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A COMPARISON OF CO_2 EXCRETION IN A SPONTANEOUSLY VENTILATING BLOOD-PERFUSED TROUT PREPARATION AND SALINE-PERFUSED GILL PREPARATIONS: CONTRIBUTION OF THE BRANCHIAL EPITHELIUM AND RED BLOOD CELL

BY STEVE F. PERRY, PETER S. DAVIE*, CHARLES DAXBOECK‡ AND DAVID J. RANDALL

Department of Zoology, The University of British Columbia, 2354–6270 University Boulevard, Vancouver, B.C., Canada V6T 2A9

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SUMMARY

(1) A spontaneously ventilating blood-perfused trout preparation and saline perfused gill preparations were utilized to investigate the role of the erythrocyte and branchial epithelium in CO_2 excretion and acid-base regulation.

(2) CO_2 excretion ($\dot{M}_{CO_2}$) in blood-perfused preparations was positively correlated with haematocrit (Hct), and was abolished completely during plasma-perfusion.

(3) Elevating HCO_3^- concentration of input blood from 10 to 25 mM significantly increased $\dot{M}_{CO_2}$ fourfold in blood-perfused preparations as a result of increased entry of HCO_3^- into the red blood cell and not into the gill epithelium. Increased HCO_3^- concentration was without effect in totally saline-perfused coho salmon (*Onchorynchus kisutch*).

(4) The addition of 4-acetamido-4′-*iso*-thiocyanatostilbene-2,2′ disulfonic acid (SITS; 10^{-4} M) to input blood significantly reduced $\dot{M}_{CO_2}$ and oxygen uptake ($\dot{M}_{O_2}$) in blood-perfused fish due to inhibition of erythrocytic HCO_3^-/Cl^-exchange.

(5) Unlike blood-perfused preparations, no saline-perfused preparation (isolated holobranchs or totally perfused rainbow trout or coho salmon) displayed measureable CO_2 excretion at physiological P_{CO_2} and pH.

(6) Increased input P_{CO_2} in both blood-perfused and saline-perfused preparations significantly increased $\dot{M}_{CO_2}$ due to enhanced branchial diffusion of molecular CO_2.

(7) It is concluded that the entry of HCO_3^- into the erythrocyte is the rate-limiting step in CO_2 excretion and that movement of HCO_3^- from plasma to gill epithelium cells in no way contributes to overall CO_2 elimination.

* Department of Physiology and Anatomy, Massey University Palmerston North, New Zealand.
‡ Pacific Gamefish Foundation, P.O. Box 25115, Honolulu, Hawaii, U.S.A. 96825

INTRODUCTION

In recent years a controversy has existed regarding the pattern of CO_2 excretion in fish. Haswell & Randall (1976, 1978) proposed a model for CO_2 excretion in fish which suggested that red blood cells are not involved. They proposed that fish erythrocytes are functionally impermeable to bicarbonate, making erythrocyte carbonic anhydrase unavailable for the dehydration of plasma bicarbonate. Instead, it was postulated that branchial carbonic anhydrase catalyses the dehydration reaction and that entry of bicarbonate from plasma to gill epithelial cells is the rate-limiting step in CO_2 excretion (Haswell, Randall & Perry, 1980). This theory, of course, drastically contrasts with the more widely accepted view, that erythrocytic carbonic anhydrase catalyses the dehydration of plasma bicarbonate in a typical mammalian fashion. In this scheme branchial carbonic anhydrase is assigned the role of rehydrating plasma CO_2 thereby furnishing the counter-ions, HCO_3^- and H^+ (NH_4^+), for exchange with Cl^- and Na^+ respectively, at the apical (water-facing) membrane of gill epithelial cells (Maetz, 1971).

The primary objective of this study was to assess the relative importance of the branchial epithelium and red blood cell in CO_2 excretion by a comparison of a blood-perfused trout preparation and other saline-perfused gill preparations under a variety of conditions. Experiments were specifically designed to test the two conflicting theories of CO_2 excretion utilizing the spontaneously ventilating blood-perfused trout preparation described in the first article of this series (Davie *et al.* 1982).

MATERIAL AND METHODS

Rainbow trout (*Salmo gairdneri*) weighing between 200–400 g were obtained from Sun Valley Trout Farm (Mission, B.C.). They were kept in large circular fibreglass tanks supplied with aerated, dechlorinated, Vancouver tap water (Na^+ = 40 μequiv. l^{-1}, Cl^- = 20 μequiv.l^{-1}, hardness = 12 ppm $CaCO_3$), at ambient temperature (7–12 °C) and photoperiod. Fish were fed daily with a commercial pelleted trout diet (Moore–Clark Co.). They were not fed for 48 h prior to, or during experiments. Experiments involving seawater were performed at Bamfield Marine Station (Bamfield, B.C.). Coho salmon (*Oncorhynchus kisutch*) weighing between 300–500 g were obtained from Pacific Biological Station (Nanaimo, B.C.). They were maintained in flowing seawater in a manner similar to rainbow trout.

