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RESPONSES OF A STENOHALINE FRESHWATER TELEOST (*CATOSTOMUS COMMERSONI*) TO HYPERSALINE EXPOSURE

I. THE DEPENDENCE OF PLASMA pH AND BICARBONATE CONCENTRATION ON ELECTROLYTE REGULATION

BY P. R. H. WILKES* AND B. R. MCMAHON

Department of Biology, University of Calgary, 2500 University Drive NW, Calgary, Alberta T2N 1N4, Canada

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SUMMARY

The effects of exposure to 0·94% (300 mosmol l^{-1}) sodium chloride on plasma electrolyte and acid–base status were examined in the freshwater stenohaline teleost *Catostomus commersoni* (Lacépède), the white sucker. Four days' exposure to this maximum sublethal salinity resulted in an increase in plasma concentrations of both sodium and chloride but a decrease in the Na^+/Cl^- ratio. Since the plasma concentrations of free amino acids and other strong ions – Ca^{2+}, Mg^{2+} and K^+ – remained unchanged, plasma strong ion difference (SID) decreased. Additionally, plasma pH and bicarbonate concentration decreased at constant P_{CO_2}. The changes in electrolyte and acid–base status that occurred after the 96 h were not appreciably altered after a further 2–3 weeks of saline exposure. The ambient calcium concentration had no influence on these results.

Haemolymph non-bicarbonate buffer capacity (β) calculated as $\Delta[HCO_3^-]/\Delta pH$, increased in saline-exposed fish. Consequently ΔH^+, the apparent proton load, was zero despite the apparent change in acid–base status. Although β was directly proportional to the haemoglobin concentration in both control and experimental fish, this could not account for the increase in β since haemoglobin remained at control values. These results can be explained solely by the change in plasma SID and serve to illustrate the dependence of plasma acid–base status on the prevailing electrolyte characteristics, weak acid concentration and P_{CO_2}.

INTRODUCTION

The extracellular electrolyte and acid–base status (pH and bicarbonate concentration) of freshwater fish are currently considered to be regulated by Na^+/H^+ and Cl^-/HCO_3^- exchange mechanisms located in the gill epithelia. These exchange mechanisms were first proposed by August Krogh (1939) after he observed that the

* Present address: Department of Internal Medicine, Health Sciences Centre, University of Calgary, Calgary, Alberta T2N 1N4, Canada.

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mechanisms of sodium and chloride uptake in the goldfish operate independently. He reasoned that the efflux of protons in exchange for sodium, and bicarbonate in exchange for chloride, was the most likely means of maintaining electroneutral charge movement across the gill epithelia. Currently it is believed that sodium uptake may utilize either protons or the ammonium ion, while chloride uptake may utilize either bicarbonate or hydroxyl ions (Kirschner, 1979; Evans, 1980*a*,*b*, 1982; Haswell, Randall & Perry, 1980). Thus, the active uptake of sodium and chloride serves not only to compensate for their diffusional loss, but is also thought to provide the means of removing acid–base constituents, protons and bicarbonate, and therefore to regulate extracellular pH.

The premise inherent in this model is that extracellular proton and bicarbonate concentrations are regulated in the same manner as sodium and chloride, i.e. the concentration of protons and bicarbonate is a function of the rate of input and rate of removal. Although intuitively appealing, this premise is simply incorrect, as can be seen from analyses from first principles (Stewart, 1978, 1981, 1983). Plasma proton and bicarbonate concentration are determined by (i) the difference between the sum of all strong cations and anions, i.e. the strong ion difference (SID); (ii) the total concentration of weak acids (A_{TOT}), essentially plasma protein concentration; and (iii) the amount of dissolved carbon dioxide, P_{CO_2}. Thus, an observed change in proton and bicarbonate concentrations can only reflect a change in one or more of these three independent variables. By extension, we propose that concepts such as non-bicarbonate buffer values ($\Delta[HCO_3^-]/\Delta pH$), the base deficit (the proton load expressed as a loss of bicarbonate equivalents), and the buffering of plasma pH by intracellular proteins (i.e. haemoglobin) cannot be used meaningfully in terms of either quantitative or cause and effect analysis of the prevailing acid–base status, since they are all expressed as changes in dependent variables – proton and bicarbonate concentration.

The purpose of the present study was to demonstrate that the above concepts can be explained solely in terms of changes in the three independent variables, SID, P_{CO_2} and A_{TOT}, which occur during acid–base disturbances. The approach adopted was to examine the changes in these independent variables and in the dependent variables, plasma pH and bicarbonate concentration, when the stenohaline freshwater teleost, *Catostomus commersoni* (the white sucker) is exposed to a hypersaline (0.94% sodium chloride) environment.

MATERIALS AND METHODS

White suckers, *Catostomus commersoni* (150–400 g) were obtained by live trapping from artificial lakes within the City of Calgary and were maintained at the University of Calgary for a minimum of 2 weeks prior to use.

Two control and two experimental regimes were used in the present study; control water contained high ($>1{\cdot}0\ mmol\ l^{-1}$) or low ($<0{\cdot}1\ mmol\ l^{-1}$) calcium (hard and soft freshwater, respectively), while saline water contained high or low calcium in 0·94% ($300\ mosmol\ l^{-1}$) sodium chloride (hard and soft saline water, respectively). These

water conditions were obtained by supplementing dechlorinated tap water with calcium nitrate for hard control water, and by supplementing tap distilled water with sodium chloride for soft control water. Concentrations of sodium, calcium, potassium and magnesium in hard and soft freshwater are given in Table 1. Hard and soft saline waters were made in a similar manner except that the sodium chloride level was increased to 0·94% (300 mosmol l^{-1}). Preliminary studies (Wilkes, 1984) demonstrated that 300 mosmol l^{-1} sodium chloride closely approximates to the maximum sublethal salinity for this species.

