J. exp. Biol. **152**, 521–547 (1990)
Printed in Great Britain © *The Company of Biologists Limited 1990*

Na^+ AND Cl^- UPTAKE KINETICS, DIFFUSIVE EFFLUXES AND ACIDIC EQUIVALENT FLUXES ACROSS THE GILLS OF RAINBOW TROUT

I. RESPONSES TO ENVIRONMENTAL HYPEROXIA

BY GREG G. GOSS* AND CHRIS M. WOOD

Department of Biology, McMaster University, 1280 Main Street West, Hamilton, Ontario, Canada L8S 4K1

Accepted 15 May 1990

Summary

Endogenous respiratory acidosis and metabolic alkalosis were induced in bladder-catheterized freshwater rainbow trout by exposure to environmental hyperoxia (72 h) and its subsequent removal. Unidirectional and net fluxes of Na^+, Cl^- and acidic equivalents across the gills were examined over 0.5 h intervals. Hyperoxia resulted in a positive Na^+ balance, negative Cl^- balance and net acidic equivalent excretion. Return to normoxia caused a negative Na^+ balance, a positive Cl^- balance and net basic equivalent excretion (=acidic equivalent uptake). Cl^-/basic equivalent exchange was more important than Na^+/acidic equivalent exchange in the homeostatic responses, and alkalosis was a more potent stimulus than acidosis for change in branchial ion fluxes. Kinetic analysis demonstrated that alterations in ion fluxes were achieved by complex changes in both the K_m (inverse of affinity) and the J_{max} (maximal transport rate) of the branchial $Cl^-/HCO_3^-(OH^-)$ and $Na^+/H^+(NH_4^+)$ transporters. K_m^{Cl} (control=165 μequiv l^{-1}) and K_m^{Na} (114 μequiv l^{-1}) were increased during hyperoxic acidosis to 250 and 445 μequiv l^{-1}, respectively. J_{max}^{Cl} (291 μequiv kg^{-1} h^{-1}) and J_{max}^{Na} (456 μequiv kg^{-1} h^{-1}) did not change significantly. During post-hyperoxic alkalosis, K_m^{Na} was further increased to 559 μequiv l^{-1}, J_{max}^{Cl} increased to 445 μequiv kg^{-1} h^{-1}, while K_m^{Cl} and J_{max}^{Na} decreased to 137 μequiv l^{-1} and 309 μequiv kg^{-1} h^{-1}, respectively. Diffusive efflux was examined using a novel method. There was no significant *differential* diffusive efflux of Na^+ and Cl^- during hyperoxia but diffusive Na^+ efflux exceeded Cl^- efflux during post-hyperoxic alkalosis, thereby serving as an additional mechanism for basic equivalent excretion.

Introduction

The ability of freshwater salmonids to alter dynamically the unidirectional Na^+

* Present address: Department of Biology, University of Ottawa, 30 George Glinski Pr., Ottawa, Ontario, Canada K1N 6N5.

Key words: hyperoxia, ion fluxes, uptake kinetics, K_m, J_{max}, efflux.

and Cl^- fluxes across the gills has now been demonstrated under several different types of acid–base disturbance (Cameron, 1976; McDonald *et al.* 1983, 1989; Wood *et al.* 1984; Perry *et al.* 1987; Wood, 1988; McDonald and Prior, 1988). These ionoregulatory changes aid in the compensation of the acidosis or alkalosis by direct or indirect exchange of Na^+ or Cl^- with acidic or basic equivalents, respectively. At present, little is known about the mechanisms involved.

A fundamental technique for understanding the function of transport systems has been to analyze their transport kinetics, an approach pioneered *in vivo* in freshwater crayfish by Shaw (1959). This involves measuring the transport rate at a variety of substrate (i.e. external ion) concentrations; the resulting Michaelis–Menten relationship in turn yields estimates of the kinetic parameters – K_m (inverse of affinity) and J_{max} (maximum transport rate). Traditionally, K_m is related to the binding affinity of the actual operational site (e.g. transport enzyme, carrier or channel) for the substrate, and J_{max} is considered to be an index of the absolute number of operational sites available. Changes in either, or both, can theoretically be responsible for observed changes in the transport rates. To date, in fish, this technique has been applied only to long-term changes in transport associated with exposure to water of altered ionic compositions (e.g. Kerstetter *et al.* 1970; Kerstetter and Kirschner, 1972; de Renzis and Maetz, 1973; Wood and Randall, 1973*a*; Laurén and McDonald, 1987; Avella *et al.* 1987) and not to the dynamic changes in Na^+/acid and Cl^-/base exchange which might occur during acid–base disturbance. In the present and the following study (Goss and Wood, 1990), a technique for the rapid determination of Na^+ and Cl^- uptake kinetics in individual rainbow trout has been applied to the study of such dynamic changes.

Recently, it has been appreciated that, in addition to influx modulation, adjustment of the differential efflux of Na^+ *vs* Cl^- across the gill may also play a significant role in acid–base regulation (Wood, 1988; McDonald *et al.* 1989). If total cation efflux (of which Na^+ is normally the major component) exceeds total anion efflux (mainly Cl^-), the extracellular strong ion difference (SID) is reduced, with a concomitant reduction in pHe (Stewart, 1978). The reverse will be true if anion efflux exceeds cation efflux. The results of several other studies (Cameron, 1976; Wood *et al.* 1984; Perry *et al.* 1987) can be interpreted as providing evidence for the involvement of differential diffusive efflux in acid–base regulation. However, in all these investigations, differential efflux measurements by radiotracer techniques may have been complicated by the presence of exchange diffusion (e.g. Maetz, 1972; de Renzis and Maetz, 1973; Wood *et al.* 1984). Therefore, a novel method, again patterned after the original technique of Shaw (1959), has been developed to measure Na^+ and Cl^- diffusive efflux rates during acid–base disturbances, without the complicating effects of exchange diffusion or changes in membrane permeability.

Earlier, environmental hyperoxia (P_{O_2}>70 kPa) was shown to be a very useful probe for determining the role of ionic exchanges in acid–base regulation (Wood *et al.* 1984). The first goal of the present study was to determine possible changes in the branchial transport kinetics of Na^+ and Cl^- during the normoxia–hyperoxia–

normoxia regime characterized by Hõbe *et al.* (1984), Wheatly *et al.* (1984) and Wood *et al.* (1984). A second objective was to assess the role of differential diffusive efflux of Na^+ and Cl^- in acid–base regulation during this regime.

Materials and methods

Experimental animals

Rainbow trout (*Oncorhynchus mykiss* (Walbaum, 1792), 250–400g) were obtained from Spring Valley Trout Farm, Petersburg, Ontario, Canada, and acclimated for at least 2 weeks to 15±0.5°C in flowing dechlorinated Hamilton tapwater (composition in Table 1). The fish were starved during this period to minimize any variations in acid output due to feeding (Wood and Caldwell, 1978). The trout were then anaesthetized (MS-222 1:10000; Sigma) and fitted with a urinary bladder catheter (Wood and Randall, 1973*b*) to separate the branchial from the renal component of ion and acid–base regulation. The fish were allowed to recover for 72 h in separate, darkened, well-aerated acrylic boxes continuously supplied ($0.5\,l\,min^{-1}$) with temperature-controlled (15±0.5°C) tapwater. The small volume of the boxes (1.3–1.7 l plus the volume of fish) was necessary to determine accurately changes in each of the measured variables over short periods (see Discussion for assessment of precision), and could easily be reset to a premeasured level. Vigorous aeration, which was separated from the fish by an acrylic wall, thoroughly mixed the water without direct exposure of the fish to the bubbling. Complete equilibration time of substances added to the box was less than 2 min.

Experimental water

Acclimation and experimental series I were performed in dechlorinated Hamilton city tapwater. Experimental series II and III were performed using a NaCl-free artificial medium designed to duplicate the Ca^{2+}, Mg^{2+} and titratable alkalinity (TAlk) composition of Hamilton tapwater as closely as possible (Table 1). Ca^{2+} and Mg^{2+} are important because of their well-known role in regulating membrane permeability and transepithelial potential (TEP) (Potts, 1984; McDonald and Rogano, 1986) and TAlk in providing water buffer capacity, HCO_3^- availability and the correct pH. This NaCl-free water was made by passing dechlorinated Hamilton tapwater through a deionizing cartridge (Anderson Water Corp.) then adding back appropriate amounts of $Ca(CO_3)_2$ and $(MgCO_3)_4 . Mg(OH)_2 . 5H_2O$ to bring Ca^{2+} and Mg^{2+} to levels normally seen in Hamilton tapwater. Because these salts are relatively insoluble in water at neutral pH, the water was bubbled with 100 % CO_2 overnight to achieve complete solution. The water was then vigorously aerated for 24 h to drive off the CO_2. The resulting medium had appropriate levels of Ca^{2+}, Mg^{2+} and TAlk (titratable alkalinity to fixed endpoint pH=4.00), but $0\,mmol\,l^{-1}$ Na^+ or Cl^- (Table 1). The temperature was 15±0.5°C.

Table 1. *Comparison of the measured composition of dechlorinated Hamilton tapwater used during acclimation and in series I, and the NaCl-free artificial media used in series II and III*

	Tapwater	NaCl-free media
Na^+	0.6±0.1 mequiv l^{-1}	0 mequiv l^{-1}
Cl^-	0.8±0.1 mequiv l^{-1}	0 mequiv l^{-1}
Ca^{2+}	2.0±0.1 mequiv l^{-1}	2.1±0.1 mequiv l^{-1}
Mg^{2+}	0.3±0.05 mequiv l^{-1}	0.3±0.1 mequiv l^{-1}
TAlk*	2.1±0.05 mequiv l^{-1}	2.4±0.1 mequiv l^{-1}
pH	8.00±0.05	8.10±0.04
Temperature	15.0±0.5°C	15.0±0.5°C
N	16	19

* Fixed endpoint (pH 4.00) titratable alkalinity.
Values are mean±1 S.E.M.

Exposure regime

After 72 h of recovery from catheterization, a series of control measurements was made under normoxia (P_{O_2}=19.2±0.3 kPa). The aeration source to the boxes was then replaced with pure O_2, which elevated the water P_{O_2} within 0.33 h to 70.9±1.9 kPa. Measurements were then made over several periods during the next 72 h of maintained hyperoxia. At 72 h, the O_2 was replaced with air, quickly returning P_{O_2} to normoxic levels, and a further series of measurements was made.

To avoid confusion, the term outflux (unidirectional outflux) is used to refer to outflux determined as the measured difference between influx (J^X_{in}) and net flux (J^X_{net}) in series I and III. The term efflux (diffusive efflux) is used to refer to the novel measurement technique in series II which did not involve radioisotopic measurements.