Experimental protocol

(1) *Spontaneously ventilating, blood-perfused trout preparation*

A spontaneously ventilating, blood-perfused trout preparation was prepared as described by Davie *et al.* (1982). Fish were perfused for 2–3 h, allowing recovery from the acute effects of anaesthesia (Houston, Madden, Woods & Miles, 1971), before experimentation commenced. Experiments involved manipulation of input blood haematocrit (Hct), P_{CO_2}, and total CO_2 (C_{CO_2}) as well as the addition of the anion transport inhibitor, SITS (4-acetamido-4′-*iso*-thiocyanatostilbene-2,2′ disul-

[illegible]onic acid). Typically, 'normal' blood samples were withdrawn from tonometer (input), dorsal aorta and venous return and analyzed immediately for C_{CO_2}, C_{O_2}, pH, P_{O_2} and Hct (see Davie *et al.* 1982). Input blood then was changed by switching to another tonometer which had been prepared appropriately. Following a 5 min adjustment period, blood samples again were withdrawn and analyzed. A normal period of 5 min and a sample always preceded and followed any experimental period.

Input blood Hct was adjusted either by adding known volumes of red blood cells or plasma, obtained from donor fish. Three categories of blood were utilized: 'normal' Hct (approx. 10%), high Hct (approx. 20%), and low Hct (approx. 4%). P_{CO_2} was doubled by changing the gas mixture equilibrating the blood from 0·4% to 0·8% CO_2 in 40% air (remainder N_2). These mixtures were provided by gas mixing pumps (Wösthoff). Blood C_{CO_2} was increased by the addition of known quantities of $NaHCO_3$. SITS (British Drug House) was added to a final concentration of 10^{-4} M. Blood and buccal pressures were monitored continuously as described previously (Davie *et al.* 1982).

(2) *Isolated, saline-perfused rainbow trout holobranch preparation*

Approximately 30 min prior to surgical procedures, fish were injected intraperitoneally with sodium heparin (5000 USP units.kg^{-1}). Following this period, fish were anaesthetized in a solution of 1:15000 MS 222 (pH adjusted to 7·0–7·5) and transferred to an operating table (Smith & Bell, 1967) where 1:20000 MS 222 was recirculated over the gills. The heart and ventral aorta were exposed by a ventral, midline incision. The bulbus arteriosus/ventral aorta was cannulated with a short length of polyethylene tubing (PE 160; 1·14 mm × 1·57 mm) and secured in place. The gills were cleared of blood by perfusing manually with filtered (Millipore, 0·45 μm), heparinized (10 USP units.ml^{-1}) Cortland saline (Wolf, 1963). The branchial basket was removed and individual holobranchs dissected free and stored in aerated saline on ice, until required.

Afferent and efferent arch vessels were exposed and cannulated with blunt 20 or 21 guage hypodermic needles depending on the diameter of the vessels (Farrell, Daxboeck & Randall, 1979). Holobranchs remained submerged in ice-cold saline during these procedures. The gill arch was ligated as near to the catheter as possible, leaving a minimal amount of cut tissue perfused. Cannulated holobranchs were suspended in an aerated, well-mixed water bath at constant temperature and perfused at constant flow (2·2 ml.min^{-1}.$arch^{-1}$.kg^{-1} body weight) with gas equilibrated Cortland saline (0·5% CO_2 in 40% air, remainder N_2) using a syringe pump (Harvard). Input pressure was monitored with a Harvard pressure transducer and displayed on a chart recorder. Experiments did not commence until input pressure had stabilized (usually 20–30 min).

Protocol consisted of sampling afferent and efferent saline while perfusing with saline equilibrated with normal (0·5%) and high (2·0%) CO_2. Gas mixtures were supplied by gas mixing pumps (Wösthoff). C_{CO_2} was determined using the method of Cameron (1971) with a Radiometer PHM-71 acid-base analyser and associated CO_2 electrode (E5036/0) maintained at 45 °C to speed electrode response. pH measurements were made using the same acid-base analyzer and micro pH electrode (G297/

G2). P_{CO_2} was calculated using the measured pH and C_{CO_2} values and a re-organizati[illegible] of the Henderson–Hasselbalch equation as follows:

$$P_{CO_2} = \frac{C_{CO_2}}{[\text{anti-log}\,(\text{pH-p}K')\,(\alpha CO_2)] + \alpha CO_2}. \quad (1)$$

The operational pK' values of carbonic acid were obtained from Severinghaus, Stupfel & Bradley (1956) and the solubility coefficients of CO_2 (αCO_2) were obtained from Albers (1970).

(3) *Totally saline-perfused rainbow trout and coho salmon*

Totally perfused preparations were prepared as described by Wood, McMahon & McDonald (1978). Briefly, the ventral aorta of pre-heparinized fish (2000 USP units.fish^{-1}) was exposed, sectioned and cannulated orthograde and retrograde using polyethylene tubing (PE 190; 1·19 mm × 1·70 mm). Filtered Cortland saline (Millipore, 0·45 μm) was infused orthograde with 100 ml syringe until the venous effluent appeared free of blood (approx. 10 min). A tube for ventilation was placed into the mouth and sewn into position. Fish then were transferred to a darkened, rectangular Perspex box and perfused with Cortland saline (equilibrated with 0·4% CO_2 in 40% air, remainder N_2) at constant flow (17 ml.min.$^{-1}$.kg^{-1}) (Kiceniuk & Jones, 1977) using a pulsatile pump (Watson–Marlow) and artificially ventilated at 500 ml.min^{-1}. Input samples were taken from a T-junction in the infusion line near the ventral aorta and arterial samples were taken from a dorsal aortic cannula (Smith, 1978) implanted 24 h previously. C_{CO_2} and pH were determined as described above.

