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WHOLE BODY CALCIUM FLUX RATES IN FRESHWATER TELEOSTS AS A FUNCTION OF AMBIENT CALCIUM AND pH LEVELS: A COMPARISON BETWEEN THE EURYHALINE TROUT, *SALMO GAIRDNERI* AND STENOHALINE BULLHEAD, *ICTALURUS NEBULOSUS*

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SUMMARY

The exchange of calcium between the blood and external medium following intravascular injection of ^{45}Ca was investigated in adult freshwater rainbow trout (*Salmo gairdneri*) acclimated to ambient $[Ca^{2+}]$ of 0·18, 0·65 and 5·0 mequiv l^{-1}, and in bullheads (*Ictalurus nebulosus*) at external $[Ca^{2+}]$ of 0·17 mequiv l^{-1}, each at near-neutral ambient pH, 11–12 °C and constant Na^+ and Cl^- levels of 0·06 and 0·03 mequiv l^{-1}, respectively.

The dispersal volume of isotope exhibited a slow exponential increase with 8–9 h required for 95 % of the radiospace to be filled, irrespective of the acclimation medium or fish species. Equilibrium radiospaces in trout (range 1600–2000 ml kg^{-1}) were independent of ambient $[Ca^{2+}]$ but 1·4-fold higher than in bullheads. In all cases, whole body exchangeable calcium content was low, representing ≈3 % of total body calcium content. The whole body calcium exchange rates (mean ranges; influx or efflux) in trout of 7–22 μequiv kg^{-1} h^{-1} were largely independent of ambient Ca^{2+} levels; lower values of 2–10 μequiv kg^{-1} h^{-1} were found in bullheads.

Acute exposure (24 h) to low ambient pH 4·0–4·2 at external $[Ca^{2+}] \simeq$ 0·18 mequiv l^{-1} resulted in whole body ion loss in both species. Net Ca^{2+} losses in trout were due to a reduction in influx whereas both increased efflux and depressed influx accounted for the response in bullheads. These changes were transitory (12 h) and minor in comparison with concomitant net Na^+ and Cl^- losses which unlike Ca^{2+}, exhibited no recovery within 24 h of acid exposure.

Possible mechanisms of Ca^{2+} regulation in freshwater fish are discussed.

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Key words: Fish, calcium, radiospaces, unidirectional exchanges, low pH.

INTRODUCTION

Calcium ions play a vital role in several physiological phenomena including membrane stability, muscular contraction, hormonal release and cellular structure/composition (see papers in Duncan, 1976). In higher vertebrates, calcium homeostasis is achieved mainly by a balance between absorption in the intestine and excretion by the kidney (Urist, 1976). Fish, on the other hand, are in continual intimate contact with the external medium wherein calcium concentrations may range from 0·01 (dilute fresh water) to 20 mequiv l^{-1} (sea water) and therefore, face unfavourable gradients for calcium movement either into or out of the body. Radiotracer studies using isolated perfused fish gill and head preparations have implicated the gill as the major site of calcium transport (Milhaud, Rankin, Bolis & Benson, 1977; Milet, Peignoux-Deville & Martelly, 1979; Payan, Mayer-Gostan & Pang, 1981; Mugiya & Ichii, 1981). However, few attempts have been made to measure unidirectional transfer rates of calcium (influx and efflux) in intact fish, particularly freshwater forms. More commonly, fish have been exposed to ^{45}Ca-labelled water for various lengths of time (hours to weeks) from which whole body accumulation and distribution of isotope in various body compartments (e.g. muscle, bone, scales, fins) have been determined (see reviews by Fleming, 1968; Simmons, 1971; Simkiss, 1974; Dacke, 1979). Notable exceptions include studies by Pang, Griffith, Maetz & Pic (1980) and Mayer-Gostan *et al.* (1983) in which calcium influx rates were measured in euryhaline marine fish species either maintained in sea water and/or transferred to fresh water. Furthermore, no estimates have been made of the apparent volume of the fish body compartment through which calcium is dispersed and from which exchange with the external medium occurs.

The present investigation was therefore designed to determine the kinetics and dimensions of calcium spaces together with whole body influx, efflux and net flux rates of calcium following intravascular injection of ^{45}Ca into the euryhaline teleost, *Salmo gairdneri* (rainbow trout), acclimated to natural soft water containing 0·18, 0·65 and 5·0 mequiv l^{-1} of calcium at near-neutral ambient pH. Since whole body net calcium loss has been demonstrated in the stenohaline freshwater fish, *Catostomus commersoni* (white sucker), during acute exposure to ambient pH 4·3 in natural soft water ($[Ca^{2+}] = 0·19$ mequiv l^{-1}; Hōbe, Wood & McMahon, 1984), a secondary aim was to establish the extent of this response (and the mechanisms involved) in acid-exposed rainbow trout and in another stenohaline freshwater species, *Ictalurus nebulosus* (brown bullhead), both acclimated to low calcium media.

MATERIALS AND METHODS

This study was conducted during April–June 1983 at the Centre National de la Recherche Scientifique (CNRS) in Strasbourg, France.

Experimental animals and media

Adult rainbow trout (*Salmo gairdneri*) were obtained from a natural soft water (see Table 1) hatchery (Hans Pisciculture), located in the Vosges Mountains, Soultzeren, France and immediately placed into acclimation tanks (see below). Adult bullheads

(*Ictalurus nebulosus*) were obtained from a hard water pond (Etang du Dett; Chaumont Haute Marne, France) and maintained in laboratory tap water (see Table 1) for months before use. Fish were not fed in the acclimation tanks or during experimentation.

Natural soft water (see Table 1), collected 2–3 times weekly from the inflow stream of Lac du Forlet (Vosges Mountains, altitude 1100 m, Soultzeren, France) was used for both acclimation and experimentation. The desired $[Ca^{2+}]$ was achieved by slowly dissolving solid calcium sulphate in natural soft water so that $[Na^+]$ and $[Cl^-]$ remained constant. Fish were acclimated in recirculating reservoirs equipped with gravel-based biological filters. Trout were acclimated to high or intermediate calcium media in 90-l Plexiglas tanks (5–10 l per 100 g fish) while both trout and bullhead were kept in low calcium conditions in a 700-l PVC tank (10 l per 100 g fish). Water samples were collected daily and analysed for $[Na^+]$, $[Cl^-]$ and $[Ca^{2+}]$ (Table 1). In view of the low buffering capacity of Vosges Mountain water (alkalinity $\simeq$60–100 μequiv l^{-1}), each tank was replenished every 2 days so that ammonia levels did not exceed 200 μmol l^{-1}. Acidified low Ca^{2+} water was prepared in 60-l reservoirs by titration to pH $\simeq$ 4·0 with H_2SO_4 and vigorous aeration for 24 h to remove CO_2.

