Mechanisms of aluminium extraction and accumulation at the gills of rainbow trout, *Oncorhynchus mykiss* (Walbaum), in acidic soft water

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Rainbow trout were fitted with latex masks for the measurement of ventilatory flow rate ($\dot{V}_w$) and with opercular catheters for sampling expired water from close to the branchial surface. Fish were exposed for 6 h to pH 5·1, 4·7 or 4·1 in the presence (138 µg l^{-1}) or nominal absence (1 µg l^{-1}) of Al. Inspired and expired Al concentrations and water pH were measured via the opercular catheters. Gills were sampled for accumulated Al at the end of the experiments. $\dot{V}_w$ increased during Al exposures at all three pHs. However, in the absence of Al, $\dot{V}_w$ increased only at pH 4·1. Aluminium extraction from the water and Al accumulation on the gills were highest at inspired pH 5·1 and lowest at pH 4·1, and correlated well with expired pH, which was 0·2–0·7 pH units higher than inspired pH. Gill Al accumulations amounted to only about one-tenth of deposition calculated from Al extraction from the water and $\dot{V}_w$, and gill Al was tightly bound to the branchial surface.

Calculations of Al solubility, oversaturation, and species composition were made using measured expired pH values, and were compared with Al extraction from the water and measured gill Al concentrations. In general, these analyses indicated that reduced Al solubility near the gills is a reasonable explanation of Al extraction from the water, and that Al(OH)$_2$$^+$ and Al(OH)$_3$$^\circ$ are the Al species most likely to interact initially at the gills. It is suggested that mucus sloughing removes most precipitated Al, and that only the charged form persists, bound to structural elements on the gill surface. A model incorporating these results, and pH changes in the fish gill micro-environment in general, is presented to explain previously-reported ionoregulatory and respiratory effects of Al.

Key words: aluminium; gills; *Oncorhynchus mykiss*; accumulation; mechanism; acidic soft water.

I. INTRODUCTION

An earlier study (Playle & Wood, 1989*b*) indicated that pH changes in the branchial micro-environment could be important in Al interactions on fish gills. Accumulation of Al on the gills is thought to be central to its toxic effects on respiratory and ionoregulatory function (Karlsson-Norggren, *et al.*, 1986*a,b*; Wood & McDonald, 1987; Booth *et al.*, 1988; Handy & Eddy, 1989; Dietrich & Schlatter, 1989*a*). As acidic water containing Al is rendered more basic by ammonia and base released at the gills (Playle & Wood, 1989*a*), Al could theoretically precipitate from solution onto branchial surfaces as the solubility of Al is reduced as pH rises. Shifts from one Al species to another could also be responsible for Al binding to the gills: the various positively charged species could theoretically bind with differing affinities to negatively charged gills. Branchial surfaces are presumed to be negatively charged because of carboxyl and other groups on mucus (Satchell, 1984). In addition, shifts to neutral species, such as Al(OH)$_3$$^\circ$, might favour the formation of Al polymers and precipitates (e.g. Dentel & Gossett, 1988).

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The present study was designed to examine in further detail the mechanisms of Al interactions with fish gills, using latex ventilation masks and opercular catheters which draw expired water from near the branchial surface. Expired pH determined in this way is thought to provide a reasonable estimate of mean water pH close to the gill surface (Wright *et al.*, 1986). Frequent measurements were made of expired pH, Al extraction at the gills, and ventilatory flow rate at three different inspired pHs (pH 5·1, 4·7 and 4·1) over relatively short (6 h) exposures to Al. The Al exposure used (138 µg l^{-1}) is an environmentally realistic concentration for the moderate to extremely acidic experimental conditions. Measured Al extractions from water passing over the gills and measured concentrations of Al on the gills after 6 h exposures were compared with inspired and expired pH to examine their pattern of variation with respect to water pH. Expired pH was also used to calculate Al solubility and speciation, to which Al extractions and gill Al concentrations were compared. These analyses had the potential to indicate whether Al solubility changes could be responsible for Al toxicity at fish gills, or, if speciation changes are important, the Al species most likely involved in Al–gill interactions.

II. MATERIALS AND METHODS

Rainbow trout, *Oncorhynchus mykiss* (Walbaum); (227 ± 8 g; $n = 30$) were obtained from Spring Valley Trout Farm, New Dundee, Ontario. Before all experiments, fish were acclimated at 15–16° C for at least 2 weeks to synthetic soft water. Soft water was produced by reverse osmosis (Culligan MP1000) followed by ionic addition as described by Playle & Wood (1989a). Acclimation and experimental water composition was: Ca^{2+} ~ 50 µequiv.l^{-1}, Na^+ ~ 50 µequiv.l^{-1}, Cl^- ~ 100 µequiv.l^{-1}, Al ~ 2 µg l^{-1}, titratable alkalinity to pH 4·0 ~ 130 µequiv.l^{-1}, pH ~ 6·5, 15° C.

Most methods and measurement techniques were identical to those described by Playle & Wood (1989a,b). In brief, fish were anaesthetized with MS222 (Sigma, Saint Louis, MS, U.S.A.) on an operating table, then fitted with latex ventilation masks and two opercular catheters each, one per operculum. Trout were placed in divided fish ventilation boxes, and allowed to recover for 24 h before an experiment was started. Two sets of initial measurements of ventilatory flow rate and pH were made at circumneutral pH before exposure to acid or acid plus Al. Water in the aerated headtank was acidified with 0·5 M H$_2$SO$_4$; Al was added by peristaltic pump as a concentrated solution of AlCl$_3$·6H$_2$O (Sigma). Six separate experiments were run: five fish at a time were exposed to pH 5·1, 4·7 or 4·1 in the presence (138 ± 1 µg l^{-1}) or absence (1 µg l^{-1}) of Al for 6 h, using the flow-through system described previously (Playle & Wood, 1989b). Measurements of ventilatory flow rate (V_w, the volume of water overflowing from the posterior section of the ventilation box per kg fish, wet weight, in 1 min), and samples of inspired and expired water Al were taken approximately hourly. Water Al was measured by graphite furnace (see below).

