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Water chemistry changes in the gill micro-environment of rainbow trout: experimental observations and theory

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Summary. Soft water of low buffer capacity was drawn from near the branchial surface of rainbow trout (*Salmo gairdneri*) at 15 °C, using opercular catheters, to determine pH changes in water passing over the gills. Latex masks allowed measurement of ventilation volume, and concentrations of carbon dioxide, oxygen, ammonia, and titratable base in expired water were compared to concentrations in inspired water. Water passing over the gills was more basic than inspired water if the inspired water was pH 4–6 (maximum increase: +0.7 pH units near pH 5). Expired water was more acidic than inspired water if the inspired water was pH 6–10 (maximum decrease: −1.7 pH units near pH 9). Ventilation volume ($\sim 0.37\ l \cdot kg^{-1} \cdot min^{-1}$) and oxygen consumption ($\sim 1.7\ mmol \cdot kg^{-1} \cdot h^{-1}$) were constant in the pH range 4.6–10.1, but both increased by 1.6–2.4 × near pH 4. Carbon dioxide transfer near the gills was about 100 μ*M*, ammonia transfer about 15 μ*M*, and titratable base added at the gills was about 30 μ*M*. A theoretical model using CO_2, titratable base, and ammonia added at the gills, the titration characteristics of the defined soft water medium, and aquatic equilibria for CO_2 and ammonia, adequately explained the experimentally observed changes in pH near trout gills. Our observations and predictive model indicate that any gill contaminant whose toxicity varies with pH may be more or less toxic at the gills than predicted from bulk water chemistry alone.

Key words: Trout – Gills – pH – Water chemistry – Model

Introduction

Fresh water fish modify the water they breathe by extracting oxygen and ions, and releasing carbon dioxide, ammonia, and other metabolic end products at the gills. Transfers of carbon dioxide and ammonia may acidify or alkalinize expired water, respectively, depending on water pH (Lloyd and Herbert 1960; Randall and Wright 1989). In well-buffered water the changes in pH at the gills may be small, but in poorly-buffered soft water the pH changes at the gills may be large enough to help protect fish gills from damage in very acidic or very basic water. In addition, any environmental contaminant whose toxicity varies with pH may be more or less toxic at the gills than would be predicted from bulk water pH, because the different pH at the gills could change toxicant species or solubility. This situation was originally proposed to explain the toxicity of ammonia (Lloyd and Herbert 1960; Szumski et al. 1982), and later aluminum (Neville 1985; Wood et al. 1988; Playle et al. 1989) at fish gills, although the relevant experimental measurements have not yet been made.

Recently, Wright et al. (1986) developed a method to measure water pH near the gills of rainbow trout during normal ventilation. The objectives of our present study were (i) to use this technique to quantify the changes in pH occurring at rainbow trout gills over a wide range of inspired water pH (4.0–10.1) in a defined soft water of low buffer capacity, (ii) to determine the accompanying carbon dioxide, ammonia, and titratable base transfers at the gills, and (iii) to model the system using classical aquatic chemistry and the measured acid and base transfers. The model presented adequately explains the experimentally observed pH changes at the gills, and should be useful for improving predictions of the effects of toxicants on fresh water fish.

Abbreviations: pH_{ex} expired pH; pH_{in} inspired pH

Materials and methods

Adult rainbow trout (*Salmo gairdneri*) of both sexes, weight 281 ± 6 g (mean ± 1 SEM, n = 52), were purchased from Spring Valley Trout Farm, New Dundee, Ont. They were held in dech-

lorinated Hamilton city tapwater (hard water; $Ca^{2+} \sim 2$ mequiv·L^{-1}, $Na^+ \sim 0.6$ mequiv·L^{-1}, $Cl^- \sim 0.8$ mequiv·L^{-1}, titratable alkalinity to pH 4.0 ~ 1.9 mequiv·L^{-1}, pH ~ 8.0) at 15–20 °C and were fed floating trout pellets (Martin Feed Mills, Elmira, Ont., Canada) twice weekly. At least two weeks before an experiment the fish were placed in a flowing soft water acclimation tank and feeding was suspended. Soft water was produced from dechlorinated tapwater passed through a reverse osmosis unit (Culligan MP1000) or through deionising resin cannisters (J.W. Anderson Co. Ltd., Dundas, Ont., Canada). Appropriate amounts of analytical grade NaCl and $CaCl_2$ (BDH, Toronto, Ont., Canada) were added by peristaltic pump. Acclimation conditions in the standard soft water were $Ca^{2+} \sim$ 47 μequiv·L^{-1}, $Na^+ \sim 68$ μequiv·L^{-1}, $Cl^- \sim 95$ μequiv·L^{-1}, titratable alkalinity ~ 130 μequiv·L^{-1}, pH ~ 6.7, at 15 °C. This water composition is typical of natural, poorly buffered soft waters from the Pacific coast of Canada, in which *Salmo gairdneri* is endemic, much of northeastern North America, and Scandinavia. Water pH was monitored daily (Radiometer PHM82 pH meter and a Radiometer GK2401C electrode), and Ca^{+2} and Na^+ concentrations were measured every few days (atomic absorption spectrophotometry; Varian 1275); Cl^- and titratable alkalinity were measured occasionally by methods outlined below.

For the operations, fish were initially anaesthetised with 0.5 mg·L^{-1} MS222 (Sigma, Saint Louis, Missouri, USA) buffered to pH 6.5 with KOH, then three-quarter strength anaesthetic was used during the rest of the operation. A latex surgical glove with thumb and fingers removed was sewn around each fish's mouth to serve as a ventilation mask; the thumb hole fitted over the fish's head (see Cameron and Davis 1970, and Wright et al. 1986 for more details). After this operation was completed, a hole was punched with a 2 mm O.D. trochar about 1 cm from the posterior margin of an operculum, and an 85-cm length of Clay-Adams PE-190 polyethylene tubing was threaded through the hole. The tubing was heat-flared, and the flange rested against the inside of the operculum. A 0.5-cm flanged piece of PE-240 tubing was placed against the outside of the operculum, and was held tightly in place by a right angle bend in the catheter near the operculum and by a knot of surgical silk. The opercular catheters stayed in place well and did not appear to hinder normal opercular movements. Affixing a mask and catheter took a total of about 45 min.

Fish fitted with latex masks and opercular catheters were placed individually in one of 5 darkened and compartmentalised Plexiglas boxes. These ventilation collection boxes were identical in design to those described by Cameron and Davis (1970). The latex masks were fitted over pegs on a retaining ring which formed a seal between anterior and posterior chambers of the boxes. Water breathed by the fish passed from the anterior chamber, over the gills, into the posterior chamber. Standpipes in each chamber were set so that there was no pressure differential between the two chambers. Vigorously-aerated water flowed from a head tank into the anterior chamber of each fish box at a rate greater than the fish's ventilatory demand, then overflowed to waste. After 24 h the opercular catheters were tested to ensure they could provide an adequate flow (2–4 ml·min^{-1}) by siphon, and to ensure they were sampling from a site which provided a representative oxygen extraction (cf. Davis and Watters 1970). If the difference between inspired and expired O_2 concentrations was below about 20 μ*M* the catheter was repositioned on the operculum (a 15-min operation); this was done to avoid using a catheter that was drawing water from an anatomical dead space. The fish were allowed to recover for ~ 48 h after the initial operations, and ~ 24 h after any catheter reimplantations, before an experiment was begun.

