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

II. TRANSEPITHELIAL FLUX OF SODIUM, CHLORIDE AND 'ACIDIC EQUIVALENTS'

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SUMMARY

The effects of exposure to 300 mosmol l^{-1} sodium chloride on the transepithelial movement of sodium, chloride and 'acidic equivalents' was examined in the stenohaline freshwater teleost *Catostomus commersoni* (Lacépède), the white sucker. The transepithelial potential (TEP) was negative in control fish acclimated to soft freshwater ($[Ca^{2+}] < 0{\cdot}1$ mmol l^{-1}) but positive in control fish acclimated to hard-water ($[Ca^{2+}] > 1{\cdot}0$ mmol l^{-1}). Using permeability coefficients calculated from measured unidirectional effluxes of sodium and chloride for both groups above, the Goldman equation predicts the observed change in polarity of the TEP. During saline exposure the ability of external calcium to influence the TEP was greatly attenuated, and the TEP remained positive throughout 96 h exposure to either hard or soft saline water. As a consequence of the reduced (and reversed) chemical potential and of the prevailing TEP, the electrochemical difference for sodium was directed out from the fish while that for chloride was directed inwards. Thus, the passive movement of sodium and chloride in opposite directions could potentially account for the previously reported decrease in plasma strong ion difference (SID), and therefore the prevailing acid–base status. Unfortunately, the existence of possible exchange diffusion processes for sodium and chloride observed in saline-exposed fish prevented a more detailed examination of this hypothesis. Since the change in plasma SID occurred gradually over a 96-h period, there was no measurable change in the net flux of 'acidic equivalents' which could have been associated with the active and passive transbranchial movement of sodium and chloride. The significance (or lack of it) of the $Na^+/H^+–NH_4^+$ and $Cl^-/HCO_3^-–OH^-$ exchange pumps to systemic acid–base balance is discussed.

INTRODUCTION

Wilkes & McMahon (1986) demonstrated that exposure of the stenohaline freshwater teleost, *Catostomus commersoni* (the white sucker) to a hypersaline

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environment (0·94 % sodium chloride, 300 mosmol l^{-1}) caused an increase in plasma osmolality, and a decrease in the plasma ratio of sodium to chloride (related to the strong ion difference, SID), pH and bicarbonate concentration. The external calcium concentration had no apparent influence on the effects of saline exposure. Those experiments, however, provided no information concerning the possible mechanism(s) which brought about the changes in plasma electrolyte, and therefore acid–base status.

Current theories on the mechanisms of electrolyte and acid–base regulation of freshwater fish stress the importance of separate, active branchial $Na^+/H^+-NH_4^+$ and $Cl^-/HCO_3^--OH^-$ exchange mechanisms (see Payan, Girard & Mayer-Gostan, 1984 for a recent review). Thus, an increased rate of active chloride uptake over active sodium uptake during saline exposure would contribute to a decrease in plasma SID, and therefore the decrease in plasma pH. However, it is also important to examine the consequences of passive diffusion of sodium and chloride on plasma electrolyte status since the direction and magnitude of the electrochemical gradients would differ during freshwater and saline exposure.

Kerstetter, Kirschner & Rafuse (1970), Eddy (1975), McWilliams & Potts (1978) and Potts (1984) have shown that the transepithelial potential (TEP) component of the electrochemical gradient in freshwater fish is a diffusion potential established predominantly by the relative permeability of the branchial epithelia to sodium and chloride. Furthermore, the permeability is strongly influenced by the external calcium concentration. Thus, the apparent lack of a significant effect of water hardness on plasma electrolyte status during saline exposure is somewhat surprising.

The purpose of the present study was four-fold. First, we examined the electrochemical gradients for sodium and chloride across the branchial epithelia in fish exposed to both hard and soft freshwater and saline conditions in order to determine the extent to which the passive diffusion of ions could contribute to the observed change in plasma electrolyte status. Second, we examined the effects of independent changes in concentration of external calcium, sodium and chloride on the TEP in order to investigate the lack of an effect of water hardness on the responses to saline exposure. Third, we examined the consequences of saline exposure on the flux of 'acidic equivalents' calculated as the resultant of the net flux of total ammonia ($NH_3 + NH_4^+$) and 'titratable protons'. Finally, the significance of these latter measurements (or lack of it) to the prevailing acid–base status is discussed.

MATERIALS AND METHODS

White suckers (*Catostomus commersoni*, Lacépède), 150–400 g, were obtained by live trapping from artificial lakes in Calgary. All animals were maintained at the University of Calgary for a minimum of 2 weeks prior to use. Fish were fed daily on pellet trout food (Silver Cup). Experimental facilities consisted of a recirculating system on line with a wet table, a 150 l holding tank and a biological filter (Wilkes, 1984). All fish were acclimated in the holding tanks to either hard (Ca^{2+} >

$1{\cdot}0\,mmol\,l^{-1}$) or soft ($Ca^{2+} < 0{\cdot}1\,mmol\,l^{-1}$) freshwater (see Table 1 in Wilkes & McMahon, 1986) for 2 weeks prior to hard, or soft saline exposure (0·94 % sodium chloride, $300\,mosmol\,l^{-1}$).

Measurement of the transepithelial potential

The transepithelial potential (TEP) was measured by a method developed from Potts & Eddy (1973) in which a saline-filled cannula (PE 50) was inserted through the abdominal wall into the extracellular space. The cannula could then be connected to a silver/silver chloride electrode *via* a KCl–agar bridge. The potential between the indwelling electrode and the external reference was measured with a d.c. amplifier (WPI, Model 4MA). Input resistance was $10^{10}\,\Omega$. Both bridges were placed in the external solution immediately prior to and following each TEP measurement. Bridge potentials, if present initially, were offset by applying an equal voltage in the opposite direction through the amplifier. The TEP measurement was repeated if a bridge potential of greater than $\pm 0{\cdot}5\,mV$ had developed over the measurement period (30–60 s).

