Physiological disturbances in rainbow trout (*Salmo gairdneri*) during acid and aluminum exposures in soft water of two calcium concentrations

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Rainbow trout (*Salmo gairdneri*) fitted with dorsal aortic cannulae were exposed in a flow-through soft water system to three acidities (pH 5.2, 4.8, or 4.4) and two concentrations of Ca (45 or 410 μequiv. $\cdot L^{-1}$), in the presence (105 $\mu g \cdot L^{-1}$) or absence of Al. Blood was sampled for respiratory gases, ions, metabolites, and hematology before and at 4, 18, 28, 42, and 66 h exposure. Two toxic mechanisms of Al and acidity were seen: (*i*) ionoregulatory toxicity, which was caused by Al at pH 5.2 and 4.8 and by acidity at pH 4.4, and (*ii*) respiratory toxicity, which was caused solely by Al, and was greatest at higher pH. Ionoregulatory toxicity involved decreases in plasma Na^+ and Cl^-, red cell swelling, and hemoconcentration. Respiratory toxicity involved reduced blood oxygen tension, elevated blood carbon dioxide tension, and increases in blood lactate. Blood acidosis was a combination of respiratory acidosis (due to CO_2 accumulation in the blood; higher pH exposures) and metabolic acidosis (probably due to differential Na^+ and Cl^- loss into the external, acidic environment; lower pH exposures). Higher water Ca reduced ionoregulatory disturbances due to acidity alone but not those due to Al at higher pH. Higher water Ca also reduced respiratory disturbances at lower pH but not at higher pH. The results are discussed with reference to the chemistry of Al and changes in the gill epithelium associated with acid and Al exposure.

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Des Truites arc-en-ciel (*Salmo gairdneri*) munies d'une canule aortique dorsale ont été exposées à trois acidités différentes (pH 5,2, 4,8 et 4,4) et à deux concentrations de Ca (45 ou 410 μéquiv. $\cdot L^{-1}$) dans un système d'eau douce à circulation continue, en présence (105 $\mu g \cdot L^{-1}$) ou en l'absence d'Al. Le sang a fait l'objet d'un certain nombre de mesures, gaz respiratoires, ions, métabolites, hématologie, avant l'expérience et au bout de 4, 18, 28, 42 et 66 h d'exposition. Deux effets toxiques de l'Al et de l'acidité ont pu être observés : (*i*) toxicité ionorégulatrice, causée par l'Al à pH 5,2 et 4,8, et par l'acidité à pH 4,4, et (*ii*) toxicité respiratoire, causée uniquement par l'Al, maximale à pH plus élevé. La toxicité ionorégulatrice s'est manifestée par la diminution des concentrations de Na^+ et de Cl^- dans le plasma, par le gonflement des érythrocytes et par l'hémoconcentration. La toxicité respiratoire a causé dans le sang une diminution de la pression d'oxygène, une augmentation de la pression de gaz carbonique et une augmentation de la concentration de lactate. L'acidose du sang est en partie une acidose respiratoire (due à l'accumulation de CO_2 dans le sang à des pH élevés) et en partie une acidose métabolique (probablement attribuable à des pertes différentielles de Na^+ et de Cl^- dans le milieu externe acide aux pH plus faibles). Une augmentation de la concentration de Ca dans l'eau diminue les effets ionorégulateurs dus à l'acidité seule, mais ne modifie pas les effets de l'Al à des pH élevés. L'augmentation de Ca dans l'eau diminue aussi les effets respiratoires à pH faible, mais pas à pH élevé. Ces résultats sont examinés à la lumière de la chimie de l'Al et des modifications de l'épithélium branchial causées par des expositions à l'acide ou à l'Al.

[Traduit par la revue]

Introduction

The acidification of natural soft water by acidic precipitation is an ecological concern in many countries, and Al leached from soil and rock by hydrogen ions is an additional stress to fish and other aquatic organisms (for reviews see Howells *et al.* 1983; Dillon *et al.* 1984; Havas and Jaworski 1986; Schindler 1988). Hydrogen ions reduce active sodium and chloride uptake at the gills and increase effluxes, resulting in net ion losses (McDonald 1983*a*). Al may exacerbate ion losses in acid-exposed fish (Muniz and Leivestad 1980; Neville 1985; Witters 1986). Ca reduces ion losses caused by H^+ alone (McDonald 1983*a*) but may (Muniz and Leivestad 1980) or may not (Witters 1986) reduce ion losses caused by Al. It is also unclear whether Al protects against ion losses caused by H^+ in very acidic water (Muniz and Leivestad 1980), protects temporarily (Neville 1985), or has no protective effect (Witters 1986).

A second aspect of Al toxicity at low pH is respiratory disturbance (Rosseland 1980), an effect not seen with acid exposures alone (McDonald *et al.* 1980). Neville (1985) found that hypoxia was the main toxic mechanism in rainbow trout exposed to Al at pH 6.1, whereas ionic depletion predominated at pH 4.5 and 4.0, and a combination of the two effects was seen at pH 5.5 and 5.0. Malte (1986) and Jensen and Weber (1987) observed hypoxia in rainbow trout and tench, respectively, exposed to very high Al concentrations in hard water (very high Ca concentrations) at pH 5.0, although the environmental relevance of their observations is doubtful because waters of such high Ca concentrations rarely become acidified. Impaired gas diffusion at the gills could be caused by Al precipitation at the gills, mucus accumulation on the gills, or gill damage.

Blood acidosis is another aspect of acid and Al toxicity. Blood acidosis does not occur in rainbow trout exposed to acid alone at low Ca concentrations, but may occur at higher Ca concentrations (Neville 1979; Wood 1988). In Wood (1988), but not Neville (1979), the acidosis at higher Ca concentrations was caused by greater Na^+ loss than Cl^- loss at the gills, which resulted in net H^+ uptake from the water. Blood acidosis has been seen in fish exposed to acidity and Al. Depending on the conditions, Al-caused decreases in blood pH have been attributed to respiratory acidosis due to CO_2 accumulation or to metabolic acidosis due to lactic acid accumulation as a result of anaerobic respiration, but in some conditions could not be explained by either mechanism (Neville 1985; Malte 1986; Jensen and Weber 1987). The

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effects of Ca on respiratory and acid–base disturbances caused by Al and acidity in combination are not known.

To date, there has been no systematic study of the interactive effects of water pH, Al, and Ca in causing ionoregulatory, respiratory, and acid–base disturbances in fish. The goal of this investigation was to perform such a study under environmentally relevant conditions. In particular, we wanted to separate the effects of acidity from those of Al, to examine the influence of acidity on Al toxicity (or *vice versa*), and to assess the protective effects of Ca. Rainbow trout were cannulated to allow repetitive blood sampling with minimal disturbance, and were exposed to conditions designed to simulate those occurring during acidic pulses such as snowmelt (e.g., Gunn and Keller 1984) or rainstorm runoff (Harvey 1980). The acidities used (pH 5.2, 4.8, 4.4) represent moderate to highly acidic conditions. An Al exposure of 105 $\mu g \cdot L^{-1}$ was chosen because at pH 5.2 this concentration is close to the solubility limit of Al, yet is still a representative concentration of Al in moderately acidic water (Dillon *et al.* 1984). The Ca concentrations used represent very soft water (45 μequiv. $\cdot L^{-1}$ Ca) and moderately soft water (410 μequiv. $\cdot L^{-1}$ Ca). We used a flow-through system to minimize the complexation and precipitation of Al that can occur in static exposures.

Methods

Experimental protocol

Adult rainbow trout (*Salmo gairdneri*) of both sexes, weighing 420 ± 10 g (mean ± SE, n = 101), were purchased from Spring Valley Trout Farm, New Dundee, Ont. They were held in dechlorinated Hamilton city tapwater (hard water; Ca ≈2 mequiv. $\cdot L^{-1}$; Na ≈ 0.6 mequiv. $\cdot L^{-1}$; pH ≈8.0) at 15–20°C and were fed floating trout pellets (Martin Feed Mills, Elmira, Ont.) twice weekly. At least 2 weeks before an experiment the fish were placed in a flowing soft water acclimation tank and feeding was suspended. Soft water was produced from dechlorinated tapwater passed through a reverse osmosis unit (Culligan MP1000) or through deionizing resin canisters (J. W. Anderson Co. Ltd., Dundas, Ont.). Appropriate amounts of analytical grade NaCl and $CaCl_2$ (BDH, Toronto, Ont.) were added by peristaltic pump. Acclimation conditions were approximately pH 6.5 at 15°C; we used Ca concentrations of 45 or 410 μequiv. $\cdot L^{-1}$ and Na concentrations of 55 μequiv. $\cdot L^{-1}$; background Al concentrations were 5 $\mu g \cdot L^{-1}$. Water pH was measured daily (Radiometer PHM82 pH meter and a Radiometer GK2401C electrode) and ion concentrations were measured every few days (atomic absorption spectrophotometry (AAS); Varian 1275). Total aqueous Al concentrations were determined using the pyrocatechol violet method (Dougan and Wilson 1974).