The inspired pH of the standard soft water was varied using either 0.5 *M* H_2SO_4 or 1 *M* KOH, delivered to the head tank by a magnetic valve controlled by a Radiometer PHM82 pH meter and Radiometer GK2401C combination electrode. The water was vigorously aerated to ensure that inspired P_{O_2} and P_{CO_2} remained constant. Five fish at a time were exposed to basic or acidic soft water for 2–3 h, measurements taken, then to water at another pH for a further 2–3 h. The order of exposures was random, apart from extreme pHs. In general, fish recovered from moderate acid or base exposures quickly (pH 5.0–9.0); as a precaution exposures to extreme pHs (<5.0, >9.0) were made at the end of the day, followed by a return to circumneutral pH overnight, as a recovery period. Soft water Ca^{2+} and Na^+ concentrations averaged 54 ± 1 and 63 ± 1 μequiv·L^{-1} (± 1 SEM; $n = 97$), respectively, over the course of the experiments; experimental temperatures were 15–16 °C.

The opercular catheters siphoned water from near the gills at a rate of 2–4 ml·min^{-1}; inspired water was similarly siphoned from the anterior chamber of each fish box using PE-190 tubing. Siphoned water flowed continuously through a closed 7.5-ml polyethylene vial into which was sealed a Radiometer GK2401C pH electrode; the actual volume of water surrounding the pH electrode was about 4.5 ml. A Radiometer PHM82 pH meter was used. Siphoned water was stirred continuously with a magnetic flea and flowed through the vial and out another port. Alternate inspired and expired water pH measurements were made using the same pH electrode. After drainage of the vial for a new sample, and the vial had refilled, three minutes were allowed to elapse for thorough flushing. Usually the pH reading stabilized within one minute, which corresponded to total delay in the system, including flow from gills to vial. Mean pH in a continually flowing system was measured so electrode response time was not a complication.

Ventilation volume ($\dot{V}_w$) for each fish was measured as the water volume overflowing from the posterior chamber of the ventilation collection boxes in 1 min. Oxygen tension of inspired and expired water was measured immediately on samples drawn anaerobically from the pH vial and injected into a Radiometer E5046 micro-electrode unit kept at 15 °C and connected to a Radiometer PHM72 acid-base analyzer. Water P_{O_2} values (torr) were converted to O_2 concentrations (μ*M*) using the solubility of O_2 at 0% salinity (Boutilier et al. 1984). Inspired water was always near saturation at 15 °C (~ 300 μ*M*). Ammonia samples were collected by filling 7.5-ml polyethylene vials from the inspired and expired siphons. Samples were frozen and later thawed and analyzed using the salicylate-hypochlorite method (Verdouw et al. 1978).

Samples for carbon dioxide analysis were collected via the siphons into 2-ml glass vials, capped tightly, and analyzed within 4 h. 1-ml aliquots of sample were mixed in a 5-ml glass syringe with 0.5 ml HCl (0.1 *M*) and 4.5 ml helium, to liberate all CO_2 as gas. The gas was injected into a Shimadzu GC-8A gas chromatograph with Shimadzu C-R3A integrator. 0.0, 0.1, 0.2, and 0.5 m*M* $NaHCO_3$ standards were used. There was no indication of CO_2 production or consumption during sample storage (<4 h) in the glass vials. Titratable base (acid neutralizing capacity) of inspired and expired water samples was measured by titrating 10 ml samples to pH 4.0 with 0.02 *M* HCl using Gilmont microburettes. The samples were at room temperature and were bubbled with air during the titrations. Total titration time was 14 min to allow adequate time for the conversion of HCO_3^- to CO_2 and its subsequent diffusive loss.

In this study the difference between expired and inspired values for a given parameter are usually reported, and are referred to as "Δ" or "transfer", i.e., ΔpH, ammonia transfer. Oxygen transfer, ammonia transfer, and ΔpH were determined in all experiments. Measurements of CO_2 transfer and titratable

base are more difficult and time-consuming, so were done only in a subset of experiments representative of the inspired pH range as a whole.

Titration curves in vitro of the acidic or basic water at 15 °C were determined on 10-ml, stirred samples using 0.02 *M* HCl or NH_4OH, delivered by Gilmont microburettes. An electrode equilibration time of 3 min between each addition of titrant was used. Samples at all pH_{in} starting values were titrated down by about 2 pH units with HCl. Samples at pH_{in} values below ~6.5 were also titrated up with NH_4OH by about 2 pH units, to determine the effects of both base and acid additions at these pHs. Stirring, instead of aerating samples was done because here we wanted to assess all the buffering in the water, including HCO_3^-. Inspired and expired water samples from three fish were also titrated, to assess buffering effects of substances released at the gills. In this instance the inspired (circumneutral) water and expired water were brought to pH 4.0 quickly with HCl, then the titration curves determined up to pH ~8.5 with NH_4OH additions, as above. Expired and inspired water samples from these three fish were also analysed for anions released at the gills. High pressure liquid chromatography (HPLC; Waters 510 pump, Waters 430 conductivity detector, and Waters IC-Pak anion exchange column) was used to assay for phosphate (PO_4^{3-}, HPO_4^{2-}, $H_2PO_4^-$), Cl^-, NO_2^-, NO_3^-, and SO_4^{2-} in 100-µl filtered samples of inspired or expired water.

In order to determine the effect of CO_2 additions on the pH of the standard soft water in vitro (in relation to model predictions), samples at various pH_{in} values were bubbled for 1–3 min with 0.3% CO_2 (in air) at 15° C. A Wösthoff 301-AF gas mixing pump was used. The aim was to achieve a ΔCO_2 approximately equivalent to that observed in vivo (~100 µ*M*; see Results), and of course this was reached more quickly at the higher starting pHs. In practice, a variety of gassing durations were tried, and those producing a ΔCO_2 closest to 100 µ*M* were used. The CO_2 samples and the pH readings were taken simultaneously. Carbon dioxide in the water was measured in the usual manner by gas chromatography; ΔpH was measured using a Radiometer GK2401C pH electrode and Radiometer PHM82 pH meter.

Experimentally observed data are presented as means ± 1 standard error (SEM). "*n*" represents the number of different fish contributing to each mean. When experimental data were recalculated for the modelling exercise, or when model-predicted curves were compared with experimentally observed values, 95% confidence limits around the means of the observed data have been used. For out data, 95% confidence limits are 2.1–2.8× larger than 1 SEM. The least squares method was used for linear regression calculation.