Experimental series

Series I

Series I was designed to characterize the time course and extent of changes in net and unidirectional ion fluxes (Na^+, Cl^-) and net acid–base fluxes [titratable acidity (TA)], ammonia (Amm) and net acidic equivalent (J^H_{net}) fluxes occurring during the hyperoxic regime. Wood *et al.* (1984) reported only average flux values over 4–12 h periods, whereas the present study was designed to resolve fluxes over successive 0.5 h intervals. A particular goal was to detect periods when Na^+ and Cl^- influxes and net acidic equivalent fluxes might be relatively stable for 4 h, to allow the planned kinetic measurements of series III. Problems of radioisotopic backflux and specific activity limitations precluded continuous 0.5 h measurements over the entire 5 day experiment. Based on the results of Hõbe *et al.* (1984) and Wood *et al.* (1984), the following periods were anticipated to be of greatest interest and therefore investigated in 0.5 h detail: normoxic control; the first 8 h of hyperoxia (0–8 h), representing the time of maximal acidosis; the final 7 h of

hyperoxic exposure (65–72 h), representing a time when internal pH had been restored almost to control values; and the first 8 h of return to normoxia, representing the time of maximal alkalosis.

At the start of each measurement segment, the boxes were closed and either $^{22}Na^+$ (1.0 μCi) or $^{36}Cl^-$ (1.0 μCi) was added. These amounts were doubled in the normoxic recovery period to prevent error that might be caused by isotopic loading, increased internal specific activity and the associated backflux of radioactivity. The $^{22}Na^+$ (N=8) and $^{36}Cl^-$ (N=8) experiments were performed on different sets of fish; in all cases, the same individual fish were followed through all four measurement segments. After an initial 10 min mixing period, water samples (40 ml) were taken at 0.5 h intervals for analysis of external $[Na^+]$ ($[Na^+]_e$), $[Cl^-]_e$, total ammonia (Amm), TA and $^{22}Na^+$ or $^{36}Cl^-$ cts min^{-1}. The boxes were flushed after each 3 h of closure with water of the appropriate P_{O_2} to ensure that ambient ammonia levels did not exceed 200 μequiv l^{-1}. Following the flush, the appropriate amount of isotope was re-added to the box. In the period between the measurement segments, the boxes were served with flowing dechlorinated tapwater of the correct P_{O_2}.

Series II

In series II, a novel technique inspired by the original approach of Shaw (1959) was developed to measure the simple diffusive effluxes of Na^+ and Cl^- from trout (N=12) in the absence of any possible exchange diffusion component (cf. Shaw, 1959) (see Discussion for an assessment of the validity of this approach). Two control efflux measurements (−3 h, −1 h), five hyperoxia measurements (1, 4, 8, 68 and 71 h after start of hyperoxia) and three measurements during normoxic recovery (73, 76 and 80 h; i.e. 1, 4 and 8 h after return to normoxia) were performed. At each time period, the boxes were flushed with NaCl-free water, to lower the external NaCl concentration ($[NaCl]_e$) to below 10 μequiv l^{-1}, and closed at a precise volume. Note that the pH, Ca^{2+}, Mg^{2+} and TAlk concentrations were maintained at normal levels (Table 1). Because this $[NaCl]_e$ was far below the K_m of either the Na^+ or Cl^- transporter, J_{in}^{Na}, J_{in}^{Cl} and exchange diffusion should not occur owing to the unavailability of the external ion. Therefore, the initial appearance of Na^+ and Cl^- in the external water should reflect the simple diffusive efflux of these ions. After an initial 2 min mixing period, samples were taken at 5 min intervals for 0.5 h and analyzed for $[Na^+]_e$ and $[Cl^-]_e$. Diffusive efflux was calculated from these values at each interval, and in most cases remained constant up to 0.5 h. The value obtained from 0 to 10 min was used in the calculation of the mean.

A separate experiment was performed to determine if there were any changes in transepithelial potential [TEP: blood relative to water as zero (0)] during exposure to NaCl-free media (Table 2). Fish were fitted with dorsal aortic catheters (see Goss and Wood, 1990) to allow TEP measurement without disturbance, and then held in Hamilton tapwater (Table 1) under control, normoxic condition. TEP between blood and external water was monitored *via* the catheter using standard

Table 2. *Comparison of the changes in transepithelial potential (TEP) during 10 min of exposure to NaCl-free artificial media used in series II and III with those occurring during exposure to pure distilled water*

	NaCl-free media	Distilled water
Control	−0.6±1.3	−1.2±0.4
2 min	−1.5±1.1	−13.5±3.1*
4 min	−1.7±0.8	−11.8±1.9*
6 min	−2.7±0.6	−12.1±1.9*
8 min	−1.9±0.8	−12.6±0.9*
10 min	−1.7±1.0	−10.9±1.0*
N	7	5

All measurements in mV.
Values are mean±1 S.E.M.
Asterisks indicate significantly different from control, $P<0.05$.

techniques (Perry and Wood, 1985). KCl (3 mol l^{-1})–agar bridges were connected *via* Ag/AgCl electrodes to a high-impedance voltmeter (Radiometer pHM 82). Control determinations were made in normal Hamilton tapwater, and then the box was flushed with either the NaCl-free medium ($N=7$) used in the diffusive efflux measurements or with distilled water ($N=5$) for comparison. Measurements were made at 2 min intervals for 10 min, representing the period used for diffusive efflux measurements.

Series III

Series III was designed to measure the influx kinetics of either Na^+ ($N=12$) or Cl^- ($N=11$) over increasing $[NaCl]_e$ in the water at various times in the exposure regime. It also investigated relationships between $[NaCl]_e$ and unidirectional efflux rates of the ion in question, Amm fluxes, TA fluxes and net acidic equivalent fluxes. Four periods were regarded as reasonably stable from the results of series I: 'control' normoxia; 4–8 h of hyperoxia ('initial'); 68–72 h of hyperoxia ('final'); and 4–8 h after return to normoxia (i.e. 76–80 h; 'recovery').

In each period, flow was stopped and the boxes were flushed with NaCl-free water so that $[NaCl]_e$ was below 10 μequiv l^{-1}. Again, pH, Ca^{2+}, Mg^{2+} and TAlk remained at normal levels. At this point, NaCl was added to the boxes from a common 1 mol l^{-1} NaCl stock solution containing approximately 1.0 μCi ml^{-1} of either $^{22}Na^+$ or $^{36}Cl^-$. This allowed for the maintenance of a relatively constant specific activity over the entire range of $[NaCl]_e$. After the stock had been added, 10 min was allowed for mixing and then a sample was withdrawn to begin the flux. After 0.5 h, another sample was taken to end the flux and more stock was added to the box to increase the water $[NaCl]_e$ further. Six flux periods of increasing $[NaCl]_e$ (nominally 50, 150, 300, 600, 1200 and 2400 μequiv l^{-1}) were measured. Each lasted 0.5 h, except for the final flux period lasting 0.67 h. Samples (20 ml for $^{36}Cl^-$

experiments, 40 ml for $^{22}Na^+$ experiments) were analyzed for Amm, TA (Amm and TA in $^{22}Na^+$ experiments only), $[Na^+]_e$, $[Cl^-]_e$ and $^{22}Na^+$ or $^{36}Cl^-$ cts min^{-1}

Analytical techniques and calculations

Water $[Na^+]_e$ was measured by atomic absorption (AAS, Varian AA1275), water $[Cl^-]_e$ by the mercuric thiocyanate method (Zall *et al.* 1956) and water ammonia by the salicylate–hypochlorite method (Verdouw *et al.* 1978). Titratable alkalinity (TAlk) was determined as described by McDonald and Wood (1981). Duplicate 5 ml $^{36}Cl^-$ or $^{22}Na^+$ water samples were measured directly by scintillation counting (LKB Rackbeta, model 1217).

Unidirectional influxes (J_{in}), outfluxes (J_{out}) and net fluxes (J_{net}) were calculated as described by Wood (1988). Correction for radioisotope backflux (Maetz, 1956) was not necessary because internal specific activity (SA) never exceeded 5 % of the external SA.

The effect of the $[NaCl]_e$ on the influx of either Na^+ or Cl^- showed distinctive saturation kinetic curves that were characterized by Michaelis–Menten analysis. The curves (see Results, Figs 6, 7) fitted to the uptake kinetic data were generated by substitution into the Michaelis–Menten equation:

$$J_{in}^X = \frac{J_{max}[X]_e}{K_m + [X]_e}, \quad (1)$$

using the mean values for K_m and J_{max} from all individual fish in a particular treatment. Here X is the ion in question (Na^+ or Cl^-), J_{in}^X is the rate at a particular external $[X]_e$, K_m is the inverse of affinity and J_{max} is the maximal rate of the transporter. K_m and J_{max} were determined for each fish *via* transformation of the data by Eadie–Hofstee regression analysis (Michal, 1985). Eadie–Hofstee analysis was used because it magnifies departures from linearity which might not be apparent from a Lineweaver–Burke plot.

Net acidic equivalent flux was calculated as the sum of the TA flux and the Amm flux, signs considered, as outlined by Maetz (1973). This method does not distinguish ammonia movement as either NH_3 or NH_4^+, or net acidic equivalent flux as a result of either acidic or basic equivalent movement (i.e. basic equivalent loss=acidic equivalent uptake and *vice versa*) but this does not matter in terms of the net acid–base status of the fish.

Statistical analysis

All values are presented as means±1 S.E.M. (N). The significance of difference for individual means was assessed by the paired Student's two-tailed t-test, using each fish as its own control. Comparisons between multiple means were performed by a one-way analysis of variance (ANOVA), followed by Duncan's new multiple-range test to determine individual differences in cases where the F-value of the ANOVA indicated significance. Linear regression relationships were generated by the method of least squares, and the strength of the relationship was assessed by a Fisher (z) transformation of Pearson's simple correlation coefficient. This trans-

formation is necessary for a bivariate normal distribution. Tests of significance for the slope and intercept were performed as outlined in Remington and Schork (1985). A significance level of $P<0.05$ was employed throughout.

Results

Unidirectional and net branchial fluxes

Exposure to hyperoxia – series I

Exposure to hyperoxia caused a significant 50 % reduction in J_{in}^{Na} over 65–72 h of exposure (Fig. 1). This decrease in J_{in}^{Na} was not significant in the initial 8 h period of exposure. Concurrently, J_{out}^{Na} was significantly depressed by about 60 %, resulting in the maintenance of a small positive J_{net}^{Na} throughout the exposure period.

Like J_{in}^{Na}, chloride influx (J_{in}^{Cl}) was also significantly reduced by 50 % upon exposure to hyperoxia (Fig. 2). However, this reduction took only 3–4 h to develop fully; J_{in}^{Cl} remained depressed for the duration of the exposure. J_{out}^{Cl} was unchanged during the initial exposure to hyperoxia, resulting in a significantly negative J_{net}^{Cl} at this time. However, by 65–72 h, J_{out}^{Cl} was also significantly reduced with respect to control levels, resulting in a J_{net}^{Cl} not significantly different from 0.

