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## **<sup>31</sup>P-NMR STUDIES ON ACID–BASE BALANCE AND ENERGY METABOLISM OF ACID-EXPOSED FISH**

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### **Summary**

Tilapias (*Oreochromis mossambicus*), acclimated to 25°C and water with a Ca<sup>2+</sup> content of 0.68 mmol l<sup>-1</sup>, were subjected to gradual water acidification (from pH 7.6 to 4.0 in 4 h), followed by 10 h of exposure to low pH (pH 4.0), gradual environmental alkalization (from pH 4.0 to 7.6 in 2 h) and 6 h of recovery at normal pH (pH 7.6). Intermediates of energy metabolism were measured in perchloric acid extracts of gill, muscle and blood. In a separate series of experiments, the intracellular pH (pHi) and the levels of high-energy phosphate compounds were continuously monitored by *in vivo* <sup>31</sup>P-NMR spectroscopy. We used a 10 mm surface coil, which was positioned above the gill arches or the epaxial white muscle. With the coil above the gill, splitting of the inorganic phosphate peak indicated that the signal was picked up from three different compartments. These were tentatively identified as plasma, muscle and gill epithelium. Water acidification induced a transient pH drop of the plasma (0.24 units) and the gill (0.19 units) but the pH of both compartments slowly recovered during the 10-h exposure to acid water. In contrast, the pHi of muscle tissue was only slightly affected. Alkalization of the environment caused a surprising transient decline of the plasma pH, which was not due to lactic acidosis, but may be related to the precipitation of a buffer compound like CaCO<sub>3</sub> in scales and bone. The high-energy phosphate stores in the tissues were unchanged during the whole experimental protocol.

### **Introduction**

The disappearance of many fish species from strongly acid waters (pH < 5) has aroused much interest in the physiological effects of acid exposure. Most studies have focused on electrolyte (Na<sup>+</sup>, Cl<sup>-</sup>) losses and acidaemia due to increased

Key words: *Oreochromis mossambicus*, intracellular pH, high-energy phosphates, acid exposure.



permeability of the gill membrane. In contrast, reports on disturbances of energy metabolism have been relatively scarce. Lowering of the environmental pH is known to elevate blood glucose levels (Audet *et al.* 1988; Audet and Wood, 1988; Brown *et al.* 1984; Lee *et al.* 1983; Tam *et al.* 1988), plasma amino acid levels (Fugelli and Vislie, 1982; Tam *et al.* 1988) and protein levels (Audet *et al.* 1988; Brown *et al.* 1984; Tam *et al.* 1988). Blood lactate levels are sometimes increased (Höbe *et al.* 1984; Ultsch *et al.* 1981), but are usually unaffected (Neville, 1979; Ultsch *et al.* 1981). Ammonia excretion is enhanced (Audet and Wood, 1988; King and Goldstein, 1983; Ultsch *et al.* 1981), cortisol turnover increases (Balm, 1986), amino acid catabolism is activated (Balm, 1986) and more glycogen is stored in the liver (Lee *et al.* 1983; Murthy *et al.* 1981b). This pattern of changes suggests an increase in the importance of protein as a metabolic fuel and an increase in gluconeogenesis (Murthy *et al.* 1981a,b; Tam *et al.* 1988). Environmental acidification can also impair oxidative metabolism and reduce high-energy phosphate content. A decline in ATP levels and a decreased adenylate energy charge have been observed in the tissues of the gulf killifish (*Fundulus grandis*), with the gill epithelium showing the most prominent loss of nucleoside triphosphate (MacFarlane, 1981).

The toxicity of environmental acid is dependent on additional factors. Water hardness reduces H<sub>2</sub>SO<sub>4</sub> toxicity (Graham and Wood, 1981; McDonald, 1983; McDonald *et al.* 1980, 1983). The physiological effects are more severe in fish transferred directly to low-pH water than in animals gradually exposed to acid (Stuart and Morris, 1985; Wendelaar Bonga *et al.* 1987).

The present paper seeks to evaluate the short-term effects of acid stress on freshwater fish using *in vivo* <sup>31</sup>P-NMR spectroscopy and enzymatic methods. The animals were acclimated to water of relatively low calcium content (0.68 mmol l<sup>-1</sup>) and gradually exposed to acid to simulate natural environmental conditions. Transient declines of pHi were observed upon acid exposure without any major disturbance of energy metabolism.

## Materials and methods

### *Animals*

Laboratory-reared tilapias (*Oreochromis mossambicus*, 80±15 g body mass, length 14±2 cm) were used in all experiments. They were acclimated to 25°C, a 16 h light period, normal oxygen levels ( $P_{O_2}$  17–21 kPa) and 'diluted tap water' (1 vol of copper-free tap water: 3 vols of demineralized water; pH 7.6–7.8, Ca<sup>2+</sup> 0.68, Mg<sup>2+</sup> 0.16, Na<sup>+</sup> 0.83, K<sup>+</sup> 0.07, NO<sub>3</sub><sup>-</sup> 0.03, HCO<sub>3</sub><sup>-</sup> 1.11, SO<sub>4</sub><sup>2-</sup> 0.29, SiO<sub>2</sub> 0.06, Cl<sup>-</sup> 0.92 mmol l<sup>-1</sup>). The experimental animals were fed daily with cichlid food in flake form (Lapis, Europet, Nürnberg, FRG). The anaesthetic ethyl-*m*-aminobenzoate methanesulphonate (MS222) and Tris buffer were products of Sigma (St Louis, MO).

