

BRANCHIAL AND RENAL ACID AND ION FLUXES IN THE RAINBOW TROUT, *SALMO GAIARDNERI*, AT LOW ENVIRONMENTAL pH

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SUMMARY

Rainbow trout were exposed for 4 days to an environmental pH averaging 4.2, an exposure which resulted in a continuous net branchial influx of acid. The influx provoked a progressive depression in blood pH and $[\text{HCO}_3^-]$, virtually complete by 48 h, and a marked increase in renal acid excretion, also complete by 48 h. The increase in the latter was sufficient to remove, at maximum, about half of the protons entering at the gills; those remaining were buffered in body fluids. The low pH exposure also impaired gill ion regulation as indicated by continuous net branchial losses of Na^+ , Cl^- and K^+ and by a progressive decline in plasma Na^+ and Cl^- levels. Evidence is presented which indicates that there was a significant contribution by the intracellular compartment both to the total body ion losses and to the buffering of the body acid load.

INTRODUCTION

There is now an extensive literature on the interaction of low pH environments with the physiology of freshwater fish (see Fromm, 1980, for a recent review). Effects on normal blood physiology are particularly well known and disturbances to acid-base state (Packer & Dunson, 1970; Neville, 1979*a*; Packer, 1979; McDonald, Høbe & Wood, 1980), ion levels (Mudge & Neff, 1971; Leivestad & Muniz, 1976; Neville, 1979*b*; McDonald *et al.* 1980) and oxygen transport (Vaala & Mitchell, 1970; Packer, 1979) have now been reported. Less well understood are effects on the actual mechanisms of ionoregulation. Whole-body unidirectional Na^+ fluxes have been examined in acid environments (Packer & Dunson, 1970; McWilliams & Potts, 1978) but no attempt has been made to separate the branchial and renal components of these fluxes nor has the regulation of other major body ions (e.g. Cl^- and K^+) been assessed. Also largely unknown are the effects of acid environments on the mechanisms of acid-base regulation. Such regulation has been largely thought to occur at the gills via Na^+ /proton and Cl^- /basic anion exchange mechanisms (cf. Maetz, 1974) but the recent demonstration (Wood & Caldwell, 1978) of a considerable renal competence in the excretion of an injected acid load suggests that the kidney may also play an important role. Thus in order to explore more fully these aspects of low pH exposure, we have undertaken a study of the effects of such exposure on branchial and renal function in the rainbow trout.

Table 1. *Ion composition and pH of test water (T = 16 ± 1 °C)*

	Day 0 (acid series)	Days 1-4 (acid series)	Days 0-4 (control series)
pH	7.5 (7.4-7.7)	4.2 (3.7-5.1)	7.9 (7.6-8.1)
Na ⁺	0.78 (± 0.06)	0.86 (± 0.02)	1.44 (± 0.01)
Cl ⁻	0.80 (± 0.02)	0.86 (± 0.03)	0.75 (± 0.01)
K ⁺	1.42 (± 0.01)	0.73 (± 0.10)	0.98 (± 0.02)
Ca ²⁺	1.94 (± 0.02)	1.93 (± 0.03)	1.94 (± 0.03)
Number of flux periods	2	8	10

Values are means; range in brackets for pH, ± S.E.M. for ions: Ions are in m-equiv/l. Acid series values are combined averages for the two replicates.

MATERIALS AND METHODS

Experimental animals

Rainbow trout (*Salmo gairdneri*) were obtained from Spring Valley Trout Farm, Petersburg, Ontario, and held in large polyethylene tanks continuously supplied with well aerated, dechlorinated tap water. Trout were fed *ad libitum* with commercial trout pellets while in the holding facilities. Prior to experimentation, trout (mean weight 290 g, range 192-430 g) were acclimated for 2 weeks to water of a temperature (16 ± 1 °C) and ionic composition very similar to that subsequently employed in the acid exposure and control experiments (Table 1). Trout were starved during this acclimation period to remove the influence of diet on renal acid output (Wood & Caldwell, 1978).

Test conditions

All acid exposure and control experiments were conducted in a thermostatted recirculating water system described in McDonald *et al.* (1980). This system consisted of eight individual fish chambers (2 l vol.) each supplied at a flow rate of 500 ml/min from either one of two volume-calibrated 100 l reservoirs. One reservoir supplied water at a normal pH while the other supplied acidified water; the switchover from one to the other could be effected without disturbance to the fish. The calibration marks in these reservoirs allowed the determination of the total experimental volume to within ± 50 ml. The water to fish ratio was maintained throughout at approximately 40 l/kg.

Prior to use, the water in each reservoir was decarbonated by acidification to pH 2.5 with H₂SO₄, 24 h aeration, and then back titration with KOH and NaOH to the appropriate pH (7.5 in the control reservoir, 4.0 in the acid reservoir). The resulting ionic composition is shown in Table 1.

Experimental protocol

Following acclimation, trout were implanted with chronic cannulae in the dorsal aorta (Smith & Bell, 1964) and urinary bladder (Wood & Randall, 1973) while under MS-222 anaesthesia (1:10 000 dilution in the acclimation water). They were then transferred to the recirculating system and allowed to recover for 36 h. This recovery

Period was followed by a 5 day experimental period. The experimental period for acid exposure (conducted as two experimental replicates, $N = 6$ and $N = 8$) consisted of one day at a normal water pH (i.e. Day 0) followed by four days of acid exposure (i.e., Days 1-4, Table 1). The control experiment (Table 1) was conducted to determine the influence of the surgical and blood sampling procedures on branchial fluxes and urine flow. In this experiment, trout ($N = 8$) were maintained at normal pH throughout but were otherwise treated identically to the trout undergoing acid exposure.

Arterial blood samples (0.6 ml) were drawn from the dorsal aortic cannula without disturbing the fish and were replaced with an equal volume of heparinized (100 i.u./ml) Cortland saline (Wolf, 1963). Blood samples were drawn, in the acid exposure series, at the midpoint of Day 0 (control sample) and Day 1, and 2-4 h following the end of Days 1, 2, 3 and 4. Blood samples were analysed for pH, C_{CO_2} (whole blood and plasma), Na^+ , Cl^- , K^+ and Ca^{2+} (see below). The mid-point Day 1 sample was analysed for pH only. In order to maintain similar experimental conditions in the control series, a blood sampling procedure identical to the above was employed. Analysis of these collected samples was not required however, as previous studies (McDonald *et al.* 1980) have established that repetitive sampling has no effect on blood acid-base and ion parameters.

