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EXTRACELLULAR CARBONIC ANHYDRASE AND AN ACID–BASE DISEQUILIBRIUM IN THE BLOOD OF THE DOGFISH *SQUALUS ACANTHIAS*

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Summary

The electrometric ΔpH method and an *in vitro* radioisotopic HCO_3^- dehydration assay were used to demonstrate the presence of true extracellular carbonic anhydrase (CA) activity in the blood of the Pacific spiny dogfish *Squalus acanthias*.

An extracorporeal circulation and stopflow technique were then used to characterise the acid–base disequilibrium in the arterial (postbranchial) blood. During the stopflow period, arterial pH (pHa) *decreased* by 0.028±0.003 units (mean ± S.E.M., *N*=27), in contrast to the *increase* in pHa of 0.029±0.006 units (mean ± S.E.M., *N*=6) observed in seawater-acclimated rainbow trout *Oncorhynchus mykiss* under similar conditions. The negative disequilibrium in dogfish blood was abolished by the addition of bovine CA to the circulation, while inhibition by benzolamide of extracellular and gill membrane-bound CA activities reversed the direction of the acid–base disequilibrium such that pHa increased by 0.059±0.016 units (mean ± S.E.M., *N*=6) during the stopflow period. When the CA activity of red blood cells (rbcs) was additionally inhibited using acetazolamide, the magnitude of the negative disequilibrium was increased significantly to −0.045±0.007 units (mean ± S.E.M., *N*=6). Blockage of the rbc Cl^-/HCO_3^- exchanger using 4,4′-diisothiocyanostilbene-2,2′-disulphonic acid (DIDS) also increased the magnitude of the negative disequilibrium, in this case to −0.089±0.008 units (mean ± S.E.M., *N*=6). Exposure of dogfish to hypercapnia had no effect on the disequilibrium, whereas the disequilibrium was significantly larger under hypoxic conditions, at −0.049±0.008 units (mean ± S.E.M., *N*=6).

The results are interpreted within a framework in which the absence of a *positive* CO_2 excretion disequilibrium in the arterial blood of the spiny dogfish is attributed to the membrane-bound and extracellular CA activities. The *negative* disequilibrium may arise from the continuation of Cl^-/HCO_3^- exchange in the postbranchial blood and/or the hydration of CO_2 added to the plasma postbranchially. Two possible sources of this CO_2 are discussed; rbc CO_2 production or the admixture of blood having 'low' and 'high' CO_2 tensions, i.e. the mixing of postbranchial blood with blood which has bypassed the respiratory exchange surface.

Key words: *Squalus acanthias*, spiny dogfish, acid–base disequilibrium, red blood cell, Cl^-/HCO_3^- exchanger, acetazolamide, benzolamide, carbonic anhydrase, DIDS, quaternary ammonium sulphanilamide, hypoxia, hypercapnia.

Introduction

In freshwater rainbow trout, *Oncorhynchus mykiss*, the relatively slow rate of the uncatalysed plasma HCO_3^- dehydration reaction in comparison with the rate of other steps in CO_2 excretion gives rise to an acid–base disequilibrium in the postbranchial blood (Gilmour *et al.* 1994). Owing to the inaccessibility of carbonic anhydrase (CA) activity to plasma reactions in the gills of teleost fish (Henry *et al.* 1988, 1993; Perry and Laurent, 1990), the plasma HCO_3^- dehydration reaction continues in the postbranchial blood and can be observed as an increase in pH of 0.02–0.04 units when the flow of arterial blood through an external circuit is stopped (Gilmour *et al.* 1994; Gilmour and Perry, 1994, 1996). The addition of bovine CA to the circulation of the trout allows plasma HCO_3^- dehydration to come to completion during transit of the blood through the gills, so that no disequilibrium is detected in the postbranchial blood.

In contrast to the situation in teleosts, plasma $CO_2/HCO_3^-/H^+$ reactions in the spiny dogfish *Squalus acanthias* may be catalysed by gill membrane-bound CA activity exposed to the plasma (Swenson *et al.* 1995; Wilson,

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This work is dedicated to the memory of John Boom, who died in a tragic accident on 26 February 1996.

1995). Furthermore, recent studies on the lesser spotted dogfish, *Scyliorhinus canicula*, have demonstrated that true extracellular CA activity is present in the blood plasma of this species (Wood *et al.* 1994; Perry *et al.* 1996). It might, therefore, be predicted from the trout studies that no acid–base disequilibrium should exist in the postbranchial blood of the dogfish. The main objective of the present study was to test whether CO_2 excretion reactions are in equilibrium in the postbranchial blood of the spiny dogfish and to characterise any disequilibrium that might exist despite the presence of pillar cell membrane-associated and extracellular CA activities. An approach involving the use of an extracorporeal preparation in combination with a stopflow technique (Gilmour *et al.* 1994) was employed. The CA inhibitor benzolamide was used to examine the role of non-erythrocytic CA activity in disequilibrium events; low doses (1–2 mg kg^{-1}) of benzolamide have been shown to cause selective inhibition of gill CA activity without inhibiting red blood cell CA activity to a physiologically significant extent (Swenson and Maren, 1987). In addition, blood samples from the spiny dogfish were tested using the [^{14}C]HCO_3^- dehydration assay of Wood and Perry (1991) and the electrometric ΔpH method (Henry, 1991) to confirm that the blood of this species, like that of the lesser spotted dogfish, contained significant extracellular CA activity.

A second objective of the present study was to examine the potential contribution of red blood cell (rbc) Cl^-/HCO_3^- exchange to disequilibrium events. The continuation of rbc anion exchange in the postbranchial blood, a possibility given the similarity in both trout and dogfish of the residence time of blood in the gills and the reaction time of rbc anion exchange (Gilmour *et al.* 1994; Wood *et al.* 1994), should give rise to a negative disequilibrium (Crandall and Bidani, 1981; Crandall *et al.* 1981; Gilmour *et al.* 1994). A protocol involving the *in vivo* injection of the anion exchange inhibitor DIDS was developed for the spiny dogfish to allow the effect of Cl^-/HCO_3^- exchange on disequilibrium events to be evaluated.

Materials and methods

Experimental animals

Pacific spiny dogfish (*Squalus acanthias* L.; 800–2900 g; experimental *N*=47) were netted off the coast of Vancouver Island, Canada, in May and June 1995, and held at Bamfield Marine Station (BMS; Bamfield, British Columbia, Canada) for up to 4 weeks in large circular tanks provided with flowing sea water at 12 °C. Fish were maintained without feeding under conditions of natural photoperiod. Freshwater rainbow trout [*Oncorhynchus mykiss* (Walbaum); 534–900 g; experimental *N*=18] obtained from West Creek Trout Farms (Mission, BC, Canada) were transported to BMS, where they were acclimated to sea water for a minimum of 2 weeks before use. Trout were held outdoors in large circular tanks provided with flowing sea water at 12 °C.

All fish were anaesthetized using ethyl-*m*-aminobenzoate (MS-222; 0.1 g l^{-1}), then transferred to an operating table that permitted continuous irrigation of the gills with aerated anaesthetic solution. In the 27 dogfish used for extracorporeal experiments, two cannulae (Clay-Adams, PE50) were implanted into the coeliac artery (Graham *et al.* 1990) in the orthograde and retrograde directions. In addition, a cannula (PE160) was inserted into each spiracle and sutured in place. The remaining dogfish were used to provide blood samples; blood was obtained by caudal puncture, by cardiac puncture or by withdrawal from a single cannula inserted into the coeliac artery. Trout were fitted with a dorsal aortic cannula (PE50) for blood sampling according to the method of Soivio *et al.* (1975). For extracorporeal experiments, an additional, return cannula (PE50) was implanted into the caudal vein (see Axelsson and Fritsche, 1994), and a catheter (PE160) was placed in each opercular cavity.