The following terms are used to describe Al interactions at the gills. ' Al *extraction* at the gills $= \Delta Al$ ' (in µg l^{-1}) is the *measured difference* between inspired and expired Al concentrations in the water. ' Aluminium *deposition* at the gills ' (in µg) is the *calculated cumulative amount* of Al removed from the water by one set of gills (branchial arches plus filaments from one side of the fish) during the course of an experiment. Deposition was calculated as $\Delta Al \times (V_w \times \text{fish weight} \div 2) \times \text{time}$. ' Gill Al *concentration* ' (µg g^{-1} wet tissue) is the Al concentration of a sample of gill digest, determined by graphite furnace. ' Aluminium *accumulation* on the gills ' (in µg) is the amount of Al on one set of gills at the end of an experiment, calculated as gill Al concentration multiplied by weight of the left set of gills (g wet tissue). ' Aluminium *precipitation* at the gills ' refers to Al which is deposited on the gills due to loss of Al solubility. ' Aluminium *binding* at the gills ' refers to Al which chemically reacts with ligands on gill surfaces (e.g. ionic binding of positively charged Al species with negatively charged organic molecules on the gill surface).

Expired pH was measured ~ 0.5 h before the experiments and at 3 and 5·5 h during the experiments, using a Radiometer GK2401C combination pH electrode sealed in a polyethylene vial (4·5 ml water volume), through which an opercular catheter siphoned water at a rate of 2–4 ml min^{-1}. Previous tests have shown that pH measurements obtained by this system are at, or close to equilibrium, and that the ionic strength error is small (Wright *et al.*, 1986; Playle & Wood, 1989*a,b*). Inspired pH was measured at each time using the same method.

At the end of each 6 h experiment, fish were removed from their boxes without anaesthetic and killed with a blow to the head. A 2·3 cm diameter circle of Whatman 3 MM qualitative filter paper was placed on the left gills with forceps for ~ 15 s to collect, in a semi-quantitative manner, mucus-bound or precipitated Al on gill surfaces. Filter papers were placed in 7 ml of experimental water in polyethylene vials (pH 5·1, 4·7 or 4·1, as appropriate; no Al), shaken for 5 s, then left standing overnight at 4° C. Filter papers were removed from the vials the next day, water samples were frozen ($-20°$ C), and the Al content of the 7 ml water was later measured. Vials were not acid washed; samples from fish not exposed to Al contained very little Al (see Results), indicating no Al contamination from the vials.

After the left gills were blotted, portions of the third right gill were removed to determine gill Al concentration. To assess the effects of sampling protocol on the results of Al analysis, one gill portion was not rinsed, a second portion was placed in 7 ml of experimental water (appropriate pH, no Al) for 1 min, and a third portion was held with forceps and agitated in three successive 7 ml rinses of experimental water (no Al), 20 s agitation per rinse. Gill portions were stored frozen at $-20°$ C, later thawed, digested in five times their weight of 0·05 M H_2SO_4 for 8 h at 80° C, diluted $100\times$, and analysed for Al by graphite furnace. Finally, the left set of gills (arches with filaments) was removed from each fish and weighed.

For all water Al analyses, 7 ml samples were acidified with 20 µl concentrated HCl, then analysed without dilution for total Al using a Varian AA-1275 atomic absorption spectrophotometer with GTA-95 graphite tube atomizer. Ten µl of water sample—or diluted gill sample—were analysed against standards at 309·3 nm. Operating parameters were as follows: 5 s at 80° C, 35 s at 95°, 10 s at 110°, 12 s at 1200° and 2·7 s at 2500° C, N_2 gas.

DATA ANALYSIS

To determine whether Al solubility or species changes were responsible for interactions of Al with fish gills, ΔAl and gill Al concentrations were first compared with inspired pH (pH 5·1, 4·7, 4·1), then with measured expired pH (variable pHs). For each opercular catheter, the mean of the 2 and 3·5 h ΔAl values (Fig. 2) was compared to pH_{in} or pH_{ex} taken at 3 h; likewise, for each catheter the mean of ΔAl for 5 and 6 h was compared to the 5·5 h pH_{ex}. Water samples for ΔAl determinations were taken more often than pH_{ex} readings to increase accuracy of mean ΔAl measurements; pH_{ex} values were approximately constant during the exposures (see Results). Gill Al concentration for each fish was compared to the mean pH_{ex} of both gills over the course of the experiment (three or four pH_{ex} values); the three rinsing protocols were considered separately. Expired pH was then used to calculate Al solubility (the predicted amount of Al that can be held in solution), using the pH-solubility relationship for micro-crystalline gibbsite given by Roberson & Hem (1969), and Al species using the speciation scheme of Dryssen (1984).

Correlation coefficients were calculated to determine the degree of association of ΔAl or gill Al concentration with pH_{ex}, log Al solubility, Al oversaturation (the difference between inspired Al concentration and calculated Al solubility), and the five Al species (Al^{3+}, $Al(OH)^{2+}$, $Al(OH)_2^{+}$, $Al(OH)_3^{\circ}$, $Al(OH)_4^{-}$). Correlation coefficients were calculated because expired pH, from which all these comparisons were based, was varied indirectly by changing inspired pH (i.e. pH_{ex} was a dependent variable), and because pH_{ex} can be affected by Al, through fish ventilation changes (Playle & Wood, 1989*b*). That is, Al extraction and pH_{ex} are sometimes interdependent.