Experiments involved manipulating P_{CO_2} (rainbow trout) and C_{CO_2} (coho salmon) while monitoring input and dorsal aortic pH and C_{CO_2}. C_{CO_2} was adjusted by addition of $NaHCO_3$ and P_{CO_2} was adjusted using gas mixing pumps (Wösthoff).

All experimental values are presented in tables as means ± S.E.M. Results were statistically analysed using Student's t-test between sample means where appropriate, and 5 or 10% was taken as the fiducial limit of significance (see Tables).

RESULTS

(1) *Spontaneously ventilating, blood-perfused rainbow trout*

The effects of Hct on respiratory and acid-base status in the spontaneously ventilating, blood-perfused rainbow trout are shown in Fig. 1 and Table 1. CO_2 excretion across the gills ($\dot{M}_{CO_2}$) increased as Hct was raised. Input blood CO_2 content (C_{CO_2}) remained virtually constant in all three groups indicating little or no carbamino-CO_2 formation in the blood. Net hydrogen ion flux (ΔH^+) in all cases was in the direction of water to blood but decreased significantly in the high Hct group. The effect of Hct on branchial and systemic haemodynamics has been discussed previously (see Davie *et al.* 1982) and cannnot explain the observed effects on blood respiratory and acid-base status. In two instances fish were perfused with plasma; during this condition $\dot{M}_{CO_2}$ was abolished completely while dorsal aortic P_{O_2} remained unchanged.

Increasing HCO_3^- concentration of input blood to approximately 25 mM significantly increased $\dot{M}_{CO_2}$ four-fold (Table 2). C_{O_2} was not affected although $\dot{M}$·

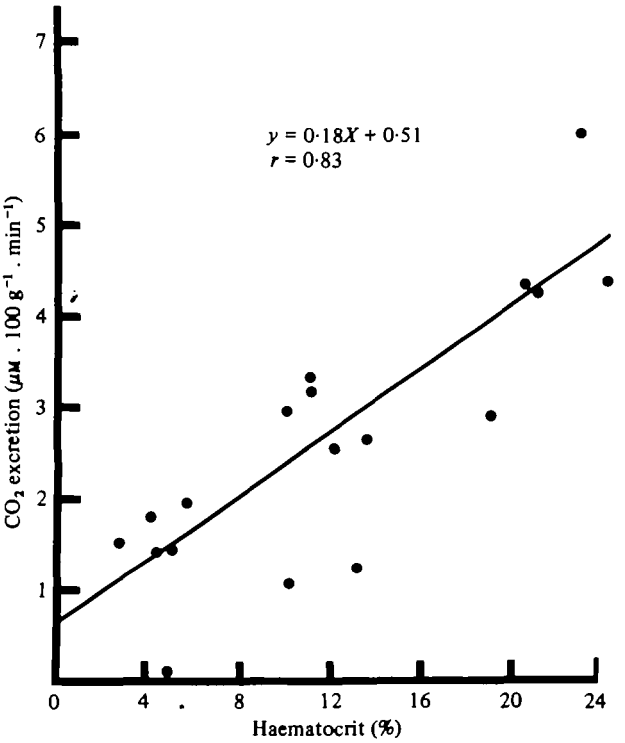

Fig. 1. The effect of haematocrit on CO_2 excretion in the spontaneously ventilating, blood-perfused rainbow trout, *Salmo gairdneri*. See text for further details.

Table 1. *Summary of blood carbon dioxide and acid-base status in the spontaneously ventilating, blood-perfused rainbow trout perfused with three different haematocrits; low, normal and high*

(n = 6 fish ± S.E.M.)

	Hct	C_{CO_2} (mM)	[H$^+$] (nM)
		Input blood	
(I) Low	4·3 ± 0·4	10·87 ± 0·6	15·78 ± 1·1 (7·81)†
(II) Normal	11·3 ± 0·5	10·73 ± 0·5	16·87 ± 1·3 (7·78)
(III) High	20·2 ± 1·6	11·30 ± 0·6	18·39 ± 1·4 (7·75)
		Dorsal aortic blood	
(I) Low	3·9 ± 0·5	10·06 ± 0·5	19·58 ± 2·2 (7·72)
(II) Normal	9·3 ± 0·5	9·12 ± 0·4	18·27 ± 2·0 (7·75)
(III) High	16·5 ± 1·2	8·90 ± 0·3	18·42 ± 1·9 (7·75)
		Δ Input – Dorsal Aorta	
	Hct (%)	$\dot{M}_{CO_2}$ (μmol/100 g/min)	[H$^+$] (pmol/100 g/min)
(I) Low	−0·4	1·34 ± 0·3	6·00 ± 3·5
(II) Normal	−2·0	2·62 ± 0·3	2·07 ± 1·5
(III) High	−5·2	3·87 ± 0·7	0·13 ± 1·7

† Corresponding pH value.

Table 2. *Effect of blood* $[HCO_3^-]$ *on blood respiratory and acid-base status in the spontaneously ventilating, blood-perfused rainbow trout*

(n = 6 fish ± S.E.M.)

