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DISTURBANCES IN HAEMATOLOGY, FLUID VOLUME DISTRIBUTION AND CIRCULATORY FUNCTION ASSOCIATED WITH LOW ENVIRONMENTAL pH IN THE RAINBOW TROUT, *SALMO GAIRDNERI*

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SUMMARY

Exposure of adult rainbow trout to low pH (4·0–4·5) for 3 days resulted in progressive increases in heart rate, mean arterial blood pressure and haematocrit. The haematocrit increase resulted from erythrocyte swelling, a reduction in plasma volume and a mobilization of erythrocytes from the spleen. Erythrocyte swelling probably resulted from plasma acidosis and a reduction in plasma ions (McDonald & Wood, 1981). There was an associated redistribution of body water from extracellular to intracellular compartments, but maintenance of total body water content. Erythrocyte mobilization was reflected in an increase in reticulocyte and erythrocyte counts, and a depletion of splenic blood reserves. Haemoconcentration caused large increases in blood viscosity, which contributed to the rise in blood pressure. Pharmacological analysis revealed an adrenergic component to the cardiovascular disturbance. These events are thought to play a key role in the toxic syndrome.

INTRODUCTION

The effects of low environmental pH on the physiology of freshwater fish are now known in some detail (see Fromm, 1980; Spry, Wood & Hodson, 1981, for recent reviews). The most extensive research has focussed on disturbances in blood oxygen transport (e.g. Vaala & Mitchell, 1970; Packer, 1979), acid-base regulation (e.g. Packer & Dunson, 1970; Neville, 1979*a*; McDonald, Hōbe & Wood, 1980; McDonald & Wood, 1981) and iono-regulation (Neville, 1979*b*; McDonald *et al.* 1980; McDonald & Wood, 1981). Of these three factors, impairment of O_2 uptake and/or delivery appears to be a key toxic mechanism at lower pH (3·0–3·5; see Spry *et al.* 1981) while ionoregulatory failure is more important at higher pH (4·0–6·0; McDonald *et al.* 1980; McDonald & Wood, 1981). Concomitant with these disturbances, at both higher and lower pH, is an elevation in haematocrit. Reasons offered for this elevation include an increase in erythropoeisis in response to tissue hypoxia (Vaala & Mitchell, 1970; Neville, 1979*b*) and a reduction in plasma volume (McDonald *et al.* 1980). It is the intent of this work to examine the cause(s) and consequence(s) of this haematocrit

elevation, as well as other cardiovascular and fluid balance disturbances, and to determine their role in the overall toxic syndrome. Experimental conditions have been chosen to duplicate as closely as possible those used in the recent study of McDonald & Wood (1981) on acid-base and ionoregulatory disturbance in rainbow trout under acid stress, in order to facilitate comparisons.

MATERIALS AND METHODS

Experimental animals

Rainbow trout (*Salmo gairdneri*; 150–400 g) were obtained from Spring Valley Trout Farm, Petersburg, Ontario. Fish were held in large fibre-glass tanks continuously supplied with aerated dechlorinated Hamilton city water (hardness $\simeq$ 140 mg/l $CaCO_3$; $[Na^+] \simeq 1{\cdot}0$, $[Cl^-] \simeq 1{\cdot}0$, $[K^+] \simeq 0{\cdot}05$, $[Ca^{2+}] \simeq 2{\cdot}0$ mEq/l; temperature = 4–17 °C, seasonal fluctuations) and fed with commercial trout pellets.

One week prior to experimentation, fish were acclimated in groups of 10 to experimental temperature, $14{\cdot}0 \pm 1{\cdot}5$ °C, in decarbonated water (pH = 7·0–7·5) in a large (500 l) recirculating system. During acclimation and the subsequent experimental period, fish were starved in order to reduce diet-induced variability in haematological parameters (Weinberg *et al.* 1976).

In experimental series II (see below) the fish were not subjected to surgery, but in series I and III, the trout were chronically cannulated to allow repetitive blood sampling and measurement of cardiovascular parameters. These fish were anaesthetized in acclimation water (MS 222 1:10000; Sigma) and surgically implanted with dorsal aortic catheters filled with Cortland saline (Wolf, 1963), following the method of Smith & Bell (1964). The fish were allowed to recover for 24–48 h prior to experimentation in decarbonated water at pH 7·0–7·5. Day 0 (i.e. control) measurements were then taken from each animal. Subsequently, the fish were either exposed to acidified decarbonated water (pH = 4·0–4·5) for 72 h or maintained in decarbonated water of normal pH (7·0–7·5) to serve as controls.

Test conditions

All experiments were carried out in a temperature controlled ($14{\cdot}0 \pm 1{\cdot}5$ °C) 100 l recirculating system, supplying eight darkened 2 l plexiglass fish boxes, in which fish were isolated. Each box was individually aerated and received a water flow of at least 600 ml/min. The switch-over from control water to acid water was achieved without disturbance to the fish.

Water was decarbonated by titrating dechlorinated Hamilton city water to a pH < 4·0 with 1 N-H_2SO_4, aerating for 24 h, then back titrating with 1 N-KOH to either normal pH (7·0–7·5) or experimental pH (4·0–4·5). Sulphuric acid was used as it is a common mineral acid pollutant in the wild (Beamish & Harvey, 1972; Schofield, 1976). Decarbonation of the water was carried out to eliminate complicating effects of high P_{CO_2} (Neville, 1979*b*). During acid exposure, water pH was monitored 2–3 times daily and readjusted to a pH of 4·0 by addition of 1 N-H_2SO_4. The overall mean pH (calculated as mean pH) in 14 different experimental runs was $4{\cdot}31 \pm 0{\cdot}11$ ($\bar{x} \pm$ 1 S.E.M., N = 177). After 3 days acid exposure, the average $[SO_4^{=}]$ added to the water was $1{\cdot}6 \pm 0{\cdot}3$ (14) mEq/l.

Experimental series

Three series of experiments were carried out, using a total of 146 fish. The effects of 3 days acid exposure (pH = 4·0–4·5) on cardiovascular function, haematology and fluid volume distribution were examined.

(1) *Series I: cardiovascular function*

Fish were cannulated as previously described. After recovery from surgery, control measurements were taken and the animals were either exposed to acid for 3 days (n = 25) or left at normal pH (n = 22). Haematocrit, plasma [protein], [haemoglobin], dorsal aortic blood pressure, and heart rate were measured each day in the acid group. Fish in both groups were then subjected to either one or the other of the two drug injection protocols outlined immediately below. Drugs were injected through the dorsal aortic cannula in a volume of 0·5 ml/kg Cortland saline and washed in with a further 1 ml/kg saline. After a further 3–4 h to allow compensation of the injected fluid load, terminal blood samples were drawn for blood and plasma viscosity measurements.

