Blood Gases, Acid–Base Status, Ions, and Hematology in Adult Brook Trout (*Salvelinus fontinalis*) under Acid/Aluminum Exposure

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Wood, C. M., R. C. Playle, B. P. Simons, G. G. Goss, and D. G. McDonald. 1988. Blood gases, acid–base status, ions, and hematology in adult brook trout (*Salvelinus fontinalis*) under acid/aluminum exposure. Can. J. Fish. Aquat. Sci. 45: 1575–1586.

The relative importance of ionoregulatory and respiratory disturbances in brook trout (*Salvelinus fontinalis*) under acid/Al stress in soft water is dependent upon water pH and Ca^{2+} levels. Trout acclimated to Ca^{2+} = 25 or 400 μequiv/L were fitted with arterial catheters and exposed to acid/Al for 10 d under flow-through conditions. Parameters monitored included pHa, Pa_{O_2}, Pa_{CO_2}, HCO_3^-, ΔH^+m, Na^+, Cl^-, K^+, Ca^{2+}, protein, lactate, glucose, hemoglobin, and hematocrit. Exposure to pH = 4.8 (no Al) at Ca^{2+} = 25 μequiv/L caused no mortality and negligible physiological disturbance. Addition of Al (333 μg/L or 12.3 μmol/L) resulted in >80% mortality (LT50 = 39.0 h) preceded by a marked decrease of plasma Na^+ and Cl^-, a moderate disturbance of blood gases, but no acidosis. At higher Ca^{2+} (400 μequiv/L), this same exposure (pH = 4.8, Al = 333 μg/L) caused similar mortality (LT50 = 38.5 h) but smaller ionic disturbances, much larger decreases in blood O_2, increases in blood CO_2, and respiratory acidosis. Exposure to pH = 4.4 (no Al) at Ca^{2+} = 25 μequiv/L caused 60% mortality (LT50 = 170.0) preceded by marked ionic disturbances and metabolic acidosis, but little change in blood gases. Addition of Al (333 μg/L) increased mortality to >80% (LT50 = 78.2 h) with smaller ionic but greater respiratory disturbances.

L'importance relative de perturbations ionorégulatoires et respiratoires chez l'omble de fontaine (*Salvelinus fontinalis*) soumis à un stress acide/Al en eau douce dépend du pH et des teneurs en Ca^{+2} de l'eau. Des ombles acclimatés à des teneurs en Ca^{+2} de 25 ou 400 μequiv/L ont été munis de cathéters artériels puis exposés pendant 10 d à diverses conditions acides/Al dans un milieu à débit continu. Les paramètres suivants ont été quantifiés : pHa, Pa_{O_2}, Pa_{CO_2}, HCO_3^-, ΔH^+m, Na^+, Cl^-, K^+, Ca^{+2}, protéine, lactate, glucose, hémoglobine et hématocrite. L'exposition au pH 4,8 (absence d'Al) et à une teneur en Ca^{+2} de 25 μequiv/L a entraîné une faible perturbation physiologique mais aucune mortalité. L'apport d'Al (333 μg/L ou 12,3 μmol/L) a entraîné un taux de mortalité supérieur à 80 % (TL50 = 39,0 h); la mort a été précédée d'une baisse marquée des teneurs en Na^+ et Cl^- du plasma, d'une perturbation modérée de l'équilibre des gaz sanguins mais d'aucune acidose. À une teneur plus élevée en Ca^{+2} (400 μequiv/L), cette même exposition (pH = 4,8, Al = 333 μg/L) a entraîné un taux de mortalité semblable (TL50 = 38,5 h) mais de plus faibles perturbations des ions, de plus grandes diminutions de la teneur en O_2 du sang, des augmentations de la teneur en CO_2 du sang et une acidose respiratoire. L'exposition au pH 4,4 (absence d'Al) à une teneur en Ca^{+2} de 25 μequiv/L a donné un taux de mortalité de 60 % (TL50 = 170,0 h) précédée de perturbations de l'équilibre ionique et d'une acidose métabolique marquées mais de faibles variations de l'équilibre des gaz sanguins. L'apport d'Al (333 μg/L) a porté le taux de mortalité à plus de 80 % (TL50 = 78,2 h) et a été accompagné de plus faibles perturbations de l'équilibre ionique mais de plus grandes perturbations respiratoires.

Received March 31, 1987
Accepted December 21, 1987
(J9212)

Reçu le 31 mars 1987
Accepté le 21 décembre 1987

It is now clear that low environmental pH, ambient calcium level (Ca^{2+}), and aluminum concentration (Al) interact with one another in an extremely complex fashion in causing toxicity to fish (Schofield and Trojnar 1980; Muniz and Leivestad 1980a, 1980b; Baker and Schofield 1982; Wood and McDonald 1982; Howells et al. 1983; Brown 1983; Howells 1984; Neville 1985; McDonald et al. 1988; Wood 1988). In broad overview, the major trends appear to be as follows: (i) increases in toxicity are associated with decreasing pH, with increasing Al, and with decreasing Ca^{2+}; (ii) the effect of Al varies with pH, changing from a greatly exacerbating influence under moderately acidic conditions to a neutral or even protective one at very low pH; (iii) there is a general protective effect of Ca^{2+} against both Al and acid toxicity, but the degree of protection is dependent upon the levels of these two toxicants; (iv) the toxic mechanism of pure acid stress involves only ionoregulatory disturbance, at least under environmentally realistic conditions of pH and Ca^{2+}; (v) the toxic mechanism of Al involves either ionoregulatory or respiratory disturbance or both, the severity and relative contribution of each being critically dependent upon the pH and absolute Al level.

This final conclusion is based on only a limited amount of physiological work involving measurements of ionic flux rates (Witters 1986; Booth et al. 1988), respiratory parameters (Rosseland 1980; Neville 1985), and blood gases, ions, and other circulating substances in single terminal blood samples (Muniz and Leivestad 1980a, 1980b; Leivestad et al. 1980; Neville 1985; Rosseland et al. 1986; Witters 1986; Booth et al. 1988). As yet there is no information on progressive changes in blood parameters in chronically catheterized animals during

acid/Al exposure. In the preceding paper (Booth et al. 1988), we have described the ion flux responses of noncannulated brook trout (*Salvelinus fontinalis*) to 10 d of exposure to a defined pH/Ca^{2+}/Al matrix in flowing soft water. In the present study, we report progressive changes in blood gases, acid–base status, electrolytes, metabolites, and hematology of chronically cannulated brook trout at selected levels of pH, Ca^{2+}, and Al. These levels were chosen to reflect those monitored during episodic acid surge events in the field while ensuring that the Al level (333 μg/L) was high enough to exert definite toxic effects but low enough to remain below the solubility limit at the tested pH's (Muniz and Leivestad 1980a; Schofield and Trojnar 1980; Johnson et al. 1981; McDonald et al. 1988). A pH of 4.4 is about the most severe acidity seen in the field, while 4.8 is more commonly encountered, but of little toxicity to brook trout by itself. However, these two pH's are far enough apart to greatly alter Al speciation. The Ca^{2+} levels chosen (25 and 400 μequiv/L) bracket the range of natural soft water.

The objectives were to correlate internal changes in blood composition with the external flux rate effects described by Booth et al. (1988) and to understand the toxic mechanism(s) of acid and Al action in relation to water pH and Ca^{2+} levels. Specifically we tested whether either lowered pH or elevated Ca^{2+} protected against the toxic effects of Al and whether the balance between respiratory and ionoregulatory effects could be shifted by changes in pH and Ca^{2+}.

Materials and Methods

Experimental Animals

Experiments were performed on 81 adult brook trout (150–350 g) at McMaster University, Hamilton, Ontario (altitude = 100 m). The fish were obtained, held, and acclimated to artificial soft water (Ca^{2+} = 25 or 400 μequiv/L, i.e. 0.5 or 8 mg/L; Na = 50–100 μequiv/L, i.e. 1.1–2.3 mg/L; pH = 6.3–6.7) as described by Booth et al. (1988). Feeding was stopped 7 d before cannulation to minimize possible effects on measured parameters of the unavoidable starvation during the experiments. All fish were fitted with chronic indwelling arterial catheters while under MS-222 anaesthesia (Sigma; 50 mg/L, buffered to pH ≃ 6.5). In preliminary trials, we found that dorsal aortic cannulation through the buccal cavity, as traditionally used for rainbow trout (*Salmo gairdneri*) (Soivio et al. 1972), had a lower success rate in brook trout because of slightly different anatomy. Instead, the caudal artery was exposed by a small incision in the peduncle, and a catheter (Clay–Adams PE50) was inserted into the dorsal aorta to about the level of the coeliac branch. The wound was dusted with the antibiotic Prefuran (Nifurpirinol, Argent Laboratories) and closed with silk suture. The catheter was filled with heparinized Cortland saline (Wolf 1963; Sigma sodium heparin, 50 IU/mL). After cannulation, fish were transferred to darkened Plexiglas chambers (volume = 2.7 L) of the design of McDonald and Rogano (1986) as used in the preceding study (Booth et al. 1988). Forty-eight to 72 h in flowing acclimation water was allowed for recovery before withdrawal of control blood samples.