Urinary bladders were drained via the cannulae with a siphon of 7 cm into covered vials, allowing continuous urine collection. Urine vials were changed at 12 h intervals throughout the duration of the experiments. Each 12 h collection for the acid series was analysed for volume, pH, NH_4^+ , $TA-HCO_3^-$, Na^+ , K^+ , Cl^- , Ca^{2+} , and phosphate (see below). Collections in the control series were analysed for volume only, as previous studies (Wood & Caldwell, 1978; Kobayashi & Wood, 1980) have established that repetitive blood sampling has no effect on renal ion and acid excretion.

Water samples (10 ml) for ion analysis (Na^+ , K^+ , Cl^- and Ca^{2+}) were collected at 12 h intervals. The first water sample on Day 1 of acid exposure was collected 2 h after the water supply to the fish boxes was switched to the acid reservoir; this delay allowed for complete mixing of water between the fish boxes and the supply reservoir. Water samples for titration (10 ml) and ammonia analysis (5 ml) were collected five times daily; at time 0, +2 h, +12 h, +14 h and +24 h. During the 0-+2 h and the +14-+16 h intervals on Days 1-4 in the acid exposure series, water pH was returned to $pH \approx 4.0$ by addition of 1 N- H_2SO_4 and distilled water was added to replace water lost by evaporation (~ 1 l/day). No water pH adjustments were made on Day 0 in the acid series nor throughout the control series since pH variations were very minor (Table 1).

At the termination of the acid-exposure and control experiments, animals were killed by a blow on the head and duplicate 1 g samples of epaxial muscle were removed from below and slightly caudal to the dorsal fin. These samples were then frozen for subsequent ion analysis.

Analytical techniques

1. Blood acid-base parameters

Arterial blood pH (pH_a) and total CO_2 (C_{a,CO_2}) were determined immediately on collected samples by methods described in McDonald *et al.* (1980). Measurements

of C_{a,CO_2} and pH_a were used to calculate P_{a,CO_2} (in mmHg) and HCO_3^- (m-equiv/l in whole blood and plasma) from the Henderson-Hasselbalch equation. The quantity of H^+ ions (in m-equiv/l of blood) added to blood buffers by non-respiratory (i.e. non-volatile) acids (ΔH_b^+) was calculated from whole blood HCO_3^- , pH_a and haematocrit according to equations 2 and 3 of McDonald *et al.* (1980).

2. Ions

Na^+ , K^+ and Ca^{2+} levels in plasma, urine and water were determined (cf. McDonald *et al.* 1980; Kobayashi & Wood, 1980) by flame photometry (EEL Mark II for Na^+ and K^+ , Coleman 20 for Ca^{2+}). Chloride levels in these fluids were determined by coulometric titration (Radiometer CMT-10). Ammonia levels in urine and water were determined by colorimetric assay using a micro-modification of the phenolhypochlorite method of Solorzano (1969). Phosphate levels in urine were determined colorimetrically by phosphomolybdate reduction using Sigma reagents (Sigma, 1974).

For ion analysis, muscle samples were dried (95 °C, 48 h), homogenized and extracted with 1.0 N-nitric acid (10:1, 37 °C, 24 h). Aliquots were analysed for Na^+ , K^+ and Cl^- levels in a fashion similar to plasma. Intracellular concentrations (m-equiv/kg cell water) were calculated according to the equation:

$$[IC] = \frac{\text{total tissue ion} - \text{extracellular ion content}}{\text{total tissue water} - \text{ECFV}}, \quad (1)$$

where extracellular ion content was taken as the product of the plasma [ion] and ECFV (extracellular fluid volume) (a Donnan equilibrium factor of 1 was assumed; Houston & Mearow, 1979). ECFV (l/kg body weight) was estimated as the chloride-potassium space, calculated according to the equation given in Conway (1957). Houston & Mearow (1979) have shown that this readily determined parameter gives values for muscle ECFV that are closely comparable to those measured more rigorously with a radiolabelled ECF marker ($[^{14}C]$ polyethylene glycol).

3. Urine acidity

Urine pH and titratable acidity (TA- HCO_3^-) were determined immediately after collection as described in Wood & Caldwell (1978) and Kobayashi & Wood (1980). TA- HCO_3^- was determined as a single value in the double titration procedure recommended by Hills (1973) using a Radiometer micro pH electrode (Type E5021) coupled to a Radiometer PHM-71 acid-base analyser. Titrants used were 0.02 N-HCl and 0.02 N-NaOH. In contrast to previous studies (e.g. Kobayashi & Wood, 1980) the final end point of the titration for all samples was the blood pH on Day 0 rather than the mean blood pH for the collection period. The latter represents the titratable acid content of the urine relative to the blood at the time of collection whereas the former more closely reflects the titratable acid content of the urine relative to a constant value. It is thus more appropriate for assessing changes in absolute acid output over time and for comparisons of branchial and renal acid fluxes. Total renal acid output was calculated as the sum of this titratable acid efflux ($[TA-HCO_3^-] \times$ urine flow rate) and the ammonium efflux ($[NH_4^+] \times$ urine flow rate).

4. Branchial ion and acid fluxes

Net fluxes of Na^+ , K^+ , Cl^- and Ca^{2+} (in $\mu\text{equiv/kg.h}$) were calculated from changes in their concentrations in the water.

Net branchial acid fluxes (in $\mu\text{equiv H}^+/\text{kg.h}$) were determined according to procedures similar to those of DeRenzi & Maetz (1973) and Cameron (1980). 10 ml water samples, collected at the beginning and end of each flux period and thermostatted to the experimental temperature, were titrated to an end point pH of 4.0 with 0.02 N-HCl. For these titrations pH was measured with a Radiometer G202C pH electrode coupled to a PHM28 pH meter. Samples were aerated 1 h prior to titration to remove respiratory CO_2 and aeration was maintained through the titration both to provide mixing and to remove additional CO_2 liberated by the titration of HCO_3^- . The end-point titrant concentrations determined by this procedure were used to calculate the titratable component of the net acid flux. This component reflects but does not equal the net acid flux since protons may have been excreted by the gills in the form of NH_4^+ and/or may have been trapped from the medium as the result of branchial NH_3 excretion, i.e. branchial ammonia excretion, regardless of its form, results in protons in the medium which will escape titration. Thus the net branchial acid flux is the sum of the titratable net flux and the ammonia excretion (signs considered). It should be pointed out that this procedure does not distinguish between a net excretion of acid and a net uptake of base or *vice-versa*. Fortunately, this does not matter in terms of the acid-base status of the animal.