The TEP was measured in two control groups acclimated to hard and soft freshwater conditions, and in the same groups after 20 min, 24 h and 96 h exposure to hard or soft saline water respectively, and also in a third group exposed to hard saline water for 3–4 weeks.

Calculations of the electrochemical differences for sodium and chloride

The electrochemical difference for a given ionic species, ΔG, across the gill epithelia was calculated as the sum of the concentration terms (established from Wilkes & McMahon, 1986) and the TEP:

$$\Delta G = RT\ln(C_i/C_o) + EzF, \qquad (1)$$

where R = the universal gas constant ($1{\cdot}987\,cal\,°K^{-1}\,mol^{-1}$), T = K, E = the TEP (volts), z = the valency of the ion in question, F = the Faraday constant (23 060 $cal\,V^{-1}\,equiv^{-1}$), C = the ion concentration ($mol\,l^{-1}$) inside (i) and outside (o) respectively. A positive value of ΔG indicates the existence of an outwardly-directed energy difference, negative values, on the other hand, demonstrate the presence of an inwardly-oriented energy difference. The absolute magnitude of ΔG represents the amount of energy which must be expended by an active pump (in $cal\,mol^{-1}$, 1 cal = 4·184 J), in order to maintain steady state in the face of the existing electrochemical difference.

Ion flux analyses

The unidirectional efflux of sodium and chloride was measured using the radiotracers ^{22}Na and ^{36}Cl. Each fish was fitted with an intraperitoneal cannula, as described above, and placed in an individual flux box 24–36 h prior to flux analysis. Flux boxes were constructed from dark Plexiglas after McDonald, Walker & Wilkes

(1983). A known load (2–3 μCi) of each isotope was injected into the fish *via* the cannula. The appearance of the isotope in the water was determined at 1-h intervals for four consecutive hours following a 1·5-h post-injection period required to ensure complete dispersion of the tracer throughout the fish. This time period has been shown to be adequate for trout of similar size (D. G. McDonald, personal communication). At the end of the 4-h flux period, the fish was anaesthetized with MS-222 (1:10 000) and a blood sample obtained by cardiac puncture. The blood samples and the 1-h water samples were analysed for radioactivity as well as for sodium and chloride concentration.

Total gamma (γ) counts were measured on 5-ml aliquots of the water samples placed in borosilicate glass test tubes in an LKB 1282 Compugamma Counter. Total beta (β) counts (from both ^{22}Na and ^{36}Cl) were also measured on 5-ml aliquots of the water samples in 10 ml of fluor (Aquasol, Fisher) with a Tracer Analytic Mark III Liquid Scintillation Counter. Plasma samples (100–500 μl) were placed in 5 ml of distilled water and treated as for water samples. The counting efficiency of each system for ^{22}Na was measured in order to correct the total β counts for ^{22}Na in the mixtures, thus obtaining the β counts due to ^{36}Cl.

The rate of sodium and chloride efflux was calculated from:

$$\text{Efflux} = (\text{c.p.m. } 100\text{g}^{-1}\,\text{h}^{-1})\ (\text{specific activity, c.p.m. } \mu\text{equiv}^{-1}),$$

where (c.p.m. $100\text{g}^{-1}\,\text{h}^{-1}$) is the rate of isotope appearance in the water corrected for water volume, fish weight and time. The specific activity is:

$$\{[\text{ion}, \mu\text{equiv}\,\text{l}^{-1}]\ (\text{ion space, L})\} / (\text{counts injected into the fish} - \text{counts lost to the water}).$$

The volume of the ion space within the fish was calculated as:

$$\text{Ion space} = (\text{c.p.m. injected} - \text{c.p.m. lost to water}) / (\text{c.p.m. ml}^{-1}\text{ in plasma})\ (100 \times \text{weight}^{-1}).$$

Isotope efflux analyses were performed on two control groups of fish acclimated to hard or soft freshwater, in four groups of hard-water acclimated fish subsequently exposed to hard saline conditions for 4 h, 24 h, 96 h and 2–3 weeks (chronic) respectively, and in a group of soft-water-acclimated fish exposed to soft saline water for 96 h. From analyses of the change in ambient sodium and chloride concentrations in the flux boxes during the 4-h control flux period, i.e. the net flux, it is possible to calculate the unidirectional influx from:

$$J^{\text{influx}} = J^{\text{net}} - J^{\text{efflux}}. \qquad (2)$$

Analyses of net flux and unidirectional influx were not possible during saline exposure

Calculations of the permeability coefficients for sodium and chloride

Unfortunately, biological membranes, let alone epithelia, are far too complex to allow a formal description of the permeability coefficient, P, from the properties of the ion and those of the diffusion barrier (Schultz, 1980). However, it is possible to derive a representative value of P_{Cl}/P_{Na} by rearrangement of the Nernst-Planck diffusion equation:

$$\frac{P_{Cl}}{P_{Na}} = \frac{J_{Cl}^{efflux} \times [Na]pl \times (e^{EzF/RT} - 1)}{J_{Na}^{efflux} \times [Cl]pl \times (e^{-EzF/RT} - 1)}, \qquad (3)$$

where E = the TEP (V), F = the Faraday constant (96 486 coulombs equiv^{-1}), R = the universal gas constant ($8{\cdot}314\,J\,K^{-1}\,mol^{-1}$), T = K, z = the valency, J = the rate of diffusion (μequiv $100g^{-1}\,h^{-1}$) from Kirschner & Howe (1981) and Kirschner (1982). It is assumed that the measured efflux is only diffusional, i.e. there is no active component or exchange diffusion.