Duncan's Multiple Range test was used to compare gill Al data. Paired Student's *t*-tests were used for analysis of fish ventilation, comparing each fish's response to its ventilation before exposure to acid or acid plus Al. Unpaired Student's *t*-tests were used for remaining comparisons. Unless indicated otherwise, the level of significance for all tests was $P < 0.05$.
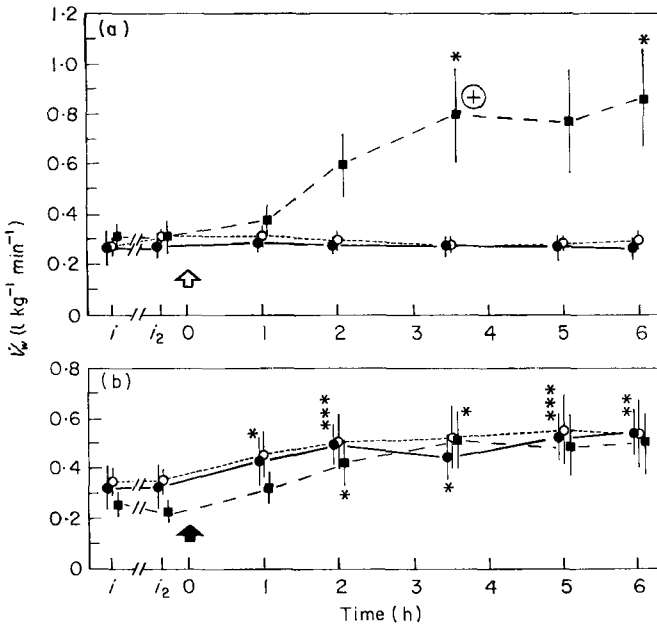
FIG. 1. (a) Ventilatory flow rates ($\dot{V}_w$) of rainbow trout exposed to pH 5·1 (●), 4·7 (○) and 4·1 (■) soft water for 6 h. Means ± 1 s.e.m. Five fish per experiment. Open arrow, start of acid additions. i, i_2, 1·5, 0·5 h before start of acid additions. ⊕, One fish death. *, **, *** = $P < 0.05$, $P < 0.01$, $P < 0.001$ respectively, paired t-test compared to $\dot{V}_w$ at time i_2. (b) as (a) but in the presence of 138 ± 1 µg l^{-1} Al. Black arrow, start of acid and Al additions.

III. RESULTS

Rainbow trout fitted with latex masks and opercular catheters were exposed for 6 h to acidic soft water (pH 5·1, 4·7 or 4·1) in the presence (138 ± 1 µg l^{-1}) or absence of Al. Ventilatory flow rates ($\dot{V}_w$) of the fish did not change in response to moderately acidic conditions alone (pH 5·1, 4·7), but doubled in 2 h and tripled by 6 h during the pH 4·1 exposure [Fig. 1(a)]. One fish died at about 3·5 h at pH 4·1. In contrast, in the presence of Al, $\dot{V}_w$ generally increased over the first 2 h of exposure at pH 5·1 and 4·1, then stayed constant [Fig. 1(b)]. Ventilation did not increase as much during the pH 4·1, Al exposure as during the pH 4·1, no Al exposure: Al apparently reduced the irritating effects of extreme acidity.

EXTRACTION OF Al AT THE GILLS

Mean Al extraction at the gills (ΔAl), the difference between inspired and expired water Al concentrations, was consistently highest in the pH 5·1 plus Al exposure, intermediate for pH 4·7, and lowest for pH 4·1 (Fig. 2). There was a tendency for ΔAl to decrease over 6 h, probably a result of increased $\dot{V}_w$ [Fig. 1(b)]. Deposition of Al for one set of gills of each fish was calculated by multiplying ΔAl by one-half the volume of water breathed by the fish in 1 min ($\dot{V}_w$ × fish weight ÷ 2), then by the elapsed time from the previous sample. Mean Al depositions over the 6 h exposures to 138 µg l^{-1} Al were 730 µg Al for pH 5·1, 310 µg for pH 4·7, and 180 µg Al for the pH 4·1 exposure (Table I; 730 µg significantly different from 180 µg).

Expired pH for all fish was measured before the start of acid or Al additions (time i_2 on figures) and at 3 and 5·5 h after the exposures started. At ambient pH
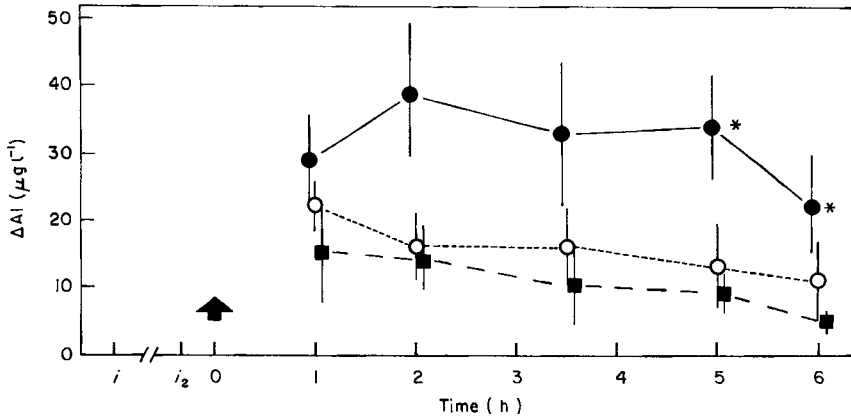
FIG. 2. Mean Al extraction ($\Delta Al = [Al]_{in} - [Al]_{ex}$) for rainbow trout exposed to 138 ± 1 µg l^{-1} Al for 6 h at pH 5·1 (●), 4·7 (○) and 4·1 (■). * = Significant difference between pH$_{in}$ 5·1 and pH$_{in}$ 4·1 exposure, unpaired *t*-test. ΔAl for pH$_{in}$ 5·1 at 1 h was low because [Al]$_{in}$ was only 105 ± 6 µg l^{-1} at that time. Other details as in caption to Fig. 1.