Statistical analyses

For the two acid-exposure replicates the experimental conditions, including the fluctuations in the acid pH regime (see Fig. 1) were nearly identical and preliminary data analysis indicated no significant differences between their mean values. Consequently, the results from the replicates have been pooled. Means ± 1 S.E.M. are reported for blood and urine parameters and means only for branchial flux data. The latter were weighted according to the number of fish in each replicate. The N values for branchial fluxes and urine parameters declined from 14 on Day 0 to 10 by the end of Day 4 because of death caused by exposure to acid (two during Day 3 and two during Day 4). N values for blood acid-base and ionic parameters were lower due to inoperative dorsal aortic catheters, declining from 10 (on Day 0) to 7 (on Day 4). Significant differences for blood and urine parameters were tested with the Student's two-tailed t test (paired design) using each fish for its own 'control' values (the Day 0 values).

RESULTS

Control measurements

In all trout the Day 0 measurements of blood acid-base status (Fig. 2), plasma ion levels (Fig. 6), urine flow rate (Fig. 5) and renal ion and acid excretion (Table 2) were closely comparable to values we have previously defined as normal for this species acclimated to similar experimental conditions (Wood & Caldwell, 1978; Kobayashi & Wood, 1980; McDonald *et al.* 1980).

Table 2. *Urinary excretion and net branchial fluxes in the rainbow trout near neutral solutions*

(pH 7.5 for Day 0, acid exposure series; 7.9 for Days 0-4, control series.)

	Urinary* excretion	Gill net fluxes		
	Day 0 (acid series)	Day 0† (acid series)	Day 0‡ (control series)	Days 1-4§ (control series)
Ammonia ($\mu\text{mol/kg.h}$)	-5.3 (± 1.4)	-325.4	-388.8	-501.5 (± 111.5)
Net acid ($\mu\text{equiv/kg.h}$)	-3.7 (± 1.9)	-166.6	-164.1	-47.7 (± 48.1)
Na ⁺ ($\mu\text{equiv/kg.h}$)	-27.4 (± 9.1)	+134.5	+85.9	+13.0 (± 22.0)
Cl ⁻ ($\mu\text{equiv/kg.h}$)	-23.1 (± 8.9)	+128.5	+51.6	+27.5 (± 25.5)

* Means ± 1 S.E.M. for 14 animals.† Means of two 12 h determinations (each determination is weighted mean of two replicates, $N = 6$ and 8).

‡ Means of two 12 h determinations (each determination is a single average value from eight animals).

§ Means ± 1 S.E.M. of eight separate 12 h determinations.

For all flux periods under control conditions the trout exhibited a net uptake of Na⁺ and Cl⁻ across the gills and a net branchial excretion of acid (Table 2). On Day 0 these net fluxes exceeded their respective urinary losses indicating that the animals were initially in positive salt balance and were clearing an acid load despite an apparently normal blood acid-base and ionic status. These disturbances were, however, transitory as indicated by the subsequent reduction in branchial fluxes in fish maintained under control conditions (control series; Days 1-4, Table 2). While variable over Days 1-4, the net influxes of Na⁺ and Cl⁻ closely matched their respective urinary losses, and branchial net acid excretion was about 30% of the Day 0 values. Branchial ammonia excretion, on the other hand, showed only a slight change from Day 0 values and was throughout, about 60- to 100-fold greater than urinary ammonia excretion.

Acid exposure

Acid exposure over a 4-day period substantially altered the direction and magnitude of the branchial ion and acid fluxes. During acid exposure the trout were not maintained at a constant pH. Rather, pH rose during each flux period due to the presence of the fish and in the 2 h intervals between flux periods was adjusted with H₂SO₄. The resulting fluctuations in pH (3.7 to 5.1, overall mean = 4.2) are shown in Fig. 1C superimposed on the titratable component of the net acid flux. This component, while variable during acid exposure, had increased substantially from that on Day 0 with its fluctuations being closely correlated ($r = 0.72$) with the variations in mean H⁺ ion concentration among the flux periods. This component, as outlined in Methods, reflects, but does not equal, net acid absorption by the gills; it overestimates this influx by an unknown amount equal to the amount of ammonia excretion in the free base form (NH₃).

Total branchial ammonia excretion (i.e. NH₃+NH₄⁺) was also variable during acid exposure but for most flux periods was slightly elevated compared to Day 0

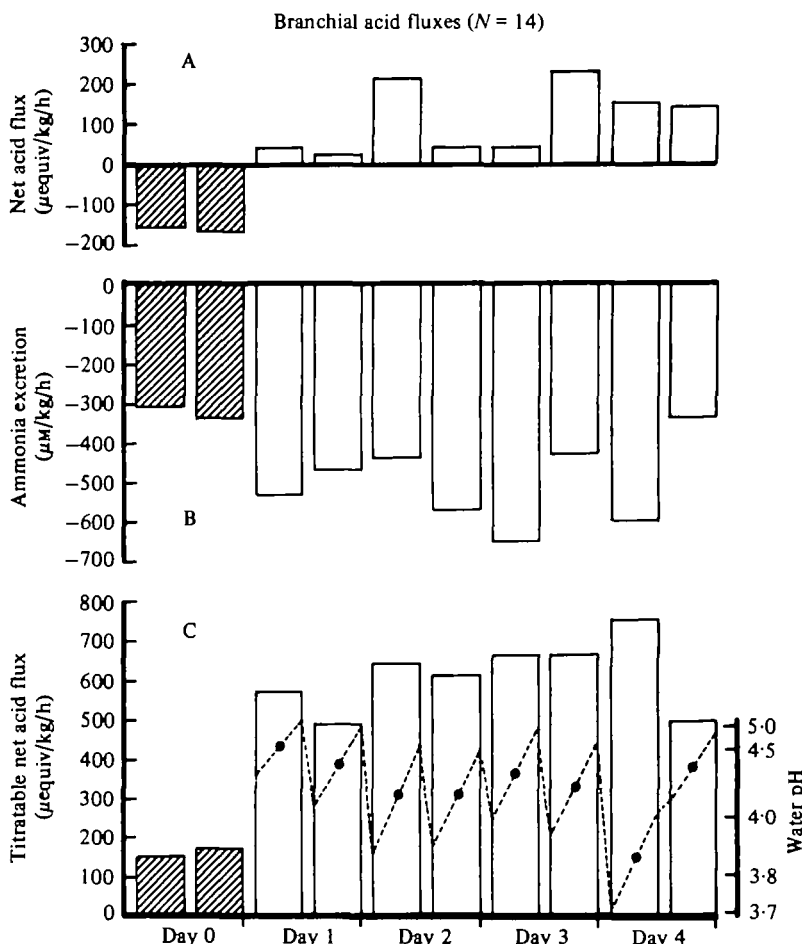


Fig. 1. Branchial net acid fluxes in the rainbow trout in near neutral (hatched bars, pH = 7.5) and acid solutions (unatched bars, mean pH = 4.2) at 16 ± 1 °C. (A) Total net acid flux. (B) Ammonia excretion. (C) Titratable acid net flux. Fluxes were determined as a single average value for groups of fish (6 and 8) in the recirculating system. Values are means of these two measurements. $N = 14$ (Day 0) declining to $N = 10$ (day 4). Dotted lines in C show fluctuations in water pH during acid exposure. ●, Mean pH for each flux period. Water pH was lowered between flux periods by addition of 1 N H_2SO_4 .