### *Conditioning*

At the onset of this study, the experimental fish were divided into two groups.



Group 1 was placed directly into a holding tank with conditions as described above. These animals were used for *in vivo*  $^{31}\text{P}$ -NMR studies of the epaxial muscle, and for metabolite measurements. The caudal part of the operculum of the animals in group 2 was surgically removed under MS 222 anaesthesia. After surgery, the fish were treated with a solution of potassium dichromate (1:25000 w/v, for 10 days) as a preventive measure against infections. Finally, they were put in a separate holding tank and treated in the same way as group 1. Fish from group 2 were used for *in vivo*  $^{31}\text{P}$ -NMR studies of the gill.

#### *In vivo* $^{31}\text{P}$ -NMR studies

For each experiment, a single fish was kept overnight in a darkened tank. On the day of the experiment, the fish was anaesthetized by addition of Tris-MS 222 (adjusted to the pH of the environment) to the water to a final concentration of 120 p.p.m. As soon as the fish lost equilibrium (<10 min), it was mounted in the Perspex flow cell of the *in vivo*  $^{31}\text{P}$ -NMR probe (Van den Thillart *et al.* 1989a) which was connected to equipment for the control of temperature, pH and  $P_{\text{CO}_2}$  (Fig. 1). During the measurements the animal was immobilized by an inflatable plastic bag filled with water. Water temperature was maintained at 25°C, using a heater with digital temperature readout and the gills were continuously irrigated with diluted tap water (see above). Since the water did not contain MS 222, the fish recovered rapidly (<2 min) and remained conscious during the experiment. The environmental pH was controlled with a pH-stat and a digital pH-meter (Metrohm, Herisau, Switzerland). The pH-stat used either  $0.5 \text{ mol l}^{-1} \text{ H}_2\text{SO}_4$  or  $1 \text{ mol l}^{-1} \text{ NaOH}$ .