J_{net}^{H} is the sum of two components, J^{Amm} and J^{TA}, signs considered. There was a 50 % reduction in J^{TA} during hyperoxia which took about 3–4 h to develop fully (Fig. 3). J^{TA} remained depressed at 65–72 h of continuous hyperoxia. There was no significant change in J^{Amm} (approx. $-200\ \mu equiv\ kg^{-1}\ h^{-1}$). A reduced J^{TA} and unchanged J^{Amm} resulted in a negative J_{net}^{H} during initial hyperoxic exposure, which continued throughout the exposure period (Fig. 3).

Return to normoxia – series I

The return to normoxia (recovery) caused a further fall in J_{in}^{Na} (Fig. 1). The overall decrease was significant compared to both the control and final time periods. J_{in}^{Na} remained depressed throughout the recovery period (Fig. 1). J_{out}^{Na} was immediately stimulated by 60 % relative to the original control value, resulting in a large negative J_{net}^{Na}. As the recovery progressed, J_{out}^{Na} was reduced and thus J_{net}^{Na} was also reduced.

Return to normoxia resulted in a highly significant threefold stimulation of J_{in}^{Cl} to a level 50 % higher than the original control value (Fig. 2). The increase was not immediate but took 3–4 h to develop fully. J_{out}^{Cl} was greatly stimulated in the first 4 h followed by stabilization over the next 4 h of recovery. The increases in J_{out}^{Cl} were significant with respect to both the final period of hyperoxia and the original control levels. The large and immediate stimulation of J_{out}^{Cl} in combination with a delayed increase in J_{in}^{Cl} resulted in a negative J_{net}^{Cl} in the initial 4 h following return to normoxia, followed by a switch to a positive J_{net}^{Cl} as J_{in}^{Cl} became fully stimulated. This changeover was statistically significant. Return to normoxia resulted in a fourfold increase in J^{TA}, which also took 3–4 h to develop fully (Fig. 3). This was significantly higher than both the original control value and the final hyperoxia value. Throughout the recovery period, J^{Amm} was again not significantly altered

(Fig. 3). The resulting effect was a highly positive J^{H}_{net} during this time of metabolic alkalosis.

In summary, exposure to hyperoxia resulted in a positive Na^+ balance, a negative Cl^- balance and a net acidic equivalent excretion, while recovery resulted in a negative Na^+ balance, a positive Cl^- balance and a large net acidic equivalent

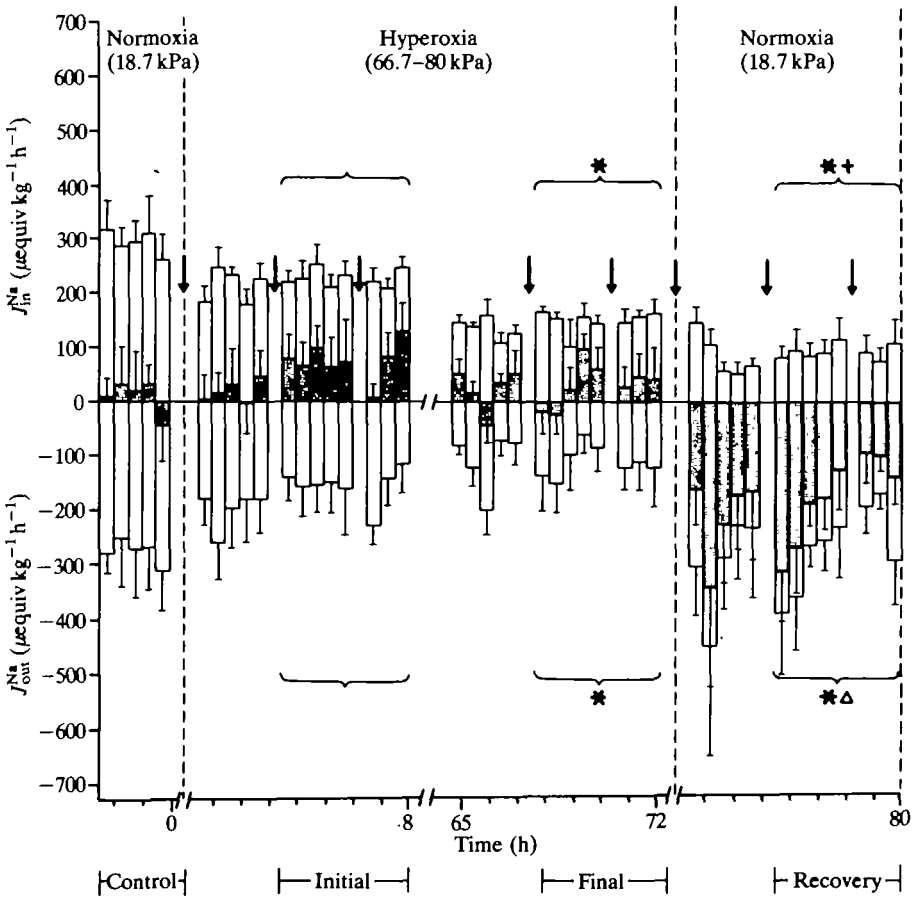

Fig. 1. Unidirectional and net flux rates for Na^+ across the gills of rainbow trout during normoxic control, and initial and final exposure to, and recovery from, 72 h of environmental hyperoxia (series I). Ion fluxes were measured in 0.5 h intervals over the experimental regime. Positive values represent movement into the fish (J^{Na}_{in}), negative values represent movement out of the fish (J^{Na}_{out}) and shaded areas indicate the net movement of Na^+ (J^{Na}_{net}) between the fish and the water. Large arrows (↓) indicate periods when the boxes were flushed to prevent ammonia build-up. The large brackets indicate the periods chosen as stable for the uptake kinetic analysis in series III (initial, final, recovery and control). Significant differences ($P<0.05$) for the entire group of data under the bracket compared to the mean control value are indicated by an asterisk (*) for J^{Na}_{in} and J^{Na}_{out} and by a triangle (Δ) for J^{Na}_{net}. In addition, significant differences in the recovery time period compared to the final time period are indicated by a plus (+). Values are means±1 S.E.M. ($N=8$).

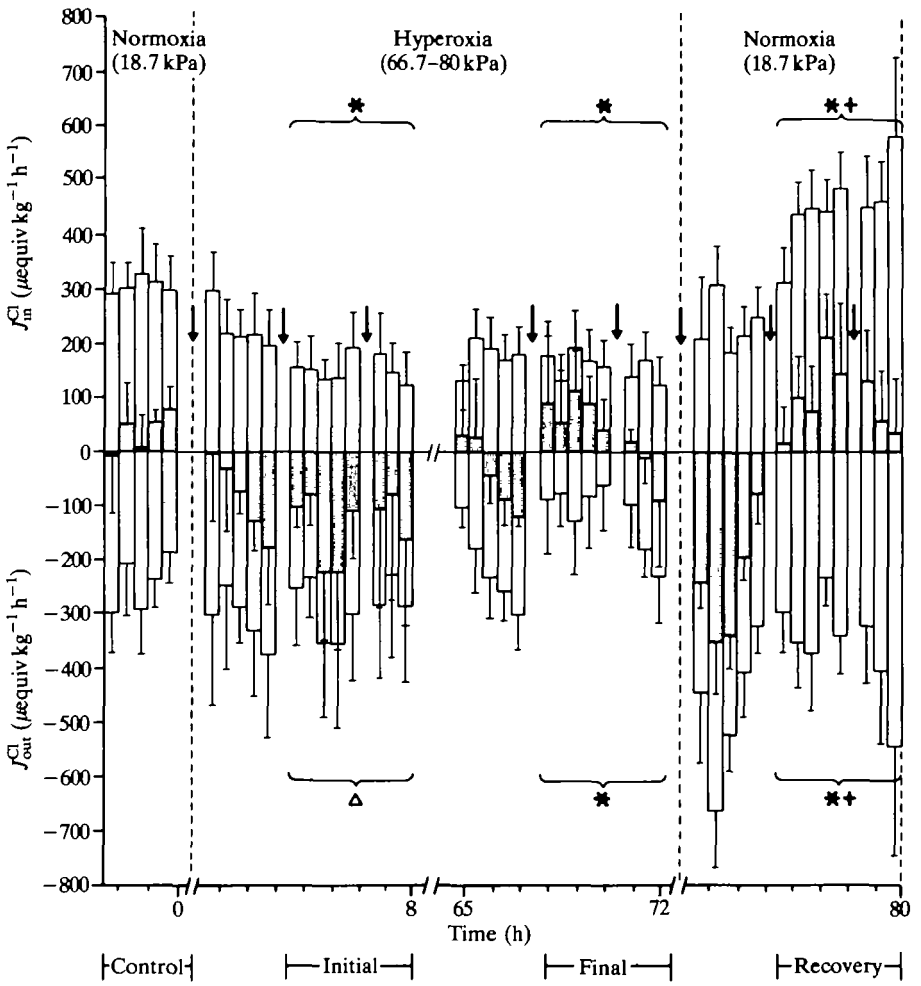

Fig. 2. Unidirectional and net flux rates for Cl^- across the gills of rainbow trout during normoxic control, and initial and final exposure to, and recovery from, environmental hyperoxia (series I). See legend of Fig. 1 for other details (N=8).

uptake. The alterations in net Na^+ and Cl^- balance resulted from complex changes in both influx and outflux components. The changes in J_{net}^{H} were accomplished by changes in the J^{TA} component only, J^{Amm} remaining invariant.

$$J_{net}^{H} \text{ versus } [J_{net}^{Na} - J_{net}^{Cl}] - \textit{series I}$$

The relationship between J_{net}^{H} and $[J_{net}^{Na} - J_{net}^{Cl}]$, based on all individual simultaneous flux measurements during initial exposure (0–8 h) to and recovery from (72–80 h) hyperoxia, is shown in Fig. 4. Based on the work of Wood *et al.* (1984) and Hõbe *et al.* (1984), these periods were chosen for analysis because they were the periods of maximal internal acid–base disturbances. The overall relationship

