arterial-venous Tamm and $\dot{M}_{Amm}$ were reduced by 40%, relative to the control period (Table 1, Fig. 3). In addition, there was a significant decrease in arterial-venous C_{O_2} and $\dot{M}_{O_2}$ (50%, Table 1, Fig. 3). The value pHa–pHv became negative with the acetazolamide treatment (Fig. 3), while pHi–pHe was reduced by 50% (Fig. 4).

In the fourth experiment, Tris was added to the water in the experimental period (amiloride was present throughout the experiment) to raise ventilatory water buffering capacity and impede water acidification in the boundary layer. This protocol resulted in a significant reduction in arterial-venous Tamm and $\dot{M}_{Amm}$ ($\sim 30\%$), in the absence of any change in arterial-venous C_{CO_2} and $\dot{M}_{CO_2}$ (Table 1, Fig. 3). There were no changes in arterial-

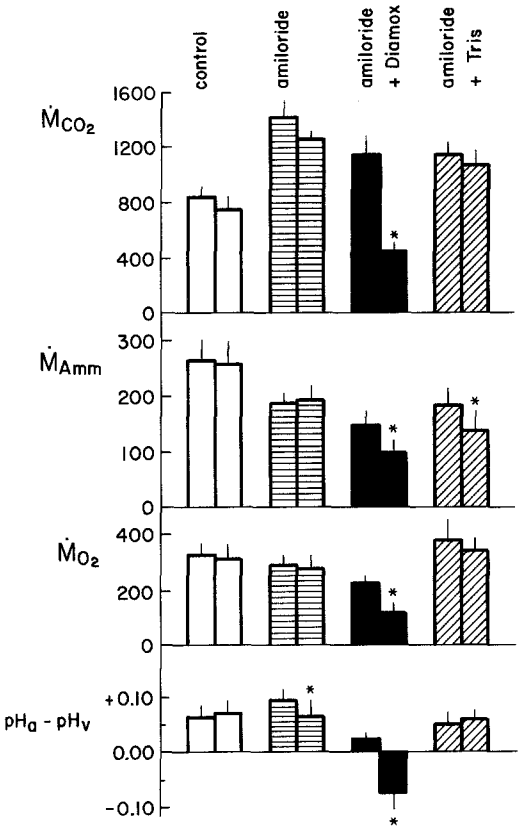

Fig. 3. The first (control) and second (experimental) measurements of carbon dioxide ($\dot{M}_{CO_2}$) and ammonia excretion ($\dot{M}_{Amm}$) and oxygen uptake ($\dot{M}_{O_2}$) in μmol·kg^{-1}·h^{-1}, are shown together with the differences between pHa and pHv (pHa–pHv), in the control ($n=10$), amiloride control ($n=8$), amiloride + acetazolamide ($n=6$), and amiloride + Tris ($n=4$) experiments. * denotes statistical significance ($P \leq 0.05$) between the first and second measurement. Means ± SEM

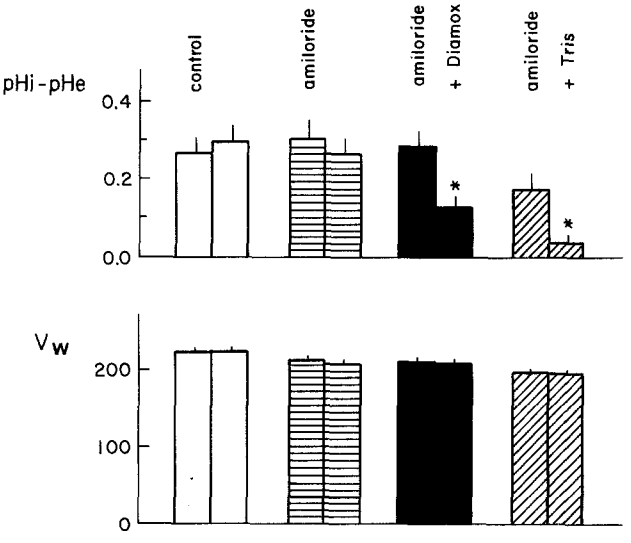

Fig. 4. The differences between the pH of inspired and expired water (pHi-pHe) and ventilation ($\dot{V}w$) in millilitres per minute per fish. See Fig. 3 legend for details

venous C_{O_2} and $\dot{M}_{O_2}$ nor in pHa–pHv (Table 1, Fig. 3). The difference between pHI and pHE was nearly eliminated with Tris buffer in the water (Fig. 4), clearly indicating the effectiveness of this protocol in preventing water acidification.

