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THE INTERACTION OF ENVIRONMENTAL CALCIUM AND LOW pH ON THE PHYSIOLOGY OF THE RAINBOW TROUT, *SALMO GAIRDNERI*

II. BRANCHIAL IONOREGULATORY MECHANISMS

BY D. G. McDONALD, R. L. WALKER* AND P. R. H. WILKES*

Department of Biology, McMaster University, Hamilton, Ontario, L8S 4K1

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SUMMARY

Adult rainbow trout, acclimated to external calcium concentrations ranging from 60–5700 μequiv/l, were exposed to pH 4·0–4·1 for 44 h. Initially, this exposure provoked massive net losses of sodium and chloride across the gills which arose through a combination of an inhibition of active transport and, more importantly, a stimulation in diffusional efflux. Subsequently, ion losses declined substantially, largely due to a rapid decline in passive efflux but also to a slower, partial recovery in sodium transport. External calcium concentration was virtually without effect on ion fluxes either prior to or following acid exposure but had a definite effect during acid exposure. This effect was initially upon the ratio of Cl^- to Na^+ loss and later upon the degree of inhibition of sodium and chloride transport. Possible mechanisms to explain the complex interactions of calcium and pH are proposed.

INTRODUCTION

The importance of external calcium in stabilizing biological membranes, maintaining the integrity of cell to cell junctions, and controlling ion and water permeability across epithelial tissues is well known (Schoeffeniels, 1967; Cuthbert, 1970; Oduleye, 1975). Thus it is not surprising that calcium is an important factor in the ecology of fish species. Fresh waters high in calcium (i.e. 'hard' waters) increase the productivity of plant and animal life (Willoughby, 1976), improve the survival of euryhaline and stenohaline marine teleosts (Pickford, Pang, Stanley & Fleming, 1966; Evans, 1975) and may reduce the energy expenditure required for the maintenance of ion balance (Eddy, 1975). With the progressive acidification of natural waters calcium has become an even more important ecological variable. Surveys of moderately acid lakes (pH 4·5–5·5) in southern Norway have indicated that calcium is as critical to fish population status as is pH (Wright & Snekvik, 1978). At more severely acid levels (pH 4·0–4·5) increased calcium extends fish survival (Brown, 1981) and reduces the rate of sodium and chloride loss from the plasma (McDonald, Hōbe & Wood, 1980; Leivestad, Muniz & Rosseland, 1980).

* Present address: Department of Biology, University of Calgary, Calgary, Alberta, T2N 1N4.
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In our previous studies (McDonald *et al.* 1980; McDonald & Wood, 1981 McDonald, 1983) we have shown that the principal sites of interaction of calcium and low pH are the gill mechanisms for the regulation of sodium and chloride, but as yet we know very little of the specific nature of this interaction. Thus the principal objectives of the present study are to examine the effects of low environmental pH on passive ion efflux and active ion transport across the gills, to determine how these parameters are altered by continued low pH exposure, and finally to assess the moderating influence of external calcium. A secondary objective was to explore further the basis for the pronounced influence of calcium on H^+ uptake at low pH (McDonald *et al.* 1980; McDonald, 1983). To enable comparisons with our previous studies, this work employed adult rainbow trout acclimated to a range of calcium concentrations representing very soft to moderately hard waters (60–5700 μequiv/l) and exposed to pH 4·0–4·1.

METHODS

Experimental animals and test conditions

Adult rainbow trout (*Salmo gairdneri* Richardson) were obtained commercially and held in running water as described in McDonald (1983). Fish were acclimated for 10–12 days to water of the temperature (16±1 °C) and ionic composition employed in subsequent experiments (Table 1). This water was prepared by supplementing partially deionized water (Ca^{2+} = 50 μequiv/l, NaCl = 70 μmol/l) from a reverse osmosis system (Culligan Aqua-Cleer 200) with Ca^{2+} [as $Ca(NO_3)_2$], NaCl, and KCl to give three different calcium levels but similar sodium and chloride concentrations (Table 1). The three levels of Ca^{2+} were: 5700 μequiv/l, (representing hard water and hereafter termed 'high Ca^{2+}') 240 μequiv/l (soft water, 'low Ca^{2+}') and 60 μequiv/l ('very low Ca^{2+}'). Low pH water required during the experiment was prepared by titration of these mixtures to a pH of 4·0 with H_2SO_4 and then aeration, prior to use, to remove CO_2.

Following acclimation, trout were surgically fitted under MS-222 anaesthesia with a catheter in the urinary bladder. Trout were then transferred to individual fish chambers held within flux chambers as described in McDonald (1983) and allowed to recover from the surgery for 36–48 h. Except for periods when branchial fluxes were being measured, the flux chambers were gravity fed from 200 l recirculated reservoirs maintained at 16 ± 1 °C.