Results

Rainbow trout were exposed to acidic and basic soft water to study the influence of inspired water pH on ventilation volume, oxygen consumption, ammonia excretion, and pH changes in water passing over the gills. Sixteen different soft water pHs in the pH range 4.0–10.1 were examined. Data from all 52 trout are included in these analyses (Figs. 1, 2). Expired water drawn from near the gills of rainbow trout was more basic than inspired soft water if the inspired water was acidic (pH_{in} = 4–6; Fig. 1). For example, expired water was about 0.7 pH units higher (i.e., pH ~5.7) than inspired water of pH ~5. Expired water was more acidic than inspired water if the inspired water was circumneutral or basic (pH_{in} = 6–10; Fig. 1). For example, if inspired water was about pH 9 then expired water was about pH 7.3 (ΔpH ~ −1.7). The ΔpH was low (+0.1) at pH_{in} ~4, most positive at pH_{in} ~5 (+0.7), zero near pH_{in} = 6, most negative at pH_{in} ~9 (−1.7), and was only about −0.7 at pH_{in} ~10 (Fig. 1).

Ventilation volume ($\dot{V}_w$) of the fish was approximately constant (~3.7 $L \cdot kg^{-1} \cdot min^{-1}$) over the inspired soft water pH range 4.6–10.1 (Fig. 2A). However, $\dot{V}_w$ increased 1.6-fold at pH_{in} = 4.4 and 2.4-fold at pH_{in} = 4.0. Oxygen consumption by the fish ($\dot{M}O_2$), measured as oxygen transfer at the gills ((inspired $[O_2]$ − expired $[O_2]$)·$\dot{V}_w$), was about 1.7 $mmol \cdot kg^{-1} \cdot h^{-1}$ over the same pH_{in} range 4.6–10.1 (Fig. 2B). Oxygen transfer at the gills was also approximately constant at about 85 µ*M*. In accord with the increase in $\dot{V}_w$ at very low pH, $\dot{M}O_2$ at pH_{in} < 4.6 was ~1.6× higher than over the rest of the pH_{in}. Oxygen transfer at the gills decreased only to 50 µ*M* at $pH_{in\ range}$ = 4.0 (Fig. 3), in spite of the 2.4-fold increase in $\dot{V}_w$, which resulted in the net increase in $\dot{M}O_2$ at this inspired pH. Ammonia excretion ($\dot{M}_{amm}$) was also approxi-

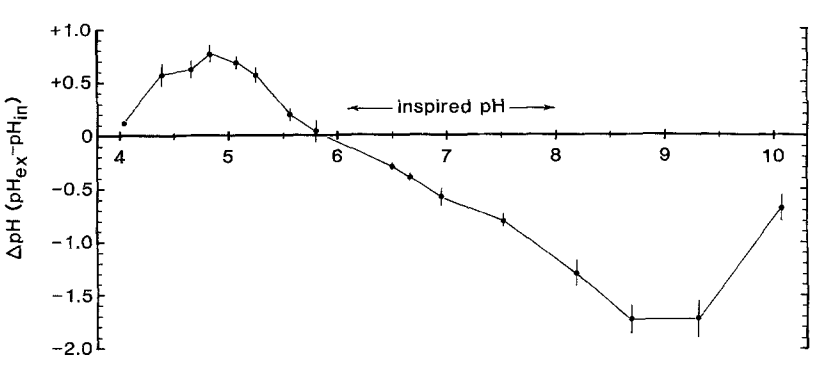

Fig. 1. The difference between pH of expired (pH_{ex}) and inspired soft water (pH_{in}) plotted against inspired water pH for rainbow trout fitted with opercular catheters and latex masks. Positive ΔpH: expired water is more basic than inspired water; negative ΔpH: expired water is more acidic than inspired water. Means ± 1 SEM are indicated. A total of 52 trout were exposed in fish boxes in a flow-through system to water of different acidities for 2–3 h. Mean number of fish represented at each point is 12; minimum number is 5 (pH_{in} = 5.2, 5.8, 7.0, 8.2); and the maximum number represented is 26 (pH_{in} = 6.5)

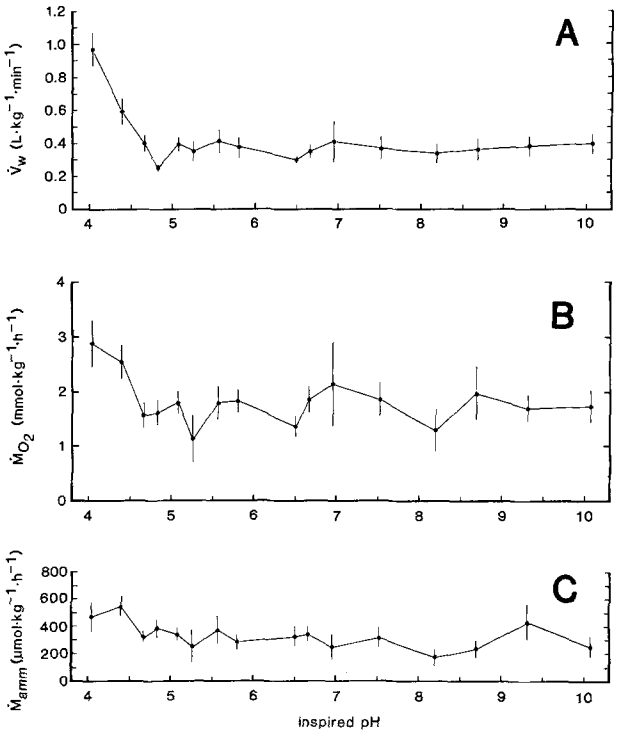

Fig. 2. A Ventilation volume ($\dot{V}_w$) of rainbow trout fitted with latex masks, in acidic and basic soft water. $\dot{V}_w$ was approximately constant in the pH_{in} range 4.6–10.1, but was about 1.6× and 2.4× higher at $pH_{in}=4.4$ and 4.0, respectively. Means ± 1 SEM; number of fish at each point as given in Fig. 1. **B** Oxygen consumption ($\dot{M}_{O_2}$) of rainbow trout in acidic and basic soft water. $\dot{M}_{O_2}$ was approximately constant at pH_{in} 4.6–10.1, but increased by about 1.6× at $pH_{in}<4.6$. Means ± 1 SEM; number of fish at each point as in Fig. 1. **C** Ammonia excretion ($\dot{M}_{amm}$) of rainbow trout in acidic and basic soft water. $\dot{M}_{amm}$ was approximately constant at pH_{in} 4.6–10.1, but was about 1.7× higher at $pH_{in}<4.6$. Means ± 1 SEM; number of fish as given in Fig. 1

mately constant ($\sim 300\ \mu mol \cdot kg^{-1} \cdot h^{-1}$) over the pH_{in} range 4.6–10.1 (Fig. 2C; ammonia transfer at the gills decreased only to 8 μM at $pH_{in}=4.0$ (Fig. 3) in spite of the 2.4× increase in $\dot{V}_w$, so a net increase in $\dot{M}_{amm}$ at this pH_{in} was seen. In summary, $\dot{V}_w$, $\dot{M}_{O_2}$, and $\dot{M}_{amm}$ were remarkably constant over a wide range of inspired pH during these relatively short (2–3 h) exposures, and increased only under very acidic conditions ($pH_{in}<4.6$).