Fish were anaesthetised with 0.5 mg $\cdot L^{-1}$ MS222 (Sigma Chemical Co., St. Louis, MO) buffered to pH 6.5 with KOH, and cannulated with Clay-Adams PE-50 polyethylene tubing via the dorsal aorta (Soivio *et al.* 1972). The catheters were filled with heparinized Cortland saline (Wolf 1963; Sigma sodium heparin, 45 IU $\cdot$ mL^{-1}). Cannulated fish were placed individually in one of 13 darkened, aerated, Plexiglas boxes (vol. ≈3 L; after McDonald and Rogano 1986), with a flow of acclimation water of about 100 mL $\cdot$ min^{-1} to each fish. Water passed through the boxes into a surrounding bath that kept the box temperature at 14–16°C, then went to waste.

After about 44 h recovery from cannulation, initial blood samples were taken. Flow to all fish boxes was then changed to acidified water by acidifying the head tank supplying the boxes. A Radiometer PHM82 pH meter with a Radiometer GK2401C combination electrode, connected to a magnetic valve, controlled delivery of 0.5 M reagent grade H_2SO_4 to the strongly aerated head tank. Flow from the head tank was split, and a concentrated stock of Al solution ($AlCl_3 \cdot 6H_2O$ (Sigma); 0.39 g $\cdot L^{-1}$; pH ≈4.0) was delivered by peristaltic pump into one-half of the flow. Fish exposed to low pH plus Al were run simultaneously with those exposed to the same low pH alone; the fish boxes were interspersed to avoid position effects. Each experiment was at one pH (pH 5.2, 4.8, or 4.4) and one Ca concentration (45 or 410 μequiv. $\cdot L^{-1}$), with (105 $\mu g \cdot L^{-1}$) and without added Al. Background Al was about 5 $\mu g \cdot L^{-1}$. Water pH in the head tank was set below the desired pH to counteract the neutralizing influence of the fish (mainly owing to ammonia excretion) on the water as it passed through the fish boxes. Water pH was monitored in the boxes near the head of the fish, using a second Radiometer electrode and meter, and was adjusted by changing water flows to individual boxes; pH was kept within ±0.1 units of the desired pH. Oxygen and carbon dioxide tensions in the fish boxes were about 140 and <1 Torr (1 Torr = 133.3 Pa), respectively.

Blood samples (1000 μL) were drawn anaerobically into gas-tight, ice-cold Hamilton syringes before the start of the acid and Al exposure (initial values) and at 4, 18, 28, 42, and 66 h thereafter, if fish death did not occur earlier. Blood removed was replaced with Cortland saline. Blood was analyzed for pH, total CO_2 (whole blood and true plasma), O_2 tension, hematocrit, hemoglobin, lactate, and plasma concentrations of Cl^-, Na^+, K^+, Ca^{2+}, protein, and glucose.

Analytical techniques

Whole blood arterial pH (pHa) and O_2 tension (Pa_{O_2}) were measured at experimental temperature using Radiometer microelectrode units (E5021, E5046) connected to a Radiometer PHM72 acid–base analyzer. Total CO_2 in whole blood and true plasma was measured using either a Cameron chamber equipped with a Radiometer E5036 PCO_2 electrode (Cameron 1971*a*) or a Corning 965 CO_2 analyzer.

Hematocrit was measured by centrifugation at ≈5000*g* for 5 min; plasma samples were then aspirated from the hematocrit tubes for plasma CO_2 analysis. Hemoglobin was measured colorimetrically as cyanmethemoglobin (Blaxhall and Daisley 1973) using Drabkin's reagent (Sigma). Lactate was measured enzymatically (L-lactate dehydrogenase – NADH method; Loomis 1961; Sigma reagents) on whole blood that had been immediately deproteinized in two volumes of ice-cold 8% perchloric acid. Remaining blood was spun at ≈9000*g* for 2 min, and the plasma was stored at −70°C for later analyses. A drop of plasma was used to determine plasma protein concentration using a hand-held refractometer (American Optical; Alexander and Ingram 1980). Frozen plasma was later thawed, and glucose was measured using the hexokinase method of Bondar and Mead (1974; Sigma reagents). Plasma Cl^- was measured either using a Radiometer CMT10 chloridometer or a mercuric–thiocyanate spectrophotometric method (Zall *et al.* 1956); the two methods gave comparable results. Plasma Na^+, K^+, and Ca^{2+} were measured by AAS after suitable dilution; 0.2% $LaCl_2$, to reduce Na interference, was used for Ca^{2+} measurements.

After 66 h, surviving fish in some treatments were stunned with a blow to the head and a section of their third right gill arch was removed for Al determinations. Each gill sample was placed for 1 min in 15 mL distilled water (to remove excess, loosely bound Al), then frozen. Filaments were later cut from the frozen gill portions, weighed, and then digested in five times their weight of 0.05 M reagent grade H_2SO_4 for 8 h at 80°C. The supernatant was analyzed for Al using the pyrocatechol violet method (Dougan and Wilson 1974). Gill supernatant was added to the Al standards to account for tissue interferences.

Calculations

Arterial CO_2 tension (Pa_{CO_2}) was calculated using the following form of the Henderson–Hasselbalch equation:

$$Pa_{CO_2} = \frac{\text{total plasma } CO_2}{\alpha CO_2 \cdot (1 + \text{antilog } (\text{pHa} - \text{p}K'))}$$

Values of αCO_2 and pK' at experimental temperatures were taken from values for trout plasma determined by Boutilier *et al.* (1984).

Whole blood and plasma bicarbonate concentrations were calculated by

$$[HCO_3^-] = \text{total plasma } CO_2 - (\alpha CO_2 \cdot Pa_{CO_2})$$

Metabolic acid load of whole blood (ΔH^+m) was calculated cumulatively (McDonald *et al.* 1980) using the following equation:

$$[\Delta H^+m] = [HCO_3^-]_1 - [HCO_3^-]_2 - \beta(pHa_1 - pHa_2)$$

Total ΔH^+m is the sum of ΔH^+m for each interval from the initial sample onwards. In this equation both $[HCO_3^-]$ and β (the non-bicarbonate buffer value) are for whole blood. β is largely a function of hemoglobin concentration (Wood *et al.* 1982), so β was calculated from hemoglobin (Hb) using the following empirical relationship determined by Wood *et al.* (1982):

$$\beta = -1.073\ [Hb] - 2.48$$

Values of ΔH^+m were calculated for whole blood rather than plasma for the sake of direct comparison to Δlactate values, which were also measured in whole blood. There is likely a small error associated with the calculation because αCO_2 values from plasma (Boutilier *et al.* 1984) were used; values for αCO_2 in whole blood are not available. We estimate this error to be less than 5%, well within the error of the overall analytical technique. Mean cell hemoglobin concentration (in $g \cdot mL^{-1}$) was calculated as the ratio of hemoglobin ($g \cdot dL^{-1}$) to hematocrit ($mL \cdot dL^{-1}$).

Treatment of data

The presentation of physiological data from toxicological experiments is complicated because different fish die at different times. Simple averaging of all data from all fish at each time can be misleading because the most sensitive fish showing the greatest physiological disturbances generally die first. Loss of their values from the mean at subsequent sample times can produce an artificial trend of group recovery. To overcome this problem, physiological data are presented in two ways in this study. For representative parameters, data from only those fish that survived at least 42 h of acid or Al exposure have been averaged at each time up to 42 h. This shows changes in parameters over time in the most resistant individuals in each experiment. For brevity, only data for the two extreme acidities (pH 5.2 and 4.4) are shown in these figures; trends at pH 4.8 were generally intermediate. Results are also presented as final minus initial values ("terminal changes") for all fish in all treatments. Here, final values represent either the 66-h sample or the last sample taken before a fish died. Presentation of "terminal changes" emphasizes, but does not change, trends in the data, and allows comparisons among all 12 treatments.