	C_{CO_2} (mM)	C_{O_2} (mM)	P_{O_2} (mmHg)	P_{CO_2} (mmHg)	$[H^+]$ (nM)
			Input blood		
(I) Normal	9·86 ± 0·5	1·18 ± 0·2	30·1 ± 4·4	3·54 ± 0·2	18·32 ± 1·7 (7·74)†
(II) High $[HCO_3^-]$	24·74 ± 1·0**	1·20 ± 0·3	23·8 ± 4·7	5·50 ± 0·4**	11·36 ± 1·1 (7·96)*
			Dorsal aortic blood		
(I) Normal	8·5 ± 0·4	1·86 ± 0·3	109·0 ± 3·9	4·36 ± 0·7	23·91 ± 3·4 (7·64)
(II) High $[HCO_3^-]$	20·58 ± 1·0**	1·71 ± 0·3	89·9 ± 4·7**	5·03 ± 0·7	12·22 ± 1·7 (7·93)**
			Δ Input – Dorsal Aorta		
	$\dot{M}_{CO_2}$ (μmol/100 g/min)	$\dot{M}_{O_2}$ (μmol/100 g/min)	P_{O_2} (mmHg)	P_{CO_2} (mmHg)	$[H^+]$ (pmol/100 g/min)	RE_g
(I) Normal	1·63 ± 0·3	1·13 ± 0·3	78·8 ± 6·5	0·82 ± 0·5	8·71 ± 3·8	1·4
(II) High $[HCO_3^-]$	6·50 ± 0·4**	0·84 ± 0·3	66·2 ± 6·9*	−0·53 ± 0·8	1·11 ± 2·7*	7·7

† Corresponding pH value.
* Significantly different from normal value at 10%
** Significantly different from normal value at 5%

Table 3. *Effect of SITS* (10^{-4}) *on blood respiratory and acid-base status in the spontaneously ventilating, blood-perfused rainbow trout*

	C_{CO_2} (mM)	C_{O_2} (mM)	P_{O_2} (mmHg)	P_{CO_2} (mmHg)	$[H^+]$ (nM)
			Input blood		
(I) Normal	11·51 ± 0·3	1·06 ± 0·2	20·9 ± 1·9	2·98 ± 0·3	12·71 ± 1·2 (7·90)†
(II) 10^{-4} M SITS	10·50 ± 0·4*	1·22 ± 0·2	27·0 ± 3·2*	3·16 ± 0·3**	14·71 ± 0·9 (7·83)*
			Dorsal Aortic Blood		
(I) Normal	9·84 ± 0·3	1·83 ± 0·4	87·7 ± 5·8	3·27 ± 0·3	16·16 ± 1·0 (7·79)
(II) 10^{-4} M SITS	9·97 ± 0·4	1·69 ± 0·3	89·3 ± 6·0	4·11 ± 0·3**	19·65 ± 0·9 (7·71)**
			Δ Input – Dorsal Aorta		
	$\dot{M}_{CO_2}$ (μmol/100 g/min)	$\dot{M}_{O_2}$ (μM/100 g/min)	P_{O_2} (mmHg)	P_{CO_2} (mmHg)	$[H^+]$ (pmol/100 g/min)
(I) Normal	2·57 ± 0·3	1·21 ± 0·3	65·6 ± 5·6	0·29 ± 0·3	2·05 ± 0·5
(II) 10^{-4} M SITS	0·80 ± 0·2**	0·70 ± 0·3*	62·3 ± 6·2	0·95 ± 0·3*	4·94 ± 1·5

† Corresponding pH value.
* Significantly different from normal value at 10%.
** Significantly different from normal value at 5%

appeared to decrease (not significant). In addition, increased $[HCO_3^-]$ was associated with significant decreases in dorsal aortic P_{O_2}, ΔP_{O_2} and ΔH^+ across the gills (Table 2). Branchial haemodynamics and ventilation were unaffected by HCO_3^- treatment.

The stilbonic acid derivative SITS has been used to inhibit chloride transport in red blood cells as well as other transporting tissues (Cabantchik & Rothstein, 1974; Shami, Rothstein & Knauf, 1978). The effects of SITS (10^{-4} M) on blood respirator

Table 4. *Effect of blood P_{CO_2} on blood respiratory and acid-base status in the spontaneously ventilating, blood-perfused rainbow trout*

(n = 6 fish ± S.E.M.)

	C_{CO_2} (mM)	C_{O_2} (mM)	P_{O_2} (mmHg)	P_{CO_2} (mmHg)	[H+] (nM)
			Input Blood		
(I) Normal	12·35 ± 0·8	0·87 ± 0·2	19·8 ± 3·1	3·31 ± 0·1	13·41 ± 0·7 (7·88)†
(II) High P_{CO_2}	13·28 ± 0·5	0·95 ± 0·2	31·1 ± 2·5	5·60 ± 0·3**	20·38 ± 1·2 (7·69)**
			Dorsal aortic blood		
(I) Normal	10·80 ± 0·7	1·78 ± 0·3	94·1 ± 8·1	3·49 ± 0·3	15·69 ± 0·9 (7·81)
(II) High P_{CO_2}	10·87 ± 0·5	1·68 ± 0·3	101·0 ± 5·3	4·46 ± 0·4**	19·68 ± 1·2 (7·71)**
			Δ Input – Dorsal Aorta		
	$\dot{M}_{CO_2}$ (μmol/100 g/min)	$\dot{M}_{O_2}$ (μmol/100 g/min)	P_{O_2} (mmHg)	P_{CO_2} (mmHg)	[H+] (pmol/100 g/min)
(I) Normal	2·56 ± 0·6	1·50 ± 0·2	74·3 ± 7·6	0·19 ± 0·3	3·68 ± 1·3
(II) High P_{CO_2}	3·97 ± 0·4**	12·0 ± 0·3	70·9 ± 5·9	−1·13 ± 0·4**	1·26 ± 2·2**

† Corresponding pH value.
* Significantly different from normal value at 10%.
** Significantly different from normal value at 5%

and acid-base status are shown in Table 3. $\dot{M}_{CO_2}$ and $\dot{M}_{O_2}$ decreased significantly following addition of SITS to the input blood. Dorsal aortic P_{CO_2} and [H+] both increased significantly although only ΔP_{CO_2} but not ΔH^+ was significantly different from normal values. Occasionally, perfusion was switched back to SITS-free blood and in these instances (three fish) $\dot{M}_{CO_2}$ and $\dot{M}_{O_2}$ were restored to normal levels. SITS treatment caused no significant effects on branchial haemodynamics or ventilation.