Experimental and control facilities consisted of constantly aerated, recirculating water on line with gravel filters. Each system embodied a small holding reservoir (approximately 150 l) in which animals could be acclimated to the desired water conditions, and a wet table on which fish could be placed in individual fish boxes. Each fish box was 2 l in volume, was darkened with opaque plastic, aerated and was supplied with a constant inflow of water from the reservoir at a rate between 0·5 and 1·0 l min^{-1}. The wet table was designed to return the overflow water from the fish boxes back through the gravel filter to the reservoir. The temperature of the systems was maintained at 15°C. Animals were fed daily on pellet trout food (Silver Cup) during holding and acclimation periods.

Experimental procedures

All fish were acclimated to either hard or soft freshwater conditions for 2 weeks prior to exposure to hard or soft saline water respectively. Some of the freshwater-acclimated fish were used as hard and soft freshwater controls. Three separate groups of hard freshwater-acclimated fish were exposed to hard saline water for 24 h, 96 h and several weeks (chronic), respectively, while a single group of soft-water-acclimated fish was exposed to soft saline water for 96 h.

Preliminary studies (Wilkes, 1984) demonstrated that caudal vessel cannulation clearly added an intolerable level of additional stress, at least to experimental groups. Blood samples were therefore taken by cardiac puncture. This factor necessarily influenced the experimental design.

Each fish was transferred, in its fish box, to the operating table where the input jack on the fish box was connected to the anaesthetic line (MS 222, 1:10000). The water used for anaesthetizing the fish was of the same temperature and electrolyte

Table 1. *Ion composition for hard and soft freshwater*

	Hard water		Soft water	
	Mean	(Range)	Mean	(Range)
Sodium	1·5	1·0–1·8	0·5	0·3–0·7
Calcium	2·4	1·3–3·3	0·1	0·03–0·2
Potassium	0·2	0·1–0·3	—	—
Magnesium	0·7	0·6–0·8	0·01	0·005–0·01

Sodium chloride was added to each to achieve hard and soft saline conditions (0·94% NaCl), respectively. All values are reported in mmol l^{-1}.

composition as that to which the fish had previously been exposed. This method permitted anaesthetization with a minimum of stress and no handling. As soon as the anaesthetic had taken effect (approximately 2–3 min) the fish was removed from the box, placed on the sling of the operating table, dorsal side up, and the gills ventilated through the mouth. The heart was exposed immediately using a mid-line incision through the cliethrum and a blood sample was drawn from the bulbus arteriosis into a heparinized syringe. Although the sample volume varied with the size of the fish it was generally possible to obtain 1–2 ml. The entire process, surgery and sampling, was completed within 2 min.

Acid–base analyses

Whole blood lactate concentration was measured enzymatically (Sigma 826 UV) on 100 μl from the first sample obtained from the heart puncture. Prior to further acid–base analyses the blood had to be equilibrated to a known P_{CO_2} since hypoperfusion and hypoventilation resulting from the method of sampling would disturb the P_{CO_2}. All samples were equilibrated in a tonometer to 1·3 Torr P_{CO_2}. Some samples were also sequentially equilibrated to 4·0, 9·1 and 13·0 Torr P_{CO_2} in order to construct *in vitro* buffer curves and to calculate β, the non-bicarbonate buffer value ($\Delta[HCO_3^-]/\Delta pH$). These P_{CO_2} levels encompass the physiological range found in blood. Carbon dioxide gas was obtained from analysed 2·0% CO_2 in air (Matheson Gas Co.). Appropriate percentages were then delivered by Wostöff gas mixing pumps. Prior to entry into the tonometer the gas was cooled to 15 °C and humidified to avoid evaporation of the blood sample. Periodic measurements of blood P_{CO_2} and pH established that equilibration was complete after 15 min for each 250 μl of sample. After equilibration to the desired P_{CO_2} a sample (approximately 200 μl) of whole blood was removed anaerobically from the tonometer. Part of this sample (60–70 μl) was injected directly into the pH capillary electrode of a BMS-3 Acid–Base Analyzer (Radiometer) in order to measure pH of true plasma. The remainder was used to fill completely 3–5 haematocrit tubes, which were subsequently sealed at both ends and centrifuged for haematocrit determination. Since the plasma fraction from the haematocrit tubes was equilibrated to the prevailing P_{CO_2} as whole blood, it represents true plasma (Davenport, 1974). The bicarbonate concentration for the true plasma was then estimated from the measured total carbon dioxide (C_{CO_2}) (Corning 965) using:

$$[HCO_3^-] = [C_{CO_2}] - (P_{CO_2})(\alpha C_{CO_2}), \quad (1)$$

where αCO_2 was taken from Severinghaus (1965) after correction for temperature.

Blood protein analyses

Total plasma protein concentration was measured with the Biuret reagent (Sigma). The haemoglobin concentration of whole blood was measured by the cyanomethaemoglobin method (Hycel). The intracellular haemoglobin concentration was estimated from ([Hb]/Ht)×100.

Plasma and tissue electrolyte analyses

Plasma was analysed for the cations Na^+, Ca^{2+}, Mg^{2+}, K^+, and the anion Cl^-, total free amino acids (ninhydrin-positive substances, NPS) and osmolality. Cations were measured either with a Jarrell Ash 850 or a Perkin Elmer 5000 atomic absorption spectrophotometer. Plasma samples were diluted in appropriate swamping agents (0·1% potassium chloride for sodium, 0·1% sodium chloride for potassium, and 1% lanthanum chloride for calcium and magnesium) prior to analyses. Plasma chloride was measured directly by coulometric titration (Buchler Chloridometer). Free amino acid concentration was calculated as the total ninhydrin-positive substances (NPS) following the method of Clark (1973). Although never more than 5% of the ninhydrin reaction, total ammonia concentration in the deproteinized sample was routinely measured (Solorzano, 1969) and subtracted from the total NPS. Plasma osmolality was measured with a vapour pressure osmometer (Wescor, 5100B).