(*a*) *Heart Rate Analysis.* Employing the protocol of Wood, Pieprzak & Trott (1979), atropine sulphate (1 μmol/kg; Sigma) was injected, followed at 30 min intervals by acetylcholine chloride (100 n-mol/kg; Sigma) and l-adrenaline bitartrate (100 n-mol/kg; Sigma). Atropine was used to produce a complete muscarinic cholinergic blockade of the heart and thereby eliminate vagal tone. Acetylcholine was used to test the effectiveness of the blockade. In all cases, the blockade was complete and no further administration of atropine was required. Adrenaline was employed to produce maximum adrenergic stimulation of the heart. Readings of mean dorsal aortic blood pressure and heart rate were taken 3–6 min after atropine injection and immediately after adrenaline injection (see Wood, Pieprzak & Trott, 1979, for additional details).

(*b*) *Blood Pressure Analysis.* Yohimbine hydrochloride (10 μ-mol/kg, Sigma) was injected in order to block α-adrenergic vasomotor tone in the circulation (cf. Wood & Shelton, 1980). In order to ensure complete blockade, the dose employed was an order of magnitude greater than that used by Wood & Shelton (1980). Adrenaline (100 n-mol/kg), which produced a very large rise in dorsal aortic blood pressure in control animals (Fig. 3), had no effect when administered after yohimbine. The yohimbine effect stabilized 3–5 min after injection; readings were taken at 10–15 min.

(2) *Series II: haematology*

The fish used in this study were not cannulated so as to avoid blood loss associated with the cannulation procedure and make a better assessment of any haematological disturbances caused by acid exposure.

The noncannulated fish were placed in the recirculating system previously described, held one day to allow recovery from handling stress, then weighed in water to an accuracy of 0·1 g by the method of Wood & Randall (1973) and exposed to either acid water (N = 8) or left at normal pH (N = 8). At the end of 3 days, fish were quickly killed (< 5 s) by a cephalic blow and reweighed in order to detect any net gain or loss of water. Blood samples were obtained by caudal haemal arch puncture

(< 1 min) and the spleen was excised immediately thereafter. Because prolonged struggling in itself caused splenic contraction and changes in haematological parameters, data were only taken from those 16 fish which were killed with a minimum of disturbance and sampled within the prescribed time. Excised spleens were weighed and analyzed for total haemoglobin content. Blood samples were analysed for haematocrit, [haemoglobin], plasma [protein], red cell and reticulocyte counts.

To assess any disturbing influence of caudal haemal arch puncture on the blood parameters measured, a preliminary experiment was performed to ascertain the effect of the sampling procedure. Four fish were cannulated, as described, allowed to recover 36 h in water of normal pH, and then blood (150 μl) was sampled via the cannula. A second blood sample was then taken from each fish via caudal puncture as described above. The blood samples were analysed for pH, haematocrit, and [haemoglobin]. The pH was measured because acidosis has been implicated as a cause of erythrocyte swelling (Murphy, 1969).

(3) *Series III: fluid volume distribution*

Fish were cannulated, as described, and after 24 h recovery, control blood samples were taken and haematocrit measured. Fish were either exposed to acid (n = 14) or held at normal pH (n = 21). After 3 days in acid, or 1–2 days at normal pH, fish were injected via the catheter with Evans' Blue dye or ^{14}C-mannitol for determination of plasma volume or total extracellular fluid volume, respectively. In some experiments, the two labels were injected together for simultaneous measurement of the two compartments. Blood samples were taken hourly for 6 h, then again at 8, 10 and 12 h after injection for analysis of haematocrit and plasma concentrations of the labels. At the end of 12 h, fish were quickly killed, and terminal blood and epaxial muscle samples were taken. Epaxial muscle was excised from below the dorsal fin for determination of white muscle ECFV. Tissue samples and whole fish were then dried at 85 °C to a constant weight for determination of muscle and total body water contents.

Analytical techniques

For determination of haematocrit, plasma [protein] and [haemoglobin], 150 μl blood samples were drawn from the dorsal aorta cannula using a gas-tight Hamilton syringe pre-rinsed with heparinized (50 i.u./ml sodium heparin; Sigma) Cortland saline. An equal volume of this saline was then returned to the animal via the cannula.

Whole blood pH was determined on 40 μl samples with a Radiometer microelectrode thermostatted to experimental temperature and connected to a Radiometer PHM-72 acid-base analyser. The pH electrode was calibrated with Radiometer precision buffers (S1500; S1510). Haematocrit was determined by centrifuging 80 μl of blood in a heparinized capillary tube (Radiometer) for 5 min at 5000 g. [Haemoglobin] was determined on 20 μl blood by the cyanmethaemoglobin method (Blaxhall & Daisley, 1973; Sigma & Hycel reagents). Mean cell haemoglobin concentration (MCHC) was calculated as [haemoglobin]/haematocrit. Plasma [protein] was determined on 20 μl plasma using a Goldberg refractometer (American Optical).

For determination of blood viscosity, blood (5–6 ml) was withdrawn either through the dorsal aortic cannulae or via haemal arch puncture into a 10 ml syringe pre-rinsed

with heparinized saline (5000 i.u./ml). The relative viscosities of blood and plasma were determined on a minimum volume of 4 ml, by comparing their flow rates with that of water through an Ostwald U-tube viscometer (capillary diameter = 1·25 mm) at experimental temperature. In some cases, insufficient blood was obtained from a single fish, so blood was pooled from two fish. Haematocrit, [haemoglobin] and plasma [protein] were measured on the pooled samples. After determination of whole blood viscosity, samples were centrifuged for plasma separation; the viscosity of plasma alone was then measured. Corrections were applied for differences in specific gravity of the fluids, densities being determined gravimetrically. Relative viscosities were converted to absolute viscosities by multiplying by the tabulated viscosity of water at 14 °C (1·169 cp).

Dorsal aortic blood pressure was recorded by attaching the dorsal aortic catheter to a Hewlett-Packard 267 BC pressure transducer. The signal was amplified by a Sanborn 150–300 carrier pre-amplifier and displayed on a Sanborn 150 recorder writing on rectilinear coordinates. Heart rate was counted visually from the pressure trace. Mean arterial pressure was calculated as (1 systolic + 2 diastolic)/3 (Burton, 1972).

Red cell and reticulocyte counts were determined on 1 ml blood samples withdrawn via haemal arch puncture. Red cell count was performed using a standard red cell diluting pipette, Hendrick's diluting fluid and a standard haemocytometer (Hesser, 1960). Mean cell haemoglobin (MCH) was calculated as haemoglobin/red cell count and mean red cell volume as haematocrit/red cell count. Reticulocyte count was determined using 1 mg/ml New Methylene Blue N stain in Cortland saline containing 30 mg/ml trisodium citrate. Stain and whole blood (2 parts stain: 1 part whole blood) were mixed gently at experimental temperature (14·0 ± 1·5 °C) for 25 min (R. Walker, personal communication). Smears were then made in the conventional manner, and the reticulocyte proportion determined as suggested by Dacie & Lewis (1975) and Cameron (1971). Reticulocytes were identified according to the description of Dawson (1932).