Increasing P_{CO_2} of input blood by 1·7 times (3·3 mmHg to 5·5 mmHg) (Table 4) significantly increased $\dot{M}_{CO_2}$ but was without effect on O_2 transfer although input blood P_{O_2} increased significantly. As in other experiments (also see Davie *et al.* 1982, and Daxboeck *et al.* 1982), normal fish showed net H^+ movements from water to blood. When input blood P_{CO_2} was elevated, a significant change in direction occurred; net H^+ movement now was from blood to water. Similarly, P_{CO_2} across the gills changed from a slight increase to a significant decrease. High input P_{CO_2} was associated with increased dorsal aortic blood [H^+] and usually was accompanied by a small, slow increase in dorsal aortic pressure.

None of the above experiments, except plasma perfusion, produced any visual signs of stress. Perfusion with plasma evoked a violent struggling response and brief pauses (2–3 s) in ventilatory movements and intrinsic heart beat. Due to the severe nature of these responses, this line of investigation was discontinued.

(2) *Saline-perfused preparations*

In contrast to spontaneously ventilating, blood-perfused fish, no saline-perfused preparation displayed measureable $\dot{M}_{CO_2}$ at physiological P_{CO_2} and pH except totally

Table 5. *Effects of perfusate P_{CO_2} and $[HCO_3^-]$ on acid-base status of various saline-perfused preparations*

(mean values ± S.E.M.)

Isolated, saline-perfused trout holobranchs ($n = 6$)

	Afferent saline	Efferent saline	Δ	Δ (%)
(I) Normal P_{CO_2}				
C_{CO_2} (mM)	8·71 ± 0·4	8·88 ± 0·5	0·17 ± 0·1	2·0
P_{CO_2} (mmHg)	4·01 ± 0·3	4·76 ± 0·5	0·75 ± 0·2	18·7
$[H^+]$ (nM)	19·04 ± 0·9	21·82 ± 1·1	2·78 ± 0·8	14·6
(II) High P_{CO_2}				
C_{CO_2} (mM)	10·82 ± 0·6	10·50 ± 0·6	−0·32 ± 0·1*	−2·6
P_{CO_2} (mmHg)	15·40 ± 0·8	13·14 ± 0·6	−2·26 ± 0·4*	−14·7
$[H^+]$ (nM)	55·64 ± 1·7	48·33 ± 1·8	−7·31 ± 1·5*	−13·1

Totally saline-perfused rainbow trout ($n = 5$)

	Input saline	Dorsal aorta	Δ	Δ (%)
(I) Normal P_{CO_2}				
C_{CO_2} (mM)	10·57 ± 0·3	11·61 ± 0·3	1·04 ± 0·3	9·8
P_{CO_2} (mmHg)	3·14 ± 0·2	4·80 ± 0·1	1·66 ± 0·1	52·9
$[H^+]$ (nM)	12·08 ± 0·6	16·37 ± 0·8	42·9 ± 0·5	35·5
(II) High P_{CO_2}				
C_{CO_2} (mM)	11·00 ± 0·4	10·46 ± 0·3	−0·54 ± 0·4*	−4·9
P_{CO_2} (mmHg)	9·16 ± 0·2	9·88 ± 0·2	0·72 ± 0·2*	7·9
$[H^+]$ (nM)	32·28 ± 1·1	36·48 ± 1·3	4·20 ± 0·9	13·0

Totally saline-perfused coho salmon ($n = 5$)

	Input saline	Dorsal aorta	Δ	Δ (%)	TMP	ΔP_{O_2} (mmHg)
(I) Normal $[HCO_3^-]$						
C_{CO_2} (mM)	11·86 ± 0·4	11·72 ± 0·4	−0·14 ± 0·2	−1·2	−21	20
(II) High $[HCO_3^-]$						
C_{CO_2} (mM)	33·15 ± 0·7	33·30 ± 0·8	0·15 ± 0·2	0·5	−21	—

* Significantly different from normal value at 5%.

perfused coho salmon; and this was not significant (Table 5). In fact, C_{CO_2} usually was higher in post-gill saline indicating a net uptake of CO_2. Only when perfusate P_{CO_2} was increased to 2% CO_2 (15 mmHg: perfused holobranchs) or 1·2% CO_2 (9 mmHg; totally perfused rainbow trout), was $\dot{M}_{CO_2}$ measurable. The differences in C_{CO_2} across the gill between normal and high P_{CO_2} groups are highly significant ($P < 0·05$). Also in contrast to blood-perfused fish, perfusate $[HCO_3^-]$ had no effect on $\dot{M}_{CO_2}$ in totally perfused coho salmon (Table 5). The trans-membrane potential (basal membrane of gill epithelial cells), as measured with microelectrodes (Maetz & Campanini, 1966), also did not vary from −21 mV (inside of cell negative) as $[HCO_3^-]$ was raised (Table 5).