Test Conditions

Fish were acclimated for at least 2 wk to the Ca^{2+} level used in the test condition. Experiments were performed at five different combinations of pH, Ca^{2+}, and Al, each involving 10 d of exposure to the test condition after sampling under the acclimation condition (control samples). The five combinations were chosen to assess Al effects at two different levels of acidity, to separate the effects of acid alone from acid plus Al stress, and to examine the influence of elevated ambient Ca^{2+} on the responses to one acid plus Al treatment. The test conditions were as follows: (1) pH = 4.8, Ca^{2+} = 25 μequiv/L, Al = 0 μg/L (N = 10); (2) pH = 4.8, Ca^{2+} = 25 μequiv/L, Al = 333 μg/L, i.e. 12.3 μmol/L (N = 19); (3) pH = 4.8, Ca^{2+} = 400 μequiv/L, Al = 333 μg/L (N = 28); (4) pH = 4.4, Ca^{2+} = 25 μequiv/L, Al = 0 μg/L (N = 11); (5) pH = 4.4, Ca^{2+} = 25 μequiv/L, Al = 333 μg/L (N = 13). Test waters were prepared daily in 1500-L batches by addition of NaCl, $CaCl_2 \cdot 2H_2O$, H_2SO_4, and $AlCl_3 \cdot 6H_20$ (all analytical grade) to deionized water generated by reverse osmosis or deionizing resin cannisters. One important difference from the protocol of Booth et al. (1988) was that acid plus Al were delivered to the fish from time 0, rather than after an initial 24 h of acid exposure alone.

The P_{O_2} of the inflowing water was maintained above 140 Torr and P_{CO_2} less than 1 Torr. The pH of inflowing water was set slightly below the desired value because of the alkalinizing influence of the fish (largely due to ammonia excretion) on the water as it passed through the chamber. Flow to each chamber was set to approximately 0.5 $L \cdot kg^{-1} \cdot min^{-1}$ and then adjusted throughout the experiment to keep the measured pH in the box within 0.1 unit of 4.4 or 4.8. The water was not recirculated, but rather led to waste after leaving the chamber so as to minimize speciation changes in Al. Total Al and Ca^{2+} levels in the inflowing and outflowing water were monitored daily; values deviated less than 10% from desired.

We avoided most of the complications associated with aging of test solutions, Al precipitation, organic complexation, and pH changes in the bulk water by using freshly prepared solutions which were moved quickly past the fish on a one-pass, flow-through basis. Al speciation can be predicted with some confidence from thermodynamic equilibrium constants for the dissolution of amorphous aluminum hydroxide at known pH and ionic strength (Johnson et al. 1981). At both pH = 4.4 and 4.8, 333 μg/L was well below the total solubility limit. At pH = 4.4, free Al^{3+} constituted about 77% of total Al, and the remainder was mainly $Al(OH)^{2+}$; at pH = 4.8, Al^{3+} was reduced to about 52%, $Al(OH)^{2+}$ was 33%, and $Al(OH)_2^+$ constituted 15%. These pH and Al levels are representative of conditions often seen during snowmelt or rainstorm runoff in acid-sensitive areas of eastern North America and Scandinavia.

Sampling Regimes

Blood samples were taken from each fish under the acclimation condition (control sample) and at 4, 18, 28, 42, 66, 114, 162, and 240 h after the start of the test condition, if death did not occur earlier. A "terminal sample" refers to the last one taken prior to death. Samples (700 μL) were drawn anaerobically into gas-tight, ice-cold Hamilton syringes via the arterial catheters, without disturbance to the animals, and replaced immediately by reinfusion of Cortland saline. Samples were analyzed for pH, O_2 tension, total CO_2 (whole blood and true plasma), hematocrit (Ht), hemoglobin (Hb), lactate, glucose, and plasma levels of Na^+, Cl^-, K^+, Ca^{2+}, and total protein.

Analytical Techniques

Arterial blood pH (pHa) and O_2 tension (Pa_{O_2}) were determined with Radiometer microelectrodes (E5021, E5046) kept

at the experimental temperature and connected to a Radiometer PHM 71 or 72 acid–base analyzer. Total CO_2 (Ca_{CO_2}) was measured on true plasma and whole blood using either a Corning 965 CO_2 analyzer or the Radiometer CO_2 microelectrode (E5036) method of Cameron (1971). Plasma was obtained by centrifuging 80 μL of whole blood in heparinized microhematocrit tubes at 5000 × *g* for 5 min. The Ht was read directly from the tube, which was then broken to allow aspiration of the plasma into a Hamilton syringe for transfer to the CO_2 analyzer or Cameron chamber. Hb was measured colorimetrically using the cyanmethemoglobin method and Sigma reagents (Blaxhall and Daisley 1973). Lactate was measured enzymatically (L-lactate dehydrogenase/NADH method; Loomis 1961; Sigma reagents) on 100 μL of whole blood which had been immediately deproteinized in 200 μL of ice-cold 8% perchloric acid. Glucose was determined by one of two methods. The colorimetric *O*-toluidine method of Hyvarinon and Nikkita (1962) used 100 μL of whole blood which had been immediately deproteinized in 900 μL of ice-cold 3% trichloroacetic acid. Alternatively, glucose was determined enzymatically on 10 μL of plasma by the hexokinase method of Bondar and Mead (1974). The two methods were cross-validated; both employed Sigma reagents.

The remainder of the blood sample was centrifuged at 9000 × *g* for 2 min to separate plasma. Plasma protein was determined with an American Optical Goldberg refractometer (Alexander and Ingram 1980). Plasma Na^+, K^+, and Ca^{2+} were appropriately diluted and measured against known standards by atomic absorption spectrophotometry (Varian 1275). Cl^- was determined by coulometric titration (Radiometer CMT10) or by the mercuric thiocyanate spectrophotometric method of Zall et al. (1956) using Sigma reagents; the two methods were cross-validated.

Water total Al levels were routinely monitored by the pyrocatechol violet method of Dougan and Wilson (1974), Na^+ and Ca^{2+} by atomic absorption, and pH by a Radiometer GK2401C electrode and PHM82 meter.

Calculations

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

$$(1)\quad Pa_{CO_2} = \frac{Ca_{CO_2}}{\alpha CO_2 \cdot (1 + \text{antilog}(pHa - pk'))}$$

where Ca_{CO_2} was measured in true plasma and values of αCO_2 and pK′ at the appropriate temperature were taken from tabulated values for trout plasma (Boutilier et al. 1984). Bicarbonate concentrations in whole blood and true plasma were calculated by

$$(2)\quad [HCO_3^-] = Ca_{CO_2} - (\alpha CO_2 \cdot Pa_{CO_2})$$

using the corresponding measurements of Ca_{CO_2}. As plasma HCO_3^- levels were always very similar to (and slightly less than) plasma Ca_{CO_2} levels, only the former have been reported. The concentration of metabolic H^+ (ΔH^+m) added to the whole blood was calculated in the cumulative fashion described by McDonald et al. (1980) using the following equation for each interval:

$$(3)\quad [\Delta H^+m] = [HCO_3^-]_1 - [HCO_3^-]_2 - \beta(pHa_1 - pHa_2)$$

and summing (taking account of sign) for each period from the control sample onwards. In this equation, values of $[HCO_3^-]$ are for whole blood, and β represents the nonbicarbonate buffer value for whole blood. The value of β is largely determined by the Hb concentration (Wood et al. 1982). Therefore, we estimated β from the measured [Hb] at each time, using a regression equation derived from in vitro P_{CO_2} equilibration of whole blood from brook trout acclimated to soft water (R. L. Walker and C. E. Booth, unpubl. results):

$$(4)\quad \beta = -0.942[Hb] - 2.84 \qquad (r = 0.97, N = 5, p < 0.01).$$

Hb-bound O_2 content per unit hemoglobin ($[O_2]/[Hb]$) was estimated from the measured Pa_{O_2} and pHa at each time, using a family of O_2 dissociation curves determined in vitro for brook trout blood at a range of P_{CO_2}'s (R. L. Walker and C. E. Booth, unpubl. results). Mean cell Hb concentration (MCHC, grams Hb per millilitre of red cells) was calculated as the ratio of the Hb and Ht measurements.

Treatment of Data

Survival curves were compared using standard log/probit analysis and nomographic methods to determine median survival times (LT50), their 95% confidence limits (CL), slope functions (*S*), and the significance of differences between treatments (Litchfield 1949; Litchfield and Wilcoxin 1949). Fish that may have died from factors unassociated with the test condition (e.g. Ht < 6%, bleeding, cannula failure) were excluded from the analysis.