Despite the 'black box' nature of the permeability coefficients for sodium and chloride, they are used in the Goldman equation to determine the possibility of the movement of other ions contributing to the observed TEP:

$$E = \frac{RT}{zF}\ln\frac{P_{[Na]_i} + P_{[Cl]_o}}{P_{[Na]_o} + P_{[Cl]_i}}, \qquad (4)$$

where R, T, z and F are as previously defined in the Nernst-Planck diffusion equation and the subscripts (i) and (o) refer to inside and outside concentrations respectively. Although there are certain assumptions concerning the use of this equation (Katz, 1966) which may not apply particularly well to epithelia (primarily a linear voltage gradient across the diffusion channels) the Goldman equation remains the most common way of analysing the diffusion potential (Eddy, 1975). If the potential, E, calculated using just sodium and chloride permeabilities agrees with the measured TEP then there is no compelling reason to postulate movement of other electrolytes contributing to the observed TEP. It is possible, however, that electrogenic transport is exactly balanced by net diffusion of other ions not accounted for in the equation (Potts, 1984).

Flux of acidic equivalents

It is not possible to distinguish the movement of protons across the epithelial barrier in one direction from the movement of bicarbonate, or hydroxyl ions, in the opposite direction. However, since the result is the same in either case, such a distinction is not necessary. Current convention is to use the term 'acidic equivalents' to describe the net flux of titratable and non-titratable protons across the branchial

epithelia. The titratable component was measured as the change in the end point titration volume of a 10-ml sample with $0{\cdot}01\,mol\,l^{-1}$ HCl to pH 4·0 as in McDonald & Wood (1981) and McDonald (1983*a*). The non-titratable component of proton flux is assumed to be associated with the transbranchial flux of ammonium. Total ammonia in water samples was measured with the phenol hypochlorite method of Solorzano (1969). The net flux of 'acidic equivalents' is calculated as the sum of these two components. Water samples for flux analyses of titratable protons and ammonia were obtained at the same time as water samples used for isotope analyses.

Effects of external calcium, sodium and chloride on the TEP

The effects of variation of external calcium, sodium and chloride concentration on the TEP were evaluated in three series of acute experiments.

Series I

Series I was designed to measure the effects of increasing the external calcium concentration on the TEP of freshwater-exposed fish. The TEP was first measured, as described above, after animals had been exposed to $1{\cdot}0\,mmol\,l^{-1}$ sodium chloride in distilled water for several hours. External calcium concentration was then increased by successive aliquots of calcium nitrate. The initial aliquot was sufficient to achieve an ambient calcium of approximately $0{\cdot}1\,mmol\,l^{-1}$. Each additional aliquot was such that the ambient concentration of calcium was doubled. Although the final concentration of calcium was usually $1–2\,mmol\,l^{-1}$, in some experiments the concentration was increased to $4–5\,mmol\,l^{-1}$. The TEP was not measured until 10–15 min after the addition of each calcium aliquot in order to ensure complete dissolution and dispersion of the salt around the fish. Results from preliminary studies demonstrated that the changes in TEP were initiated rapidly and completed within a minute or so of the addition of electrolytes, and were just as readily reversible on their removal.

Series II

Series II was similar to series I except that the effect of increasing external calcium concentration on the TEP was measured in fish previously exposed to distilled water plus $160\,mmol\,l^{-1}$ sodium (as sodium sulphate), chloride (as choline chloride) or sodium chloride.

Series III

The approach used in series III was to examine the effects of increasing external sodium sulphate, choline chloride and sodium chloride on the TEP of fish previously exposed to either hard or soft freshwater for several hours. The range of external sodium and chloride concentrations was from $0{\cdot}5\,mmol\,l^{-1}$ to approximately $180–200\,mmol\,l^{-1}$. The duration of these three studies was not sufficient to result in measurable changes in the concentration of plasma electrolytes.

Statistical treatment

Statistical difference was inferred if $P < 0.05$ using a Student's unpaired, two-tailed *t*-test, unless otherwise stated. All results are reported as the mean ± 1 S.E.M.

RESULTS

Transepithelial potential

The TEP measured in control fish acclimated to hard freshwater was $13{\cdot}3 \pm 2{\cdot}0$ mV (Fig. 1). Within minutes of exposure to hard saline water the TEP decreased significantly to $2{\cdot}4 \pm 0{\cdot}2$ mV. Although the TEP remained significantly above zero for the first 96 h, it decreased to $-0{\cdot}5 \pm 0{\cdot}3$ mV, a value not significantly different from zero, after chronic exposure to hard saline conditions. In control fish acclimated to soft freshwater, the TEP was $-7{\cdot}3 \pm 2{\cdot}6$ mV. However, within minutes of exposure to soft saline water the TEP increased significantly to $6{\cdot}7 \pm 1{\cdot}1$ mV and remained significantly above zero after 96 h exposure to soft saline conditions.

Electrochemical differences for sodium and chloride across the branchial epithelia

Analysis of the electrochemical differences for sodium and chloride (equation 1) demonstrated the presence of a large dissipative difference of 2000–3000 cal mol^{-1} for both sodium and chloride in either hard- or soft-water-acclimated control fish (Fig. 2). During saline exposure, however, the absolute magnitude of these differences was rapidly and significantly reduced to several hundred cal mol^{-1}. In fish exposed to hard saline water, the electrochemical difference for sodium remained between zero and 100 cal mol^{-1} for all but the initial period of saline exposure, when ΔG_{Na} was not significantly different from zero. Therefore, the electrochemical difference for sodium would still be outwardly directed during saline exposure. In contrast, ΔG_{Cl} for fish exposed to hard saline water fell to -364 ± 26 cal mol^{-1}, remaining significantly below zero throughout the 96-h exposure period. Thus the electrochemical gradient for chloride was directed into the fish. After chronic exposure to hard saline conditions, the electrochemical difference for chloride was not significantly different from zero, while ΔG_{Na} was only $36{\cdot}1 \pm 7{\cdot}6$ cal mol^{-1}.

