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ABSTRACT

To test the hypothesis that internal ion imbalances at high pH are caused by altered branchial ion transporting capacity and permeability, radiotracers ($^{24}Na^+$ and $^{36}Cl^-$) were used to measure ion movements across the gills of intact rainbow trout (*Oncorhynchus mykiss*) during 3 d exposure to pH 9.5. At control pH (pH 8.0), the trout were in net ion balance, but by 8 h at high pH, 60%–70% reductions in Cl^- influx (J_{in}^{Cl}) and Na^+ influx (J_{in}^{Na}) led to net Cl^- and Na^+ losses of -200 μmol kg^{-1} h^{-1}. Outflux (diffusive efflux plus renal ion losses) was not initially altered. By 72 h, net Cl^- balance was reestablished because of a restoration of J_{in}^{Cl}. Although J_{in}^{Na} remained 50% lower at this time, counterbalancing reductions in Na^+ outflux restored net Na^+ balance. One-substrate ion-uptake kinetics analyses indicated that reduced ion influx after 8 h at pH 9.5 was caused by 50% decreases in Cl^- and Na^+ maximal transport rates (J_{max}^{Cl}, J_{max}^{Na}), likely reflecting decreased numbers of functional transport sites. Two-substrate kinetic analyses indicated that reduced internal HCO_3^- and H^+ supply for respective branchial Cl^-/base and Na^+/acid transport systems also contributed to lower J_{in}^{Cl} and, to a lesser extent, lower J_{in}^{Na} at pH 9.5. Recovery of J_{max}^{Cl} after 3 d accounted for restoration of Cl^- balance and likely reflected increased numbers of transport sites. In contrast, J_{max}^{Na} remained 33% lower after 3 d, but a lower affinity of the gills for Na^+ (fourfold greater K_m^{Na}) accounted for the chronic reduction in Na^+ influx at pH 9.5. Thus, reestablishment of Cl^- uptake capacity and counterbalancing reductions in Na^+ outflux allows rainbow trout to reestablish net ion balance in alkaline waters.

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Introduction

The freshwater rainbow trout (*Oncoryhnchus mykiss*) does not normally live in alkaline water, but it may be subject to temporary upward pH surges caused by the photosynthetic processes of aquatic plants and algae (e.g., Halstead and Tash 1982; Murray and Zeibell 1984). The inability of rainbow trout to readily cope with high pH has also complicated efforts to stock this salmonid into saline-alkaline lakes scattered throughout western North America (e.g., Kucera et al. 1985; Coleman and Johnson 1988; Wagner et al. 1997). These problems may be related to the pronounced, sometimes lethal reductions in blood Na^+ and Cl^- concentration experienced by salmonids at high pH (Heming and Blumhagen 1988; Wilkie and Wood 1991; Yesaki and Iwama 1992; Wilkie et al. 1993; McGeer and Eddy 1998). Although Wilkie and Wood (1994) suggested that these disturbances may be related to lower Cl^- and Na^+ influx rates across the gills (Wilkie and Wood 1994), it is presently unclear how these ionoregulatory disturbances are initiated at high pH.

High-pH-induced reductions in gill-mediated Cl^- and Na^+ influx may result from altered ion transporter numbers and/or affinity. Traditionally, Na^+ uptake was thought to occur by electroneutral Na^+/H^+ or Na^+/NH_4^+ exchange, both of which represent Na^+/acid coupling (Perry 1997). Recently, evidence has accumulated that indicates that Na^+ uptake across the apical membranes of gill epithelia takes place by a different sort of Na^+/acid coupling, specifically by an electrochemical gradient created by active H^+ extrusion mediated by apically located H^+-ATPases on branchial pavement cells (Avella and Bornancin 1989; Goss et al. 1992; Lin et al. 1994; Sullivan et al. 1995, 1996). Chloride uptake likely takes place by electroneutral Cl^-/HCO_3^- exchange across branchial chloride cells, possibly driven by outward HCO_3^- movement down its electrochemical gradient (Marshall 1995; Perry 1997). Externally, highly alkaline water may therefore interfere with ion transport by acting directly on the transporters, but it may also decrease internal H^+ and HCO_3^- availability for the respective Na^+/acid coupling and Cl^-/HCO_3^- (Cl^-/base coupling) systems. At high pH, the ex-