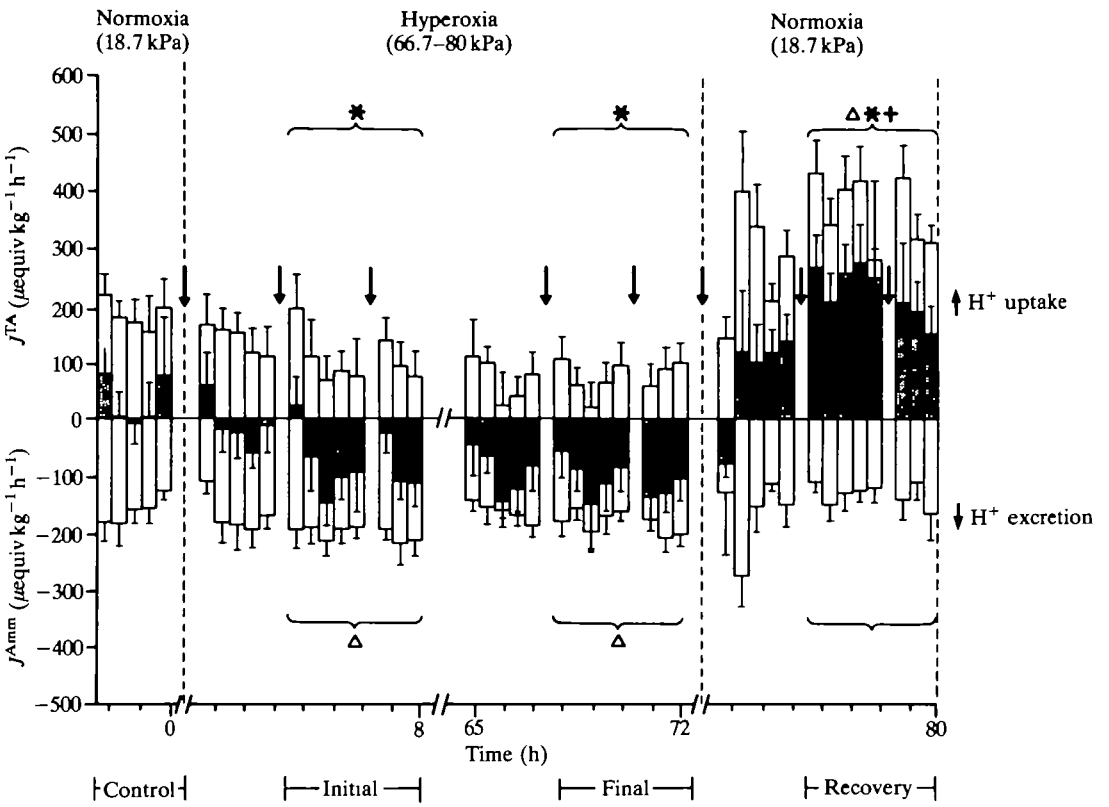

Fig. 3. The branchial flux rates of titratable acidity (J^{TA}), total ammonia (J^{Amm}) and net acidic equivalents (J^{H}_{net}) during normoxic control, and initial and final exposure to, and recovery from, hyperoxia (series I). Positive values indicate acidic equivalent uptake while negative values indicate acidic equivalent excretion. Shaded areas indicate net acidic equivalent flux (J^{H}_{net}) as the sum of the two components: J^{TA} and J^{Amm}, signs considered. Results from the $^{22}Na^+$ (Fig. 1) and $^{36}Cl^-$ (Fig. 2) experiments have been combined. See legend of Fig. 1 for other details (N=16).

was highly significant ($r=-0.535$, $N=208$, $P<0.001$) as described by the regression equation:

$$[J^{Na}_{net} - J^{Cl}_{net}] = -0.94J^{H}_{net} - 15\,. \qquad (2)$$

The slope was not significantly different from that of the line of equality and the y-intercept was not significantly different from 0, suggesting that the differential movement of Na^+ and Cl^- could fully explain the net movement of acidic equivalents across the gills.

Diffusive efflux – series II

Exposure to hyperoxia produced no significant changes in the diffusive effluxes of either Na^+ (Fig. 5A) or Cl^- (Fig. 5B). The net difference between Cl^- and Na^+ efflux was not significantly different from zero. This pattern continued throughout hyperoxia without significant change (Fig. 5).

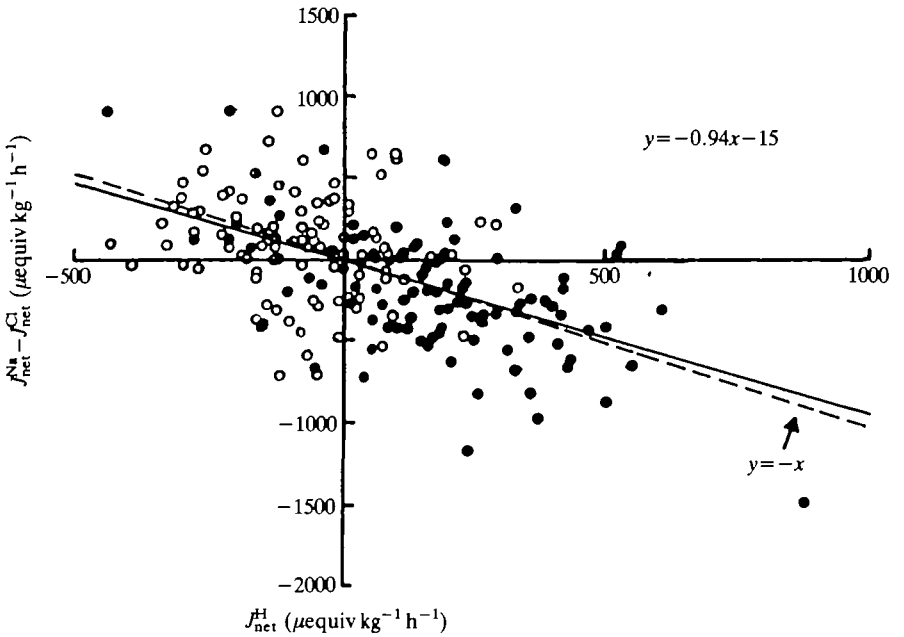

Fig. 4. The relationship between branchial net acidic equivalent flux rate (J^{H}_{net}) and the simultaneously measured differential flux rate of Na^+ (J^{Na}_{net}) and Cl^- (J^{Cl}_{net}) during initial exposure to (0–8 h), and recovery from (72–80 h), hyperoxia (series I). Open circles (○) represent individual fluxes in individual fish during the initial exposure period. Closed circles (●) represent individual fluxes in individual fish during the recovery time period. The dashed line represents the line of equivalence (1:1 ratio) while the solid line was fitted by least-squares regression analysis:

$$[J^{Na}_{net}-J^{Cl}_{net}]=-0.94(\pm0.03)J^{H}_{net}-15(\pm41)\ (r=-0.535,\ N=208,\ P<0.001).$$

Return to normoxia resulted in a significant stimulation of both Na^+ and Cl^- efflux. However, Na^+ efflux was increased to a much greater extent than Cl^- efflux, resulting in a net differential flux of about $-150\ \mu equiv\ kg^{-1}\ h^{-1}$ immediately following return to normoxia, stabilizing at $-90\ \mu equiv\ kg^{-1}\ h^{-1}$ for the remainder of the recovery period. This differential flux was significantly different from the values measured both during the original control period and during the final period of hyperoxia. Qualitatively, trends in diffusive effluxes throughout the regime were similar to those in J^{Na}_{out} and J^{Cl}_{out} (outfluxes, see Figs 1, 2) determined at comparable times in series I, but absolute values were quantitatively smaller in the former, suggesting the presence of exchange diffusion components in the radiotracer outfluxes.

Measurements of TEP during 10 min of exposure to the NaCl-free media used in the diffusive efflux determinations demonstrated that TEP remained invariant (Table 2). Thus, changes in TEP were not a factor biasing the diffusive efflux results. In contrast, 10 min of exposure to pure distilled water resulted in an immediate shift to highly negative TEP values (Table 2).

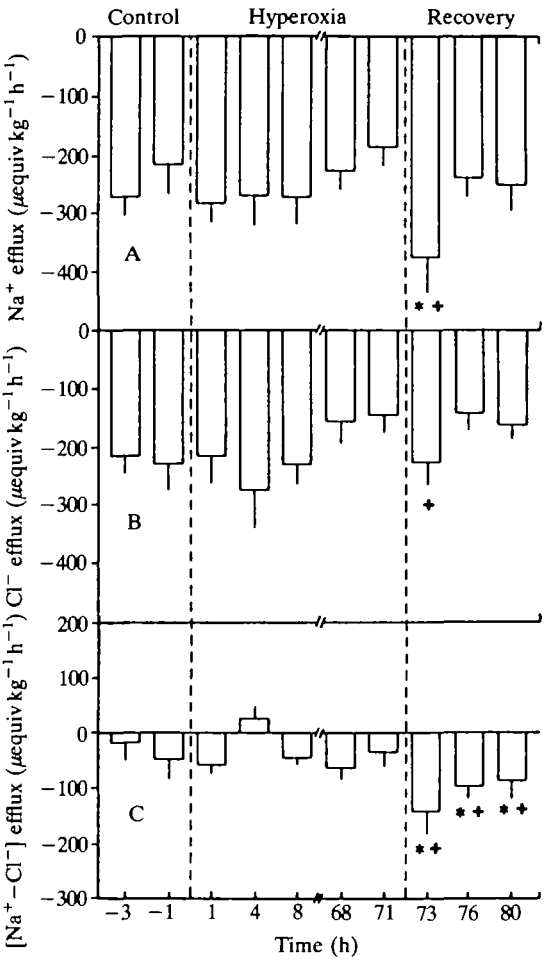

Fig. 5. Diffusive efflux of (A) Na^+, (B) Cl^- and (C) $[Na^+-Cl^-]$ across the gills of rainbow trout during normoxic control, and initial and final exposure to, and recovery from, hyperoxia (series II). Significant differences ($P<0.05$) from the mean control value are indicated by an asterisk (*). Significant differences in the recovery time period compared to the final time period are indicated with a plus (+). Values are means±1 S.E.M. ($N=12$).

Uptake kinetics – series III

A particular goal in series I had been to determine 4 h periods within the overall protocol when Na^+ and Cl^- influxes and net acidic equivalent fluxes might be reasonably stable. These periods could then be employed for the planned uptake kinetic measurements. Based on the results shown in Figs 1, 2 and 3, the following periods were selected: control (−4 to 0 h); initial hyperoxia (4–8 h); final hyperoxia (68–72 h); and recovery normoxia (76–80 h).