Spleen haemoglobin content was determined by homogenizing the spleen in 5 ml Drabkin's reagent (Sigma and Hycel) for 2 min, using an Ultra-Turmax tissuemizer. The homogenate was allowed to stand for 15 min, centrifuged for 5 min at 5000 g, then 20 μl of the supernatant was diluted in 5·0 ml Drabkin's; absorbance was read spectrophotometrically at 540 nm. Haemoglobin content was calculated assuming a specific gravity of 1·00 g/ml for the spleen.

Plasma volume was estimated using the Evans's Blue dye (CI. 23680; Sigma) dilution method outlined by Smith (1966). Dye (1 mg/ml Cortland saline) was injected in a dose of 1 mg/kg and washed in with 1 ml/kg saline. Extracellular fluid volume was estimated by following the dilution of ^{14}C-mannitol (NEN; specific activity = 45 mCi/mmol) using a dose of 7 μCi/kg (Cameron, 1980) administered in the same manner. To measure ECFV and plasma volume simultaneously, ^{14}C-mannitol was diluted in the dye solution – i.e. 7 μCi/1 mg dye/ml saline/kg.

Blood (150 μl) was sampled hourly for 6 h, then at 8, 10 and 12 h, each sample being replaced by an equal volume of Cortland saline. Blood was centrifuged in 80 μl heparinized capillary tubes, haematocrit measured, and plasma drawn off. For plasma volume determination, 40 μl of plasma was diluted 1:25 in 1·0 ml saline; absorbance

was then read spectrophotometrically at 610 nm, together with that of injected stock diluted 1:500 in saline. For extracellular fluid volume determinations, 20 μl aliquots of plasma and injected stock were added to 10 ml scintillation fluid (PCS; Amersham) and radioactivity counted on a Beckman LS-250 scintillation counter. The channels ratio method was used to check for quenching. Log plasma concentrations of labels were plotted against time. For Evan's Blue dye, the mixing phase lasted approximately 2 h, so the 3–6 h points were used to determine the back extrapolated zero time concentration. For ^{14}C-mannitol, the mixing time was about 3 h, so the 4–12 h points were used routinely to fit the extrapolation line. Compartment volumes were calculated by dividing the injected dose by the extrapolated zero time concentration. Intracellular fluid volume (ICFV) was calculated as the difference between simultaneous measurements of total body water content (by drying) and ECFV. Interstitial fluid volume (ISFV) was calculated as the difference between simultaneous measurements of ECFV and plasma volume (PV). Blood volume (BV) was estimated as:

$$BV = \frac{PV}{1-\bar{H}t} \qquad \text{where } \bar{H}t = \text{mean}$$

haematocrit (as a decimal) of samples used in calculating the plasma volume.

In order to measure ECFV in white muscle, wet, pre-weighed epaxial muscle samples ($\sim$0·5 g) and terminal plasma samples (50 μl) were digested in 5 ml tissue solubilizer (NCS; Amersham) for 8 days. The digest was then neutralized with glacial acetic acid ($\approx$150 μl) and diluted with 10 ml scintillation fluid (OCS; Amersham). After 24 h incubation in the dark (to reduce chemiluminescence) radioactivity was counted as above. Muscle ECFV was calculated by dividing the simultaneously measured concentration of the label in the muscle (cpm/g) by that in the plasma (cpm/ml). Muscle ICFV was estimated as the difference between the total water content of duplicate muscle samples (by drying) and the calculated ECFV. All compartments, both in the whole fish and in the white muscle, have been expressed on kg wet weight basis.

Statistical analysis

Means $\pm$ 1 S.E.M. are reported throughout. These means exclude values from fish that succumbed during 3-day acid exposure or control periods. Significant differences ($P < 0{\cdot}05$) within each group were tested with Student's two-tailed t test (paired design) using the Day 0 values for each fish at its own control. Comparisons between groups (i.e. control *vs.* acid) were tested with Student's two-tailed t test (unpaired design; $P < 0{\cdot}05$). Lines were fitted using the method of least squares linear regression, and the significance of the correlation coefficient (r) assessed.

RESULTS

Series I: cardiovascular function

Exposure of adult rainbow trout to low pH for 3 days resulted in progressive increases in heart rate and mean dorsal aortic pressure (Fig. 1). By day 3 of acid exposure, heart rate had increased by 16·7%, with mean dorsal aortic pressure increasing by 21%.

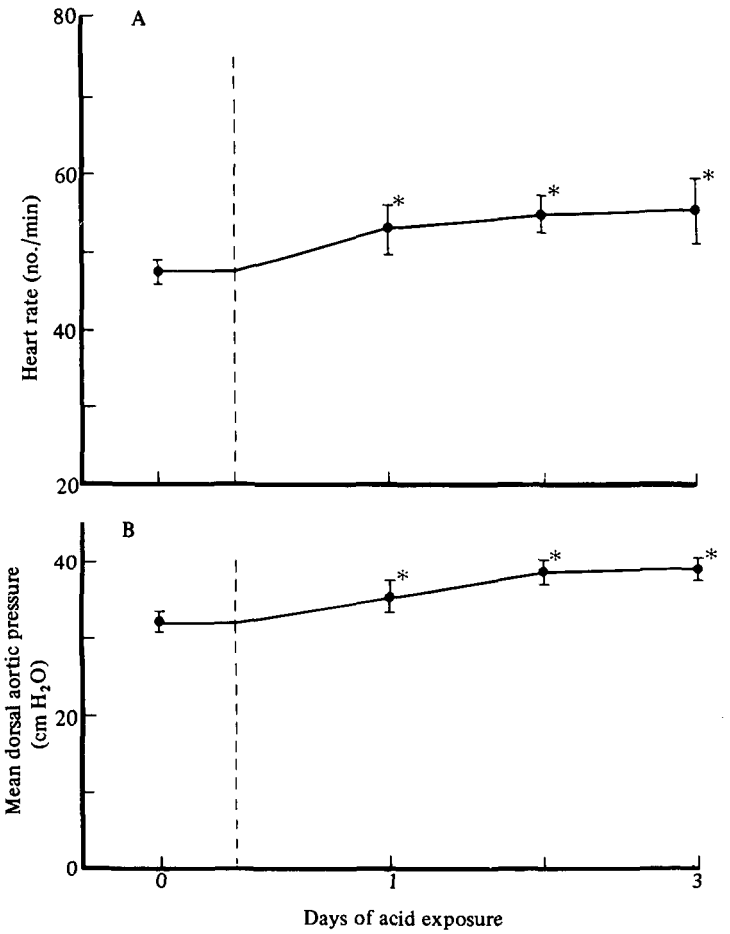

FIG. 1. Heart rate (A) and mean dorsal aortic pressure (B) in rainbow trout exposed to acid for three days. * significantly different ($P < 0.05$) from control (Day 0) values.