Changes in water chemistry at the gills were examined in greater detail in 10 trout exposed to soft water of seven different inspired pHs. In these fish CO_2 and titratable base transfers to the water were measured, as well as the usual O_2 and ammonia measurements. Mean CO_2 and O_2 transfers at the gills of these fish were about 100 and 85 μM, respectively, excluding values from $pH_{in}=4.0$ (Fig. 3). Oxygen and CO_2 transfers at the gills were lower at $pH_{in}=4.0$, associated with the higher ventilation volume at that pH. The relative decrease in CO_2 transfer (~60%) was greater than that in O_2 transfer (~40%) at $pH_{in}=4.0$. Mean titratable base added at the gills was about 30 μM, and ammonia transfer at the gills was about 15 μM (Fig. 3). Again, the notable feature of all these transfers at the gills, except at the lowest inspired pH (4.0), was their constancy over a wide range of inspired pH (4.6–10.1).

Composite titration curves of the soft water used are given in Fig. 4A. Two curves are presented because the buffer capacity of the un-modified or acidified inspired water was very low (i.e., negligible bicarbonate alkalinity), whereas the buffer capacity of alkalinized inspired water was higher in the pH range 5.5–7.0. This difference was due to the fact that addition of base (KOH) for alkalinization resulted in the fixation of atmospheric CO_2 as HCO_3^-; when the alkalinized soft water was titrated downwards, the buffering effect of the extra HCO_3^- became apparent in the pH range 7.0–5.5. Similarly, bicarbonate buffering was added to water breathed by the fish as CO_2, ammonia, and titratable base were released at the gills (cf. expired, inspired curves of Fig. 4B.

Using the titration curves appropriate to the inspired pH, as illustrated in Fig. 4A, we calculated the amount of base or acid needed to change the inspired water pH to the experimentally observed expired water pH, given in Fig. 1. The results of this analysis are presented in Fig. 5. For example, at $pH_{in}=5.0$, about 15 μM of "base" were apparently needed to raise the expired water pH to about pH 5.7 (Fig. 5); at $pH_{in}=9.0$, about 80 μM of "acid" were needed to lower the expired pH to pH 7.3. Near $pH_{in}=6$ no net base or acid addition was apparent at the gills (i.e., observed $\Delta pH=0$).

We next tried to explain the apparent base and acid additions by the fish to the water near the gills using the measured CO_2, ammonia, and titratable base transfers, and aquatic CO_2 and ammonia equilibria. In water, CO_2 dissociates to HCO_3^- and H^+ (pK ~ 6.3), then from HCO_3^- to CO_3^{2-} and H^+ (pK ~ 10.3; Stumm and Morgan 1981). Ammonia dissociation ($NH_4^+ \rightleftharpoons NH_3 + H^+$) has a pK of about 9.5 (Cameron and Heisler 1983). Using these equilibria we calculated the theoretical base and acid additions at trout gills. Five assumptions were made: (i) CO_2 released at the gills was 100 μM (Fig. 3), (ii) titratable base released at the gills was 30 μM, of which ammonia (assumed to all be released as NH_3) contributed 15 μM (Fig. 3), (iii) instantaneous reactions at the gills, (iv) con-

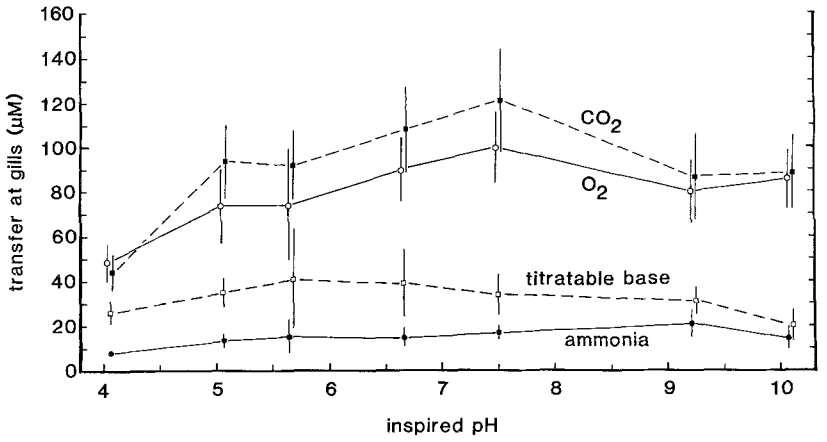

Fig. 3. Carbon dioxide, oxygen, titratable base, and ammonia transfers at the gills of rainbow trout held in soft water of various acidities. Transfers were approximately constant at pH_{in} 5.1–10.1, but were reduced at $pH_{in} = 4.0$, in accord with increased $\dot{V}_w$ at that pH. Means ± 1 SEM; $n = 10$ except at $pH_{in} = 5.6$ and $pH_{in} = 7.5$, where $n = 5$ and 6, respectively

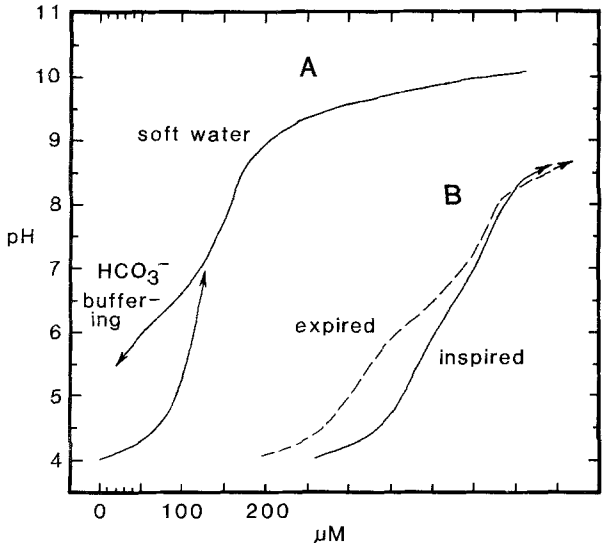

Fig. 4. A Composite titration curves for the soft water used. Buffer capacity of alkalinized water was higher in the pH range 5.5–7.0 than that of acidified or un-modified water, because of HCO_3^- buffering. Arrows indicate the direction to which the titrations apply. Horizontal axis: amount of acid or base, in μ*M* (μequiv·l^{-1}). See text for further details. **B** Titration curve of expired water of a single fish, compared with the titration curve of the inspired water it was breathing. Bicarbonate buffering was added to the expired water because of CO_2, ammonia, and titratable base transfers at the gills. Inspired water pH was 6.6. Two other fish for which we have expired and inspired titration curves showed similar added buffering in the expired water. Titration curves in B are displaced to the right of those in A for clarity only; the same vertical and horizontal scales apply to all curves

stant fish ventilation volumes, and (v) a situation in which water pH did not change as base or acid were added (i.e., a perfectly buffered system). The last assumption is clearly untrue, but simplified the modelling process.