tremely low P_{CO_2} of water would promote blood-to-water CO_2 diffusion, leading to reduced internal H^+ and HCO_3^- levels (respiratory alkalosis; Johansen et al. 1975; Lin and Randall 1990; Wilkie and Wood 1991).

With this background, this investigation tested the hypothesis that internal electrolyte imbalances in rainbow trout at high pH are caused by both inhibited ion transport across the gill and increased branchial ion permeability. Accordingly, radiotracers ($^{24}Na^+$, $^{36}Cl^-$) were used to measure the influx and outflux of Cl^- and Na^+ across the gills of rainbow trout exposed to high pH (pH 9.5) for 72 h. The maximal ion transport capacity (J_{max}^{ION}) and transporter affinity (K_m^{ION}) were also determined in trout exposed to high pH by experimentally determining kinetic curves (i.e., external substrate dependence) for Na^+ and Cl^- uptake rates (influx). The blood acid-base status of rainbow trout at high pH (taken from parallel experiments by Wilkie and Wood 1991) was also used to predict how internal H^+ and HCO_3^- supply influenced Na^+ and Cl^- transport kinetics, respectively.

Material and Methods

Experimental Animals and Setup

Domestic rainbow trout (*Oncorhynchus mykiss*; mean weight 229.0 ± 5.7 g; $N = 40$) of both sexes were acclimated to hard, dechlorinated Hamilton tap water (composition: $[Na^+] = 0.6$, $[Cl^-] = 0.8$, $[Ca^{++}] = 0.9$, $[Mg^{++}] = 0.4$, titratable alkalinity = 2.0 mmol L^{-1}) for a minimum of 6 wk. On the basis of total carbon dioxide concentration ($T_{CO_2} = CO_2 + HCO_3^- + CO_3^{--}$) measurements, water titratable alkalinity was found to be mainly (>95%) caused by HCO_3^- at pH 8.0.

One wk before experiments, the fish were transferred in batches of 10 from the 6°–10°C holding tank to a temperature-acclimation tank that paralleled the experimental temperature of 13°–15°C. Food was withheld at this time to minimize the known effects that feeding has on metabolic processes (Brett and Zala 1975). Fish were placed in individual, darkened Plexiglas flux boxes that received flowing water at 0.5 L min^{-1} 2 d before experiments. The boxes were part of a "flow-through" experimental system, fitted with a pH-stat, which has been described in detail previously (Wilkie and Wood 1991). The pH of water entering the system was independently monitored and averaged 9.56 ± 0.02. At pH 9.5, this CO_3^{--} could have accounted for up to 30% of the titratable alkalinity, but $CaCO_3$ precipitate formation at high pH made it impossible to accurately determine titratable alkalinity under these conditions. A flow-through system was employed to minimize the unavoidable decreases in water Ca^{++} that occur as $CaCO_3$ precipitates out of solution at pH 9.5 when CO_2 is added to the water by the fish or simply because of aeration (Wilkie and Wood 1991, 1994). Water Ca^{++} concentrations were ca. 0.4–0.6 mmol L^{-1} during the high pH exposure regime in this study.

