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# DOWNSTREAM pH CHANGES IN WATER FLOWING OVER THE GILLS OF RAINBOW TROUT

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## SUMMARY

We investigated the pH of interlamellar water of trout (*Salmo gairdneri*) by following changes in the downstream pH of expired water using a stopped-flow method. As water flowed over the gills of control fish, there was a significant decrease in water pH. Acetazolamide added to the water increased the $CO_2$ disequilibrium, while carbonic anhydrase (CA) eliminated the $CO_2$ disequilibrium relative to control water. Mucus excreted by the fish was found to contain CA activity by the pH-stat technique. We conclude water acidification is due to the conversion of excreted $CO_2$ to $HCO_3^-$ and $H^+$ at the gill surface.

## INTRODUCTION

The apparent mean pH gradient across the gills of fish is usually determined as the difference between bulk water pH and blood pH. The precise gradient, however, is dependent upon possible pH gradients within the interlamellar space. Water flow is laminar through the mouth and over the gills (Randall & Daxboeck, 1984). Boundary layers are undoubtedly present next to the mucous layer covering the gill surface since water flow through the gill channels in relation to their dimensions results in Reynolds numbers that have been reported by Hughes (1984) to be very small (<10). It has been suggested (Lloyd & Herbert, 1960; Szumski, Barton, Putnam & Polta, 1982) that the pH of water within the gill chamber is significantly more acidic than that of bulk water due to $CO_2$ excretion and subsequent hydration to $HCO_3^-$ and $H^+$. If this theory is correct it would have important implications for the study of water pollutants, such as ammonia, the toxicity (Thurston, Russo & Vinogradov, 1981) and excretion (Wright & Wood, 1985) of which are pH-dependent. For hydration of excreted $CO_2$ to have such an appreciable effect on interlamellar pH, $CO_2$ hydration must occur rapidly enough to alter the proton activity within the interlamellar transit time of gill water (100–400 ms, Randall, 1982*a*,*b*). This is much faster than the uncatalysed rate of $CO_2$ hydration, which is of the order of minutes (Kern, 1960) at typical fish water pH and temperatures.

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Carbonic anydrase, the enzyme responsible for catalysing $CO_2$ hydration and dehydration reactions, has been located within gill epithelial cells of fish (Lacy, 1983; Dimberg, Hoglund, Knutsson & Ridderstrale, 1981). Thus, it seems possible that the apical surface of epithelial cells and/or branchial mucus could contain carbonic anhydrase.

The aim of the present study was to investigate interlamellar water pH of trout (*Salmo gairdneri*) by following changes in the pH of expired water in order to determine the contribution of carbonic anhydrase activity to the hydration of $CO_2$ eliminated at the branchial epithelium. Downstream changes in external water pH ($pH_{ex}$) were followed using a stopped-flow apparatus. Effects of carbonic anhydrase and acetazolamide, a specific inhibitor of carbonic anhydrase, on downstream $pH_{ex}$ changes were also studied. In addition, mucus excreted by fish was assayed for carbonic anhydrase activity by the pH-stat technique (Henry & Cameron, 1982).

## MATERIALS AND METHODS

### *Experimental animals*

Rainbow trout (average weight 257 g, range 208–312 g) from the Sun Valley Trout Hatchery (Mission, BC) were held in outdoor fibreglass tanks supplied with dechlorinated Vancouver tapwater (pH approx. 7·0, $[Na^+] = 40\,\mu equiv\,l^{-1}$, $[Cl^-] = 20\,\mu equiv\,l^{-1}$, hardness = 12 p.p.m. $CaCO_3$, temperature = 8–14 °C). Fish were fed a diet of commercial trout pellets.

The experiments were divided into two series. In the first experiment (series I), fish were allowed at least 5 days to acclimate to a test solution of 40 mmol $l^{-1}$ NaCl and 0·5 mmol $l^{-1}$ $CaCl_2$ in dechlorinated tapwater (11·7 °C ± 0·2) with a $\beta$ value of 81 $\mu$equiv $l^{-1}$ pH unit$^{-1}$, prior to surgery and experimentation. Fish were starved during this acclimation period and throughout the experiment. This time is adequate for the rainbow trout to re-establish ionic, osmotic and respiratory steady-state conditions after transfer from freshwater to a balanced salt solution (Perry & Heming, 1981). The second type of experiment (series II) was an *in vitro* experiment in which mucus samples were collected from fish held in dechlorinated tapwater.