The analysis and presentation of physiological data representative of group responses is complex when individual members of the group die at different times during the test conditions, as was the case in the present study. Simple averaging of all data at a particular time can be very misleading because the fish showing the greatest disturbances are often those which die first; loss of their values from the mean at subsequent sample times gives an incorrect impression of recovery. This problem was avoided in two ways. In the first, means (± 1 SE, *N*) were presented up to the 42-h sample for all fish in a group which survived beyond this time. As 42 h was longer than the LT50 in several test conditions, this analysis showed trends representative of the most resistant fish in the group. In the second approach, the last measurements prior to death ("terminal samples") for each fish in a group were tabulated as means (± 1 SE, *N*), irrespective of when the fish died during the 10-d exposure. These terminal means were compared with the initial means for the same fish during the control period. This analysis illustrated the disturbances which immediately preceded death and therefore more clearly indicated key mechanisms of lethality under different conditions. In both cases, there was a paired design, so Student's two-tailed paired *t*-test was used to compare means back to the control mean for those particular fish. Student's two-tailed unpaired *t*-test was used for comparisons between groups. A 5% significance level was employed throughout.

Results

Mortality

Exposure to pH = 4.8, Ca^{2+} = 25 μequiv/L in the absence of Al caused no mortality over 10 d (Fig. 1). Addition of Al (333 μg/L) resulted in >80% mortality with an LT50 (± 95% CL) of 39.0 h (29.5–51.5). At higher Ca^{2+}, this same exposure (pH = 4.8, Ca^{2+} = 400 μequiv/L, Al = 333 μg/L) caused

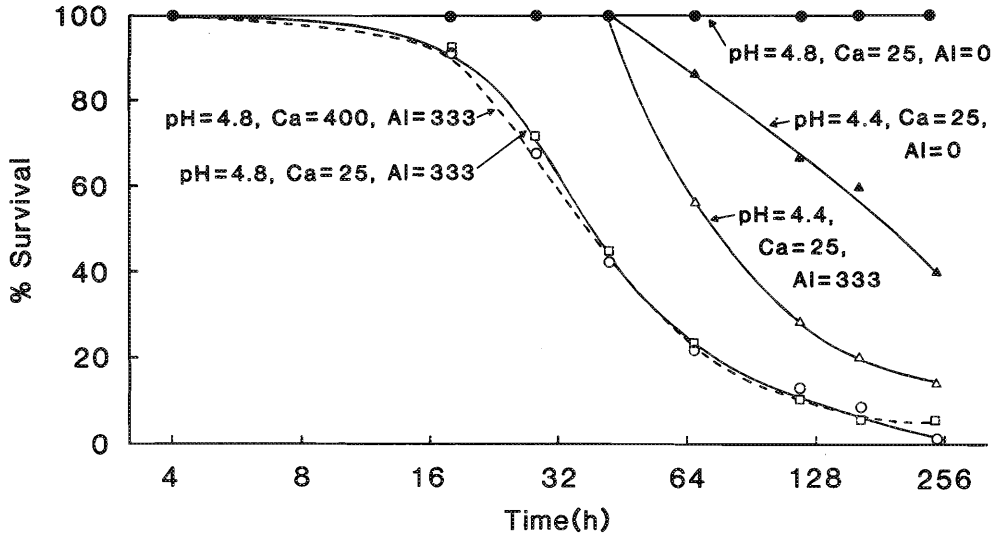

FIG. 1. Survival curves for chronically cannulated brook trout exposed to five different pH/Ca^{2+}/Al conditions for a 10-d period. The time scale is logarithmic. Ca^{2+} concentrations are in μequiv/L; Al concentrations are in μg/L.

an almost identical pattern of mortality with unchanged LT50 = 38.5 h (30.3–48.9).

At lower pH (pH = 4.4, Ca^{2+} = 25 μequiv/L), acid exposure alone caused 60% mortality with an LT50 of 170.0 h (103.6–278.8). Addition of Al (333 μg/L) increased mortality to >80% and significantly decreased median survival time to 78.2 h (50.6–120.1). This LT50 was significantly longer than in the same exposure at higher pH (pH = 4.8, Ca^{2+} = 25 μequiv/L, Al = 333 μg/L). There were no significant differences among the slope functions (*S*) of the log time/probit mortality curves in any of the five test conditions.

Relative to the noncannulated fish of Booth et al. (1988), these mortality rates were comparable in the pH = 4.8 tests at low Ca^{2+}, but significantly greater at high Ca^{2+} and in the pH = 4.4 tests. This suggests that cannulation and blood sampling may reduce tolerance.

Effects of Moderate Acidity Alone

Exposure to pH = 4.8, Ca^{2+} = 25 μequiv/L, Al = 0 μg/L had negligible influence on most measured parameters. Major plasma electrolytes (Fig. 2A; Table 1), MCHC (Fig. 3A), and lactate and glucose (Table 2) all remained unchanged throughout 10 d. The only exceptions were a very small but significant drop in pHa (<0.1 unit; Fig. 4A), which was fully corrected by 114 h, a slight rise in plasma K^+ (Table 1), and an initial increase in Pa_{O_2} (Fig. 8A). In terms of standard acid–base terminology (Davenport 1974), the acidosis was entirely of "metabolic" origin (Fig. 6A), for there was no "respiratory" component, i.e. no change in Pa_{CO_2} (Fig. 5A). Blood lactate remained constant (Table 2). The rise in Pa_{O_2} (Fig. 8A) had no effect on $[O_2]$/[Hb] (Fig. 9A) because the Hb was already saturated under control conditions.

Effects of Al in Combination with Moderate Acidity

The presence of Al (333 μg/L), at pH (4.8) and Ca^{2+} levels (25 μequiv/L), which were shown to be nonlethal in the preceding section, killed more than half the fish before the 42-h sample. Ionoregulatory disturbance was prominent, with significant decreases in plasma Na^+ and Cl^- appearing by 18 h, even in the most resistant fish (Fig. 2B). In terminal samples, Na^+ levels fell to ~109 and Cl^- to ~97 mequiv/L (Fig. 2B), and there was substantial swelling of the red cells as evidenced by the decline in MCHC (Fig. 3B). Acid–base status was little affected (Fig. 4B,5B,6B), but there was a significant rise in Pa_{CO_2} (Fig. 7B) and reciprocal fall in Pa_{O_2} (Fig. 8B) in dying fish. The latter was associated with about a 25% drop in oxygenation of the Hb (Fig. 9B) and a significant rise in blood lactate (Table 2). The rise in blood lactate was also seen in the 42-h survivors (Table 2). While the decline in plasma Na^+ and Cl^- appeared to be the most serious of these disturbances, neither this nor the respiratory effects (i.e. changes in Pa_{O_2} and Pa_{CO_2}) prior to death were as large as in other test conditions where one or the other mechanism clearly predominated (see below). The results therefore suggest a compound mechanism of toxicity.

Effects of Higher Ca^{2+} in the Presence of Al at Moderate Acidity

The time course and extent of mortality during exposure to Al (333 μg/L) at pH = 4.8 were unchanged by the presence of higher Ca^{2+} (400 μequiv/L), but there were substantial differences in blood chemistry. The loss of plasma electrolytes was reduced, with terminal levels ($Na^+ \simeq 128$, $Cl^- \simeq 112$ mequiv/L; Fig. 2C) significantly higher than at the same pH and Al levels at low Ca^{2+} (Fig. 2B). However, the respiratory disturbance was much more severe. Pa_{O_2} fell below 30 Torr, even in the most resistant trout (Fig. 8C), and this, together with an accompanying acidosis, lowered $[O_2]$/[Hb] by 50–60% (Fig. 9C). Lactate increased markedly prior to death (Table 2). Pa_{CO_2} more than doubled (Fig. 7C), in mirror image to the fall in Pa_{O_2}. This increase in Pa_{CO_2} caused a classic respiratory acidosis, pHa falling by 0.2–0.3 unit (Fig. 4C), while plasma HCO_3^- rose significantly (Fig. 5C). Despite the considerable lactate release (Table 2), there was no metabolic component to the acidosis (Fig. 6C). Other prominent effects were significant increases in plasma K^+ and protein concentrations (Table 1) and a red cell swelling which was the most marked of any of the test conditions (Fig. 3C). On balance, these results indicate

that respiratory disturbance (i.e. interference with O_2 and CO_2 exchange), rather than ionic dilution, was the cause of mortality in this test condition.