DISCUSSION

The results of the present studies clearly demonstrate an important involvement of trout erythrocytes in CO_2 excretion and as such, oppose the theory of Haswell & Randall (1978) that the teleost red blood cell is functionally impermeable to plasma bicarbonate. Much of the evidence supporting non-involvement of the teleost

erythrocyte has come from *in vivo* studies using anaemic fish (Haswell, 1978; Haswell & Randall, 1978). These experiments showed that CO_2 excretion, arterial pH and P_{CO_2} did not vary following 24 h of severe anaemia in rainbow trout. The present results however, show a highly significant positive correlation between Hct and $\dot{M}_{CO_2}$. In addition, Daxboeck *et al.* (1982) observed a similar relationship between Hct and $\dot{M}_{O_2}$, indicating a common pathway through the erythrocyte. The differences between the studies are probably a result of the profound cardiovascular adjustments associated with severely anaemic fish, particularly increased cardiac output ($\dot{Q}$) (Wood, McMahon & McDonald, 1979; Wood & Shelton, 1980) due to increased stroke volume (Cameron & Davis, 1970). These responses, by increasing the delivery of physically dissolved CO_2 to the gills, would maintain net $\dot{M}_{CO_2}$ thereby masking any effects of anaemia on CO_2 excretion. In our experiments, using the spontaneously ventilating, blood-perfused trout, we were able to maintain $\dot{Q}$ constant thereby eliminating the effects of cardiovascular changes on $\dot{M}_{CO_2}$. Branchial vascular resistance increased both during low and high Hct experiments (see Davie *et al.* 1982) so it is unlikely that changes in branchial haemodynamics contributed to the overall results. Thus we are confident that our results reflect only the concentration of circulating erythrocytes. Recently, Wood, McDonald & McMahon (1981) observed that severe experimental anaemia (1–5% Hct) in starry flounder (*Platichthys stellatus*) and rainbow trout caused respiratory acidosis (decreased pH, increased P_{CO_2}) supporting the conclusion that plasma bicarbonate is dehydrated within erythrocytes in a typical mammalian fashion (Cameron & Polhemus, 1974). Unlike the present study, however, Wood *et al.* (1981) found no effect on blood acid-base status until a Hct of between 5–10% was reached. Again, this is probably attributable to cardiovascular adjustments which can maintain $\dot{M}_{CO_2}$ during mild anaemia in intact fish. At Hcts below 10% it is likely that these compensatory adjustments are no longer sufficient to maintain $\dot{M}_{CO_2}$ and blood acid-base status.

Cameron (1978) demonstrated that teleost blood (red snapper and rainbow trout) displayed a typical chloride shift (HCO_3^-/Cl^- exchange) which could be abolished by addition of the carbonic anhydrase inhibitor, acetazolamide. Obaid, Critz & Crandall (1979) also have shown the presence of HCO_3^-/Cl^- exchange in dogfish erythrocytes which was blocked by the anion transport inhibitor, SITS. These results, together with the findings of this study, present overwhelming evidence opposing the theory of Haswell & Randall (1978) that fish erythrocytes are functionally impermeable to HCO_3^-. Results from *in vitro* experiments, which indicate the presence of a plasma inhibitor rendering erythrocytic carbonic anhydrase unavailable to catalyse plasma HCO_3^- dehydration (Haswell & Randall, 1976), can be attributed to methodological problems (see Heming & Randall, 1982).

Confirmation that teleost red blood cells are involved in CO_2 excretion does not exclude the possibility that the branchial epithelium also is involved in the dehydration of plasma HCO_3^-. In fact, movement of HCO_3^- between plasma and gill epithelial cells has been proposed (Haswell, Randall & Perry, 1980) and investigated indirectly (Perry *et al.* 1981). The data of Perry *et al.* (1981) demonstrated that pharmacological inhibition of apical branchial $Na^+/H^+(NH_4^+)$ or Cl^-/HCO_3^- exchange mechanisms results in significant decreases and increases in plasma C_{CO_2} respectively. These

authors concluded that HCO_3^- entry into the gill epithelium is in part controlled by epithelial cell pH and is an important factor determining overall CO_2 excretion and blood acid-base status. Re-evaluation of the results however reveals that these changes alternatively might be due to changing rates of proton movements between plasma and epithelial cells, thereby altering the CO_2–HCO_3^- equilibrium in plasma. Indeed, recent work by McWilliams & Potts (1978) has shown that the gill epithelium is extremely permeable to H^+ ions. Because of these uncertainties, additional experiments were performed with the blood-perfused preparation and various saline-perfused preparations in order to investigate this problem and to assess the relative contributions of red blood cells and the branchial epithelium to CO_2 excretion. If the branchial epithelium is permeable to HCO_3^- and the entry of HCO_3^- into gill epithelial cells is a major pathway for CO_2 excretion, one would expect saline-perfused gill preparations to excrete CO_2 at rates comparable to live intact fish or blood-perfused preparations. Furthermore, CO_2 excretion should be proportional to the concentration of perfusate HCO_3^-.