Statistical analysis included a χ^2 test with Yate's correction for fish mortality; a paired Student's *t*-test to determine if a parameter in a treatment changed with time; and an unpaired *t*-test to compare, within a treatment, fish exposed to Al with fish not exposed to Al. Analysis of variance followed by Duncan's multiple range test was used to compare terminal changes among treatments. Unless otherwise stated the level of significance used was $P \leq 0.05$.

Results

Mortality

Mortality associated with exposure to acidity alone in cannulated rainbow trout was 0–35%, and was not significantly different among the three acidities and two Ca concentrations (Fig. 1). Al was most toxic to cannulated rainbow trout at pH 5.2 and least toxic at pH 4.4. Higher water Ca concentrations reduced mortality owing to Al at pH 5.2 and 4.8, but had no significant effect at pH 4.4, where mortality was 0–35% in the presence or absence of Al. Most fish deaths occurred between 42 and 66 h, with the exception of the Al exposure at pH 5.2, low Ca treatment, where 4 of 10 fish died at about 30 h. In general, mortality due to Al was greater at higher pH,

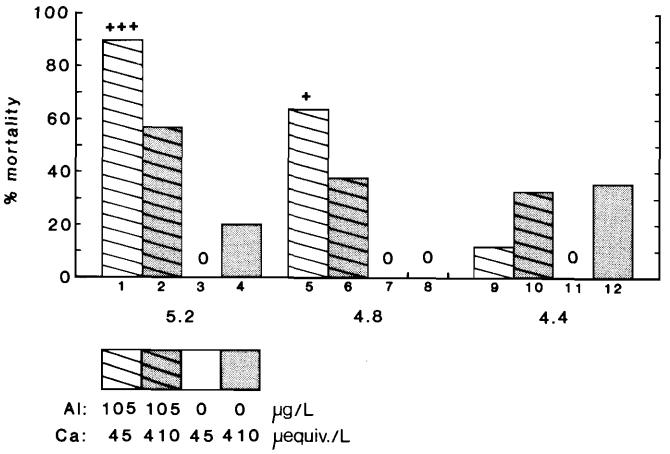

FIG. 1. Mortality in cannulated rainbow trout in the presence (105 $\mu g \cdot L^{-1}$) or absence of Al, in low (45 $\mu equiv. \cdot L^{-1}$) or higher (410 $\mu equiv. \cdot L^{-1}$) Ca, in water of three acidities (pH 5.2, 4.8, and 4.4; 66-h tests). Number of fish exposed in each treatment, from left to right: 10, 7, 8, 5, 11, 8, 6, 5, 8, 12, 10, and 11. Significant differences in mortalities between an Al treatment and the same pH and Ca concentration without Al are indicated by the following: + ($P \leq 0.05$), ++ ($P \leq 0.01$), and +++ ($P \leq 0.001$). Significant differences in mortalities between treatments are indicated below. Numbers refer to the 12 treatments, as given in the figure. Single lines underscore mortalities that are not significantly different from one another ($P > 0.05$): 1 5 2 6 12 10 4 9 8 3 7 11.

and Ca ameliorated mortality caused by Al at higher pH but not at pH 4.4.

Ionoregulatory responses

In trout surviving to 42 h, decreases in plasma Cl^- concentrations caused by acidity alone were seen only in the pH 4.4, low Ca treatment; the decreases were approximately linear over time, and were significant by 4 h (Fig. 2C). Decreases in plasma Cl^- concentrations due to the presence of Al were seen at both pH 5.2 and 4.8 (not shown), but there was no additional effect of Al at pH 4.4 (Figs. 2A, 2B, 2C, 2D). Higher Ca concentrations appeared to reduce but not eliminate Cl^- losses caused by acidity or Al (Figs. 2B, 2D). It is not known why the initial plasma Cl^- concentrations in the pH 4.4, high Ca exposures were lower than in the other treatments; initial Na^+ concentrations were normal. The summary of terminal changes emphasizes that, at pH 4.4, acidity alone caused large decreases in plasma Cl^-, whereas there was little change in plasma Cl^- at pH 5.2 and 4.8 in the absence of Al (Fig. 3A). Plasma Cl^- losses caused by Al were high at pH 5.2 and 4.8, but Al neither added to nor reduced the Cl^- losses already resulting from acidity alone at pH 4.4. Ca had little effect on Cl^- losses caused by Al at pH 5.2 or 4.8, but reduced by about half the plasma Cl^- losses caused by acidity in the pH 4.4 treatments (Fig. 3A).

In general, trout surviving to 42 h showed decreases in Na^+ ions that were similar to the plasma Cl^- losses shown in Fig. 2, but the terminal change summary revealed some subtle differences in the overall patterns. Acidity alone caused large reductions in plasma Na^+ concentrations in the pH 4.4 treatments (Fig. 3B), which agrees with the results for plasma Cl^- (Fig. 3A). However, exposure to Al appeared to worsen plasma Na^+ losses at all three acidities, whereas Al had no effect on Cl^- losses in the pH 4.4 treatments. Furthermore, higher water Ca did not reduce Na^+ losses at pH 4.4, and

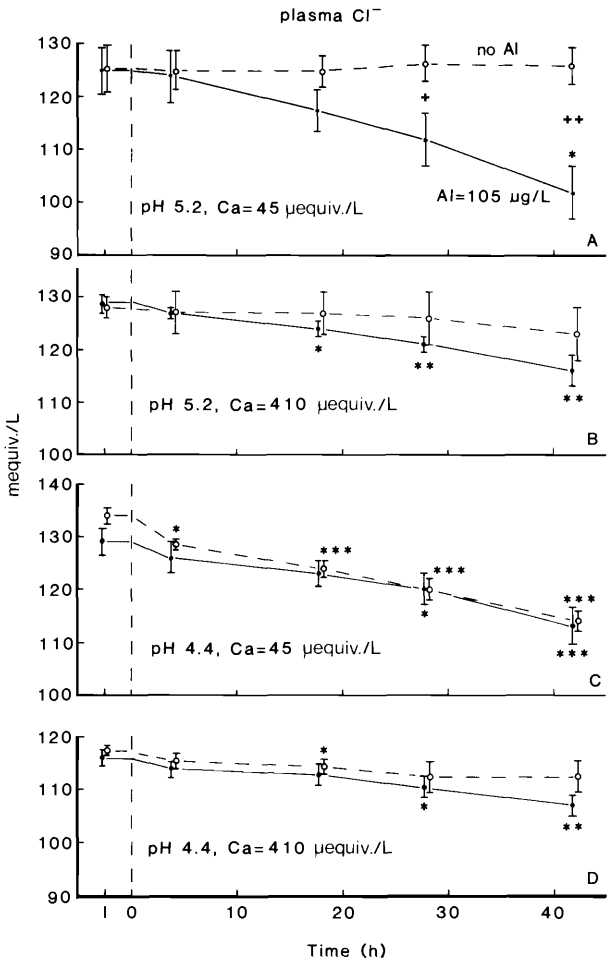

FIG. 2. Plasma Cl^- concentrations of cannulated rainbow trout during 42-h exposure to pH 5.2 or 4.4, 45 or 410 μequiv. $\cdot L^{-1}$ Ca, in the presence (105 $\mu g \cdot L^{-1}$) or absence of Al. Values are means $\pm$ 1 SEM. The number of 42-h survivors for each treatment was as follows: (A) no Al, $n = 8$; with Al, $n = 4$; (B) no Al, $n = 5$; with Al, $n = 7$; (C) no Al, $n = 10$; with Al, $n = 8$; (D) no Al, $n = 9$; with Al, $n = 12$. *, **, ***, denote significant differences ($P \leq 0.05$, ≤ 0.01, ≤ 0.001, respectively) in mean plasma Cl^- concentration compared with the same fish at time 0. +, ++, +++, indicate significant differences between fish exposed to Al and fish not exposed to Al. I, initial value, taken before acid and Al exposures started at time 0.