Since the blood sampling procedure was terminal, the same animal could be used to obtain muscle samples for analyses of ions, amino acids and the percentage hydration. Muscle ions were measured by the method developed by McDonald, Hōbe & Wood (1980). Briefly, a 2- to 3-g muscle sample was removed from the epaxial muscle mass and analysed for wet weight/dry weight. The dried tissue was ground to a fine powder with pestle and mortar to facilitate elution of ions into 1·0 equiv l^{-1} nitric acid. After 48 h of constant rotation at room temperature the nitric acid supernatant was analysed for sodium, potassium, calcium, magnesium and chloride concentrations as described above. Muscle ion concentrations were reported in terms of total tissue water, intracellular plus interstitial. An additional wet tissue sample was homogenized in distilled water (1·0 ml/0·25 g) using a Sorvall Omni mixer for measurement of free amino acids, also as described above for plasma. These muscle ion and amino acid analyses were performed only in a group of hard-water-acclimated control fish and in a group exposed to hard saline water for 96 h.

Calculations

The slope (β) of the *in vitro* buffer curves was calculated as $\Delta[HCO_3^-]/\Delta pH$ (Wood, McMahon & McDonald, 1977). The observation that the absolute magnitude of β is positively correlated to haemoglobin concentration in fish (Wood, McDonald & McMahon, 1982) is usually interpreted to mean that haemoglobin is the major non-bicarbonate buffer. The non-bicarbonate buffer value (β) is often used in calculating the base deficit from:

$$\Delta H^+ = \Delta[HCO_3^-] - \beta(\Delta pH), \quad (2)$$

where ΔH^+ is thought to represent the proton load donated by a fixed acid, expressed as the loss of bicarbonate equivalents. Although never examined, the accepted premise is that the value of β is not altered during an acid–base disturbance. The approach taken in the present study permits evaluation of this premise since *in vitro* buffer curves can be constructed for both control and saline-exposed fish.

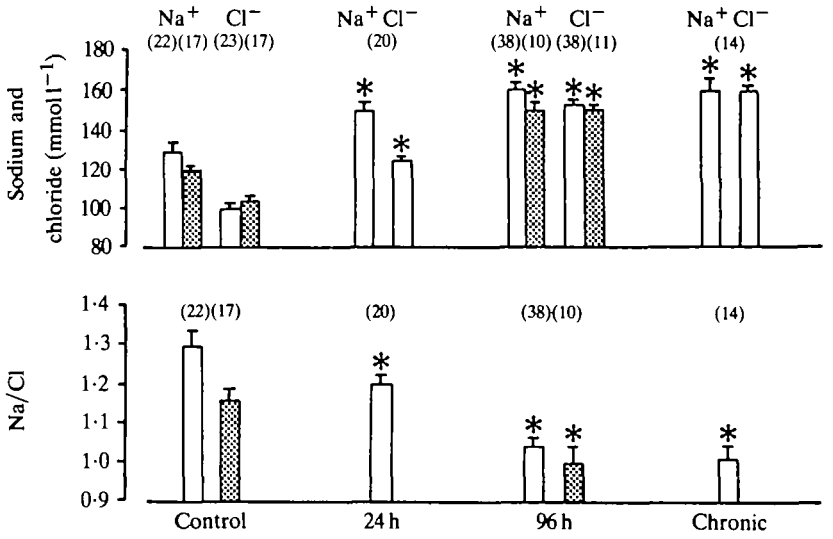

Fig. 1. *In vitro* sodium chloride concentration, and Na^+/Cl^- in plasma of fish exposed to hard (clear) and soft (stippled) freshwater and saline conditions. The number of animals used (N) is given in parentheses. Statistical significance from respective control values is demonstrated by (*). The vertical lines in this and all subsequent figures represent 1 S.E.M.

Although it was not possible to measure the change in bicarbonate concentration and pH in the same animal during saline exposure, equation 2 can be modified as follows:

$$\text{control} \quad H^+_c = [HCO_3^-] - \beta(\text{pH}) \tag{3a}$$

$$\text{saline} \quad H^+_s = [HCO_3^-] - \beta(\text{pH}). \tag{3b}$$

So that ΔH^+ can be calculated from:

$$\Delta H^+ = H^+_c - H^+_s. \tag{3c}$$

Statistical significance was inferred if $P < 0{\cdot}05$ using an unpaired, two-tailed Student's t-test, unless otherwise stated. The number of animals used in each segment is given in the appropriate results sections and all results are reported as the mean ± S.E.M.

RESULTS

Plasma and tissue electrolyte status

The only significant differences in plasma electrolyte status between control fish acclimated to hard and soft freshwater were reduced concentrations of sodium and calcium in the latter group (Figs 1, 2). A consequence of the lower plasma sodium concentration in soft-water-acclimated control fish was that the Na^+/Cl^- ratio of $1{\cdot}16 \pm 0{\cdot}03$ was significantly lower than the $1{\cdot}30 \pm 0{\cdot}04$ value found in hard-water control fish (Fig. 1).

Plasma sodium and chloride concentrations of fish initially acclimated to hard freshwater increased significantly after 24 h exposure to hard saline water. By 96 h,

the concentrations of sodium and chloride were significantly greater than control values by 20% and 33%, respectively (Fig. 1). The greater increase in plasma chloride concentration over that of sodium resulted in a significant (17%) decrease in the Na^+/Cl^- ratio from $1{\cdot}30 \pm 0{\cdot}04$ to $1{\cdot}08 \pm 0{\cdot}02$. No further significant changes in sodium and chloride concentrations, or their ratio occurred in plasma of fish chronically exposed to hard saline conditions.