Theoretical base and acid additions at rainbow trout gills, using the above five assumptions, are portrayed in Fig. 6. 30 μ*M* base are added between pH 4 and 10, of which 15 μ*M* are added as NH_3. Above pH 8.5, the base addition owing to NH_3 decreases to 7.5 μ*M* at pH 9.5 (the pK of ammonia) and decreases to zero by about pH 10.2. Acid addition by 100 μ*M* CO_2 is negligible below about pH 5, is 50 μ*M* at pH 6.3 (the first pK of CO_2), and increases to its full 100 μ*M* by pH 8. At pH 10.3 (the second pK of CO_2), 50% of the CO_2 released at the gills is further converted to CO_3^{2-}, so 150 μ*M* H^+ are produced. The theoretical sum of titratable base and CO_2-acid added at the gills is about 28 μ*M* base at pH 5, no net addition at pH 6.1, about 70 μ*M* acid added near pH 8, and 100 μ*M* acid added at pH 10.1 (Fig. 6).

The next step was to convert the theoretical acid and base added at the trout gills to predicted ΔpH at the gills by means of the soft water titration curves (Fig. 4A). To do this conversion we used an iterative calculation to account for changes in pH at the gills in the poorly buffered water as acid (CO_2) and base are released into it; changes in pH would affect further CO_2 dissociation. The iterative calculation overcame the problems with assumption (v) above. The calculation was the equivalent of adding 100 μ*M* CO_2 and 30 μ*M* base in 10 equal parts to soft water at each inspired pH. The final pH_{ex} was the cumulative effect of each small addition of acid and base on pH and therefore on dissociation of the CO_2 portion of the next small acid and base addition. A good fit was obtained between theoretically predicted and experimentally observed ΔpH at the gills (Fig. 7). We also calculated the theoretical ΔpH assuming that no base was released at the gills at $pH_{in} > 8.6$, which fits the observed data better at high inspired pH (see Discussion).

To check that CO_2 did lower water pH as predicted, we used 0.3% CO_2 to add, in vitro, about

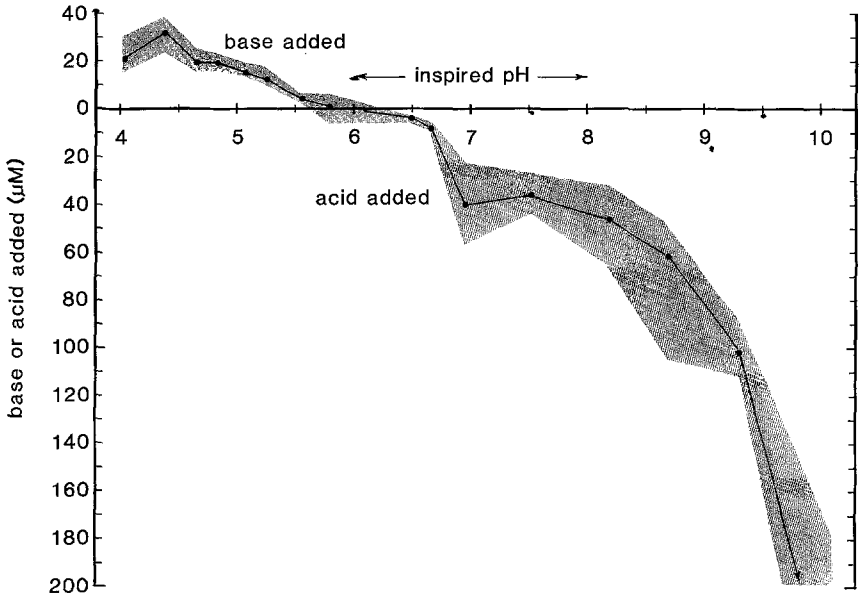

Fig. 5. Amount of base or acid needed to change inspired pH to the experimentally observed expired pH (from Fig. 1), calculated from the appropriate titration curves given in Fig. 4A. See text for further details. Above the horizontal axis base is added; below, acid is added, in μM (μequiv·L^{-1}). 95% confidence limits are indicated about the mean values

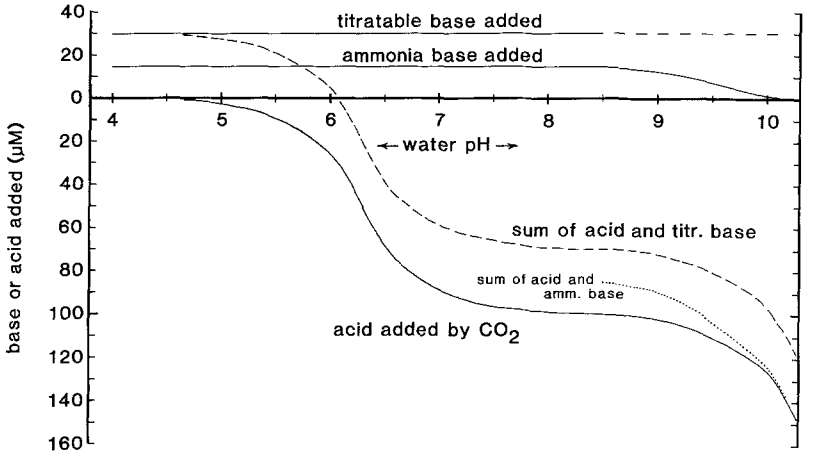

Fig. 6. Theoretical acid or base contributions of 100 μM CO_2 and 30 μM base (including 15 μM as NH_3) at equilibrium in water of various acidities. See text for further details. Above the horizontal axis base is added; below, acid is added

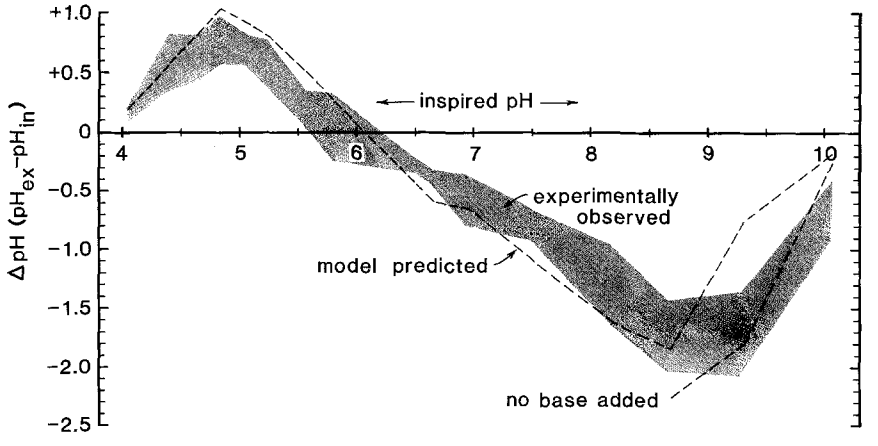

Fig. 7. Model predicted ΔpH vs pH_{in} contrasted with experimentally observed ΔpH vs pH_{in} (from Fig. 1). 95% confidence limits of the means are indicated for the observed ΔpH vs pH_{in} curve. The predicted curve was calculated from the theoretical acid and base contributions at rainbow trout gills (Fig. 6) and the appropriate titration curves of the water used in the experiments (Fig. 4A). An iterative calculation was used to account for the changes in water pH as acid or base were released into the poorly buffered water. Predicted ΔpHs assuming that no base was released at the gills are also presented for $pH_{in} > 8.6$. See text for further details