Effects of More Severe Acidity Alone

Exposure to pH = 4.4 alone (Ca^{2+} = 25 μequiv/L, Al = 0 μg/L) had more pronounced effects than to pH = 4.8 alone, resulting in 60% mortality over 10 d. Ionoregulatory failure was the prominent feature of the internal disturbance. Plasma Na^+ and Cl^- declined progressively, the trend becoming significant after only 4 h of exposure, even in the most resistant fish (Fig. 2D). Terminal ion levels ($Na^+ \simeq 96$, $Cl^- \simeq 85$ mequiv/L) were significantly lower than in any other treatment, and MCHC fell substantially prior to death (Fig. 3D). A classic metabolic acidosis developed, reflected in progressive decreases in pHa (Fig. 4D) and plasma HCO_3^- (Fig. 5D) and an accumulation of metabolic H^+ (ΔH^+m) in the blood (Fig. 6D). Lactate increased significantly (Table 2), but the increase was too small to fully account for the ΔH^+m. As Pa_{CO_2} remained unchanged (Fig. 7D), there was no respiratory component to the acidosis. Similarly, Pa_{O_2} (Fig. 8D) and $[O_2]/[Hb]$ (Fig. 9D) were virtually unaffected, even in dying fish; the slight decline in the latter was entirely attributable to acidosis. In summary, these data indicate that under pure acid stress, the toxic mechanism is closely associated with ionoregulatory failure, and perhaps exacerbated by metabolic acidosis. There appears to have been no direct disturbance of respiratory gas exchange.

Effects of Al in Combination with More Severe Acidity

The presence of Al (333 μg/L) at pH = 4.4, Ca^{2+} = 25 μequiv/L increased mortality and decreased survival time, but the LT50 was significantly longer than under the same Ca^{2+} and Al levels at pH = 4.8. In general the internal changes were similar to these seen with pH = 4.4 alone, but with a superimposed disturbance of O_2 and CO_2 levels and less ionic dilution prior to death. Decreases in major plasma electrolytes were similar to those at pH = 4.4 alone up to 42 h in surviving trout, but the absolute levels ($Na^+ \simeq 111$, $Cl^- \simeq 97$ mequiv/L) prior to death were not as low (Fig. 2E versus 2D). Indeed, these terminal ion levels were close to those in dying fish at pH = 4.8, Ca^{2+} = 25 μequiv/L, Al = 333 μg/L, where respiratory disturbance was similarly seen. Depressions of pHa (Fig. 4E) and plasma HCO_3^- (Fig. 5E) and elevations of ΔH^+m (Fig. 6E) reflected a metabolic acidosis similar to that at pH = 4.4 in the absence of Al (cf. Fig. 4D,5D,6D). O_2 and CO_2 disturbance was negligible in surviving fish up to 42 h, but was clearly seen in the terminal measurements from dying fish. Pa_{CO_2} (Fig. 7E) and lactate (Table 2) rose significantly before death, the former contributing a respiratory component to the acidosis, while Pa_{O_2} (Fig. 8E) and $[O_2]/[Hb]$ (Fig. 9E) fell. On balance, the results suggest a compound mechanism of toxicity involving both ionoregulatory and gas exchange impairment, similar to that seen at pH = 4.8, Ca^{2+} = 25 μequiv/L, Al = 333 μg/L.

Discussion

Responses to Acid Stress Alone

Salvelinus fontinalis is considered one of the most resistant of the salmonid species to environmental acidity (Grande et al. 1978), a conclusion supported by an extensive toxicological literature (e.g. Menendez 1976; Trojnar 1977; Swarts et al.

TABLE 1. Plasma potassium, calcium, and protein concentrations in 42-h surviving fish (control and 42-h values) and in fish which died at any time (initial and terminal values) during 10 d of exposure to five different pH/$[Ca^{2+}]$/[Al] conditions. Initial values were taken during the control period, and terminal values represent the last measurements prior to death. Means ± 1 SEM.

Test condition			K^+ (mequiv/L)					Ca^{2+} (mequiv/L)					Plasma Protein (g/100 mL)				
			42-h survivors			Mortalities		42-h survivors			Mortalities		42-h survivors			Mortalities	
pH	$[Ca^{2+}]$ (μequiv/L)	[Al] (μg/L)	Control	42 h	*N*	Initial	Terminal	Control	42 h	*N*	Initial	Terminal	Control	42 h	*N*	Initial	Terminal
4.8	25	0	2.58 ± 0.08	2.92 ± 0.04[a]	9	2.58 ± 0.08	2.95 ± 0.14[b]	4.57 ± 0.37	4.24 ± 0.28	9	4.57 ± 0.37	4.46 ± 0.29[b]	3.40 ± 0.30	3.28 ± 0.34	9	3.40 ± 0.30	3.60 ± 0.45[b]
4.8	25	333	3.67 ± 0.63	4.80 ± 0.68	4	3.96 ± 0.36	4.64 ± 0.48	4.65 ± 0.55	4.56 ± 0.32	7	4.41 ± 0.15	4.47 ± 0.23	4.10 ± 0.28	4.10 ± 0.30	7	3.55 ± 0.19	3.79 ± 0.28
4.8	400	333	2.72 ± 0.29	3.53 ± 0.23[a]	11	2.92 ± 0.18	3.83 ± 0.14[a]	5.86 ± 0.65	5.76 ± 0.45	12	4.62 ± 0.32	5.19 ± 0.22	3.14 ± 0.20	3.57 ± 0.32	12	2.88 ± 0.12	3.55 ± 0.20[a]
4.4	25	0	2.59 ± 0.21	2.36 ± 0.26	8	2.78 ± 0.22	2.85 ± 0.36	4.31 ± 0.26	4.02 ± 0.15	8	4.50 ± 0.19	3.93 ± 0.38	2.85 ± 0.14	2.69 ± 0.16	8	2.88 ± 0.15	3.20 ± 0.38
4.4	25	333	3.10 ± 0.39	2.74 ± 0.34	9	3.12 ± 0.59	2.62 ± 0.51	4.66 ± 0.21	3.98 ± 0.15[a]	9	4.35 ± 0.09	3.70 ± 0.15[a]	3.01 ± 0.16	3.02 ± 0.14	9	2.75 ± 0.11	2.92 ± 0.06

[a] $p < 0.05$ relative to appropriate "control" or "initial" value.
[b] Not terminal when sacrificed after 10 d of exposure.

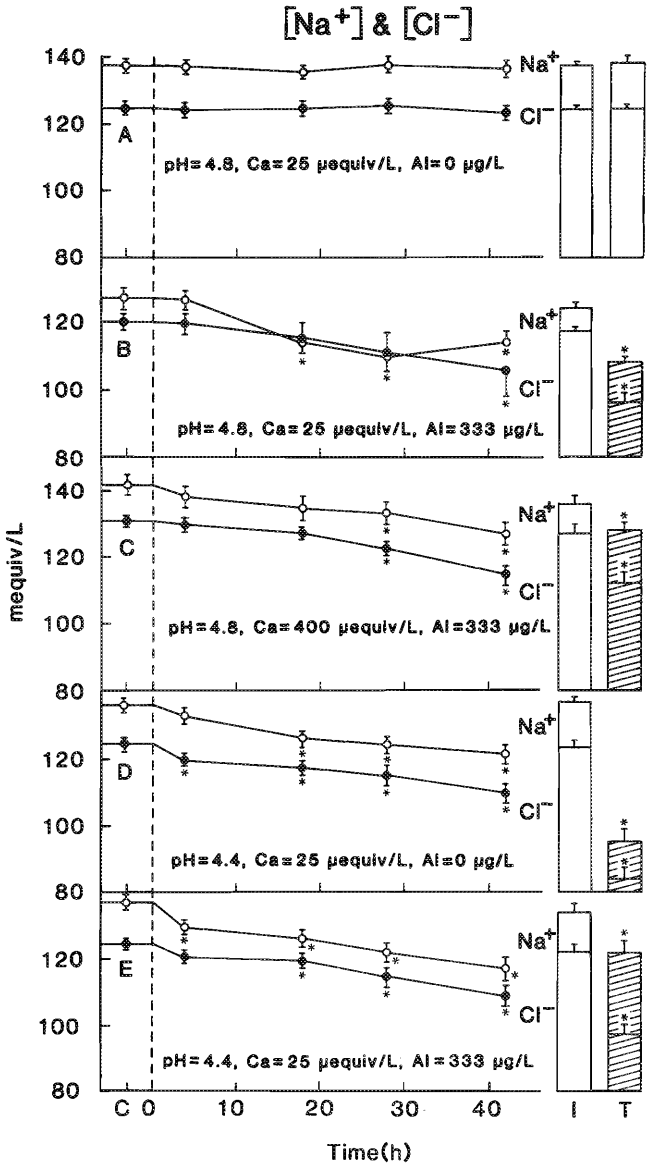

FIG. 2. Changes in plasma Na^+ (open circles, upper bar graphs) and Cl^- concentrations (closed circles, lower bar graphs) in chronically cannulated brook trout during 10 d of exposure to five different pH/Ca^{2+}/Al conditions in flowing soft water. Data are shown from the control period until 42 h for all fish which survived beyond this time in each test condition (i.e. the most resistant fish in the group). Additionally, terminal data (T; cross-hatched bars) representing the last measurements prior to death in fish dying at any time during the 10 d of exposure are shown in the bar graphs at the right and compared with initial measurements (I; open bars) during the control period for these same fish. In Fig. 2A, none of the fish died, so data taken when the fish were sacrificed at 10 d have been substituted for terminal data. Values are means ± 1 SEM for (A) $N = 9$; (B) $N = 7$ for 42-h survivors, $N = 14$ for mortalities; (C) $N = 12$ for 42-h survivors, $N = 24$ for mortalities; (D) $N = 8$ for 42-h survivors, $N = 6$ for mortalities; and (E) $N = 9$ for 42-h survivors, $N = 6$ for mortalities. Asterisks indicate means significantly different ($p < 0.05$) from the respective control mean (42-h survivors) or initial mean (mortalities) taken during the control period.