values (Fig. 1 B). In this case the variability was not correlated with the variations in the pH regime nor were the increases apparently due to the acid exposure since a similar phenomenon was observed in the controls. In fact the average branchial ammonia excretion rates (i.e. Days 1-4) were nearly identical for the two series (504.0 ± 35.6 $\mu M/kg.h$, acid series; 501.5 ± 115.5 $\mu M/kg.h$, control series). The net flux of acid across the gills (i.e. ammonia component + titratable acid component) is shown in Fig. 1 A. The variations in this flux were also not related to fluctuations in the pH regime because of the lack of correlation of the ammonia component. Nevertheless, for all flux periods during acid exposure there was a net branchial influx of acid.

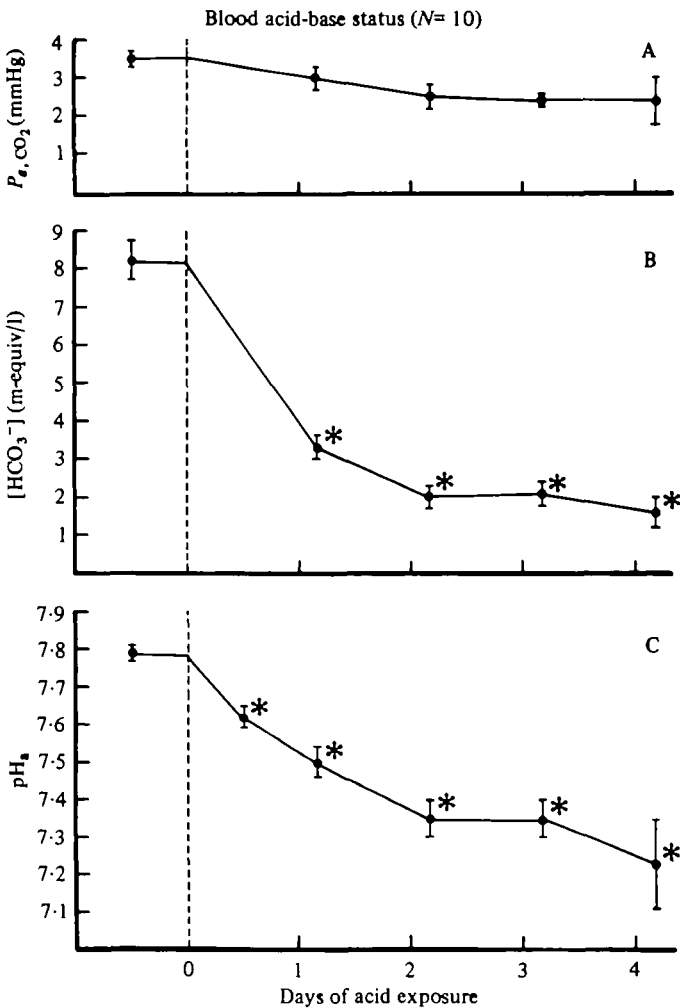


Fig. 2. Arterial blood acid-base status in the rainbow trout prior to and during acid exposure. (A) Arterial CO_2 tension. (B) Plasma bicarbonate concentration. (C) Plasma pH. Dotted line indicates beginning of acid exposure. Values are means \pm 1 S.E.M., $N = 10$ (Day 0) declining to $N = 7$ (day 4). Asterisks indicate means significantly different ($P < 0.05$) by paired t test from day 0 values.

This continuous penetration of acid across the gills resulted in a blood acid-base disturbance which progressively developed over the first 2 days of acid exposure (Fig. 2). This disturbance was characterized by a marked depression in both arterial pH (Fig. 2C) and $[HCO_3^-]$ (Fig. 2B) whereas P_{a,CO_2} remained unchanged (Fig. 2A). The nature of this disturbance is similar to that occurring in trout in HCl acidified hard water (McDonald *et al.* 1980) but was of greater magnitude. By Day 4 the total net accumulation of H^+ in blood (ΔH_b^+) was almost double that found previously (10.91 ± 1.39 m-equiv H^+ /l *v.* 5.71 ± 1.22 m-equiv/l). This difference is of uncertain genesis; it may be related to possible toxic effects of the sulphate anion or may simply reflect the higher temperature employed (16 ± 1 °C *v.* 11 ± 1 °C).