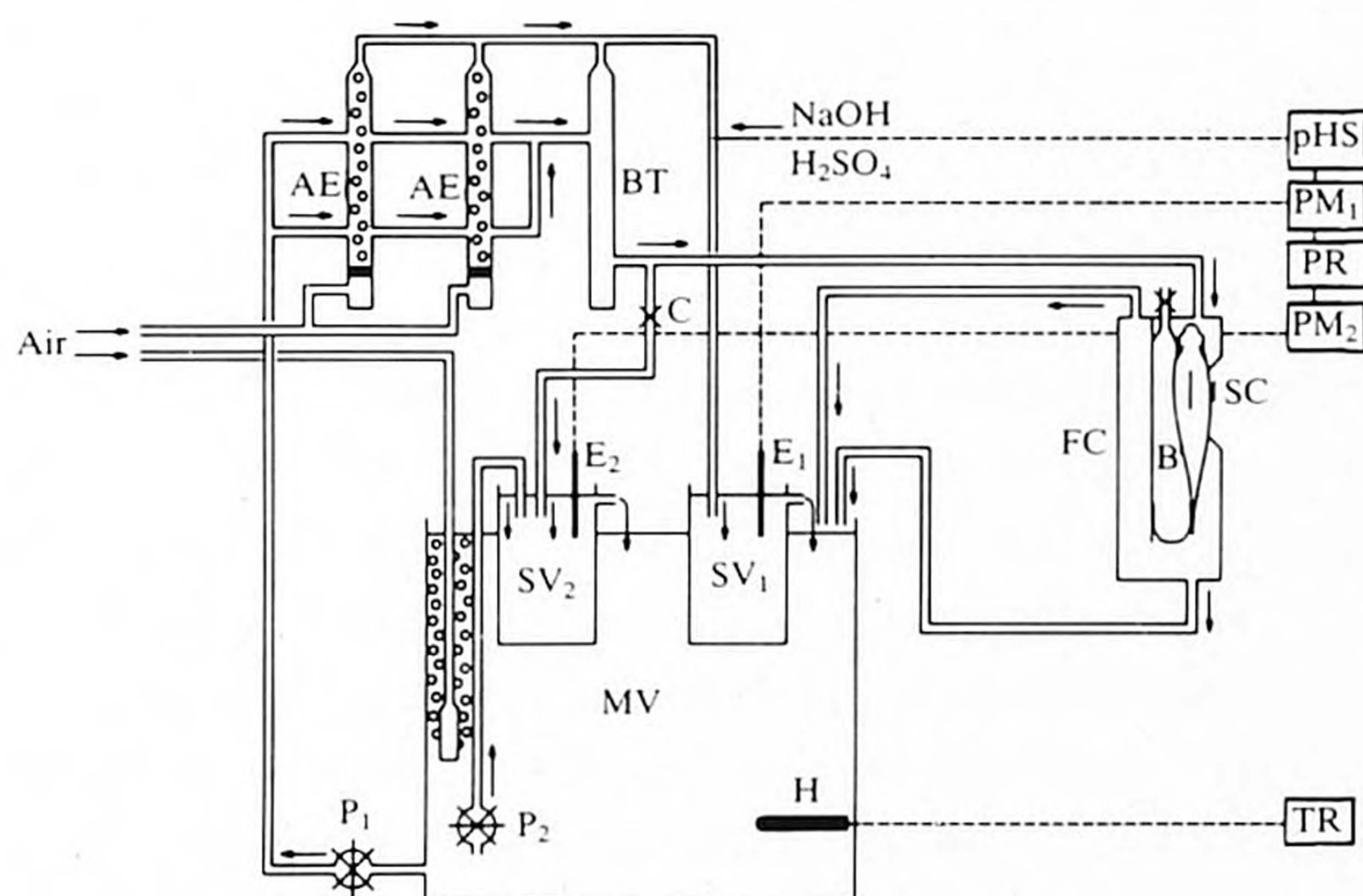


Fig. 1. Scheme of the experimental apparatus used in the *in vivo*  $^{31}\text{P}$ -NMR experiments. AE, aerators; B, inflatable plastic bag; BT, bubble trap; C, clamp;  $E_1$  and  $E_2$ , pH electrodes; FC, flow cell; H, heater; MV, main mixing vessel;  $P_1$  and  $P_2$ , pumps; pHS, pH-stat;  $PM_1$  and  $PM_2$ , pH-meters for measurement of the pH in the mixing vessel and the pH of the gill perfusion fluid, respectively; PR, penrecorder; SC, surface coil;  $SV_1$  and  $SV_2$ , secondary mixing vessels; TR, digital temperature readout.



The water flowing through the NMR probe was thoroughly aerated to avoid hypercapnia during titration with acid. We used two special aerators and a third aerator in the main mixing vessel. Preliminary experiments showed that our aeration system was effective. There was only a transient and minor rise of the  $P_{\text{CO}_2}$  (from 31 to 53 Pa) during water acidification. The water current could be adjusted with a clamp.

*In vivo*  $^{31}\text{P}$ -NMR spectra of the epaxial muscle (or gill) were acquired with a Bruker MSL-400 spectrometer. The signal of the tissue of interest was picked up with a surface coil of 10 mm diameter, which was double-tuned to the hydrogen (400 MHz) and phosphorus (162 MHz) frequencies. A microsphere, filled with a solution of methylene diphosphonate in deuterium oxide, was mounted at the centre of the coil and served as an external intensity standard. The homogeneity of the stationary magnetic field was optimized by shimming on the  $^1\text{H}$ -NMR signal of the intracellular water.  $^{31}\text{P}$ -NMR spectra (8192 data points) were accumulated over a period of 30 min and consisted of 412 individual scans, using a pulse width of  $60^\circ$  (in the sensitive volume), an acquisition time of 0.4 s and a 4 s relaxation delay. Measurements of the longitudinal relaxation time ( $T_1$ ) of phosphocreatine and ATP by the progressive saturation method indicated that the resonances of these compounds were fully relaxed [ $\exp(-t/T_1) < 0.1$ ]. Tissue pH values were calculated from the difference in chemical shift between the inorganic phosphate ( $\text{P}_i$ ) and phosphocreatine resonances in the NMR spectra. The pH measurements were standardized by titration of two different model solutions over a pH range of 5.0–8.5. The model solutions were: (1) inorganic phosphate and phosphocreatine dissolved in physiological saline; and (2) a homogenate of muscle tissue, as described previously (Van den Thillart *et al.* 1989b).

In control fish (more than 2 h elapsed after handling, exposed to well-oxygenated water of pH 7.6), the intracellular pH of the epaxial muscle was about 7.3 and the phosphocreatine/inorganic phosphate ratio was high ( $>15$ ). If the pH of the water was not altered, all NMR-observable parameters remained stable for periods greater than 36 h.

The water was not acidified until a period of more than 3 h had elapsed after handling. During the final 90 min of this period, we measured control parameters for the animal. At least three subsequent 30-min spectra at the neutral steady state were accumulated before the set-point of the pH-stat was changed. The pH was gradually lowered from 7.6 to 4.0 over a period of 4 h and maintained at 4.0 for the following 10 h. The pH was then gradually raised from 4.0 to 7.6 over a period of 2 h and maintained at 7.6 for another 6 h. Spectra of the epaxial muscle (or gill) were acquired continuously during the whole sequence of events.

#### *Metabolite measurements*

In a parallel series of experiments, fish were subjected to the same protocol of water acidification, exposure to acid water and recovery from acid stress. At various times, fish were anaesthetized with Tris-MS 222, as described above, and a blood sample (0.5–1.0 ml) was drawn by cardiac puncture. About 2 g of epaxial



white muscle was excised from each fish (below the dorsal fin) and immediately freeze-clamped. The gills were cut out and dropped into liquid nitrogen. The removal and freezing of the tissues was completed within 30 s. Gill arches were discarded after separation from the lamellae at the temperature of liquid nitrogen. Blood samples, gill lamellae and muscle pieces were extracted with ethanol/perchloric acid, as described previously (Van den Thillart *et al.* 1982). Creatine, phosphocreatine, ATP, glucose and lactate were measured in the tissue extracts (Bergmeyer, 1970; Van den Thillart *et al.* 1982).

### *Statistics*

Differences between groups were tested using the nonparametric test of Wilcoxon. A dual-tail probability of  $<0.05$  was considered statistically significant.

## **Results**

### *Appearance of NMR spectra*

When the surface coil was placed above the epaxial muscle, we acquired *in vivo* <sup>31</sup>P-NMR spectra of excellent resolution and signal-to-noise ratio. These results have been published previously (Van den Thillart *et al.* 1989a; Van Waarde *et al.* 1990) and are not reproduced here. A representative spectrum obtained with the coil above the gill is presented in Fig. 2. The most striking feature of the spectrum is the splitting of the inorganic phosphate resonance, indicating that the signal is picked up from three different compartments.