When water flow was terminated to the boxes during high-pH flux determinations, the continuous CO_2 production by the fish and aeration drove water pH down. This necessitated manual monitoring of water pH at 30-min intervals; when water pH dropped below 9.5, an appropriate amount (0.5–2.0 mL) of 1 N KOH was added to the water. The flux boxes were slightly modified versions of those described in detail by McDonald and Rogano (1986) and comprised an aerated outer chamber, thereby ensuring thorough mixing, and an inner chamber containing each fish. Boxes used in Part 1 were ca. 3.0 L in volume, while those used in Part 2 were ca. 2.0 L. The shorter flux determination periods in Part 2 necessitated the use of smaller box volumes for more accurate resolution of differences in water radioactivity and ion concentrations. The boxes were fitted with a three-way stopcock valve to which a syringe could be attached, connected to a 10-cm length of vinyl tubing that led into the inner chamber of the box. Since the boxes were covered and the syringes fitted on the externally mounted valve, water samples were drawn without apparent disturbance to the fish. Low plasma cortisol concentrations in similarly confined trout (≤10 ng mL^{-1}; Wilkie et al. 1996) indicate that this protocol causes minimal stress to these domestic rainbow trout.

Experimental Protocol

Part 1: Unidirectional Ion (Na^+ and Cl^-) Movements at High pH. Flux rates were determined on seven rainbow trout initially held at pH 8.0 and then exposed to pH 9.5 for 72 h. Measurements of Na^+ and Cl^- influx, outflux, and net flux were measured at pH 8.0 (control) and after 0–3, 8–11, 24–27, 48–51, and 72–75 h at pH 9.5. Flow to the boxes was cut off 20 min before the initiation of flux determinations and 4 μCi of $^{24}Na^+$ and 10 μCi of $^{36}Cl^-$ was added to each box and allowed to equilibrate for 15 min. A 10-mL water sample was then withdrawn for pH determination, immediately followed by a 45-mL water sample for determination of $^{24}Na^+$ and $^{36}Cl^-$ radioactivity, plus total Na^+ and Cl^- concentrations. The process was repeated at 1 and 3 h, and flow was then reestablished to the boxes. At the end of the final flux determination (72–75 h), the fish were killed with an overdose of buffered MS-222 solution (1.5 g L^{-1}; Syndel). Blood samples (1 mL) were then drawn via caudal puncture, centrifuged at 10,000 *g*, and the plasma frozen for determination of $^{24}Na^+$and $^{36}Cl^-$ radioactivity and plasma total Na^+ and Cl^- concentrations.

Part 2: High-pH Induced Changes in Ion-Uptake Kinetics. Saturation kinetics analysis was performed using a protocol developed by Goss and Wood (1990*a*, 1990*b*) in which water Cl^- and Na^+ concentrations were sequentially increased and the Cl^- or Na^+ influx rates measured at each respective concentration. This allowed determination of the apparent maximal transport rates (J_{max}^{ION}) and affinities (K_m^{ION}) for Cl^- and Na^+

across trout gills at pH 8.0 or after exposure to pH 9.5 for either 10 h, 1 d, or 3 d.

Accurate determination of ion-uptake kinetics necessitates the use of water that lacks Na^+ or Cl^- (so that external Na^+ and Cl^- concentrations can be easily and accurately manipulated; Shaw 1959), but that has a normal complement of hardness cations (e.g., Mg^{++}, Ca^{++}) and alkalinity (Goss and Wood 1990*a*). Accordingly, NaCl-free water was prepared by passing dechlorinated Hamilton tap water through a deionizing canister and then adding back the appropriate amount of Ca^{++} ($CaCO_3$ salt) and Mg^{++} ($4MgCO_3 \cdot Mg(OH)_2 \cdot 4H_2O$ salt) to mimic the composition of Hamilton tap water (see Goss and Wood [1990*a*] for additional details). The measured composition of the NaCl-free water was: $[Na^+] = 2$ μmol L^{-1}; $[Cl^-] = 1.5$ μmol L^{-1}; $[Ca^{++}] = 0.9$ mmol L^{-1}; $[Mg^{++}] = 0.3$ mmol L^{-1}; titratable alkalinity = 2.57 mmol L^{-1}.