Table 1. Comparison of observed ΔpH and predicted ΔpH when 0.3% CO_2 is gassed into poorly buffered soft water of various starting pHs (see text for details). Correlation coefficient of observed and predicted ΔpH is 0.98. Equation of the line is: observed $\Delta pH = 0.70 \cdot$(predicted ΔpH)-0.15

Initial pH	ΔCO_2, μM (measured)	ΔpH (observed)	ΔpH (predicted)
4.74	85	−0.06	−0.02
5.42	114	−0.41	−0.22
6.15	103	−0.74	−0.75
6.64	99	−0.84	−0.64
7.26	102	−0.80	−1.23
8.30	148	−1.72	−2.50
9.07	156	−2.39	−2.97
10.03	174	−0.41	−0.53

100 μM CO_2 to soft water of different starting pH. The actual ΔCO_2 added was measured, and the ΔpH was predicted using the equilibria of Fig. 6, the iterative calculation, and the appropriate soft water titration curve (Fig. 4A). Predicted ΔpHs were compared to observed ΔpHs (Table 1). Observed ΔpH and predicted ΔpH correlated well (correlation coefficient = 0.98, $P < 0.001$); the slope of the line was 0.70, not significantly different from a slope of 1.0 ($P > 0.10$).

Finally, using the titration curves of our soft water we calculated (Fig. 8) how much base or acid would have to be added to our poorly buffered water to produce the predicted ΔpH versus pH_{in} curve shown in Fig. 7. In essence Fig. 8 is a redrawing of Fig. 6, with the 100 μM CO_2 and 30 μM base added into a poorly buffered solution instead of into a perfectly buffered system (again overcoming the problems with model assumption (v)). The acid or base needed to produce the theoretically predicted ΔpH curve agrees well with the amount of acid or base needed to change the inspired water pH to the experimentally observed expired water pH (Fig. 8). For $pH_{in} > 8.6$ we also calculated the acid needed to produce the theoretical ΔpH curve assuming no base was added at the gills (see Fig. 7 and Discussion).

A potential, small source of error in this analysis could be the addition of buffer substances (besides CO_2/HCO_3^- and NH_3/NH_4^+) to the soft water as it passes over the gills. For example, it is possible that some of the change in slope and position of the titration curves of expired water relative to inspired water (e.g., Fig. 4B), attributed here to net CO_2, ammonia, and titratable base transfers, could reflect an addition of some other buffer at the gills. Inorganic phosphate, with a pK ~ 6.8, was an obvious candidate. Using HPLC we surveyed the inorganic anion content of inspired and expired water samples from three fish. Neither phosphate, NO_2^-, NO_3^-, nor SO_3^{2-} were released at the gills in greater than 1 μM concentrations. Up to 10 μM Cl^- were lost at the gills, but this ion is fully dissociated in water so would be detected in the titratable base measurement (if not matched by equimolar cation loss). The release of inorganic buffers at the gills does not appear to be a complicating factor in our analysis, although the possibility of organic buffer release at the gills (e.g., glycoproteins) cannot be eliminated at present.

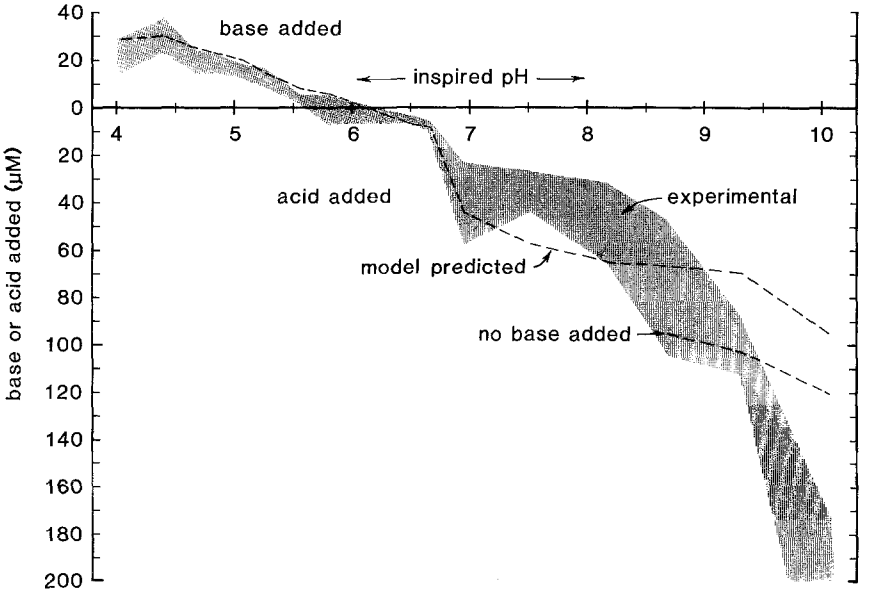

Fig. 8. Base or acid needed to change inspired pH to the model predicted expired pH, contrasted with the base or acid needed to change inspired pH to the experimentally observed expired pH (from Fig. 5). 95% confidence limits of the means are given for values derived from observed expired pHs. Above the horizontal axis base is added; below, acid is added. For $pH_{in} > 8.6$ the predicted acid added is also indicated assuming no base is released at the gills. See text for further details

Discussion

Our results clearly show that water chemistry in the gill micro-environment is different than the chemistry of the bulk water in which a fish lives and which a fish breathes. Poorly buffered soft water near the gills has a higher pH than does acidic inspired water, and water near the gills has a lower pH than circumneutral or basic inspired water, with a crossover point near $pH_{in}=6.0$. In similar soft water, Wright et al. (1986) reported a gill ΔpH of about -0.6 to -0.8 at $pH_{in}\sim 7.2$, and Holeton and Randall (1967) reported a ΔpH of about -0.2 to -0.3 for pH_{in} 6.9–7.4, close to the present values at these inspired pHs (Fig. 1).

Experimentally determined CO_2, titratable base, and ammonia transfers at the gills (about 100, 30, and 15 μM, respectively), and the in vitro titration characteristics of the defined soft water medium, were used in a model to explain the pH changes seen at the gills. A good fit between theoretical and observed values was obtained (Fig. 7). This good fit suggests that CO_2, base, and ammonia transfers at fish gills adequately account for pH changes at the gills, and that the assumptions of the model are reasonable. The model assumptions are discussed below.