### *Changes of the pHi induced by environmental acidification*

The protocol of gradual water acidification, exposure to low pH, gradual environmental alkalization and recovery at pH 7.6 induced disturbances in the pHi of the experimental fish. The pH of each compartment could be calculated from the difference between the chemical shift of its inorganic phosphate resonance and the phosphocreatine resonance in the NMR spectra (Fig. 2, Van den Thillart *et al.* 1989b). The observed pattern is illustrated in Fig. 3 and a statistical treatment of the data is presented in Table 1.

The pH of all compartments was lowered at the end of the 4-h period of environmental acidification. The pH drop was largest in compartment 1 (from 7.78 to 7.54), intermediate in compartment 3 (from 6.98 to 6.79) and small in compartment 2 (from 7.27 to 7.21). During prolonged exposure to low-pH water, the pH of all compartments returned towards the control value. After 10 h at pH 4, this return was complete in compartments 1 and 2, but only partial in compartment 3. Subsequent alkalization of the environment induced a new fall of the pH of compartment 1, whereas the pH of compartment 2 was unaffected and that of compartment 3 returned towards the control value. During 6 h of recovery in neutral water, all NMR-observed pH values returned to normal.



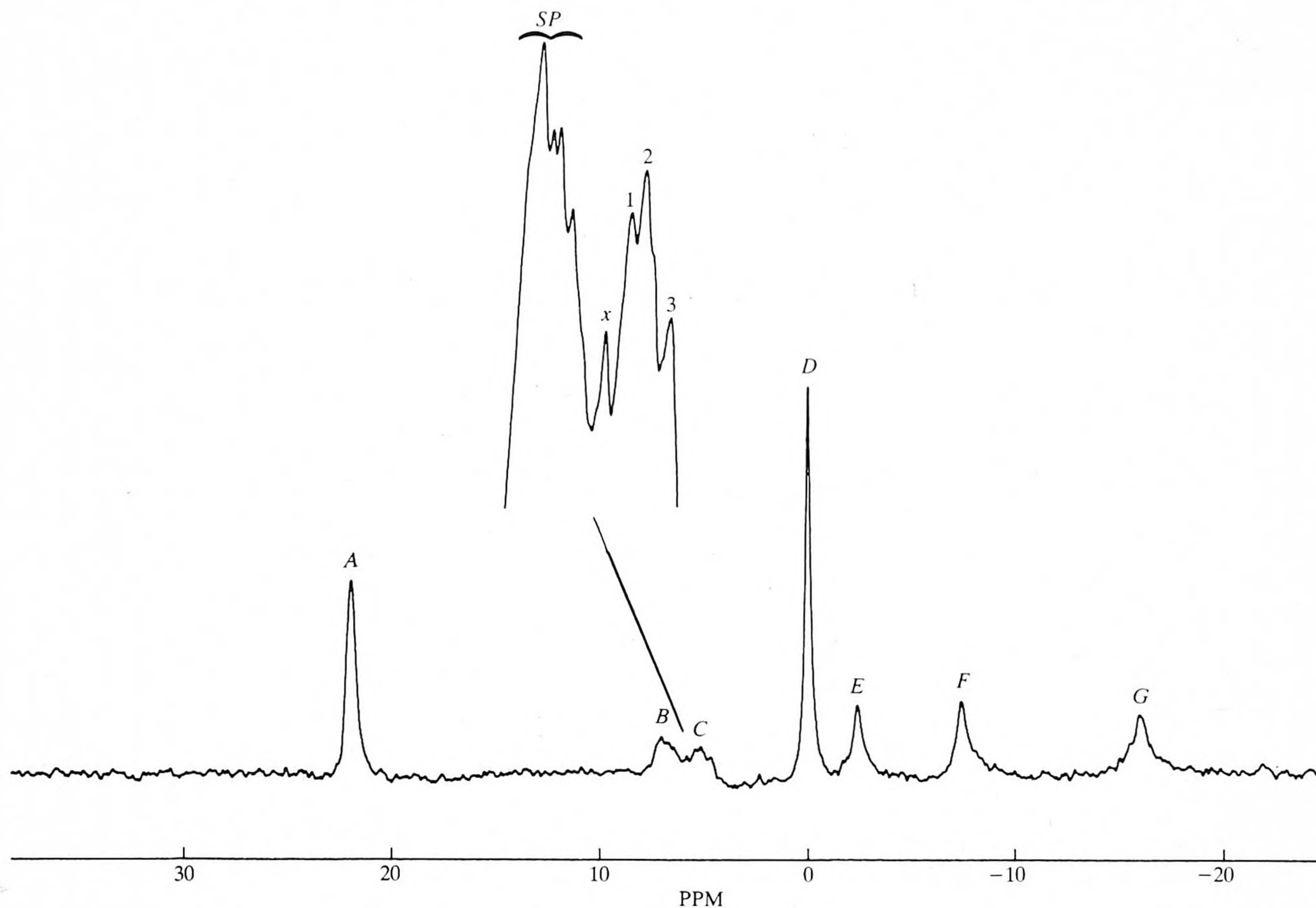


Fig. 2. *In vivo*  $^{31}\text{P}$ -NMR spectrum of *Oreochromis mossambicus*, acquired with a 10 mm surface coil placed above the gill. Labelled resonances are those of: A, methylene diphosphonate (external standard); B sugar phosphates; C inorganic phosphate; D phosphocreatine; E, F, G gamma-, alpha- and beta-phosphate atoms of ATP. The inset shows the splitting of the inorganic phosphate peak. SP, sugar phosphates; x, unknown resonance; 1, 2, 3, inorganic phosphate in three different compartments with different pH values. PPM means parts per million (1 p.p.m. is 162 Hz).