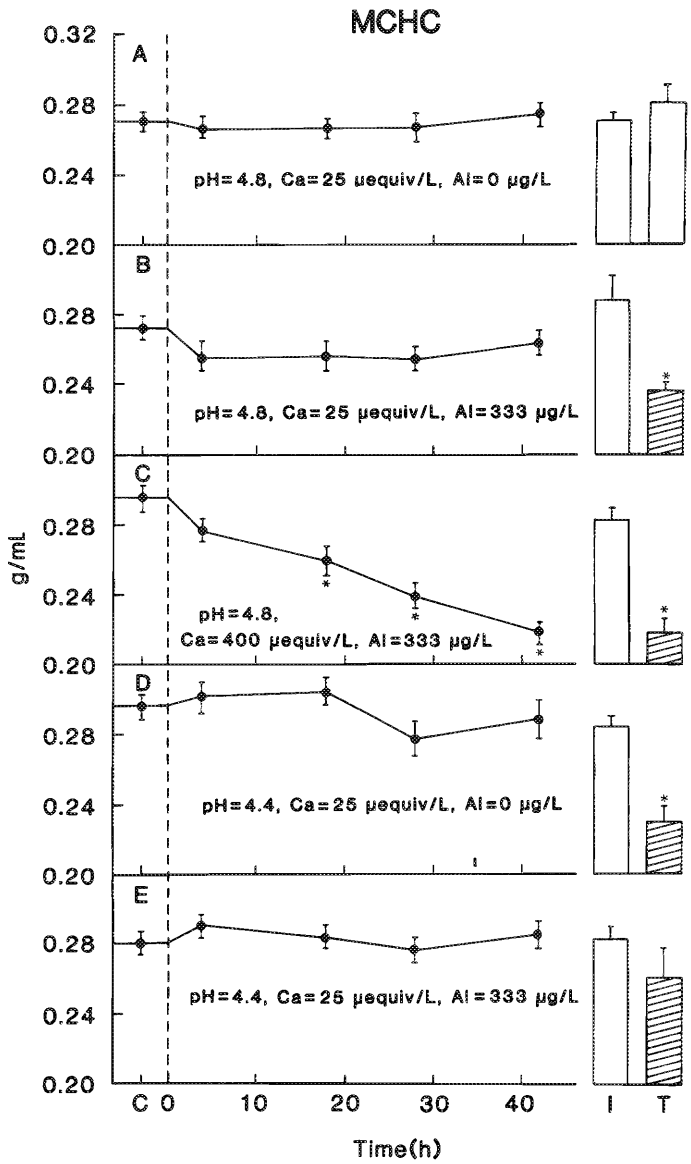

FIG. 3. Changes in mean red blood cell hemoglobin concentration (MCHC) in chronically cannulated brook trout during 10 d of exposure to five different pH/Ca^{2+}/Al conditions in flowing soft water. Other details as in legend to Fig. 2.

1978; Schofield and Trojnar 1980; Baker and Schofield 1982). Nevertheless, relevant physiological data on brook trout under acid stress are sparse, limited to studies at pH's below 4.0 and/or involving blood sampling from uncannulated fish in water of unknown composition (Vaala and Mitchell 1970; Mudge and Neff 1971; Dively et al. 1977; Packer 1979). These methodological differences are so great as to render comparison with the present data impractical. However, there exists extensive information on the more sensitive rainbow trout, obtained by comparable cannulation techniques under similar test conditions (cf. Wood and McDonald 1982; McDonald 1983a; Wood 1988 for review).

In the present study, exposure to pH = 4.4 at very low Ca^{2+} (25 µequiv/L) resulted in 60% mortality over 10 d and a severe ionic dilution of the blood plasma in dying brook trout, without evidence of respiratory disturbance. There was also a moderate metabolic acidosis, and a fall in MCHC. Responses to pH = 4.8 without Al were negligible, apart from a small, rapidly corrected acidosis, again of metabolic origin. Ionoregulatory failure as the proximate toxic mechanism in the pH range 4.0–4.5 is entirely consistent with the rainbow trout data (cf. McDonald 1983b; Wood 1988). As in rainbow trout, this effect

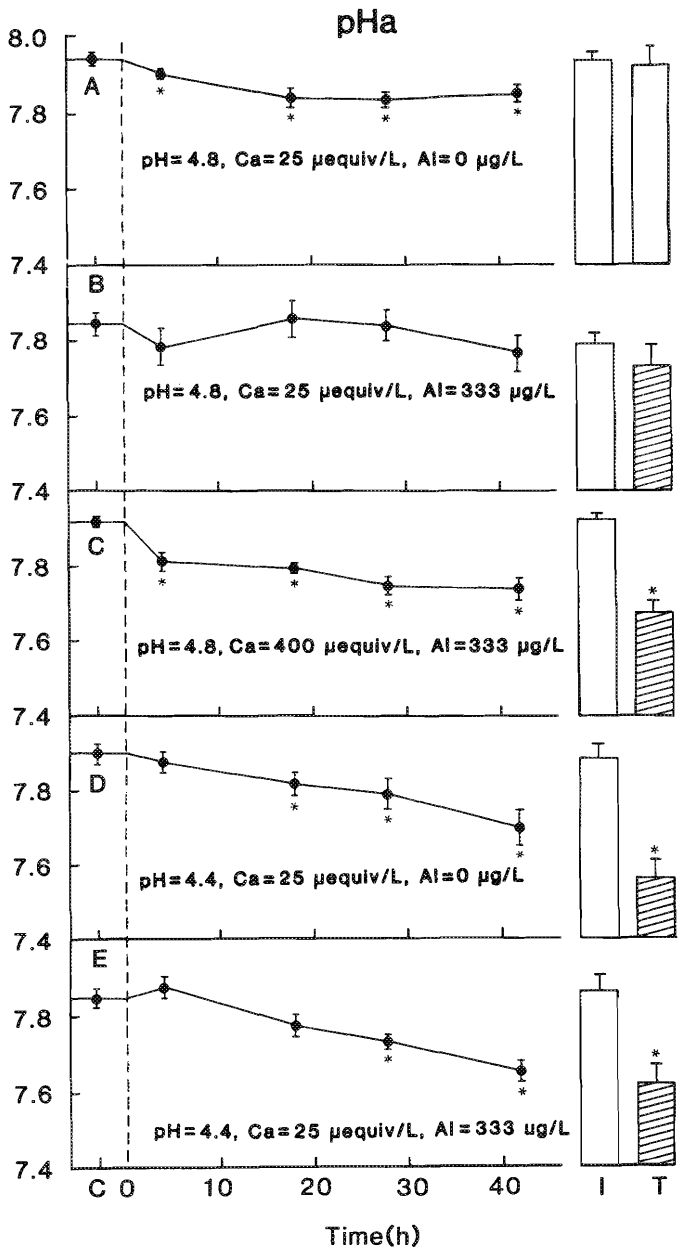

FIG. 4. Changes in arterial blood pH (pHa) in chronically cannulated brook trout during 10 d of exposure to five different pH/Ca^{2+}/Al conditions in flowing soft water. Other details as in legend to Fig. 2.