TABLE I. Aluminium deposition and Al accumulation on one set of gills of rainbow trout exposed for 6 h to 138 ± 1 µg l^{-1} Al in soft water at pH 5·1, 4·7 or 4·1, means ± 1 s.e.m. (*n*)

Exposure pH	Gill Al deposition (µg)	Gill Al accumulation (µg)	Gill Al concentration (µg g^{-1} wet tissue)	Surface Al removed from gills (µg)
5·1	730 ± 290 (5)	62 ± 8 (5)	$29·4 \pm 7·3$ (5)	$0·13 \pm 0·03$ (4)
4·7	310 ± 90 (5)	45 ± 6 (5)	$16·8 \pm 1·1$ (5)	$0·20 \pm 0·02$ (5)
4·1	180 ± 80 (5)	17 ± 5 (5)	$7·3 \pm 3·5$ (5)	$0·11 \pm 0·02$ (5)

Al deposition onto one set of gills was estimated from ΔAl, $\dot{V}_w$, and fish weight. Al accumulations for one set of gills of each fish were calculated from measured gill Al concentrations at the end of the 6 h experiments (Fig. 3; combined data from all rinse protocols) and weights of left gill arches plus filaments (mean = 2·4 g). Mean gill Al concentrations (all rinse protocols, all fish) are given, as well as surface Al removed from gills by filter paper discs. See text for more details.

(6·54 ± 0·01), expired pH for all 30 fish was $6·22 \pm 0·02$. Without added Al, expired pHs were approximately constant during the exposures, averaging pH $5·81 \pm 0·06$, pH $5·35 \pm 0·10$ and pH $4·29 \pm 0·04$ for the five fish in each group at pH$_{in}$ 5·1, 4·7 and 4·1, respectively. For fish exposed to Al, expired pHs were also constant during the exposures, averaging $5·60 \pm 0·06$, $5·08 \pm 0·07$ and $4·42 \pm 0·06$ for pH$_{in}$ 5·1, 4·7 and 4·1, respectively.

ACCUMULATION OF Al ON THE GILLS

At the end of all experiments, fish were removed from their ventilation boxes, killed, and the gills sampled. Left gill arches and filaments (= one set of gills) weighed $2·4 \pm 0·2$ g, wet weight (*n* = 15).
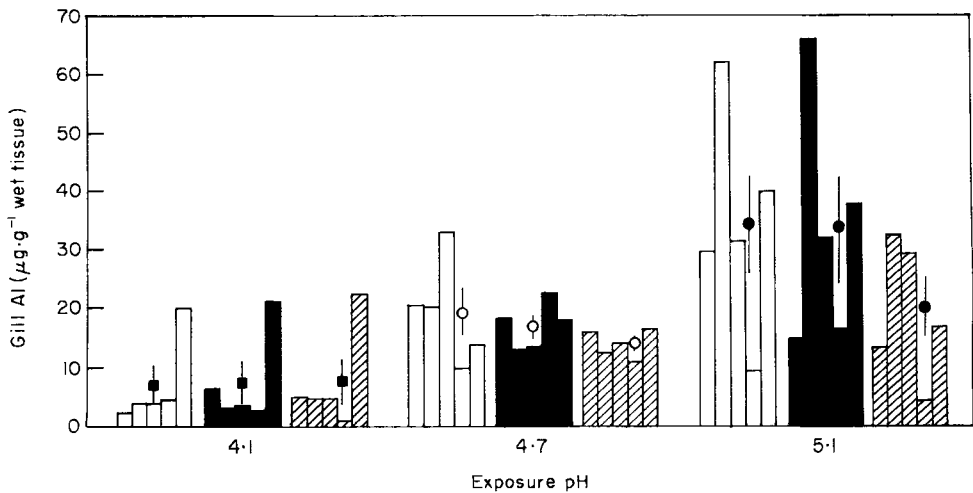
FIG. 3. Gill Al concentrations of rainbow trout exposed to 138 ± 1 µg l^{-1} Al at pH 4·1 (■), 4·7 (○) and 5·1 (●) for 6 h. Gill portions were handled with three different rinsing protocols: □, no rinse; ■, 1 min rinse; ▨, three 20 s rinses (see text for details). For each pH, values from five individual fish are shown, as well as the means ± 1 s.e.m. In each case, the order of the gills from the five fish treated three ways is given from left to right.

The third right gill filaments were assayed for Al. These filaments were either not rinsed, were placed in experimental water (Al free) for 1 min, or were agitated in three, 20-s rinses of experimental water (Al free). Effects of exposure pH on gill Al concentrations, and of the three rinsing protocols, were determined. Gill Al concentration increased as inspired pH increased (Fig. 3; $P < 0.01$ for no rinse and 1 min rinse, $P < 0.05$ for three 20-s rinses). Within each rinse protocol, gill Al concentrations for the pH 5·1 exposure were greater than for the 4·1 exposure ($P < 0.05$), but the pH 4·7 values were not significantly different from either of the other two sets (Duncan's Multiple Range test). The three rinsing protocols yielded the same results: even for the pH 5·1 exposure there was no significant difference between the three rinse protocols ($P > 0.05$, Duncan's Multiple Range test). Mean gill Al concentration for fish exposed to acidity alone was 0·1 µg Al g^{-1} (range: 0·0–1·3, all rinse protocols, $n = 14$).

Accumulations of Al for one set of gills, using gill Al concentrations (Fig. 3) and weights of left gill arches plus filaments, averaged 62, 45, and 17 µg Al for pH 5·1, 4·7 and 4·1 Al exposures, respectively (Table I; combined data from all rinse protocols; 62 and 45 µg significantly greater than 17 µg). Note that these accumulations of Al amount to only a small fraction of Al deposition calculated from ΔAl (Table I).