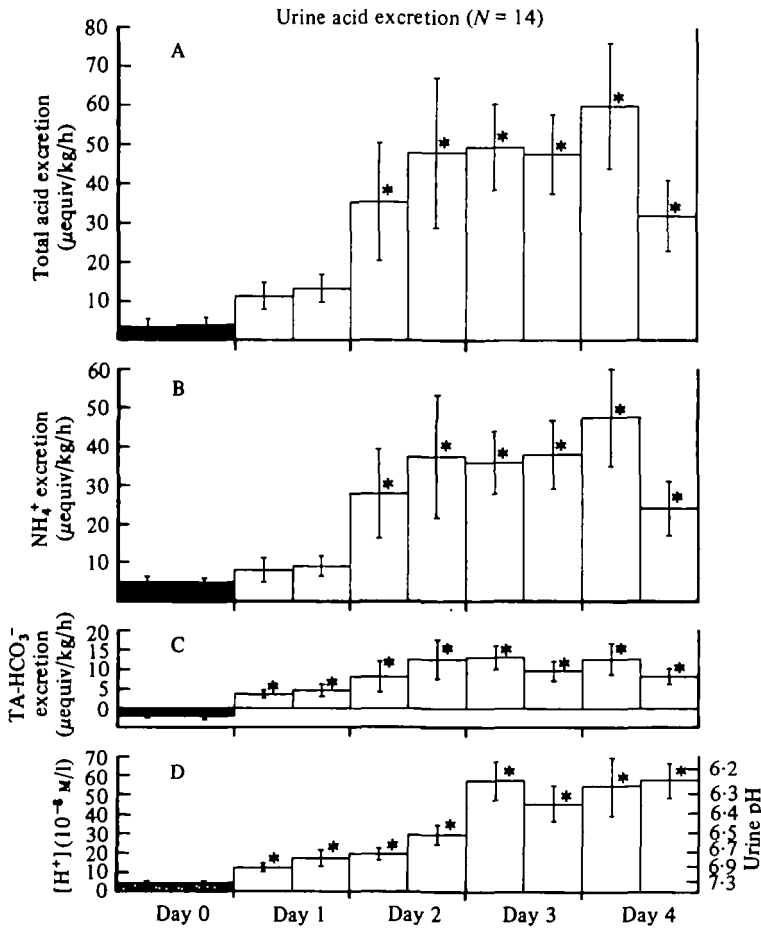


Fig. 3. Urinary acid excretion in the rainbow trout in near neutral (hatched bars, pH = 7.5) and acid solutions (unhatched bars, mean pH = 4.2). (A) Total acid excretion. (B) NH₄⁺ excretion (non-titratable component). (C) TA-HCO₃⁻ excretion (titratable component). (D) Hydrogen ion concentration in urine. Values are means ± 1 s.e.m., N = 14 (Day 0) declining to 10 (Day 4). Asterisks indicate means significantly different (P < 0.05) by paired t test from Day 0 values.

The progressive development of a blood acid-base disturbance during acid exposure was followed by a marked increase in acid excretion by the kidney (Fig. 3). The renal response to acid exposure comprised an immediate significant increase in the titratable component of the acid efflux (TA-HCO₃⁻, Fig. 3 C) and a slower though much larger increase in the non-titratable component (NH₄⁺, Fig. 3 B). The sum of the two components, the total acid excreted (Fig. 3 A), increased progressively for most of the acid exposure period, reaching a peak on Day 4, 15-fold higher than the Day 0 excretion rate. This was accompanied by a large increase in urinary acidification; by Day 4 the urine [H⁺], as assessed by pH, was 15-fold higher than Day 0 values (Fig. 3 D). This increase in acid excretion occurred against a background of a gradual

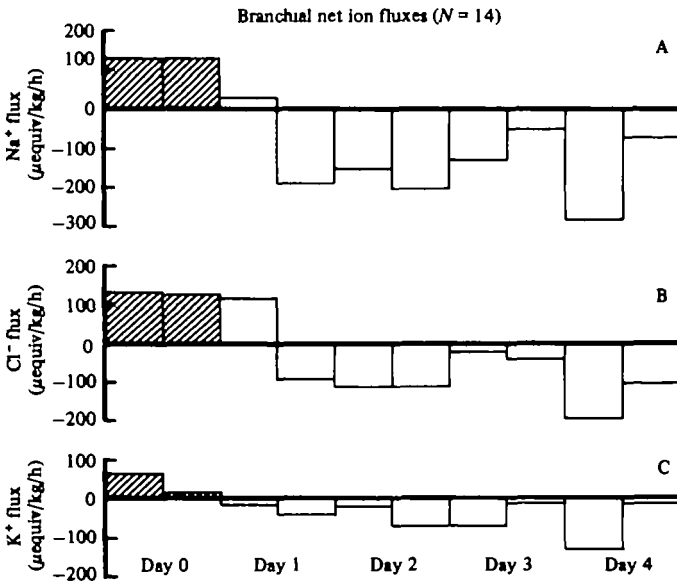


Fig. 4. Branchial net ion fluxes in the rainbow trout in near neutral (hatched bars, pH = 7.5) and acid solutions (unhatched bars, mean pH = 4.2). (A) Net sodium flux. (B) Net chloride flux. (C) Net potassium flux. Fluxes were determined as a single average value for six and eight fish at one time. Values are means of these two measurements. $N = 14$ (Day 0) declining to $N = 10$ (Day 4).

decrease in urine flow from Day 2 onward (Fig. 5F). This was particularly marked for the final collection period of acid exposure and, as a consequence, the urinary acid excretion for this period was significantly reduced compared to the previous collection period (Fig. 3A). The decrease in urine flow was apparently a real effect of acid exposure rather than an artefact, since urine flow in the controls (dotted lines in Fig. 5F) remained virtually unchanged throughout.

In addition to these substantial acid fluxes, acid exposure provoked a major alteration in the net ion fluxes across the gills (Fig. 4). For the first 12 h of acid exposure the trout continued to exhibit positive Na^+ and Cl^- net fluxes; thereafter the net fluxes of Na^+ , Cl^- and K^+ , although quite variable, were consistently negative (i.e. net losses). For all three ions there was a significant positive correlation ($r = 0.79$, 0.76 and 0.79 respectively) between the amplitude of net flux (sign considered) and the mean $[\text{H}^+]$ over the period the flux was determined, which explained much of the variability. There were also consistent differences among the ion fluxes within each flux period following the initial 12 h of acid exposure. The net Na^+ loss consistently exceeded the Cl^- loss by about 1.6-fold on average; the Cl^- loss, in turn, exceeded the K^+ loss by about 2-fold on average. Estimations of the branchial Ca^{2+} fluxes during acid exposure were relatively inaccurate because of measurement errors related to high background levels (~ 1.9 m-equiv/l) and because of uncertainties concerning the fraction of the total calcium in ionic form in the water. Nevertheless, the average net fluxes, while highly variable, were not significantly different from zero ($120 \pm$