Changes in plasma sodium and chloride concentrations which occurred in fish exposed to soft saline water were qualitatively similar at 96 h to those observed in fish similarly exposed to hard saline water. The concentration of plasma chloride again increased substantially more than that of sodium, resulting in a significant (13%) decrease in the Na^+/Cl^- ratio from $1{\cdot}16 \pm 0{\cdot}03$ to $1{\cdot}01 \pm 0{\cdot}02$ (Fig. 1).

Exposure to either hard or soft saline conditions had no significant effect on the plasma concentrations of calcium, magnesium or potassium. Free amino acid concentration, measured only in the plasma of hard-water exposed fish, was also not significantly affected by saline exposure (Fig. 2).

The concentration of ions in epaxial muscle was measured only in a hard-water control group and in a group exposed to hard saline water for 96 h. Tissue calcium and magnesium concentration, and percentage tissue hydration in the saline-exposed animals were not significantly different from control values (Table 2). However, dramatic increases in sodium, chloride and free amino acid concentrations, of 85%, 175% and 66%, respectively, were measured in muscle samples from fish exposed to hard saline conditions for 96 h. Potassium concentration also increased significantly over control values, but only by 17% (Table 2).

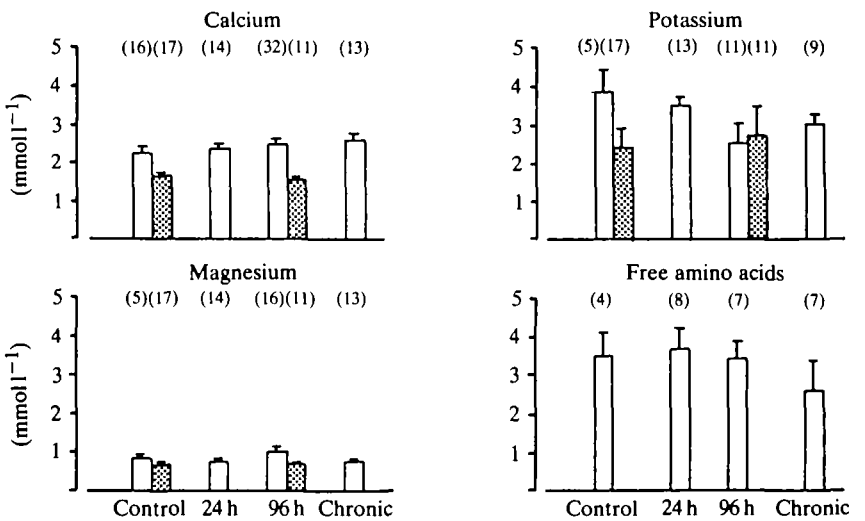

Fig. 2. *In vitro* concentrations of calcium, potassium, magnesium and free amino acids in plasma of fish exposed to hard (clear) and soft (stippled) freshwater and saline conditions. The number of animals used (*N*) is given in parentheses. Statistical significance from respective control values is demonstrated by (•).

Blood acid–base status

Both pH and bicarbonate concentration in true plasma of soft-water-acclimated control animals were significantly elevated over corresponding values measured in the hard-water control group (Fig. 3). Nevertheless, the acid–base status in blood of both groups was affected in a qualitatively similar manner by saline exposure. True plasma pH and bicarbonate concentration of fish exposed to hard saline conditions for 24 h were not significantly different from control values. However, by 96 h, pH had fallen significantly from a control value of $7{\cdot}770 \pm 0{\cdot}053$ to $7{\cdot}408 \pm 0{\cdot}022$, and fell even further to $7{\cdot}241 \pm 0{\cdot}039$ after chronic saline exposure. Initial true plasma bicarbonate concentration in hard-water control fish was $3{\cdot}7 \pm 0{\cdot}03$ mmol l^{-1}, and the 96 h value was significantly lower at $1{\cdot}3 \pm 0{\cdot}1$ mmol l^{-1}. No further statistically significant change occurred in the bicarbonate concentration during chronic saline exposure.

True plasma pH in the soft-water-exposed group fell significantly from a control value of $7{\cdot}934 \pm 0{\cdot}030$ to $7{\cdot}485 \pm 0{\cdot}043$ after 96 h exposure to soft saline water. True plasma bicarbonate concentration also decreased significantly during this period, from $7{\cdot}6 \pm 0{\cdot}07$ mmol l^{-1} to $3{\cdot}0 \pm 0{\cdot}2$ mmol l^{-1} (Fig. 3). The acid–base changes in fish exposed either to hard or soft saline water could not be attributed to a build-up of lactic acid which decreased progressively throughout saline exposure (Fig. 3).