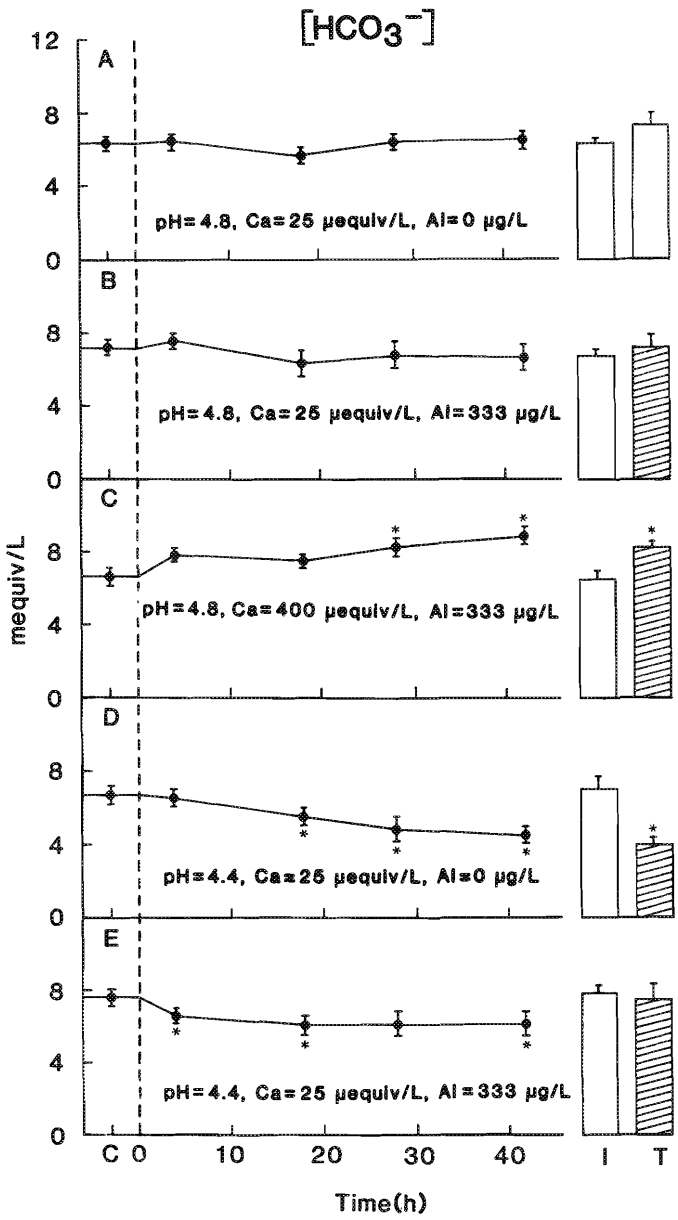

FIG. 5. Changes in the HCO_3^- concentration of arterial blood plasma in chronically cannulated brook trout during 10 d of exposure to five different pH/Ca^{2+}/Al conditions in flowing soft water. Other details as in legend to Fig. 2.

was due to a stimulation of passive Na^+ and Cl^- efflux across the gills and a more persistent inhibition of active influx, both of which were demonstrated in the preceding study (Booth et al. 1988). These results agree with the early work of Packer and Dunson (1970, 1972) on brook trout. The decrease in MCHC (Fig. 3D) was also similar to the rainbow trout response, resulting from erythrocytic swelling in response to ionic dilution of the plasma, as discussed by Milligan and Wood (1982). However, the occurrence of a moderate metabolic acidosis was surprising because rainbow trout exhibit this response only in acidified hard water. In acidified soft water, similar to the present test conditions, they show virtually unchanged pHa because at low Ca^{2+} levels the net losses of Na^+ and Cl^- are equal, preventing net H^+ "entry" (McDonald 1983b; Wood 1988). The relationship between water Ca^{2+} and net Na^+ and Cl^- losses is presumably different in brook trout.

There was no blood glucose response during acid exposure or any other test condition of the present study, even in dying fish (Table 2). Its absence may be related to the pre-experimental starvation period. Glucose mobilization is considered a general "stress" response in fish, as well as serving an osmoeffector function during acid exposure in rainbow trout (McDonald 1983b; Brown et al. 1984). Plasma Ca^{2+} levels were also generally unresponsive (Table 1), but this was in accord with rainbow trout data (McDonald et al. 1980). Plasma K^+ concentrations tended to increase (Table 1) despite increased branchial losses in many of the treatments (Booth et al. 1988). This has been commonly observed in acid-stressed rainbow trout and reflects both hemoconcentration and an efflux of K^+ from the intracellular compartment of white muscle (McDonald et al. 1980; Wood and McDonald 1982).

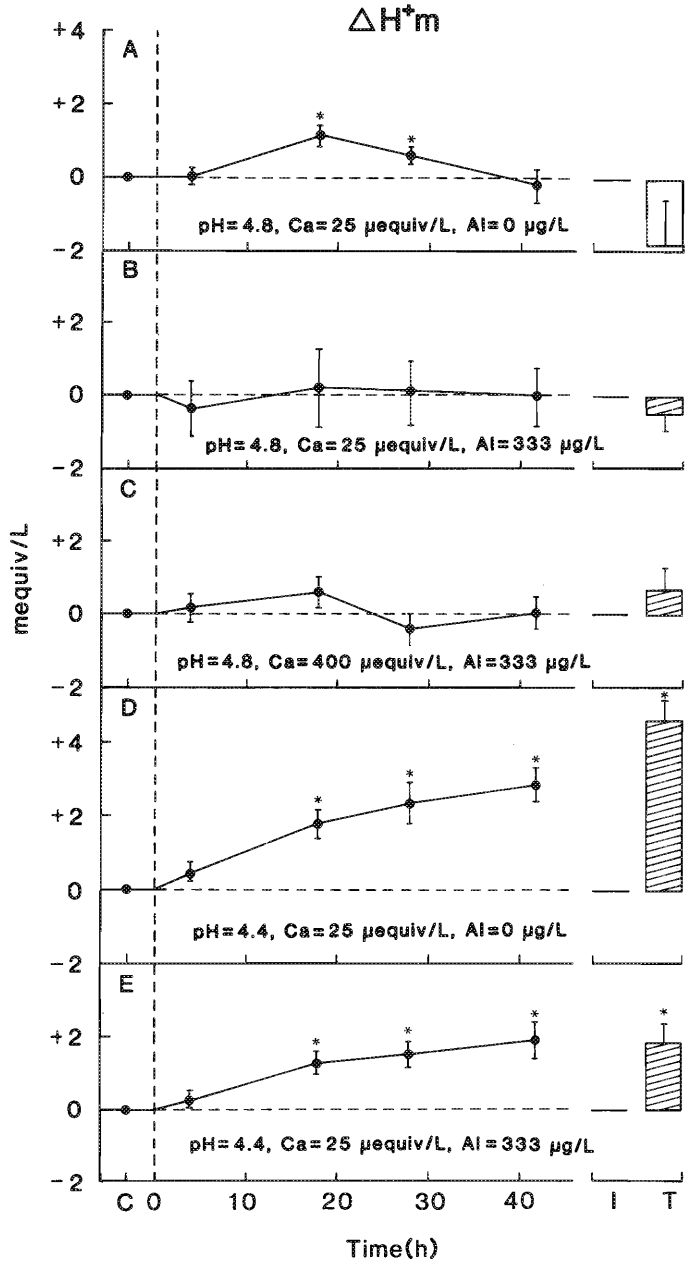

FIG. 6. Changes in the calculated metabolic acid load (ΔH^+m) in whole blood of chronically cannulated brook trout during 10 d of exposure to five different pH/Ca^{2+}/Al conditions in flowing soft water. By definition, ΔH^+m is zero in the control or initial samples. Other detals as in legend to Fig. 2.

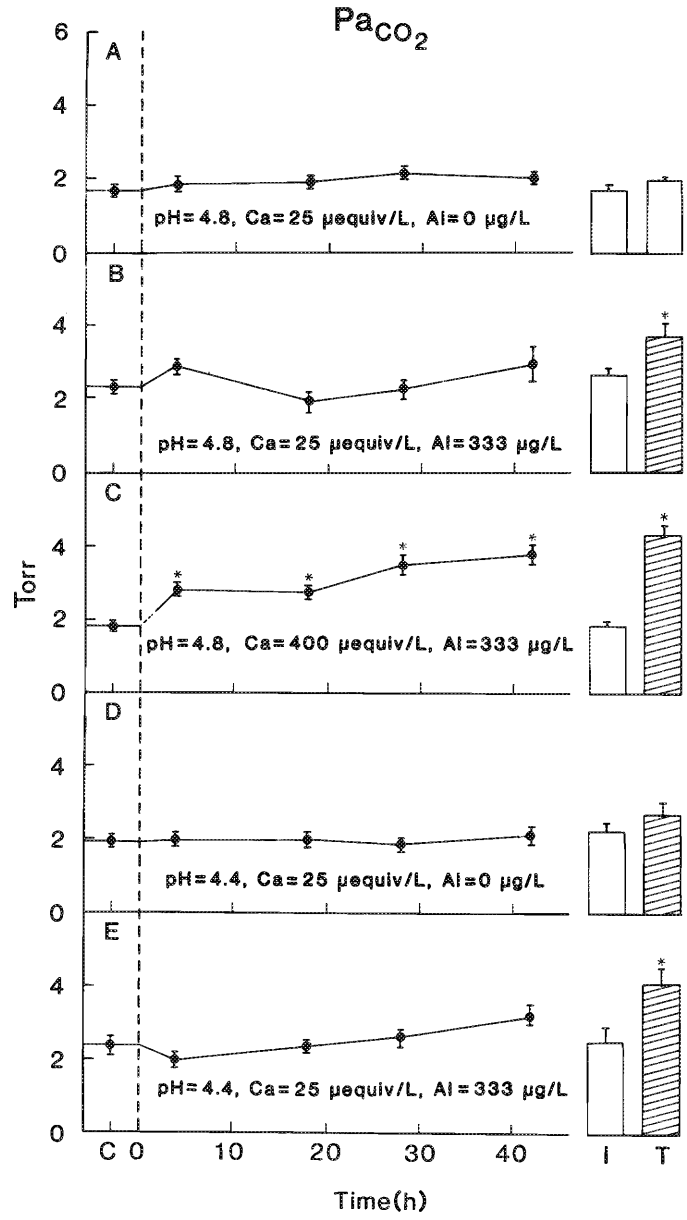

FIG. 7. Changes in the CO_2 tension (Pa_{CO_2}) of arterial blood in chronically cannulated brook trout during 10 d of exposure to five different pH/Ca^{2+}/Al conditions in flowing soft water. Other details as in legend to Fig. 2.