### *Surgical procedure*

Fish in series I were anaesthetized by immersion into buffered ($NaHCO_3$) tricanemethanesulphonate (MS 222) solutions. Concentrations of 1:10 000 and 1:20 000 were used to anaesthetize and to maintain the fish on the operating table, respectively. In order to measure pH of expired water just leaving the opercular cavity, a polyethylene cannula (PE 90) was stitched in position under and midway along the opercular opening. The presence of the cannula just inside the opercular cavity did not appear to prevent proper closure of the opercular valve and we believe there was no interference with the normal pumping of water over the gills. It has been suggested that the opercular catheterization method is a poor method for sampling mean expired water in trout (Davis & Watters, 1970; Davis & Cameron, 1971). We conducted tests to determine if water pH varied with the position of the

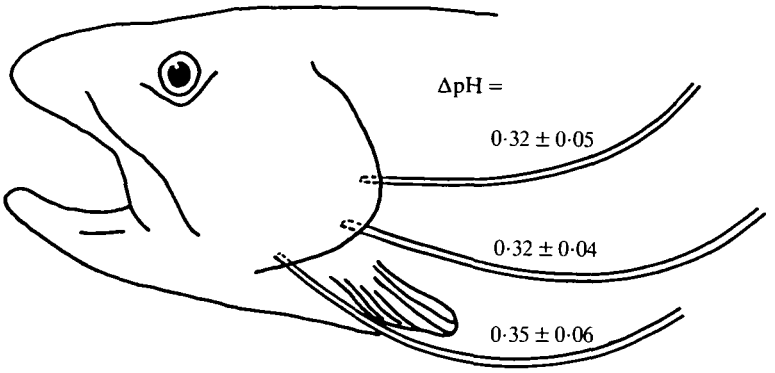


Fig. 1. Trout were surgically fitted with three cannulae placed just under the opercular valve (dotted line). The differences between inspired water pH and expired water pH at equilibrium (ΔpH) were measured on six fish. Numbers represent means ± S.E.

opercular cannula. Six fish were surgically fitted with three opercular cannulae which were positioned at different locations along the opercular valve (Fig. 1). The differences between inspired water pH and expired water pH at equilibrium were measured from individual cannulae. The unpaired *t*-test for two means was employed to compare the three possible pairs of mean values, and there was no relationship between measured pH values and the position of the cannula.

Fish were fitted with rubber dams prepared from latex surgeon gloves using the technique of Cameron & Davis (1970). This technique involves cutting the thumb of the glove to form a snug mask that is sewn posterior to the mouth and anterior to the gills of the fish. Following surgery, fish were inserted into a narrow black Perspex box in the posterior end of a two-chambered box (Fig. 2). The rubber dam was secured to a dividing Perspex O-ring so as to separate the front compartment (inspired water) from the back compartment (expired water) in this continuous flow-through apparatus. Fish were left to recover in the experimental apparatus for 48 h following surgery.

### *Measurements*

In series I, water pH was measured at two locations; inspired water pH was measured continuously with a combination glass pH electrode (Radiometer GK2401C) placed in the front chamber of the fish box, and expired water was drawn from the opercular cavity through the opercular cannula and into a glass 'stopped-flow' chamber (volume, 1 ml), housing a pH electrode (Canlab pH semi-micro-electrode, H5503-21) and micro Teflon stirring bar. The stopped-flow chamber was designed to measure pH changes in the expired water. The opercular cannula fed into the inlet port (inside diameter 1·27 mm) of the stopped-flow chamber and another cannula (PE 160), inserted into the outlet port (inside diameter 1·70 mm), carried the water out of the chamber. The pH electrode fitted tightly into a Teflon sleeve which then fitted into the glass chamber so as to form a seal to ensure the chamber was gas tight.

The pH electrodes were calibrated with standard Radiometer buffer solutions at pH 7 and 4. Vancouver tapwater has a low ionic strength (approx 0·002 mol $l^{-1}$), relative to other freshwaters, and therefore the addition of dissolved salts to the test water greatly increased the stability and decreased the lag time of the pH electrode response. The error associated with ionic strength differences between the commercial buffers and the test solution was not corrected for and is estimated to be approximately 0·05–0·25 pH units depending on pH. Although this introduces an error to the absolute pH values, the relative changes in pH were of greater importance to this study. The lag time of the Canlab microelectrode was determined for a step change in water pH at the same ionic strength and temperature as the experimental solution.

Fish were exposed to three different water regimes: test solution (control water), acetazolamide (1·6 mmol $l^{-1}$) in test solution and carbonic anhydrase (6·8 mg $l^{-1}$ or 20 400 Wilbur-Anderson units $l^{-1}$ bovine carbonic anhydrase) in test solution. The buffer values of these solutions were 81, 179 and 78 $\mu$equiv $l^{-1}$ pH unit$^{-1}$, respectively. Two complete sets of measurements were obtained while fish were in control