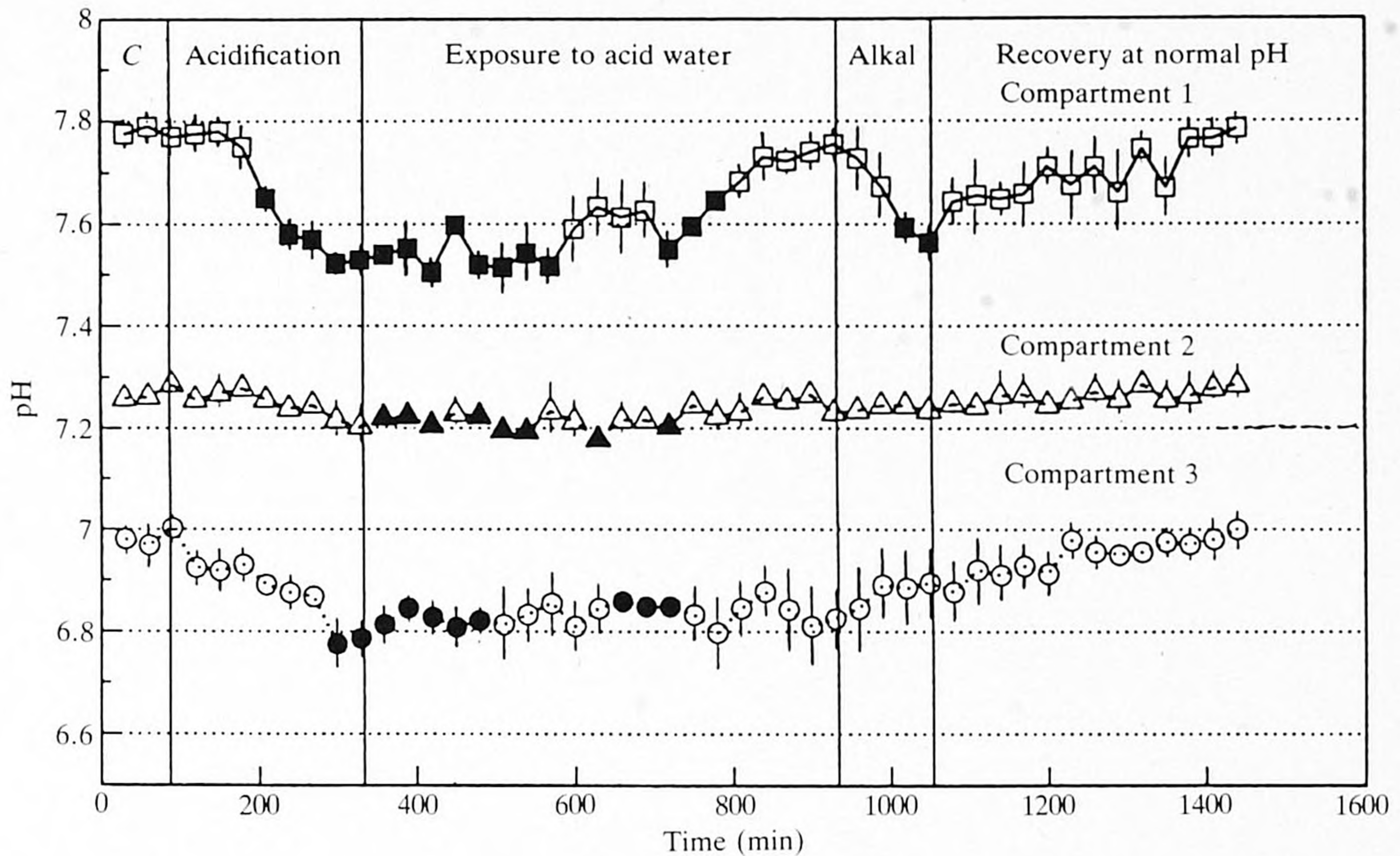


Fig. 3. pH changes in three compartments of *Oreochromis mossambicus* caused by changes in the acidity of the environment. The pH of the compartments was measured by *in vivo*  $^{31}\text{P}$ -NMR spectroscopy, using a surface coil above the gill. Each data point is the mean of four independent observations on four different animals. Significant differences between an experimental value and the value obtained before acidification (Wilcoxon's  $Q$ -test,  $P < 0.05$ ) are indicated by filled symbols. The vertical lines indicate standard errors; some values acquired between 500 and 900 min are not significantly different from the control owing to large individual variability. C, control; Alkal, alkalization.

#### *Changes in direct energy reserves, glucose and lactate induced by acid water*

During the *in vivo*  $^{31}\text{P}$ -NMR experiments on gill and muscle tissue, we never observed a significant depletion of tissue ATP and phosphocreatine. Since the origin of the phosphocreatine and ATP signals in the  $^{31}\text{P}$ -NMR spectra is not well defined, we also measured intermediates of energy metabolism in perchloric acid extracts of muscle (Table 2), gill (Table 3) and blood (Table 4).

Acid exposure had no influence on the phosphocreatine, creatine and ATP contents of white muscle, but there was a tendency towards lower lactate levels in the acid-treated group (Table 2). After 2 h in pH 4 water, and at the end of the 2-h period of alkalization, this trend was statistically significant. The lower lactate level is probably due to a suppression of routine activity. We always observed that the fish in the acid tank became quiet and swam slowly, in contrast to the control animals which showed large peaks of activity.