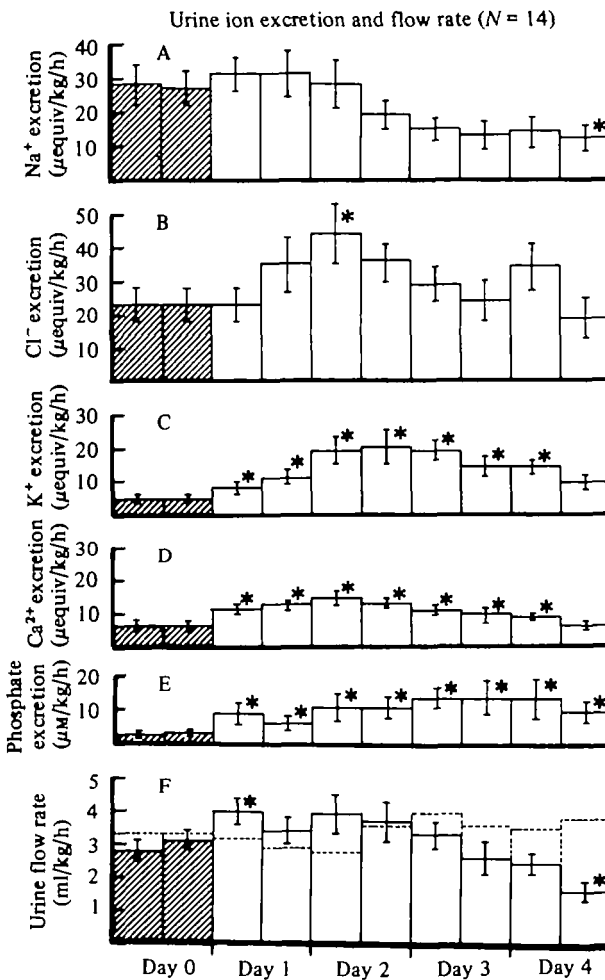


Fig. 5. Urinary ion excretion and flow rates in the rainbow trout in near neutral (hatched bars, pH = 7.5) and acid solutions (unhatched bars, mean pH = 4.2). (A) Sodium excretion. (B) Chloride excretion. (C) Potassium excretion. (D) Calcium excretion. (E) Phosphate excretion. (F). Urine flow rate. Dotted lines indicate urine flow rate in fish maintained at normal pH (means only, S.E.M.'s omitted for clarity, N = 8). Values are means \pm 1 S.E.M., N = 14 (Day 0) declining to 10 (Day 4). Asterisks indicate means significantly different ($P < 0.05$) by paired *t* test from Day 0 values.

$\mu\text{equiv/kg}\cdot\text{h}$) over the 4 day period. This does suggest that if there was a net loss or uptake of Ca^{2+} at the gills, it was of relatively small magnitude.

Compared to the branchial ion losses (Fig. 4), the renal ion losses were relatively small and relatively unaffected by acid exposure (Fig. 5). Similarly, urine flow rate (Fig. 5E) was only slightly elevated initially and was followed by a gradual decline. Renal Na^+ and Cl^- losses (Fig. 5A, B) largely followed the fluctuations in urine flow, although Cl^- losses were more variable, exhibiting an initial increase and thereafter gradually declining. There were, however, major increases in Ca^{2+} , K^+ and phosphate

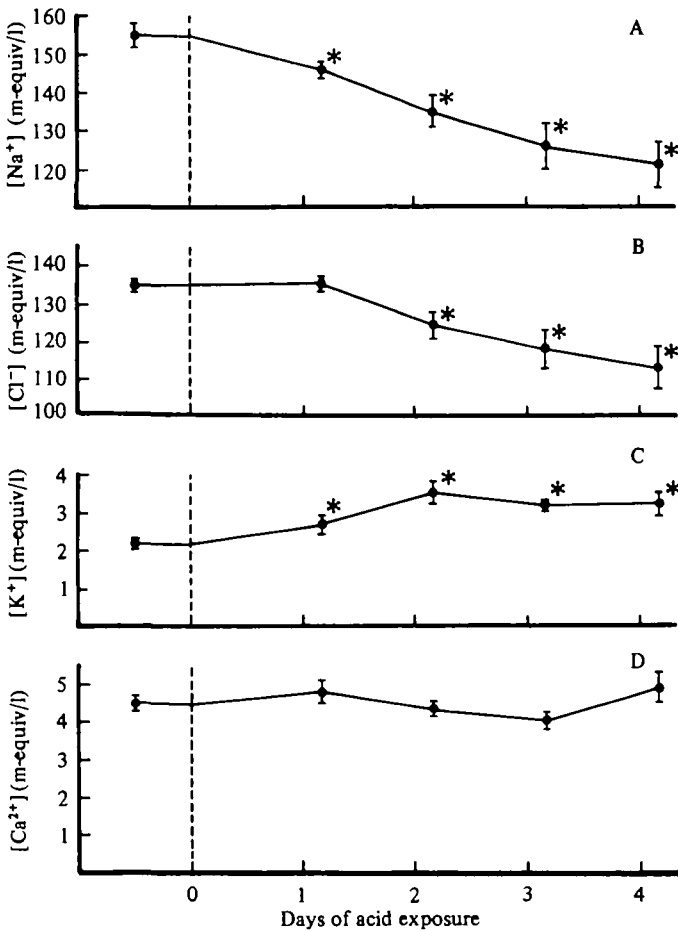


Fig. 6. Plasma ion levels in the rainbow trout prior to and during acid exposure. Dotted line indicates beginning of acid exposure. Values are means \pm 1 s.e.m., $N = 10$ (Day 0) declining to $N = 7$ (Day 4). Asterisks indicate means significantly different ($P < 0.05$) by paired t test from Day 0 values.

(H_2PO_4^- and HPO_4^{2-}) losses in the urine during acid exposure. Urinary K^+ (Fig. 5 C) increased progressively over the first 2 days of acid exposure to a level about 4-fold higher than Day 4 values, and thereafter declined coincident with the decrease in urine flow rate. Urinary Ca^{2+} (Fig. 5 D) and phosphate (Fig. 5 E) showed a similar pattern but the changes, while significant, were not as marked.

The net losses of Na^+ and Cl^- across the gills and via the urine led to a progressive reduction in their plasma levels (Fig. 6). The progressive reduction in plasma Na^+ (about 8.6 m-equiv/l. day) exceeded that of Cl^- (5.4 m-equiv/l. day), a pattern which reflected the greater branchial losses of the former. Plasma K^+ levels, on the other hand, significantly increased during acid exposure despite net branchial and urinary losses. Plasma $[\text{Ca}^{2+}]$ levels remained unaltered. These ionic disturbances were similar in nature to those occurring in the trout in HCl-acidified hard wa

Table 3. *Epaxial muscle ion levels and compartment volumes (means \pm 1 S.E.M.) in acid-exposed and control fish*

Parameter	Units	Control series (Day 4) (N = 8)	Acid series (Day 4) (N = 10)
ECF volume†	l/kg wet wt.	0.090 \pm 0.006	0.067 \pm 0.006*
ICF volume	l/kg wet wt.	0.698 \pm 0.005	0.744 \pm 0.009*
[Na ⁺]			
Plasma	m-equiv/l	152.7 \pm 2.7	115.9 \pm 5.7*
Tissue	m-equiv/kg wet wt.	16.1 \pm 1.1	9.8 \pm 1.5*
Intracellular	m-equiv/kg cell H ₂ O	3.3 \pm 1.4	1.8 \pm 1.5
[Cl ⁻]			
Plasma	m-equiv/l	134.2 \pm 1.0	108.9 \pm 5.6*
Tissue	m-equiv/kg wet wt.	13.2 \pm 0.8	9.8 \pm 0.8*
Intracellular	m-equiv/kg cell H ₂ O	1.6 \pm 0.1	2.5 \pm 0.2
[K ⁺]			
Plasma	m-equiv/l	2.1 \pm 0.1	3.3 \pm 0.2*
Tissue	m-equiv/kg wet wt.	127.0 \pm 2.0	107.5 \pm 3.0*
Intracellular	m-equiv/kg cell H ₂ O	181.7 \pm 2.9	146.8 \pm 4.0*

* Indicates means significantly different ($P < 0.05$) from corresponding control value by unpaired *t* test.