In all fish in all periods, increases in $[NaCl]_e$ resulted in increases in J_{in}^{Na} and J_{in}^{Cl} (cf. Figs 6, 7) which followed typical Michaelis–Menten curves. These data could

be linearized by Eadie–Hofstee regression analysis to obtain estimates of K_m and J_{max} of the transporters for individual fish. No consistent departures from linearity were observed. The means (Figs 8, 9) of the K_m and J_{max} values obtained from

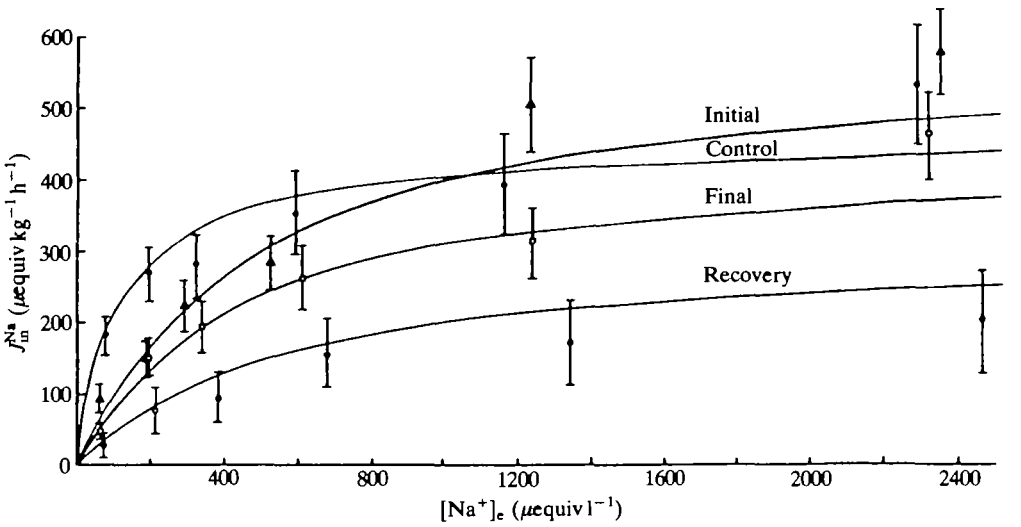

Fig. 6. The kinetics of sodium influx (J_{in}^{Na}) as a function of the external sodium concentration ([Na^+]$_e$) during normoxic control, and initial and final exposure to, and recovery from, hyperoxia (series III). Curves were drawn by Michaelis–Menten analysis from mean estimates of K_m^{Na} and J_{max}^{Na} obtained by Eadie–Hofstee regression analysis for all individual fish in the group. Mean J_{in}^{Na} values have been plotted at the mean [Na^+]$_e$ for each point. Values are means±1 S.E.M. (N=12).

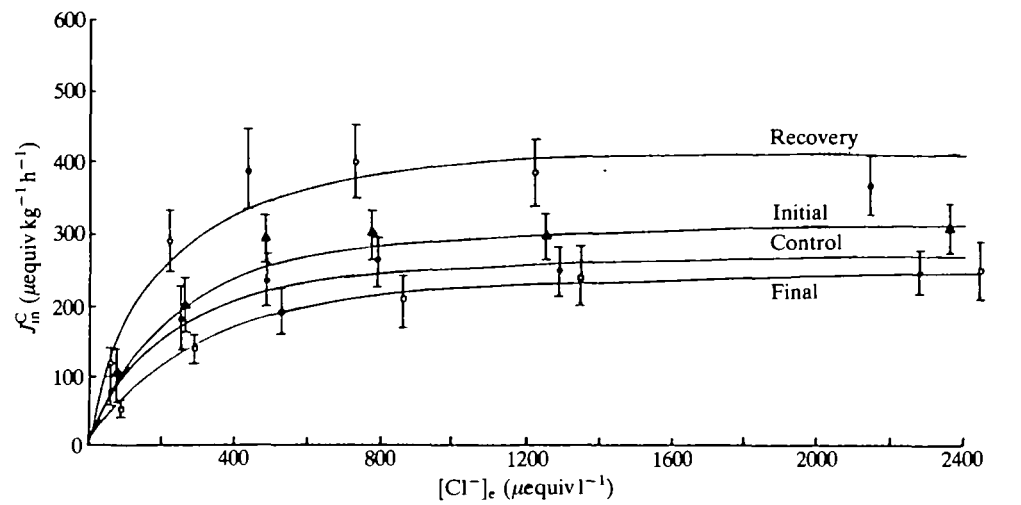

Fig. 7. The kinetics of chloride influx (J_{in}^{Cl}) as a function of the external chloride concentration ([Cl^-]$_e$) during normoxic control, and initial and final exposure to, and recovery from, hyperoxia (series III). See legend of Fig. 6 for other details (N=11).

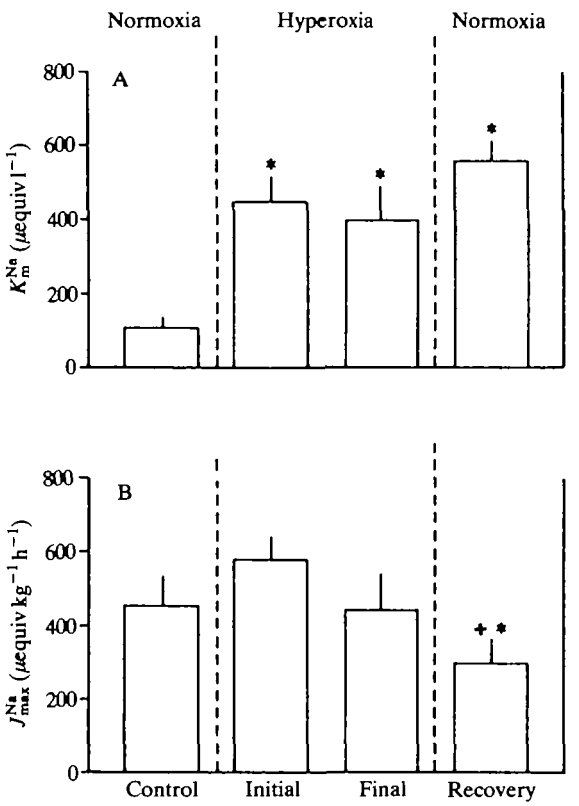

Fig. 8. Mean estimates of the affinity (K_m^{Na}) and the maximum transport rate (J_{max}^{Na}) for the sodium transporter during normoxic control, and initial and final exposure to, and recovery from, hyperoxia (series III). Values significantly different from control ($P<0.05$) are indicated with an asterisk (*). In addition, recovery values significantly different ($P<0.05$) from the final time period are indicated by a plus (+). Values are means±1 S.E.M. ($N=12$).

individual fish were substituted into equation 1 to generate the curves fitted to the mean data in Figs 6 and 7.

Exposure to hyperoxia (initial, final time periods) caused reduced values of J_{in}^{Na} at lower levels of $[Na^+]_e$, but J_{in}^{Na} was unaltered at higher $[Na^+]_e$ (Fig. 6). Return to normoxia (recovery time period) lowered J_{in}^{Na} by at least 50% at almost every $[Na^+]_e$ (Fig. 6). In contrast, J_{in}^{Cl} was not greatly altered at any $[Cl^-]_e$ during exposure to hyperoxia (Fig. 7). However, the Cl^- influx values were greatly elevated at each $[Cl^-]_e$ in the recovery period (Fig. 7). Regression analyses of individual fish yielded estimates of the average K_m and J_{max} for each ion (Figs 8, 9). The affinity of the Na^+ transporter was significantly decreased (i.e. K_m^{Na} increased fourfold from 114 to 445 μequiv l^{-1}) upon initial exposure to hyperoxia and remained so throughout the exposure period (Fig. 8A). Return to normoxia resulted in a further loss of affinity as K_m^{Na} increased to 559 μequiv l^{-1}. Concur-

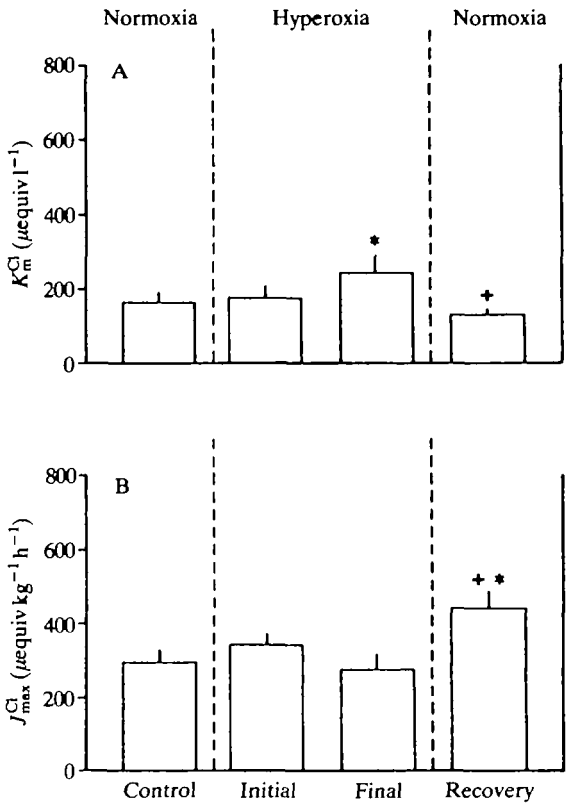

Fig. 9. Mean estimates of the affinity (K_m^{Cl}) and the maximum transport rate (J_{max}^{Cl}) for the chloride transporter during normoxic control, and initial and final exposure to, and recovery from, hyperoxia (series III). See legend of Fig. 8 for other details (N=11).

rently, J_{max}^{Na} was not significantly altered during hyperoxia but return to normoxia resulted in a decrease in J_{max}^{Na} by 30 % (Fig. 8B), which was significant compared to both the control and final periods.

Exposure to hyperoxia had no initial effect on the affinity of the Cl^- transporter, but later decreased it significantly as K_m^{Cl} increased by 50 % (Fig. 9A). Return to normoxia reversed the effect, significantly increasing the affinity compared to the final time period as K_m^{Cl} dropped from 250 to 137 μequiv l^{-1}, a value not significantly different from the original control. J_{max}^{Cl} remained unaltered at about 291 μequiv kg^{-1} h^{-1} during the hyperoxic exposure but increased by 65 % upon return to normoxia, reaching a value (445 μequiv kg^{-1} h^{-1}) that was significantly higher than both the control and the final exposure period values (Fig. 9B).