There also occurred progressive elevations in haematocrit, plasma [protein], [haemoglobin], and a progressive decrease in mean cell [haemoglobin] (MCHC) (Fig. 2). Haematocrit increased substantially, rising by 65% after 3 days of acid exposure. The elevations in plasma [protein] and [haemoglobin] were less dramatic, rising by 47% and 37%, respectively. MCHC is a measure of the amount of haemoglobin per 100 ml red cells, and is seen to decline by 21% with acid exposure. This decline in MCHC in the face of a rising haematocrit suggests that either red cell swelling and/or a mobilization of haemoglobin-poor erythrocytes from the spleen or other haemopoetic organ(s) into the circulation occurred.

Results from pharmacological analysis of the heart rate increase in acid exposed fish are shown in Fig. 3. Administration of atropine, a muscarinic cholinergic blocker caused a significant elevation in heart rate in both control and 3-day acid exposed trout (Fig. 3*A*). On both an absolute (Δ heart rate: $13{\cdot}5 \pm 3{\cdot}7$ ($n = 4$) *vs.* $13{\cdot}3 \pm 3{\cdot}6$ ($n = 10$) and relative basis (% increase: $30{\cdot}7 \pm 8{\cdot}1\%$ ($n = 4$) *vs.* $27{\cdot}4 \pm 8{\cdot}27$ ($n = 10$)), control

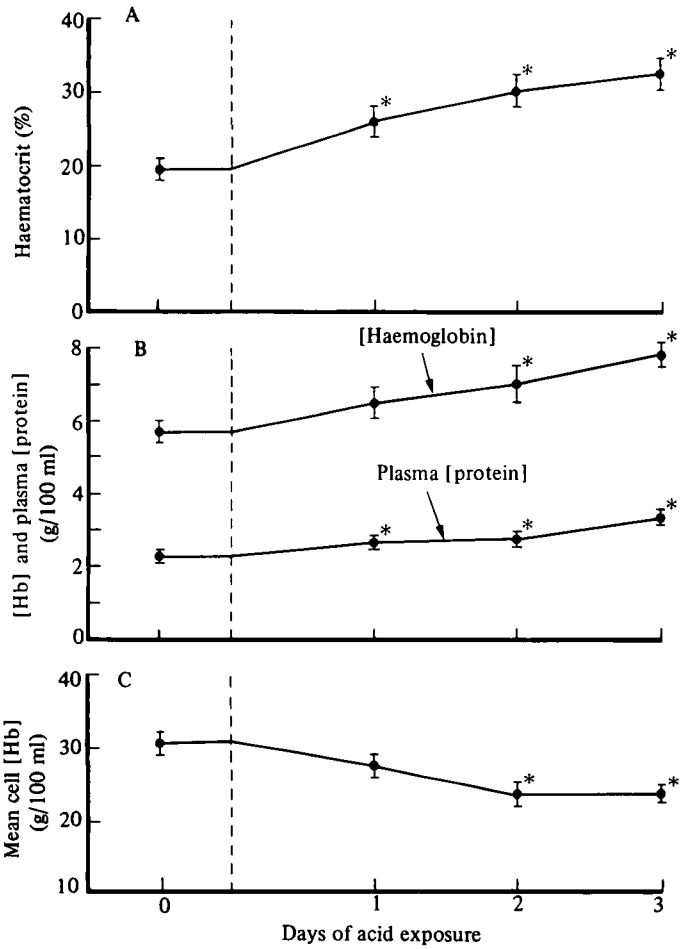

Fig. 2. Daily changes in (A) haematocrit, (B) [haemoglobin] and plasma [protein] and (C) mean cell [haemoglobin] during 3 days acid exposure. * significantly different ($P < 0.05$) from control (Day 0) values.

fish and 3-day exposed fish did not differ in their response to atropine. Atropine did not, however, have any significant effect on mean arterial pressure in either controls or 3-day acid exposed trout (Fig. 3*B*). These results suggest that the observed increases in heart rate were not due to a withdrawal of vagal cholinergic tone.

Administration of adrenaline after atropine caused a further significant elevation in heart rate in control fish ($29.2 \pm 3.0\%$, $n = 4$) equal in magnitude to that caused by atropine (Fig. 3*A*). However, the response of acid exposed fish was very different (Fig. 3*A*), with heart rate increasing a mere $3.6 \pm 1.4\%$, ($n = 10$) beyond the atropine rate, a non-significant change. Adrenaline also caused a significant elevation in mean dorsal aortic pressure in both groups (Fig. 3*B*), not surprising in light of the fact that adrenaline stimulates systemic vasoconstriction (Wood, 1976). This pressor response to adrenaline was significantly smaller in the acid group than in the control group on both a relative and absolute basis. These results suggest that the heart rate in 3-day

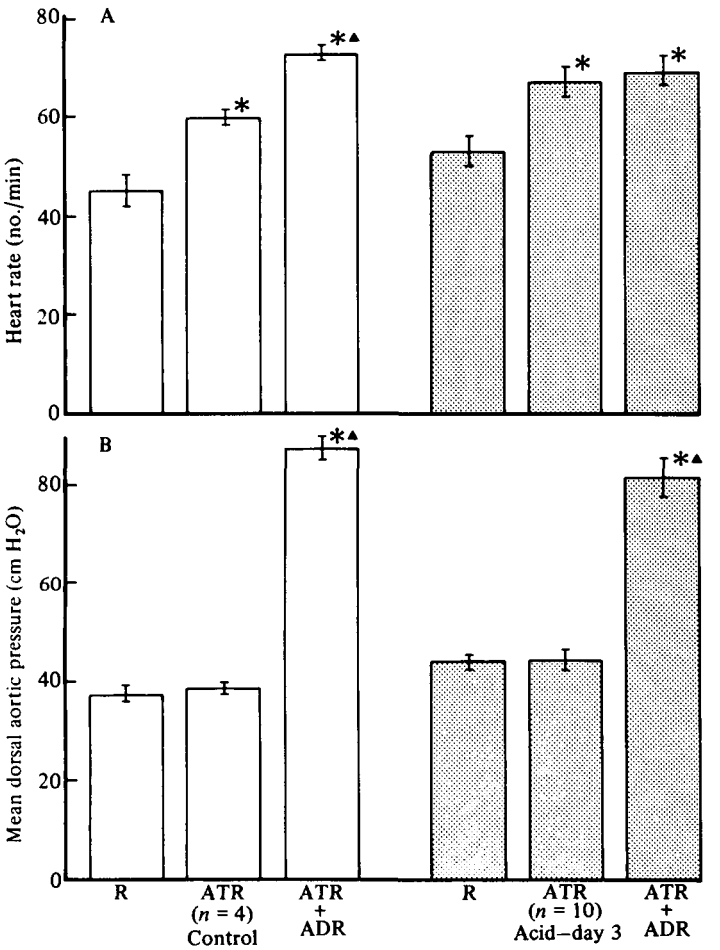

Fig. 3. Effects of atropine (1 μ-mol/kg) and adrenaline (100 n-mol/kg) after atropine on (A) heart rate and (B) mean dorsal aortic pressure in non-acid exposed trout and trout after three days of acid exposure. R, Resting; ATR, atropine; ATR+ADR, adrenaline after atropine. * Significantly different from resting values. ▲ Significantly different from value after atropine alone.

acid exposed fish was already maximally stimulated by endogenous catecholamines, a factor which may also have contributed to the progressive rise in dorsal aortic pressure.