After surgery, fish were transferred to experimental chambers of an appropriate size, supplied with aerated flow-through sea water (flow rate >2.5 l min^{-1}) at 12 °C, for a 24 h recovery period. Cannulae were flushed with heparinized (100 i.u. ml^{-1} ammonium heparin) saline; dogfish saline consisted of 500 mmol l^{-1} NaCl, while the trout saline was a Cortland saline (Wolf, 1963) adjusted for seawater salmonids by raising [NaCl] to 171 mmol l^{-1}.

Extracorporeal circulation

Detailed descriptions of the extracorporeal circulation and stopflow technique have been provided by Gilmour *et al.* (1994) and Gilmour and Perry (1994, 1996). Blood was withdrawn from the fish and passed by means of a peristaltic pump through an external circuit containing pH, P_{CO_2} and P_{O_2} electrodes, before being immediately returned to the fish; the stopflow condition was imposed by turning off the peristaltic pump. The flow rate through the external loop, which contained approximately 1 ml of blood, was 0.55 ml min^{-1}, and the transit time of the blood from the gills to the electrodes was approximately 30 s.

One opercular or spiracular catheter was used to sample water from the holding chamber for the measurement of water P_{O_2} (Pw_{O_2}). The remaining catheter was connected to a pressure transducer (Harvard), and the arithmetic difference between inspiratory and expiratory pressures was used as a measure of ventilation amplitude (V_{amp}). The output of the pressure transducer was also displayed on a chart recorder (Harvard) and used to determine breathing frequency (f_V).

Analogue measurements of pHa, Pa_{CO_2}, Pa_{O_2}, Pw_{O_2} and V_{amp} were transformed into digital output (Data Translation, Inc.) and stored using customized data acquisition software (P. Thoren; Göteborg, Sweden).

Experimental protocol

A 1 ml blood sample was withdrawn prior to the commencement of the extracorporeal loop for the determination of true extracellular CA activity using the electrometric ΔpH method (see below). The experiment was then initiated by connecting the cannulae to the external loop. Once the measured ventilatory, cardiovascular and blood gas variables had stabilized (usually within 10–30 min of starting

the extracorporeal circulation), a control stopflow period (8 min) was imposed. Experiments on trout ended at this point. Following collection of the control stopflow data, dogfish were subjected to one or more experimental treatments which included exposure to hypercapnia or hypoxia and/or the administration of drugs: carbonic anhydrase (CA) followed by acetazolamide (ACTZ) or benzolamide, acetazolamide or benzolamide alone, or 4,4′-diisothiocyanostilbene-2,2′-disulphonic acid (DIDS).

Hypercapnia was imposed by bubbling CO_2 in air through a water equilibration column supplying the experimental chamber. The percentage of CO_2 in the CO_2/air mixture was empirically adjusted using a gas-mixing pump (Wösthoff) to achieve a final $P_{a}CO_2$ of approximately 1.20 kPa in the animal, and the hypercapnic stopflow period was imposed once pHa and $P_{a}CO_2$ had stabilized at their new levels. The experimental chamber was then returned to normocapnia and the fish was allowed to recover until the measured variables had re-stabilized under normocapnic conditions before continuing the experiment. A similar protocol was followed in hypoxia experiments; the fish was subjected to hypoxia by bubbling N_2 through the water equilibration column to lower $P_{w}O_2$ to approximately 4.00 kPa, which resulted in a final $P_{a}O_2$ of approximately 1.20 kPa in the fish. Following the hypoxic stopflow period, normoxic conditions were re-established and the experiment was continued once the measured variables had re-stabilized. To take into account any changes in the acid–base disequilibrium that might have been induced by exposure to hypoxia or hypercapnia, a second 'control' stopflow was carried out under normoxic/normocapnic conditions, before any drug treatments were examined.

All drugs were administered as a bolus injection into the return cannula of the extracorporeal loop. Bovine CA [2500 Wilbur-Anderson units per mg (1 Wilbur-Anderson unit will cause the pH of a 0.02 mol l^{-1} Trizma buffer to drop from 8.3 to 6.3 in 1 min at 0 °C); 2 mg kg^{-1}] was dissolved in dogfish saline and the stopflow condition was imposed when the measured variables had re-stabilized (approximately 5 min) following the injection. Injection of acetazolamide (30 mg kg^{-1}) resulted in a brief alkalosis which was followed by a progressive acidosis (see Gilmour *et al.* 1994), and the stopflow was initiated as pHa returned to the pre-acetazolamide level during the acidotic phase. Both acetazolamide and benzolamide were initially dissolved in a stock solution of saline with added NaOH (pH approximately 10); the pH was then slowly titrated to approximately 8.5, and this stock was diluted to a working solution by adding a small sample to the dogfish saline. The stopflow period was imposed approximately 6 min after benzolamide (1.3 mg kg^{-1}) injection, to ensure that only the plasma, and not rbc, CA was inhibited (see Results). A 1 ml blood sample was withdrawn immediately prior to each benzolamide stopflow period for the determination of plasma and lysate CA activities by the electrometric CA assay. DIDS was dissolved in dogfish saline containing 2 % dimethyl sulphoxide (DMSO) and injected into the fish to achieve a final concentration in the blood of 2×10^{-5} mol l^{-1} (0.2 % DMSO). The stopflow period was initiated once the measured variables had stabilized, 12–15 min after the DIDS injection.

Measurement of true extracellular CA activity

Two techniques were used to ascertain whether true extracellular CA activity was present in the blood plasma of the spiny dogfish, the electrometric ΔpH method (Henry and Kormanik, 1985; Henry, 1991) and the radioisotopic HCO_3^- dehydration assay of Wood and Perry (1991). Although both techniques were used for the same purpose, they measure very different processes. In the electrometric assay, the degree to which the addition of a sample containing CA activity increases the rate of the uncatalysed CO_2 hydration reaction is measured under optimal conditions, i.e. unlimited proton absorptive capacity by the buffer; hence, there is no end-product inhibition and no diffusive limitation. The electrometric assay cannot, however, be used to measure the activity within intact rbcs (or whole blood) because intracellular proton accumulation results in the inhibition of the reaction (Henry *et al.* 1993). Clearly, then, it cannot take into account any involvement of band 3 Cl^-/HCO_3^- exchange, the process which is thought to limit the rate of CO_2 excretion *in vivo* (Perry, 1986; Wood and Munger, 1994). In contrast, the radioisotopic HCO_3^- dehydration assay measures the rate at which CO_2 is evolved from plasma HCO_3^- when the plasma or whole-blood sample is exposed to an external gas phase of P_{CO_2} approximately equal to 0 (Wood and Perry, 1991). It provides a measure of HCO_3^- dehydration under physiologically relevant conditions, in which Cl^-/HCO_3^- exchange is involved, but is probably limited by the rate of diffusion of CO_2 from the liquid to the gas phase, a factor that becomes increasingly important as the rate of the dehydration reaction increases. The radioisotopic HCO_3^- dehydration assay was used in addition to the electrometric CA assay to establish whether a significant capacity for HCO_3^- dehydration was present in the blood plasma of the spiny dogfish. This assay was also used to evaluate the effect of the DIDS or benzolamide treatments used in the extracorporeal experiments on whole-blood CO_2 excretion.