Experimental protocol

Eighteen trout were acclimated to each of the three Ca^{2+} levels. For each group the experiment consisted of 24 h at a circumneutral pH, followed by 44 h of exposure to an acid pH and then by 6 h at a circumneutral pH. Branchial fluxes were determined at four intervals during this experiment: (1) *control conditions*, i.e. prior to acid exposure; (2) *acute acid exposure*, 1·5 h following reduction in pH; (3) *chronic acid exposure*, 40 h after reduction in pH and (4) *recovery*, 1·5 h after the restoration of a circumneutral pH. Each flux period was conducted with at least four fish at each Ca^{2+} level and was initiated in the same manner. For 1 h before the start, the flux chambers

were flushed with fresh water of the appropriate ion composition and pH. This ensured removal of nitrogenous wastes and equilibration of pH in those circumstances where pH was altered. The flux chambers were then closed, volume was adjusted to a known value (nominally 5 l/fish) and radiosodium (10 μCi of ^{24}Na as Na_2CO_3) and radiochloride (2 μCi of ^{36}Cl as HCl) were added. The isotope was allowed to mix for 0·5 h, water samples were then collected for analysis at hourly intervals over the next 4 h. Each sample was analysed immediately for ^{24}Na activity and for titratable alkalinity. The remaining volumes were either counted later for $^{36}Cl^-$ or frozen for later analysis of ion concentrations and ammonia. During the acid exposure flux periods,

Table 1. *Ion composition (overall means for all four tests periods ± one S.E.M.) in μequiv/l and pH (means ± one S.E.M.) of test water*

T = 16 ± 1 °C.

	High calcium	Low calcium	Very low calcium
Ca^{2+}	5658(± 148)	241(± 9)	61(± 3)
Na^+	261(± 27)	266(± 55)	277(± 35)
Cl^-	264(± 15)	295(± 54)	393(± 35)
K^+	26(± 1)	31(± 10)	169(± 9)
pH Control	7·56(± 0·1)	7·76(± 0·01)	7·15(± 0·03)
Acute acid	4·00(± 0·3)	4·16(± 0·05)	4·01(± 0·04)
Chronic acid	4·12(± 0·02)	4·12(± 0·03)	4·14(± 0·04)
Recovery	7·59(± 0·02)	7·65(± 0·02)	7·23(± 0·03)

known volumes of 0·1 N-H_2SO_4 were added at hourly intervals to maintain pH. The measurements of titratable alkalinity enabled an accurate assessment of the volumes required and, as a consequence, pH was maintained within ±0·1 units (Table 1). During flux periods at circumneutral pH, pH variations were minor (Table 1) and no adjustments were necessary. For all flux periods, urine was collected from the bladder catheter by a 7 cm siphon into covered vials. This removed the renal contribution to ion and H^+ fluxes.

Analytical techniques

$^{24}Na^+$ was measured by gamma counting in a well counter (Nuclear Chicago Model 1085). After allowing for the decay of $^{24}Na^+$, $^{36}Cl^-$ was determined by liquid scintillation counting (Beckman LS 230). Concentrations of Na^+, K^+ and Ca^{2+} were determined by flame photometry (EEL and Coleman 20 flame photometers), Cl^- by coulometric titration (Radiometer and Buchler Chloridometers) and ammonia by a micro-modification of the salicylate-hypochlorite reaction of Verdouw, van Echteld & Dekkers (1978). Titratable alkalinity was determined by titrating continuously-aerated 10 ml samples to a pH below 4·0 with 0·02 N-HCl. Any acid which had been added to the flux chamber during a flux measurement was incorporated into the final calculation of titratable alkalinity. Net Na^+, Cl^- and ammonia fluxes (J_{net} in μequiv/kg/h) were determined on an hourly basis from the change in their respective concentrations in the water. Sodium and chloride influx values (J_{in}) were determined by disappearance of radioactivity from the water according to the following equation (modified from equation 5 of Kirschner, 1970):

$$J_{in} = \frac{(\ln Q^{*}_{out_o} - \ln Q^{*}_{out_t})}{t \cdot W} Q_{out}$$

where $Q^{*}_{out_o}$ and $Q^{*}_{out_t}$ were the total amounts of radioactivity in the medium at the beginning and end of the 1 h interval, Q_{out} was the average amount of ion in the medium over the 1 h interval, t was 1 h and W was body weight in kg. Analysis at hourly intervals minimized variations in Q_{out}; necessary since this equation assumes that Q_{out} remains constant. Radioisotope back flux over the 4 h flux period was calculated according to equation 3 of Cuthbert & Maetz (1972) and was found to be negligible. J_{out} for Na^+ and Cl^- was calculated by subtracting J_{net} from J_{in}. The net H^+ uptake/excretion (J^{H+}_{net}) was determined as the difference between the change in titratable alkalinity (apparent H^+ uptake) and the ammonia excretion (apparent H^+ excretion) as described in detail in McDonald & Wood (1981), McDonald, Walker, Wilkes & Wood (1982) and McDonald (1983).

Statistical analysis

All data are expressed as means ± one S.E.M.. Means were based on measurements from at least four fish with four separate, hourly measurements per fish. Statistical differences among the fish from the three different calcium levels were tested by the Students two-tailed '*t*' test (unpaired design). The chosen level of significance was $P < 0.01$.

RESULTS

At a circumneutral pH there were relatively small differences among the fish acclimated to the high, low and very low calcium environments (Fig. 1). Chloride turnover rates (Fig. 1B) were similar in all three groups although the sodium turnover in high Ca^{2+} (Fig. 1A) was almost double that observed in the two lower Ca^{2+} environments. Ammonia excretion (J^{Amm}_{net}, Fig. 2B) was also significantly higher in this group. Slight differences in J^{H+}_{net} (Fig. 2A) were also observed; in high Ca^{2+} there was a net excretion of H^+ while in very low Ca^{2+} there was a net uptake of H^+. These were significant differences but may have been caused by the lower pH in the very low calcium environment (7·2 *vs* 7·6, Table 1) rather than by the difference in external Ca^{2+} concentrations.