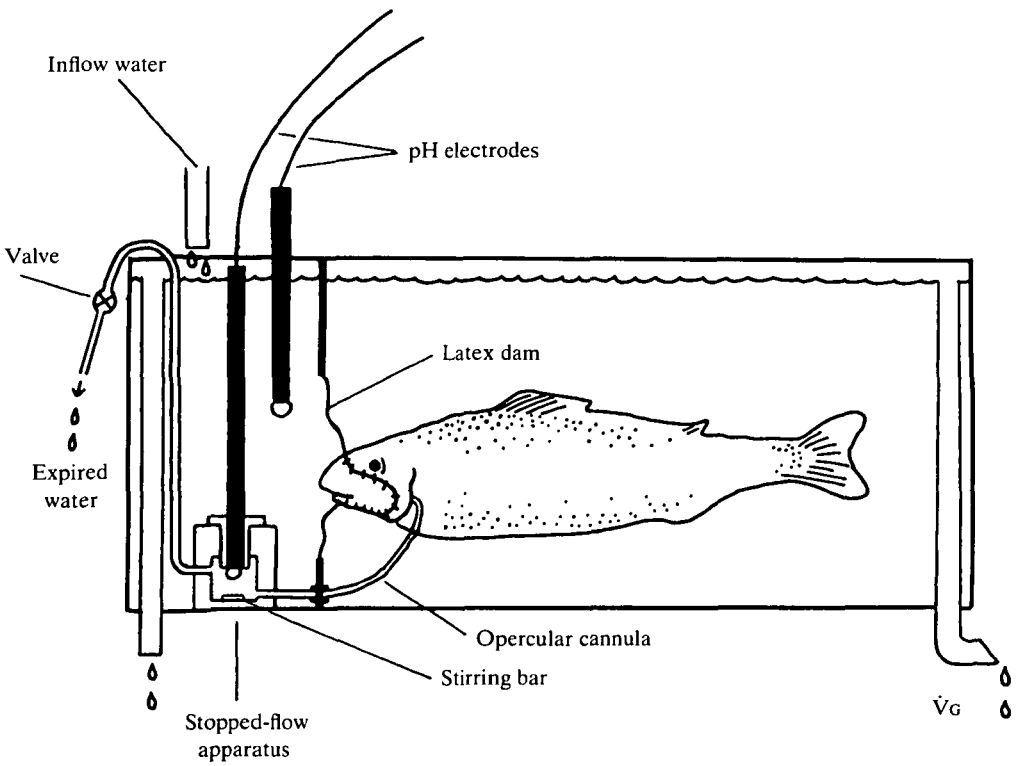


Fig. 2. Fish were placed in the two-chambered Plexiglas box. A latex dam was stitched around the mouth of the fish to separate inspiratory water from mixed expiratory water. The opercular cannula was stitched against the body of the fish and positioned just inside the opercular cavity. The small, glass, stopped-flow chamber contained a pH electrode and magnetic stirring bar. Expired water flowed through the opercular cannula, past the pH electrode and out through the outlet valve by gravitational forces.

water. The inflow was then changed over to either the acetazolamide or the carbonic anhydrase test solution (fish were exposed to one or the other, but not to both) and the same duplicate measurements were repeated after 30 min.

Inspired and expired water pH were recorded for 1 h before the experiment to ensure that the water pH was stable over time. Inspired water pH ($pH_{in}$) and expired water continuous flow pH ($pH_{ex}$) were measured simultaneously and recorded immediately before the flow of water through the stopped-flow chamber was stopped. The rate of water flow through the chamber (8–10 ml $min^{-1}$) was such that water transit time through the opercular cannula to the pH electrode was less than 2 s. Once the flow of water had been stopped, expired water pH was recorded over an 8-min period with a Hewlett Packard data acquisition unit and a strip chart recorder. If a chemical disequilibrium existed in the water, then a change in pH with time (dpH/dt) would be recorded. The final equilibrium pH after 8 min was referred to as the expired stopped-flow pH ($pH_{st}$). After the response time of the electrode had been accounted for, the half-time ($t_{1/2}$) of the equilibrium reaction was calculated.

To ensure that inspired water was in complete pH equilibrium, water was vigorously bubbled with compressed air in a 100-l tank before it flowed into the experimental apparatus. The pH equilibrium of inspired water was periodically checked in the stopped-flow chamber as described above.

Immediately before the flow of water was stopped in the chamber, ventilation ($\dot{V}_G$) and carbon dioxide content of the inspired ($C_{I_{CO_2}}$) and expired ($C_{E_{CO_2}}$) water were measured. $\dot{V}_G$ was determined by collecting the outflow water from the standpipe in the back chamber of the experimental chamber. Collections were made over 1-min periods and volumes determined by weight. Measurements of water $C_{I_{CO_2}}$ and $C_{E_{CO_2}}$ were made by gas chromatography with a Carle gas chromatograph (Model III) containing a $CO_2$ discriminating column (porapak Q). Sample preparation, calibration and the principle of the technique are given in Boutilier, Iwama, Heming & Randall (1985) and in Lenfant & Aucutt (1966), respectively. The rate of carbon dioxide excretion ($\dot{M}_{CO_2}$) was calculated by application of the Fick equation.