A dramatic decline of muscle phosphocreatine and ATP occurred in both the control and acid-exposed fish during the 6 h recovery period (Table 2). The reason



Table 1. Influence of water acidification on intra- and extracellular pH of tilapia

Condition	Surface coil above the gill			Surface coil above the epaxial muscle
	Compartment 1	Compartment 2	Compartment 3	Single compartment
Control (pH 7.6)	7.78±0.06 (12)	7.27±0.03 (12)	6.98±0.07 (12)	7.30±0.05 (8)
End of acid titration	7.54±0.07* (12)	7.21±0.05† (12)	6.79±0.08* (12)	7.21±0.04† (8)
10 h of acid exposure (pH 4)	7.74±0.07 (12)	7.25±0.04 (12)	6.90±0.03† (12)	7.31±0.05 (8)
End of base titration	7.56±0.07* (8)	7.24±0.04 (8)	6.95±0.08 (8)	7.29±0.06 (8)
6 h of recovery (pH 7.6)	7.76±0.08 (12)	7.27±0.06 (12)	6.97±0.07 (12)	7.30±0.06 (8)

All pH values were determined by *in vivo* <sup>31</sup>P-NMR spectroscopy.

Compartments 1, 2 and 3 are tentatively identified as blood plasma, muscle and gill epithelium (see Discussion).

\*† Significant difference between the experimental pH value and the control value before acidification (\* *P*<0.001; † *P*<0.01; Wilcoxon's *Q*-test, dual-tail probability). Values are mean±s.d.

Values in brackets refer to the number of observations on four different animals.

10 h of acid exposure and 6 h of recovery mean the end (i.e. the final hour) of these periods, not the average value for the entire period.

Table 2. Influence of water acidification on energy metabolism of white muscle

Condition	Phosphocreatine		Creatine		ATP		Lactate	
	Control	Acid	Control	Acid	Control	Acid	Control	Acid
Control (pH 7.6)	11.82±1.13	12.31±1.65	14.80±2.07	16.54±0.52	4.10±0.59	4.13±0.24	6.28±0.49	6.73±1.74
End acid titration	10.06±4.00	8.26±0.76	19.34±2.22	18.93±1.14	3.33±0.73	3.11±0.95	7.52±2.20	5.73±0.86
2 h of acid exposure	10.96±3.61	12.87±2.16	21.07±2.92	16.28±1.10	3.38±1.07	3.82±1.00	10.11±1.09	5.96±1.34*
10 h of acid exposure	13.33±0.15	10.88±1.55	12.58±1.43	11.27±3.86	3.89±0.24	3.29±1.16	5.11±1.15	3.93±1.97
After base titration	11.86±3.38	14.03±0.92	15.15±1.63	14.20±4.20	3.87±0.27	3.88±0.35	8.91±3.00	2.99±0.75*
6 h of recovery	4.70±1.05	5.95±3.28	19.41±2.52	18.86±4.22	2.33±0.45	2.72±1.00	9.55±1.61	7.77±1.65

The acid group was exposed to the sequence of experimental conditions listed on the left; the control group was kept continuously in pH 7.6 water.

Control animals were killed simultaneously with acid-exposed individuals to compensate for diurnal effects. Values are mean±s.d. of four observations on four different animals; concentrations are expressed as  $\mu\text{mol g}^{-1}$  wet mass.

\* Significant difference between control and acid-exposed animals. 2 h or 10 h of acid exposure actually means 4 h of acid titration followed by 2 h or 10 h of exposure to pH 4 water.



Table 3. Influence of water acidification on energy metabolism of gill tissue

Condition	Phosphocreatine	Creatine	Lactate
Control (pH 7.6)	0.71±0.17 (8)	0.71±0.27 (8)	0.55±0.32 (8)
10 h of acid exposure (pH 4)	0.51±0.18 (3)	0.70±0.31 (3)	0.45±0.14 (3)
After base titration	0.53±0.17 (3)	0.75±0.22 (3)	0.38±0.17 (3)

Values are mean±s.d.

Numbers in brackets indicate the number of independent observations on different fish. Concentrations are expressed as  $\mu\text{mol g}^{-1}$  wet mass.

No significant differences between the control and experimental groups were detected.

10 h of acid exposure actually means 4 h of acid titration followed by 10 h of exposure to pH 4 water.

Table 4. Influence of water acidification on blood glucose and lactate

Condition	Glucose		Lactate	
	Control	Acid	Control	Acid
Control (pH 7.6)	3.09±0.57	3.41±0.41	0.86±0.39	0.72±0.19
End of acid titration	4.73±0.45	4.36±1.47	0.66±0.35	0.97±0.33
2 h of acid exposure	3.93±0.42	5.98±2.22	0.63±0.18	0.40±0.04
10 h of acid exposure	3.64±0.13	4.44±1.01	0.40±0.18	0.49±0.17
End of base titration	5.38±0.58	3.64±0.93*	1.01±0.42	0.45±0.11*
6 h of recovery (pH 7.6)	5.75±1.65	3.93±0.68	1.88±0.60	1.08±0.54

The acid group was exposed to the sequence of experimental conditions listed on the left; the control group was kept continuously in pH 7.6 water.

Control animals were killed simultaneously with acid-exposed individuals to compensate for diurnal effects.