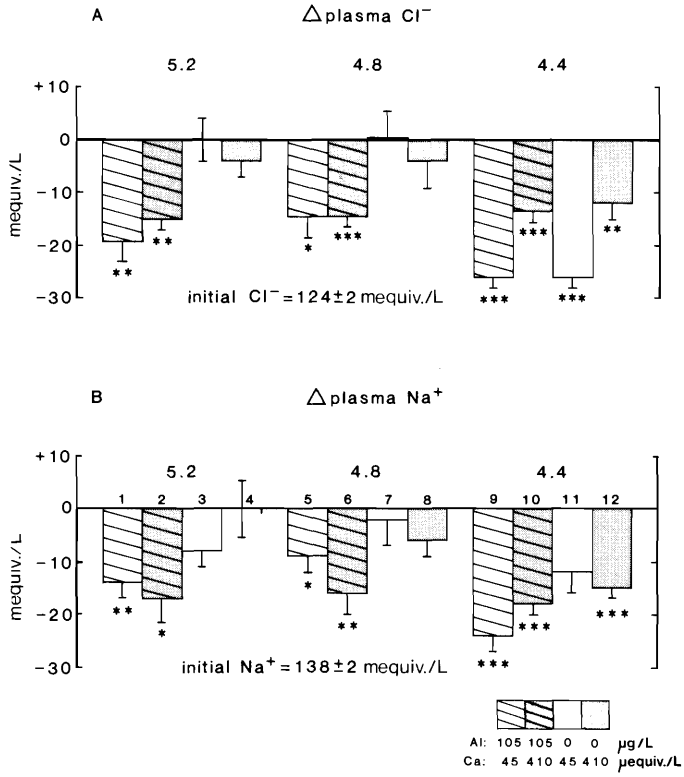

FIG. 3. Terminal changes in plasma concentrations of Cl^- and Na^+ for cannulated rainbow trout exposed to pH 5.2, 4.8, or 4.4, 45 or 410 μequiv. $\cdot L^{-1}$ Ca, in the presence (105 $\mu g \cdot L^{-1}$) or absence of Al. Values are means $\pm$ 1 SEM, n values are as in Fig. 1. *, **, ***, denote significant differences ($P \leq 0.05$, ≤ 0.01, ≤ 0.001, respectively) between terminal and initial values for the same treatment. The mean initial concentrations for all 12 treatments are given in each panel. Significant differences in terminal changes between treatments are indicated below. Numbers refer to the 12 treatments, as given in the figure. Single lines underscore terminal changes that are not significantly different from one another ($P > 0.05$):

Cl^-: 11 9 1 6 2 5 10 12 8 4 7 3

Na^+: 9 10 2 6 12 1 11 5 3 8 7 4

tended if anything to worsen Na^+ losses due to Al at pH 5.2 and 4.8 (Fig. 3B). Overall, Al caused decreases in plasma Cl^- and Na^+ ion concentrations in the pH 5.2 and 4.8 treatments, where Cl^- and Na^+ losses were low in the absence of Al. Decreases in plasma Cl^- and Na^+ ions at pH 4.4 were caused mainly by acidity alone.

Plasma K^+ concentrations generally increased when Na^+ and Cl^- ions were lost from the plasma (Fig. 4A), although there were only two statistically significant changes. There was an overall trend towards decreasing plasma Ca^{2+} concentrations over time in most exposures, likely a result of repetitive blood sampling (i.e., McDonald *et al.* 1980), but the effect was reduced or even reversed at pH 5.2 in the presence of Al (Fig. 4B). Plasma protein concentration (Fig. 4C) tended to increase and mean cell hemoglobin concentration (Fig. 4D) tended to decrease, as plasma Cl^- and Na^+ ions decreased (Fig. 3A, 3B). At all three acidities these effects were more pronounced in the presence of Al, and were not systematically affected by Ca (Figs. 4C, 4D). The glucose data were rather variable, but a significant increase in plasma glucose concentration associated with acidity alone was seen in the pH 4.4, low Ca treatment (Fig. 4E). Plasma glucose also increased significantly in the presence of Al in the pH 4.4, low and high Ca treatments (Fig. 4E).

Respiratory responses

Acidity alone had little effect on arterial oxygen tension (Pa_{O_2}) in 42-h survivors (Fig. 5), but Al caused large and rapid decreases in Pa_{O_2} (significant by 4–18 h) in the pH 5.2 treatments (Figs. 5A, 5B). Similarly, in the pH 4.8, low Ca treatment, Al caused a drop in Pa_{O_2} from 100 to 40 Torr in 42 h (not shown). Calcium did not reduce the effect of Al on Pa_{O_2} at pH 5.2 (Figs. 5A, 5B), but the decrease in Pa_{O_2} caused by Al at pH 4.8 was eliminated by higher Ca (not shown); a similar protective effect of Ca occurred at pH 4.4 (Figs. 5C, 5D).

Responses in arterial carbon dioxide tension (Pa_{CO_2}) in 42-h survivors generally mirrored those in Pa_{O_2}. The Pa_{CO_2} was little affected by acidity alone: in the pH 4.4, no Al treatments, Pa_{CO_2} increased by only about 0.5 Torr (Figs. 6C, 6D). Alu-

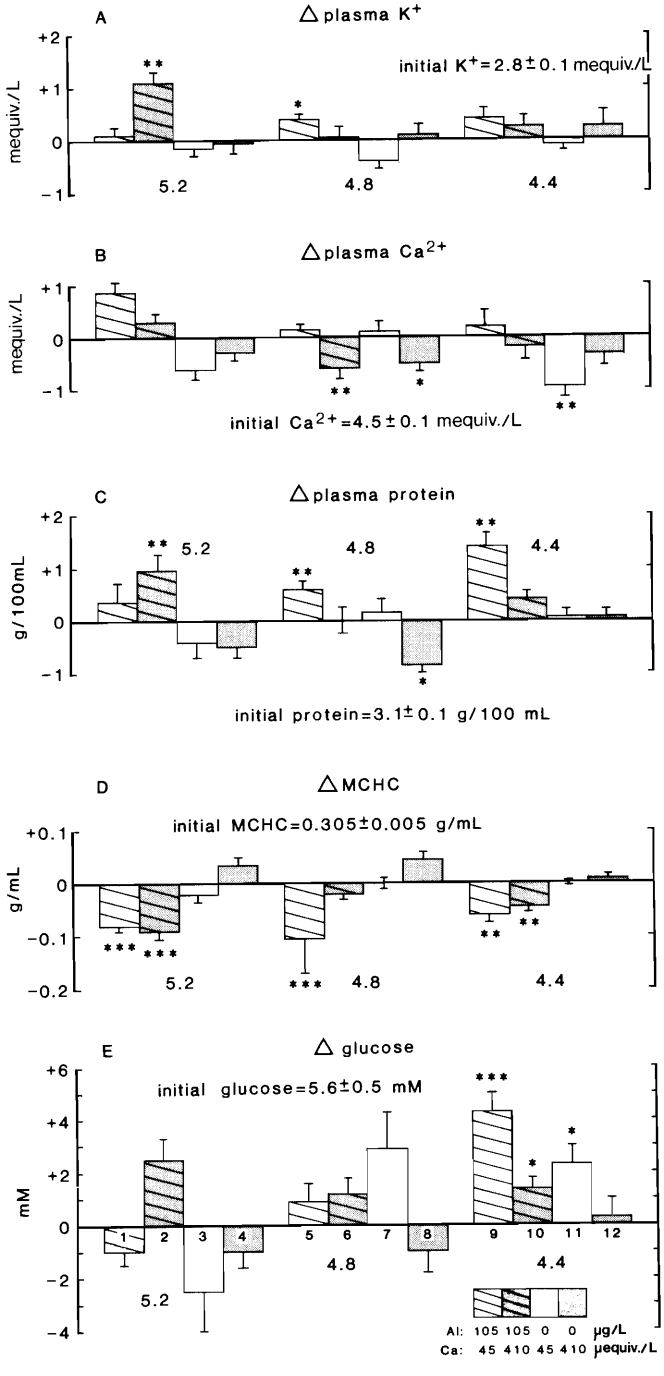

FIG. 4. Terminal changes in plasma concentrations of K^+, Ca^{2+}, protein, and glucose, and mean cell hemoglobin concentration (MCHC) for cannulated rainbow trout exposed to pH 5.2, 4.8, or 4.4, 45 or 410 μequiv. $\cdot L^{-1}$ Ca, in the presence or absence of Al. Significant differences in terminal changes among treatments are indicated below. See legend of Fig. 3 for other details.