The slopes (β) of whole blood buffer curves constructed from blood of fish exposed to hard and soft control conditions, to 96 h hard and soft saline water, and to 3 weeks hard saline conditions, were calculated from the change in true plasma bicarbonate concentration per unit change in pH, $\Delta[HCO_3^-]/\Delta pH$, which occurred after equilibration of whole blood to increasing levels of P_{CO_2}. The mean and standard error for β at haemoglobin concentrations of 2, 4, 6 and 8 g% were calculated by analysis of variance of the β-haemoglobin regressions according to Zar (1974). The results of these analyses demonstrated that the absolute magnitude of β increased with increasing haemoglobin concentration in blood of both hard- and soft-water-acclimated control fish, and hard and soft saline-exposed fish. Additionally, β was observed to increase independently of the prevailing haemoglobin concentration in saline-exposed fish ($P<0{\cdot}05$ at mean haemoglobin concentrations). This latter

Table 2. *Tissue electrolytes, free amino acid concentration (FAA) (mmol kg^{-1} muscle water) and percentage muscle water in control fish acclimated to hard freshwater and in fish exposed to hard saline water for 96 h*

		Na^+	Cl^-	K^+	Ca^{2+}	Mg^{2+}	FAA	% H_2O
Control	$\bar{x}$	26·1	11·9	132·1	6·7	13·5	40·5	81·62
	S.E.M.	1·5	1·1	5·2	0·4	0·7	2·9	0·49
	(*N*)	(6)	(6)	(6)	(6)	(6)	(5)	(6)
96 h	$\bar{x}$	48·2	32·7	155·5	7·2	13·1	67·3	80·38
	S.E.M.	4·5	4·0	7·9	1·1	1·2	2·4	0·52
	(*N*)	(10)	(10)	(11)	(12)	(12)	(11)	(12)
	P<	0·001	0·001	0·05	NS	NS	0·001	NS

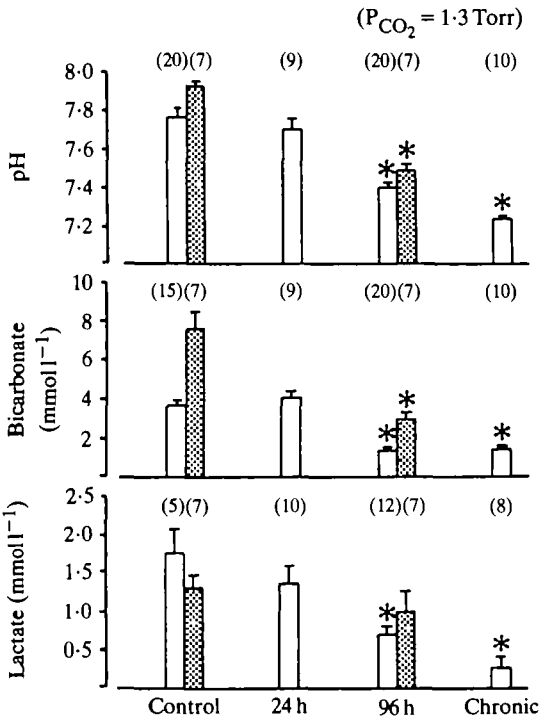

Fig. 3. *In vitro* acid–base status for fish exposed to hard (clear) and soft (stippled) control and experimental conditions. The number of animals used (*N*) is given in parentheses. Statistical significance from respective control values is demonstrated by (*).

effect was influenced more by the duration of saline exposure rather than water hardness (Fig. 4).

A consequence of the haemoglobin-independent increase in β that occurred during saline exposure was that a negative value for ΔH^+ was calculated for fish exposed to hard or soft saline conditions (Table 3) despite the decrease in pH and bicarbonate concentration. However, the ΔH^+ values are in fact zero since there were no significant differences between H^+_c and H^+_s (Table 3).

Blood protein characteristics

Plasma protein concentration, whole blood haemoglobin concentration, and haematocrit of soft-water-acclimated control fish were all significantly below values measured in hard-water-acclimated control fish (Table 4). Exposure to soft saline water for 96 h had no effect on plasma protein concentration. However, by 96 h and after chronic exposure to hard saline conditions, plasma protein concentration had fallen significantly below values found in hard-water-acclimated control fish. The only statistically significant effect of either hard or soft saline water on whole blood haemoglobin concentration was a lower value in the group chronically exposed to hard saline conditions. Haematocrit values were significantly higher than control

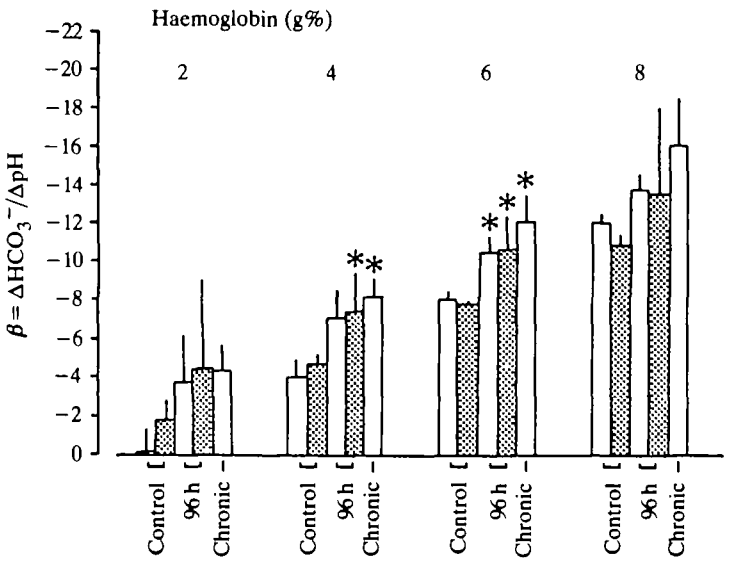

Fig. 4. The non-bicarbonate buffer values of whole blood, β, for control fish, experimental fish after 96 h, and chronic (3–4 weeks) saline exposure. Stippled bars represent results from soft-water-exposed fish (control and experimental). Clear bars represent results from hard-water-exposed fish (control and experimental). Statistical significance from respective control values in freshwater is demonstrated by (*).