Accumulation of Al on gills was also assessed semi-quantitatively by blotting the surface of intact left gills. Filter paper discs picked up significantly more Al from the gills of Al-exposed fish in the pH 4·7 exposure than in the pH 4·1 exposure, while the pH 5·1 results were intermediate (Table I). However, these amounts were minute, less than 1% of gill Al accumulations at these pHs. Aluminium blotted from the gills of fish not exposed to Al (pH 5·1, 4·7, 4·1) was ≤0·02 µg.
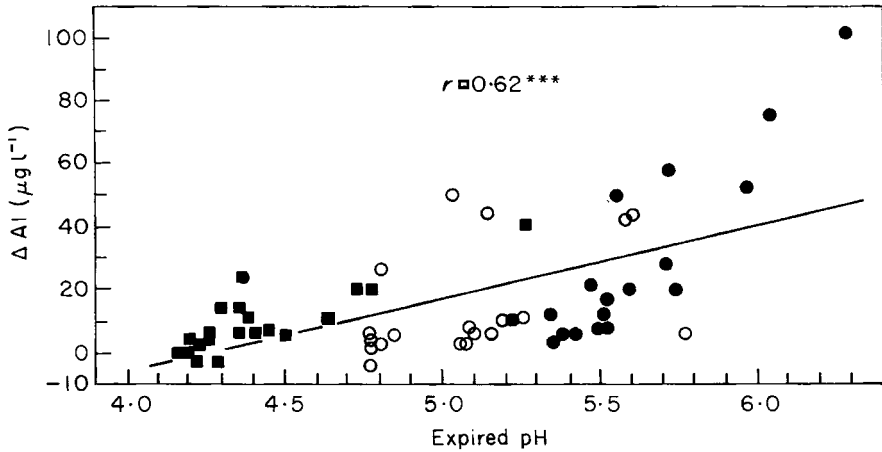
FIG. 4. Mean extraction of Al (ΔAl) at rainbow trout gills plotted against corresponding measured expired pHs (at 3 and 5·5 h exposure to $138 \pm 1\ \mu g\,l^{-1}$ Al). *** $= P < 0\cdot001, n = 57$. Line fitted by least squares linear regression. ●, pH 5·1; ○, pH 4·7; ■, pH 4·1.

MECHANISMS OF Al INTERACTIONS WITH THE GILLS

Expired pH and ΔAl (mean of two ΔAl determinations) of Al-exposed fish were used to analyse the mechanisms of Al interactions with fish gills. Expired pH was dependent on inspired pH ($pH_{ex} = 1\cdot19\ pH_{in} - 0\cdot55$, $P < 0\cdot001$, $n = 57$; $r = 0\cdot87$; regression analysis by least squares method), as was mean ΔAl ($\Delta Al = 20\cdot9$ $pH_{in} - 79\cdot3$, $P < 0\cdot01$, $n = 60$; $r = 0\cdot38$). As inspired pH increased, so did ΔAl and expired pH. These two dependent variables were also associated, as shown by their correlation coefficients: as expired pH increased, so did ΔAl ($r = 0\cdot62$, $P < 0\cdot001$; Fig. 4). ΔAl correlated better with expired pH than with inspired pH ($r = 0\cdot62$ v. $0\cdot38$, respectively).

Solubility

To dissect further the relationship between ΔAl and pH_{ex}, mean ΔAl was plotted against the log of Al solubility calculated for each measured expired pH. There was a highly significant, negative correlation between ΔAl and Al solubility [Fig. 5(a)]. That is, the lower the Al solubility, the higher the Al extraction at the gills. Similarly, ΔAl plotted against oversaturation of Al yielded a highly significant correlation [Fig. 5(b)]. Here, oversaturation of Al was taken as the difference between Al solubility (Roberson & Hem, 1969) and the inspired Al concentration ($138\ \mu g\,l^{-1}$), if Al solubility was less than this value. Precipitation of Al from oversaturated solution onto the gills is implied by these correlations.

Speciation

However, higher ΔAl at higher expired pH could also be a result of greater affinity of various species of Al for gill surfaces. To assess this possibility, concentrations of the five Al species contained in the speciation scheme of Dryssen (1984) were calculated from measured expired pHs, and mean ΔAl was compared to these values. Total Al for the calculations was the mean exposure value, $138\ \mu g\,l^{-1}$ Al. Mean ΔAl showed a highly significant negative correlation with Al^{3+} [Fig. 6(a)],
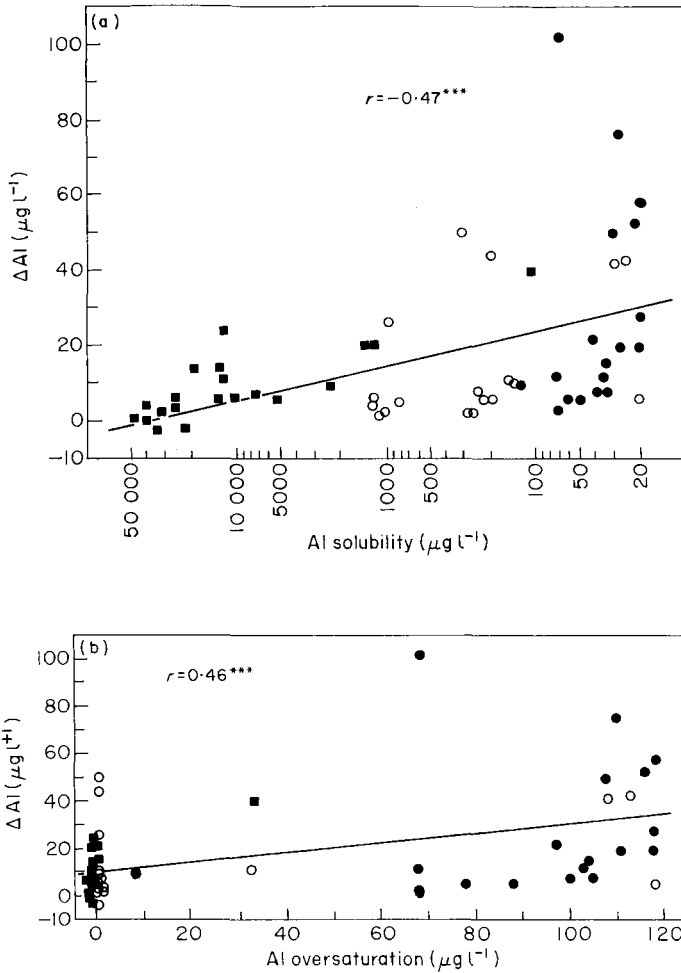
FIG. 5. (a) Mean extraction of Al (ΔAl) at rainbow trout gills v. Al solubility (log scale) for the same data set in Fig. 4. Total Al $= 138 \pm 1$ μg l^{-1}. Solubility of Al was calculated for each expired pH (see Fig. 4), using the solubility diagram of Roberson & Hem (1969). $*** = P < 0.001$, $n = 57$. (b) Mean ΔAl at rainbow trout gills v. calculated Al oversaturation. See text for details. $\bullet$, pH 5.1; $\bigcirc$, pH 4.7; $\blacksquare$, pH 4.1.

and a significant negative correlation with $AlOH^{2+}$ [Fig. 6(b)], suggesting that Al extraction at the gills was not related to either of these Al species. Mean ΔAl showed a significant correlation with $Al(OH)_2^+$, and a highly significant correlation with the neutral $Al(OH)_3^\circ$ species [Fig. 6(c),(d)]. There was also a highly significant correlation between ΔAl and the Al anion, $Al(OH)_4^-$ [Fig. 6(e)].