Metabolites were determined in neutralized perchloric acid extracts of whole blood; concentrations are expressed as  $\mu\text{mol g}^{-1}$  wet mass. Values are mean±s.d. of four observations on four different animals.

\* Significant difference between control and acid-exposed individuals ( $P < 0.05$ , Wilcoxon's  $Q$ -test).

2 h or 10 h of acid exposure actually means 4 h of acid titration followed by 2 h or 10 h of exposure to pH 4 water.

for this change is not clear. The decrease of high-energy phosphate content coincided with the large activity peak in the morning after the lights in the aquarium room had been switched on. However, hydrolysis of phosphocreatine is unlikely, since there was no accompanying increase of creatine content.

Surprisingly, lactate, phosphocreatine and creatine levels in the gill lamellae were not affected by the experimental protocol (Table 3). Blood glucose and lactate levels also showed only minor changes. There was a significant difference between control and acid-exposed individuals at the end of alkalization, fish recovering from acid stress having lower levels of glucose and lactate (Table 4).



### Discussion

#### *Identification of the compartments observed by NMR*

When the surface coil of the *in vivo*  $^{31}\text{P}$ -NMR probe was above the gill, we observed splitting of the inorganic phosphate peak in the NMR spectra, indicating that the signal was picked up from three different compartments (Fig. 1).

The first compartment had a control pH of  $7.78 \pm 0.06$  (Table 1). The following evidence suggests that it represents plasma. (i) The value of 7.78 is a normal pH for blood plasma of relaxed fish at 25°C. Cameron and Kormanik (1982) measured values of 7.93 at 15°C, 7.83 at 22°C and 7.70 at 31°C. (ii) We performed a parallel experiment in which two cannulated tilapias were gradually exposed to environmental acid, using the same protocol of acidification. Although we could not sample very frequently, we measured a similar transient pH drop (from 7.80 to 7.55) and recovery of the arterial pH during prolonged exposure to pH 4, as was observed in compartment 1 of the present study. (iii) It may be argued that the alkaline inorganic phosphate peak in the NMR spectra represents mitochondrial phosphate, since mitochondria functioning normally have a lower internal  $\text{H}^+$  concentration than the surrounding cytoplasm. It is generally believed that mitochondrial phosphates are NMR-invisible (Murphy *et al.* 1988), but in some studies, using isolated mitochondria (Ogawa *et al.* 1978; Ogawa and Lee, 1984), hepatocytes (Cohen *et al.* 1978) and perfused rat liver (Thoma and Ugurbil, 1988), distinct signals from cytoplasmic and mitochondrial phosphates have been observed. The intramitochondrial pH was reported as 7.86 at 8°C (Ogawa and Lee, 1984). Nevertheless, we do not consider it likely that the first  $\text{P}_i$  resonance represents mitochondrial phosphate. In  $^{31}\text{P}$ -NMR spectra of the lateral red muscle of fish, no alkaline  $\text{P}_i$  resonance was visible, although the tissue contains many mitochondria (Van den Thillart *et al.* 1989b). Resonances of intramitochondrial phosphate are usually 2.5–3 times broader than those of cytosolic phosphate owing to interaction with divalent metal ions (Ogawa *et al.* 1978; Ogawa and Lee, 1984), but the inorganic phosphate peak of compartment 1 was relatively narrow (Fig. 2). (iv) We measured inorganic phosphate levels in tilapia plasma and found a value of  $1.2 \pm 0.1 \text{ mmol l}^{-1}$ . Such a concentration would indeed give rise to a small peak in the  $^{31}\text{P}$ -NMR spectrum.

The inorganic phosphate resonance of the second compartment indicated a control pH of  $7.27 \pm 0.03$  (Table 1). We think this compartment is muscle (with a contribution from erythrocytes) for the following reasons. (i) The  $\text{pH}_i$  in myotomal muscle of relaxed tilapias is 7.27–7.30 at 25°C (Van den Thillart *et al.* 1989a). (ii) In a parallel series of experiments using the same protocol, we placed the surface coil above the epaxial muscle and monitored changes in  $\text{pH}_i$  during acid exposure and recovery from acid stress. We found a similar, transient and minor pH drop (from 7.30 to 7.21) during acidification followed by stability of the intracellular pH (Table 1). (iii) Since the intraerythrocyte pH of teleost fish is 7.22 at 25°C (Cameron and Kormanik, 1982, see also below) and the signal from plasma is being picked up, it is likely that red blood cells contribute to the  $\text{P}_i$  resonance of compartment 2.



The  $P_i$  resonance of the third compartment indicated an initial pH value of  $6.98 \pm 0.07$  (Table 1). We have tentatively identified this compartment as gill epithelium, since other possible sources of the NMR signal could be excluded. Compartment 3 cannot be the epithelium or bone of the gill cover, since this tissue was found to have a relatively high  $pH_i$  of 7.4 (these measurements were performed with an oval surface coil of  $20 \text{ mm} \times 5 \text{ mm}$ ; accumulation times of several hours were necessary owing to a low signal-to-noise ratio). Skin is not the source of the acid-shifted  $P_i$  resonance either, since this  $P_i$  resonance was not observed when the surface coil was above the myotomal muscle. Red blood cells are not causing the  $P_i$  resonance of compartment 3. In freshly drawn blood from an anaesthetized fish, we measured an intracellular pH of 7.29 and a plasma pH ( $pH_e$ ) of 7.57 by  $^{31}\text{P}$ -NMR (the low  $pH_e$  is probably due to the lactic acidosis induced by anaesthesia). After lysis of the cells by the freeze-thaw method, the directly measured pH values were 7.23 and 7.52, respectively. The hypothesis that compartment 3 represents the environmental water is unlikely, since the water did not contain inorganic phosphate and the environmental pH was changed from 7.6 to 4.0, rather than 7.0 to 6.8.