Irrespective of these differences among the groups, the overall J^{ion}_{net} and J^{H+}_{net} were lower than we have previously reported for this species (Table 2). In particular, the fish were closer to ionic equilibrium with respect to Na^+ and Cl^-, were not excreting appreciable quantities of H^+, and had a lower branchial ammonia excretion. These phenomena may be related to the absence of the dorsal aorta catheter and of repetitive blood sampling, since these were the only major differences from the previous study. If this is the case, then it is probable that the fish were under less stress than previously. The lower ammonia excretion rates (particularly in the two low Ca^{2+} environments, Fig. 2B) provide support for this conclusion, as stress causes a cortisol-mediated increase in branchial ammonia excretion in fish (Chan & Woo, 1978).

Further evidence for a lower basal stress level in this present study comes from the

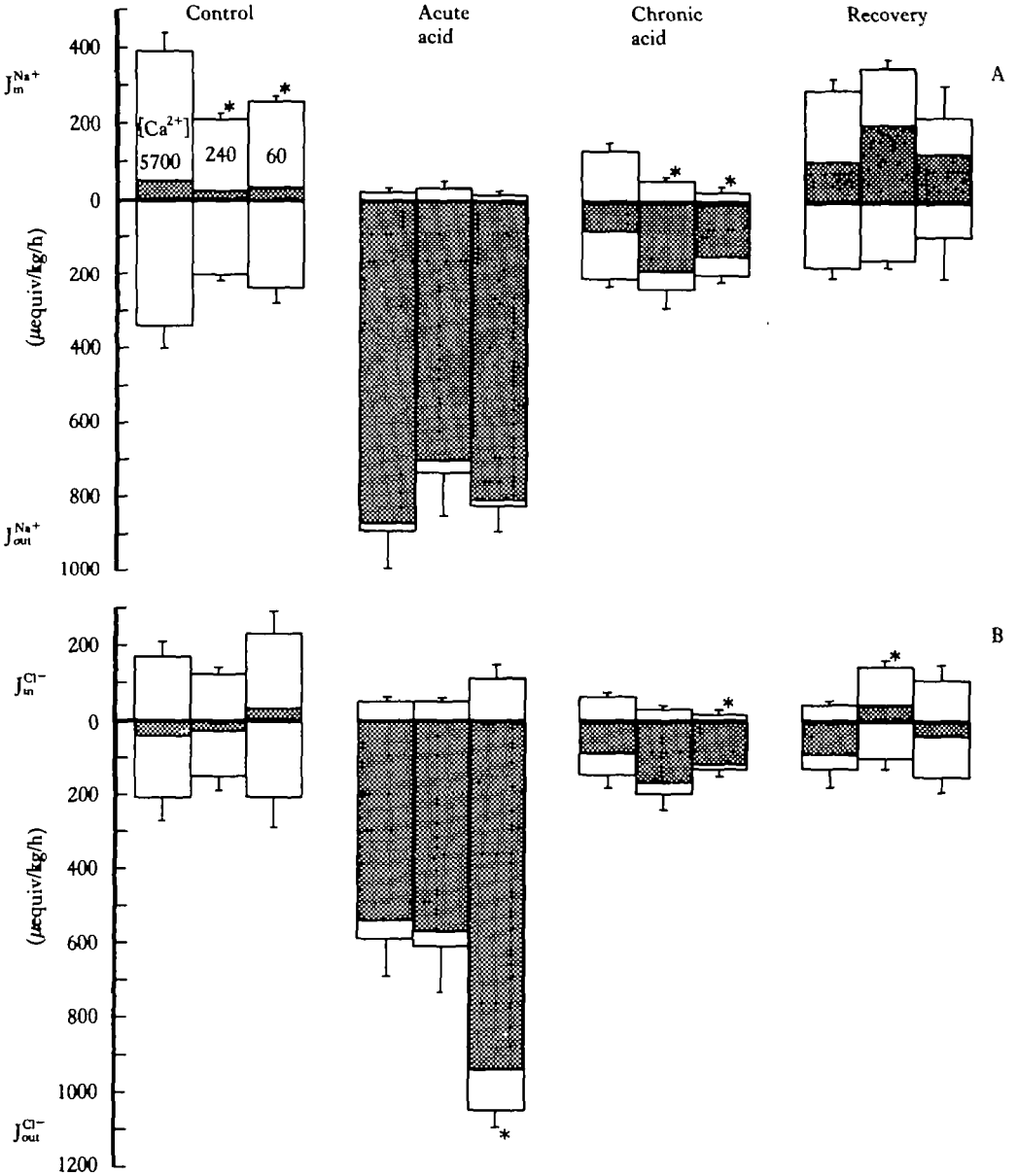

Fig. 1. (A) Sodium influx (J_{in}^{Na+}), efflux (J_{out}^{Na+}) and net flux (shaded area); (B) Chloride influx (J_{in}^{Cl-}), efflux (J_{out}^{Cl-}) and net flux (shaded area) across the gills of rainbow trout under four conditions: prior to acid exposure, *control*; during *acute acid* exposure, flux period (4 h in duration) started 1·5 h after reduction in water pH to 4·0: during *chronic acid* exposure, flux period started after 40 h of acid exposure; during *recovery*, flux period started 1·5 h after return to circumneutral pH. All flux periods were 4 h in duration, T = 16±1 °C. For each condition the values for animals acclimated to 5700, 240 and 60 μequiv/l Ca^{2+} are shown left to right. All values are means ± one S.E.M. ($N = 4$ fish, 4 hourly measurements per fish). Asterisks indicate means significantly different ($P < 0·01$) from corresponding high calcium value by '*t*' test, unpaired design.