It was noted earlier that gill Al concentrations increased as inspired water pH increased (Fig. 3). Gill Al concentrations can also be compared to expired pH, and to calculated Al solubilities and Al species at those expired pHs. These comparisons for all three gill rinse protocols are summarized in Table II. Gill Al concentrations correlated significantly with expired pH (more gill Al at higher pH_{ex}), with log Al solubility (more gill Al at lower Al solubility), and with oversaturation (more gill Al at greater oversaturation). Gill Al concentrations showed a negative
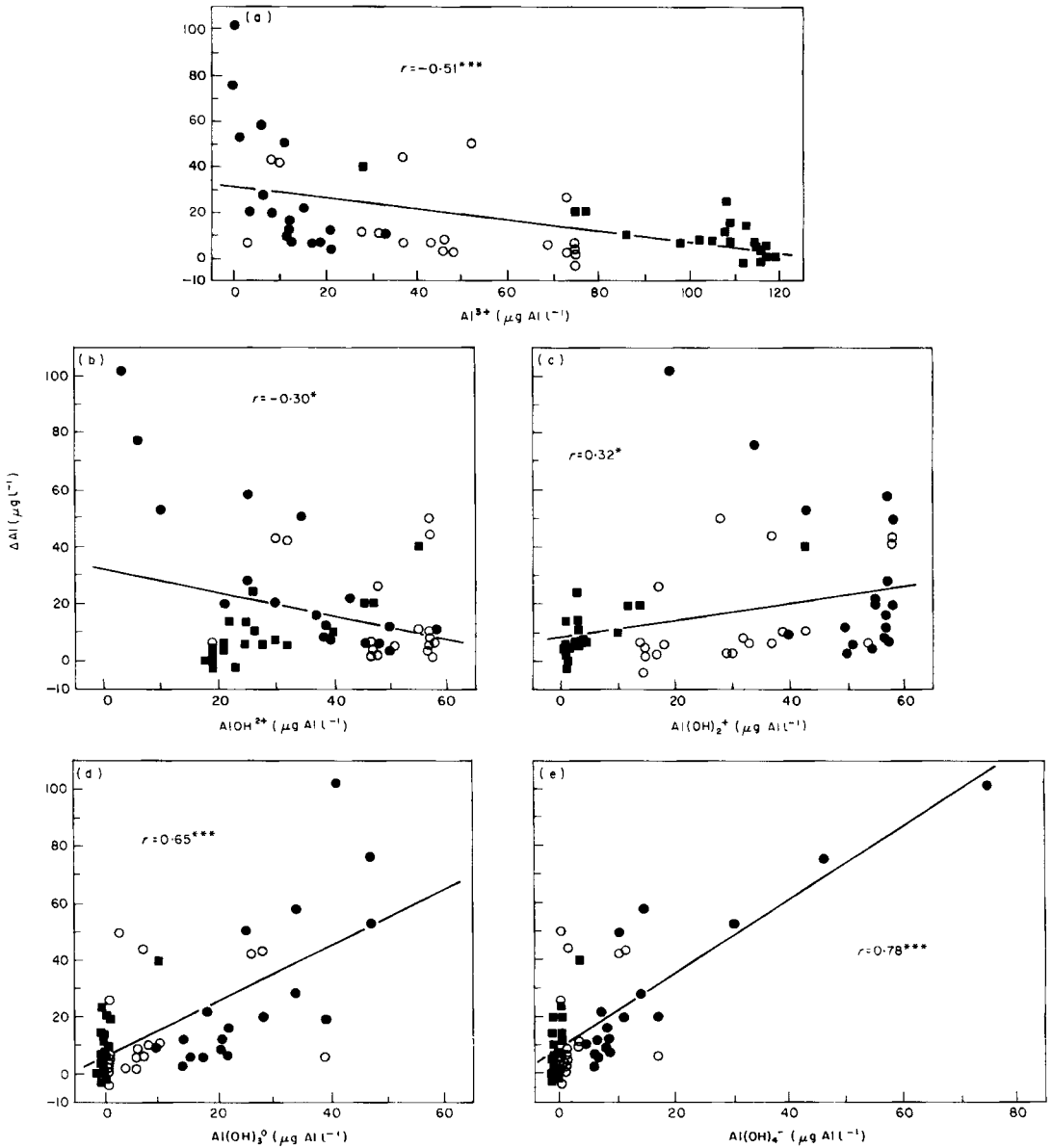
FIG. 6. Mean extraction of Al (ΔAl) at rainbow trout gills *v*. calculated concentrations of individual species of Al, from the same data set as in Fig. 4. Species were determined from measured expired pHs and the speciation scheme of Dyrssen (1984). Total Al = 138 ± 1 μg l^{-1}. * = $P < 0.05$; *** = $P < 0.001$ for correlation coefficient, $n = 57$. (a) Mean ΔAl *v* Al^{3+}. (b) Mean ΔAl *v*. AlOH^{2+}. (c) Mean ΔAl *v*. Al(OH)$_2$$^+$. (d) Mean ΔAl *v*. Al(OH)$_3$$^\circ$. (e) Mean ΔAl *v*. Al(OH)$_4$$^-$. ●, pH 5·1; ○, pH 4·7; ■, pH 4·1.

correlation with Al^{3+} and no correlation with AlOH^{2+}. The Al species showing the most significant correlation was Al(OH)$_2$$^+$ (Table II). Correlations between gill Al concentrations and Al(OH)$_3$$^\circ$ and Al(OH)$_4$$^-$ were not significant (Table II), despite the fact that these two species had highly significant correlations with ΔAl [Fig. 6(d),(e)].