Electrometric ΔpH method

Plasma samples (100 μl) were added to a reaction medium (6 ml of a buffer containing, in mmol l^{-1}, 225 mannitol, 75 sucrose, 10 Tris, pH 7.40, 4 °C), and the reaction was initiated by the addition of CO_2-saturated water (100 μl, 4 °C). The initial velocity of the reaction was measured over a change of approximately 0.15 pH units. The CA activity of lysate samples (10 μl of lysate diluted 400- to 500-fold) was measured in a similar fashion, allowing quantification of rbc CA activity. Lysate was obtained by sonicating the rbc pellet in a set volume of EGTA.

[^{14}C]HCO_3^- dehydration assay

The assay was carried out as described by Wood and Perry

(1991). Samples (0.7 ml) of blood or separated plasma were equilibrated with a humidified gas mixture consisting of 0.5 % CO_2 in air for at least 60 min. The gas mixture was provided by a gas-mixing pump (Wösthoff). Acetazolamide and DIDS were dissolved in dogfish saline containing 2 % DMSO and added to the blood at 5 % by volume to achieve final concentrations of 1×10^{-4} mol l^{-1} for acetazolamide and 2×10^{-5} mol l^{-1} for DIDS; the final DMSO concentration did not exceed 0.1 %. Acetazolamide and DIDS were added to the blood or plasma samples for the final 30 min of the equilibration period. Benzolamide, dissolved in dogfish saline, was added to the blood or plasma samples (final concentration 1.3×10^{-5} mol l^{-1}) 6 min before the assay was carried out to mimic the *in vivo* protocol.

Following the 3 min assay, filter paper and plasma ^{14}C activities were determined by liquid scintillation counting (LKB RackBeta). The plasma (50 µl) or filter papers were counted in 10 ml of ACS (Amersham) scintillation cocktail. Plasma total CO_2 was measured on 50 µl samples using the method of Cameron (1971). The HCO_3^- dehydration rate for each vial was calculated by dividing filter paper ^{14}C activity by plasma specific activity and time.

In vitro *mixing experiments*

The objectives of the mixing experiments were twofold: (i) to confirm the presence of extracellular CA activity in the blood of dogfish and its absence from the blood of trout; and (ii) to examine the possible role of this extracellular CA activity in disequilibrium events. Blood samples (3.0 ml) from a trout or dogfish were heparinized (100 i.u. ml^{-1}) and equilibrated, for 30 min, with either humidified air or a humidified mixture of 0.5 % CO_2 in air. A gas-mixing pump (Wösthoff) supplied the gas mixture. Using a peristaltic pump, blood was then drawn at the same rate from the two samples into a mixing chamber and from there passed to a pH electrode (Metrohm) housed in a thermostatted cuvette (volume approximately 0.1 ml). The transit time of the blood from the mixing chamber to the pH electrode was approximately 30 s. Once a stable pH had been obtained, the pump was turned off and pH was monitored for a 6 min stopflow period using the Radiometer PHM 73 analyzer connected to the data acquisition system. Quaternary ammonium sulphanilamide (QAS; final concentration 1 mmol l^{-1}), a CA inhibitor which does not penetrate the rbc (Henry, 1987), was then added to the blood, the samples were re-equilibrated and the experiment repeated.

Statistical analyses

Data are presented as means ±1 standard error of the mean (S.E.M.). Statistical differences between control and treatment values were determined by paired *t*-tests or by one-way analysis of variance (ANOVA) followed by Fisher's LSD test for multiple comparisons, as appropriate. One-sample *t*-tests were used in some cases to judge whether changes during the stopflow period were significantly different from zero (Zar, 1984). The fiducial limit of significance was 5 %.

Results

Extracellular carbonic anhydrase activity

The blood of the spiny dogfish contained significant extracellular CA activity as measured using either the radioisotopic HCO_3^- dehydration assay or the electrometric ΔpH method (Table 1). The rate of $[^{14}C]HCO_3^-$ dehydration in separated plasma was reduced by 44 % in the presence of the CA inhibitor acetazolamide (Table 1). The results of the electrometric CA assay indicated that dogfish plasma was generally capable of increasing the uncatalysed reaction rate by 10- to 15-fold, whereas all trout plasma samples had no measurable CA activity.

The acid–base disequilibrium

A *negative* acid–base disequilibrium, consisting of a decrease in pHa of 0.028 units, was detected in the arterial blood of the spiny dogfish (Fig. 1; Table 2). This contrasted with the *positive* disequilibrium (pHa increased by 0.029 units) measured for seawater-acclimated rainbow trout (Fig. 1; Table 2). $P_{a_{CO_2}}$ increased significantly during the stopflow period in both species, although the increase was larger in rainbow trout than in dogfish (Table 2). The acid–base disequilibrium in the arterial blood of the dogfish was essentially eliminated by the injection of bovine CA (Table 3); ΔpH during the stopflow

Table 1. *A comparison of results obtained using the radioisotopic HCO_3^- dehydration assay and the electrometric ΔpH carbonic anhydrase assay*

	Spiny dogfish	Rainbow trout
$[^{14}C]HCO_3^-$ dehydration assay		
Plasma rate (µmol ml^{-1} h^{-1})	8.1±0.3 (15)*	3.5±0.1 (4)
Plasma+ACTZ (µmol ml^{-1} h^{-1})	4.5±0.2 (15)	4.1±0.3 (4)
RF (%)	43.6±2.0 (15)	−16.4±8.7 (4)
Electrometric ΔpH CA assay		
V_u (µmol ml^{-1})	1.91±0.07 (52)	1.83±0.11 (17)
Plasma activity (µmol CO_2 ml^{-1} min^{-1})	5.97±1.12 (34)	1.81±0.12 (17)
EF	12.1±0.9 (33)	0

$[^{14}C]HCO_3^-$ dehydration rates (µmol ml^{-1} h^{-1}) are for separated plasma from dogfish in the presence or absence of 10^{-4} mol l^{-1} acetazolamide (ACTZ). The reduction factor (RF) is the percentage reduction in the plasma HCO_3^- dehydration rate in the presence of acetazolamide. Corresponding rates for rainbow trout, as reported by Wood *et al.* (1994), are shown for comparison.

* indicates a significant difference between plasma and plasma+ ACTZ values (paired *t*-test, $P<0.05$).

The carbonic anhydrase (CA) activity (µmol CO_2 ml^{-1} min^{-1}) of plasma obtained prior to the initiation of the extracorporeal loop was determined using the electrometric CA assay for samples from dogfish and trout. V_u (µmol ml^{-1}) is the uncatalysed reaction rate in the absence of the plasma sample, and the enhancement factor (EF) is the factor by which 1 ml of plasma increases V_u.

Values are means ± 1 S.E.M. (*N*).