The 96-h values of ΔG for sodium and chloride of fish exposed to soft saline conditions were still significantly different from zero at 43 ± 10 and -43 ± 10 cal mol^{-1}, respectively.

Ion flux

Control fish in hard or soft freshwater were in steady state with respect to the transbranchial movement of sodium and chloride since the respective net fluxes were not significantly different from each other or from zero. The only statistically significant effect of variation in the degree of water hardness on the flux rates of sodium and chloride in control fish was an increase in sodium uptake from $9{\cdot}0 \pm 1{\cdot}5$

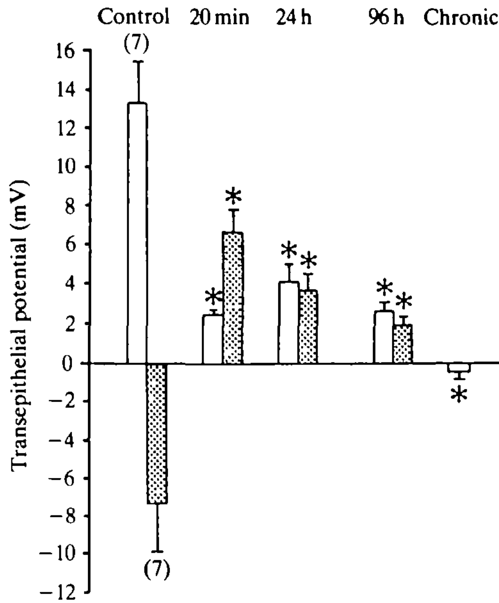

Fig. 1. The transepithelial potential (TEP) in fish exposed to hard (clear) and soft (stippled) freshwater and saline conditions. The number of animals used (N) is given in parentheses. Statistical significance from respective control values is demonstrated by (*). The vertical lines in this and all subsequent figures represent 1 S.E.M.

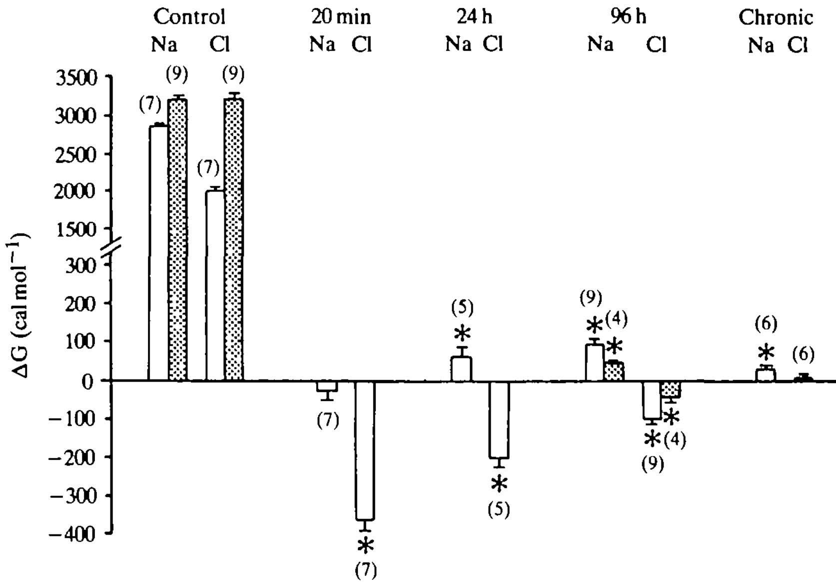

Fig. 2. ΔG for sodium and chloride for fish exposed to hard freshwater and hard saline conditions (clear), and soft freshwater and soft saline conditions (stippled). The number of animals used (N) is given in parentheses. ΔG_{Na} and ΔG_{Cl} for all saline-exposed animals were significantly lower than respective control values. Statistical difference from zero is demonstrated by (*).

in hard-water-acclimated control fish to $18 \cdot 1 \pm 3 \cdot 7\ \mu$equiv $100g^{-1}\ h^{-1}$ in soft-water-acclimated control fish (Fig. 3).

On initial exposure to hard saline water, the measured efflux rates for sodium and chloride increased significantly by 74 % and 52 % respectively (Fig. 3). Both efflux rates continued to increase so that by 24 h of saline exposure the sodium efflux rate was 10-fold, while that for chloride was 7·5-fold, greater than respective control values. Both efflux rates remained significantly elevated throughout exposure to hard saline water.

Sodium and chloride efflux rates from fish in soft saline water were measured only after 96 h exposure (Fig. 3). As noted in fish exposed to hard saline water, the rates of both sodium and chloride efflux were significantly increased by an order of magnitude over control values.

In one experiment involving three fish previously exposed to hard saline conditions for 4 days, the rates of ^{22}Na and ^{36}Cl appearance in the water were measured over a single continuous 9-h flux period which incorporated a return to control (hard freshwater) conditions (Fig. 4). For the first 4 h the fish were in saline conditions, following which the salt water was displaced with hard freshwater over a 15-min period. The rates of isotope appearance from the same fish now exposed to

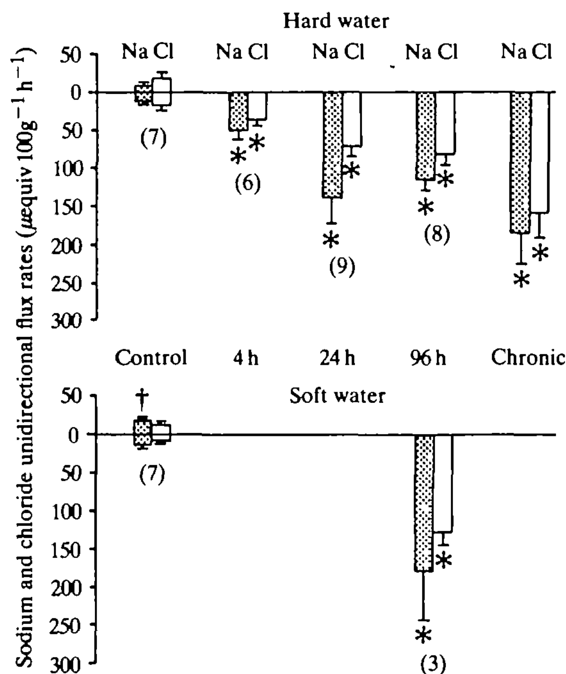

Fig. 3. Unidirectional fluxes for sodium (stippled) and chloride (clear) for fish in hard and soft freshwater control conditions and unidirectional efflux of sodium (stippled) and chloride (clear) during exposure to hard and soft saline water. The number of animals used (N) is given in parentheses. Statistical significance from respective control values is demonstrated by (*). Statistical differences between sodium and chloride flux rates in control fish are demonstrated by (†).