We assumed, based on our experimental measurements, that CO_2 transfer at rainbow trout gills was about 100 μM (Fig. 3). Expressed as CO_2 excretion ($\dot{M}CO_2$), 100 μM CO_2 for our fish is 2.2 $mmol \cdot kg^{-1} \cdot h^{-1}$ (using $\dot{V}_w$ of 0.37 $l \cdot kg^{-1} \cdot min^{-1}$; Fig. 2A). This number agrees well with previously reported $\dot{M}CO_2$ values for rainbow trout (fitted with masks) of 2.4–3.9 $mmol \cdot kg^{-1} \cdot h^{-1}$ (Wright et al. 1986), and about 1.1–2.7 $mmol \cdot kg^{-1} \cdot h^{-1}$ (Iwama et al. 1987). Conversely, using average reported fish weights and ventilation volumes, these published $\dot{M}CO_2$ values can be converted to CO_2 transfers: 95–109 μM (Wright et al. 1986) and 52–127 μM (Iwama et al. 1987). It appears then, both from our own data and other published values, that 100 μM CO_2 transfer at the gills is a reasonable estimate to use in our model.

Although it will have little effect on the model, a puzzling aspect of our results is the higher than expected ratio between CO_2 and O_2 transfers at the gills. From standard metabolic theory the predicted $CO_2:O_2$ ratio would be 0.7–1.0, depending on the aerobic substrate used, but our respiratory exchange ratio was approximately 1.2 (i.e., 100/85; Fig. 3). Iwama et al. (1987) reported a respiratory quotient (RQ) of 0.87 for rainbow trout, in agreement with theory, as did Kutty (1968; RQ = 0.96). However, RQ values of >1 have been reported for hypoxic rainbow trout (RQ = 1.4) and trout at the beginning of exercise (RQ = 1.2; Kutty 1968), and in resting coho salmon and starry flounder (RQ $\sim$ 1.1 for both; Milligan and McDonald 1988). Causes of the discrepancies between observations and theory in resting fish in well oxygenated water are unknown, but could be related to nutritional status of the fish (Kutty 1972) or to stress-induced anaerobic respiration (Kutty 1968). We cannot eliminate the possibility that the restraints involved in our experiments caused increases in plasma cortisol and catecholamine concentrations, resulting in CO_2, ammonia, and acid-base fluxes across the gills different from a true resting situation. However, an alternative explanation for the elevated $CO_2:O_2$ ratio is that differential CO_2 and O_2 exchange elsewhere on the fish – perhaps the skin – compensated for the seemingly abnormal ratio at the gills. Thus, on a whole body basis the RQ would be closer to unity. All previous measurements of the $CO_2:O_2$ ratio in trout have been on a whole animal basis, not just at the gills as in our study, so we cannot separate these possibilities.

For the predictive model, titratable base released at the gills was assumed to be about 30 μM, of which ammonia contributed 15 μM, based on our experimental observations (Fig. 3). Net base release (titratable base minus ammonia transfer, = acid entry), the value normally reported for the acid-base status of fish, would therefore be about 330 $\mu mol \cdot kg^{-1} \cdot h^{-1}$ (using $\dot{V}_w = 0.37\ l \cdot kg^{-1} \cdot min^{-1}$). Our fish had been acclimated to soft water ($Ca^{2+} \sim 50\ \mu equiv \cdot l^{-1}$) for about 2 weeks, and were not fed. McDonald (1983a) and Audet et al. (1988) reported net base releases of 70–80 $\mu mol \cdot kg^{-1} \cdot h^{-1}$ for rainbow trout acclimated to Ca^{2+} = 50–60 $\mu equiv \cdot l^{-1}$. Holeton et al. (1983) reported net base release of 330 $\mu mol \cdot kg^{-1} \cdot h^{-1}$ in rainbow trout in hard water, as opposed to the usual small net base uptake (acid excretion) in non-feeding animals in hard water (e.g., Wood 1988). They attributed the net base release to lack of recovery after arterial cannulation of their fish. The results of McDonald (1983a) with starved trout at a range of Ca concentrations suggest, however, that low Ca concentrations in water may also be responsible for the observed base releases at fish gills. The long acclimation period (2–4 mo) of fed fish (Audet et al. 1988) suggest that base release to the water is a steady state condition of rainbow trout in very soft water.

15 μM of the 30 μM titrable base were added as ammonia (from our experimental observations), assuming that all ammonia was released as NH_3

at the gills. Ammonia excretion in soft water acclimated rainbow trout has been reported at about 200 $\mu mol \cdot kg^{-1} \cdot h^{-1}$ (McDonald 1983; Audet et al. 1988), which is similar to the 15 μM (330 $\mu mol \cdot kg^{-1} \cdot h^{-1}$) value used in our model. Ammonia transfer in trout lies between 45% and 100% NH_3, the remainder occurring as NH_4^+ (Cameron and Heisler 1983; Wright and Wood 1985). Since ammonia transfer is included within the 30 μM titratable base transfer, the ratio of NH_3:NH_4^+ is immaterial, except at $pH_{in} > 8.6$, where our assumption of 100% ammonia transfer as NH_3 becomes important (see below).

Instantaneous reactions at the gills were assumed in the model. The NH_3 to NH_4^+ and HCO_3^- to CO_3^{2-} reactions are essentially instantaneous (i.e., milliseconds; Stumm and Morgan 1981). The CO_2 to HCO_3^- reaction is slow in the absence of carbonic anhydrase (i.e., minutes), which probably explains why acidification of water near the gills by CO_2 transfer, as originally proposed by Lloyd and Herbert (1960), was not generally accepted; it was unclear how an instantaneous reaction could occur, unless carbonic anhydrase was present at the external gill surface (see Wright and Wood 1985; Wright et al. 1986). Szumski et al. (1982) speculated that carbonic anhydrase was indeed present at the gills, speeding CO_2 dissociation, but gave no evidence to support this idea. Now, however, carbonic anhydrase has been localized in fish mucus (Wright et al. 1986) and in gill epithelia (Conley and Mallatt 1988; Rahim et al. 1988). Wright et al. (1986) concluded that CO_2 disequilibrium was negligible in water near the gills. With regards to bases released at the gills, we can only speculate whether or not their reactions are instantaneous, because we do not know the precise bases involved.

Constant fish ventilation volumes were assumed for the model, a valid assumption at inspired pHs greater than about 4.6 (Fig. 2A). Ventilation increased greatly below $pH_{in} = 4.6$, presumably due to irritation of the gills by H^+ ions and accumulation of mucus on the gills (McDonald 1983b). In spite of increased $\dot{V}_w$ at low inspired pH, the ΔpHs predicted by the model are very close to the observed values (Fig. 7), because the buffer capacity of the very acidic water is so high in this region (cf. Fig. 4A) that increased $\dot{V}_w$ is inconsequential to ΔpH. Where constant $\dot{V}_w$ is most important is between pH 5 and 9, where buffering in the soft water is low (Fig. 4A). Our experimental results show approximately constant ventilation in that pH range, so assuming constant $\dot{V}_w$ in our model is reasonable.