Table 2. *Resting branchial ion and H^+ fluxes (means ± one S.E.M.) in μequiv/l in rainbow trout catheterized in the urinary bladder (N = 12) compared to animals catheterized in both the dorsal aorta and urinary bladder (N = 13, data of McDonald, 1983)*

Water pH ≈ 7·5 and T = 16 ± 1 °C for both groups.

	Present study	McDonald, 1983
J_{net}^{H+}	− 6(± 20)	−200(± 47)
J_{net}^{Amm}	−306(± 14)	−419(± 47)
J_{net}^{Na+}	+ 30(± 18)	+ 67(± 24)
J_{net}^{Cl-}	− 15(± 18)	+ 52(± 35)

much lower mortalities in response to acid exposure. This was particularly evident at the lower Ca^{2+} levels where the mortalities by 48 h of exposure at 240 μequiv/l and at 60 μequiv//l were each 17 % (2 out of 12 in each case) compared to 62 % at 230 μequiv/l and 83 % at 69 μequiv/l in dorsal aorta catheterized trout (McDonald, 1983).

Despite these differences the effects of acid exposure on branchial ion and H^+ fluxes were qualitatively similar to those observed previously (McDonald, 1983). Acid exposure prompted large net losses of Na^+ and Cl^- and a net uptake of H^+, all of which diminished with continued acid exposure (Figs 1, 2, 3). Furthermore, this same pattern was observed at all three external Ca^{2+} levels.

Acute acid exposure

During acute acid exposure (pH = 4·0, Table 1), the net losses of sodium and chloride (shaded areas, Fig. 1) arose predominantly from massive increases in passive efflux (J_{out}^{Na+} and J_{out}^{Cl-}, Fig. 1A, B). These effluxes, however, rapidly declined during the acute 4 h exposure from about 6 to about 2 times the control rates (Fig. 3A). Although this trend was evident in all animals there were differences which are attributable to external calcium. In particular, J_{out}^{Cl-} was significantly larger at the lowest calcium concentration (Fig. 1B). Furthermore, there was a significant effect of calcium on the ratio of J_{out}^{Cl-} to J_{out}^{Na+}. With decreasing Ca^{2+}, chloride diffusional losses increased relative to sodium; the efflux ratios were 0·66 ± 0·10, 0·83 ± 0·05 and 1·27 ± 1·0 respectively. External calcium, on the other hand, had no significant effect on J_{out}^{Na+}.

A pronounced inhibition of active sodium and chloride transport accompanied the large passive efflux of these ions (Fig. 1A, B). J_{in}^{Na+} was not significantly different from zero for most of the acute exposure period (Fig. 3A), whereas J_{in}^{Cl-} was reduced, on average, to about 30 % of control rates (Fig. 3A). There were no significant effects of calcium on either J_{in}^{Cl-} or J_{in}^{Na+} during this period (Fig. 1A, B).

There was also a large net uptake of H^+ across the gills (J_{net}^{H+}, Fig. 2A) at all calcium levels. This uptake declined during acute exposure but the trend was not as marked as that for passive ion efflux (Fig. 3A). Branchial ammonia excretion was relatively unaffected by acute acid exposure (Fig. 2B) although it significantly declined in high Ca^{2+} relative to control rates; slight decreases at the lower calcium levels were not significant. For the animals in high calcium the decline in J_{net}^{Amm} was