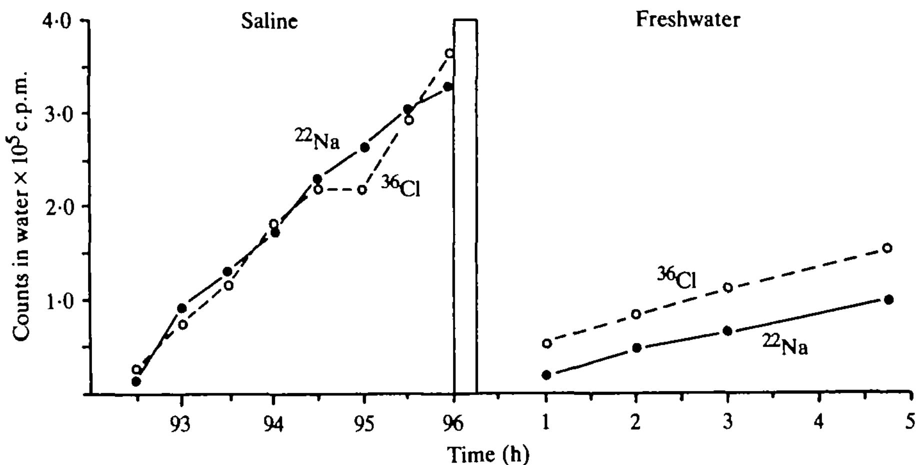

Fig. 4. Appearance of isotope ^{22}Na (closed circles) and ^{36}Cl (open circles) for one individual fish after 4 days exposure to hard saline water followed by a 15-min rinse (bar) and then a subsequent 5 h in hard freshwater.

freshwater were then measured for an additional 5-h period. At no time were the fish handled or disturbed. The transition to freshwater resulted in a 61 % and 54 % reduction in the sodium and chloride efflux rates respectively (Fig. 4).

P_{Cl}/P_{Na} ratio

The P_{Cl}/P_{Na} ratios for control fish were calculated from equation 3 using individual isotope efflux values and mean TEP values of 13·3 and −7·0 mV respectively for hard- and soft-water conditions. The P_{Cl}/P_{Na} ratio for fish acclimated to hard freshwater was 4·11 ± 0·55 (6), whereas that for fish acclimated to soft freshwater was significantly lower ($P < 0.002$) at 0·58 ± 0·14 (6).

Using these calculated P_{Cl}/P_{Na} ratios and concentration differences for sodium chloride across the gill epithelia, the Goldman equation (equation 4) predicts a negative TEP of −19·8 ± 6·7 mV in fish acclimated to soft freshwater, and 23·9 ± 3·1 mV for fish acclimated to hard freshwater.

Flux of 'acidic equivalents'

There were no significant differences in the flux of 'acidic equivalents' or its components in the two control groups of fish. Exposure to soft saline water for 96 h had no significant effect on the net flux of 'titratable protons', total ammonia or their resultant, the net flux of 'acidic equivalents'. Exposure to hard saline water caused statistically significant increases in the net flux of 'acidic equivalents' within 24 h, and the 'titratable proton' flux and 'acidic equivalents' flux after chronic exposure (Table 1). Although statistically different from control values, these changes were small when compared to the increases of which this species is potentially capable (Hōbe, 1985); therefore, they are probably of minor physiological importance.

Effects of external calcium, sodium and chloride on the TEP

Results from series I show that the TEP was always negative, between −10 and −25 mV (Fig. 5) when fish were exposed to zero-calcium freshwater (distilled water plus 1·0 mmol l^{-1} sodium chloride). However, the polarity changed to 10–25 mV positive once the external calcium concentration exceeded 0·2–0·4 mmol l^{-1}. Further increases in external calcium concentration to 4–5 mmol l^{-1} had no appreciable additional affect on the TEP.

Table 1. *The net flux of titratable protons, $J^{net}_{titratable\,protons}$, the net flux of total ammonia, $J^{net}_{([NH_3]+[NH_4^+])}$ and their resultant, the net flux of 'acidic equivalents', $J^{net}_{acidic\,equivalents}$ for control fish acclimated to hard or soft freshwater and for experimental fish exposed to hard or soft saline water respectively*