In series II, mucus samples taken from the body of trout were assayed for carbonic anhydrase (CA). Initially we tried to obtain mucus samples from the gill surface; it was impossible, however, to collect samples that were large enough for the assay technique. The pH-stat technique was chosen to measure CA activity in preference to other methods (modified boat and esterase assay) because previous work had shown that the pH-stat method exhibited the lowest limit of detection and the highest degree of sensitivity (Heming, 1984; see also Henry & Cameron, 1982). A mixture of phosphate buffer and a bicarbonate solution initiates the conversion of $HCO_3^-$ and $H^+$ ions to $CO_2$ gas. As $CO_2$ is liberated from the reaction solution, the pH will increase and the volume of acid (HCl) that is required to maintain the reaction mixture at a given pH will be proportional to the production of $CO_2$, since the stoichiometry of $H^+$:$CO_2$ is 1:1. The reaction rate (in mol $CO_2$ $min^{-1}$) will be greater in the presence of the catalyst, CA, than in its absence.

Mucus samples (approx. 0·5 ml) were collected from the bodies of six fish by lightly stroking the fish with a metal spatula, and these samples were then assayed for

CA. The addition of mucus to the reaction vessel caused foaming, a problem which was eliminated with a defoaming agent (50 $\mu$l octan-2-ol). The dehydration reaction rate was not affected by the addition of octan-2-ol. The catalysed (mucus) reaction rate was compared to the uncatalysed rate, with or without acetazolamide. Anhydrous acetazolamide (5 mmol $l^{-1}$) was added directly to the phosphate buffer solution. To test for the presence of cellular material, mucus samples were stained with eosin and crystal violet and examined microscopically.

### *Determination of half-time values*

Half-time values ($t_{1/2}$) were calculated by taking several points along individual kinetic curves (dpH/dt) and converting these values to percentage changes in pH (assuming that the $pH_{st}$ value at 8 min was the equilibrium pH or very close to it). These points were then plotted on semilogarithmic paper as log % change in pH *versus* time, giving straight lines over the first 60 % of the reaction. The $t_{1/2}$ values were determined graphically.

### *Statistics*

Data are presented as means ± S.E. Student's two-tailed *t*-test was used to compare relationships in the data, with a 5 % level of rejection taken as the statistical limit of significance.

## RESULTS

An important factor in the lag time of the pH electrode was the degree of mixing in the stopped-flow chamber. To test the effects of mixing under similar conditions to those in the experiment, a small volume of pure $CO_2$ gas was introduced into the test solution before it flowed into the stopped-flow chamber. The flow of water past the electrode was then stopped, and the $CO_2$ hydration reaction was followed over time, with and without a stirring bar in the chamber. Fig. 3 depicts the change in pH with time for the mixed ($t_{1/2} = 76$ s) and the unmixed ($t_{1/2} = 300$ s) solutions. The difference of 224 s was presumably due to slow diffusion of protons through the unmixed solution. To reduce the effects of mixing on the lag time of the pH electrode, a stirring bar was placed in the stopped-flow chamber and a magnetic stirrer maintained a constant rotation of the stirring bar. This test also demonstrated that our system detects pH differences due to $CO_2$ hydration, and the $t_{1/2}$ value for the mixed reaction is in approximate agreement with values given by Kern (1960) at the test pH and temperature.

The results from series I are presented in Tables 1 and 2. Fig. 4. shows representative stopped-flow traces from two fish. As water flowed over the gills of control fish, there was a significant decrease in pH from inspired water ($pH_{ex}$) to expired water at equilibrium ($pH_{st}$) (Tables 1, 2). Expired control water was almost completely equilibrated by the time it reached the pH electrode, because $pH_{ex}$ was not significantly different from $pH_{st}$ in both the first and second control sets. Even though the overall expired water pH disequilibrium in control water was small, in

11/30 stopped-flow pH traces there was a significant decrease in pH once water flow had been stopped in the chamber, in a few cases pH increased after the flow had been stopped in the chamber (2/30 stopped-flow pH traces), and in the remainder of traces there was no measurable change in water pH (16/30 traces). The addition of acetazolamide significantly increased the $CO_2$ disequilibrium relative to control