The effect of increasing $[NaCl]_e$ on J_{net}^{Na}, J_{out}^{Na}, J_{net}^{Cl}, J_{out}^{Cl} and J_{net}^{H} and its components was also examined in the kinetics experiments (series III). As $[NaCl]_e$ was increased, there were no detectable changes in either J_{out}^{Na} or J_{out}^{Cl}, and J_{net}^{Na} and J_{net}^{Cl} became increasingly positive (data not shown). This suggests that the

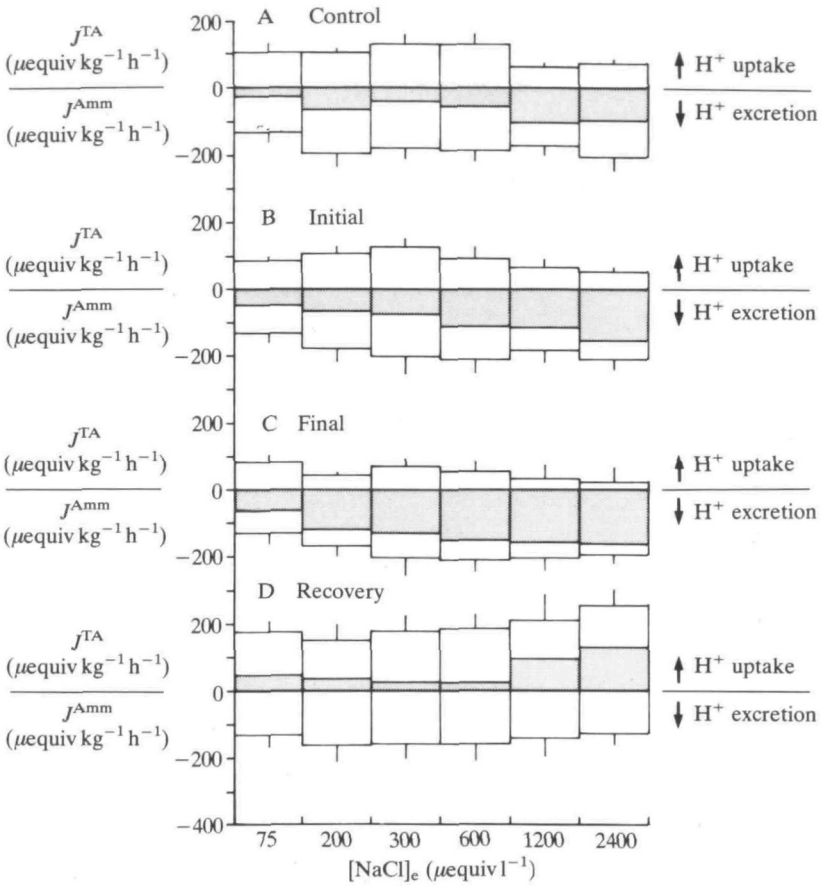

Fig. 10. Titratable acidity (J^{TA}), total ammonia (J^{Amm}), and net acidic equivalent (J^{H}_{net}) (stippled bars) flux across the gills of rainbow trout as a function of external NaCl concentration ([NaCl]$_e$) during normoxic control, and initial and final exposure to, and recovery from, hyperoxia (series III). Standard error bars have been omitted from J^{H}_{net} for clarity. Values are means±1 S.E.M. (N=11).

exchange diffusion components saturated at very low values of [NaCl]$_e$ or else were absent. However, it must be noted that the accuracy of J^{Na}_{out} determinations progressively decreases at higher substrate concentrations. Small changes in J_{out} would be undetectable with the short flux periods used here.

J^{Amm}, J^{TA} and J^{H}_{net} in each of the four exposure periods were also not significantly altered as [NaCl]$_e$ was increased (Fig. 10). However, in all treatments except recovery, there was a tendency for J^{Amm} to increase with [NaCl]$_e$ at the three lowest concentrations and for a general increase in acidic equivalent excretion (negative J^{H}_{net}) over the whole range of [NaCl]$_e$ tested. Interestingly, during recovery, when net acidic equivalent uptake predominated, there was a similar tendency for positive J^{H}_{net} to increase at higher [NaCl]$_e$.

Discussion

Validity of new methods

Shaw (1959) developed a method for measuring the diffusive efflux of Na^+ and Cl^- from an aquatic animal which involved replacing the external water with deionized water and monitoring the appearance of the ion in question. As both influx and exchange diffusion are theoretically eliminated, the measured net flux should represent the true diffusive efflux, provided that influx and exchange diffusion blockade are complete and that changes in TEP and gill permeability are not induced by the changes in external water NaCl. In series II of the present study, a modification of this approach has been employed for the first time to measure the *differential* diffusive efflux of Na^+ and Cl^- and its possible contribution to acid–base homeostasis. Shaw's original method has been specifically modified to avoid possible changes in membrane permeability and TEP associated with the use of distilled water (e.g. Potts, 1984; McDonald and Rogano, 1986). An artificial medium was developed providing normal levels of Ca^{2+}, Mg^{2+}, TAlk and pH, but lacking Na^+ and Cl^- (Table 1). The maintenance of Ca^{2+} and Mg^{2+} in this NaCl-free medium allowed for measurements of efflux in the absence of any detectable changes in TEP (Table 2). In contrast, exposure to distilled water resulted in an immediate highly negative TEP (Table 2), predictable from the absence of hardness cations (Potts, 1984).

Recently, it has been shown that rainbow trout body mucus may have up to 10 times the concentration of ions compared to the bulk water, although the activity coefficients are much lower (Handy, 1989). It is not known whether similar relationships apply to gill mucus, or to what levels these concentrations would be lowered during exposure to NaCl-free water. Thus, it is possible that the present method may not completely eliminate the influx or the exchange diffusion component owing to incomplete removal of the ions from the gill microenvironment. It is also possible that Na^+ and Cl^- removal from the apical surface may have an effect on the tight junction permeability, although we are not aware of any evidence that this effect occurs for the very small absolute decreases in $[NaCl]_e$ used in the present study. Furthermore, the lack of change in TEP when the fish were placed in NaCl-free media (Table 2) suggests that such alterations in permeability did not occur.

The experimental protocols for both steady-state and kinetic analyses used in the present study required determination of both ion and isotopic fluxes over very short periods (0.5 h) to minimize errors and resolve trends resulting from the changing dynamic state of the fish during acid–base disturbance. These short flux determinations therefore placed great demand on analytical precision. Exceptional care was taken in sampling and analysis to determine accurately the small changes in concentrations and radioactivity occurring over these very short periods. The final protocols arose out of preliminary experiments with various experimental periods and analytical techniques.

In determining changes in total Na^+ and Cl^- concentrations, which entered directly into net flux calculations, the most important precautions were: (i) to read

the samples undiluted, wherever practical; (ii) to use background correction and bracketing standards in the range of measurement; (iii) to replicate (duplicates for Na^+ and triplicates for Cl^-); and (iv) to read the start and end samples for a flux period *as pairs immediately after one another*. In practice, the latter was the most important precaution, because it is the difference between start and end values, not the absolute values, which enters into the net flux calculation. To illustrate, the AAS had a reproducibility of ±about 1% when calibrated over the range 550–650 μequiv l^{-1} $[Na^+]_e$. A typical net flux (80 μequiv kg^{-1} h^{-1}) over 0.5 h (Fig. 1) might change the external Na^+ concentration by 9 μequiv l^{-1}. While this difference was only 1.5% of the total background of 600 μequiv l^{-1}, it was 9% of the calibration range and, in practice using the pairing approach, could be reproduced to 8–10 μequiv l^{-1}. Therefore, the overall reproducibility of the net flux measurements was about ±10%.

For influx determinations, the influence of such errors in the total 'cold' concentration measurements was small; the critical measurement was again the difference, this time in radioactivity, between start and end samples. Here counting statistics and pipetting were the major sources of error. Statistically, the error associated with radioactive counting can be reduced through increasing the counting time (and thus the total counts per sample), replicating the samples, repeating the count procedure on the same samples, and generating weighted means from the results. A complete description of the error calculations is beyond the scope of this paper, but can be found in Wang *et al.* (1975) and Remington and Schork (1985). In practice, we counted at least duplicate water samples each twice for 10 min. Statistically, error in count separation accounted for variations in influx determination from 2 to 12% in the kinetic series (series I) and 5 to 10% in the steady-state flux determinations. Pipetting error was reduced by using individual volumetric pipettes for the beginning and end of each flux period. As a result, overall reproducibility of about ±10% was achieved for influx measurements using 0.5 h periods.

Branchial ion and acidic equivalent fluxes

The results of series I confirm and extend those of Wood *et al.* (1984), who found that branchial Na^+ *vs* acidic equivalent and Cl^- *vs* basic equivalent exchanges were dynamically adjusted to achieve acid–base homeostasis during the hyperoxic regime. A positive Na^+ balance and negative Cl^- balance were associated with net acidic equivalent excretion, while a negative Na^+ balance and positive Cl^- balance were associated with net basic equivalent excretion (=acidic equivalent uptake). While the general agreement of the two studies was good, some points of difference with the result of Wood *et al.* (1984) are noted subsequently.

An important new contribution of the present study was the precise characterization of the time course of these responses, for fluxes were measured over 0.5 h intervals. Changes in J_{in}^{Na} occurred slowly during hyperoxia but rapidly during recovery (Fig. 1), while overall changes in J_{in}^{Cl} took 3–4 h to develop fully in both

situations (Fig. 2). Similarly, J^{TA} and J^{H}_{net} also took 3–4 h to respond to either internal acidosis or alkalosis (Fig. 3). The importance of the time course of these changes with reference to the mechanisms involved will be discussed below.

Theoretically, changes in J^{TA} could result from changes in NH_3 (but not NH_4^+), H^+ and/or $HCO_3^-(OH^-)$ fluxes. However, the constancy of the total ammonia flux (J^{Amm}) in these experiments in the face of large changes in J^{H}_{net} (Fig. 3) and the non-correspondence of J^{TA} changes with J^{Na}_{in} (Fig. 2) suggest that the alterations in J^{TA} mainly reflected changes in $HCO_3^-(OH^-)$ fluxes. The time course and pattern of changes in J^{TA} and J^{Cl}_{in} followed each other very closely, presenting strong evidence for the linkage between influx of Cl^- and the outflux of HCO_3^-. Throughout the experiment, changes in the net flux of acidic equivalents appeared to be largely the result of changes in J^{TA}, with no significant changes in J^{Amm}. This lack of an increase in J^{Amm} during hyperoxia contrasts with several previous studies on freshwater teleosts (Wood *et al.* 1984; Claiborne and Heisler, 1986; Perry *et al.* 1987), which found that respiratory acidosis in the arterial blood was accompanied by increases in the flux of ammonia to the water. The reasons for this difference are unknown. In any event, it must be emphasized that the ammonia and titratable components of J^{H}_{net} are not necessarily dependent or independent parameters, for the latter may or may not be affected by the former.

During exposure to hyperoxia, both J^{Na}_{in} and J^{Cl}_{in} were reduced significantly. While the decrease in J^{Cl}_{in} was in accord with the results of Wood *et al.* (1984), the reduction in J^{Na}_{in} was not. Another difference in the present study was the continued negative J^{H}_{net} (acidic equivalent excretion) at 72 h, rather than complete compensation by this time. We speculate that lamellar vasoconstriction and gill hypoventilation was larger in the present study, resulting in greater respiratory acidosis and reduced mixing in the gill boundary layer. This could decrease Na^+ and Cl^- availability in the interlamellar water, thereby reducing the overall ion turnover rates and speed of acid–base correction. This non-specific effect may have been the explanation for the slow decrease in J^{Na}_{in} observed during hyperoxia (Fig. 1).