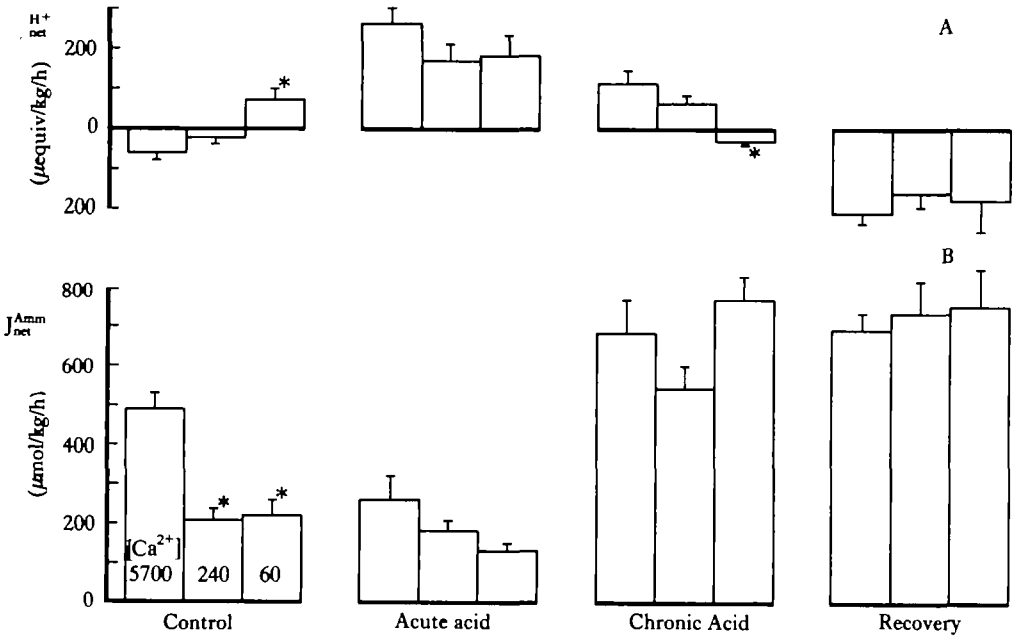

Fig. 2. (A) Net H^+ flux ($J_{net}^{H^+}$); (B) ammonia excretion (J_{net}^{Amm}) across the gills of rainbow trout under the same conditions as in Fig. 1. For each condition the values for animals acclimated to 5700, 240 and 60 μequiv/l calcium are shown left to right. All values are means ± one S.E.M. ($N = 4$ fish, 4 hourly measurements per fish). Asterisks indicate means significantly different ($P < 0{\cdot}01$) from corresponding high calcium value by unpaired 't' test.

progressive, falling from 392 ± 12 μmol/kg/h for the first hour of exposure to 242 ± 32 μmol/kg/h for the fourth hour.

Chronic acid exposure

After 40 h of acid exposure there were major changes in the patterns of ion movement across the gills. Firstly, the net losses of sodium and chloride were substantially reduced relative to acute exposure values (Fig. 1A, B). Secondly, these losses arose predominantly from the persistent inhibition of $J_{in}^{Na^+}$ and $J_{in}^{Cl^-}$ rather than diffusional efflux, which had returned in all animals to values indistinguishable from control rates. Thirdly, the effect of external calcium was no longer on ion efflux or efflux ratios ($J_{out}^{Cl^-} \div J_{out}^{Na^+}$) but rather on active transport mechanisms. At the high calcium level there was a significant recovery in $J_{in}^{Na^+}$ to about 40 % of control rates, while at the lower calcium levels $J_{in}^{Na^+}$ was not significantly changed from levels during acute exposure. $J_{in}^{Cl^-}$, on the other hand, showed no recovery at any calcium level. Indeed, it was significantly inhibited at the lowest calcium level relative to its value during acute exposure. Furthermore, $J_{in}^{Cl^-}$ at the lowest calcium level was significantly lower than its high calcium value. Thus for both sodium and chloride there were definite effects of external calcium on their transport mechanisms.

There was also a clear effect of external calcium on $J_{net}^{H^+}$ (Fig. 2A). At the high calcium level a net uptake of H^+ persisted, whereas at the lowest calcium level there was a slight, but significant, net excretion of H^+. Ammonia excretion increased by at

least 3-fold in all animals (Fig. 2B). Here, however, there was no significant effect of external calcium.

Recovery

Unlike the response to acute acid exposure, the acute response to an increase in water pH was constant over the flux period; adjustments in J_{in} and J_{out} were made immediately and remained constant thereafter (Fig. 2B). A common response was observed in all animals. There were substantial increases in $J_{in}^{Na^+}$, and smaller increases in $J_{in}^{Cl^-}$. The latter, however, were only significantly increased relative to the chronic acid exposure values in the low and very low calcium environments. $J_{out}^{Na^+}$ and $J_{out}^{Cl^-}$ were, in contrast, virtually unaffected by the restoration of a circumneutral pH. The overall result was a significant net uptake of Na^+ in all animals and some reduction in net Cl^- loss at the lower calcium levels. Ammonia excretion remained elevated and there was a substantial net H^+ excretion in all fish.

DISCUSSION

Acclimation to environmental calcium

In freshwater fish an acute reduction in external calcium causes a substantial increase in the efflux of sodium and chloride at the gills (Cuthbert & Maetz, 1972; Eddy, 1975; McWilliams, 1982). For the rainbow trout this disturbance is sufficiently prolonged that a plasma ionic imbalance results (McDonald *et al.* 1980), but eventually $J_{out}^{Na^+}$ and $J_{out}^{Cl^-}$ return to normal (Fig. 1A, B) and plasma ion levels are restored (McDonald *et al.* 1980). Wendelaar Bonga (1978) has shown that calcium reduction is accompanied by prolactin secretion and has proposed that this pituitary hormone is largely responsible for counteracting the rise in gill permeability and ionic efflux. The mechanism of action of prolactin in this circumstance is not certain but various suggestions have been made including a limitation upon diffusion due to enhanced mucus secretion, a direct restriction of diffusion channel permeability, and a stimulation of the calcium binding capacity of the gills (Bern, 1975; Wendelaar Bonga, 1978; Lahlou, 1980). Whatever the mechanism, low calcium acclimation is obviously a complex phenomenon and this means that caution must be exercised in the interpretation of the effects of Ca^{2+} and low pH upon fish. In previous studies (e.g. Eddy, 1975; McWilliams & Potts, 1978; McWilliams, 1982), fish were acutely exposed to changes in both parameters, while in the present study animals were Ca^{2+}-acclimated prior to exposure. The latter is not only more likely to occur in nature but is also likely to give qualitatively different results, particularly considering the slow time course of the adaptive response (at least 1 week for trout exposed to a 10-fold reduction in Ca^{2+}; McDonald *et al.* 1980).