TABLE II. Correlation coefficients between gill Al concentration after 6 h exposure to 138 ± 1 µg l^{-1} Al (three rinse protocols) and mean expired pH (measured), log Al solubility and oversaturation (calculated), and calculated concentrations of five Al species

Gill Al concentration	Correlation coefficient							
	Mean pH_{ex}	Log Al solubility	Over-saturation	Al^{3+}	$AlOH^{2+}$	$Al(OH)_2^+$	$Al(OH)_3^\circ$	$Al(OH)_4^-$
No rinse	0·60 *	−0·63 *	0·54 *	−0·61 *	0·11	0·58 *	0·50	0·29
1-min rinse	0·58 *	−0·66 **	0·65 **	−0·64 *	0·16	0·71 **	0·46	0·14
Three 20-s rinses	0·54 *	−0·60 *	0·50	−0·57 *	0·16	0·57 *	0·44	0·16

*, ** $= P < 0.05$, $P < 0.01$ respectively. Solubility of Al was calculated for each mean expired pH using the solubility diagram of Roberson & Hem (1969). Concentrations of Al species were calculated using the speciation scheme of Dyrssen (1984). $n = 15$ for each comparison.

IV. DISCUSSION

Rainbow trout fitted with ventilation masks and opercular catheters showed elevations in ventilatory flow rate ($\dot{V}_w$) during 6 h exposures to 138 µg l^{-1} Al [Fig. 1(b)], similar to those seen previously (Playle & Wood, 1989b). Moderately low pH alone (pH 5·1, 4·7) did not alter $\dot{V}_w$, but extreme acidity (pH 4·1) caused a marked stimulation. Increases in $\dot{V}_w$ were actually lower in the presence of Al than in its absence during the pH 4·1 exposures. Amelioration of the effects of extreme acidity by Al has been reported before (e.g. Muniz & Leivestad, 1980; Neville, 1985). It is likely the Al^{3+} cation reduces the effects of very acidic conditions by competing with H^+ ions for gill binding sites (e.g. Pagenkopf, 1983).

As expected from previous studies (Wright et al., 1986; Playle & Wood, 1989a; Randall & Wright, 1989; Lin & Randall, 1990), expired pH was dependent on inspired pH. The relationship is complex, and is determined by titration characteristics of the water, buffering action of Al, relative ammonia, titratable base, and CO_2 outputs of the fish, and the pKs of the relevant aqueous reactions. It should be noted that at acidic inspired pHs, measured expired pH may underestimate the extent of alkalinization in some areas of the gills because of deadspaces and surface heterogeneity. Further, conditions in unstirred layers right next to the gills are not sampled by opercular catheters. Nevertheless, pH_{ex} measurements are thought to provide a reasonable estimate of overall mean water pH close to the gill surface. This pH is obviously very different from inspired bulk water pH, and will determine initial interactions of Al at fish gills.

Aluminium extraction at the gills (ΔAl), gill Al concentrations, and Al deposition and accumulation on the gills were highest at inspired pH 5·1, intermediate at pH_{in} 4·7, and lowest at pH_{in} 4·1 (Figs 2, 3; Table I). In turn, ΔAl and gill Al concentrations showed positive correlations with expired pH (Fig. 4, Table II); ΔAl correlated better with pH_{ex} than with pH_{in}. Solubility of Al decreases exponentially as pH increases from pH 4·0 to pH 5·8 (e.g. Roberson & Hem, 1969), and Al chemistry

changes from predominantly Al^{3+} at pH 4·0 to a mixture of Al-hydroxides and the $Al(OH)_4^-$ anion near pH 6 (e.g. Dyrssen, 1984). Comparing ΔAl and gill Al concentrations with calculated Al solubility and Al species yielded some insights into likely mechanisms of Al interactions at fish gills.

Both extraction of Al at the gills and gill Al concentrations increased as Al solubility decreased [Fig. 5(a), Table II] and oversaturation increased [Fig. 5(b), Table II]. While the Al solubility curve for microcrystalline gibbsite (Roberson & Hem, 1969) was used for these calculations, any Al solubility curve would give broadly similar results. These relationships support the idea that increased pH in the gill micro-environment, reducing Al solubility, results in Al precipitation onto the gills. Aluminium precipitating onto the gills might then cause the ionoregulatory and severe respiratory disturbances that resulted in higher mortality at pH > 5·2 than at pH 4·8 or 4·4 (Playle et $al.$, 1989; see also Neville, 1985). Chemical simulations of the partial neutralization of Al solutions at fish gills, in which fast filtration was used to identify newly-formed polymeric Al and precipitated Al, have demonstrated that Al precipitation is possible during the short (<2 s; Randall, 1970) contact time of water at the gills (Playle & Wood, 1990).

Some Al extraction from the water and Al accumulations on the gills occurred even at acidic expired pHs when Al solubility was still high (Fig. 4; Table I), suggesting that binding of Al species to the gill surface might also be involved in initial interactions of Al at the gills. We used the speciation scheme of Dyrssen (1984) to analyse this situation in relation to expired pH. Note that many other speciation schemes for Al exist (e.g. May et $al.$, 1979; Helliwell et $al.$, 1983; Bache, 1986), some of which consider different Al species besides the five presented by Dyrssen. However, general trends would be similar with most schemes. From our calculations, Al^{3+} and $AlOH^{2+}$ were unlikely to be responsible for much of the Al interactions at the gills, judging by their negative or non-significant correlations with ΔAl and gill Al concentrations [Fig. 6(a),(b); Table II]. However, low but measurable Al extractions and gill Al accumulations for fish in very acidic conditions may be related to adsorption of these positively charged species to negatively charged gill surfaces and mucus (Satchell, 1984).