After 10–14 days of acclimation, fish were anaesthetized (MS 222; tricaine methanesulphonate; 1:10 000) with aerated neutral water (1·0 N-KOH added to maintain ambient pH $\simeq$ 7·0) of appropriate ion composition, for surgical implantation of catheters into either the dorsal aorta through the roof of the buccal cavity (DA; trout) or the caudal artery *via* the caudal peduncle (CA; bullhead) using a modification of the method of Soivio, Nyholm & Westman (1975) and a trocar catheter designed by B. Vincent (CNRS, personal communication). In each case, a 70–80 cm long catheter (with a tapered tip) was prepared by heating and pulling gas-impermeable PVC tubing (o.d. = 11 mm) to the appropriate diameter (o.d. = 1 mm). The sharpened point of a stainless steel 8-cm long trocar was inserted through the wall of the catheter, about 4 cm from the tip, as illustrated in Fig. 1. A 5-mm length of silastic tubing formed a seal at the point of trocar entry into the catheter. The catheter was filled with heparinized saline (50 i.u. ml^{-1}). Once the trocar penetrated the blood vessel, it was removed and the cannula fed about 3 cm into the DA and 5 cm into the CA. No blood loss occurred during this procedure and cannulations were always successful.

During recovery from surgery (48–60 h) and experimentation, trout were confined individually in Lucite boxes (28 cm×5 cm×9 cm; 1·3 l) which were contained in

Table 1. *Concentrations of ions in various water systems*

Type	$[Ca^{2+}]$	$[Na^+]$ (mequiv l^{-1})	$[Cl^-]$
Laboratory tap water	4·1	1·15	0·54
Vosges mountain stream (spring)	0·04	0·04	0·02
Soultzeren hatchery	0·12	0·09	0·05
Acclimation tanks*			
High calcium	4·8 (4·6–5·0)	0·05 (0·04–0·06)	0·03 (0·02–0·04)
Intermediate calcium	0·66 (0·59–0·72)	0·07 (0·06–0·08)	0·02 (0·01–0·03)
Low calcium	0·15 (0·12–0·18)	0·06 (0·04–0·08)	0·03 (0·02–0·04)

* Mean values and ranges in the experimental tanks during the acclimation period.

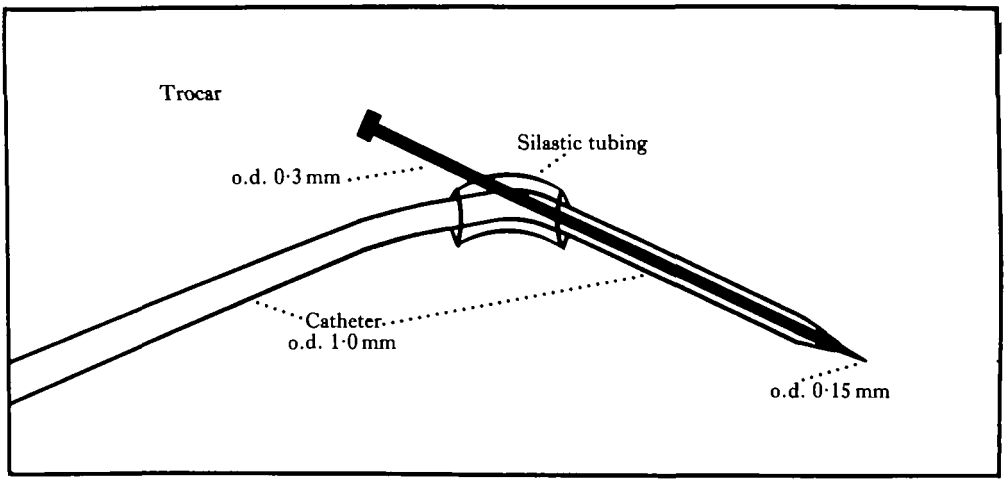

Fig. 1. A schematic diagram of the trocar catheter used for cannulating the dorsal aorta in *Salmo gairdneri* and caudal artery in *Ictalurus nebulosus* (designed by B. Vincent; personal communication).

larger PVC flux chambers (40 cm×8 cm×30 cm; 9·6 l); an air-lift circulated water through the Lucite box while airlines at the circumference of the larger chamber provided mixing and aeration (cf. McDonald, 1983). Bullheads were maintained individually in the larger chambers because of their docile behaviour.

All aspects of this investigation were conducted in a temperature-regulated cold room (11–12 °C).

Experimental protocol

Four experimental series were performed. The first three studied the effects of ambient $[Ca^{2+}]$ on unidirectional flux rates of Ca^{2+} in rainbow trout at ambient pH 6·8–7·0. Whole body rather than separate branchial and renal ion fluxes were measured in order to avoid stress induced by urinary cannulation (Hōbe *et al.* 1984). Trout were monitored in high $[Ca^{2+}] = 4{\cdot}95$ mequiv l^{-1} (series 1: mean wt = 200·9 ± 3·6 g; $N = 7$), intermediate $[Ca^{2+}] = 0{\cdot}65$ mequiv l^{-1} (series 2: mean wt = 61·3 ± 14·6 g; $N = 4$) and low $[Ca^{2+}] = 0{\cdot}18$ mequiv l^{-1} (series 3: mean wt = 204·0 ± 4·7 g; $N = 8$). The latter group were subsequently exposed to ambient pH 4·0–4·2 for 24 h. The fourth series examined Ca^{2+} flux rates in low Ca^{2+}-acclimated bullheads ($[Ca^{2+}] = 0{\cdot}17$ mequiv l^{-1}; mean wt = 134·6 ± 12·5 g; $N = 8$) at near-neutral ambient pH (control) and during 24 h of acid exposure, for comparison with data on trout (series 3).