Effects of low pH on sodium and chloride regulation

Much of the previous work on the effects of acid exposure on fish ionoregulatory mechanisms has involved acute studies on sodium regulation (Packer & Dunson, 1970, 1972; Maetz, 1973; McWilliams & Potts, 1978) and in this respect the present study is largely confirmatory. Acute low pH stimulated Na^+ efflux and inhibited

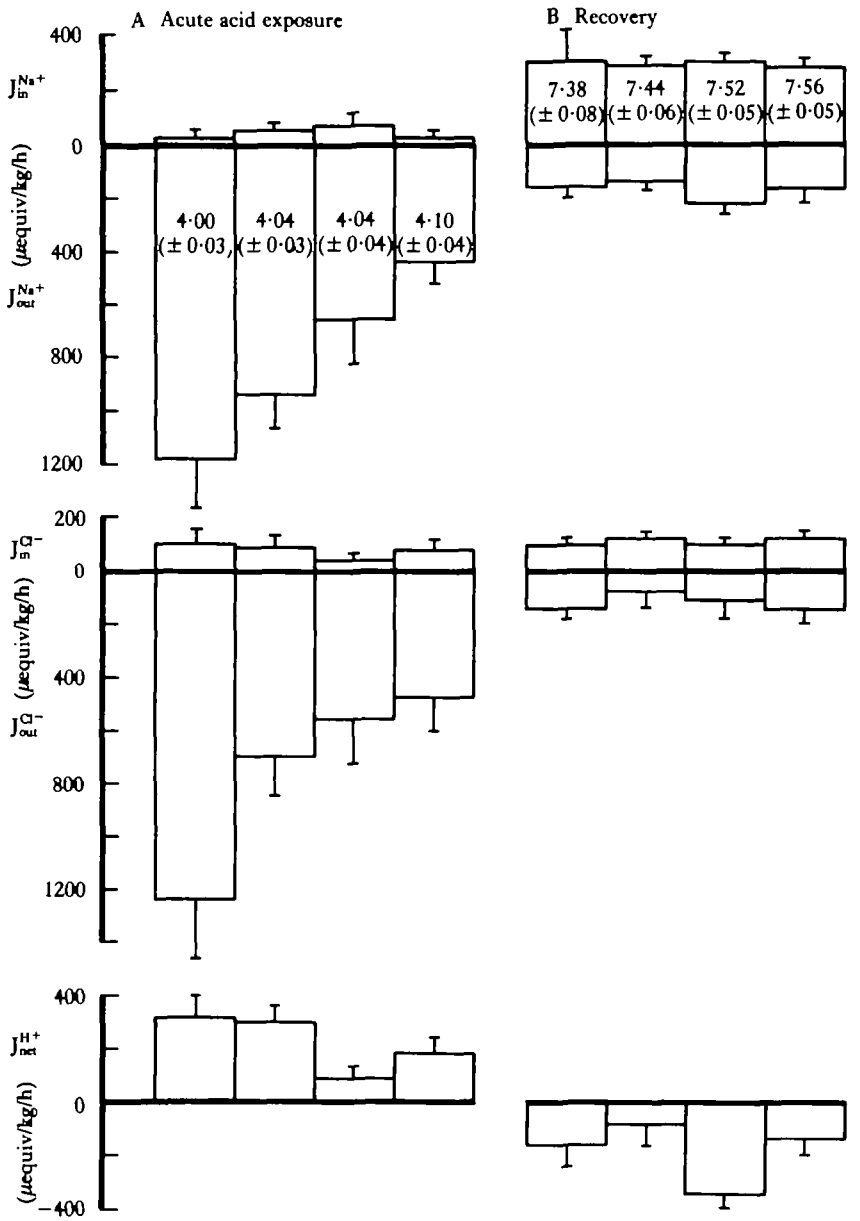

Fig 3. Hourly mean ion and H^+ fluxes (± one S.E.M., $N = 12$; pooled values from all three calcium levels) during (A) acute acid exposure and (B) recovery from acid exposure. Mean pH values (± one S.E.M.) for each hour are shown in the top panel.

influx; inhibition being complete at a pH of about 4·0. Similar studies have not been made on Cl^- regulation but by inference from studies on frog skin, Cl^- transport may be less sensitive to a decrease in pH. With this epithelium, pH 4·0 exposure either stimulated $J_{in}^{Cl^-}$ (*in vitro*, Ussing, 1949) or was without effect (*in vivo*; Garcia-Romeu, Salibian & Pezzani-Hernandez, 1969). These different responses of Na^+ and Cl^-

transport to low pH have been interpreted (Motais & Garcia-Romeu, 1972; Maetz 1973; Erlij & Ussing, 1978) by considering the apical (i.e. external) membrane to be a mosaic of negatively (cation exchange) and positively charged (anion exchange) channels. By reducing the external pH, the negative charges on the membrane channels would be titrated and the access of Na^+ to its transport mechanism would therefore be reduced. The access of Cl^-, on the other hand, would be increased. This would explain the initial differences between J_{in}^{Na+} and J_{in}^{Cl-} in the trout at pH 4·0 (Fig. 1A, B), but it is clear that the effect did not persist. After 40 h at low pH, J_{in}^{Cl-} was either equal to or less than J_{in}^{Na+} ; phenomena attributable to a further inhibition of the former and a partial recovery of the latter. Clearly then, the effect of low pH on branchial ion transport is more complex than can be explained by interference with the access of ions to their transport mechanisms (see below).

Judging from the massive increases in J_{out} with acute exposure (Fig. 1A, B), low pH increased the ionic permeability of the gills. Previous studies have indicated that the pH at which this occurred was much lower than that which inhibited influx. Maetz (1973), for example, showed a significant reduction in J_{in}^{Na+} in the goldfish at pH 6·0 but no effect on J_{out}^{Na+}. Similarly, Schoeffeniels (1956) showed a reduction in J_{in}^{Na+} across frog skin below pH 6·0 but no effect on J_{out}^{Na+} until a pH of 3·8. These observations suggest that H^+ ions affect diffusional channels that are separate from those involved in ion transport. Where are these channels and what effect does low pH have upon them?