Discussion

Methodology

Several modifications were made to the original blood-perfused trout head preparation (Perry et al. 1985a, b) which enhance the simulation of in vivo conditions. Inspired water was delivered through a mouth tube tightly secured in a central position inside the buccal chamber, and at a rate comparable to that in exercising fish (Kiceniuk and Jones 1977). The pattern of water flow was assessed visually before and after measurements by adding a non-toxic dye to the inspired water to ensure that both opercular chambers were irrigated evenly. In other perfused-head preparations (for review see Perry et al. 1984a), a mouth tube is simply placed in the opening of the mouth. In preliminary tests (see Materials and methods), this technique was shown to be far less effective in producing an even flow of water through both opercular chambers. Also, previous investigators have typically set $\dot{V}_W$ at 500–1000 ml·min^{-1} (Payan and Matty 1975; Payan 1978; Perry et al. 1984b; Bornancin et al. 1985; Perry et al. 1985a, b), which is 2.5 times $\dot{V}_W$ in the present study and far in excess of the physiological range (Kiceniuk and Jones 1977; Wright et al. 1986; Iwama et al. 1987). The perfused head in the present study was irrigated with a large recirculating water reservoir (10 l) or flow-through water (control), in contrast to other studies which report a recirculating volume of 100–200 ml (Payan and Matty 1975; Payan 1978; Perry et al. 1984b; Bornancin et al. 1985; Perry et al. 1985a, b). These volumes are too small to maintain optimal O_2, CO_2, and NH_3 gradients across the gills over the experimental period.

In a detailed study, Wright and Perry (submitted) have compared gas transfer variables in vivo with those in the blood-perfused trout head. In brief, CO_2 excretion is 40–60%, and oxygen is 80–90% lower in the perfused head preparation compared to intact rainbow trout (Wright et al. 1986; Iwama et al. 1987; Wright and Perry, submitted). The lower $\dot{M}_{CO_2}$ and $\dot{M}_{O_2}$ values in the blood-perfused head, do not reflect abnormal gill function (i.e. impaired diffusive conditions), but rather are the result of reduced blood-to-water diffusion gradients, which in turn are largely set by venous gas tensions (Wright and Perry, submitted). In contrast to $\dot{M}_{O_2}$ and $\dot{M}_{CO_2}$, the rate of ammonia excretion was similar to in vivo values, which normally range between 200 and 350 μmol·kg^{-1}·h^{-1} (McDonald and Wood 1981; Cameron and Heisler 1983; Wright and Wood 1985; Vermette and Perry 1987). The ratio of ammonia to CO_2 excretion of resting, starved trout is approximately 0.10 (Wright, unpublished data), but following feeding, where ammonia excretion increases 3-fold (Brett and Zala 1975), it is likely that the $\dot{M}_{Amm}:\dot{M}_{CO_2}$ ratio may be closer to 0.3. In the present study, the control $\dot{M}_{Amm}:\dot{M}_{CO_2}$ ratio was 0.3.

The RE values ($\dot{M}_{CO_2}:\dot{M}_{O_2}$) in perfused trout preparations are consistently greater than 1.0 (this study, 2.9; Perry et al. 1982, 1.7; Perry et al. 1985a, 2.5), while in vivo values range between 0.8 and 1.0 (Iwama et al. 1987). The RE values in vivo reflect the rate of CO_2 production relative to O_2 consumption, while RE values in perfused preparations reflect blood-to-water O_2 and CO_2 gradients (see above). Hence, assuming that the conditions for CO_2 diffusion are similar to those for O_2 in the perfused head, our relatively high RE values presumably reflect the atypical O_2 (~3-fold greater than normal) and/or CO_2 (~1.5-fold lower than normal) venous tensions (Wright and Perry, submitted).