Table 3. *H^+_c for hard- and soft-water-acclimated control fish, and H^+_s for fish exposed to hard and soft saline water, respectively*

	H^+_c (mequiv l^{-1})	H^+_s (96 h) (mequiv l^{-1})	$\Delta H^+_{c\to s}$ (96 h) (mequiv l^{-1})	H^+_s (chronic) (mequiv l^{-1})	$\Delta H^+_{c\to s}$ (chronic) (mequiv l^{-1})
			Hard water		
$\bar{x}$	74·6	86·8	−12·2	81·0	−6·4
S.E.M.	5·6	9·2		16·9	
(*N*)	(15)	(12)		(10)	
P<		NS		NS	
			Soft water		
$\bar{x}$	65·9	70·8	−4·9	—	—
S.E.M.	5·1	8·5			
(*N*)	(7)	(7)			
P<		NS			

H^+ values were calculated for individual fish using pH and true plasma bicarbonate concentrations measured in a whole blood sample equilibrated *in vitro* to P_{CO_2} 1·3 Torr, and the β value ($\Delta[HCO_3^-]/\Delta pH$) from the individual *in vitro* buffer curve according to equations 3a, 3b.

The 'proton load', ΔH^+, for saline-exposed fish was calculated as the difference between the mean H^+_s and mean H^+_c according to equation 3c.

values in the two groups exposed to hard and soft saline water for 96 h. Haematocrit values of fish chronically exposed to hard saline water were not significantly different from control values. The changes in haemoglobin concentration and haematocrit were such that the mean red cell haemoglobin concentration was significantly reduced in fish exposed to hard saline conditions for 96 h, and after chronic exposure to hard saline water. However, the decrease in mean red cell haemoglobin concentration could not be attributed to consistent changes either in whole blood haemoglobin concentration or in haematocrit.

DISCUSSION

Numerous studies have examined the effects of sublethal saline exposure on the plasma electrolyte levels of stenohaline freshwater fish (Davis & Simco, 1976;

Table 4. *Plasma protein concentration (g%), haemoglobin concentration (g%), haematocrit (%) and red cell haemoglobin concentration (g%) in control fish acclimated to hard and soft freshwater, after 96 h in soft saline water, and after 24 h, 96 h and chronic exposure to hard saline water*

		Plasma protein (g%)	Haemoglobin (g%)	Haematocrit (%)	Red cell haemoglobin (g%)
			Hard water		
Control	$\bar{x}$	2·37	6·82	27·8	25·0
	S.E.M.	0·14	0·29	1·0	1·0
	(*N*)	(35)	(21)	(26)	(21)
24 h	$\bar{x}$	2·43	7·66	30·4	25·1
	S.E.M.	0·18	0·41	1·8	0·8
	(*N*)	(11)	(10)	(10)	(10)
	P<	NS	NS	NS	NS
96 h	$\bar{x}$	1·90	7·25	31·9	22·7
	S.E.M.	0·14	0·34	1·4	0·4
	(*N*)	(41)	(20)	(20)	(20)
	P<	0·05	NS	0·05	0·05
Chronic	$\bar{x}$	1·85	4·78	23·9	19·7
	S.E.M.	0·18	0·43	2·0	0·5
	(*N*)	(12)	(14)	(14)	(14)
	P<	0·05	0·001	NS	0·001
			Soft water		
Control	$\bar{x}$	1·74	4·58	19·6	23·3
	S.E.M.	0·11	0·38	1·5	1·0
	(*N*)	(17)	(17)	(17)	(17)
96 h	$\bar{x}$	1·68	5·30	24·6	21·5
	S.E.M.	0·12	0·40	1·8	0·6
	(*N*)	(9)	(11)	(11)	(11)
	P<	NS	NS	0·05	NS

Norton & Davis, 1977; Kilambi & Zdinak, 1980; Maceina, Nordlie & Shireman, 1980; Hegab & Hanke, 1982). Although it is difficult to establish a specific maximum sublethal salinity, results from the above studies, and from Wilkes (1984), indicate that stenohaline freshwater fish cannot survive salinities in excess of 300–400 mosmol l^{-1}. A common finding in all the above studies, as well as the present results, is that stenohaline freshwater fish could survive a saline stress only so long as plasma osmolality was above ambient. Hegab & Hanke (1982) suggested that an inwardly directed osmotic gradient is required in order to obtain the water influx necessary to ensure continued renal function.

On initial exposure to a hypersaline environment some osmotic loss of water was expected, but this seemed to be minimal in *C. commersoni* since both plasma and tissue levels of calcium and magnesium remained constant (Fig. 2). Therefore, the observed increase in plasma osmolality was more a function of a net influx of sodium and chloride than of water loss. In either case, intracellular osmolality must match that of extracellular fluid in order to avoid cell shrinkage. Current theories on cell volume regulatory mechanisms state that both inorganic, essentially sodium and potassium, and organic electrolytes, principally non-essential amino acids and taurine (Gilles, 1979; Rorive & Gilles, 1979) are used to maintain the intracellular environment isosmotic with surrounding fluids. Although sodium, chloride and, to a lesser extent, potassium concentrations in tissue increased during saline exposure (Table 2), these concentrations are expressed in terms of total tissue water, intracellular plus interstitial. Therefore, some of the increase in sodium and chloride concentration was attributable to an increase in overall extracellular osmolality. However, the plasma (and therefore interstitial fluid) concentration of free amino acids did not change during 96 h of saline exposure, indicating that the measured 66% increase in tissue free amino acid concentration was predominantly intracellular.