Ion-uptake kinetics experiments were performed on seven fish at pH 8.0 (control) and on separate groups of six to seven fish exposed to pH 9.5 for either 10 h, 1 d, or 3 d. Determinations of Na^+ and Cl^- influx were made over five 30-min intervals with nominal water NaCl concentrations of 80, 200, 350, 600, and 1,200 μmol L^{-1}, respectively, in each experiment. These fluxes were followed by a final 60-min flux period at 2,200 μmol L^{-1} of NaCl, which was needed to detect small changes in radioactivity against the high absolute activities of $^{24}Na^+$and $^{36}Cl^-$.

Each ion-uptake kinetics experiment was preceded by an initial flushing of each box (3/4 replacement × 7) with the NaCl-free water at the control (pH 8.0) or experimental pH (pH 9.5). A known amount of a 1 mmol L^{-1} NaCl solution radiolabeled with 4 μCi $^{24}Na^+$ mL^{-1} and 2 μCi $^{36}Cl^-$ mL^{-1} was then added to each box to yield the appropriate initial nominal water NaCl concentration of 80 μmol L^{-1}. Water samples (10 mL + 45 mL, respectively) were then taken at the start and end of this initial 30-min flux. An equal volume of NaCl-free water (at the appropriate pH and temperature) and a suitable volume of radiolabeled NaCl stock solution was then added back to each box for the next flux determination. Following each experiment, water flow was briefly reestablished to each box and then the fish were killed and sampled as previously described. Plasma was analyzed for $^{24}Na^+$ and $^{36}Cl^-$ radioactivity, plus total Na^+ and Cl^- concentrations.

Analytical Techniques and Calculations

Unidirectional Flux Rates. Unidirectional fluxes of Na^+ and Cl^- were determined using well-established protocols (e.g., Wood 1988; Wilkie and Wood 1994). Briefly, Na^+ and Cl^- influx rates (J_{in}^{Cl}, J_{in}^{Na}) were based on decreases in water radioactivity during each flux period, the known box volume, and the fish's weight. Net ion fluxes (J_{net}^{Na}, J_{net}^{Cl}) were based on changes in the external total ion concentrations, not radioactivity, and outward ion movements (outflux; J_{out}^{Cl}, J_{out}^{Na}) were calculated by subtracting ion influx from net ion flux rates.

Since $^{24}Na^+$ emits both gamma and beta rays and $^{36}Cl^-$ is a pure beta emitter, samples (5 mL) were first analyzed (in triplicate) for total gamma counts on a Packard 5000 series gamma counter. After the samples decayed for 40 $^{24}Na^+$ half-lives ($t_{1/2} = 15$ h), 10 mL ACS (Amersham) fluor was added to each vial for measurements of $^{36}Cl^-$ activity on a LKB 1217 Rackbeta scintillation counter. Concentrations of Na^+, in water and plasma, were determined by atomic absorption (Varian 1275 AA). Water and plasma Cl^- concentrations were measured using the mercuric thiocyanate assay (Zall et al. 1956) and coulometric titration (Radiometer CMT10 chloridometer), respectively.

Saturation Kinetics. Detailed calculations used to describe the saturation kinetics for Cl^- and Na^+ across trout gills are presented in Goss and Wood (1990*a*, 1990*b*) and are summarized here. Briefly, data generated in Part 2 indicated that Na^+ and Cl^- influx both followed first-order saturation kinetics and could therefore be described by the Michaelis-Menten relationship,

$$J_{in}^{ION} = \frac{J_{max}^{ION} \times [ION]_e}{K_m^{ION} + [ION]_e},$$

where J_{max}^{ION} is the apparent maximal Na^+ or Cl^- uptake rate, $[ION]_e$ is the external Na^+ or Cl^- concentration, and K_m^{ION} is an inverse index of the affinity of each respective transporter for Na^+ or Cl^-. Specifically, K_m^{ION} represents the external ion concentration at which J_{in}^{ION} is exactly equal to 50% of J_{max}^{ION}. The curves displayed in Figure 2 were fitted using the Michaelis-Menten equation. Estimates of J_{max}^{ION} and K_m^{ION} were determined on individual fish by Eadie-Hofstee regression analysis (Michal 1983), where J_{in}^{ION} was plotted against $J_{in}^{ION}/[ION]_e$. The *y*-intercept and the negative slope of the relationship yielded respective J_{max}^{ION} and K_m^{ION} estimates.