The following sampling regime was used. Once each chamber was thoroughly flushed (40 l per chamber) with water of appropriate composition (see Table 2) and adjusted to set levels (5 l) by drainage, a dose of ≃160 μCi ^{45}Ca (as calcium chloride; New England Nuclear) per ml saline per kg fish was infused *via* either the DA (trout) or CA catheter (bullhead) over 1 min, followed by an additional 2 ml saline kg^{-1} which ensured that no radioactivity remained in the catheter. Each pre-injection (0 h) and post-injection sample (2 h intervals for 12 h) of blood (150–250 μl) and water (40–50 ml), analysed for ^{45}Ca radioactivity and total $[Ca^{2+}]$, was taken with a fresh disposable plastic syringe to avoid contamination. An equal volume of saline was also

injected at each sampling time to replace blood volume lost. Water [Na^+] and [Cl^-] were measured in the 0 h, 6 h and 12 h samples.

Before switching to ambient pH 4·0–4·2 in series 3 and 4, the volume of experimental water (near-neutral ambient pH) in each chamber was increased to 8–10 l for another 12 h period (no measurements). Each chamber was then flushed for 15 min with acidified water, set to 5 l and a second dose of ^{45}Ca ($\simeq$160 μCi) was injected so that the specific activity of plasma remained at least 25-fold greater than that in the external medium. Data were collected for 12 h and analysed as described above. For the second 12 h of acid exposure, the water volume in each chamber was again increased by 3–4 l to dilute accumulated ammonia. Post-injection samples of plasma and water were then taken at 18 h and 24 h. Measured amounts (10–30 ml) of 0·02 N-H_2SO_4 were added to each chamber over the 24 h, as required, to maintain water pH.

Analytical techniques and calculations

Cation levels (Na^+, Ca^{2+}) in water and plasma were measured by atomic absorption spectrophotometry (Perkin Elmer Model 2380) after appropriate swamping and dilution. Water Cl^- levels were assessed by coulometric titration (American Instrument Co. Inc. No. 951) using a NaCl-spiked acid reagent (see Hōbe *et al.* 1984). Water pH was monitored manually (every hour) with a pH electrode (Ingold) and Radiometer digital readout (PHM 64).

Aliquots of ^{45}Ca injection stock (20 μl in triplicate), plasma (20 μl in duplicate) and water (5 ml in duplicate) were added to 10 ml scintillation fluid (Aquasol; New England Nuclear) and ^{45}Ca radioactivity was measured by liquid scintillation spectroscopy (Intertechnique, Model 30). To establish steady-state distribution time of ^{45}Ca, radiospaces were calculated for each sampling time from the ratio of total radiocalcium in the fish (in c.p.m.) relative to radioactivity per ml plasma (C_p) (Mayer & Nibelle, 1969), according to the equation:

$$\text{Radiospace} = \frac{(\text{Total dose } ^{45}Ca \text{ injected}) - (\text{Total } ^{45}Ca \text{ lost to water and removed by sampling})}{C_p \times W},$$

where W is the fish weight in kg. Whole body Ca^{2+} efflux rates (J_{out}^{Ca}) were measured over each 2-h interval flux period, using the equation:

$$J_{out}^{Ca} = \frac{(C_i - C_f)_w \times V_w}{SA_p \times (t_f - t_i) \times W},$$

where $(C_i - C_f)_w$ represents the change in radioactivity (c.p.m. ml^{-1}) in the external medium and SA_p, the average specific activity in plasma (c.p.m. μequiv μequiv^{-1}), from the beginning (i) to the end (f) of each flux period ($t_f - t_i$ in hours), and V_w is average experimental water volume in ml. Backflux corrections were not necessary since the external specific activity in water never exceeded more than 4 % of the values in plasma. Net fluxes of ions (e.g. Ca^{2+}) were estimated from measured concentrations in water, according to the equation:

$$J_{net}^{Ca} = \frac{[Ca^{2+}]_i - [Ca^{2+}]_f \times V_w}{(t_f - t_i) \times W}.$$

Unidirectional Ca^{2+} influxes (J_{in}^{Ca}) were then calculated using the conservation equation:

$$J_{in}=J_{net}+J_{out}.$$

Whole body exchangeable Ca^{2+} content was estimated as the product of total plasma concentration (assuming ^{45}Ca equally distributed between bound and free calcium) and calculated radiospaces (Bath & Eddy, 1979), once steady-state ^{45}Ca distribution had been achieved.

Data have been expressed throughout as means (±S.E.; N) unless otherwise stated. Differences between means in each series were tested using a Student's two-tailed t-test (unpaired design). Time-dependent radiospaces, calcium fluxes and responses to acid exposure were evaluated using a repeated measures analysis of variance and Dunnett t-statistic, as outlined in Höbe *et al.* (1983). A significance level of $P < 0{\cdot}05$ was used.

RESULTS

Near-neutral ambient pH

Salmo gairdneri, during acclimation at slightly lower ambient $[Na^+]$ and $[Cl^-]$ than the water from which they were obtained (Table 1), typically experienced net body losses of Na^+ and Cl^- over the initial 24 h of acclimation (data not shown). After 10–14 days, however, whole body J_{net}^{Na} and J_{net}^{Cl} (two 6-h flux periods averaged) were not significantly different from zero in trout kept in ambient $[Ca^{2+}]$ of 5·0 (series 1), 0·65 (series 2) or 0·18 (series 3) mequiv l^{-1} (Table 2). Similarly, *Ictalurus nebulosus*, maintained in CNRS tap water (Table 1) and then transferred to 25-fold lower $[Na^+]$ and 30-fold lower $[Cl^-]$, also exhibited a transitory net loss of Na^+ and Cl^-, but by 10–14 days acclimation, J_{net}^{Na} and J_{net}^{Cl} were again not significantly different from zero (Table 2). Estimates of whole body J_{net}^{Ca} were limited by analytical sensitivity such that flux intervals of less than 6 h and 12 h could not be reliably detected at ambient $[Ca^{2+}]$ of 0·18 and 0·65 (or 5·0) mequiv l^{-1}, respectively. Mean values given in Table 2 for trout at all three ambient calcium levels were not significantly different from zero whereas low Ca-acclimated bullheads (series 4) were in net positive balance with J_{net}^{Ca} significantly above zero.