Responses to Acid plus Al Stress

The presence of Al clearly increased toxicity relative to acid alone at both pH = 4.8 and 4.4 at very low Ca^{2+} (25 μequiv/L). In terms of mortality and LT50, this effect was much more dramatic at pH = 4.8 (Fig. 1), but changes in blood chemistry were similar at the two pH's. Ionoregulatory failure occurred at both pH's in the presence of Al (while it did not at pH = 4.8 in the absence of Al), but the terminal plasma Na^+ and Cl^- levels in dying fish were not as low as at pH = 4.4 in the absence of Al (Fig. 2). There are two related reasons for this difference.

Firstly, in the presence of Al, the fish died more quickly (Fig. 1) and lost Na^+ and Cl^- more rapidly to the water, as demonstrated in the preceding flux study (Booth et al. 1988). This suggests that the rate, rather than the absolute loss, may be the more important factor involved in lethality, probably because of the resultant internal fluid shifts and circulatory collapse accompanying high Na^+ and Cl^- efflux rates (Milligan and Wood 1982; McDonald 1983b; Wood 1988). At pH = 4.4 in the absence of Al, the fish lost Na^+ and Cl^- more slowly and therefore could withstand lower absolute concentrations of these ions in the blood plasma prior to death.

Secondly, pure ionoregulatory failure was not the sole cause of death, for there was also clear evidence of disturbance to respiratory gas exchange. This was reflected in elevations of Pa_{CO_2} (Fig. 7B,7E) and lactate (Table 2) and depressions of Pa_{O_2} (Fig. 8B,8E) which resulted in 25–35% decreases in the O_2 saturation of the Hb (Fig. 9B,9E). Respiratory problems probably accelerated the death of the fish, so again, plasma ion

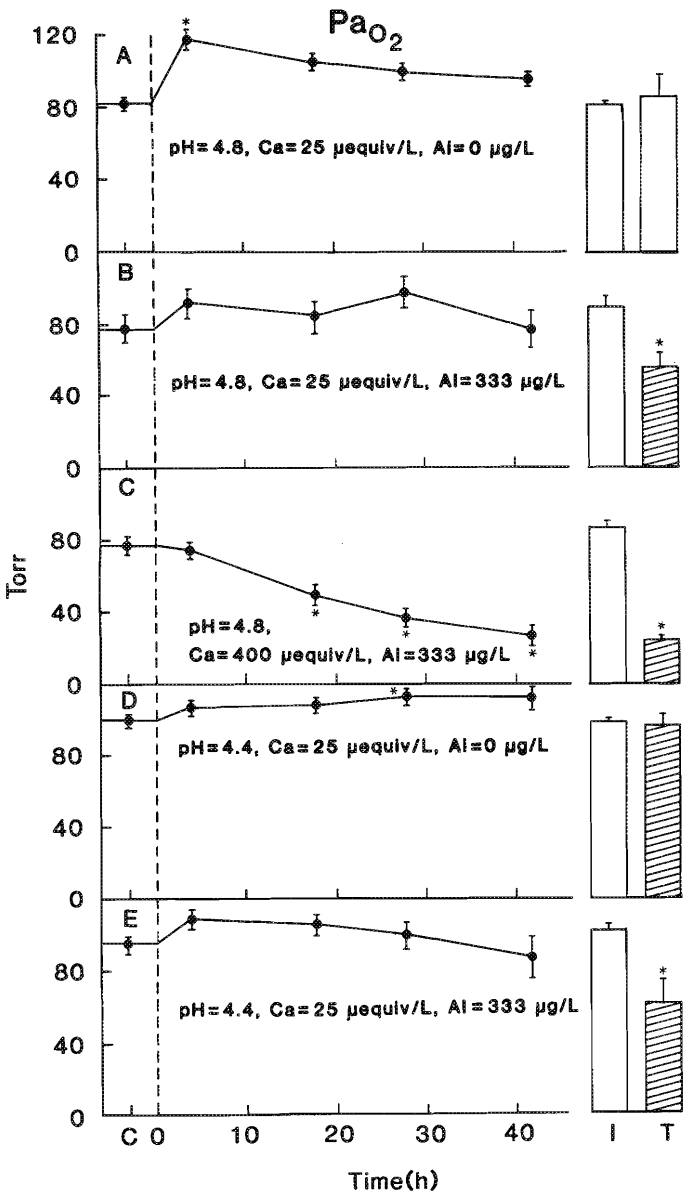

FIG. 8. Changes in the O_2 tension (Pa_{O_2}) of arterial blood in chronically cannulated brook trout during 10 d of exposure to five different pH/Ca^{2+}/Al conditions in flowing soft water. Other details as in legend to Fig. 2.

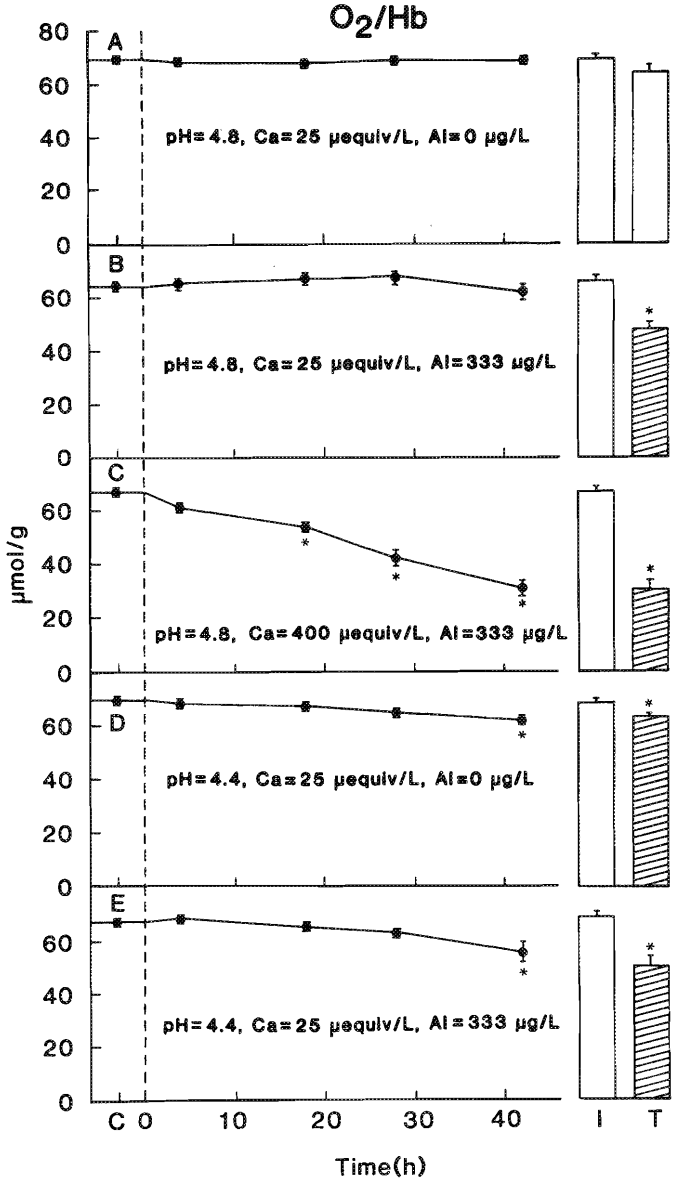

FIG. 9. Changes in the hemoglobin-bound O_2 content per unit hemoglobin ($[O_2]/[Hb]$) of arterial blood in chronically cannulated brook trout during 10 d of exposure to five different pH/Ca^{2+}/Al conditions in flowing soft water. Other details as in legend to Fig. 2.

levels did not fall as low as when fish died more slowly from pure ionoregulatory failure.