To further investigate the pharmacological basis for the increase in mean dorsal aortic pressure, yohimbine, an α-adrenergic blocking agent was used. In both control and acid exposed fish, there occurred a significant depression in mean dorsal aortic pressure in response to yohimbine. In 3-day acid exposed fish, the blood pressure fell from an average of $37{\cdot}45 \pm 3{\cdot}8$ cm H_2O to a mean post-yohimbine level of $11{\cdot}76 \pm 1{\cdot}02$ cm H_2O (n = 10); compared to a decline in control fish from a resting mean pressure of $25{\cdot}39 \pm 1{\cdot}46$ cm H_2O to $17{\cdot}33 \pm 1{\cdot}17$ cm H_2O (n = 9). The depression of mean pressure in 3-day acid exposed fish was therefore nearly twice the size of that observed in control fish, with stabilization at a significantly lower post-yohimbine level. These results support the idea that elevated α-adrenergic systemic vasoconstriction occurred

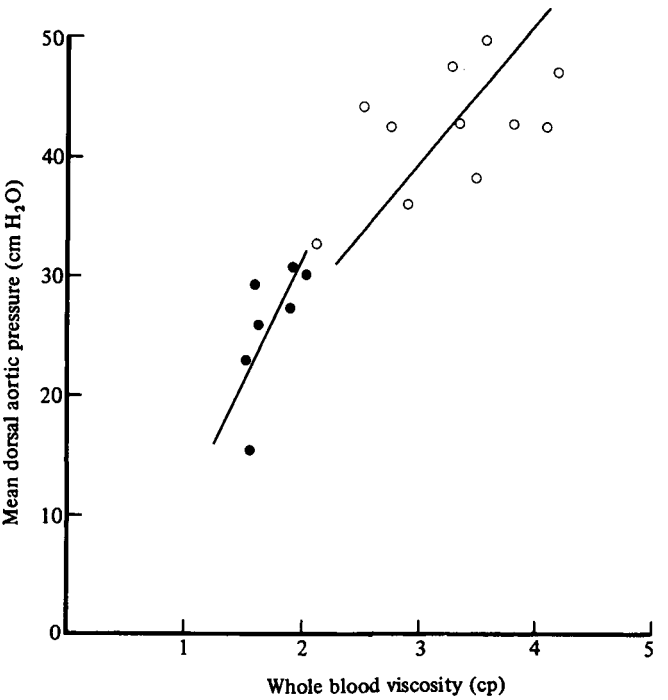

Fig. 4. Relationship between mean dorsal aortic pressure and blood viscosity in non-acid exposed trout (●) and in trout after three days acid exposure (○). Regression lines as determined by the method of least-squares. Day 0, $y = 18{\cdot}78x - 6{\cdot}92$; ($n = 7$) ($r = 0{\cdot}71$; $P < 0{\cdot}05$); Day 3, $y = 11{\cdot}8x - 3{\cdot}81$; ($n = 11$) ($r = 0{\cdot}68$; $P < 0{\cdot}05$).

in acid exposed fish, mediated by an endogenous catecholamine release. This catecholamine release may also have stimulated a small β-dilatory response. When the α-vasoconstrictory response was blocked by yohimbine, the β-vasodilatory response would be unmasked. The lower post-yohimbine level in acid exposed fish may have been a result of the unmasked β-dilatory response. Unfortunately, selective blockade of β-adrenoreceptors could not be employed to confirm this point (see Discussion).

Although α-adrenergic vasoconstriction occurred during acid exposure, it cannot be considered as the sole cause of the observed pressor effect. The increase in heart rate (Fig. 1*A*) may also have been a contributing factor. More importantly, 3 days acid exposure resulted in a near doubling of whole blood viscosity (acid: $3{\cdot}63 \pm 0{\cdot}291$ cp ($n = 15$); control: $1{\cdot}864 \pm 0{\cdot}061$ cp ($n = 31$)), as measured *in vitro*. Plasma viscosity also rose, (acid: $1{\cdot}486 \pm 0{\cdot}074$ cp, $n = 10$; control: $1{\cdot}298 \pm 0{\cdot}016$ cp, $n = 28$), but the change, while significant, was not as dramatic as the whole blood viscosity increase. In a simple constant flow system, resistance, and therefore pressure, are direct functions of viscosity. The situation *in vivo* is much more complex (see Discussion); nevertheless, increasing blood viscosity might be expected to cause a rise in blood pressure. The significant positive correlation between the two parameters in both control and 3 day acid exposed fish (Fig. 4) suggests that this was indeed the case, though correlation need not imply causation.

In Fig. 5, blood viscosities from control and 3-day acid exposed fish are plotted

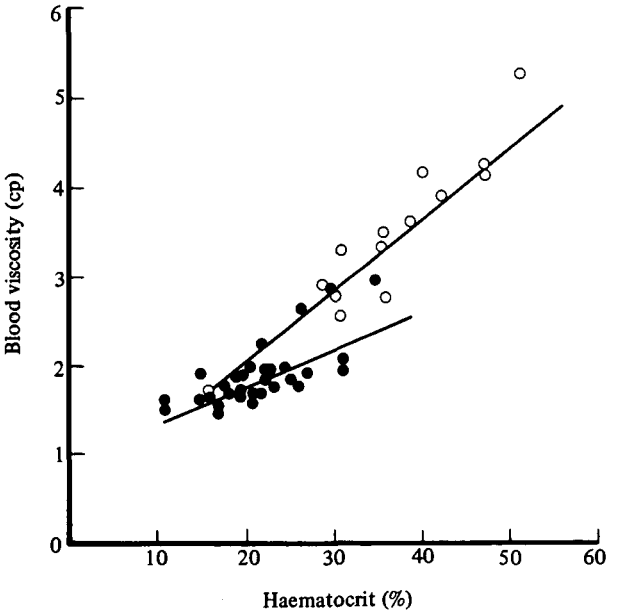

Fig. 5. Relationships between haematocrit and blood viscosity in control fish (●) and in fish after three days of acid exposure (○). Regression lines as determined by the method of least-squares. Day 0, $y = 0{\cdot}04x + 0{\cdot}93$; ($n = 31$) ($r = 0{\cdot}72$; $P < 0{\cdot}001$); Day 3, $y = 0{\cdot}08x + 0{\cdot}39$; ($n = 14$) ($r = 0{\cdot}93$; $P < 0{\cdot}001$).

against haematocrits. In both groups, the relationship is strongly correlated ($r = 0{\cdot}72$, $r = 0{\cdot}98$, respectively; $P < 0{\cdot}001$), suggesting that the blood viscosity increase was due to the haematocrit increase, abetted by the rise in plasma protein levels (Fig. 2*B*). The change in slope between the two groups is due to the nature of the haematocrit-viscosity relationship, which becomes alinear with increasing haematocrit (Burton, 1972).