K^+: 2 5 9 10 12 1 6 8 4 11 3 7

Ca^{2+}: 1 2 5 9 7 10 12 8 4 3 6 11

protein: 9 2 5 10 1 7 11 12 6 3 4 8

MCHC: 5 2 1 9 10 6 3 7 4 12 11 8

glucose: 9 7 11 2 10 6 5 12 1 8 4 3

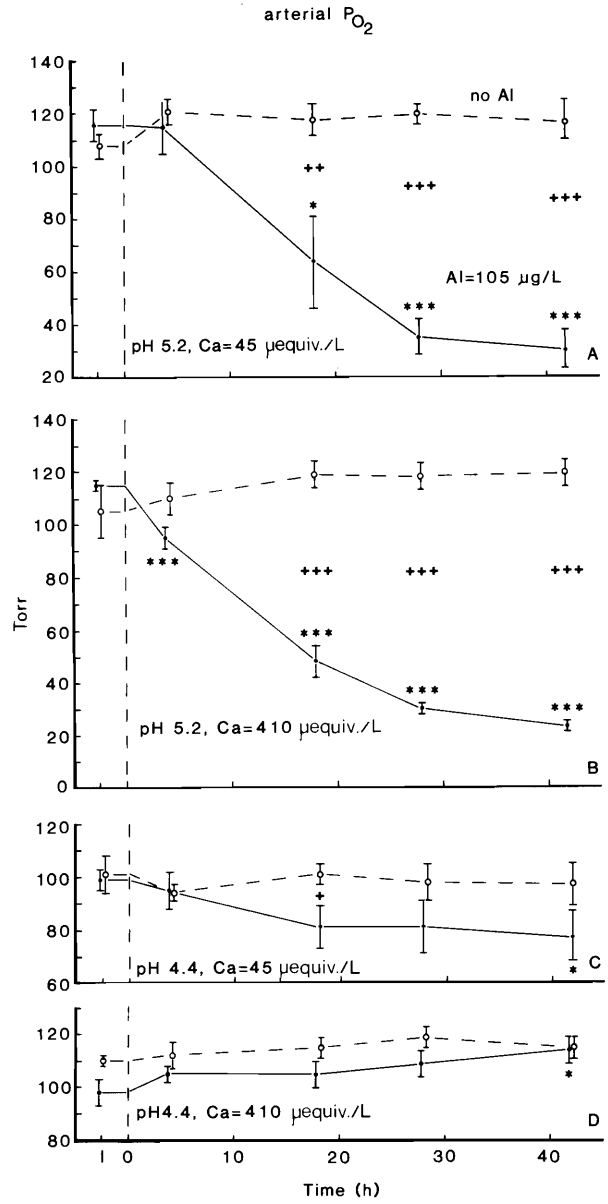

FIG. 5. Arterial oxygen tension of cannulated rainbow trout during 42-h exposure to pH 5.2 or 4.4, 45 or 410 μequiv. $\cdot L^{-1}$ Ca, in the presence (105 $\mu g \cdot L^{-1}$) or absence of Al. 1 Torr = 0.133 kPa. See legend of Fig. 2 for other details.

minum caused large, rapid increases in Pa_{CO_2} at pH 5.2 (Figs. 6A, 6B) and pH 4.8, low Ca (not shown), and increased Pa_{CO_2} to a lesser degree in the pH 4.4, low Ca treatment (Fig. 6C). Calcium did not alter the effect of Al on Pa_{CO_2} at pH 5.2 (Figs. 6A, 6B), but reduced it by half at pH 4.8 (not shown), and eliminated the effect at pH 4.4 (Figs. 6C, 6D). The reciprocal nature of the Pa_{O_2} and Pa_{CO_2} responses is well illustrated by the summary of terminal changes in all treatments (Figs. 7A, 7B).

In broad overview, acidity alone had little effect on blood gases, but Al caused severe respiratory toxicity in rainbow trout at pH 5.2 (shown by the large decreases in Pa_{O_2} and increases in Pa_{CO_2}; Figs. 7A, 7B), caused less severe respiratory toxicity at pH 4.8, and induced only moderate respiratory distress at pH 4.4. Calcium reduced the respiratory effects of Al at pH 4.4 and especially at pH 4.8, but had no effect at pH

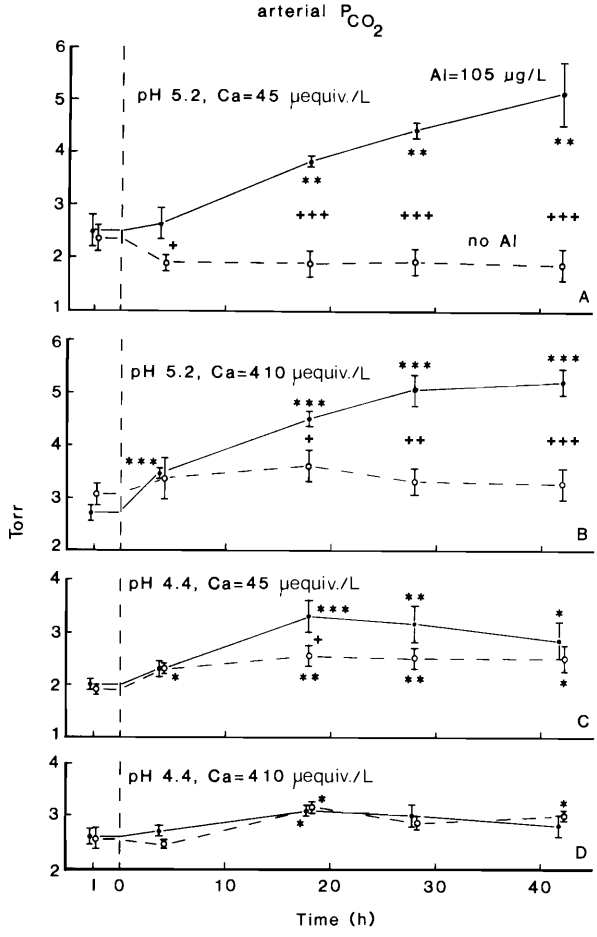

FIG. 6. Arterial carbon dioxide tension of cannulated rainbow trout during 42-h exposure to pH 5.2 or 4.4, 45 or 410 μequiv. · L^{-1} Ca, in the presence (105 μg · L^{-1}) or absence of Al. 1 Torr = 0.133 kPa. See legend of Fig. 2 for other details.

5.2. Increases in blood lactate (Fig. 9B) correlated well with the extent of respiratory toxicity.

Acid–base responses

In 42-h survivors, arterial pH (pHa) decreased quickly owing to acidity alone in the pH 4.4, high Ca treatment (significant by 18 h; Fig. 8D), but not in the pH 4.4, low Ca treatment (Fig. 8C). Acidity alone had no effect on pHa at pH 5.2 (Figs. 8A, 8B). Aluminum had little effect on pHa in the pH 5.2 treatments (Figs. 8A, 8B), but caused large, linear decreases in pHa in the pH 4.4, low Ca treatment (Fig. 8C), and worsened the blood acidification already present in the pH 4.4, high Ca treatment without added Al (Fig. 8D). Arterial pH decreased from about 7.8 to 7.6–7.7 in 42-h survivors in all four pH 4.8 treatments (not shown).