Statistics

All data are expressed as means ± 1 SEM (N). Data generated in Part 1 were analyzed by repeated-measures ANOVA; if significant differences were detected, a Bonferroni post-test followed. Data generated in Part 2 were tested by a simple one-way ANOVA and the Tukey-Kramer post-test. The level of statistical significance was at $P < 0.05$.

Results

Unidirectional Ion Movements at High pH

At control pH (8.0), Cl^- and Na^+ influx rates (J_{in}^{Cl}, J_{in}^{Na}) were both ca. 220 μmol kg^{-1} h^{-1}. Since Cl^- and Na^+ outflux (J_{out}^{Cl},

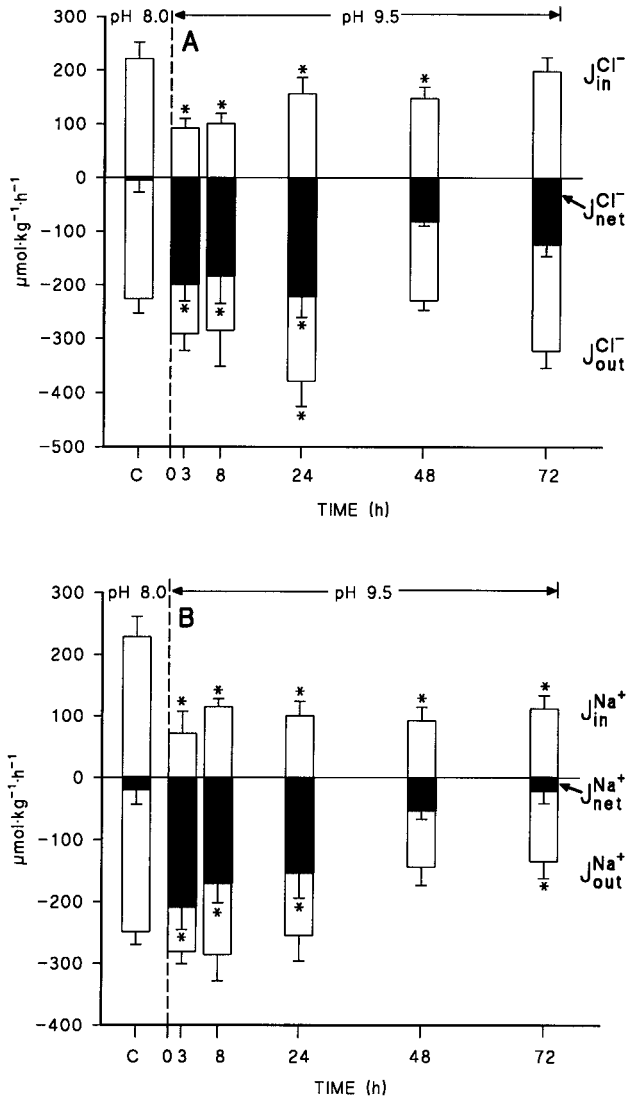

Figure 1. Influx (J_{in}^{ION}, *upward facing bars*), outflux (J_{out}^{ION}, *downward facing bars*), and net movements (J_{net}^{ION}, *shaded bars*) of (*A*) Cl^- and (*B*) Na^+ by rainbow trout during exposure to pH 9.5 for 72 h. Means ± 1 SEM; $N = 7$. Asterisks demonstrate statistical significance ($P < 0.05$) from control measurements at pH 8.0.