It is evident that saline-perfused preparations do not excrete CO_2 at physiological P_{CO_2} and pH whereas blood-perfused fish do, at rates comparable to published *in vivo* data (see also Davie *et al.* 1982). Although this may be due, in part, to increased diffusion barriers to get transfer in saline-perfused preparations, we feel this is unlikely to explain the absence of $\dot{M}_{CO_2}$ for the following reasons. Totally saline-perfused coho salmon displayed no significant CO_2 excretion but did not show an increase in dorsal aortic P_{O_2} above input levels of approximately 20 mmHg (Table 5). Nor do we believe that the absence of $\dot{M}_{CO_2}$ in saline-perfused preparations is due to artificial ventilation. In another series of experiments (unpublished observations) blood-perfused fish were ventilated artificially by providing a pressure head of water (no longer spontaneously ventilating) and $\dot{M}_{CO_2}$ and $\dot{M}_{O_2}$ were unaffected. Furthermore, perfusion of spontaneously ventilating fish with plasma completely abolished $\dot{M}_{CO_2}$ but was without effect on dorsal aortic P_{O_2}. Clearly, the differences observed between saline and blood-perfusion must be due to the presence or absence of erythrocytes.

Increasing the concentration of HCO_3^- in the input blood of perfused fish caused a dramatic increase in $\dot{M}_{CO_2}$. This is due to increased flux of HCO_3^- into red blood cells and not into the gill epithelium. We conclude this for two reasons; first, because of the accompanying effect on oxygen transport and secondly, because of the lack of an effect of increased HCO_3^- in totally saline-perfused fish. That HCO_3^- is without effect in saline-perfused fish indicates that the branchial epithelium (basal membrane) is impermeable to HCO_3^- and that its movement from plasma to epithelium cannot constitute a major pathway for CO_2 excretion. Measurements of a constant trans-membrane potential (basal membrane) of gill epithelial cells at all concentrations of perfusate HCO_3^- support this conclusion. The decrease in dorsal aortic P_{O_2} during high HCO_3^- blood perfusion is not related to lower input P_{O_2} because fluctuations of input P_{O_2} in this range ($\sim$ 10 mmHg) did not affect dorsal aortic P_{O_2} of normal fish (see Davie *et al.* 1982). An alternative explanation is increased entry of HCO_3^- into the red blood cell thereby facilitating O_2 binding to haemoglobin, and reducing the amount in solution.

Whereas saline [HCO_3^-] had no effect on $\dot{M}_{CO_2}$, increasing P_{CO_2} in saline-perfused

bulobranchs and totally saline-perfused trout did stimulate CO^2 excretion which was significantly increased from normal values. Similar results were obtained from blood-perfused fish with increased P_{CO_2}. Thus, increasing the amount of dissolved CO_2 in blood or saline can increase $\dot{M}_{CO_2}$, probably by enhanced diffusion of molecular CO_2 across the branchial epithelium. Haswell & Randall (1978) perfused fish with saline equilibrated with 1 % CO_2 in air (pH 7·5). This may account for the discrepancy between their results, showing CO_2 excretion in saline-perfused fish, and the results of the present study.

The results of studies comparing blood-perfused and saline-perfused preparations indicate that the gill epithelium is impermeable to HCO_3^- and that movement of HCO_3^- from plasma to gill epithelial cells does not contribute to CO_2 excretion or acid-base balance. Moreover, it is clear that the entry of HCO_3^- into the erythrocyte is the rate limiting step in CO_2 excretion and that the only contribution of the branchial epithelium to this process is via diffusion of molecular CO_2 and apical Cl^-/HCO_3^- exchange (Fig. 2).

Results from this study and others (McWilliams & Potts, 1978; van den Thillart & Randall, in preparation) have shown that net H^+ ion movement across the gill is related to the $[H^+]$ ion gradient between blood and water. Increasing $[HCO_3^-]$ of input blood certainly increases this gradient, yet net H^+ ion influx is reduced significantly (Table 2). This can be explained by enhanced H^+ ion excretion via combination with HCO_3^- forming CO_2 which diffuses into the water. Normally, O_2 binding to haemoglobin will provide protons to maintain an RQ of 0·7 if all CO_2 is derived from bicarbonate (German & Wyman, 1937). RE_g changed from 1·4 to 7·7 during high HCO_3^- perfusion. Clearly, to maintain an RE_g of 7·7 requires a source of protons other than that derived from haemoglobin oxygenation. Two possible sources are first those released from proteins, especially haemoglobin if pH rises, and second protons that diffuse into the blood from other compartments. Blood pH did not rise during passage through the gills, thus the proton source must have come from another compartment, either the gill tissue or the water and not from blood proteins. Given that the gill epithelium is highly permeable to H^+ ions, plus the large number of protons required, influx from the water is the most probable source.

SITS is a potent inhibitor of anion movements across the mammalian red blood cell (Cabantchik & Rothstein, 1974; Cabantchik, Knauf & Rothstein, 1978; Shami *et al.* 1978). Again, we believe the inhibitory action of SITS on $\dot{M}_{CO_2}$ in blood-perfused fish is due to inhibition of erythrocytic HCO_3^-/Cl^- exchange and not to inhibition of HCO_3^- movement into gill epithelial cells. The decreases in $\dot{M}_{O_2}$ associated with SITS treatment must be due to abolition of the chloride shift which decreases red blood cell pH and reduces blood O_2 capacity via the Root shift. The increase in input blood P_{O_2} also can be attributed to a Bohr shift to the right (due to decreased rbc pH).