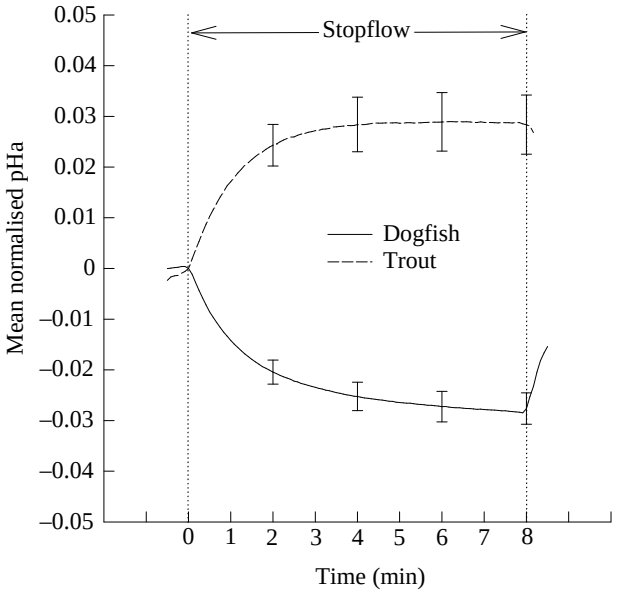

Fig. 1. Continuous, mean normalized pHa values for dogfish (N=27) and rainbow trout (N=6) during the control stopflow period. Because the magnitude of pH changes in the stopflow period was small relative to individual variability in pHa, data for individual fish were normalized by subtracting from each point in a response the value at the beginning of the stopflow period. Error bars represent ±1 S.E.M. and are shown only every 2 min for clarity.

period after CA injection was not significantly different from zero (one-sample t-test, P>0.05).

The magnitude of the *negative* disequilibrium in dogfish, as well as the starting $P_{a_{CO_2}}$ and the change in $P_{a_{CO_2}}$, were increased significantly by injection of acetazolamide, which inhibits both intracellular and extracellular/membrane-bound CA activity (Table 3). In contrast, when the extracellular and membrane-bound CA activities were selectively inhibited using a low dose of benzolamide, the direction of the acid–base disequilibrium was reversed (Fig. 2A; Table 3). Benzolamide injection itself, prior to the stopflow, resulted in a significant decrease in arterial pH and a non-significant increase in $P_{a_{CO_2}}$ (Table 3). Once arterial pH had stabilized following the pHa decrease (approximately 6 min), the stopflow was carried out and a *positive* disequilibrium was observed (Fig. 2A). At this

Table 2. *Ventilatory variables, absolute values of* $P_{a_{O_2}}$, $P_{a_{CO_2}}$ *and pHa at the beginning of the control stopflow period and magnitudes of* $P_{a_{O_2}}$, $P_{a_{CO_2}}$ *and pHa changes (*$\Delta P_{a_{O_2}}$, $\Delta P_{a_{CO_2}}$ *and* Δ*pHa, respectively) during the control stopflow period for dogfish and trout*

	Spiny dogfish	Rainbow trout
V_{amp} (kPa)	0.20±0.03 (25)	0.09±0.01 (6)
f_V (min^{-1})	31±1 (27)	72±2 (6)
$P_{a_{O_2}}$ (kPa)	18.7±0.4 (19)	13.9±1.6 (6)
$\Delta P_{a_{O_2}}$ (kPa)	–4.8±0.3 (27)*	–4.7±0.7 (6)*
$P_{a_{CO_2}}$ (kPa)	0.17±0.01 (24)	0.31±0.04 (6)
$\Delta P_{a_{CO_2}}$ (kPa)	0.01±0.003 (26)*	0.04±0.01 (6)*
pHa	7.722±0.019 (27)	7.870±0.028 (6)
ΔpHa	–0.028±0.003 (27)*	0.029±0.006 (6)*

V_{amp}, ventilation amplitude; f_V, breathing frequency.

Values are means ± 1 S.E.M. (N).

* indicates a magnitude significantly different (one-sample t-test, P<0.05) from zero.

The variability in the N values for dogfish was a result of occasional equipment failure.

Table 3. *Ventilatory variables, absolute values of* $P_{a_{O_2}}$, $P_{a_{CO_2}}$ *and pHa at the beginning of the stopflow period and magnitudes of* $P_{a_{O_2}}$, $P_{a_{CO_2}}$ *and pHa changes (*$\Delta P_{a_{O_2}}$, $\Delta P_{a_{CO_2}}$ *and* Δ*pHa, respectively) during the stopflow period, for control (upper data set in each case) and treatment stopflows for dogfish*

Treatment	f_V (min^{-1})	V_{amp} (kPa)	$P_{a_{O_2}}$ (kPa)	$\Delta P_{a_{O_2}}$ (kPa)	$P_{a_{CO_2}}$ (kPa)	$\Delta P_{a_{CO_2}}$ (kPa)	pHa	ΔpHa
CA (4)	32±2	0.14±0.01	17.3±0.7	–3.7±0.5	0.17±0.05	0.01±0.01	7.771±0.061	–0.070±0.013
	33±2	0.14±0.02	17.5±0.8	–3.9±0.4	0.19±0.06	0.01±0.004	7.744±0.063	–0.012±0.007*
Acetazolamide (6)	28±2	0.30±0.13	19.1±0.7	–5.6±0.3	0.17±0.02	0.001±0.004	7.746±0.040	–0.019±0.006
	28±2	0.27±0.09	18.3±1.5	–6.5±0.7	0.43±0.06*	0.07±0.02*	7.661±0.065	–0.045±0.007*
Benzolamide (6)	31±2	0.20±0.05	17.2±0.5	–3.9±0.4	0.17±0.03	0.01±0.004	7.749±0.030	–0.053±0.012
	33±2*	0.27±0.06	17.3±0.9	–3.4±0.3	0.20±0.05	0.01±0.01	7.624±0.040*	0.059±0.016*
DIDS (6)	35±1	0.20±0.03	16.4±1.7	–5.3±0.5	0.18±0.01	0.02±0.004	7.645±0.045	–0.025±0.006
	33±2	0.29±0.12	18.1±1.3	–7.1±1.2	0.20±0.02	0.03±0.01	7.710±0.049*	–0.089±0.008*
Hypercapnia (7)	31±2	0.17±0.02	18.7±0.8	–5.7±0.7	0.16±0.01	0.01±0.004	7.798±0.026	–0.021±0.008
	34±3	0.46±0.20	14.8±2.1	–3.9±0.7	1.18±0.10*	0.04±0.02*	7.286±0.046*	–0.016±0.005
Hypoxia (6)	32±2	0.19±0.03	18.5±0.5	–4.1±0.4	0.19±0.04	0.01±0.01	7.731±0.028	–0.028±0.005
	33±2	0.25±0.03	1.2±0.1*	0.3±0.7*	0.11±0.03	0.003±0.003	7.865±0.025*	–0.049±0.008*

f_V, breathing frequency; V_{amp}, ventilation amplitude; CA, carbonic anhydrase.

Values are means ± 1 S.E.M.; N values are given in parentheses for each treatment.

* indicates a significant difference (paired t-test, P<0.05) between the treatment value and its associated control.