J_{out}^{Na}) counterbalanced influx, the fish were in net ion balance (Fig. 1). Over the first 8 h of exposure to pH 9.5, J_{in}^{Cl} was reduced by 60% but J_{out}^{Cl} was unaltered, leading to net Cl^- losses of −200 μmol kg^{-1} h^{-1} (Fig. 1*A*). Although J_{in}^{Cl} had partially recovered at 24 h, net Cl^- losses persisted because of a twofold elevation of J_{out}^{Cl} (Fig. 1*A*). By 72 h, the return of J_{in}^{Cl} and J_{out}^{Cl} to control values accounted for a reestablishment of net Cl^- balance (Fig. 1*A*).

In contrast to J_{in}^{Cl}, J_{in}^{Na} was chronically depressed at pH 9.5 (Fig. 1*B*). In the absence of any significant change in J_{out}^{Na}, reduced Na^+ influx led to net losses of −150 to −200 μmol kg^{-1} h^{-1} during the first 24 h of high pH exposure (Fig. 1*B*). By 48 h, however, the fish had reestablished net Na^+ balance because of 50% decreases in J_{out}^{Na}, which counterbalanced persistently reduced J_{in}^{Na} (Fig. 1*B*).

High-pH Induced Changes in Ion-Uptake Kinetics

At pH 8.0 and 9.5, Cl^- and Na^+ uptake closely followed first-order saturation kinetics (Fig. 2). High pH exposure resulted in a temporary downward shift in the Cl^- kinetics curve after 10 h, which was characterized by Cl^- uptake rates that were ca. 50%–60% lower than the respective control measurements at pH 8.0 (Fig. 2*A*). Subsequent recovery was evident after 1 d and 3 d of pH 9.5 exposure, when the kinetics curve of J_{in}^{Cl} was not significantly different from control estimates (Fig. 2*A*). Control J_{max}^{Cl} was ca. 360 μmol kg^{-1} h^{-1}, whereas J_{max}^{Cl} was 50% lower in fish held at pH 9.5 for 10 h (Fig. 3*A*). After 1 and 3

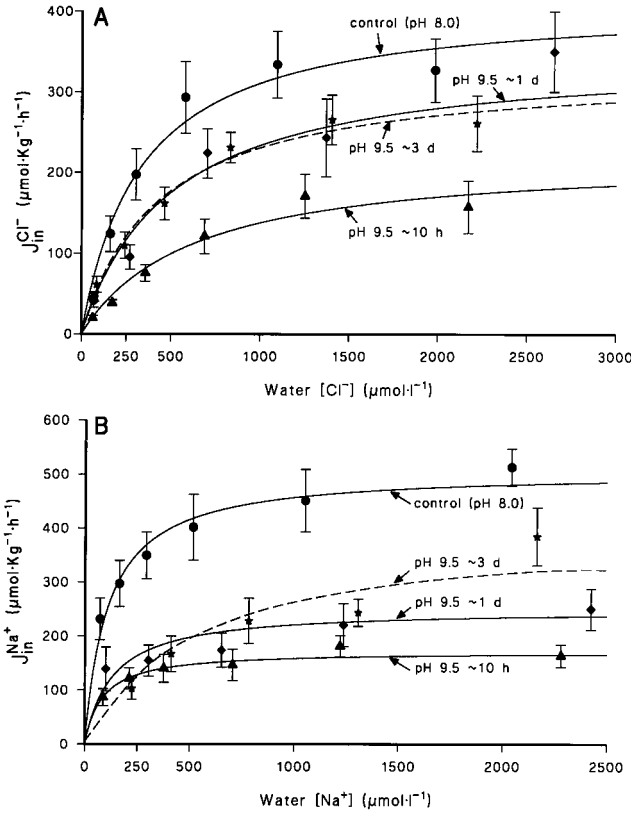

Figure 2. Saturation kinetic curves of (*A*) Cl^- influx (J_{in}^{Cl}) and (*B*) Na^+ influx (J_{in}^{Na}) for rainbow trout at sequentially higher external Cl^- or Na^+ concentrations under control pH conditions (*circles*; pH 8.0; $N = 7$) or after 10 h (*solid triangles*; $N = 7$), 1 d (*diamonds*; $N = 6$), and 3 d (*stars* and *broken line*; $N = 7$) exposure to pH 9.5. Data expressed as means ± 1 SEM.