In conclusion, the blood-perfused trout head is a stable preparation because there were no significant differences between values in the first and second measurement period of the control experiment. $\dot{M}_{O_2}$ and $\dot{M}_{CO_2}$ were lower, while $\dot{M}_{NH_3}$ was similar to in vivo published values. The $\dot{M}_{Amm}:\dot{M}_{CO_2}$ ratio was in the range expected for fed rainbow trout. The absolute gas transfer rates were not critical but the $\dot{M}_{Amm}:\dot{M}_{CO_2}$ ratio was important because the objective of the study was to identify a link between CO_2 and ammonia excretion in the external gill boundary layer.

The linkage between carbon dioxide and ammonia excretion

In our experiments, it was essential to isolate NH_3 from NH_4^+ excretion in order to determine if NH_3 diffusion across the gill was linked to CO_2 excretion. In the amiloride control experiment, mean ammonia excretion decreased by 30% relative to the control experiment (i.e. 30% of ammonia is excreted as NH_4^+), the same as reported by Kirschner et al. (1973) for the semi-anaesthetized, artificially-ventilated trout, and compares well with the 23% reduction reported in vivo (Wright

and Wood 1985). The stimulation of carbon dioxide excretion with amiloride may be related to removal of CO_2 from the gill epithelium due to acidification caused by inhibition of H^+ excretion (H^+ ions may be excreted directly by Na^+/H^+ exchange, or may be excreted as NH_4^+ which is also linked to Na^+ uptake). The significant reduction in the value of pHa–pHv indicates that amiloride inhibited branchial H^+ ion excretion, but the effect on the blood compartment was delayed and only apparent in the second amiloride measurement.

Acetazolamide dramatically reduced $\dot{M}_{CO_2}$ (Fig. 3); inhibition of red cell carbonic anhydrase slows the flux of HCO_3^- into, and CO_2 out of, the red cell resulting in reduced CO_2 excretion across the gills (Swenson and Maren 1987). Moreover, acetazolamide also resulted in a significant decrease in $\dot{M}_{Amm}$ (Fig. 3) which demonstrates chemical coupling between ammonia and carbon dioxide excretion. The concommitant decrease in oxygen uptake was probably a direct result of the decrease in $\dot{M}_{CO_2}$, since O_2 and CO_2 gas transfer are related through chemical reactions involving haemoglobin in the red cell (Maren and Swenson 1980). The reversal of the pHa–pHv difference with acetazolamide is attributable to higher P_{CO_2} levels in arterial blood due to the inhibition of M_{CO_2}. It should be noted that the decrease in arterial pH did not affect NH_3 diffusion gradients because the calculated arithmetic arterial-venous mean P_{NH_3} values did not change significantly between the control (214 ± 23 μTorr) and acetazolamide (190 ± 20 μTorr) measurements. The difference between pHI and pHE (Fig. 4) was reduced as a direct result of the depression in CO_2 excretion and subsequent reduction in CO_2 hydration in the expired water.

It is possible that the link between CO_2 and ammonia excretion occurs internally, similar to the coupling between CO_2 and O_2 in the red cell, rather than in the boundary layer on the external surface of the gill. Tris buffer was added to the water in the final experiment to inhibit acidification of boundary layer water and test whether the link between CO_2 and ammonia was indeed due to acidifying chemical reactions in the boundary layer. Tris caused a significant reduction in ammonia excretion in the absence of any change in CO_2 excretion. Since CO_2 excretion and O_2 uptake did not change with the Tris treatment, it is unlikely that the change in $\dot{M}_{Amm}$ was due to direct effects of Tris on gill cell membrane permeability. Rather, we suggest that the decrease in $\dot{M}_{Amm}$ occurred because H^+ ions, normally available in the boundary layer from the hydration of CO_2, were buffered by Tris. This is also demonstrated by the fact that the difference between pHI and pHE was virtually eliminated with Tris in the water (Fig. 4). These experiments establish that NH_3 diffusion is linked to CO_2 excretion (acetazolamide experiment) and this linkage occurs in the gill boundary layer (Tris experiment). The model we propose is shown in Fig. 1. In it H^+ ions produced from the catalyzed CO_2 hydration reaction are used to protonate NH_3, which in turn facilitates NH_3 excretion.