Accumulation of CO_2 in the blood due to respiratory toxicity should shift the carbon dioxide – bicarbonate equilibrium, resulting in increases in arterial concentrations of HCO_3^- (which were seen in several treatments; data not presented) and H^+ ions, thereby decreasing blood pH (respiratory acidosis; Davenport 1974). Although pHa fell in almost every treatment where Pa_{CO_2} increased (Fig. 7C), the relationship was not proportional. Fish showed greater acidosis at pH 4.4 in the presence of Al, where Pa_{CO_2} elevations were smallest, than at pH 5.2, where Al caused large accumulations of CO_2 in the

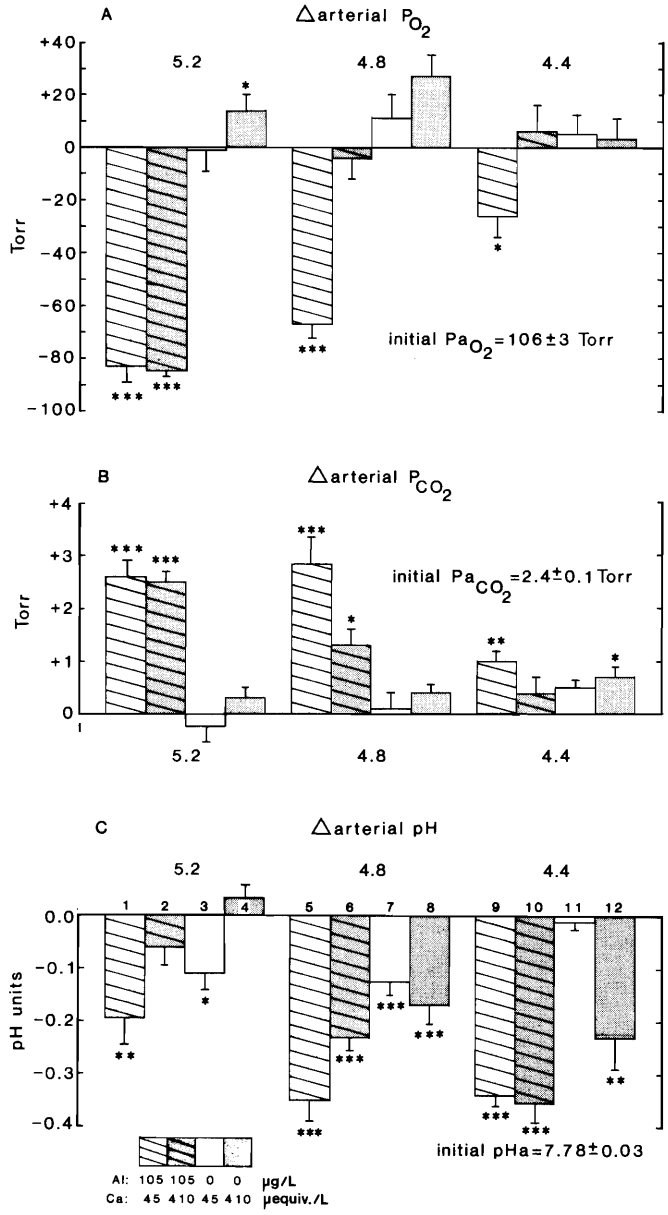

FIG. 7. Terminal changes in arterial oxygen tension, carbon dioxide tension, and pH in cannulated rainbow trout exposed to pH 5.2, 4.8, or 4.4, 45 or 410 μequiv. · L^{-1} Ca, in the presence (105 μg · L^{-1}) or absence of Al. 1 Torr = 0.133 kPa. Significant differences in terminal changes among treatments are indicated below. See legend of Fig. 3 for other details.

Pa_{O_2}: 2 1 5 9 6 3 10 12 7 11 4 8

Pa_{CO_2}: 5 1 2 6 9 12 8 10 11 7 4 3

pHa: 5 9 10 6 12 1 8 7 3 2 11 4

blood. Blood acidification at pH 4.8 was worse than expected if caused solely by respiratory acidosis.

Metabolic acid load (ΔH^+m) was calculated to separate the metabolic component of acidosis from the respiratory component. This calculation indicates that at pH 4.4, in the presence or absence of Al, blood acidification was caused mainly by entry of metabolic H^+ ions into the blood (Fig. 9A), and that this factor also made a substantial contribution to acidosis at pH 4.8. This "entry" was probably from the external, acidic water. ("Entry" is used here in its broadest sense; acid

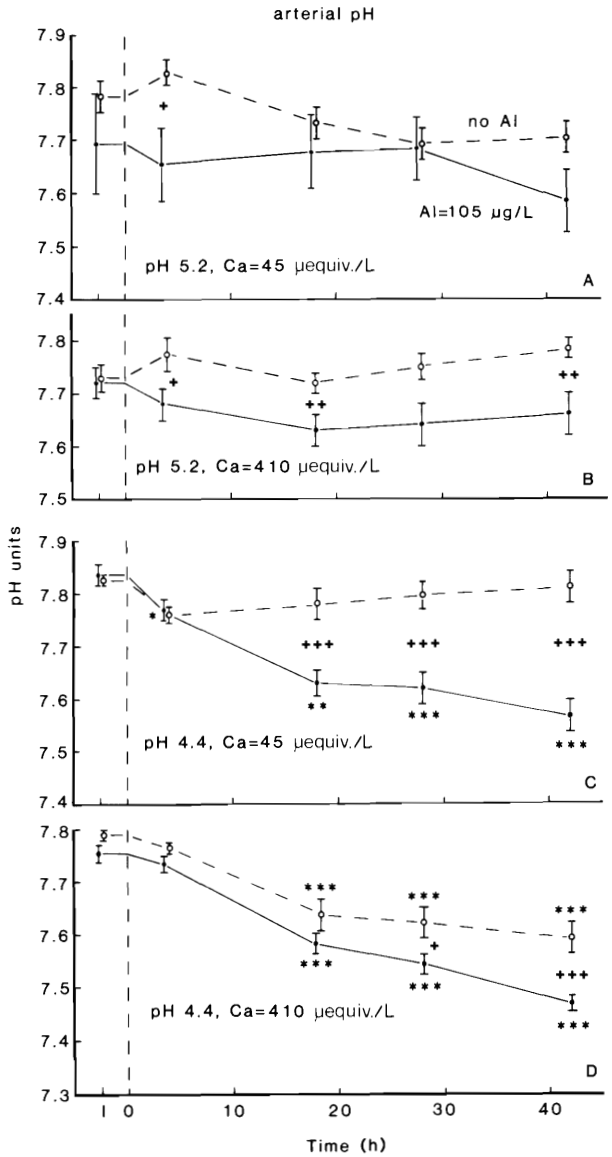

FIG. 8. Arterial pH of cannulated rainbow trout during 42-h exposure to pH 5.2 or 4.4, 45 or 410 μequiv. $\cdot L^{-1}$ Ca, in the presence (105 $\mu g \cdot L^{-1}$) or absence of Al. See legend of Fig. 2 for other details.

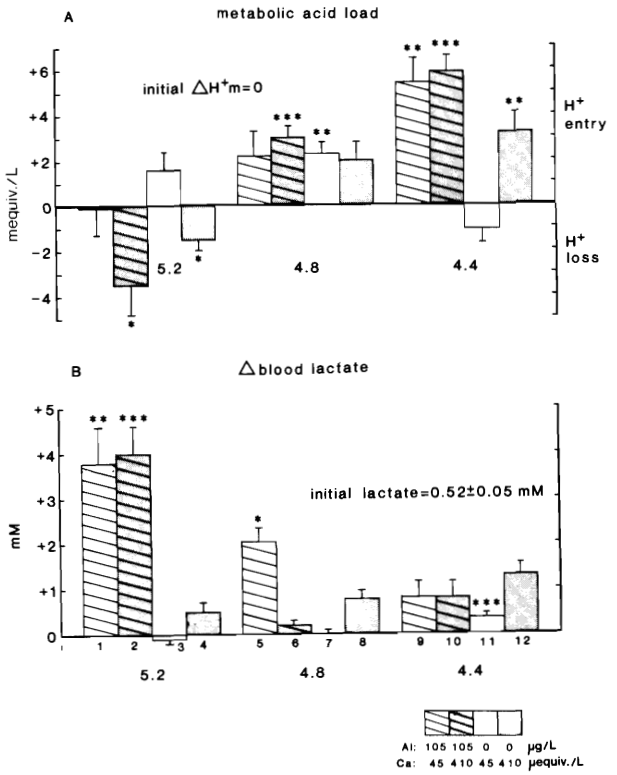

FIG. 9. Terminal metabolic acid load and terminal changes in blood lactate for cannulated rainbow trout exposed to pH 5.2, 4.8, or 4.4, 45 or 410 μequiv. $\cdot L^{-1}$ Ca, in the presence (105 $\mu g \cdot L^{-1}$) or absence of Al. Significant differences between treatments are indicated below. See legend of Fig. 3 for other details.

ΔH^+m: 10 9 12 6 7 5 8 3 1 11 4 2

lactate: 2 1 5 12 9 10 8 11 4 6 7 3

entry cannot be distinguished from base efflux with current technology.) Changes in blood lactate and ΔH^+m showed very different trends, and the ΔH^+m exceeded the elevations in blood lactate in most treatments (Figs. 9A, 9B). Thus H^+ ions from lactic acid production were generally not responsible for blood metabolic acidosis. For example, blood lactate increased by about 4 mM overall in the pH 5.2 plus Al exposures (Fig. 9B), yet blood acidification was low (Fig. 7C), and ΔH^+m was zero or negative. Here, lactate accumulation in the blood reflected anaerobic metabolism due to low Pa_{O_2}, but did not result in blood acidification.