The last assumption of our model, that water pH did not change as base or acid were added at the gills, is clearly not valid for our poorly buffered water. We used this invalid assumption solely as a necessary first step in preparing the model as presented in Fig. 6. When applying the model to our poorly buffered system we used the actual titration curves for our system (Fig. 4A) and an iterative calculation that accounted for the pH changes in the soft water as base and CO_2-acid were added.

In general the fit between model-predicted and experimentally observed ΔpH at rainbow trout gills is good (Fig. 7). The major discrepancy is at pH_{in} 9.3 and 10.1, where predicted ΔpH is less negative than observed. Likewise, theoretical acid added at pH_{in} 9.3 and 10.1 is less than the observed acid added (Fig. 8). A possible modification of the model to better reflect reality is to assume that no effective base is released at the gills if inspired pH is greater than $\sim$8.5 (Figs. 7, 8). The rationale for this modification is that the titratable base transfer (30 μM) was determined by comparing the titratable base of inspired and expired water titrated to pH 4.0; the unknown products released (or taken up) at the gills to add base to the water would have no alkalinizing effect at $pH_{in} > 8.5$ if their (unknown) pKs were anywhere between pH 4 and pH 8, but *would* show up as titratable base using our methodology. Above $pH_{in} \sim 8.5$ the postulated 15-μM ammonia addition at the gills would also have less alkalinizing effect at the gills than predicted (Fig. 6) because rainbow trout may decrease the proportion of ammonia excreted as NH_3 at high pH (i.e., more excreted as NH_4^+; Wright and Wood 1985). Without better knowledge of the nature of the bases released at trout gills in soft water it is difficult to decide whether 0 μM, 30 μM, or an intermediate value for base transfer should be used in the model for $pH_{in} > 8.5$.

An alternative explanation of the discrepancy between predicted and observed ΔpH at high inspired pH is that the second pK of CO_2 is less than 10.3 in the gill micro-environment, resulting in more H^+ released at $pH_{in} > 9$ than we have modelled. The pKs of CO_2 vary with ionic strength, decreasing as ionic strength increases (Stumm and Morgan 1981). We assumed CO_2 pKs of 6.3 and 10.3 as reasonable for low ionic strength fresh water; release of electrolytes and ammonia at the gills might decrease these pKs in the gill micro-environment as ionic strength is increased. If, for example, the second pK of CO_2 was 10.0 instead of 10.3 in the gill micro-environment then 25–30 μM more acid would be released near

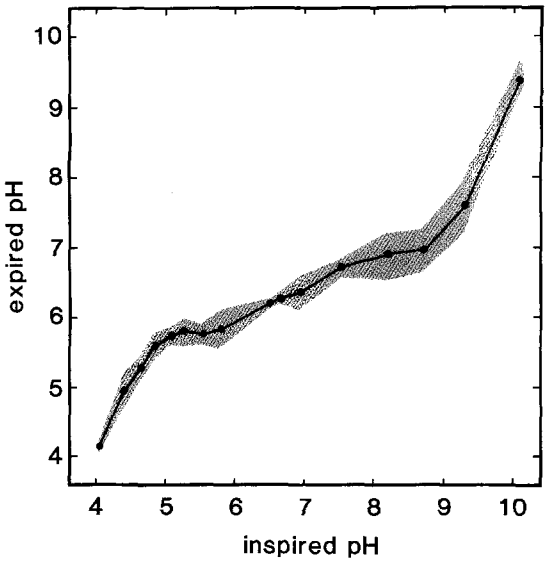

Fig. 9. Buffering of the gill micro-environment of rainbow trout held in soft water of various acidities. Buffering at the gills is good over the inspired pH range 4.8–9.3, but breaks down under more acidic or more basic conditions. 95% confidence limits are indicated about the mean expired pHs

$pH_{in} = 10.1$ than is indicated in Fig. 8. Which of the two modifications to the model at high inspired pH is more valid is difficult to resolve at present; in any case the model quantitatively mimics experimentally observed results well in the pH_{in} 4.0–8.5 range, and qualitatively follows the observed trends above $pH_{in} = 8.5$. The assumption that no base is released at the gills at high inspired pH is a reasonable method of fine-tuning the model fit.

In our poorly buffered soft water the release of CO_2, base, and ammonia at the gills kept expired pH between 5.6 and 7.6 over the inspired water pH range 4.8–9.3 (Fig. 9). Below $pH_{in} = 4.8$ and above $pH_{in} = 9.3$ the "buffering" of the gill micro-environment breaks down, and eventually the pH near the gills approaches inspired water pH (i.e., $pH_{in} = 4.0$, 10.1). Excretion of metabolic end products at the gills may allow fish to survive in acidic or basic conditions that might otherwise damage gill epithelia. Our results provide no evidence that CO_2, titratable base, or ammonia transfers at the gills are actively manipulated by the fish for their protective value during short-term exposures to acidic or basic conditions. Indeed, end product transfers at the gills were remarkably constant over a wide range of inspired pH (Figs. 2, 3). Rather, it appears that the protective value of buffering the gill micro-environment is a fortuitous by-product of excretion, not the primary reason for end product release at the gills.

Our experimental observations and predictive model are important for fish toxicant studies. Any gill contaminant whose toxicity varies with pH may be more or less toxic than expected from bulk water chemistry alone. For example, the solubility of aluminum varies exponentially with pH, reaching a minimum between about pH 5.5 and 6.0. Acidic water ($pH < 5.5$) containing Al may become super-saturated with Al near the gills where the pH is higher, and Al precipitating onto the gills may cause the ionoregulatory and respiratory problems we and others have documented (Neville 1985; Wood et al. 1988; Playle et al. 1989). The toxicity of ammonia is pH dependent, and Lloyd and Herbert (1960) were correct in equating the observed pHdependence of ammonia toxicity with CO_2 transfer at the gills. Szumski et al. (1982) were able to construct curves to predict toxic concentrations of ammonia considering water pH, alkalinity, and temperature, and assuming CO_2-induced pH changes in branchial water, but these workers were hindered by not having direct measurements of the actual pH changes occurring at fish gills. Pagenkopf (1983) presented a gill surface interaction model to explain pH and water chemistry dependent trace metal toxicity in fish, but did not consider water chemistry changes in the branchial micro-environment: incorporation of pH changes near the gills into such a model would make it more complete. In theory, by measuring pH changes near the gills using opercular catheters, or by calculating pH changes using measured CO_2, titratable base, and ammonia transfers and an appropriate titration curve, the toxicity of any pH dependent gill contaminant can be better predicted.

To summarise, the pH of water near rainbow trout gills is different than the bulk water in which the fish lives. The pH of the gill micro-environment is higher in acidic water, and lower in basic water, with a crossover point near pH 6. Carbon dioxide, base, and ammonia transfers at the gills adequately explain the experimentally observed pH changes. The gill micro-environment is essentially buffered over a wide range of environmental pHs, which perhaps allows fish to live in acidic or basic conditions which might otherwise harm gill epithelia. Knowledge of the pH near the gills is important when pH dependent gill toxicants are considered, because their toxicity may be different at the gills than would be predicted from bulk water pH alone.

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