In 'tight' epithelia (frog skin, fish gill, etc.) the transcellular pathway (i.e. through apical and basal membranes) is thought to be the major route for ion losses at circumneutral pH with, perhaps, less than 10% occurring by the paracellular pathway (i.e. via the apical tight junctions between cells, Erlij & Martinez-Palomo, 1978). Lowering the external pH has, however, been shown to open specifically the paracellular pathway to ion diffusion in frog skin *in vitro* (González, Kirchhausen, Linares & Whittenbury, 1976). To explain this phenomenon González *et al.* (1976) proposed that the paracellular channels were opened because of displacement of calcium from binding sites in the skin, particularly at the apical tight junctions and, indeed, were able to detect an efflux of calcium from the skin when external pH was lowered. A similar phenomenon may be occurring in the trout acutely exposed to low pH, as recently suggested by McWilliams (1983). If the large increase in gill sodium and chloride permeability was, in fact, due to the leaching of epithelial-bound Ca^{2+} then this would explain why external calcium had only a small effect on initial ion efflux relative to the effect of pH, i.e. low pH may be operating in a manner similar to a chelating agent (cf. Cuthbert & Maetz, 1972). The effect of acute low pH is not, however, identical to that of acute calcium removal. Under the latter circumstance, gill sodium permeability increases more than chloride permeability (Eddy, 1975; McWilliams & Potts, 1978). In the present study this was observed only at high external calcium, i.e. $J_{out}^{Na+} > J_{out}^{Cl-}$. In trout acclimated to low calcium, J_{out}^{Na+} and J_{out}^{Cl-} either increased equally (at Ca^{2+} of 240 μequiv/l; Fig. 1A, B) or J_{out}^{Cl-} increased relatively more than J_{out}^{Na+} (at Ca^{2+} of 60 μequiv/l; Fig. 1A, B). These results suggest that H^+ does more to the gill than simply displace epithelial-bound Ca^{2+} and/or that acclimation to low Ca^{2+} involves adjustments in addition to those promoting an increase in calcium-binding capacity. Whatever their interpretation, these results

To emphasize the qualitative differences likely to exist between animals acclimated to low Ca^{2+} and those that are not. For example, when brown trout were acutely exposed to both low pH *and* low calcium they became relatively more permeable to Na^+ than to Cl^-, the same response as that occurring at high Ca^{2+} (McWilliams & Potts, 1978).

A striking feature of the acid-stimulated increase in ion permeability in all fish was the speed with which it subsequently declined (Fig. 2). The basis for this rapid compensation is uncertain. One possibility is a change in the perfusion pattern of the branchial epithelium. One of the immediate responses of the trout to acid exposure would have been an increase in metabolic rate (Hargis, 1976; Ultsch, Ott & Heisler, 1980), which would have led to a recruitment of secondary lamellae and thereby promoted branchial ion losses (Wood & Randall, 1973*a*). Any subsequent reduction of ionic efflux may thus, in part, reflect a return of oxygen consumption and blood perfusion to a resting condition. Also, since diffusional ion losses can decrease while metabolic rate remains elevated (Wood & Randall, 1973*b*) the possibility of a hormonal modulation of membrane permeability cannot be ruled out. Another possibility is the progressive re-binding of calcium to the gill surfaces (as suggested by McWilliams, 1980); a phenomenon in which an elaboration of the mucous coat is likely to play an important role (Chartier, 1973). Certainly, increased mucus secretion is a well known (Westfall, 1945) and rapid response to acid exposure. For example, in the carp, *Cyprinus carpio*, a visible mucus secretion was evident within 30 min of exposure to a pH of 3·5 (Ultsch & Gros, 1979). Whatever the basis for the rapid compensation it was a quite effective adjustment as ion effluxes across the gills were indistinguishable from control rates by 40 h of acid exposure (Fig. 1A, B).

A slower and less complete compensation to acid exposure was the partial restoration of sodium transport (Fig. 1A), a phenomenon which was Ca^{2+}-dependent (Fig. 1B). The role of calcium here is uncertain but an interaction simply with the access of these ions to their respective transport mechanisms can probably be ruled out. If anything, low Ca^{2+} should have increased carrier access, since the acute effect of Ca^{2+} removal (at circumneutral pH) is a stimulation of both $J_{in}^{Na^+}$ (Cuthbert & Maetz, 1972) and $J_{in}^{Cl^-}$ (Eddy, 1975; Eddy & Bath, 1979). Thus, the interaction of chronic low pH and calcium must be directly with the transport mechanisms themselves; either with the number of transport sites or with their transport affinity. How this might be brought about is unknown and must await further information on the location (i.e. apical or basal) and nature (e.g. Ca^{2+}-dependence of transport ATPases) of the transport mechanisms in the fish gill.