Gill aluminum accumulation

To assess whether gill Al accumulation was correlated with physiological disturbances, we sampled gills from surviving fish at the end of some experiments. The number of samples was low, and some treatments were not sampled (i.e., pH 5.2 and 4.8, low Ca treatments, with or without Al). Nevertheless, at all three acidities gill Al concentrations were elevated in the presence of Al (Table 1). Higher water Ca reduced gill Al accumulation in the one available comparison, at pH 4.4 (Table 1).

Discussion

In our experiments mortality in cannulated rainbow trout exposed to Al and acidity in combination was greatest at pH 5.2 and least at pH 4.4. Mortality was caused by a combination of respiratory and ionoregulatory toxicity. Respiratory toxicity was caused solely by Al and was greatest at higher pH, in contrast with ionoregulatory toxicity which was due to Al at pH 5.2 and 4.8 but was caused mainly by acidity at pH 4.4. In general, Ca reduced both ionoregulatory and respiratory toxicity at lower pH, but not at pH 5.2.

Ionoregulatory responses

Ionoregulatory effects of acidity and Al on rainbow trout can be described using a simple model proposed by Wood and McDonald (1987). The presence of H^+ in the external environment inhibits active uptake of Na^+ and Cl^- at the gills, and stimulates passive effluxes through paracellular channels, perhaps by displacement of Ca^{2+} from the tight junctions. These changes lead to net plasma Na^+ and Cl^- losses as seen in our study in the pH 4.4 treatments. Acid-induced decreases in plasma ions have been reported in adult rainbow trout exposed to pH 4.0–4.8 in both soft and hard

TABLE 1. Gill Al concentrations ($\mu g \cdot g^{-1}$ wet tissue) in cannulated rainbow trout surviving 66 h of exposure to pH 5.2, 4.8, or 4.4, 45 or 410 μequiv. $\cdot L^{-1}$ Ca, in the presence (105 $\mu g \cdot L^{-1}$) or absence of Al

	pH 5.2		pH 4.8		pH 4.4			
Al ($\mu g \cdot L^{-1}$):	105	0	105	0	105		0	
Ca (μequiv. $\cdot L^{-1}$):	410	410	410	410	45	410	45	410
	20±18 (2)	5±2 (4)	18±3* (4)	1±1 (3)	17±4** (5)	4±4† (6)	2±1 (5)	3±2 (7)

NOTE: Values are means ± 1 SEM (n). Significant differences among treatments are indicated by the following numbers, which refer to the eight treatments, numbered from left to right (see legend of Fig. 3 for other details): 3 5 1 2 6 8 7 4. *, **, significantly different ($P \leq 0.05$, $P \leq 0.01$; t-test, log(x + 1) transformed data) from the comparable mean in the absence of Al; †, significantly different ($P \leq 0.05$; t-test, log(x + 1) transformed data) from the comparable mean at lower Ca (45 μequiv. $\cdot L^{-1}$).

water (McDonald *et al.* 1980; McDonald and Wood 1981; McDonald 1983*b*; Lee *et al.* 1983; Holeton *et al.* 1983; Giles *et al.* 1984; Neville 1985), but not at more neutral pH.

In this model, Al also reduces active Na^+ and Cl^- uptake and increases Na^+ and Cl^- efflux, resulting in net ion losses at moderate pH where acidity alone causes little or no decrease in these ions (i.e., pH 5.2 and 4.8 treatments, Fig. 3). Previous reports of Na^+ and Cl^- losses in adult salmonids exposed to Al in soft water at physiologically "safe" pHs include Muniz and Leivestad (1980), Neville (1985), Goss and Wood (1988), and Wood *et al.* (1988). We suggest that, at moderate acidities (pH ≈5), Al accumulation at the gills leads to ion losses because of inflammation, cell swelling, and distortion of the branchial epithelium, resulting in increased paracellular permeability. Gill damage of this nature attributed to Al in the pH range 5–6 has been observed through both light and electron microscopy in several fish species (Chevalier *et al.* 1985; Malte 1986; Karlsson-Norrgren *et al.* 1986*a*, 1986*b*; Youson and Neville 1987). Our study showed little if any effect of Al on plasma Na^+ and Cl^- losses due to acidity alone (pH 4.4 treatments, Fig. 3). The transition between harmful effects of Al on ionoregulation at higher pH to benign effects at lower pH may be due to greater Al precipitation at the gills at pH 5–6 where Al solubility is lowest (Roberson and Hem 1969), or to a change in toxicity as the Al species shift from aluminum hydroxides (higher pH) to the Al^{3+} cation (lower pH). Indeed, Al^{3+} can be protective at very low pH (e.g., Muniz and Leivestad 1980; Neville 1985; pH 4.0), perhaps through its ability to mimic the effects of Ca^{2+} on limiting membrane permeability (Baker and Schofield 1982). However, in our experiments, as in those of Neville above pH 4.0 (Neville 1985) and Witters (1986) using rainbow trout, the presence of Al never reduced ionoregulatory disturbances caused by acidity alone. Besides depending on pH, the protective effects of Al in acidic water may vary with species or be related to stage of fish development (Baker and Schofield 1982).

Calcium reduced Cl^- losses owing to acidity alone (pH 4.4), but did not reduce Cl^- losses caused by Al at higher pH (Fig. 3A). Calcium did not reduce Na^+ losses caused by acidity alone, and may even have worsened Na^+ losses caused by Al (Fig. 3B). The differential effect of Ca in reducing acid-induced Cl^- loss more than acid-induced Na^+ loss has been reported before (summarized by McDonald 1983*a*; Wood 1988), and is attributed largely to differential effects on the passive efflux components rather than the active uptake components of branchial Na^+ and Cl^- exchange. The inability of Ca to reduce the effects of Al on ion losses is likely related to the specific toxic action of Al at the gill membranes: we think that gill inflammation and damage caused by Al accumulation at the gills are responsible for Al-induced ion losses, effects of Al that are probably not ameliorated by Ca.

Na^+ and Cl^- ion losses are accompanied by fluid volume shifts out of the plasma into muscle because extracellular osmolarity decreases faster than intracellular osmolarity (Milligan and Wood 1982). Observed increases in plasma concentrations of K^+, Ca^{2+}, and protein (Figs. 4A, 4B, 4C) were likely a result of decreased plasma volume, which would translate into higher concentrations of these parameters. Increased plasma K^+ may also be related to acidosis, because intracellular K^+ is released from muscle as H^+ enters (Ladé and Brown 1963). Mean cell hemoglobin concentration (MCHC) usually decreased as plasma Na^+ and Cl^- decreased (Fig. 4D). The decrease in MCHC was likely associated with entry of fluid into the red blood cells in response to osmotic disequilibrium caused by the decrease in plasma ions. In addition, mobilization of catecholamines into the blood may also have promoted red cell swelling (Vermette and Perry 1988). Preliminary work of our own has shown large but transitory increases in plasma epinephrine and norepinephrine in rainbow trout exposed to 105 $\mu g \cdot L^{-1}$ Al at pH 4.8 (G. G. Goss, R. C. Playle, and C. M. Wood, unpublished results). Such increases in plasma catecholamines were probably responsible for the observed elevations of plasma glucose concentrations (Fig. 4E; Perry *et al.* 1988). Glucose mobilization is a commonly observed response to general stress in fish, and during acid and Al exposures glucose may be particularly useful as a method for supplementing plasma osmolarity in the face of ion loss (McDonald 1983*b*; Goss and Wood 1988).

Respiratory responses

Respiratory toxicity in rainbow trout was caused by Al but not by acidity alone, and was worse at higher pH (Figs. 7A, 7B). In the simple model (Wood and McDonald 1987), the branchial epithelium becomes inflamed, swollen, and coated with mucus as Al precipitates on the gills. Inflammation and cell swelling would decrease gas transfer because of increased diffusion distance across the gill. Mucus accumulation would also decrease gas transfer because of lower diffusion through mucus and an increased boundary layer (Ultsch and Gros 1979). Accumulation of Al on gills has been reported by Neville (1985), Chevalier *et al.* (1985), Harvey and McArdle (1986), Lee and Harvey (1986), Karlsson-Norrgren *et al.* (1986*a*, 1986*b*), Jensen and Weber (1987), Youson and

Neville (1987), and us (Table 1). Accumulation of mucus on gills during exposures to Al has been reported by Muniz and Leivestad (1980), Rosseland (1980), Harvey and McArdle (1986), Lee and Harvey (1986), Karlsson-Norrgren *et al.* (1986*a*), and Jensen and Weber (1987). Overall, these studies suggest that gill mucification, Al accumulation, and damage are worst between pH 5 and 6, where Al solubility is low (Roberson and Hem 1969) and Al precipitation on the gill would be expected to be high. Furthermore, ammonia excretion at the gill probably raises the pH of the branchial microenvironment (Wright and Wood 1985), intensifying Al precipitation as the solubility of Al is exceeded.