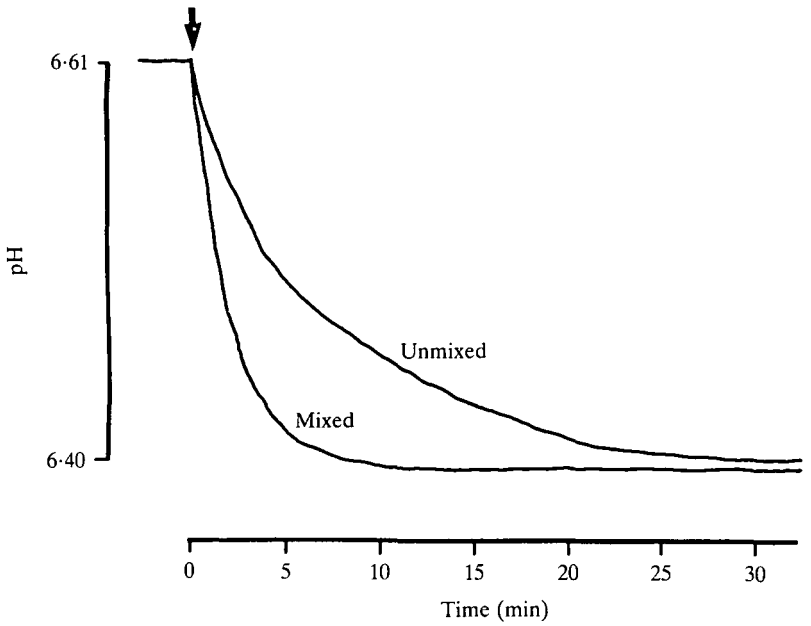


Fig. 3. Water flow in the stopped-flow chamber was stopped (arrow) and the kinetics of the $CO_2$ hydration reaction were recorded. In one case a Teflon-coated, magnetic stirring bar continuously mixed the solution (mixed), while in the other solution no stirring bar was present (unmixed).

Table 1. *Water pH values (inspired water, $pH_{in}$; expired water immediately leaving the opercular valve, $pH_{ex}$; equilibrated expired water, $pH_{st}$, values given as $\bar{x} \pm$ S.E.) and half-time values for $CO_2$:$HCO_3^-$ interconversions measured on rainbow trout in control water and after acetazolamide (1·6 mmol $l^{-1}$) had been added to the water*

| | | Control | | Acetazolamide |
|---|---|---|---|---|
| Temperature (°C) | | 10·4 ± 0·1 | | 10·7 ± 0·2 |
| Direction of pH change† (*N*) | ↓ (7) | ↑ (2) | no change (5) | ↓ (12) |
| $pH_{in}$ | 7·41 ± 0·01 | 7·39 ± 0·00 | 7·41 ± 0·02 | 7·42 ± 0·02 |
| $pH_{ex}$ | 6·84 ± 0·06 | 6·70 ± 0·05 | 6·60 ± 0·01 | 7·38 ± 0·02* |
| $pH_{st}$ | 6·82 ± 0·06 | 6·72 ± 0·05 | 6·60 ± 0·01 | 7·31 ± 0·02* |
| $pH_{ex}-pH_{st}$ | 0·03 ± 0·00 | 0·02 ± 0·01 | 0·00 ± 0·00 | 0·07 ± 0·01* |
| $t_{1/2}$ (s) | 90 ± 6 | 66 ± 4 | — | 88 ± 3 |

† Direction of the pH change refers to whether pH increased or decreased once the flow of water was stopped in the stopped-flow chamber.

* Significantly different from pH control, $P \leq 0{\cdot}05$.

water (Fig. 4; Table 1). In contrast, carbonic anhydrase eliminated the $CO_2$ disequilibrium except in one stopped-flow pH trace where a small pH change ($pH_{ex} - pH_{st} = 0{\cdot}01$) was observed (Fig. 4; Table 2).

There was no significant change in ventilation and $CO_2$ excretion rates between control I and acetazolamide measurements and between control II and carbonic anhydrase values (Table 3). It is interesting to note, however, that fish in the first experiment (April–May, 10·5°C) had considerably lower $\dot{V}_G$ and $\dot{M}_{CO_2}$ rates compared to fish in the second experiment (September–October, 13·0°C), and these differences may be related to seasonal variations.

Mucus excreted by the fish contained carbonic anhydrase activity. Addition of mucus doubled the $CO_2$ dehydration reaction rate relative to the uncatalysed rate and acetazolamide reduced the reaction rate to the uncatalysed rate (Table 4). There was a small difference in the reaction rate between control and acetazolamide uncatalysed reactions. In addition, mucus samples were found to contain cellular material.

## DISCUSSION

This study presents the first documented measurement of the pH change in water flowing over the gills of freshwater fish. Holeton & Randall (1967) reported a rough estimate of the inspired to expired water pH difference of about 0·2–0·5 pH units in similar Vancouver tapwater. The difference measured in this study, however, was 0·7–0·9 pH units. In both cases these differences were measured at point sources from the opercular outflow but preliminary tests in this study, to determine if water pH varied with the position of the cannula, showed that there were no significant differences between the pH measured in three locations along the opercular valve. Changes in water sampled from a single point, therefore, are representative of changes in expired water in general. Differences in the magnitude of the pH changes