† ECFV estimated as chloride-potassium space (see Methods). ICFV estimated as total tissue water volume-ECFV.

(McDonald *et al.* 1980) but, like the blood acid-base disturbances, were of somewhat greater magnitude. The mean reduction in plasma Na⁺ levels by Day 4 of acid exposure was almost double that seen previously (-36.8 ± 5.8 m-equiv/l *v.* -20 ± 2.5 m-equiv/l) while the Cl⁻ reduction was about 5-fold greater (-25.3 ± 5.7 *v.* -5.4 ± 3.1 m-equiv/l.)

The terminal (Day 4) values for Na⁺, Cl⁻ and K⁺ in epaxial muscle (in m-equiv/kg wet weight) were significantly lower in the acid-exposed fish than in the control fish (Table 3). However, the calculated intracellular ion concentrations (in m-equiv/kg cell H₂O) indicate that only K⁺ fell in this compartment; the reduction in Na⁺ and Cl⁻ levels was largely due to their losses from the extracellular fluid compartment. According to these calculations (Table 3), there was a minor and not significant decrease in intracellular Na⁺ whereas Cl⁻ actually increased slightly. Intracellular K⁺ had, on the other hand, decreased by about 20% and the muscle extracellular fluid volume, estimated as the Cl-K space, had contracted by about 26%. Correspondingly, the ICFV (intracellular fluid volume) increased significantly by about 6%.

DISCUSSION

Validity of branchial flux estimates

The conclusions of this study are based to a large extent on the estimates of net fluxes of ions and protons across the gills of trout in near-neutral and acid solutions. Since these estimates were determined as a single average value for a group of fish (6-8) they are perhaps more subject to error than are averages computed from individual determinations. Thus, it is worthwhile to first compare the fluxes estimated in

Table 4. *Total net acid and ion fluxes (in $\mu\text{equiv/kg}$) via the gills and kidney of rainbow trout over the 4 days of acid exposure*

	Branchial	Renal	Total	Estimated ECF \dagger load of H $^+$ or loss of ions	Estimated ICF load or loss
H $^+$	+10440	-3110	+7330	+1670	+5660
Na $^+$	-12580	-1950	-14530	-7800	-6700
Cl $^-$	-6480	-2920	-9400	-5500	-3900
K $^+$	-3900	-1360	-5260	+230	-5490
*Net charge	+440				

* Net charge = (Na $^+$ + K $^+$ + H $^+$) - Cl $^-$ = -6040 - (-6480).

\dagger See text for details.

the present study with those previously obtained on individuals maintained under similar experimental conditions. In terms of ammonia excretion and branchial net Na $^+$ flux the agreement is quite good. Fromm & Gillette (1968) reported branchial ammonia excretion rates in the fasted rainbow trout at 13 °C of $380 \pm 90 \mu\text{M/kg.h}$, a mean value closely comparable to values obtained in the present study (Table 2). Similarly, the branchial Na $^+$ fluxes for Days 1-4 on the control series ($+13.0 \pm 22.1 \mu\text{equiv/kg.h}$, Table 2) are closely comparable to values reported by Wood & Randall (1973) for the resting trout at 15 °C ($+19.3 \pm 37.3 \mu\text{equiv/kg.h}$). While the initial values for net Na $^+$ flux (i.e. Day 0, acid and control series, Table 2) were substantially higher than the subsequent measurements in the controls, the extent of the elevation was not unusual compared to previously published values. Wood & Randall (1973), for example, have reported net Na $^+$ fluxes of similar magnitude in trout recovering from mild exercise ($+118 \pm 52 \mu\text{equiv/kg.h}$). Similar direct comparisons for branchial Cl $^-$, K $^+$ and proton fluxes are not possible because of a lack of comparable published data. However, there is a very good agreement between the total net flux of positive charges (Na $^+$, K $^+$ and H $^+$) and the net flux of negative charges (Cl $^-$) (Table 4). This does suggest that there was no substantial inaccuracy in any of the flux estimates since the law of maintenance of electroneutrality was not violated. These branchial flux values when considered together with the renal values (also in Table 4) provide the basis for an analysis of net acid uptake and net ion losses from the body of the trout during acid exposure.

Net acid loading

Despite the pronounced renal response to acid exposure (Fig. 3) the kidney removed, on average, slightly less than one-third of the total amount of acid entering the animal via the gills (Table 4). This fraction, of course, obscures the fact that the renal response to acid exposure was not immediate. However, even at maximum excretion ($60 \mu\text{equiv/kg.h}$, Fig. 3A) the renal acid output amounted to about one half of the average branchial influx during acid exposure ($108.8 \pm 29.6 \mu\text{equiv/kg.h}$; mean \pm s.e.m. for the eight influx determinations, Fig. 1A). Furthermore, the kidney was apparently incapable of maintaining acid excretion at this level because of the decline in urine flow rate (Fig. 5F). Thus, this substantial and persistent discrepancy

Between uptake and removal of acid led, by Day 4 of acid exposure, to a net body acid load amounting to 7330 $\mu\text{equiv H}^+/\text{kg}$ (Table 4).

By making some reasonable assumptions, it is possible to calculate, from the depression in plasma pH and $[\text{HCO}_3^-]$ (Fig. 2), the portion of this load buffered in extracellular fluids and thus, by difference, the amount buffered by the intracellular compartment. These ECF estimates (Table 4) are based on the assumptions that the extracellular fluid volume (ECFV) for the whole animal was 190 ml/kg (cf. Eddy & Bath, 1979; Cameron, 1980) partitioned as 50 ml/kg blood volume (BV; Stevens, 1968) and 140 ml/kg interstitial fluid (IFV), that interstitial fluids were in equilibrium with plasma with respect to pH and HCO_3^- , and that the non-bicarbonate (i.e. protein) buffering capacity of interstitial fluids was similar to that of plasma (Hargens, Millard & Johansen, 1974). The equation used for this calculation was thus:

$$\Delta\text{H}_{\text{ECFV}}^+ = \text{BV} \cdot \Delta\text{H}_\beta^+ + \text{IFV} \cdot \Delta\text{H}_{\text{IF}}^+,$$

where ΔH_β^+ and $\Delta\text{H}_{\text{IF}}^+$, the quantities of buffered H^+ , were calculated from equation 2 of McDonald *et al.* (1980). For the calculation of ΔH_β^+ , the required β value (the whole blood buffer value) was calculated from equation 3 of McDonald *et al.* 1980; for $\Delta\text{H}_{\text{IF}}^+$, a β of -2.70 mequiv/l. pH unit (the buffer capacity of separated plasma; D. G. McDonald and C. M. Wood, unpublished results) was assumed.