Recovery

An alternative explanation for the calcium-dependent nature of active transport at low pH is that low calcium may destabilize the outer gill membranes and thus render the gills more susceptible to general epithelial damage (e.g. separation of epithelial layers and oedema similar to that occurring with heavy metal poisoning; Hughes, 1976). However, the rapid recovery of active transport at all calcium levels with return to neutral pH (Fig. 2B) indicates that such damage, if it occurred, was of a minor nature. $J_{in}^{Na^+}$, in particular, recovered in all fish to levels similar to or higher than pre-exposure values (Fig. 1A). Furthermore, it was very close to its estimated maximum for the rainbow trout (330 μequiv/kg/h; Kerstetter, Kirschner & Rafuse, 1970),

which further indicates negligible damage, at least to gill sodium transport. Chloride transport may be another matter. Here, there were smaller increases in $J_{in}^{Cl^-}$ with the return to normal pH (Fig. 1B); recovery values being about 50% lower than pre-exposure. This may not, however, reflect damage to branchial chloride transport mechanisms but rather ionic adjustments to acid-base state (see below).

Calcium effects on H^+ uptake/excretion

There is now considerable evidence (McDonald *et al.* 1980; McDonald & Wood, 1981; McDonald, 1983) that fish in low calcium have a lower rate of branchial H^+ uptake at low pH and thus develop a minimal blood acidosis. To explain this phenomenon, a passive mechanism was proposed (McDonald, 1983); namely, that the net H^+ flux was, in effect, determined by the differential permeability of the gills to sodium and chloride. An animal acclimated to low calcium would experience an increased chloride loss relative to sodium loss and would therefore face a lower passive H^+ influx. The present data provide qualified support for this hypothesis. There was an increase in $J_{out}^{Cl^-}$ relative to $J_{out}^{Na^+}$ with progressively lower calcium concentration (Fig. 1A, B) and there was a significantly lower $J_{net}^{H^+}$ at the very low external Ca^{2+} level (Fig. 2A). Unfortunately, these phenomena did not occur simultaneously as the model would predict. The former was evident only during acute low pH exposure while the latter only during chronic exposure. This does not necessarily invalidate the model, however, as measurement imprecision and the small fraction of the acid exposure period actually examined (8 out of 44 h) may have obscured real differences. Certainly, when total fluxes over 4 days of acid exposure were examined (Fig. 5 of McDonald, 1983) the total sodium and chloride net losses were more nearly equimolar and the net H^+ uptake much smaller in low *vs* high Ca^{2+} acclimated animals. In any case, the present results quite clearly rule out active H^+ excretion as limiting $J_{net}^{H^+}$ at low Ca^{2+} at least to the extent that this might involve the coupling of acid excretion to active sodium transport, i.e. Na^+/H^+ and Na^+/NH_4^+ exchange mechanisms (Maetz, Payan & de Renzis, 1976). Instead, the reverse situation was apparent. During chronic low pH exposure, the change in $J_{net}^{H^+}$ from a net uptake to a net excretion with decreasing water calcium was correlated with a decrease rather than an increase in $J_{in}^{Na^+}$ (Figs 1A, 2A).

H^+ excretion during recovery may be another matter. Here, there was a large net excretion of H^+, significantly larger than prior to acid exposure. This was correlated with a substantial $J_{in}^{Na^+}$ relative to $J_{in}^{Cl^-}$; a result which would be predicted if putative Na^+/H^+ and Cl^-/HCO_3^- exchange mechanisms (Maetz *et al.* 1976; Girard & Payan, 1980) were being manipulated to correct a blood acidosis. Internal acid-base disturbances have been shown to provoke alterations in the relative intensities of sodium and chloride exchanges (de Renzis & Maetz, 1973; Cameron, 1976) and, in fact, may be more important stimuli to these alterations than disturbances to internal ion concentrations (de Renzis & Maetz, 1973). It is interesting to note in this regard that $J_{in}^{Cl^-}$ was higher in low *vs* high calcium during recovery (Fig. 1B). If, as expected, these animals had developed a smaller blood acidosis than animals in high calcium (McDonald *et al.* 1980), then there would have been less stimulus to the modulation of $J_{in}^{Na^+}$ relative to $J_{in}^{Cl^-}$.

In conclusion we are now able to propose a mechanism for the ionic imbalances in

fish that arise from exposure to acidified hard and soft waters. We propose that upon low pH exposure there is an immediate and pronounced increase in sodium and chloride diffusion via the paracellular pathway. This may be caused in part, by a displacement of membrane-bound Ca^{2+}. External calcium has relatively little influence upon this initial response except to somewhat moderate Cl^- loss relative to Na^+ loss (Fig. 1A, B). Rapidly thereafter the gill permeability to Na^+ and Cl^- is reduced, irrespective of calcium concentration, by mechanism(s) as yet unknown, until normal diffusional loss rates are achieved (Figs 1, 3). This suggests that the more severe ionic disturbances which develop in chronically acid exposed fish from low *vs* high calcium environments (McDonald *et al.* 1980; Leivestad *et al.* 1980; McDonald, 1983) are more likely to arise from the relatively greater inhibition of uptake apparent in these animals (Fig. 1) than from elevated diffusional losses. The precise nature of this interaction of calcium with active transport mechanisms in the gills requires further study but the implication of these findings to natural fish populations is clear. At pH levels of 4·0 and above the principal cause of adult fish mortalities is ionic imbalances [see Wood & McDonald (1982) for review]. Thus, the more moderate the acid exposure, the longer will be the resistance of the population to acid stress and the more likely that ionic imbalances will arise from an inhibition of ionic uptake. Since it is here that calcium exerts its greatest effect, even apparently minor differences in water-borne calcium may spell the difference between survival and eventual extinction of fish populations in the wild.

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