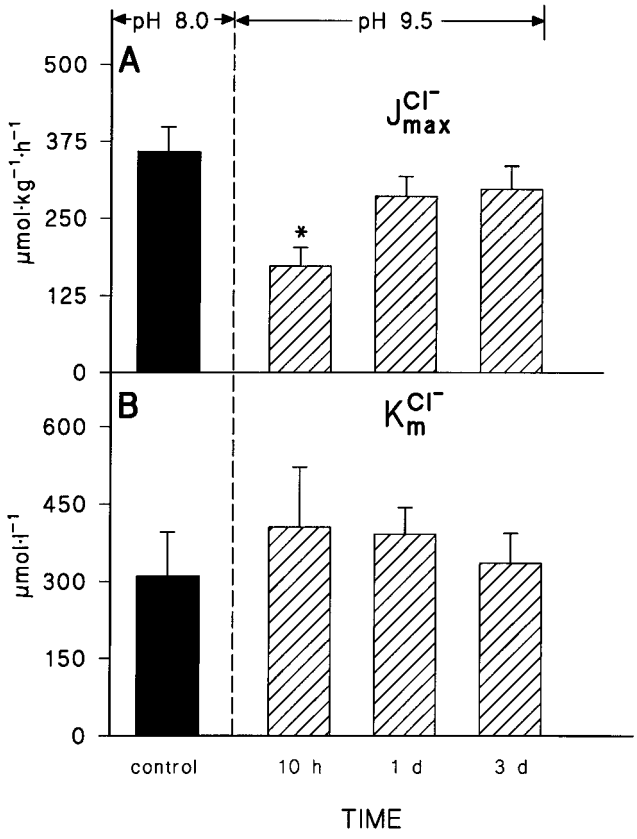

Figure 3. (*A*) The apparent maximal Cl^- transport rate (J^{Cl}_{max}) and (*B*) inverse of Cl^- transporter affinity (K^{Cl}_m) of rainbow trout sampled at control pH ($N = 7$) or after 10 h ($N = 7$), 1 d ($N = 6$), and 3 d ($N = 7$) at pH 9.5. Data are expressed as means ± 1 SEM. Asterisks indicate statistically significant differences from pH 8.0 values ($P < 0.05$).

d at high pH, J^{Cl}_{max} approached control values (Fig. 3*A*), but K^{Cl}_m remained unaltered (Fig. 3*B*).

After 10 h at pH 9.5, the Na^+ kinetics curve shifted downward and was characterized by 60%–70% lower Na^+ influx rates. After 1 and 3 d, the kinetic curves were shifted back toward the control curve, but J^{Na}_{in} was still 50% lower than the control measurements (Fig. 2*B*). At pH 8.0, J^{Na}_{max} was ca. 480 μmol kg^{-1} h^{-1}, but it was 70% lower after 10 h at pH 9.5 and remained 46% and 33% below control levels after 1 and 3 d, respectively (Fig. 4*A*). The K^{Na}_m at pH 8.0 was 88 μmol L^{-1} and was unaltered after 10 h, but by 3 d it was fourfold greater than pH 8.0 values (Fig. 4*B*).

Plasma Cl^- concentrations were significantly reduced in trout subjected to pH 9.5 for 1 d and 3 d, while Na^+ concentrations were not significantly different from measurements made in control trout sampled at pH 8.0 (Table 1).

Discussion

The whole-body unidirectional fluxes measured here largely represent branchial fluxes because the majority of ion uptake and ion loss takes place across the gill epithelium in freshwater-adapted rainbow trout (e.g., McDonald and Wood 1981). Significant (≈10%) ion loss also occurs via renal routes (e.g., McDonald and Wood 1981), however, and these were not separated from branchial routes of diffusive ion efflux in the present study. Accordingly, outward ion movements across the fish's body surface are termed "outflux" rather than "diffusive efflux" throughout this article.