Fish, unlike mammals, do not utilize changes in ventilation to achieve pH regulation during acid-base disturbances. Instead, plasma HCO_3^- levels are adjusted and the P_{CO_2} gradient between blood and water remains constant (Cameron & Randall, 1972; Janssen & Randall, 1975; Cameron, 1978). For plasma bicarbonate levels to rise during hypercapnic acidosis, either H^+ excretion must be enhanced or carbon dioxide

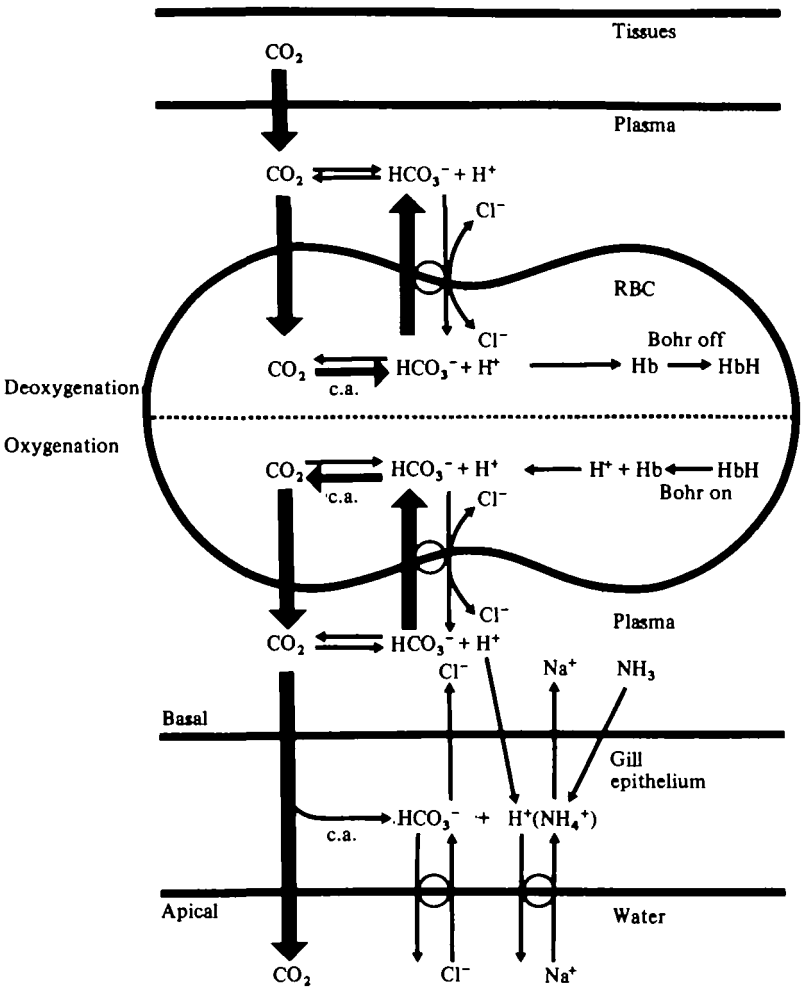

Fig. 2. A diagrammatic representation of carbon dioxide excretion and ion movements in the rainbow trout, *Salmo gairdneri*. See text for further details.

excretion reduced. It is unlikely that intracellular buffering can account for the large increase in plasma HCO_3^- observed during hypercapnic acidosis. We believe that H^+ ion excretion is independent of CO_2 excretion and that the movement of H^+ ions from plasma to gill epithelial cells is related to intracellular pH which in turn is controlled by the rates of the apical ion exchange mechanisms (Fig. 2). Modulations of these exchange mechanisms have been shown to affect blood acid-base balance of freshwater trout (de Renzis & Maetz, 1973; Perry *et al.* 1981). Cameron (1976) has observed changes in apical $Na^+/H^+(NH_4^+)$ and Cl^-/HCO_3^- exchanges during hypercapnic acidosis in Arctic grayling (*Thymallus arcticus*) sufficient to inhibit net movement of H^+ ions into gill epithelial cells, causing plasma HCO_3^- levels to rise. Thus pH compensation during hypercapnic acidosis may be accomplished without grossly affecting CO_2 excretion. How these regulatory mechanisms are controlled is

t well understood. Recent work however (S. F. Perry, P. Payan & J. P. Girard, in preparation) has shown that Cl^-/HCO_3^- exchange in isolated, saline-perfused head preparations of rainbow trout is under adrenergic control. β-receptors inhibit while α-receptors stimulate Cl^-/HCO_3^- exchange. Similarly, Girard & Payan (1977) found that β-receptors stimulate $Na^+/H^+(NH_4^+)$ exchange in the same preparation. It is possible that levels of circulating catecholamines increase during hypercapnic acidosis, as they do during periods of imposed stress (Nakano & Tomlinson, 1967), thereby causing the appropriate modulations of branchial ion exchanges. Another possible explanation for the compensatory increase in plasma HCO_3^- is inhibition of CO_2 excretion. Knowing that the branchial epithelium is impermeable to HCO_3^-, the most likely controllable process in CO_2 excretion is erythrocytic HCO_3^-/Cl^- exchange. Preliminary investigation has demonstrated that HCO_3^- entry into rainbow trout erythrocytes is inhibited by adrenaline (T. A. Heming, & S. F. Perry, in preparation). It is possible that both branchial and erythrocytic HCO_3^-/Cl^- exchanges are controlled in a similar manner. The possible role of catecholamines in regulating acid-base disturbances by these mechanisms is being investigated currently.

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