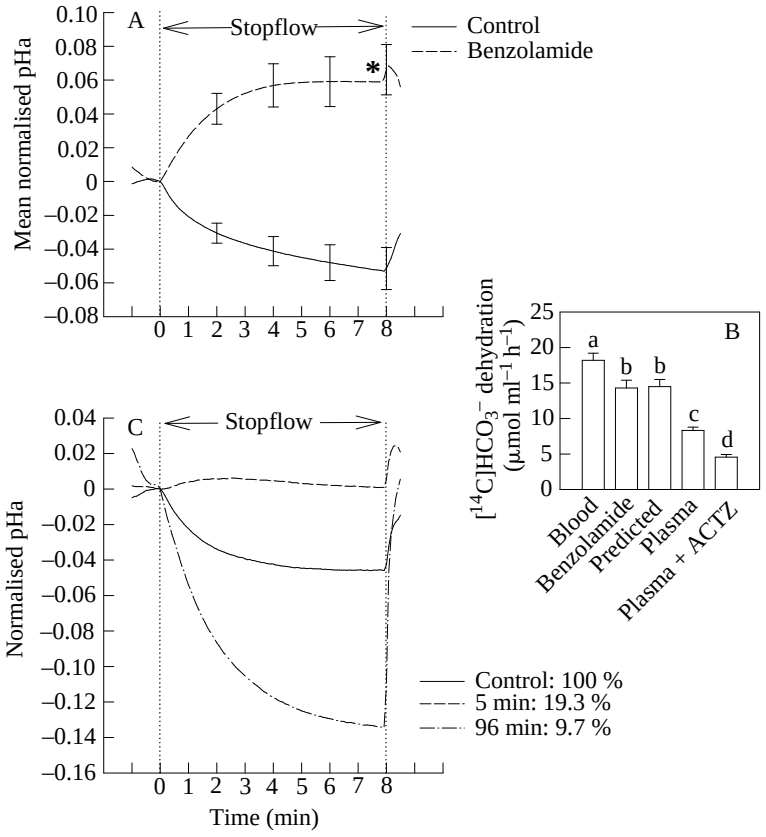

Fig. 2. (A) Mean normalized pHa values for dogfish postbranchial blood during sequential stopflow periods under control conditions and following a low dose (1.3 mg kg^{-1}) of benzolamide (N=6). * indicates a significant difference (paired t-test, P<0.05) at the end of the stopflow period (8 min) from the control value. Error bars represent ±1 S.E.M. (B) [^{14}C]HCO_3^- dehydration rates for dogfish whole blood, blood incubated for 6 min with 1.3×10^{-5} mol l^{-1} benzolamide, separated plasma and separated plasma incubated for 30 min with 10^{-4} mol l^{-1} acetazolamide (plasma + ACTZ). A predicted HCO_3^- dehydration rate was calculated for each set of samples based on the assumption that benzolamide would inhibit only non-erythrocytic carbonic anhydrase (CA) activity (see text). Values are means +1 S.E.M. (N=5). Means sharing the same letter are not significantly different from one another (one-way ANOVA followed by Fisher's LSD multiple comparison test, P>0.05). (C) Representative, continuous normalized pHa values during consecutive stopflow periods for a single dogfish under control conditions and at two times (5 min and 96 min) after the infusion of a high dose (13 mg kg^{-1}) of benzolamide. Erythrocyte CA activity was evaluated by carrying out the electrometric CA assay on lysate derived from blood samples collected immediately prior to initiating the stopflow periods. Lysate CA activity is given as a percentage of the control lysate activity for each stopflow period.

point, only the extracellular and membrane-bound CA activities were assumed to be inhibited. This assumption was evaluated using both the radioisotopic HCO_3^- dehydration assay and the electrometric CA assay. The HCO_3^- dehydration rate of blood to which benzolamide had been added *in vitro* (6 min incubation) was found not to differ significantly (Fig. 2B) from a 'predicted' value based on the assumption of no rbc CA inhibition and calculated from the HCO_3^- dehydration rates for blood, plasma, and plasma incubated with acetazolamide *in vitro* (plasma$_{ACTZ}$):

$$\text{predicted rate} = (\text{blood rate} - \text{plasma rate}) + \text{plasma}_{ACTZ}\ \text{rate}.$$

Furthermore, use of the electrometric CA assay demonstrated an 88±7 % (N=6) reduction in plasma CA activity following benzolamide injection (blood sample withdrawn immediately before stopflow period), while rbc CA activity was reduced by only 7±40 % (N=6). It should be noted that sample dilution prior to and during the electrometric assay results in dissociation of the inhibitor from the enzyme, such that the measured values are likely to be conservative estimates of CA inhibition *in vivo*. Thus, plasma CA activity was probably fully inhibited *in vivo*, while rbc CA activity was well within the range required for its usual function. Injection of a higher dose of benzolamide (13 mg kg^{-1}) *in vivo* initially also resulted in a *positive* disequilibrium, but when the incubation was permitted to continue, the direction of the acid–base disequilibrium was reversed once more and the magnitude of the *negative* disequilibrium increased progressively with time (Fig. 2C). Measurement, using the electrometric CA assay, of rbc lysate CA activity under this protocol demonstrated a progressive inhibition of rbc CA activity (Fig. 2C).

The potential involvement of Cl^-/HCO_3^- exchange in the generation of the acid–base disequilibrium in dogfish was examined by measuring the magnitude of the disequilibrium following injection of the Cl^-/HCO_3^- inhibitor DIDS. To establish the efficacy of the *in vivo* DIDS injection, the HCO_3^- dehydration rates of blood samples withdrawn immediately prior to the stopflow period were compared with those of blood samples collected before the experiment and with those of samples to which DIDS or acetazolamide had been added *in vitro*. The whole-blood HCO_3^- dehydration rate for blood incubated with DIDS *in vitro* did not differ significantly from that for blood withdrawn from dogfish following DIDS injection and, in both cases, the dehydration rates were significantly lower than that of control blood (Fig. 3A). While addition of DIDS *in vitro* reduced the whole-blood rate to that of a blood sample incubated with acetazolamide *in vitro*, the inhibition produced by an *in vivo* injection of DIDS was not this severe (Fig. 3A). *In vivo* injection of DIDS, prior to the stopflow, resulted in a significant increase in pHa (Fig. 3C; Table 3) and elicited a nearly fourfold increase in the magnitude of the *negative* disequilibrium (Fig. 3B; Table 3). $P_{a_{CO_2}}$ was not altered significantly, although there was a tendency for an increase in $P_{a_{CO_2}}$ following DIDS injection (Table 3).