Table 2. *Whole body net ion fluxes in* Salmo gairdneri *and* Ictalurus nebulosus *in each test media at near-neutral ambient pH*

Fish	Series no.	Test media $[Ca^{2+}]$	Test media $[Na^+]$ (mequiv l^{-1})	Test media $[Cl^-]$	N	Net ion fluxes J_{net}^{Ca}	Net ion fluxes J_{net}^{Na} (μequiv kg^{-1} h^{-1})	Net ion fluxes J_{net}^{Cl}
Trout	1	4·95	0·058	0·029	7	0·00 ± 0·00	+ 0·06 ± 8·11	+ 3·47 ± 3·94
	2	0·65	0·058	0·029	4	−7·26 ± 4·64	−14·79 ± 7·45	−15·36 ± 7·40
	3	0·18	0·044	0·022	8	−3·20 ± 2·32	+ 7·06 ± 8·76	+ 2·92 ± 10·61
Bullhead	4	0·17	0·057	0·025	8	+7·12 ± 1·59	− 4·13 ± 7·39	−14·56 ± 7·94

Values are expressed as means ± S.E. See text for details.

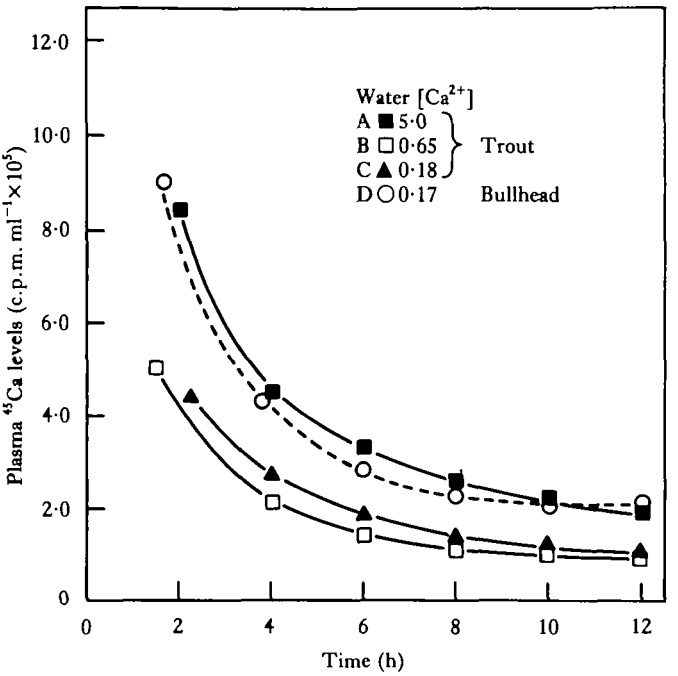

Fig. 2. Plasma ^{45}Ca concentrations as a function of time following single intravascular injections of isotope. Values are means for *Salmo gairdneri* at ambient calcium levels of 5·0 (A; $N = 5$), 0·65 (B; $N = 4$) and 0·18 mequiv l^{-1} (C; $N = 8$) and for *Ictalurus nebulosus* at 0·17 mequiv l^{-1} (D; $N = 8$).

Mean plasma isotope concentrations as a function of time following single injections of ^{45}Ca into the arterial blood of either trout or bullheads are illustrated in Fig. 2. About 5–7 % of the ^{45}Ca dose was retained in plasma at ≈2 h post-injection irrespective of the fish species or ambient [Ca^{2+}]. The exponential decline in plasma radioactivity probably corresponded mainly to the dispersal of ^{45}Ca throughout the extracellular and intracellular compartments since the observed exchanges between the fish and the external medium were comparatively small (see below). The relative position of each curve simply reflected slight differences between fish in both the absolute quantity of isotope injected and body weights.

The evolution of calcium radiospace as a function of time in each experimental series is plotted in Fig. 3; for the sake of clarity, mean values without standard errors are illustrated. The apparent distribution kinetics in both trout (Fig. 3A,B,C) and bullheads (Fig. 3D) followed an exponential curve. Ninety-five per cent of the distribution volume was reached by trout in ≈9 h compared with 8 h in bullheads. Equilibrium radiospaces (10 h and 12 h samples averaged), defined as the apparent volume occupied by exchangeable calcium when uniformly distributed at the same concentration as that of plasma (Mayer & Nibelle, 1969; a theoretical approximation), in trout maintained in the three ambient calcium regimes were not significantly different but 1·4-fold higher than those in bullheads (Table 3). Samples taken at 24 h post-injection in low-Ca trout (series 3) and bullheads (series 4) gave radiospaces which were also not significantly different from those given in Table 3 (data not shown), confirming that isotopic equilibrium had been achieved as early as 10–12 h

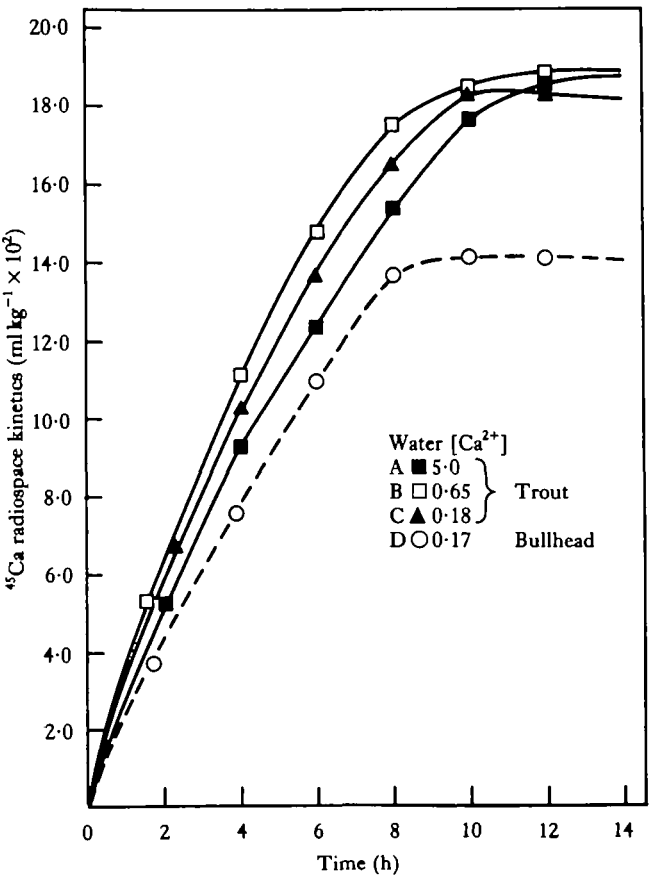

Fig. 3. Evolution of calcium radiospaces as a function of time in *Salmo gairdneri* at ambient calcium levels of 5·0 (A; $N = 5$), 0·65 (B; $N = 4$) and 0·18 mequiv l^{-1} (C; $N = 8$) and in *Ictalurus nebulosus* at 0·17 mequiv l^{-1} (D; $N = 8$). For the sake of clarity, mean values without standard errors have been plotted.