Series II: haematological disturbance

In this series of experiments, non-cannulated fish were employed to assess haematological changes associated with acid exposure. Results from a preliminary experiment showed that while blood samples obtained via caudal haemal arch puncture were slightly acidic compared to samples obtained from the same fish via the dorsal aorta cannula (pH = $7{\cdot}599 \pm 0{\cdot}061$ *vs.* $7{\cdot}765 \pm 0{\cdot}024$, $n = 4$), there was no significant difference in either [haemoglobin] ($4{\cdot}26 \pm 0{\cdot}5$ *vs.* $4{\cdot}21 \pm 0{\cdot}76$ g/100 ml, $n = 4$) or haematocrit ($21{\cdot}9 \pm 2{\cdot}7\%$ *vs.* $20{\cdot}2 \pm 1{\cdot}1\%$, $n = 4$). We conclude therefore that sampling by caudal haemal arch puncture would have negligible disturbing influence on the parameters measured.

The haematocrit increase observed after 3-days acid exposure in noncannulated fish was much greater than that in cannulated fish (122% *vs.* 65%; Fig. 1 and Table 1), a difference most likely reflecting blood loss due to the cannulation procedure. Similarly, increases in plasma [protein] and [haemoglobin] during acid exposure were also greater in noncannulated fish. Investigation into other haematological parameters

Table 1. *Changes in various haematological parameters after 3-day acid exposure in noncannulated fish*

	Control ($n = 8$)	Acid – day 3 ($n = 8$)
Haematocrit %	21·3 ± 2·9	47·3 ± 7·8*
Plasma [protein] (g/100 ml)	2·08 ± 0·11	3·94 ± 0·28*
[Haemoglobin] (g/100 ml)	5·95 ± 0·76	8·91 ± 0·95*
Red blood cell count ($\times 10^4$/mm^3)	65·6 ± 6·6	108·5 ± 13·1*
Reticulocyte count ($\times 10^3$/mm^3)	18·95 ± 5·45	83·55 ± 15·76*
Mean RBC volume ($\times 10^{-12}$ l/cell)	0·325 ± 0·016	0·420 ± 0·040*
Mean cell [haemoglobin] (g/100 ml RBC)	29·4 ± 3·7	16·7 ± 1·5*
Mean cell haemoglobin (pg/cell)	92·26 ± 9·45	67·49 ± 2·79*
Spleen weight† (g)	0·546 ± 0·071	0·340 ± 0·060*
Spleen haemoglobin (g/spleen)	0·18 ± 0·04	0·06 ± 0·01*

* Significantly different ($P < 0·05$) from control values.
† Mean body weight: controls – 180·3 ± 15·3 g; day 3 acid – 198·0 ± 20·2 g.

(Table 1) revealed that acid exposure resulted in significant increases in the number of circulating erythrocytes and reticulocytes, in addition to red cell swelling, as indicated by the significant increase in mean red cell volume. Mean cell haemoglobin concentration, (MCHC) and mean cell haemoglobin (MCH) were both significantly reduced. Splenic stores of blood were depleted as indicated by a reduction in spleen weight, and most notably, in spleen haemoglobin content.

The reduction in mean cell haemoglobin (MCH) is surprising in light of simultaneous increases in whole blood haemoglobin levels and red cell count. In order for MCH to be reduced by this extent (Table 1), new red cells entering the circulation must contain approximately one-half the haemoglobin content per cell (i.e. MCH) as those cells already present in the circulation. Indeed, the quadrupling of the reticulocyte count suggests a mobilization of immature erythrocytes. If it is assumed that all haemoglobin released from the spleen is contained in cells, then, based upon the difference in red cell count and spleen haemoglobin content after 3 days acid exposure, it is estimated that the 'new' cells entering the circulation have an MCH of only 47·1 pg/cell. The uncertainty in this estimation lies in the fact that possible mobilization of cells from sources other than the spleen is not taken into account.

In summary, the reduction in MCHC appears to result from both red cell swelling and a mobilization of new, haemoglobin-poor erythrocytes into the circulation. The extent to which these factors contribute to the haematocrit increase is discussed below.

Table 2. *Fluid compartments in non-acid exposed fish and fish after 3 days of acid exposure*

(Means ± 1 S.E.M., (*N*)).

	Control	Day-3 acid
Weight (g)	275·5 ± 12·5 (27)	273·1 ± 14·5 (14)
Haematocrit (%)	21·9 ± 1·1 (27)	29·7 ± 2·6* (14)
Plasma volume (ml/kg)	43·5 ± 1·5 (26)	31·4 ± 1·7* (13)
Blood volume (ml/kg)	55·8 ± 1·6 (26)	46·1 ± 2·5* (13)
ISFV (ml/kg)	233·2 ± 8·9 (12)	169·9 ± 6·8* (12)
ECFV (ml/kg)	273·5 ± 8·8 (12)	199·9 ± 6·8* (12)
ICFV (ml/kg)	459·1 ± 9·4 (11)	524·8 ± 10·8* (10)
Total body water (ml/kg)	730·8 ± 4·5 (20)	731·1 ± 5·6 (10)

* Significantly different ($P < 0{\cdot}05$) from corresponding control values.

Series III: fluid volume distribution

While there was no significant change in total body water in 3-day acid exposed fish (Table 2), there was a marked redistribution of fluid from the extracellular compartment to the intracellular compartment. This maintenance of total body water is also reflected in the fact that neither control nor acid exposed fish in Series II exhibited a significant weight gain ($\Delta = 11{\cdot}35 \pm 6{\cdot}81$ ($n = 8$) g/kg; $\Delta = 32{\cdot}02 \pm 15{\cdot}48$ ($n = 8$) g/kg respectively), over the 3-day period.

The two components of the extracellular compartment, the plasma volume (PV) and interstitial fluid volume (ISFV) were reduced by the same relative extent ($\sim 27\%$; Table 2). This reduction in ECFV represents a net shift of 60–70 ml/kg into the intracellular compartment (ICFV) resulting in cell swelling, as has already been demonstrated for the erythrocytes (Table 1). Because of this erythrocyte swelling, as well as erythrocyte mobilization (Table 1), the reduction in total blood volume was only $\sim 17\%$.