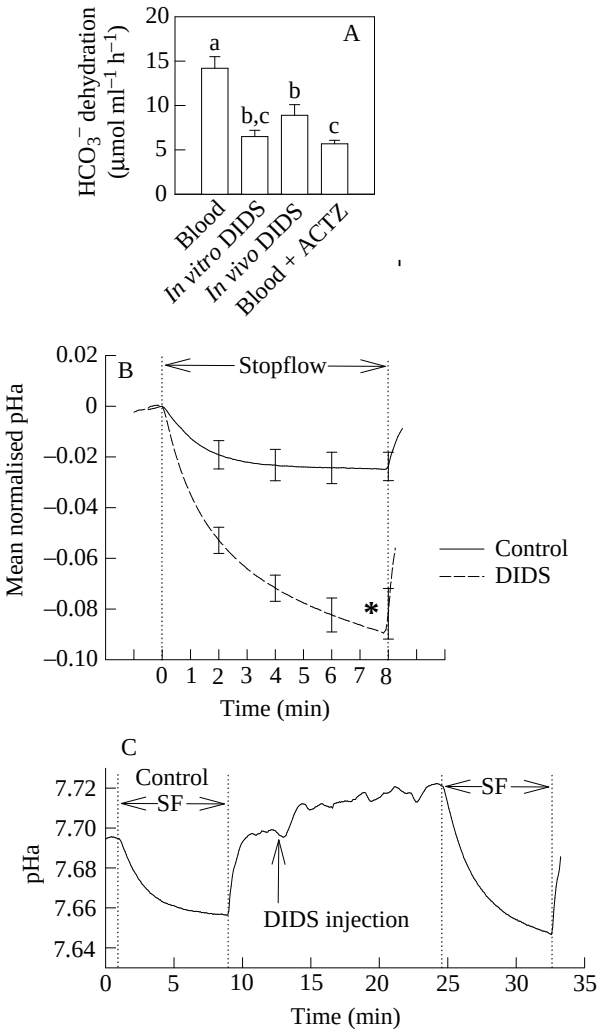

Fig. 3. (A) The effect of DIDS (2×10^{-5} mol l^{-1}) on the [^{14}C]HCO_3^- dehydration rate for blood samples from spiny dogfish incubated with DIDS *in vitro* and for blood samples from spiny dogfish treated with DIDS *in vivo*. The effect of *in vitro* incubation with ACTZ is also shown. Values are means +1 S.E.M. (N=4). Means sharing the same letter are not significantly different from one another (one-way ANOVA followed by Fisher's LSD multiple comparison test, P>0.05). (B) Mean normalized pHa values for dogfish postbranchial blood during successive stopflow periods under control conditions and following DIDS injection (2×10^{-5} mol l^{-1}) (N=6). Blood samples drawn from four of these fish before initiating the extracorporeal circulation and immediately before beginning the DIDS stopflow period were used to generate the data presented in A. * indicates a significant difference (paired t-test, P<0.05) at the end of the stopflow period (8 min) from the control value. Error bars represent ±1 S.E.M. (C) A representative, continuous recording of pHa to illustrate the changes in pHa associated with DIDS injection and stopping the flow of blood. Stopflow (SF) periods are marked by the dotted lines.

Exposure of dogfish to hypercapnic conditions that raised Pa_{CO_2} to approximately 1.20 kPa decreased arterial pH on average by 0.5 units, but had no significant effect on the magnitude of the pH disequilibrium (Table 3). Dogfish subjected to hypoxia (final Pa_{O_2}=1.20 kPa) exhibited an alkalosis, and the magnitude of the disequilibrium was increased significantly (Table 3). Although both ventilation frequency and amplitude tended to increase during hypercapnia and hypoxia, the changes in fV and V_{amp} were not significant (Table 3), probably because of individual variability in the responses together with the low numbers of dogfish used. Perry and Gilmour (1996) have reported significant increases in fV and V_{amp} in dogfish under identical conditions of hypercapnia and hypoxia.

Table 4. *Absolute magnitudes of the* in vitro *mixing disequilibria, in the presence and absence of the extracellular carbonic anhydrase inhibitor QAS, for blood samples from dogfish and trout*

	Spiny dogfish	Rainbow trout
Control ΔpH	0.012±0.002	0.037±0.007†
QAS ΔpH	0.043±0.009*	0.031±0.011

QAS, quaternary ammonium sulphanilamide.
Values are means ± 1 S.E.M., and N=5 for each group.
* indicates a significant difference between the *actual* magnitudes (see text) of the control and QAS values for dogfish or trout (paired t-test, P<0.05).
† indicates a significant difference between the *absolute* magnitudes of dogfish and trout values under control or QAS conditions (two-sample t-test, P<0.05).

In vitro *mixing experiments*

An acid–base disequilibrium was generated *in vitro* by mixing blood samples, from either trout or dogfish, which had been equilibrated to two different P_{CO_2} levels [air (approximately 0.03 kPa) and 0.51 kPa]. This disequilibrium was eliminated by the addition of bovine CA (3 mg ml^{-1}) to the blood samples and re-established by the addition of QAS (0.3 mg ml^{-1}), a selective inhibitor of extracellular CA activity. No disequilibrium occurred when blood samples equilibrated to the same P_{CO_2} were mixed. Because it was difficult to ensure that the two samples entered the mixing chamber at exactly the same rate, and because the direction of the mixing disequilibrium depended on which sample was added at the higher rate, the results are reported as *absolute* magnitudes in Table 4. Comparisons between control and QAS values, however, were performed in a pairwise fashion on the *actual* magnitudes (i.e. magnitudes including a positive or negative sign to indicate the direction of disequilibrium). The absolute magnitude of the *in vitro* mixing disequilibrium for dogfish blood under control conditions was significantly smaller than that for trout blood (Table 4). In the presence of the inhibitor QAS, which decreased the plasma CA activity by 83±14 % (N=5) (electrometric CA assay), the actual magnitude of the mixing disequilibrium in dogfish blood was increased significantly to a value close to that of trout blood. The actual magnitude of the trout-blood mixing disequilibrium was unchanged in the presence of QAS (Table 4).

Discussion

Extracellular carbonic anhydrase activity

The results of the present study have confirmed that the blood of the spiny dogfish, like that of the lesser spotted dogfish (Wood *et al.* 1994; Perry *et al.* 1996), contains significant true extracellular CA activity. $[^{14}C]HCO_3^-$ dehydration from separated plasma was reduced by 44 % in the presence of acetazolamide, a value similar to the 31 % reduction measured for the lesser spotted dogfish (Wood *et al.* 1994). Further evidence was provided by the *in vitro* mixing experiment, in which the magnitude of a disequilibrium state generated by mixing two blood samples of different CO_2 tensions was significantly larger for dogfish blood following addition of the extracellular CA inhibitor QAS (Table 4). The similarity between the absolute magnitude of the mixing disequilibrium in QAS-treated dogfish blood and that of trout blood was striking. Precautions similar to those described by Wood *et al.* (1994) were taken during sampling from several dogfish to avoid the possibility that the CA activity measured was an artefact introduced by sampling-related haemolysis. The care taken during sampling in both studies, the use of two different techniques in the present study, and the similarity of the results for two different species of dogfish, all lend weight to the conclusion that significant CA activity is present in dogfish plasma *in vivo*. While the origin of the extracellular CA activity remains unclear, one possibility is that it arises from endogenous lysis of rbcs; Butler and Metcalfe (1989) discuss the potential role of the corpus cavernosum, an irregular structure which is adjacent to the afferent filament artery and is unique to elasmobranch fish, as a site of erythrocyte destruction.

In addition to the true extracellular CA activity in dogfish blood, CA activity has also been located in the gills (Swenson and Maren, 1987; Wilson, 1995; R. P. Henry, K. M. Gilmour, C. M. Wood and S. F. Perry, in preparation), and some experimental evidence for *Squalus acanthias* suggests the presence of gill membrane-bound CA activity which is exposed to the plasma. Swenson *et al.* (1995) have used benzolamide and an extracellular CA inhibitor, polyoxyethylene-aminobenzolamide, to distinguish between the roles of cytosolic and membrane-bound branchial CA activities in the correction of a metabolic alkalosis *in vivo*. The absence of a postbranchial disequilibrium when gills of *Squalus acanthias* were perfused *in situ* with a mixture of acidic and basic salines provided additional evidence for pillar cell membrane-associated CA activity in the gills (Wilson, 1995). This dual availability of CA activity to plasma reactions during the passage of the blood through the gills of the dogfish (Fig. 4) is in complete contrast to the situation in teleost fish, which not only lack functional quantities of pillar cell membrane-associated CA activity (Henry *et al.* 1988, 1993; Perry and Laurent, 1990), but some species of which also possess a plasma CA inhibitor (Dimberg, 1994; R. P. Henry, K. M. Gilmour, C. M. Wood and S. F. Perry, in preparation). Possible reasons for the absence of plasma-accessible CA activity in teleost fish have been presented by Lessard *et al.* (1995). The physiological roles of the membrane-bound and extracellular CA activities in disequilibrium events in dogfish blood were the focus of this study.