Table 3. *Plasma calcium levels, radiospaces, and whole body exchangeable calcium content in* Salmo gairdneri *and* Ictalurus nebulosus *in each test media at near-neutral ambient pH*

Fish	Ambient [Ca^{2+}] (mequiv l^{-1})	Plasma [Ca^{2+}] (mequiv l^{-1})	Radiospaces (ml kg^{-1})	Exchangeable Ca^{2+} (mequiv kg^{-1})
Trout	4·95	3·96 ± 0·10 (7)	1809·1 ± 103·5 (5)	7·09 ± 0·24 (5)
	0·65	3·39 ± 0·24 (4)	1863·8 ± 75·4 (4)	6·01 ± 0·25 (4)
	0·18	3·23 ± 0·14 (8)	1823·0 ± 72·4 (8)	5·98 ± 0·27 (8)
Bullhead	0·17	2·88 ± 0·14 (8)	1405·5 ± 72·3 (8)	4·37 ± 0·12 (8)

Values are expressed as means ± S.E. (N). See text for details.

post-injection. A comparison of plasma calcium levels (six samples taken over 12 h averaged) in fish of each series revealed marginally but significantly elevated values in high-Ca trout (Table 3). Whole body exchangeable Ca^{2+} contents were lowest in low-Ca bullheads (Table 3).

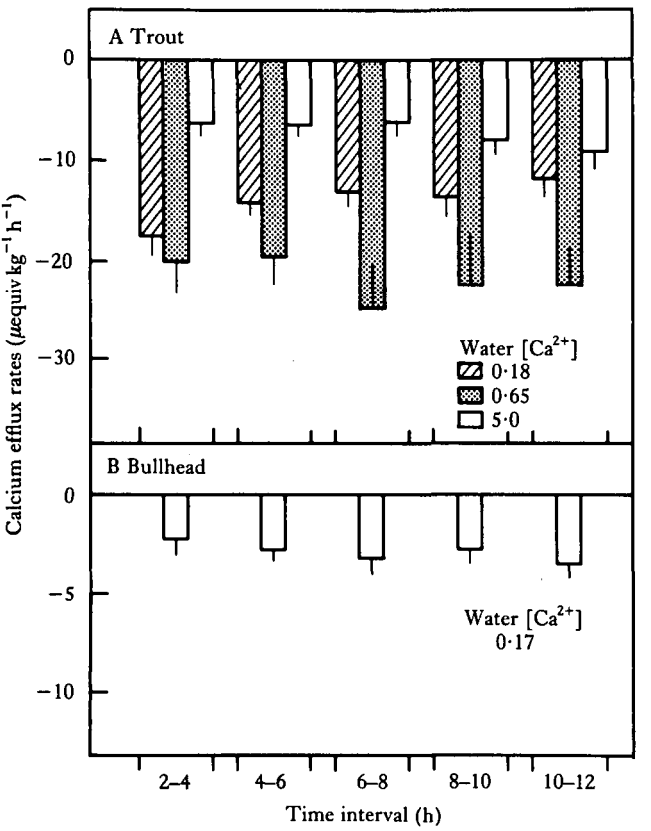

Fig. 4. Whole body efflux rates of calcium at near-neutral ambient pH and 11–12 °C in *Salmo gairdneri* acclimated to three ambient calcium levels (A) and *Ictalurus nebulosus* at $[Ca^{2+}]$ = 0·17 mequiv l^{-1} (B). Values are means ± S.E. for J_{out}^{Ca} calculated from the appearance of ^{45}Ca in the external medium relative to the average specific activity of ^{45}Ca in plasma at each 2-h sampling interval.

The rate of appearance of injected isotope in the external medium was logarithmic over the 12-h measurement period (data not shown) and thus yielded relatively constant whole body J_{out}^{Ca} rates determined at 2-h intervals in both trout (at each ambient calcium regime; Fig. 4A) and bullhead (low-Ca medium; Fig. 4B). However, the absolute magnitude of mean J_{out}^{Ca} (five 2-h flux periods averaged) varied slightly but significantly; the lowest values were seen in bullheads (−2·88 ± 0·55 μequiv kg^{-1} h^{-1}; $N = 8$) compared with rates in trout ranging from −7·08 ± 1·18 ($N = 5$), −13·80 ± 1·59 ($N = 8$) and −22·9 ± 4·15 ($N = 4$) μequiv kg^{-1} h^{-1} in ambient $[Ca^{2+}]$ of 5·0, 0·18 and 0·65 mequiv l^{-1}, respectively.

Whole body J_{in}^{Ca} were then calculated from measured J_{net}^{Ca} (Table 2) and J_{out}^{Ca} (Fig. 4; five 2-h flux periods averaged). Values for trout at ambient $[Ca^{2+}]$ of 0·18 and 0·65 mequiv l^{-1} were 10·60 ± 2·56 ($N = 8$) and 15·03 ± 4·01 ($N = 4$) μequiv kg^{-1}, respectively; these were not significantly different from each other or from those in bullheads at $[Ca^{2+}]$ = 0·17 mequiv l^{-1} (10·01 ± 1·41 μequiv kg^{-1} h^{-1}; $N = 8$). J_{in}^{Ca} could not be calculated in trout at $[Ca^{2+}]$ = 5·0 mequiv l^{-1} due to analytical problems in J_{net}^{Ca} estimation (discussed above). Thus, two methods were used to measure J_{in}^{Ca}

directly. About 35 μCi of ^{45}Ca was added to 5 l of high-Ca water in individual experimental chambers and J_{in}^{Ca} values determined from (1) the disappearance of radioactivity from the external medium over 12 h (2-h interval samples; methodology in Hōbe *et al.* 1984) and (2) the amount of radioactivity in the fish at 12 h (terminal plasma c.p.m. ml^{-1} times radiospace of 1810 ml kg^{-1} from Table 3) relative to the average specific activity of ^{45}Ca in the experimental water over 12 h (Maetz, 1956). For two uncannulated trout, the disappearance of radioactivity from the water over 12 h yielded a mean $J_{in}^{Ca} = 91{\cdot}00 \pm 2{\cdot}13$ μequiv kg^{-1} h^{-1}. However, in the absence of a fish (one empty chamber), ^{45}Ca disappeared from the medium at a considerable rate ($\simeq 6{\cdot}81$ c.p.m. ml^{-1} h^{-1}). Taking this large (55 %) chamber adsorption into consideration, a mean fish $J_{in}^{Ca} = 40{\cdot}26 \pm 2{\cdot}28$ μequiv kg^{-1} h^{-1} was obtained. When this value was compared with the $J_{in}^{Ca} = 15{\cdot}35 \pm 0{\cdot}49$ determined from the amount of isotope accumulated in the fish, it was still too high, indicating that significant