		$J^{net}_{titratable\,protons}$ (μequiv 100g^{-1} h^{-1})	$J^{net}_{([NH_3]+[NH_4^+])}$ (μequiv 100g^{-1} h^{-1})	$J^{net}_{acidic\,equivalents}$ (μequiv 100g^{-1} h^{-1})
		Hard water		
Control	x̄	16·0	−21·5	−5·5
	S.E.M.	2·6	2·4	3·2
	(*N*)	(11)	(11)	(11)
4 h	x̄	22·6	−19·2	−3·5
	S.E.M.	2·1	4·5	3·2
	(*N*)	(11)	(11)	(11)
	P<	NS	NS	NS
24 h	x̄	29·2	−24·5	4·7
	S.E.M.	6·8	5·8	2·5
	(*N*)	(9)	(9)	(9)
	P<	NS	NS	0·05
96 h	x̄	18·6	−20·8	−2·0
	S.E.M.	6·6	4·6	3·4
	(*N*)	(8)	(8)	(8)
	P<	NS	NS	NS
Chronic	x̄	31·5	−20·5	10·9
(2 consecutive	S.E.M.	5·1	3·3	3·4
days)	(*N*)	(9)	(9)	(9)
	P<	0·02	NS	0·005
	x̄	36·4	−22·5	13·9
	S.E.M.	3·8	3·4	2·2
	(*N*)	(9)	(9)	(9)
	P<	0·005	NS	0·001
		Soft water		
Control	x̄	22·7	−27·6	−4·9
	S.E.M.	3·5	3·5	2·7
	(*N*)	(10)	(10)	(10)
96 h	x̄	19·1	−15·6	3·5
	S.E.M.	4·2	2·6	2·8
	(*N*)	(4)	(4)	(4)
	P<	NS	NS	NS

NS, not significant.

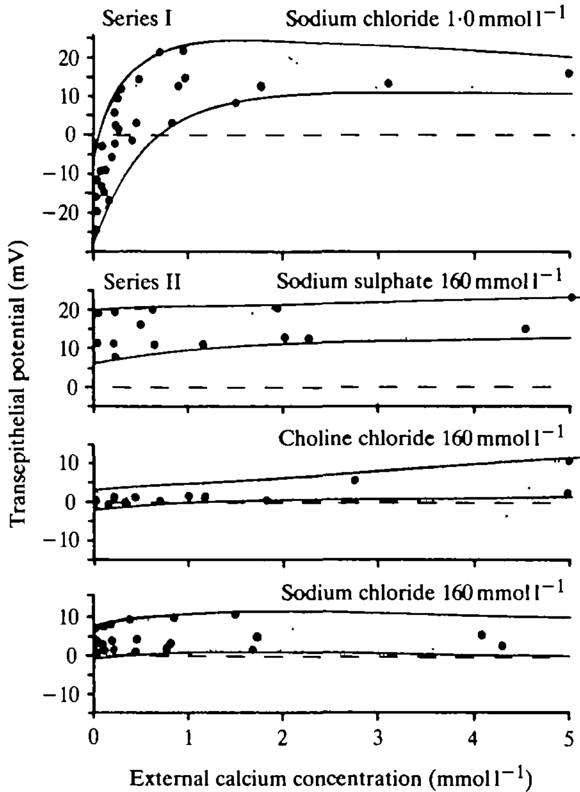

Fig. 5. The transepithelial potential (TEP) for fish in series I and II in which external calcium concentration was increased from 0 to $5\,\mathrm{mmol\,l^{-1}}$. The lines and enclosed stippling were drawn by hand to encompass the range of values and are intended only as a visual aid.

In series II, fish were exposed to distilled water plus either $160\,\mathrm{mmol\,l^{-1}}$ sodium (as sodium sulphate), chloride (as choline chloride) or sodium chloride for several hours prior to the addition of calcium nitrate. In contrast to results from series I, the initial TEP values were $16{\cdot}8 \pm 1{\cdot}5\,\mathrm{mV}$, $1{\cdot}4 \pm 0{\cdot}8\,\mathrm{mV}$ and $5{\cdot}4 \pm 0{\cdot}9\,\mathrm{mV}$, respectively, for the three salt solutions (Fig. 5). Furthermore, the TEP for fish in series II was unresponsive to change in external calcium concentration over the range of 0–$5\,\mathrm{mmol\,l^{-1}}$.

In series III, fish had been previously exposed to either hard or soft freshwater for several hours. The TEP was measured prior to and following addition of successive aliquots of either sodium sulphate, choline chloride or sodium chloride up to final concentrations of approximately $200\,\mathrm{mmol\,l^{-1}}$. Increasing sodium sulphate concentration had no effect on the TEP in hard-water exposed fish (Fig. 6). However, when choline chloride or sodium chloride concentrations were increased, the TEP fell from +(10 to 20) mV to +(0 to 10) mV at $160\,\mathrm{mmol\,l^{-1}}$ chloride. Essentially opposite responses were observed in soft water; as external sodium concentration was increased (either as sodium sulphate or sodium chloride) the TEP increased from −(10 to 20) mV to +(10 to 20) mV (Fig. 6). Although increasing

external choline chloride concentration had minor effects on the TEP, this may have been due to some diffusion of choline (J. W. Hanrahan, personal communication).

DISCUSSION

The influence of external calcium on the polarity of the TEP of *C. commersoni* is clearly demonstrated by the different potentials measured in hard and soft freshwater-acclimated fish (Fig. 1). Although these results are qualitatively comparable to the findings of Eddy (1975) for goldfish, and Kerstetter *et al.* (1970) and McWilliams & Potts (1978) for trout, there is a clear quantitative difference. The maximum response of the TEP for *C. commersoni* was achieved at approximately