A gradual decrease in J^{Na}_{in} was not the appropriate response for correction of a respiratory acidosis by Na^+/acidic equivalent exchange. Wood *et al.* (1984) found only a very small increase in J^{Na}_{in} during initial exposure to hyperoxia and a decrease later in the exposure period. Perry *et al.* (1987) found a similar decrease in J^{Na}_{in} during hypercapnic acidosis. Based on these studies, the relative contribution of Na^+/acidic equivalent exchange during compensation from respiratory acidosis appears to be minimal. However, inhibition of this exchange may have played an important role following return to normoxia (Fig. 1), in agreement with the results of both Wood *et al.* (1984) and Perry *et al.* (1987). The observed reduction in J^{Na}_{in} at this time is the expected response, as it would result in the retention of acidic equivalents and aid in compensation of the metabolic alkalosis present during normoxic recovery. The pattern of changes in J^{Cl}_{in} was in general agreement with those of previous workers (Cameron, 1976; Wood *et al.* 1984; Perry *et al.* 1987), who found that J^{Cl}_{in} was similarly inhibited during acidosis and

greatly stimulated during alkalosis. This is the expected response if the exchange of Cl^- was for basic equivalents (HCO_3^-, OH^-).

In summary, these results suggest that J_{in}^{Na} and its associated exchange with acidic equivalents play a minor role relative to J_{in}^{Cl} and its exchange with basic equivalents during compensation from hyperoxia-induced respiratory acidosis. To compensate an acidosis, the fish increases the plasma SID, thereby retaining plasma [HCO_3^-]. This change in SID is usually not accomplished by increasing plasma [Na^+]. Therefore, manipulation of the Cl^-/HCO_3^- exchanger offers the most direct method for compensation. However, to compensate an alkalosis, such as during post-hyperoxic recovery, both J_{in}^{Na} and J_{in}^{Cl} are adjusted. Thus, post-hyperoxic alkalosis is a more powerful stimulant than hyperoxic acidosis for eliciting changes in ion/acidic equivalent fluxes, in agreement with the findings of Wood *et al.* (1984).

Relationship between net branchial ion flux and net acidic equivalent flux

The flux of acidic equivalents across the branchial surface was stoichiometrically (−1:1) proportional to the difference between the net Na^+ and Cl^- fluxes over a wide range of the two variables (Fig. 4). This excellent correlation agrees with the findings of many (but not all, see Goss and Wood, 1990, for references) previous workers. Heisler (1982), McDonald (1983), McDonald *et al.* (1983, 1989) and Wood *et al.* (1984) all found a similar stoichiometry. According to SID theory popularized by Stewart (1978), the differential flux of strong ions from one compartment to another will necessarily constrain a change in acid–base status in that compartment. However, the −1:1 stoichiometry says nothing about the mechanisms involved in these fluxes, only that Na^+ and Cl^- are the major ions involved.

Diffusive efflux and acid–base regulation

Exposure to hyperoxia produced no significant changes in the diffusive efflux of either Na^+ or Cl^-, and the $Na^+ - Cl^-$ efflux difference was not significantly different from 0 (Fig. 5). During recovery from hyperoxia, there was a large stimulation of both Na^+ and Cl^- efflux. This stimulation of Cl^- efflux was paradoxical, given that retention of anions would aid in correcting the acid–base disturbance. It is probable that a general non-specific stimulation of both Na^+ and Cl^- efflux was the result of increased gill perfusion and ventilation upon return to normoxia. There was, however, a greater increase in Na^+ efflux compared to Cl^- efflux at this time (net cation loss) *via* the diffusive efflux mechanism (Fig. 5C). A net loss of cations would act to decrease SID, thereby decreasing pH. Therefore, there appears to be a definite *differential* diffusive efflux mechanism for acid–base regulation across the branchial surface during recovery from hyperoxia. Diffusive efflux measurements (series II) and acidic equivalent flux measurements (series I) were made on different batches of fish, so direct comparison is difficult. Nevertheless, it is apparent that the *differential* efflux during normoxic recovery was large enough to account for up to 50 % of the positive J_{net}^{H} at this time (cf.

Figs 3 and 5). The mechanisms controlling the rate of diffusive efflux are not known but evidence is mounting that paracellular passage of ions across many epithelia is an active, controlled process (Madera, 1988).

Na^+ and Cl^- uptake kinetics – changes during acid–base disturbances

The present study is only the third to report measurements of K_m and J_{max} for Na^+ transport in intact, unanaesthetized rainbow trout (Wood and Randall, 1973*a*,*b*; Laurén and McDonald, 1987) and the first to report comparable values for Cl^- transport. However, there exist several previous determinations of these parameters in perfused head preparations (Perry *et al.* 1985; Avella *et al.* 1987) and irrigated/anaesthetized whole-trout preparations (Kerstetter *et al.* 1970; Kerstetter and Kirschner, 1972), while the data of McDonald and Rogano (1986) can be re-analyzed to estimate these parameters in intact trout. Table 3 compares the present measurements with those in the literature.

Table 3. *Comparisons of the kinetic parameters for Na^+ and Cl^- transport measured in the present study under normoxic control conditions with previous literature reports of these values in rainbow trout*

Reference	External $[Na^+]/[Cl^-]$ (μequiv l^{-1})	K_m (μequiv l^{-1})	J_{max} (μequiv kg^{-1} h^{-1})	Comments
Na^+ transport				
Kerstetter *et al.* (1970)	900	450	333	Irrigated gill
Wood and Randall (1973*a*)	28	20	616	*in vivo*
Perry *et al.* (1985)	590	340	454	Perfused head preparation
Laurén and McDonald (1987)	600	57	540	*In vivo* (juveniles)
McDonald and Rogano (1986)	580	155	375	*In vivo**
Avella *et al.* (1987)	148	422	544	Perfused head preparation
Present study	600	114	456	*In vivo*
Cl^- transport				
Kerstetter and Kirschner (1972)	900	250	290	Irrigated gill
Perry *et al.* (1985)	590	1740	193	Perfused head preparation
McDonald and Rogano (1986)	580	174	504	*In vivo**
Present study	600	165	291	*In vivo*

* Calculated from original data (D. G. McDonald, personal communication).

External $[Na^+]$ and $[Cl^-]$ are the concentrations in which the trout were held prior to experimentation.

Affinity values obtained *in vivo* are clearly higher (i.e. lower K_m) than those obtained using either the perfused head technique or irrigated gills in the anaesthetized trout. This may be the result of limitations in the 'artificial' preparations. Incomplete ventilation of the gill lamellae could cause a depletion of $[NaCl]_e$ in the boundary layer, resulting in reduced apparent affinity. K_m values could also be elevated by the presence of the high concentrations of epinephrine (10^{-7} mol^{-1}) necessary to maintain the perfused head preparation (Perry *et al.* 1984). However, estimates of J_{max} for both the Na^+ and Cl^- transporters appear to be relatively uniform using all techniques.

Previous descriptions of branchial transport kinetics have been concerned only with long-term adjustments to altered environmental ion levels (see Introduction). The present study (series III) is the first to employ specific internal acid–base disturbances as possible stimuli for *dynamic* changes in the transport kinetics of Na^+ and Cl^-, and to relate the latter to the measured changes in J_{in}^{Na} and J_{in}^{Cl} during acid–base compensation. The results demonstrate that alterations in Na^+/acidic equivalent and Cl^-/basic equivalent exchanges (Figs 1, 2, 3) occur through complex adjustments in both K_m and J_{max} for the two transport systems. Specifically, decreased Cl^- uptake/base excretion during the compensation of hyperoxic respiratory acidosis was achieved by decreased Cl^- affinity (increased K_m) without alterations in J_{max}^{Cl} (Fig. 9). However, increased Cl^- uptake/base excretion during the compensation of post-hyperoxic metabolic alkalosis was mediated by both decreases in K_m^{Cl} and increases in J_{max}^{Cl}. The opposite changes in Na^+ uptake/acid excretion at this time were achieved by exactly opposite alterations of K_m^{Na} and J_{max}^{Na}.

Changes in the K_m of branchial Na^+ (Maetz, 1972; Avella *et al.* 1987) and Cl^- transporters (de Renzis and Maetz, 1973) have been observed in several previous kinetic studies. The explanation generally offered has been that alterations in K_m resulted from changes in the Ca^{2+} levels of the external water, acting as a competitive or uncompetitive inhibitor. This cannot be the explanation for the observed K_m shifts in the present study, for normal levels of water Ca^{2+} were maintained throughout all experimental treatments. The results instead point to direct or indirect effects of acid–base status on carrier affinity, though non-specific effects of increased oxygen should not be discounted. For example, it has been suggested earlier that decreased ventilation and perfusion of the gill during hyperoxia may have resulted in decreased Na^+ and Cl^- availability in the interlamellar water. This could well have contributed to the observed increases in K_m at this time (Figs 8, 9).

There are many possible mechanisms for acid–base effects on K_m, all of which are highly speculative. These include structural changes in the transport molecules themselves, or changes in the charge of the channels. There could be direct effects of changes in pHi, or in pHe *via* an intracellular messenger, or there might be a hormonal or neural level of control. Large numbers of innervated neuroepithelial cells have been described in both elasmobranch and teleost gills (Dunel-Erb *et al.* 1982; Donald, 1987). These cells occur over the entire region of the gill filament

and respond to hypoxia by degranulation (Laurent, 1985), though their exact role remains to be elucidated. Frain (1987) has suggested that K_m changes may reflect the existence of fundamentally different populations of transporters which are activated under different conditions. This whole topic of carrier affinity regulation in relation to acid–base status is clearly worthy of further detailed study.

Large changes in J_{max} also occurred, but only during the metabolic alkalosis of the recovery period (Figs 8, 9). Furthermore, these changes were in the opposite direction for the Na^+ and Cl^- transporters. Classically, changes in J_{max} have been interpreted as increases in the number of transport sites available (Shaw, 1959). Theoretically many possible mechanisms could be involved. Laurent *et al.* (1985) showed that there was an increase in the number of chloride cells present in fish adapted to more dilute water. However, it seems unlikely that the actual number of chloride cells might increase or decrease in the relatively short time (4–8 h) over which responses occurred in the present study. Avella *et al.* (1987) demonstrated a strong correlation between increased relative surface area of the chloride cells and an elevated Na^+ uptake, though the time course of these changes was not evaluated. Further study is clearly warranted to see whether these changes can occur over such a short time course. The possibility of non-specific effects resulting from the perfusive and convective changes occurring during hyperoxia (Wood and Jackson, 1980), or from the action of the oxygen itself, cannot be eliminated. Such non-specific mechanisms could act to alter the number of chloride cells actually perfused or ventilated on their apical exposure, thereby changing J_{max} for the Na^+ and Cl^- transporters. However, one important argument against all these explanations is that the fish must simultaneously reduce the J_{max} of one transporter while increasing that of the other. Clearly, this would be difficult if changes in chloride cell number, morphology or ventilation/perfusion were the only mechanisms available. An alternative possibility recently suggested by Frain (1987) is the production of new transporters or activation/deactivation of nascent transporters already present on the cell membrane.