Two prime candidates for Al species responsible for greater ΔAl and gill Al accumulations at higher expired pHs are $Al(OH)_2^+$ and $Al(OH)_3^\circ$. These species showed significant to highly significant correlations with ΔAl [Fig. 6(c),(d)] and $Al(OH)_2^+$ showed a significant correlation with gill Al concentrations (Table II). The gill interaction mechanism of netural $Al(OH)_3^\circ$, and probably $Al(OH)_2^+$, is likely different from those of Al^{3+} and $AlOH^{2+}$. That is, $Al(OH)_2^+$ and $Al(OH)_3^\circ$ probably would not bind to negatively charged gill surfaces to the same degree as the more positively charged species. A precipitation phenomenon may occur instead, especially for neutral $Al(OH)_3^\circ$, where charge repulsion is minimal and polymerization of Al could result (Dental & Gossett, 1988).

The Al anion, $Al(OH)_4^-$, showed no correlation with gill Al concentrations (Table II), but showed a highly significant correlation with ΔAl [Fig. 6(e)]. $Al(OH)_4^-$ represents the major form of dissolved Al in alkaline conditions, and its correlation with ΔAl may be an artifact of the many acidic expired pHs (with low ΔAl) having calculated concentrations of $Al(OH)_4^-$ of $0\ \mu g\ l^{-1}$ [Fig. 6(e)]. In addition, two or three data points have a disproportionate effect on the correlation. $Al(OH)_4^-$ is unlikely to contribute substantially to initial Al interactions

with fish gills, because charge repulsion would prevent its adsorption to negatively charged gill surfaces.

Shifts in Al species are likely to be rapid (a few msec; Wakeman, 1986), which brings up the point made by Neville & Campbell (1988), of whether calculations of Al species present near the gills are valid. If species of Al are in rapid equilibrium, and there is enough free metal available (Pagenkopf, 1983), any individual species could be responsible for Al interactions at the gills, because as that species is removed from solution it is quickly replaced at the expense of other Al species. In the absence of any experimental data, this is a difficult issue to resolve at present. With regards to binding of positively charged Al species to the negatively charged gill surfaces, it is not known if this process can occur within the water–gill contact time (< 2 s). However, adsorption of Al onto negatively charged clays is complete in < 30 s (Walker *et al.*, 1988), and complexation of Al with carboxyl groups of humic and fulvic acids (Lewis *et al.*, 1988; Bache, 1986; Plankey & Patterson, 1987) may have reaction half times of as little as 5 s (Mak & Langford, 1982). From these studies, it seems likely that binding reactions are fast enough to occur at fish gills, but there is a clear need for more data on this topic.

Whatever the processes involved, accumulation of Al on the gills was only about one-tenth the Al deposition calculated from ΔAl (Table I). This result agrees well with similar but somewhat less accurate calculations in Playle & Wood (1989*b*) for trout subjected to longer Al exposures (44 h).

The fact that the three rinsing protocols (Fig. 3) did not affect gill Al concentrations suggests that most Al remaining on the gills was tightly bound. Blotting gills with filter paper discs collected less than 1% of the gill Al accumulation (Table I), which also suggests, in a semi-quantitative manner, that most Al retained on the gills was bound intimately. Presumably, sloughing of mucus at the gill surface removes most ($\sim 90\%$) of the Al which is continuously extracted from the water passing over the gills. Mucus-bound Al would probably fall to the bottom of the opercular cavity and be expelled there, instead of being siphoned down the opercular catheters. Using background whole-body mucus secretion rates of rainbow trout from Lock & van Overbeeke (1981), fish used in our study would have produced about 15 mg (dry weight) of mucus over 6 h (~ 0.3 g wet weight). Stimulated mucus secretion at the gills in response to Al would likely be similar; this amount of mucus appears ample to slough most Al deposition from the gills.

We suggest that Al initially collects on gills through precipitation phenomena, is mostly sloughed off with mucus, and that the small proportion of Al remaining is positively charged Al (perhaps $Al(OH)_2^+$) bound to negative charges on branchial surfaces. This may explain why gill Al concentrations correlated best with $Al(OH)_2^+$ (Table II), whereas ΔAl was best correlated with $Al(OH)_3^\circ$ [Fig. 6(d)]. Furthermore, the distinction between Al precipitation on the gill, most of which appears to be rapidly sloughed off, and the small amount which persists on the gill, may offer an explanation for the separate respiratory and ionoregulatory aspects of Al toxicity which have been documented repeatedly.

Ionoregulatory disturbances predominate at more acidic pHs (< 4.8–5.0), whereas a respiratory disturbance is added and may become predominant at more moderate pHs (> 4.8–5.0; e.g. Neville, 1985; Witters, 1986; Malte & Weber, 1988; Wood *et al.*, 1988; Playle *et al.*, 1989; Dietrich & Schlatter, 1989*b*). These results indicate a fundamental difference in toxic mechanism of Al between moderately

acidic and very acidic pHs. We suggest that respiratory problems largely result from Al precipitation phenomena, which are clearly favoured by more moderate pHs, as the solubility of Al is reduced or exceeded in the more alkaline gill micro-environment. Gill mucification and inflammation in response to Al deposition would increase the diffusion barrier to oxygen and carbon dioxide (Karlsson-Norrgren *et al.*, 1986*a,b*; Harvey & McCardle, 1986; Wood & McDonald, 1987; Gossenaerts *et al.*, 1988; Dietrich & Schlatter, 1989*b*; Handy & Eddy, 1989; Mueller *et al.*, 1990).

Ionoregulatory problems, on the other hand, would result from direct toxic effects of the persistent charged Al species which become chemically bound to structural elements of the gill surface. These species would be favoured by more acidic pHs, and their toxic actions would include displacement of bound Ca^{2+}, opening up of tight junctions, resultant increases in diffusive permeability to Na^+ and Cl^-, and chemical interference with active Na^+ and Cl^- uptake mechanisms (Staurnes *et al.*, 1984; Wood & McDonald, 1987; Booth *et al.*, 1988).

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