The overall disturbance in body fluid distribution was reflected in the expaxial white muscle as an increase in total muscle water and intracellular fluid volume (ICFV) and a decrease in ECFV (Table 3). Looking at the data in Table 3, it would appear that the net shift of fluid of 20–30 ml/kg from the ECFV to the ICFV in muscle was not adequate to explain the total whole body shift of 60–70 ml/kg. However, this is misleading because the total muscle water was changing as well as ICFV and ECFV, a phenomenon which tended to mask the true fluid shift.

In view of the fact that there was an increase in total muscle water, a 1 kg (wet weight) piece of muscle in a 3-day acid exposed fish would contain relatively less dry cellular material (214·0 g/kg *vs.* 228·8 g/kg) and more water (786·0 ml/kg *vs.* 771·2 ml/kg) than a 1 kg piece of tissue from a control fish. Since the values in Table 3 are

Table 3. *Fluid compartments in epaxial white muscle of control fish and fish after 3 days acid exposure*

(Means ± 1 S.E.M.)

	Control (n = 8)	Day 3 acid (n = 10)	†Corrected Day 3 acid
Total muscle water (ml/kg)	771·2 ± 2·5	786·0 ± 4·3*	839·7
ECFV (ml/kg)	73·2 ± 2·6	59·5 ± 1·9*	63·5
ICFV (ml/kg)	698·0 ± 3·5	726·4 ± 4·5*	776·2

* Significantly different ($P < 0.05$) from corresponding control values.
† See text for details.

expressed per kg wet weight, error is introduced. In control fish, the ratio of water: dry cellular material is 3·37 ml/g; this increases after 3 days acid exposure to 3·67 ml/g. After 3 days acid exposure, the original 1 kg control muscle tissue now contains 3·67 ml/g × 228·8 g/kg = 839·7 ml of water, yielding a tissue swollen to a weight of 1068·5 g. In this swollen tissue, the ECFV is 63·6 ml and the ICFV is 776·2 ml. The real increase in muscle ICFV then is from 698·0 ml/kg of original wet weight to 776·1 ml/kg of original wet weight; a shift adequate to explain the whole body fluid shift (Table 3).

DISCUSSION

Exposure of adult rainbow trout to a mean pH of 4·31 resulted in disturbances in cardiovascular function, basic haematological homeostasis, and fluid volume distribution.

Cardiovascular disturbance was manifested as an increase in both heart rate and mean dorsal aortic pressure. Pharmacological studies revealed that the cardioacceleration was due to increased endogenous adrenergic stimulation to the heart and not a withdrawal of vagal cholinergic tone (cf. Wood *et al.* 1979). This stimulation could result from direct sympathetic innervation (Gannon & Burnstock, 1969) or release of catecholamines into the circulation. The latter is a common stress response in fish (Mazeaud, Mazeaud & Donaldson, 1977). Similarly endogenous adrenergic activity also caused α-adrenergic systemic vasoconstriction, contributing to the observed increase in mean dorsal aortic blood pressure during acid exposure. It should be noted that selective β-adrenoreceptor blocking agents (e.g. propranolol) could not be employed in these experiments as effective blocking doses have non-specific effects on both the heart and systemic vasculature of the trout (Bennion, 1968; Wood, 1976; Wood & Shelton, 1980).

Adrenergic effects on heart rate and systemic vascular resistance cannot be considered the only causes of the rise in blood pressure. Blood viscosity may also be an important factor (cf. Cameron & Davis, 1970). Whole blood viscosity increased greatly during acid exposure, undoubtedly a direct result of the rise in haematocrit, abetted by the increase in plasma protein concentration. While blood viscosity nearly doubled as measured *in vitro*, the actual *in vivo* increase was probably smaller, as it is known, at least in mammals, that *in vivo* viscosity for any given haematocrit is usually less than

in vitro viscosity, due to the Fahraeus–Lindqvist effect (Whittaker & Winton, 1933; Burton, 1972). It is this anomalous viscosity of blood which complicates its role as a determinant of mean dorsal aortic blood pressure. Until pressure-viscosity relationships *in vivo* and *in vitro* are better understood, it can only be said that the increased mean dorsal aortic pressure has a complex origin, involving both blood viscosity and increased endogenous catecholamine levels.

Several investigators have reported a variety of haematological responses to acid stress in fish. Increases in haematocrit have been seen in rainbow trout exposed to pH of 4·0–4·5 (McDonald *et al.* 1980; Neville, 1979*a*) and brook trout exposed to a pH of 4·2 (Dively *et al.* 1977). Neville (1979*a*) also reported significant elevations in [haemoglobin] and red blood cell count in rainbow trout exposed to pH 4·0, as did Vaala & Mitchell (1970) in brook trout exposed to pH 3·0–4·0. It has been suggested by Fromm (1980) that these haematological disturbances are a haemopoietic or erythrocyte mobilization response to hypoxemia induced by acid-stress. However, the results of these studies tend to conflict with that hypothesis, as no substantial decrease in arterial P_{O_2} nor increase in lactate content of the blood, which would be indicative of tissue hypoxia, have been reported in acid exposed (pH = 4·0–4·5) trout (brook trout, Vaala & Mitchell, 1970; Dively *et al.* 1977; rainbow trout, Neville, 1979*a*; McDonald *et al.* 1980). However, at lower pH (3·0–3·5), there may be impaired O_2 uptake and delivery. This could reflect diffusional limitations at the gill by mucus accumulation (Ultsch & Gros, 1979), and/or interference with haemoglobin O_2 transport by blood acidosis via the Bohr and Root effects (Packer, 1979).

The rise in haematocrit reported in the present study was quite pronounced (65% in cannulated fish, 122% in noncannulated fish) and accompanied by mobilization of erythrocytes from the spleen, manifested as increases in the number of circulating erythrocytes and reticulocytes as well as a reduction in spleen haemoglobin. Release of cells from the spleen was most likely due to splenic contraction in response to increased circulating catecholamines (Nilsson & Grove, 1974). Swelling of red cells and a reduction in plasma volume also accompanied the haematocrit increase.

Fish exposed to low pH in hard water experience a reduction in plasma [Na^+] and [Cl^-] as well as a pronounced blood acidosis (see recent reviews by Fromm, 1980; Spry *et al.* 1981). McDonald & Wood (1981) have exposed rainbow trout to low pH under virtually identical conditions to those used here. They found that large Na^+ and Cl^- losses occurred primarily at the gill and were a result of low pH interference with gill ionoregulatory mechanisms. Substantial plasma acidosis resulted from H^+ entry at the gill inadequately compensated by H^+ excretion at the kidney (McDonald & Wood, 1981). Red cell swelling most probably results from this ionic dilution of the plasma, which also affects body fluid distribution (see below), and is potentiated by plasma acidosis (Ferguson & Black, 1941; Murphy, 1969).