Kirschner (1988), using isolated frog skins, has suggested that apparent saturation of Na^+ influx (J_{in}^{Na}) is not due to saturation of the Na^+ element in the transporter, but instead is the result of a limitation in the flux of the counterion. Indeed, stimulation of proton efflux was found to increase J_{in}^{Na}. If this idea is substantiated, it suggests that the one-substrate Michaelis–Menten model which we and all previous workers on freshwater fish have employed may be overly simplistic. A model taking into account both the external substrate (Na^+, Cl^-) and the internal substrates (acidic, basic equivalents) will be required. As a first step in this process, we are currently examining the possibility that the availability of the acidic and basic equivalent counterions determines the rates of J_{in}^{Na} and J_{in}^{Cl} during acid–base disturbances in trout.

This study was supported by a NSERC operating grant to CMW. Many thanks to M. Kovacevic for excellent technical assistance. Dr D. G. McDonald is thanked for his helpful comments and access to original data.

References

AVELLA, M., MASONI, A., BORNANCIN, M. AND MAYER-GOSTAN, N. (1987). Gill morphology and sodium influx in the rainbow trout (*Salmo gairdneri*) acclimated to artificial freshwater environments. *J. exp. Zool.* **241**, 159–169.

CAMERON, J. N. (1976). Branchial ion uptake in Arctic grayling: resting values and the effects of acid–base disturbance. *J. exp. Biol.* **64**, 711–725.

CLAIBORNE, J. B. AND HEISLER, N. (1986). Acid–base regulation and ion transfers in the carp (*Cyprinus carpio*): pH compensation during graded long- and short-term environmental hypercapnia, and the effect of bicarbonate infusion. *J. exp. Biol.* **126**, 41–62.

DE RENZIS, G. AND MAETZ, J. (1973). Studies on the mechanism of chloride absorption by the goldfish gill: relation with acid–base regulation. *J. exp. Biol.* **59**, 339–358.

DONALD, J. A. (1987). Comparative study of the adrenergic innervation of the teleost gill. *J. Morph.* **193**, 63–73.

DUNEL-ERB, S., BAILLY, Y. AND LAURENT, P. (1982). Neuroepithelial cells in fish gill primary lamellae. *J. appl. Physiol.* **53**, 1342–1353.

FRAIN, W. J. (1987). The effect of external sodium and calcium concentrations on sodium fluxes by salt-depleted and non-depleted minnows, *Phoxinus phoxinus* (L.). *J. exp. Biol.* **131**, 417–425.

GOSS, G. G. AND WOOD, C. M. (1990). Na^+ and Cl^- uptake kinetics, diffusive effluxes and acidic equivalent fluxes across the gills of rainbow trout. II. Responses to bicarbonate loading. *J. exp. Biol.* **152**, 549–571.

HANDY, R. D. (1989). The ionic composition of rainbow trout body mucus. *Comp. Biochem. Physiol.* **93A**, 571–575.

HEISLER, N. (1982). Transepithelial ion transfer processes as mechanisms for fish acid–base regulation in hypercapnia and lactacidosis. *Can. J. Zool.* **60**, 1108–1122.

HÖBE, H., WOOD, C. M. AND WHEATLY, M. G. (1984). The mechanisms of acid–base and ionoregulation in the freshwater rainbow trout during environmental hyperoxia and subsequent normoxia. I. Extra- and intracellular acid–base status. *Respir. Physiol.* **55**, 139–154.

KERSETTER, T. H., KIRSCHNER, L. B. AND RAFUSE, D. (1970). On the mechanisms of sodium ion transport by the irrigated gills of rainbow trout (*Salmo gairdneri*). *J. gen. Physiol.* **56**, 342–359.

KERSTETTER, T. H. AND KIRSCHNER, L. B. (1972). Active chloride transport by the gills of rainbow trout (*Salmo gairdneri*). *J. exp. Biol.* **56**, 263–272.

KIRSCHNER, L. B. (1988). Basis for apparent saturation kinetics of Na^+ influx in freshwater hyperregulators. *Am. J. Physiol.* **254**, R984–988.

LAURÉN, D. J. AND MCDONALD, D. G. (1987). Acclimation to copper by rainbow trout, *Salmo gairdneri*: physiology. *Can. J. Fish. aquat. Sci.* **43**, 1488–1496.

LAURENT, P. (1985). Organization and control of the respiratory vasculature in lower vertebrates: are there anatomical gill shunts? In *Cardiovascular Shunts* (ed. K. Johansen and W. Burrgren), pp. 58–70. Alfred Benzon Symposium 21, Copenhagen: Munksgaard.

LAURENT, P., HÖBE, H. AND DUNEL-ERB, S. (1985). The role of environmental sodium chloride relative to calcium in gill morphology of freshwater salmonid fish. *Cell Tissue Res.* **240**, 675–692.

MADERA, J. L. (1988). Tight junction dynamics: Is paracellular transport regulated? *Cell* **53**, 497–498.

MAETZ, J. (1956). Les échanges de sodium chez le poisson *Carassius auratus* L. Action d'un inhibiteur de l'anhydrase carbonique. *J. Physiol., Paris* **48**, 1085–1099.

MAETZ, J. (1972). Branchial sodium exchange and ammonia excretion in the goldfish *Carassius auratus*. Effects of ammonia loading and temperature changes. *J. exp. Biol.* **56**, 601–620.

MAETZ, J. (1973). Na^+/NH_4^+, Na^+/H^+ exchanges and NH_3 movement across the gills of *Carassius auratus*. *J. exp. Biol.* **58**, 255–275.

McDonald, D. G. (1983). The interaction of environmental calcium and low pH on the physiology of the rainbow trout, *Salmo gairdneri*. I. Branchial and renal net ion and H^+ fluxes. *J. exp. Biol.* **102**, 123–140.

McDonald, D. G. and Prior, E. T. (1988). Branchial mechanisms of ion and acid–base regulation in the freshwater rainbow trout, *Salmo gairdneri*. *Can. J. Zool.* **66**, 2699–2708.

McDonald, D. G. and Rogano, M. S. (1986). Ion regulation by the rainbow trout in ion-poor water. *Physiol. Zool.* **59**, 318–331.

McDonald, D. G., Tang, Y. and Boutilier, R. G. (1989). Acid and ion transfer across the gills of fish: mechanisms and regulation. *Can. J. Zool.* **67**, 3046–3054.

McDonald, D. G., Walker, R. L. and Wilkes, P. R. H. (1983). The interaction of environmental calcium and low pH on the physiology of the rainbow trout, *Salmo gairdneri*. II. Branchial ionoregulatory mechanisms. *J. exp. Biol.* **102**, 141–155.

McDonald, D. G. and Wood, C. M. (1981). Branchial and renal acid and ion fluxes in the rainbow trout, *Salmo gairdneri*, at low environmental pH. *J. exp. Biol.* **93**, 101–118.

Michal, G. (1985). Determination of Michaelis constants and inhibitor constants. In *Methods of Enzymatic Analysis*, vol. 1 (ed. H. U. Bergmeyer), pp. 86–103. Weinheim: Verlag Chemie.

Perry, S. F., Booth, C. E. and McDonald, D. G. (1985). Isolated perfused head of rainbow trout II. *Ionic fluxes*. *Am. J. Physiol.* **249**, R255–R261.

Perry, S. F., Kinkead, R., Gallaugher, P. and Randall, D. J. (1989). Evidence that hypoxemia promotes catecholamine release during hypercapnic acidosis in rainbow trout (*Salmo gairdneri*). *Respir. Physiol.* **77**, 351–364.

Perry, S. F., Malone, S. and Ewing, D. (1987). Hypercapnic acidosis in the rainbow trout (*Salmo gairdneri*). I. Branchial ion fluxes and blood acid–base status. *Can. J. Zool.* **65**, 888–895.

Perry, S. F., Payan, P. and Girard, J. P. (1984). Adrenergic control of branchial chloride transport in the isolated perfused head of the freshwater trout (*Salmo gairdneri*). *J. comp. Physiol.* **154**, 269–274.

Perry, S. F. and Wood, C. M. (1985). Kinetics of branchial calcium uptake in the rainbow trout: effects of acclimation to various external calcium levels. *J. exp. Biol.* **116**, 411–433.

Potts, W. T. F. (1984). Transepithelial potentials in fish gills. In *Fish Physiology*, vol. XB (ed. W. S. Hoar and D. J. Randall), pp. 105–128. New York: Academic Press.

Randall, D. J. and Jones, D. R. (1973). The effect of deafferentation of the pseudobranch on the respiratory response to hypoxia and hyperoxia in the trout (*Salmo gairdneri*). *Respir. Physiol.* **17**, 291–301.

Remington, R. D. and Schork, M. A. (1985). *Statistics with Applications to the Biological and Health Sciences*. 2nd edn. New Jersey: Prentice-Hall Inc.

Shaw, J. (1959). The absorption of sodium ions by the crayfish *Astacus pallipes* (Lereboullet). I. The effect of external and internal sodium concentration. *J. exp. Biol.* **36**, 126–144.

Stewart, P. A. (1978). Independent and dependent variables of acid–base control. *Respir. Physiol.* **33**, 9–26.

Verdouw, H., van Echteld, C. J. A. and Dekkers, E. M. J. (1978). Ammonia determinations based on indophenol formation with sodium salicylate. *Water Res.* **12**, 399–402.

Wang, C. H., Willis, D. L. and Loveland, W. D. (1975). *Radiotracer Methodology in the Biological, Environmental and Physical Sciences*. Town: Prentice Hall.

Wheatly, M. G., Hõbe, H. and Wood, C. M. (1984). The mechanisms of acid–base and ion regulation in freshwater rainbow trout during environmental hyperoxia and subsequent normoxia. II. The role of the kidney. *Respir. Physiol.* **55**, 155–173.

Wood, C. M. (1988). Acid–base and ionic exchanges at gills and kidney after exhaustive exercise in the rainbow trout. *J. exp. Biol.* **136**, 461–481.

Wood, C. M. and Caldwell, F. H. (1978). Renal regulation of acid–base balance in a freshwater fish. *J. exp. Zool.* **205**, 301–307.

Wood, C. M. and Jackson, E. B. (1980). Blood acid–base regulation during environmental hyperoxia in the rainbow trout (*Salmo gairdneri*). *Respir. Physiol.* **42**, 351–372.

Wood, C. M. and Randall, D. J. (1973*a*). Sodium balance in the rainbow trout (*Salmo gairdneri*) during extended exercise. *J. comp. Physiol.* **82**, 235–256.

Wood, C. M. and Randall, D. J. (1973*b*). The influence of swimming activity on water balance in the rainbow trout (*Salmo gairdneri*). *J. comp. Physiol.* **82**, 257–276.

WOOD, C. M., WHEATLY, M. AND HÖBE, H. (1984). The mechanisms of acid–base and ionoregulation in the freshwater rainbow trout during environmental hyperoxia and subsequent normoxia. III. Branchial exchanges. *Respir. Physiol.* **55**, 175–192.

ZALL, D. M., FISHER, M. D. AND GARNER, Q. M. (1956). Photometric determination of chlorides in water. *Analyt. Chem.* **28**, 1665–1678.