On this basis we estimate that by 4 days of acid exposure 1670 $\mu\text{equiv/kg}$ of protons were buffered in the ECF. Thus a large fraction of the total body load (77% or 5660 $\mu\text{equiv/kg}$) was, by this time, buffered by the intracellular compartment. Similar estimates for the first 2 days of acid exposure indicate that the participation of ICF to total buffering was progressive; by 2 days the net body load was 2427 $\mu\text{equiv/kg}$ of which 1540 $\mu\text{equiv/kg}$ was buffered in the ECF, i.e. ICF buffering, at this point, amounted to only 37% of the total. Further evidence for this progressive penetration of the intracellular compartment by H^+ is provided by the significant elevation in plasma K^+ levels (Fig. 6C) and in urinary K^+ excretion (Fig. 5C). In mammals, similar changes accompany a chronic metabolic acidosis and are attributed to H^+ penetration of the ICF in exchange for K^+ loss (Woodbury, 1974). The elevation of Ca^{2+} and phosphate excretion in the urine (Fig. 5D, E) further suggests that bone minerals may also make a significant contribution to intracellular buffering. Such changes occur in man during and following sustained acid loading (Lemann, Litzow & Lennon, 1966) and are attributed to the dissolution of the major mineral components of bone; $\text{Ca}_3(\text{PO}_4)_2$ and CaCO_3 (Lemann *et al.* 1966; Burnell & Teubner, 1971). Titration of the latter can, in fact, account for the major fraction (40–60%) of the total body buffering of an acid load (Burnell & Teubner, 1971). While the extent of the participation of bone in buffering in the present study is unknown, spinal deformities attributable to skeletal demineralization have been reported for chronically acid-stressed fish in the wild (Beamish, 1974).

Net ion losses

From Table 4 it can also be seen that the gills constitute the major route for Na^+ , Cl^- , and K^+ losses during acid exposure. Furthermore, despite the substantial differences in the magnitude of the losses, the percentages of the total loss occurring via

the gills were similarly large for all three ions; 87, 69 and 74 %, respectively. Thus, the ionic disturbances are due mainly to the effects of low pH on gill iono-regulatory mechanisms rather than on the renal reabsorption of filtered ions. Such disturbances may arise both via a stimulation of the passive branchial efflux of the ion in question and/or by an inhibition of its active inward transport. For branchial Na^+ fluxes, previous studies have shown that both phenomena are important. At pH 4.0, Na^+ influx was virtually completely inhibited in the trout (Packer & Dunson, 1970; McWilliams & Potts, 1978), while passive Na^+ efflux had increased substantially. These studies have also shown that the magnitudes of the unidirectional fluxes are correlated with pH, a result confirmed by the correlation in the present study between the net flux and the mean $[\text{H}^+]$ over the flux period (Figs. 1 C, 4 A). Less is known of the effects of low pH on gill Cl^- fluxes. Maetz (1973) reported that Cl^- net flux in the goldfish was unaffected by acid pH but the reduction in pH employed in this study was small (7.2 to 6.1). McWilliams & Potts (1978), while not directly measuring Cl^- fluxes at low pH, recorded a marked change in transepithelial potential in the trout transferred from pH 7.0 to 4.0. This, they estimated, would have resulted in an actual reduction in passive Cl^- efflux by about 50%. This phenomenon may be the basis for the lower net Cl^- losses reported in the present study. No information is presently available on the effect of low pH on branchial K^+ fluxes.

By assuming that plasma levels of Na^+ , Cl^- and K^+ (Fig. 6) reflect those of the whole body extracellular fluid space (190 ml/kg) than it can be estimated that 7800 $\mu\text{equiv/kg}$ of Na^+ and 5500 μequiv of Cl^- were lost from the ECF, and 230 $\mu\text{equiv/kg}$ of K^+ were gained by the ECF by Day 4 of acid exposure (Table 4). Thus, all of the body K^+ losses and slightly less than one-half of the body Na^+ and Cl^- losses came from intracellular compartments. Since muscle intracellular Na^+ and Cl^- levels were low and not appreciably affected by acid exposure (Table 3), the losses of these ions must have arisen largely from the remaining body tissues, particularly those with relatively high intracellular concentrations (e.g. cardiac muscle, gut and brain; Houston & Mearow, 1979). Muscle did, however, make a major contribution to the total K^+ loss. Indeed, if it is assumed that the muscle K^+ values (Table 3) are representative of the total white muscle mass ($\sim 55\%$ of the body weight; Cameron, 1974) then it can be estimated that the muscle K^+ loss is 11 847 $\mu\text{equiv/kg}$, a value approximately double the total body loss (Table 4). For this to have occurred, a substantial portion of the muscle K^+ loss must have been taken up by other body tissues, possibly those which sustained relatively high Na^+ and Cl^- losses.

These considerations suggest that, at the cellular level, the ionic disturbance has a complex and heterogeneous nature, the physiological consequences of which cannot be predicted with certainty. There are, however, some obvious possibilities. Since intracellular K^+ loss is linked to H^+ gain (Woodbury, 1974), the intracellular acidosis (Table 4) may be particularly severe in muscle tissue, leading to weakness and eventual paralysis. In turn, this K^+ efflux from muscle may provoke a hyperkalemia in blood (Fig. 6 C, Table 4) and other tissues. Increases in intracellular K^+ would lead, in tissues so affected, to membrane hyperpolarization and intracellular alkalosis (Woodbury, 1974). Major fluid shifts may also accompany the ion losses. A decrease in ECF osmotic pressure resulting from the reduction in plasma Na^+ and Cl^- levels

Fig. 6) would tend to reduce branchial water entry and increase ICFV at the expense of ECFV (Table 3). A general contraction of the ECFV would result and would affect circulatory efficiency. Recent studies in this laboratory have found evidence for such cardiovascular disturbances (C. L. Milligan and C. M. Wood, unpublished results). Further work will be needed to confirm these phenomena and to ascertain their relative importance in the overall toxic syndrome.

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