Table 2. *Water pH values (inspired water, $pH_{in}$; expired water immediately leaving the opercular valve, $pH_{ex}$; equilibrated expired water, $pH_{st}$, values given as $\bar{x} \pm$ S.E.) and half-time values for $CO_2$: $HCO_3^-$ interconversions, measured on rainbow trout in control water and after carbonic anhydrase (6·8 mg $l^{-1}$) had been added to the water*

| | Control | | Carbonic anhydrase |
|---|---|---|---|
| Temperature (°C) | 12·7 ± 0·1 | | 13·1 ± 0·1 |
| Direction of pH change† (*N*) | ↓ (4) | no change (11) | no change (12) |
| $pH_{in}$ | 7·35 ± 0·10 | 7·13 ± 0·03 | 7·19 ± 0·06 |
| $pH_{ex}$ | 6·44 ± 0·05 | 6·35 ± 0·03 | 6·39 ± 0·03 |
| $pH_{st}$ | 6·43 ± 0·04 | 6·35 ± 0·03 | 6·39 ± 0·03 |
| $pH_{ex} - pH_{st}$ | 0·02 ± 0·01 | 0·00 ± 0·00 | 0·00 ± 0·00 |
| $t_{1/2}$ (s) | 48 ± 13 | — | — |

† See Table 1.

Experimental values were not significantly different from control values using the Student's paired *t*-test.

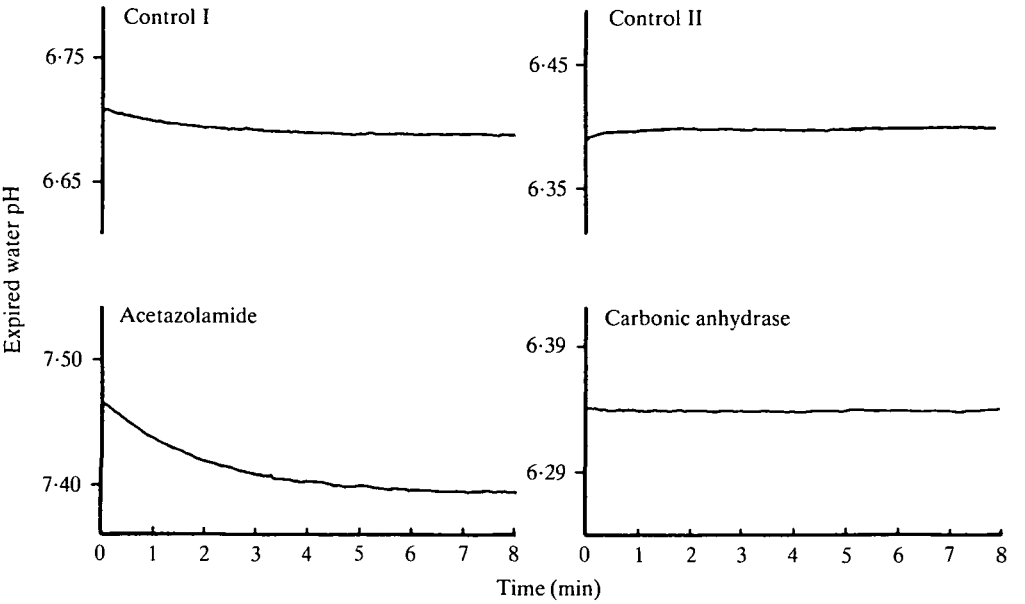


Fig. 4. Representative changes in expired water pH of two fish (control I and acetazolamide; control II and carbonic anhydrase) after water flow past the pH electrode had been stopped at time 0.

Table 3. *Ventilation ($\dot{V}_G$) and carbon dioxide excretion ($\dot{M}_{CO_2}$) rates from experiment one (control I and acetazolamide) and experiment two (control II and carbonic anhydrase)*

| | Temperature (°C) | $\dot{V}_G$ ($l\,h^{-1}$) | $\dot{M}_{CO_2}$ ($\mu mol\,100\,g^{-1}\,h^{-1}$) |
|---|---|---|---|
| Control I | 10·4 ± 0·1 | 6·45 ± 0·37 | 239·39 ± 27·28 |
| Acetazolamide | 10·7 ± 0·2 | 6·18 ± 0·18 | 261·56 ± 28·26 |
| Control II | 12·7 ± 0·1 | 9·95 ± 0·97 | 380·83 ± 38·96 |
| Carbonic anhydrase | 13·1 ± 0·1 | 10·21 ± 0·77 | 393·11 ± 42·02 |

Experimental values (acetazolamide, carbonic anhydrase) were not significantly different from the respective control values using the Student's paired *t*-test.