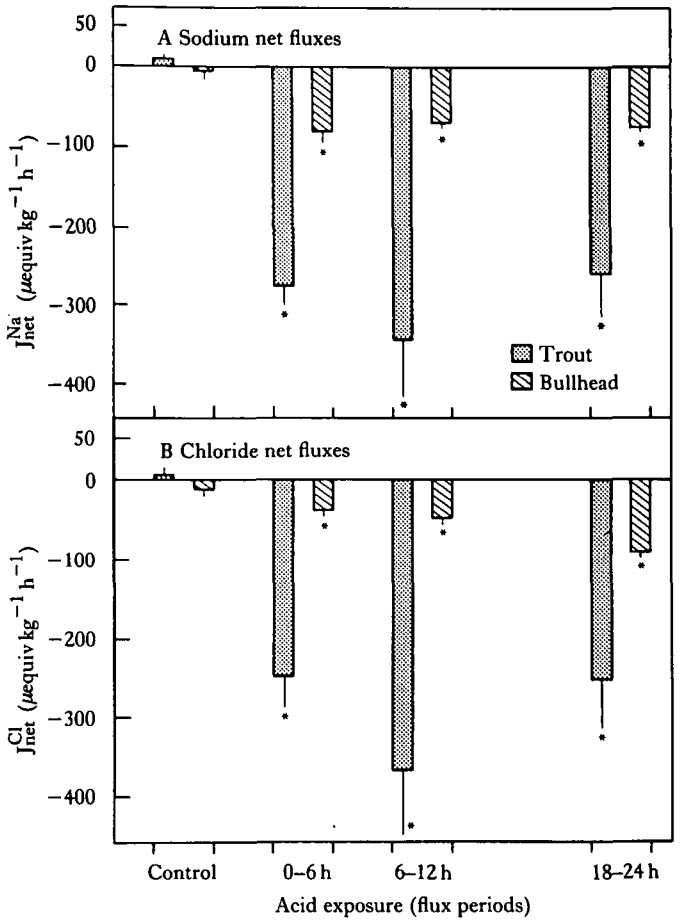

Fig. 5. Whole body net sodium (A) and chloride (B) flux rates in low calcium-acclimated *Salmo gairdneri* ($N = 8$) and *Ictalurus nebulosus* ($N = 8$) prior to (control; two 6-h flux periods averaged) and during 24 h (flux periods of 0–6 h, 6–12 h, 18–24 h) of acid exposure (ambient pH 4·0–4·2 at 11–12 °C). Values are means ± S.E. Asterisks represent significance ($P < 0{\cdot}05$) obtained by a within-group comparison of means.

amounts of isotope also adhered to the surface of the fish. Relative to measured values of J_{out}^{Ca} in high-Ca trout (Fig. 4A), the latter mean J_{in}^{Ca} value seemed most reasonable. Thus, J_{in}^{Ca} values in trout in each of the ambient calcium regimes were not significantly different.

Low ambient pH

Both trout and bullheads acclimated to low-Ca media survived 24 h exposure to low ambient pH 4·0–4·2.

Acid exposure resulted in significant whole body net losses of both Na^+ and Cl^- (Fig. 5). Over the initial 6 h, both J_{net}^{Na} (Fig. 5A) and J_{net}^{Cl} (Fig. 5B) in trout became highly negative at rates of $-275{\cdot}96 \pm 28{\cdot}10$ ($N = 8$) and $-247{\cdot}01 \pm 36{\cdot}05$ (8) μequiv kg^{-1} h^{-1}, respectively; this pattern of equimolar Na^+ and Cl^- losses persisted over the remaining period with no evidence of either J_{net}^{Na} or J_{net}^{Cl} recovery. In bullheads, however, both the magnitude and the pattern of Na^+ and Cl^- losses were different. Over the initial 6 h, J_{net}^{Na} was only $-79{\cdot}99 \pm 8{\cdot}21$ (8) μequiv kg^{-1} h^{-1}, remaining at this rate throughout the 24 h of acid exposure (Fig. 5A). J_{net}^{Cl}, on the other hand, exhibited

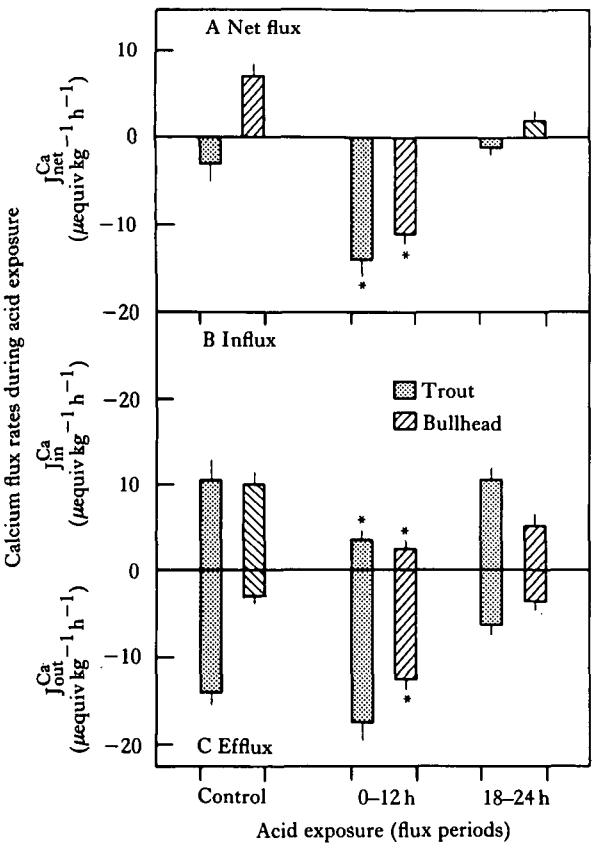

Fig. 6. Whole body flux rates of calcium in low calcium-acclimated *Salmo gairdneri* ($N = 8$) and *Ictalurus nebulosus* ($N = 8$) prior to (control) and over 12 h and 18–24 h of acid exposure (ambient pH 4·0–4·2) at 11–12 °C. Values are means ± S.E. for calcium net flux (A; 6-h flux periods averaged), influx (B; estimated from the difference between measured J_{net}^{Ca} and J_{out}^{Ca}), and efflux (C; five 2-h flux periods averaged). Asterisks represent significance as described in Fig. 5.

a gradual increase, reaching a rate of $-90{\cdot}48 \pm 10{\cdot}0$ (8) μequiv $kg^{-1} h^{-1}$ by 24 h of acid exposure compared with $-37{\cdot}22 \pm 7{\cdot}12$ (8) μequiv $kg^{-1} h^{-1}$ over the initial 6 h (Fig. 5B); in other words, the pattern of $J_{net}^{Na} > J_{net}^{Cl}$ over 12 h had reversed by 24 h.