*The mechanism underlying the transient drops of  $pH_e$  and  $pH_i$  during environmental acidification*

During titration of the surrounding water with acid, we observed a transient fall of the pH in all three compartments (Fig. 3). In theory, three mechanisms may have caused the decline of the pH in the animal: (i) accumulation of lactic acid in the tissues due to hypoxic stress or an escape response; (ii) an increase of the  $\text{CO}_2$  tension in the fish body due to a suppression of breathing or hypercapnia; (iii) influx of protons from the surrounding water across the gill epithelium.

We measured the lactate concentration in muscle (Table 2), gill (Table 3) and blood (Table 4) to find out whether the pH drops were due to lactic acidosis. No significant increase of the lactic acid level occurred. The transient declines of pH are therefore not a side effect of an escape reaction or caused by hypoxic stress. The second explanation for the observed acidosis does not seem very probable either. It is true that free-swimming tilapias respond to acid water with a decreased amplitude of ventilation, but the animals in this study were artificially ventilated, resulting in very efficient gas exchange. Also, there was no macroscopically visible accumulation of mucus on the gills and the  $P_{\text{CO}_2}$  of the water showed only a minor rise during titration with acid (see Materials and methods).

It can therefore be safely assumed that the transient acidosis is due to  $\text{H}^+$  influx from the surrounding water. McWilliams and Potts (1978) observed a shift from negative to positive charge over the gill epithelial membrane upon exposure of trout to acid water, which suggests an influx of  $\text{H}^+$ . Other authors have measured a net influx of protons in acid-exposed fish (Höbe *et al.* 1984; Holeton *et al.* 1983; McDonald and Wood, 1981; McDonald, 1983).

The acidosis that we observed in tilapias was transient (Table 1, Fig. 3). It is thus necessary to explain why the decline in pH came to an end and was actually



reversed in compartments 1 and 2 during acid exposure. Two possible mechanisms may be involved: (i) increased sodium pumping in the gills and kidneys (since  $\text{Na}^+$  uptake is coupled to  $\text{H}^+$  extrusion) and (ii) a decrease of the ion permeability of the gill membrane, causing a suppression of proton influx. Both phenomena are known to occur. Rainbow trout showed a marked increase in renal acid excretion during 1–4 days of exposure to pH 4 (McDonald and Wood, 1981). Balm (1986) measured an increase of the  $\text{Na}^+/\text{K}^+$ -ATPase activity in gill tissue of tilapias during a 5-day exposure to pH 3.3, but at pH 4.5 this response did not occur (Wendelaar Bonga *et al.* 1990). During 1–4 days of exposure of rainbow trout to pH 4, McDonald and co-workers demonstrated a substantial decrease of net  $\text{Na}^+$  losses and  $\text{H}^+$  uptake, which suggests alterations in the ion permeability of the gill membrane (McDonald, 1983; McDonald *et al.* 1983). Dramatic increases of ion pumping cause partial depletion of ATP and phosphocreatine and lowering of the adenylate energy charge in the gill epithelium (MacFarlane, 1981). The fact that we did not observe such changes in acid-exposed tilapias (Table 3) may therefore indicate that permeability changes were more important than changes in the activity of  $\text{Na}^+/\text{H}^+$ - and  $\text{Na}^+/\text{NH}_4^+$ -ATPases under the conditions of this study.

*The mechanism underlying the transient decline of pHe during environmental alkalization*

Our most intriguing observation is the transient decline of the pH of compartment 1 (i.e. plasma) during titration of the water with base (Fig. 3). This phenomenon does not occur in the other compartments. In a study on the recovery of cannulated rainbow trout from acid stress (Holeton *et al.* 1983), no transient decline of the blood pH was observed. However, these authors did not sample earlier than 4–6 h after the beginning of base titration, so an initial decline of the blood pH may have been overlooked.

It appears that protons that have entered the fish during the initial 4 h of acid exposure are temporarily stored in a buffer. When the environmental pH starts to rise, stored acid equivalents are released into the blood and finally excreted. It is possible that calcium carbonate from the skeleton participates in pH buffering (Holeton *et al.* 1983; McDonald and Wood, 1981; Wendelaar Bonga and Dederen, 1986; Wendelaar Bonga *et al.* 1984). Resynthesis of calcium carbonate upon the return to a neutral environment will be accompanied by the formation of acid equivalents. It is conceivable that such repletion of a depleted buffer compound is the cause of the transient decline of the plasma pH.

In conclusion, this paper shows that *in vivo*  $^{31}\text{P}$ -NMR spectroscopy can be used to monitor the responses of pHi and pHe in fish to pH changes of the environmental water. The use of the NMR technique has provided interesting new information on disturbances of the internal milieu during water acidification and recovery from acid stress. First, tilapias were able to restore the pH of muscle and blood to normal values within a few hours of the onset of acid exposure. Second, gradual exposure to acid did not give rise to impairment of energy metabolism in muscle and gill. Finally, there was a transient decline of the plasma pH during the



gradual return to a neutral environment, which may be related to resynthesis of a buffer compound.

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