Basis of the negative disequilibrium

A *positive* acid–base disequilibrium, consisting of an increase in pHa of 0.02–0.04 units under stopflow conditions, has been observed in the postbranchial blood of freshwater rainbow trout (Gilmour *et al.* 1994; Gilmour and Perry, 1994, 1996), and was also detected in this study in the postbranchial blood of seawater-acclimated rainbow trout (Fig. 1). This disequilibrium exists because the uncatalysed plasma HCO_3^- dehydration reaction does not reach completion before the blood leaves the gills. It is eliminated by the addition of bovine CA to the circulation (Gilmour *et al.* 1994). It was, therefore, predicted that an equilibrium condition should exist in the postbranchial blood of the spiny dogfish, owing to the presence of extracellular and gill membrane-bound CA activities available to plasma reactions. In fact, a disequilibrium was observed in the dogfish but, in contrast to the *positive* disequilibrium found in rainbow trout arterial blood, pHa *decreased* when the flow of postbranchial blood through an external circuit was stopped (Fig. 1). Wilson (1995) observed a similar *negative* disequilibrium in the blood of the spiny dogfish under stopflow conditions using a caudal extracorporeal loop.

It is possible that this disequilibrium is a result of continuing Cl^-/HCO_3^- exchange in the postbranchial blood (see also below) which, in theory, is expected to yield a *negative* disequilibrium (Crandall and Bidani, 1981; Gilmour *et al.* 1994). The magnitude of the disequilibrium in dogfish blood was significantly reduced by the addition of bovine CA to the circulatory system (Table 3). Indeed, the pH change during the CA stopflow period was not significantly different from zero. Inasmuch as all HCO_3^- dehydration would be expected to occur through the plasma in the presence of excess plasma CA activity, thereby bypassing the Cl^-/HCO_3^- exchanger, the absence of a disequilibrium following the administration of bovine CA does not exclude the possibility that continuing Cl^-/HCO_3^- exchange is responsible for the negative disequilibrium in dogfish blood (Fig. 4). However, the *negative* direction of the pH change during the stopflow period, coupled with the effect of bovine CA on the magnitude of the pH change, might also suggest that the disequilibrium in dogfish blood results from the postbranchial addition of CO_2 to the plasma and its hydration (Fig. 4). Since the true extracellular CA activity in dogfish is low relative to the rbc CA activity, Table 1 shows the enhancement factor for plasma was 12-fold while it was nearly 20 000-fold for rbc lysate (R. P. Henry, K. M. Gilmour, C. M. Wood and S. F. Perry, in preparation), chemical equilibrium still lags behind CO_2 diffusion. Evidence to support this explanation of the disequilibrium was obtained from the *in vitro* mixing experiment. A small, but significant, pH change was measured in control dogfish blood samples under stopflow conditions,

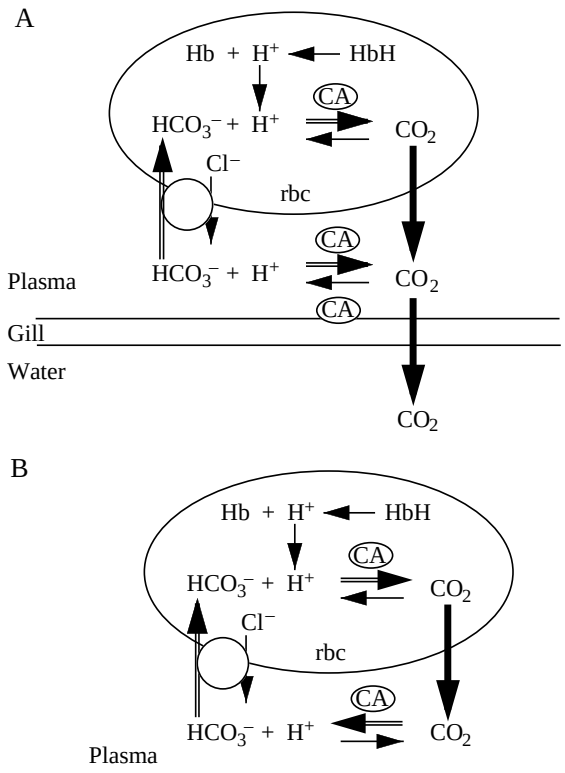

Fig. 4. Schematic model of processes involved in (A) CO_2 excretion at the gill and (B) the generation of an acid–base disequilibrium in the postbranchial blood of the spiny dogfish. In A, plasma $CO_2/HCO_3^-/H^+$ reactions are catalysed by both true extracellular carbonic anhydrase (CA) and gill membrane-bound CA exposed to the plasma. The buffering capacity of the plasma is low relative to that of the red blood cell (rbc); haemoglobin (Hb) buffering capacity is high in elasmobranchs (Jensen, 1989). Note also that the Haldane effect appears to be very small or absent in dogfish (Wood *et al.* 1994). Two possible explanations of the disequilibrium are illustrated in B, namely continuing Cl^-/HCO_3^- exchange and re-equilibration of H^+ and CO_2 across the red blood cell membrane (see text). A third possibility, involving the postbranchial admixture of blood having 'low' and 'high' CO_2 tensions (see text), is not shown.

demonstrating that the true extracellular CA activity was not sufficient to establish an equilibrium state (Table 4).

A situation analogous to that in dogfish arises in trout treated with acetazolamide. Acetazolamide, methazolamide and benzolamide in high concentrations all result in significant inhibition of rbc CA activity in fish (agnathans, elasmobranchs and teleosts) when given sufficient time to equilibrate across the red cell membrane and, under these conditions, all three inhibitors cause a respiratory acidosis to develop in the blood (Swenson and Maren, 1987; Henry *et al.* 1988, 1995). A large, *negative* disequilibrium is measured in the arterial blood of acetazolamide-treated rainbow trout under stopflow conditions (Gilmour *et al.* 1994). Owing to the reduced CA activity, CO_2 continues to be formed after the blood leaves the gills. This CO_2 diffuses into the plasma, driving plasma $CO_2/HCO_3^-/H^+$ reactions towards CO_2 hydration and the generation of protons. Thus, in both dogfish and acetazolamide-treated trout, the *negative* disequilibrium results from the postbranchial addition of CO_2 to the plasma (Fig. 4). The situation in dogfish infused with acetazolamide resembled that in acetazolamide-treated trout; $P_{a_{CO_2}}$ was elevated and the magnitude of the *negative* disequilibrium was increased significantly (Table 3). Inhibition of the extracellular CA activity together with continued postbranchial rbc CO_2 production were probably responsible for the increase. Similarly, a *negative* disequilibrium was observed when the concentration and incubation time of benzolamide were increased so as to produce significant inhibition of rbc CA activity (Fig. 2C). Wilson (1995), however, measured a *positive* acid–base disequilibrium in dogfish blood following acetazolamide injection. The lower dose of acetazolamide used by Wilson (1995), 10^{-4} mol l^{-1} based on a blood volume of 5 % of body mass, or 1 mg kg^{-1}, in comparison with that used in the present study (30 mg kg^{-1}), may not have resulted in significant inhibition of rbc CA activity (see also discussion of benzolamide below).