Another possible explanation of greater respiratory toxicity of Al at higher pH (i.e., pH 5.2 and 4.8, Figs. 7A, 7B) than at lower pH (i.e., pH 4.4) would be higher binding affinity to the gills of aluminum hydroxides compared with Al^{3+}. Alternatively or additionally, aluminum hydroxides attached to the gill might impair gas transfer more than would bound Al^{3+} cations. By these explanations, the species shift would be more important than decreased Al solubility at higher pH. The effects of Al precipitation in near-saturated conditions are difficult, if not impossible, to separate from those of Al species shift and, in any case, are not mutually exclusive.

The effect of Ca on Al accumulation at the gills is unclear. Ca reduced respiratory disturbances in the pH 4.8 and 4.4 treatments but not at pH 5.2 (Figs. 7A, 7B). As discussed earlier, Ca did not ameliorate ionoregulatory disturbances caused by Al at higher pH (Fig. 3). Our gill Al data for the pH 4.4 treatments (Table 1) suggest that Ca may have reduced respiratory disturbances caused by Al at lower pH by reducing Al accumulation on the gills. Rainbow and brook trout fingerling data from our laboratories also suggest that Ca can reduce gill Al accumulation (D. G. McDonald and C. M. Wood, unpublished results). In addition, brook trout yolk-sac fry and swim-up fry at pH 4.8 and 5.2 accumulated less Al in water of higher Ca than lower Ca (Wood *et al.* 1989*a*, 1989*b*). How Ca reduces Al accumulation at the gills is unknown, but perhaps Ca competes with Al for its binding sites. Curiously, this postulated effect of Ca on Al binding at the gills did not reduce the respiratory effects of Al in the pH 5.2 treatments, possibly because Al precipitation at that pH may simply be too great to be ameliorated by Ca. Equally curious is why the respiratory toxicity of Al in the pH 4.8 treatments was reduced by Ca but the ionoregulatory toxicity was not. Perhaps only a small amount of precipitated Al is needed to cause gill inflammation and thereby ion losses, and larger amounts of precipitated Al are necessary to impair gas transfer through increased diffusion distance owing to cell swelling and mucus accumulation. A further complication is that 400 μequiv. $\cdot L^{-1}$ Ca worsened the respiratory effects of 330 $\mu g \cdot L^{-1}$ Al at pH 4.8 in brook trout (Wood et al. 1988) but reduced the respiratory effects of 105 $\mu g \cdot L^{-1}$ Al at pH 4.8 in rainbow trout (this study). Whether there is some basic difference between gills of the two species (brook trout are more resistant to acidity and Al; Grande *et al.* 1978) or whether this contrast is solely a result of the different Al concentrations used remains to be seen.

Acid–base responses

The calculation of metabolic acid load (Fig. 9A) indicated that blood acidification in the pH 4.4 and 4.8 treatments was caused mostly by metabolic acid entry, probably from the acidic water. In agreement with theory (Wood 1988) and previous experimental results on rainbow trout (McDonald *et al.* 1980; McDonald and Wood 1981; McDonald 1983*b*), apparent H^+ entry at pH 4.4 (no Al) was associated with higher water Ca concentration. This effect is explained by a differential action of Ca^{2+} on Na^+ and Cl^- losses at low pH, which constrains net H^+ entry through the "strong ion difference" relationship (Stewart 1978). In simple terms, any excess of Na^+ over Cl^- loss to the water is made up by H^+ entry, resulting in blood acidification. In the high Ca, pH 4.4 treatment, Al worsened metabolic acid load, and therefore blood acidification (Figs. 7C, 9A), possibly because Al enhanced the Ca-induced effect of greater Na^+ over Cl^- losses (Fig. 3). Aluminum also worsened metabolic acid load and blood acidification in the low Ca, pH 4.4 treatment, but Na^+ and Cl^- losses from the blood were of similar magnitude.

Impaired gas transfer in Al-exposed rainbow trout caused CO_2 accumulation in the blood, especially at higher pH (Fig. 7B), which by itself would decrease arterial pH (respiratory acidosis; Davenport 1974). Blood acidification in the Al treatments at pH 5.2 was due solely to respiratory acidosis, and respiratory acidosis added to the metabolic acidosis already present in the pH 4.8 plus Al treatments and in the pH 4.4, low Ca, Al treatment (Fig. 7B, 7C). Neville (1985), Malte (1986), Jensen and Weber (1987), and Wood *et al.* 1988) have also demonstrated respiratory acidosis caused by Al. The pH 5.2, 105 $\mu g \cdot L^{-1}$ Al, high Ca treatment is interesting because a metabolic *alkalosis* (Fig. 9A) counteracted the respiratory acidosis owing to arterial CO_2 build-up (Fig. 7B), resulting in only minor blood acidification (Fig. 7C). Metabolic alkalosis was also seen in this treatment in the absence of Al. The causes of these alkaloses remain unknown. Small increases in Pa_{CO_2} in fish not exposed to Al (Figs. 6C, 6D, 7B) were not due to excess CO_2 in acidified water because the head tank and fish boxes were well aerated and measured water PCO_2 stayed below 1 Torr. Increased Pa_{CO_2} in these fish may have been a result of catecholamine mobilization (Milligan and Wood 1982), which would raise both Pa_{O_2} and Pa_{CO_2}, the latter possibly by inhibiting HCO_3^- dehydration through the red blood cells (Wood and Perry 1985; Vermette and Perry 1988).

In our experiments lactate production reflected anaerobic metabolism caused by hypoxemia during Al exposures. For example, in the pH 5.2 plus Al treatments blood lactate concentrations increased sharply when Pa_{O_2} fell below 40 Torr and Pa_{CO_2} increased above 4 Torr, conditions in which the blood was probably only 40–70% saturated with oxygen (Cameron 1971*b*). However, metabolic acid load was lowest when blood lactate was highest (Fig. 9). Lactate production did not cause blood metabolic acidosis, probably because H^+ ions from lactic acid were retained in muscle and not released into the bloodstream (Wood and Perry 1985). Neville (1985) also reported high (5 mM) blood lactate concentrations, probably a result of anaerobic metabolism (O_2 saturation was about 40%) in her pH 6.1 plus Al treatments.

In summary, we have demonstrated that rainbow trout exposed to an environmentally realistic level of Al in soft water develop Na^+ and Cl^- losses at pH 5.2 and 4.8, pHs where ion losses due to acidity alone are negligible. However, at pH 4.4 the presence of Al does not add to ion losses caused by acidity alone. We have also shown that Al causes respiratory disturbances that are not seen with acidity alone; these disturbances are greatest at higher pH. At higher pH, Al

precipitation at the gills likely causes gill inflammation and damage, leading to ion losses and gas transfer impairment. These effects of Al at the gill surface (inflammation and damage) are distinct from the effects of H^+ alone, which probably increases gill permeability by displacing Ca^{2+} from the tight junctions. Acid–base disturbances are a combination of metabolic acidosis caused by entry of acidic equivalents from the water and, at higher pH, respiratory acidosis caused by Pa_{CO_2} build-up. Ca reduces Cl^- losses caused by acidity alone, but worsens blood acidosis at pH 4.4 through unequal effects on net Na^+ and Cl^- fluxes. Ca does not ameliorate ion losses caused by Al at higher pH. Ca also reduces the respiratory effects of Al at lower pH but not at higher pH, where Al precipitation may be too high to be ameliorated by Ca. The next step to a better understanding of the effects of acidity and Al at the gill is to study the gill microenvironment itself, concentrating on how the pH at the gill affects Al precipitation and speciation. Knowledge of reactions of Al at the gill microenvironment may help explain physiological changes in fish exposed to pulses of acid and Al that occur during events such as snowmelt.

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