McDonald *et al.* (1980) also exposed rainbow trout to low pH (HCl) under similar conditions to those employed in this study. By assuming an initial blood volume of 50 ml/kg, they calculated from changes in plasma protein concentration that total blood volume was reduced by 17·5% on average in rainbow trout exposed to an average pH of 4·3 for 5 days. Results from the present study corroborate this, as blood volume, as calculated from measured plasma volume and haematocrit changes was

Table 4. *Independent relative contributions of three factors to the haematocrit increase observed during 3 days acid exposure*

(See text for details.)

RBC swelling	29%
Reduction in plasma volume	23%
Mobilization of RBC's from spleen	12%
Total	64%

reduced, on average, by 17·4% over 3 days from an initial value of 55·8 ± 1·6 ml/kg to 46·1 ± 2·5 ml/kg (Table 2). The reduction in plasma volume reflects an overall shift of fluid from the extracellular compartment to the intracellular compartment. This fluid shift can be viewed as a consequence of ion loss from the plasma across the gills (McDonald & Wood, 1981) which generates an osmotic and ionic gradient favouring the entry of water into the intracellular space and electrolyte flux in the opposite direction.

The relative contributions of red cell swelling, mobilization of red cells from the spleen and plasma volume reduction to the elevation of haematocrit were determined as outlined by Yamamoto, Itazawa & Kobayashi (1980) and are shown in Table 4. The calculation assumes that the effects occur independently. However, if the effects are compounded, as must occur *in vivo*, then about 75% of the observed haematocrit increase is accounted for. The 25% apparently unaccounted for may be a result of experimental artifact, as data from 2 groups of experimental fish (Series II, uncannulated, and III, cannulated) were used in the calculation. It may also reflect the release of red cells from other haemopoietic organs (such as the head kidney).

In the acid exposed fish, two major events are occurring which are primary consequences of acid exposure: firstly a reduction of plasma ions, and secondly, an extracellular acidosis (McDonald & Wood, 1981; see Fromm, 1980; Spry *et al.* 1981, for a review). The effects of these two events on fluid balance cannot be considered in isolation. At the whole body level, the shift of fluid from the extracellular space to the intracellular space can be viewed as a consequence of the fall in plasma ions. However, intracellular ion responses are themselves more complex. In test conditions similar to those employed in this study, McDonald & Wood (1981) reported a significant decline in intracellular K^+ in epaxial muscle, no apparent changes in intracellular Na^+ or Cl^-, and an apparent shift of fluid from ECFV to ICFV, based on chloride-potassium space calculations. The latter is confirmed by the present results. The reduction in intracellular K^+ can be seen as both a consequence of ionic dilution and a result of K^+/H^+ exchange as a mechanism for buffering acid intracellularly (cf. McDonald & Wood, 1981).

Fugelli & Vislie (1980) reported a reduction in intracellular K^+ and taurine in heart ventricle muscle in acid exposed brown trout (*Salmo trutta*), but no significant changes in ventricular muscle ECFV, despite a significant decline in plasma Cl^- levels. ECFV did decline non-significantly, however, so this apparent discrepancy may be artifact due to the small number of fish examined (control, $N = 3$; acid-exposed, $N = 4$). These authors suggest that K^+ and taurine are acting as osmo-effectors in an attempt

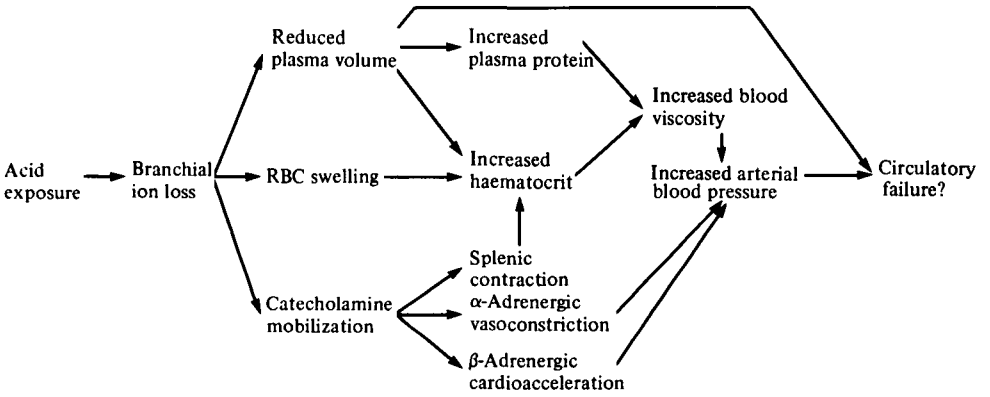

Fig. 6. Proposed sequence of events through which acid may exert its toxic effect(s). See Discussion for details.

to maintain osmotic equilibrium in the heart muscle (i.e. prevent cell swelling). However, they estimated that the cellular losses of K^+ and taurine accounted for only about 50% of the intracellular solute reduction required to totally prevent swelling. If the mechanisms of cell volume regulation in response to ion losses proposed by Fugelli & Vislie (1980) prove correct, then some form of tissue selective osmoregulation may be occurring in an attempt to 'save' certain vital organs (e.g. heart), perhaps to prolong survival in the face of acid stress.

In summary, most of the cardiovascular, haematological, and fluid compartment disturbances observed in the present study can be related to the primary ionoregulatory failure. The ultimate consequences of ionoregulatory failure may be circulatory failure due to the combination of increasing blood pressure and declining plasma volume. As the loss of ions continues, the circulatory effects would become progressively more severe, leading finally to circulatory collapse. Figure 6 outlines the proposed sequence of events initiated by ionoregulatory failure and ending in death. Values reported in this paper are only from fish which survived the 3 days of acid exposure. Mortality rates of about 40% were observed during 3 days of acid exposure (*vs.* 11% in control fish). As death is an unpredictable event, it is very difficult to obtain measurements at the point of death, which are obviously required to substantiate this model. The few that we have indicate haematocrits as high as 75%, enormous increases in blood viscosity and reductions in plasma volume to less than half the control levels.

It is recognized that acid precipitation primarily affects soft water lakes (Leivestad *et al.* 1976), and conclusions drawn in the present study are based upon results obtained in hard water. The proposed model, nevertheless, holds true for acid exposed fish in soft water. Recently it has been shown that as the level of environmental Ca^{2+} falls (i.e. the softer the water), acid exposed trout (mean pH 4·27) experience a greater net branchial ion loss (D. G. McDonald, in preparation). With increased branchial ion loss, the haematological changes occur more quickly in soft water. It seems probable that the circulatory effects would be exacerbated and circulatory collapse accelerated.

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