Before analysing the effects of saline exposure on acid–base status, a brief discussion of the approach used in the present study is required. Wilkes, Walker, McDonald & Wood (1981) demonstrated that cannulation of a caudal vessel can result in decreased plasma protein concentration and haematocrit, which may in turn perturb the acid–base status. Cannulated fish rarely survived beyond 48 h in 300 mosmol l^{-1} sodium chloride and experienced an acid–base disturbance which was often characterized by an increase in lactic acid concentration 24 h prior to death. However, before becoming lactacidotic and moribund, blood pH and bicarbonate concentration fell while P_{CO_2} (measured directly by a method adopted from Boutilier, Randall & Toews, 1978, and DeFur, Wilkes & McMahon, 1980) remained constant. These preliminary results indicate that the initial decreases in pH and bicarbonate concentration were consistent with a decrease in plasma strong ion differences (SID) brought about by a reduction in the plasma Na^+/Cl^- ratio. The *in vitro* methods described in the present study allow evaluation of the blood pH and bicarbonate concentration at constant P_{CO_2}, without the complications brought on by surgical manipulation. The actual P_{CO_2} levels used, 1·3, 4·0, 9·1 and 13·0 Torr, were chosen

so as to encompass the range of normal values but are not meant to be specifically representative of arterial or venous blood.

In agreement with preliminary *in vivo* results (Wilkes, 1984), the use of *in vitro* methods demonstrates that the acid–base disturbance can be accounted for by the decrease in plasma SID. However, the finding that ΔH^+ was zero indicates that ΔH^+ does not reflect, either in quantitative or qualitative terms, the observed change in acid–base status. Since the prevailing proton and bicarbonate concentrations are determined solely by the independent variables, SID, P_{CO_2} and A_{TOT}, of the system, so too is the slope of the non-bicarbonate buffer line, β. Therefore, any explanation of both the haemoglobin-independent and haemoglobin-dependent increases in β, and therefore ΔH^+, must also be expressed in terms of change in one or more of the independent variables. Using the following equation from Stewart (1981):

$$(H^+)^4+(Ka+SID)(H^+)^3+[Ka(SID-A_{TOT})-(Kc\times P_{CO_2}+Kw)](H^+)^2- [Ka(Kc\times P_{CO_2}+Kw)+K3\times Kc\times P_{CO_2}](H^+)-Ka\times K3\times Kc\times P_{CO_2}=0, \quad (4)$$

it can be demonstrated that the lower the plasma SID: (i) the greater the proton concentration, (ii) the lower the bicarbonate concentration, and (iii) the greater the magnitude of β for a given increase in P_{CO_2}.

Therefore, the haemoglobin-independent increase in β observed in saline-exposed fish is a direct consequence of the lower plasma SID. The means by which the plasma SID decreases, i.e. whether through a decrease in the sodium chloride ratio as in the present study, or through a lactacidosis during exercise, is not important.

The underlying reason behind the haemoglobin-dependent increase in β is readily explained by the chloride shift as follows. When the level of molecular carbon dioxide in plasma increases, it diffuses along its partial pressure gradient, across the plasmalemma, and into the red blood cell, where intracellular carbonic anhydrase catalyses its hydration to bicarbonate and protons. The protons are buffered by haemoglobin *via*:

$$KHb+H^+ \longleftrightarrow HHB+K^+, \quad (5)$$

(Harper, 1975; Selkurt, 1976). Since the intracellular potassium activity increases, so too does intracellular SID, which in turn allows more bicarbonate to exist at the prevailing P_{CO_2}. Most of the bicarbonate, however, is carried in plasma not in the intracellular compartment of the red blood cell (Harper, 1975; Selkurt, 1976). In order to move bicarbonate between the two compartments, the SID of the plasma must increase relative to that of the intracellular environment of the red blood cell. This effect is brought about by the inward movement of chloride from the plasma into the red blood cell. The SID of the red blood cell is thus maintained, while that of the plasma increases. Whether bicarbonate moves out of the red cell so as to maintain electroneutral exchange with chloride, or a proton moves in with the chloride ion, is not important; the same final state will be achieved in each case. The critical point is that the change in plasma bicarbonate concentration can only occur because of the increase in P_{CO_2} and the resulting increase in plasma SID due to the chloride shift.

The consequences of the chloride shift to plasma proton and bicarbonate concentrations are greatly magnified by *in vitro* methodology. The greater the haematocrit of an isolated blood sample, the greater the chloride shift and the greater the increase in plasma SID for a given increase in P_{CO_2}. In the whole animal, the effect of the chloride shift on plasma SID is diluted throughout the entire extracellular compartment, not just the plasma volume. Additionally, any compensatory effects of strong ion movement between the interstitial and intracellular compartments which would normally occur *in vivo* are also eliminated.

It is possible to use equation 4 above from Stewart (1981) to demonstrate quantitatively the consequences of an increase in P_{CO_2} on the β values for two hypothetical blood samples. The first plasma sample has an initial SID of 35 mequiv l^{-1} (represented by the stippled bars in Fig. 5). The second sample has an initial SID of 20 mequiv l^{-1} (represented by the clear bars in Fig. 5). These two hypothetical plasma samples are then subjected to an increase in P_{CO_2} (from 10 to 20 Torr) in the following three conditions. Condition I represents the case of plasma without red cells so that there is no chloride shift and SID does not change from its initial value when P_{CO_2} increases. Conditions II and III represent the same plasma samples but with red blood cells added to cause a 1 and 2 mequiv l^{-1} increase in plasma SID, respectively, to occur *via* the chloride shift as the P_{CO_2} is raised from 10 to 20 Torr. Note that by using these SID and P_{CO_2} values in equation 4 it is readily apparent (Fig. 5) that even these small, almost immeasurable, increases in plasma SID can have a pronounced effect on the proton and bicarbonate concentrations such