By contrast, whole body net losses of calcium were less marked (Fig. 6). In trout, J_{net}^{Ca} increased over 12 h of acid exposure to a mean rate of $-14{\cdot}06 \pm 1{\cdot}94$ (8) μequiv $kg^{-1} h^{-1}$ but returned to the control rate by 24 h (Fig. 6A). This transitory net loss of Ca^{2+} was due solely to a 65 % reduction in J_{in}^{Ca} (Fig. 6B) since the slight elevation in J_{out}^{Ca} was not significant (Fig. 6C). In bullheads, a similar transitory whole body net loss of Ca^{2+} was seen over the initial 12 h with a rate of $-10{\cdot}81 \pm 1{\cdot}18$ (8) μequiv $kg^{-1} h^{-1}$ (Fig. 6A). However, unlike trout, the change in J_{net}^{Ca} was due both to a 75 % reduction in J_{in}^{Ca} (Fig. 6B) and a 4·4-fold rise in J_{out}^{Ca} (Fig. 6C).

DISCUSSION

Behaviour of radiocalcium

The behaviour of ^{45}Ca administered intravascularly (Fig. 2) was similar to that documented for monovalent radioisotopes (Maetz, 1956; Motais, 1967) but isotopic equilibrium proceeded comparatively slowly, reaching completion in ≃10–12 h for ^{45}Ca (Fig. 3) *versus* ≃3 h for ^{22}Na (Mayer & Nibelle, 1969). The size of the steady-state Ca^{2+} space was also about 8-fold higher (Table 3) than reported Na^+ and Cl^- spaces (range 200–300 ml kg^{-1}; Bath & Eddy, 1979; Hōbe *et al.* 1984), presumably a reflection of the magnitude of respective whole body ion contents (trout ≃ 250, 50 and 30 mequiv kg^{-1} for Ca^{2+}, Na^+ and Cl^-, respectively; H. Hōbe, unpublished results). The extremely low exchangeable Ca^{2+} pool in both trout and bullhead (Table 3) was noteworthy since it amounted to only ≃3 % of total body calcium content compared with ≃70 % for monovalent ions (estimated from values given above). Assuming a whole body extracellular fluid volume of 250 ml kg^{-1} (Milligan & Wood, 1982) and plasma $[Ca^{2+}] \simeq 4{\cdot}0$ mequiv l^{-1} (Table 3), it was estimated that about 20 % of the exchangeable Ca^{2+} pool resided in the ECF compartment. The remainder is presumably localized in soft tissues (e.g. muscle) since calcium mobilization both from fish bone and scales, which constitute up to 79 % and 18 % of total body calcium respectively (Brehe & Fleming, 1976), have been shown to be slow processes, requiring weeks to months (Fleming, 1968; Simmons, Simmons & Marshall, 1970; Mugiya & Watabe, 1977; Weiss & Watabe, 1978).

Unidirectional Ca^{2+} exchange rates

Calcium entry into body fluids at ambient Ca^{2+} levels below blood (i.e. 0·18 and 0·65 mequiv l^{-1}) would be against moderate concentration gradients (8–15; [ion] medium/[ion]blood) with the reverse situation prevailing at ambient $[Ca^{2+}] = 5{\cdot}0$ mequiv l^{-1}. While greater J_{in}^{Ca} would be anticipated in the former (Baldwin & Bentley, 1980), rates in each of the three experimental media were not significantly different (mean range 10–15 μequiv $kg^{-1} h^{-1}$). Thus, soft water hatchery trout, exposed to elevated ambient Ca^{2+} levels, precisely regulated J_{in}^{Ca} in order to achieve steady-state conditions by ≃2 weeks of acclimation. A similar status has been found to be achieved by the marine *Fundulus heteroclitus* acclimated to fresh wate

containing reduced levels of Ca^{2+} by 3 weeks (Mayer-Gostan *et al.* 1983).

The small magnitude of J_{in}^{Ca} in both trout and bullhead, however, was surprising since it amounted to less than one-tenth of those reported in other intact animal studies (Fleming, 1968; Pang *et al.* 1980; Mayer-Gostan *et al.* 1983). This large discrepancy could be attributed to several factors such as the nutritional status of fish, experimental temperature, fish body weight or species differences. More probably, differences in methodology between this and previous studies may be involved, since overestimates of J_{in}^{Ca} would be substantial if ^{45}Ca absorption (adsorption) by the experimental chamber and/or surface of the fish were not accounted for (see Results). Both the capacity of mucus to bind Ca^{2+} (Chartier, 1973) and radioaccumulation of ^{45}Ca from the external medium into skin and scales (Fleming, 1968) have been documented. The observed J_{in}^{Ca} therefore seems reasonable and presumably reflects gill influx since values fell in the middle of the mean range found by Payan *et al.* (1981) using isolated, perfused, trout head preparations in the presence (40 μequiv kg^{-1} h^{-1}) and absence (5 μequiv kg^{-1}) of 10^{-6} M-adrenalin in the perfusate. However, in comparison with Na^+ and Cl^- transport which must take place against massive concentration gradients (>2000) at the low external [Na^+] and [Cl^-] of the present study (Table 2), the magnitude of J_{in}^{Ca} was relatively minor, representing about 3–10 % of typical J_{in}^{Na} and J_{in}^{Cl} in freshwater fish (range 100–300 μequiv kg^{-1} h^{-1}; Kirschner, 1979; Evans, 1979).