Table 4. *$CO_2$ dehydration reaction rate in catalysed (mucus samples) and uncatalysed solutions, with or without acetazolamide at 22 °C*

| | Control ($CO_2$, $\mu mol\,min^{-1}$) | Acetazolamide (5 $mmol\,l^{-1}$) ($CO_2$, $\mu mol\,min^{-1}$) |
|---|---|---|
| Catalysed (mucus) | 48·0 ± 7·7* | 20·7 ± 0·8** |
| Uncatalysed | 26·0 ± 1·6 | 21·4 ± 0·7** |

Mucus samples taken from the body of six fish.
* Significantly different from control.
** Significantly different from uncatalysed control.

reported in our study and that of Holeton & Randall (1967) are probably related to several factors, including differences in temperature, the presence or absence of latex masks, fluctuations in calcium carbonate and buffering capacity of the water, and differences in the amount of $CO_2$ eliminated by the fish.

Evidence for the presence of carbonic anhydrase in the mucus is supported by the fact that expired water was almost completely equilibrated as it left the opercular cavity. Water is only in contact with the gill for about 100–400 ms (Randall, 1982*a*,*b*) and the time required for complete $CO_2$ hydration is of the order of minutes (Kern, 1960), therefore the $CO_2$ reaction must be catalysed at the gill surface. Acetazolamide inhibited CA and increased the expired water $CO_2$ disequilibrium, whereas the addition of CA to the water completely eliminated the small disequilibrium that was observed in the expired control water of some fish. The cause of this small disequilibrium downstream from the gills in control water will be discussed later, but is probably not related to any nonequilibrium state at the gill surface.

Carbonic anhydrase activity is present in mucus covering the epidermis of rainbow trout. Although it was not feasible to measure CA activity in mucus covering the gill surface, it is probable that CA activity is present in the branchial mucus excreted by gill epithelial cells. Fletcher, Jones & Reid (1976) compared epidermal (body) and gill filament mucus-secreting goblet cells of rainbow trout and found that cell density and glycoprotein composition (predominantly acid glycoproteins) were very similar. Branchial epithelium contains high levels of carbonic anhydrase (Haswell, Randall & Perry, 1980), which appears to be concentrated in the apical regions of the epithelial cells (Dimberg *et al.* 1981). CA has also been located in the cytoplasm of chloride cells, vesicular cells, supportive cells, in the cytoplasm and mucous granules of goblet cells, and in the extracellular spaces between vesicular and supportive cells of the opercular membrane of the teleost, *Fundulus heteroclitus* (Lacy, 1983). It is reasonable to assume, therefore, that carbonic anhydrase activity is also present in the mucus covering the gill filaments of rainbow trout. Mucus samples were found to contain cellular material, which is not surprising considering that the epidermal cells are regularly sloughed off to allow for new growth. The presence of cells does not invalidate the experimental findings. Enzyme activity in the mucus may originate from CA expelled from mucous cells into the mucus covering the gills and body of the fish, or from epithelial cell fragments which have been sloughed off into the mucous layer.

The elimination rate of $CO_2$ far exceeds that of other branchially excreted molecules and it is well established that $CO_2$ diffusion is facilitated in the presence of CA (Longmuir, Forster & Wu, 1966; Enns, 1967; Zborowska-Sluis, L'Abbate & Klassen, 1974; Gutknecht, Bisson & Tosteson, 1977; Burnett, 1984). Carbon dioxide is primarily excreted in the gaseous form (Perry, Davie, Daxboeck & Randall, 1982), with approximately 10 % of total excretion as $HCO_3^-$ exchanging for $Cl^-$ (Cameron, 1976). Gutknecht *et al.* (1977) used artificial lipid bilayer membranes to study properties of $CO_2$ flux and found that at pH 7–8 CA (1–1·6 mg $ml^{-1}$) caused a 10- to 80-fold stimulation of $CO_2$ flux. They suggested that the presence of

CA in the unstirred layer renders the $CO_2$ hydration–dehydration reaction so fast that chemical equilibrium between $CO_2$ and $HCO_3^-$ exists throughout the unstirred layer. Furthermore, the rate-limiting step in $CO_2$ transport in a situation where $HCO_3^-$ cannot readily cross the membrane and the solutions are poorly buffered is the diffusion of $HCO_3^-$ and $H^+$ through the unstirred layer.

It is interesting that a small disequilibrium was observed in expired control water, even though we postulate complete equilibration of the $CO_2/HCO_3^-$ reaction at the gill surface. The fact that CA eliminated the pH disequilibrium observed downstream of the gills indicates that it was, in fact, due to a $CO_2/HCO_3^-$ disequilibrium in the water. $CO_2$ may be in complete equilibrium with $HCO_3^-$ and $H^+$ in the mucus and associated unstirred layer next to the gill, but the differential diffusion rates of these molecules across the unstirred layer may create a small disequilibrium in the mainstream of water flowing over the gills. In addition, mixing of waters of different carbon dioxide content may result in $CO_2/HCO_3^-$ disequilibrium downstream of the gills; indeed this may account for the two traces collected from one fish which showed a reverse reaction, that is the expired water pH increased slightly when the flow of water was stopped in the chamber. It may be that respiratory water at equilibrium was mixed with nonrespiratory 'shunt' water just after it entered the opercular cavity. This would result in a small increase in pH as the shunt water would contain a lower $H^+$ activity compared to the respiratory water. In one trace, slow pH changes were observed in the presence of CA, presumably due to the uncatalysed $CO_2$ hydration reaction. $\dot{M}_{CO_2}$ was extremely high in this fish and therefore the level of CA in relation to the concentration of $CO_2$ was probably inadequate to catalyse the reaction completely.