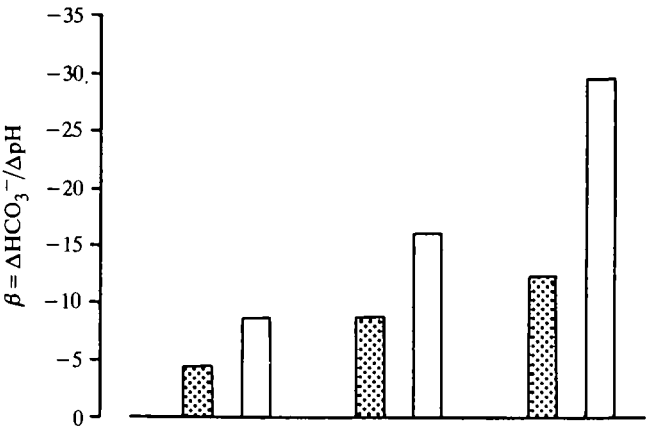

Fig. 5. Non-bicarbonate buffer values (β) calculated for the hypothetical blood sample with a high SID (stippled) and a low SID (clear) used in conditions I, II and III (see text). The pH and bicarbonate values used for each determination of β were derived from equation 4. Values for pertinent independent variables, A_{TOT}, SID and P_{CO_2} are given in the text.

that the lower the SID the greater the value of β. Additionally, the greater the red cell concentration in both of these samples, the greater the increase in β.

The above explanation for the haemoglobin-independent and haemoglobin-dependent increases in β observed in the present study points out the inability of the ΔH^+ calculation to determine a 'proton load' (Table 3). Indeed, the term 'proton load' is meaningless since the proton concentration within a system is not a simple function of the number of protons added to it (Stewart, 1978, 1981, 1983). Since similar statements are equally true for bicarbonate, it follows that calculations based solely on the change in dependent variables (i.e. β and ΔH^+) are of no functional use in determining the cause, or extent of a given change in acid–base status.

The initial differences in acid–base status in the two control groups of fish acclimated to hard and soft freshwater (Fig. 3) can probably be explained by the differences in plasma protein concentration (Table 4). Although the plasma SID was higher in fish acclimated to soft freshwater than in those acclimated to hard freshwater, the plasma protein concentration (A_{TOT}) was much lower. Using equation 4 (from Stewart, 1981) it is possible to show that the influence of A_{TOT} on proton concentration is greatly enhanced at low P_{CO_2} levels. Thus, the reduced plasma protein concentration in soft-water-acclimated control fish has a more pronounced effect on proton concentration than the low SID. It is difficult to determine from these results whether the differences in protein concentration, and therefore acid–base status, observed in the two control groups is a function of water calcium concentration. Although Hōbe, Wood & McMahon (1984) reported that blood pH and bicarbonate concentration of *C. commersoni* reared in natural soft-water is higher than in *C. commersoni* reared in natural hard-water conditions, the plasma protein concentration in the former population was, in fact, higher than the levels reported in the present study for either control group. The lower plasma protein concentration observed in the present study may be attributable to a combination of the effects of several months' captivity and of an artificial diet. Interestingly, the acid–base status of trout blood is not affected by water hardness of neutral pH (McDonald *et al.* 1980).

Relatively few studies have examined the effects of a change in external salinity on acid–base status of fish. DeRenzis & Maetz (1973) demonstrated that goldfish maintained in dilute choline chloride developed an acidosis, while dilute sodium sulphate exposure effected an alkalosis. Farrell & Lutz (1975) reported that when brackish water adapted eels were exposed to freshwater there was an increase in the SID in association with an increase in plasma bicarbonate concentration. Finally, Smatresk & Cameron (1982) reported the development of a metabolic acidosis, which they attributed to a decrease in SID, in the spotted gar after transfer from freshwater to 50% seawater. The results of these studies indicate that species which cannot readily tolerate or adapt to large changes in external salinity are subject to acid–base disturbances during such treatment. In all of the above studies the observed change in plasma pH is directly related to the change in plasma SID.

Apparently, euryhaline species are not subject to the same acid–base disturbances when external salinity is altered. While Perry & Heming (1981) showed a statistically

significant alkalosis in trout transferred from freshwater to 25% seawater, the acid–base disturbance was, physiologically, quite small. Similarly, Milne & Randall (1976) and Bath & Eddy (1979*a,b*) showed no change in blood pH or bicarbonate concentration in rainbow trout after an abrupt change from freshwater to full-strength seawater. Apparently, the ionoregulatory mechanisms of trout are capable of controlling both absolute and relative concentrations of plasma electrolytes during saline stress.

The results of the present study indicate that the Na^+/H^+–NH_4^+ and Cl^-/HCO_3^-–OH^- of acid–base regulation is too simplistic. This model proposes that it is the rate of active uptake of sodium and chloride which is responsible for the removal, and therefore regulation, of acid–base constituents, protons and bicarbonate. However, as pointed out by Stewart (1978, 1981, 1983), the prevailing proton and bicarbonate concentrations have nothing to do with their rate of input to or removal from a given compartment. The proton and bicarbonate concentrations are dependent variables which are determined by the prevailing SID, A_{TOT} and P_{CO_2}. If: (i) the plasma SID is determined predominantly by the difference between sodium and chloride concentration, and (ii) the sodium and chloride concentrations are functions of their rate of input (i.e. as determined by the magnitude of the rate constants for the active pumps and the external substrate concentration), and their rate of efflux (i.e. as described by the Nernst–Planck diffusion equation), then it follows that the only way in which the transepithelial movement of sodium and chloride can influence plasma acid–base status is if active and/or passive movement is such that the sodium to chloride ratio in the plasma is altered. A corollary of this is that any measured transepithelial flux of protons and/or bicarbonate (acidic equivalents) is a reflection of the change of SID in the external compartment brought about by the transepithelial movement of strong ions. The transepithelial flux of 'acidic equivalents' should not be interpreted as a factor responsible for causing or correcting the blood pH. Changes in transbranchial ion fluxes in *C. commersoni* during saline exposure are examined in the following paper.

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