J_{out}^{Ca} also exhibited some variability with slightly higher rates seen in low and intermediate Ca^{2+} conditions relative to high Ca^{2+} (Fig. 4). While this may simply reflect between-fish variability, it clearly points out the need to measure both J_{in}^{Ca} and J_{out}^{Ca} in order to establish firmly the Ca^{2+} status of the animal. Further support comes from the species differences seen in J_{out}^{Ca} (Fig. 4A,B) but not J_{in}^{Ca}. The lower values in bullheads suggested a reduced permeability of the exchangeable surfaces to Ca^{2+} which in turn, coincided with a slightly lower whole body exchangeable Ca^{2+} content in this species (Table 3).

Responses to low ambient pH

In natural soft water hatchery trout, acute exposure to ambient pH 4·0–4·2 resulted in equimolar whole body Na^+ and Cl^- losses (Fig. 5A,B) similar to those found in acid-exposed rainbow trout reared in a hard water hatchery but acclimated in the laboratory to artificial soft water (McDonald, 1983; McDonald, Walker & Wilkes, 1983). By contrast, in bullheads, both the pattern and magnitude of monovalent ion losses (Fig. 5A,B) more closely paralleled those reported in another stenohaline fish species, *Catostomus commersoni* (white sucker; Hōbe *et al.* 1984), confirming earlier views (Hōbe *et al.* 1983, 1984) that euryhaline and stenohaline fish species differ in their responses to external acid stress.

The present study further demonstrated significant net whole body Ca^{2+} losses in both trout and bullhead although these (Fig. 6A) were of lesser magnitude than found in acid-exposed white suckers (Hōbe *et al.* 1984). The mechanisms involved have not been previously examined and once again varied between species. While the J_{in}^{Ca} inhibition seen in both trout and bullhead may have resulted from a blockage in a transport process involved in Ca^{2+} entry (see below), the cause of the marked stimulation of J_{out}^{Ca} in bullhead but not in trout is less clear. Since elevated renal Ca^{2+} efflux

has not been found in acid-exposed stenohaline fish (Hōbe *et al.* 1983), enhanced Ca^{2+} efflux *via* the gill seemed a more likely explanation especially because of the increase in gill permeability which is thought to be generated by increased external $[H^+]$ (McWilliams & Potts, 1978). Alternatively, Ca^{2+} may have been liberated into the external medium by low ambient pH titration (González, Kirchhausen, Linares & Whittenbury, 1976) of the extensive mucous coating observed on the gills and body surface of bullheads but not trout.

The complete recovery of J_{net}^{Ca} within 24 h of acid exposure (Fig. 6A) in the absence of corresponding changes in monovalent ion J_{net} (Fig. 5A,B) was also interesting. The pituitary hormone, prolactin, may be implicated because of its independent involvement in either Ca^{2+} or Na^+ (Pang *et al.* 1980; Bentley, 1980; Feinblatt, 1982) but not Cl^- homeostasis (Fortner & Pickford, 1982). Enhanced prolactin secretion has been reported in brook trout at pH 4·0 by Notter, Mudge, Neff & Anthony (1976).

Mechanisms of Ca^{2+} regulation

The present study provides several insights into the possible mechanisms of Ca^{2+} regulation in freshwater fish. Firstly, the 65–75 % reduction in J_{in}^{Ca} (Fig. 6B) at low ambient pH, either in the presence (bullhead) or absence (trout) of a significant change in J_{out}^{Ca} (Fig. 6C), suggests that inward transport of Ca^{2+} across the gill occurs by pathways which are separate from those involved in efflux. Secondly, the absence of any parallel between the observed pattern in J_{in}^{Ca} over 24 h of acid exposure relative to those reported for J_{in}^{Na} and J_{in}^{Cl} (McDonald *et al.* 1983; Hōbe *et al.* 1984), suggests that the exchange mechanisms involved in Ca^{2+} entry *via* the gill may be independent of those known for Na^+ and Cl^- transport (i.e. Na^+/H^+ or NH_4^+; Cl^-/HCO_3^- or OH^-; Kirschner, 1979; Evans, 1979; Wood, Wheatly & Hōbe, 1984).

Thirdly, it is proposed that a Ca^{2+}/H^+ exchange may be involved in modulating Ca^{2+} entry since a blockage of this mechanism would explain the observed J_{in}^{Ca} inhibition (Fig. 6B), which in turn correlates with the increased base efflux (or reduced H^+ efflux) reported in previous studies of acid-exposed fish (McDonald *et al.* 1983; Hōbe *et al.* 1984). Indeed, this transport process exists in several other biological membranes and has been shown to be suppressed in low pH media (Adams & Duggan, 1981). However, the possibility of a Ca^{2+}-HCO_3^- co-transport mechanism cannot be excluded since the present analytical techniques employed in measuring the movement of acidic equivalents across the fish gill cannot differentiate between H^+ entry and HCO_3^- loss. A Ca^{2+}-ATPase has also been found in the gills of several fish species (reviewed by Feinblatt, 1982) but it is not possible to implicate this enzyme since no commonly-based relationship between its activity and external calcium levels has been established, as would be expected if the enzyme was directly involved in active Ca^{2+} transport. Mayer-Gostan *et al.* (1983) recently concluded that J_{in}^{Ca} in *Fundulus heteroclitus* was a passive process even though their data for low Ca-acclimated animals exposed to acute changes in ambient Ca^{2+} levels exhibited saturation kinetics. Thus the possibility of either a Ca^{2+}/H^+ or Ca^{2+}-HCO_3^- exchange mechanism in the freshwater fish gill clearly warrants future consideration.

Finally, ion efflux typically comprises two independent outflows in freshwater fish with part of the loss occurring across the gills and another fraction *via* the kidney (Kirschner, 1979). Although these components were not analysed separately in th

present study, renal calcium efflux rates of 1 (stenohaline sucker; Hōbe *et al.* 1984) and 5 μequiv kg^{-1} h^{-1} (euryhaline trout; Wheatly, Hōbe & Wood, 1984) have been reported, representing up to 50 % of the observed whole body J_{out}^{Ca} (Fig. 4) in bullhead and trout, respectively, which seems substantial when compared with a 1–2 % renal contribution for whole body J_{out}^{Na} and J_{out}^{Cl} (Evans, 1979). Thus, contrary to earlier views (Hickman & Trump, 1969), the role of the kidney in regulating whole body calcium efflux of freshwater fish may indeed be significant and requires future re-examination.

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