The present finding that Al exacerbates acid toxicity at both pH = 4.4 and 4.8, but to a greater extent at the higher pH, is in general accord with previous toxicological studies on brook trout (Schofield and Trojnar 1980; Baker and Schofield 1982). There have been no previous physiological studies on brook trout under combined acid plus Al stress, but several studies have been done on other salmonids (Muniz and Leivestad 1980a, 1980b; Leivestad et al. 1980; Rosseland 1980; Neville 1985; Rosseland et al. 1986; Witters 1986). While none of these studies are directly comparable, there does seem to be general agreement that Al causes both ionoregulatory and respiratory disturbances and that these effects are more intense at higher (but still acidic) pH's, in accord with the present data. The most detailed of these investigations, that of Neville (1985) on rainbow trout, concluded that the fish died from electrolyte loss at pH = 4.0–4.5 but from hypoxia at pH = 6.1, with a transition in causation between these levels. Taking into account the greater tolerance of brook trout for low pH, our data do not conflict with Neville's (1985) interpretation.

In the preceding study (Booth et al. 1988), we have shown that the effect of Al on branchial ion balance, over and above that due to acidity alone, was primarily increased efflux of Na^+ and Cl^-, combined with a small further inhibition of the active uptake of these ions. The effect was greater at pH = 4.8 than at 4.4 and was associated with the accumulation of Al on the gills; dying fish showed the greatest branchial Al burdens. Other studies have demonstrated branchial mucification, edema, and even lamellar fusion in Al-exposed fish (Muniz and Leivestad 1980a, 1980b; Schofield and Trojnar 1980; Tandjung 1982; Karlsson-Norrgren et al. 1986). A present working hypothesis is that precipitation of aluminum hydroxides and/or binding of Al to organic anions occurs on the gill surface, inducing an inflammatory response. In turn, this stimulates mucus produc-

TABLE 2. Blood lactate and glucose concentrations in 42-h surviving fish (control and 42-h values) and in fish which died at any time (initial and terminal values) during 10 d of exposure to five different pH/[Ca^{2+}]/[Al] conditions. Initial values were taken during the control period, and terminal values represent the last measurements prior to death. Means ± 1 SEM.

Test condition			Lactate (mequiv/L)						Glucose (mmol/L)					
			42-h survivors			Mortalities			42-h survivors			Mortalities		
pH	[Ca^{2+}] (μequiv/L)	[Al] (μg/L)	Control	42 h	*N*	Initial	Terminal	*N*	Control	42 h	*N*	Initial	Terminal	*N*
4.8	25	0	0.48 ±0.06	0.79 ±0.17	9	0.48 ±0.06	1.07 ±0.42[b]	9	3.84 ±0.76	2.91 ±0.47[b]	9	3.84 ±0.76	3.18 ±0.34[b]	9
4.8	25	333	1.10 ±0.26	2.39 ±0.69[a]	6	1.15 ±0.15	3.63 ±0.92[a]	14	7.96 ±1.37	11.46 ±2.35	7	8.22 ±0.99	7.57 ±1.20	16
4.8	400	333	0.67 ±0.27	2.86 ±0.73[a]	12	0.77 ±0.13	5.04 ±0.69[a]	22	5.64 ±0.49	5.83 ±1.01	12	5.75 ±0.32	5.57 ±0.55	22
4.4	25	0	0.70 ±0.09	1.45 ±0.31[a]	8	0.68 ±0.15	1.74 ±0.43[a]	5	8.13 ±1.17	5.78 ±0.87	12	8.20 ±1.32	11.95 ±4.10	6
4.4	25	333	0.60 ±0.11	1.52 ±0.66	9	0.68 ±0.16	2.25 ±0.90[a]	6	6.61 ±0.70	5.08 ±0.71	9	7.35 ±0.91	6.88 ±1.58	6

[a] $P < 0.05$ relative to appropriate "control" or "initial" value.
[b] Not terminal when sacrificed after 10 d of exposure.

tion, thickens and distorts the branchial epithelium, decreases its transcellular permeability to O_2 and CO_2, yet simultaneously increases the permeability of paracellular channels through which the majority of electrolyte loss is thought to occur (McDonald 1983a; Wood 1988). At low water pH's, the lamellar microenvironment is undoubtedly less acidic than the bulk medium, due mainly to NH_3 efflux (Wright and Wood 1985). As gill water flow encounters this more basic milieu, the formation of aluminum hydroxide complexes will be favoured. Depending on the actual pH shift and Al concentration, supersaturating conditions may occur, resulting in direct precipitation of Al on the gill. This interpretation would explain the greater toxic effect of Al at pH = 4.8 than at 4.4, for at the latter pH, solubility is higher and aluminum hydroxide concentrations are lower. Alternatively, the greater toxicity at pH = 4.8 may reflect a greater reactvity of cationic aluminum hydroxides than Al^{3+} with surface binding sites. While this interpretation views precipitation as secondary to binding, the two explanations are certainly not mutually exclusive.

Fish kills in the field may occur at pH's greater than 4.8 (Dickson 1983; Henriksen et al. 1984), and field surveys suggest that loss of fish populations is progressive as pH's fall from 6.5 to 5.0 (Magnuson et al. 1984). Neville (1985) found severe physiological responses in rainbow trout exposed to low levels of Al at pH = 6.1, responses which lessened at both 6.5 and 5.0. The chemistry and solubility of Al are poorly understood at these higher pH's, and there is little experimental information on fish responses. There is a clear need for physiological, toxicological, and water chemistry studies with Al in this ecologically significant pH range (4.8–6.1).

In the present study, we did not test the effects of Al at circumneutral pH (i.e. Al in the absence of acid stress) because our objective was to evaluate the effects of different pH and Ca^{2+} levels on the responses to a constant level of *dissolved* Al (333 μg/L). Al solubility is extremely low at circumneutral pH and would have been greatly exceeded by Al = 333 μg/L, resulting in unstable, supersaturated solutions with suspended particles (Johnson et al. 1981; Neville 1985).

Influence of Higher Ca^{2+} on the Responses to Acid plus Al Stress

At higher water Ca^{2+} (400 μequiv/L), a level still well within the softwater range, the physiological response to Al = 333 μg/L at pH = 4.8 was fundamentally altered to one where respiratory disturbances clearly predominated. Ionoregulatory effects were greatly attenuated (Fig. 2C). Pa_{O_2} fell precipitously (Fig. 8C) and Pa_{CO_2} rose reciprocally (Fig. 7C) to levels representative of *venous* blood in cannulated brook trout (Walker et al. 1988). These changes resulted in respiratory acidosis (Fig. 4C,5C), arterial O_2 saturations less than half the control levels (Fig. 9C), and substantial lactate release (Table 2). The rise in Pa_{CO_2} undoubtedly contributed to the marked drop in MCHC (Fig. 3C), for this is known to potentiate erythrocytic swelling (Wood et al. 1982).

The protective effect of Ca^{2+} on ion balance agrees with the results of the flux experiments (Booth et al. 1988). It also agrees with general theory (McDonald 1983a; McDonald et al. 1988), for Ca^{2+} is known to reduce paracellular channel permeability and to compete with metals for surface ligands. Elevations in Ca^{2+} across a comparable range similarly attenuated plasma Na^+ and Cl^- depressions in Al-exposed brown trout (*Salmo trutta*) (Muniz and Leivestad 1980a, 1980b) but not in Al-exposed rainbow trout (Witters 1986). However, this discrepancy is probably due to the acutely low pH (4.1) and short duration (3.5 h) of the tests in the latter study.

The exacerbating effect of higher Ca^{2+} on respiratory failure has not been reported previously. It presumably explains why ion loss rates did not predict mortality in the same way as at low Ca^{2+} and why there was no clear relationship between pH and ion losses in the presence of Al at Ca^{2+} = 400 μequiv/L (Booth et al. 1988). This toxic effect at the gill surface must in some way reflect a greater increase in resistance to O_2 and CO_2 diffusion, but we can only speculate as to mechanism. Possibilities include a greater inflammatory response, more mucus production, a promotion of Al polymerization or precipitation by Ca^{2+}, and more Al-binding sites in fish acclimated and

exposed at higher water Ca_{2+}. However, we could detect no difference in net branchial accumulation of Al between Ca^{2+} = 25 and 400 μequiv/L treatments (Booth et al. 1988). The problem clearly requires detailed morphological study.

The observation of respiratory toxicity at higher Ca^{2+} concentration may have considerable environmental significance. A number of fish kills in the wild associated with ameliorative liming of lakes and streams have been reported (e.g. Dickson 1978, 1983). Liming increases both the water Ca^{2+} level and pH without altering total Al, at least in the short term. The present observations and the data of Neville (1985), when taken together, suggest that increases in Ca^{2+} and pH should both promote respiratory failure, while simultaneously reducing ionoregulatory disturbance. Thus under some circumstances, liming may simply exchange one toxic effect of environmental acidity and Al for another.

Acknowledgements

This work was supported by a contract ("Lake Acidification and Fisheries," RP-2346-01) from the Electric Power Research Institute, Environmental Assessment Department, through a subcontract from the University of Wyoming. We thank Dr. J. Mattice, EPRI project manager, for his advice and encouragement and Drs. R. L. Walker and C. E. Booth for access to their unpublished data.

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