Variations in $t_{1/2}$ values for a given reaction are the result of differences in temperature, ionic strength and buffering capacity. Ionic strength influences the ionic mobility of the solution, as well as affecting the stability of the pH electrode. The $t_{1/2}$ values for the control experiments ranged from 48 to 90 s. These variations in $t_{1/2}$ may be due to ionic strength differences related to small changes in the ionic compositions of the Vancouver tapwater at different times of the year. These seasonal variations in water composition are also reflected in the slightly different $pH_{in}$ values between the two control experiments. Increases in the buffering capacity of a solution result in longer $t_{1/2}$ values for the uncatalysed $CO_2$ hydration reaction (Gray, 1971). Even though acetazolamide increased the $\beta$ value in this study, the mean $t_{1/2}$ value was not altered, which may be a reflection of the complexity of pH changes in water downstream of the gills.

The final equilibrium pH for acetazolamide-treated fish was significantly higher than the final control pH. The addition of acetazolamide altered the ionic strength of the test solution slightly. The error in the absolute pH value associated with ionic strength differences was estimated to be no more than 0·01 pH units, and this cannot account for the relatively high expired water pH value. The three parameters that would affect the expired water pH value are $\dot{M}_{CO_2}$, $\dot{V}_G$ and the $\beta$ value. There were no changes in either $\dot{M}_{CO_2}$ or $\dot{V}_G$ and the results can be explained only in terms of

changes in the $\beta$ value with the addition of acetazolamide, a weak acid. Acetazolamide increased the $\beta$ value two-fold compared to that of control water.

Heming (1985) performed similar experiments to this study on rainbow trout acclimated to a saline test solution (in mmol $l^{-1}$: NaCl, 40; KCl, 1·6; $CaCl_2$, 0·47; $MgSO_4$, 0·62; $NaHCO_3$, 5·5; $NaH_2PO_4$, 0·95) with different results and conclusions. He found expired pH was almost identical to inspired pH, and water downstream from the gill became progressively more acidic at the uncatalysed rate of $CO_2$ hydration, and concluded that CA was not present on the gill surface. An explanation for the discrepancy between the two studies may be related to the fact that the fluid in Heming's stopped-flow chamber was static after water flow had stopped, whereas in the present study the fluid was continually mixed with a stirring bar. We have found that dpH/dt in static fluids has a much larger $t_{1/2}$ than in mixed fluids and this accounts for the slow responses observed.

Carbon dioxide is not the only molecule eliminated at the gill which will influence water pH. Fish excrete $NH_3$, $NH_4^+$, $H^+$ and $HCO_3^-$ in addition to $CO_2$ across their gills. All of these molecules will affect the water pH and the magnitude of the effect will depend on the relative rates of excretion, the rates of chemical equilibrium, the water-buffering capacity, and the rate of water flow past the gills. Protonation of $NH_3$ is extremely rapid, so $NH_3$ excretion will result in the immediate elevation of water pH at the gill surface. Alternatively, $NH_4^+$ excretion will have a negligible effect on water pH. Efflux of $H^+$ (or $OH^-$ influx) will alter the pH of water instantaneously, but the gradient for $H^+$ ions is generally very small [$H^+$ gradient from blood (pH 8) to expired water (pH 6) = $9{\cdot}9\times10^{-7}$ equiv], thus net excretion of $H^+$, in exchange for $Na^+$ or $NH_4^+$, will be low under most conditions, even if the gills are very permeable to protons (McWilliams & Potts, 1978).

It is clear that interlamellar water pH is significantly lower than that of bulk water in the gills of rainbow trout. Water acidification is due to the catalysed conversion of excreted $CO_2$ to $HCO_3^-$ and $H^+$ at the gill surface. Several authors (Lloyd & Herbert, 1960; Szumski *et al.* 1982) have modelled the observed effects of bulk water pH on the aquatic toxicity of ammonia in terms of a large $CO_2$-induced acidification of interlamellar water. Such models are valid under the water conditions tested in this study. However, water acidification at the gill may not occur in all situations. For example, in very well buffered lakes and rivers $CO_2$ excretion may have very little effect on interlamellar water pH. Furthermore, the degree of acidification at the gill surface may vary greatly in fish depending on the environmental water pH, temperature, the relative branchial molecular excretion rates and the rate of water flow past the gills. All these factors must be accounted for before one can assess the effect of $CO_2$ excretion by the fish on interlamellar water pH in a given body of water.

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