In theory, it can be predicted that the effect of changes in CO_2 excretion on NH_3 diffusion in the intact animal would be more pronounced at lower $\dot{V}_W$ rates because boundary layer thickness will be increased. Piiper et al. (1986) have estimated the thickness of the boundary layer in dogfish (*Scyliorhinus stellaris*) at rest ($\dot{V}_W = \sim 200$ ml·min^{-1}·kg^{-1}) and during swimming, where $\dot{V}_W$ rates were similar to the present study ($\dot{V}_W = \sim 900$ ml·min^{-1}·kg^{-1}). They found that at rest the thickness of the boundary layer was about 20% greater than that in swimming fish. A 20% increase in the boundary layer would increase the diffusion pathway for NH_3 and accentuate the importance of chemical removal of NH_3 through interactions with the CO_2 hydration reaction in the boundary layer.

For ammonia excretion to be facilitated by the catalysed CO_2 hydration reaction in the gill water boundary layer, the apical epithelial membrane must be relatively impermeable to ion species. If the apical membrane was highly permeable to NH_4^+, HCO_3^-, or H^+ ions, then NH_3 levels in the water boundary layer would always be low, and there would be no need of a link between carbon dioxide and ammonia excretion. Our experimental demonstration of this linkage indicates that the gill water boundary layer is a distinct microenvironment and that the apical membrane is relatively impermeable to ions. This is consistent with accepted theory of freshwater gill epithelium ion permeabilities (for reviews see Girard and Payan 1980; Potts 1984).

The significance of the link between carbon dioxide and ammonia will be greatest for fish in alkaline waters. For instance, several species of tilapia inhabit the alkaline lakes of the Kenyan Great Rift Valley where water pH may be between 9.6 and 10.5 (Johansen et al. 1975). In these waters, the pK of ammonia is approximately 9, hence the majority of ammonia will exist as NH_3 and levels in the gill water boundary layer may be extremely high. Therefore, CO_2 excretion may be essential to maintain an acidic boundary layer adjacent to the gill which in turn facilitate NH_3 removal from

the boundary layer and enhance clearance from the blood.

In acidic environments, there will be little HCO_3^- formed as CO_2 is excreted into the gill water boundary layer. Under these conditions NH_3 excretion may alkalinize the boundary layer relative to the bulk water. The transition from an acidic to a more alkaline water boundary layer will depend on the relative excretion rates of CO_2 and NH_3 and the pK of the reactions involved.

Is the reverse situation i.e. that NH_3 diffusion into the boundary layer facilitates CO_2 excretion by buffering H^+ ions and enhancing the blood-to-water P_{CO_2} gradients, also possible? The fact that $\dot{M}_{CO_2}$ did not change with an approx. 3-fold increase in inspired water buffering capacity (Tris experiment), implies that under these conditions, increases in $\dot{M}_{Amm}$ would probably not affect the rate of CO_2 excretion. It is conceivable, however, that NH_3 acts as an important buffer in the boundary layer under other conditions, such as in poorly buffered or acidic waters, at maximal CO_2 production rates, at low $\dot{V}_W$ rates, and when boundary layer thickness is increased.

In summary, we have demonstrated a linkage between CO_2 and ammonia excretion in the blood-perfused trout head. The ammonia: CO_2 excretion ratios in our preparation are similar to those expected in intact feeding fish. Moreover, at lower ventilatory flow rates (rest), one would predict that changes in CO_2 excretion will cause even greater effects on NH_3 diffusion across the gills. For these reasons, we consider it highly likely that a linkage between CO_2 and ammonia excretion also exists in the intact trout.

Acknowledgments. Our thanks to G. Haddad (University Tavern) for his generous gift of the non-toxic dye and Drs. Moon, Mommsen, Walsh, Hyde and Boutilier for helpful criticisms on earlier versions of the manuscript. Financial support was provided by Natural Sciences and Engineering Research Council (NSERC) to SFP and the U.S. Environmental Protection Agency (project # CR812397) to DJR.

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