Whereas acetazolamide inhibits rbc CA, the low dose and low diffusibility of benzolamide effected only insignificant inhibition of rbc CA activity and, hence, selective inhibition of extracellular and membrane-bound CA activities (Fig. 2B; Swenson and Maren, 1987). The use of a low dose of benzolamide, therefore, converted the dogfish into a trout with respect to CO_2 excretion and resulted in the establishment of a *positive* acid–base disequilibrium similar to that found in the postbranchial blood of rainbow trout (Figs 1, 2A), albeit somewhat larger in magnitude (Tables 2, 3). A significant acidosis was also measured in dogfish blood following benzolamide injection (pHa; Table 3). Owing to the establishment of a CO_2 excretion disequilibrium, blood must be leaving the gills at the non-equilibrium pHa value, which should be approximately 0.112 units less than the pre-benzolamide pHa value. That is, assuming that the equilibrium pH value (pH at the end of the stopflow period) is the same in both cases, then pHa at the beginning of the post-benzolamide stopflow period must be 0.053 units (control ΔpH) plus 0.059 units (ΔpH post-benzolamide) lower than pHa at the beginning of the control stopflow period (Fig. 2A). The actual pHa difference was approximately 0.125 units (Table 3), in close agreement with this prediction. It is noteworthy that Swenson and Maren (1987) found no significant effect of benzolamide (1 or 2 mg kg^{-1}) on arterial pH; the sampling protocol they employed probably resulted in the measurement of equilibrium values (see Gilmour and Perry, 1996).

The origin of any CO_2 added to the plasma postbranchially remains uncertain. One possibility is that the combination of true extracellular and membrane-bound CA activities results in a significant portion of HCO_3^- dehydration occurring in the plasma, i.e. bypassing the rbc, as the blood passes through the gills. Because plasma buffering capacity is low compared with that of the rbc (Wood *et al.* 1994), plasma $[H^+]$ is probably lowered relative to rbc $[H^+]$ by the time the blood leaves the gills. In addition, HCO_3^- dehydration *via* the rbc is probably limited by the rate of Cl^-/HCO_3^- exchange, so that plasma

P_{CO_2} may be lower than rbc P_{CO_2} postbranchially. The re-equilibration of CO_2 and protons (by the Jacobs–Stewart cycle) across the rbc membrane in the postbranchial blood would result in the addition of CO_2 to the plasma (Fig. 4). This explanation is similar to the theoretical model for mammalian systems put forward by Crandall and Bidani (1981). In mammals, lung endothelial CA provides a secondary site for HCO_3^- dehydration (reviewed by Bidani and Crandall, 1988) and Crandall and Bidani (1981) predict a negative postcapillary disequilibrium in the presence of large amounts of lung endothelial CA activity. Alternatively, the CO_2 added to the plasma postbranchially might originate from a shunt which enables blood that has bypassed the respiratory gas exchange surface, and hence has a higher P_{CO_2}, to mix with low-P_{CO_2}, postlamellar blood. Anatomical evidence for such a shunt exists (Laurent, 1984), but the generally accepted view is that the physical connections do not constitute a functional bypass (Butler and Metcalfe, 1989). Nevertheless, a functional shunt resulting from ineffective ventilation or the mismatch of ventilation and perfusion conductances remains a possibility (Piiper and Schumann, 1967; Piiper and Baumgarten-Schumann, 1968; Piiper and Scheid, 1984), although the generally high Pa_{O_2} values measured for the dogfish in this study (Table 3) imply good equilibration with the water and hence a good match between ventilation and perfusion.

The possible sources of postbranchial CO_2 addition to the plasma cannot be distinguished on the basis of the present study. The evidence obtained from the hypercapnia and hypoxia experiments is consistent with either explanation, as well as with the possibility that continuing Cl^-/HCO_3^- exchange contributes to the disequilibrium. Hypercapnia had no effect on the magnitude of the disequilibrium (Table 3), presumably because once the blood gas and acid–base status had stabilized at the new water P_{CO_2}, the situation with respect to CO_2 excretion corresponded to that under normocapnic conditions. Hypoxia, in contrast, resulted in a significant increase in the magnitude of the *negative* disequilibrium (Table 3). Hyperventilation and a consequent lowering of the arterial P_{CO_2} may have produced a larger venous–arterial P_{CO_2} difference, which, by the shunt hypothesis, would result in a larger *negative* disequilibrium. A decrease in the residence time of the blood in the gills elicited by hyperventilation-linked increases in gill perfusion would have a similar effect, by reducing the time available for CO_2 reactions to reach equilibrium.

DIDS treatment and the physiological role of extracellular carbonic anhydrase in CO_2 excretion

The similarity between the reaction time of Cl^-/HCO_3^- exchange (T_{67}=0.4 s; Cameron, 1978) and the residence time of blood in the gills (0.5–2.5 s; Cameron and Polhemus, 1974) in rainbow trout gives rise to the possibility that continuing anion exchange in the postbranchial blood could contribute to disequilibrium events (Gilmour *et al.* 1994; Gilmour and Perry, 1994). Elimination of the CO_2 excretion disequilibrium using bovine CA did not reveal an underlying *negative* disequilibrium, implying that Cl^-/HCO_3^- exchange reaches completion during the gill transit period under control conditions (Gilmour *et al.* 1994). However, a potential contribution under circumstances in which the gill transit time was reduced was not ruled out. As in trout, the half-time of Cl^-/HCO_3^- exchange in dogfish blood (Obaid *et al.* 1979) may be comparable to the residence time of blood in the gills (Butler and Metcalfe, 1989). The results of this study have clearly demonstrated that inhibition of the rate of Cl^-/HCO_3^- exchange in dogfish blood *in vivo* leads to the establishment of a large, *negative* disequilibrium (Fig. 3B). This result is in accordance both with theory (Crandall and Bidani, 1981; Gilmour *et al.* 1994) and with the results of experiments carried out on isolated, perfused rat lungs (Crandall *et al.* 1981). Furthermore, the observation that DIDS administration led to a significant increase in the magnitude of the negative disequilibrium in dogfish blood supports the possibility that this disequilibrium is due, at least in part, to continuing Cl^-/HCO_3^- exchange in the postbranchial blood (Fig. 4). The significant increase of 0.065 units in arterial pH measured following DIDS injection *in vivo* (pHa; Table 3) is a consequence of the larger negative disequilibrium. Given the assumption that the same equilibrium pH (pH at the end of the stopflow period) is reached both before and after DIDS treatment, the pHa at the beginning of the post-DIDS stopflow period must be 0.089 units (ΔpH following DIDS) minus 0.025 units (control ΔpH), or 0.064 units, higher than that at the beginning of the control stopflow (Fig. 3B,C); a similar argument was used to explain the difference in pHa following benzolamide injection.

Interestingly, Pa_{CO_2} was not increased significantly by the DIDS treatment (Table 3). While DIDS injection *in vivo* reduced the whole-blood $[^{14}C]HCO_3^-$ dehydration rate by only 38±3 % (N=4) (Fig. 3A), if Cl^-/HCO_3^- exchange is actually the rate-limiting step to CO_2 excretion *in vivo* (Perry, 1986; Wood and Munger, 1994), any decrease in the rate of this process might be expected to inhibit CO_2 excretion significantly. The fact that it did not suggests that there is a reserve capacity in the CO_2 excretion system, which might be provided by the extracellular and gill membrane-bound CA activities. Similarly, inhibition of these CA activities using benzolamide did not result in a significant increase in arterial P_{CO_2} (Table 3; see also Swenson and Maren, 1987), implying that they are not essential for CO_2 excretion under normal conditions. A significant increase in Pa_{CO_2} might be expected, however, with a combined DIDS and benzolamide treatment; this aspect of the present study certainly warrants further investigation.

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