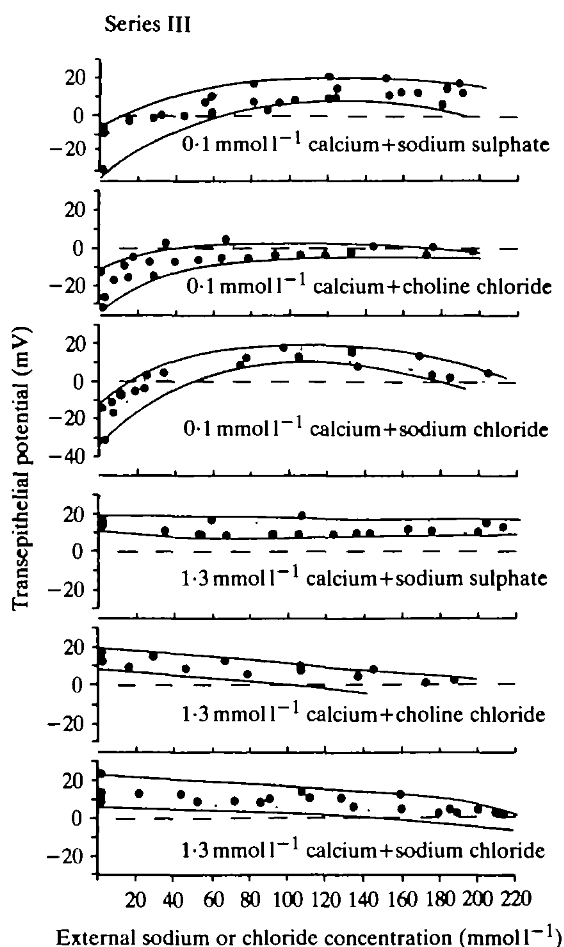

Fig. 6. The transepithelial potential (TEP) in soft- and hard-water-exposed fish in series III involving progressive increases in sodium (as sodium sulphate), chloride (as choline chloride) and sodium chloride in both soft- and hard-water conditions. The lines and enclosed stippling were drawn by hand to encompass the range of values and are intended only as a visual aid.

$0{\cdot}8\,\text{mmol}\,\text{l}^{-1}$ external calcium concentration as opposed to 4–5 $\text{mmol}\,\text{l}^{-1}$ in the other studies. The TEP of freshwater fish is most often viewed as a diffusion potential established primarily by the concentration difference and permeability coefficients of the branchial epithelia to sodium and chloride (Eddy, 1975; Potts, 1984). The ability of calcium ions in the external compartment to influence the polarity of the TEP can thus be explained as a change in the physico-chemical properties of the diffusion channels in the gill epithelia (McDonald, 1983*b*; Potts, 1984). Although the Goldman equation correctly predicts the change in polarity of the TEP in hard- and soft-water-acclimated fish, using the P_{Cl}/P_{Na} ratios of 4·1 and 0·6 respectively, there is a quantitative discrepancy between the measured and calculated potentials. Part of the reason for the discrepancy may stem from the fact that the TEP and efflux measurements were not made on the same animals. There are at least two other possible explanations. First, other electrolytes, not accounted for in equation 4, may contribute to the diffusion potential. If sodium and chloride diffusion were the only determinants of the TEP, the expected P_{Cl}/P_{Na} ratios would be closer to 2·2 and 1·0 for hard- and soft-water-acclimated fish respectively. Second, part of the measured TEP may be due to an electrogenic component (Maetz, 1974).

During saline exposure the external calcium concentration no longer has an influence on the TEP (Figs 1, 5, 6). When the external sodium chloride concentration is increased, the equilibrium potentials for sodium and chloride both tend to zero. If we use the 96-h plasma concentrations of sodium and chloride in the Nernst equation, since they should closely approximate steady-state conditions, E_{Na} and E_{Cl} values are 5 mV and -11 mV respectively. Thus the measured TEP is more a reflection of E_{Na} than a diffusion potential, i.e. the Goldman equation reduces to the Nernst equation for sodium. Therefore the P_{Cl}/P_{Na} ratio has only a minor influence on the TEP. In addition, it could be argued that exposure to high external concentrations of electrolytes would alter the electrostatic environment of the diffusion channels by titrating any fixed charges and thus 'swamp' any influence of external calcium.

The small positive TEP observed in fish exposed to either hard or soft saline conditions had a dramatic effect on the transepithelial electrochemical differences for sodium and chloride. Although there was an inwardly directed chemical potential for both sodium and chloride during the first 96 h of saline exposure, the prevailing electrical potential served to enhance the chloride chemical potential difference, but to act against that for sodium. The resultant positive ΔG_{Na} indicated the presence of a small but persistent outwardly-directed energy difference, while the negative ΔG_{Cl} was somewhat larger in absolute magnitude than that for sodium (Fig. 2) and was directed inward.

The high affinity of the active uptake pumps for sodium and chloride (under 20 $\text{mequiv}\,\text{l}^{-1}$ for a variety of species, Kirschner, 1970; Evans, 1984) suggests that they would both be operating at maximum rates during exposure to approximately 160 $\text{mequiv}\,\text{l}^{-1}$ sodium chloride. Thus the continued active uptake, in conjunction with the greatly reduced passive efflux of sodium and chloride, is probably the underlying reason for the observed increase in plasma osmolality (Wilkes &

McMahon, 1986). Furthermore, if the maximum rate of chloride uptake exceeded that of sodium, the activity of these pumps would also be contributing to the observed decrease in plasma SID. However, without direct measurement of unidirectional influx rates and analysis of the kinetic information relating to the substrate affinity (K_m) and number of functional pumps (V_m) as described by the Michaelis-Menten equation, further analysis along this line is precluded. Indeed, the kinetic information is required in order to determine whether the external calcium concentration was responsible for the observed increase in sodium uptake in hard-water-acclimated fish over that of soft-water-acclimated fish (Fig. 3).

Although the electrochemical potentials for sodium and chloride were much attenuated during saline exposure, the measured rates of sodium and chloride efflux were greatly increased (Fig. 3). Efflux rates increased within minutes of saline exposure, prior to any increase in plasma osmolality (established in preliminary studies), and decreased just as rapidly on recovery from 96 h saline exposure (Fig. 4) when plasma osmolality was still elevated. This demonstrates a pronounced trans effect. Two possible explanations are (1) the presence of exchange diffusion, i.e. Na^+/Na^+ and Cl^-/Cl^- exchange (Ussing, 1947; Motais, Garcia-Romeu & Maetz, 1966); and (2) an increase in branchial permeability (Zadunaisky, 1984). Exchange diffusion mechanisms have often been invoked to account for the high turnover rates of salts in marine fish (Potts, 1976) and in goldfish placed in a saline environment (Lalhou, Henderson & Sawyer, 1969; DeRenzis, 1975). Although the functional significance of the exchange mechanism is not entirely clear, the 1:1 stoichiometry dictates that it cannot account for a change in solute concentration on either side of the pump. Zadunaisky (1984) has recently suggested that much of the trans effect observed in flux studies can be explained by an increase in permeability. These latter studies were carried out on isolated opercula of *Fundulus*, a preparation which permits a more detailed and rigorous examination of transport mechanisms than is currently possible using whole animal preparations. Nevertheless, care must be taken in extrapolating results from euryhaline to stenohaline species; intuitively one would expect fundamental differences in transport mechanisms and kinetics.

Research on acid-base regulation in fish has often been concerned with the active $Na^+/H^+-NH_4^+$ and $Cl^-/HCO_3^--OH^-$ exchange. Inherent to this approach is the idea that the rate of proton input to (or removal from) a compartment is a determining factor in the extent to which proton concentration (pH) is altered. This has been the rationale behind a number of studies which have examined the relationship between blood acid-base status and the transbranchial flux of 'acidic equivalents' (see Cameron, 1978; Girard & Payan, 1980; Evans *et al.* 1982; Heisler, 1984 for reviews). However, as Stewart (1978, 1981, 1983) points out, the only way in which the proton (and bicarbonate) concentration can be altered is by a change in one or more independent variables – strong ion difference (SID), P_{CO_2}, and the total concentration of weak acids and weak bases. Therefore, any observed change in proton concentration is simply the result of a change in one or more of these independent variables. The transbranchial movement of protons and/or bicarbonate, which is no doubt associated with the movement of sodium and chloride,

cannot cause, or correct, an observed change in pH in either compartment. Th. question then becomes, what is the transbranchial flux of 'acidic equivalents' a measure of, and how can it be accounted for in terms of independent and dependent variables?

In order to answer these questions it is necessary to examine the two components of the 'acidic equivalent' flux separately, i.e. the net flux of total ammonia ($NH_3 + NH_4^+$) and that of 'titratable protons'. The former is a net efflux in virtually all circumstances, and is commonly viewed as a net efflux of protons. One of the major end products of amino acid breakdown is thought to be NH_3. Since the pK for the $NH_3 \leftrightarrow NH_4^+$ reaction is approximately 9·2, most of the free base would be ionized by surrounding protons to NH_4^+. This view has recently been challenged by Atkinson & Camien (1982). They point out that at physiological pH, the products of hydrolysis of glutamine are not glutamic acid and NH_3, but glutamate and NH_4^+. Therefore, the efflux of ammonium, the predominant species, cannot be a proton removing mechanism. In either case, a net ammonium efflux into neutral water (i.e. SID = 0) will cause a proportionately larger increase in water SID than a decrease in extracellular SID since (i) plasma ammonium concentration is no more than a few μequiv l^{-1}, while the plasma SID is of the order of 20 mequiv l^{-1}, and (ii) there is continual production of ammonium by the fish so that plasma concentrations would remain relatively constant while the external ammonium concentration would increase several-fold.

The second of the 'acidic equivalent' flux components is termed the 'titratable proton' flux and is measured by the change in the endpoint titrant volume. Obviously, if the SID of the water increases, because of the net NH_4^+ efflux, then the volume of acid (strong anions) required to titrate the water to a given pH (SID) will also increase, resulting in an apparent increase in the flux of 'titratable protons'. If the relative concentrations of sodium and chloride in the external water are also altered, *via* enhanced uptake or efflux of one or the other, then the 'titratable proton' flux will also vary accordingly, i.e.:

$$J^{net}_{titratable\ protons} = J^{net}_{NH_4^+} + J^{net}_{Na^+} + J^{net}_{cations} - J^{net}_{Cl^-} - J^{net}_{anions}. \quad (5)$$

Rearranging, and ignoring the net flux of cations and anions other than sodium, chloride and ammonium, results in:

$$J^{net}_{titratable\ protons} - J^{net}_{NH_4^+} = J^{net}_{Na^+} - J^{net}_{Cl^-}. \quad (6)$$

Support for the validity of equation 6 is provided by the results of Wood, Wheatly & Hōbe (1984) who arrived, by empirical means, at the same equation in trout exposed to hyperoxia.

Thus, the net efflux of ammonium is of little significance to the plasma pH because of its minor effect on plasma SID, and the so-called flux of 'titratable protons' could be explained as the rate of change of the SID of the external medium due to the relative movement of strong anions and cations into and out of that compartment. The 'titratable proton' flux does not indicate which, or how, electrolyte concentrations are being altered. In the control fish, the net flux rates for sodium and

chloride were not significantly different from zero. Therefore, the net flux of 'titratable protons' can be accounted for almost completely by the net efflux of ammonium (Table 1).

In conclusion, we suggest that during 96 h of saline exposure a net influx of sodium and chloride had to occur in order to account for the increase in plasma osmolality (Wilkes & McMahon, 1986). Additionally, the net influx of chloride was greater than that of sodium. The resulting decrease in plasma SID was responsible for the observed changes in acid–base status (Wilkes & McMahon, 1986). Unfortunately, it was not possible to clarify the mechanism(s) by which the extracellular electrolyte status was altered during saline exposure. Nevertheless, the observation that the electrochemical differences for sodium and chloride were oriented in opposite directions suggests that passive sodium and chloride diffusion may have been partly responsible. Since the strong ion difference is largely determined by the difference in sodium and chloride concentrations, we conclude that extracellular proton and bicarbonate concentrations are not in themselves regulated variables. By extension, it is not necessary to ascribe an acid–base regulatory function to the active branchial uptake pumps for sodium and chloride, i.e. $Na^+/H^+–NH_4^+$ and $Cl^-/HCO_3^-–OH^-$ exchange. These are in fact simply one portion of the overall electrolyte regulatory mechanism. The electrolyte status in turn is simply one of the independent variables which determine proton and bicarbonate concentration.

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