Initial exposure of rainbow trout to pH 9.5 clearly interfered with Na^+ and Cl^- influx but had surprisingly little influence on respective outfluxes. Thus, previously observed high-pH-induced reductions in plasma Cl^- and Na^+ concentrations (cf. Wilkie and Wood 1991) likely resulted from reduced bran-

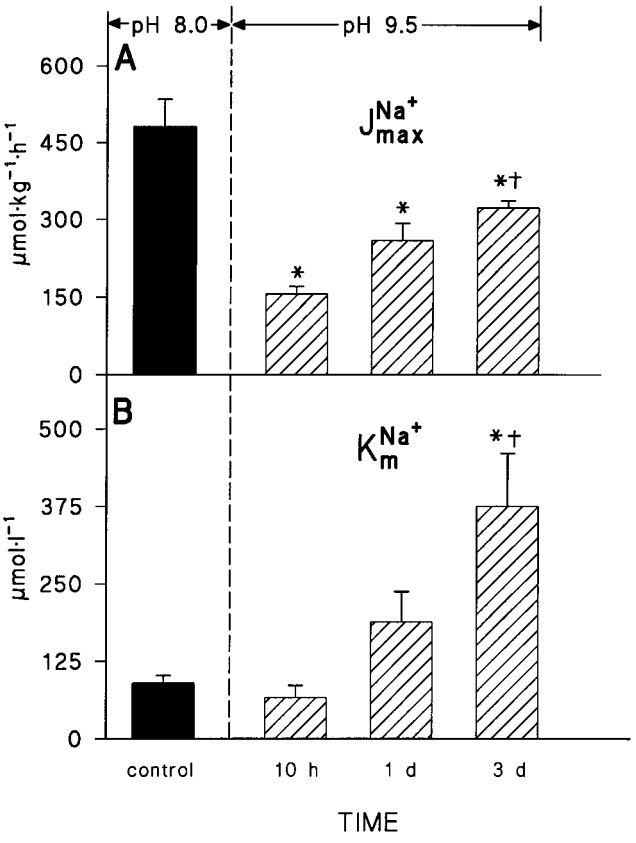

Figure 4. (*A*) The apparent maximal Na^+ transport rate (J^{Na}_{max}) and (*B*) inverse of Na^+ transporter affinity (K^{Na}_m) for rainbow trout sampled at control pH (pH 8.0; $N = 7$) or after 10 h ($N = 7$), 1 d ($N = 6$), and 3 d ($N = 7$) at pH 9.5. Data expressed as means ± 1 SEM. Asterisks indicate statistically significant differences from pH 8.0 values ($P < 0.05$), while daggers represent significant differences from measurements made after 10 h.

Table 1: Plasma ion concentrations following high pH (pH 9.5) exposure in Part 2

		pH 9.5		
	pH 8.0 (Control)	10 h	1 d	3 d
$[Na^+]$ (mmol L^{-1})	150.5 ± .8	144.1 ± 2.3	146.8 ± 3.0	144.1 ± 1.6
$[Cl^-]$ (mmol L^{-1})	136.6 ± 1.2	128.3 ± 1.9	123.1 ± 5.7[a]	122.9 ± 3.3[a]

[a] Significantly lower than corresponding values measured at pH 8.0 ($P < 0.05$).

chial Cl^- and Na^+ influx, not greater diffusive or renal ion losses. Indeed, model calculations, using J_{net}^{Cl} and J_{net}^{Na} measurements over the first 24 h of pH 9.5 exposure, indicate that the observed fluxes were sufficient to explain the plasma ion reductions reported in the present and earlier articles (Wilkie and Wood 1991). For instance, the present fish would have lost ca. 4.8 and 4.4 mmol kg^{-1} body weight of Cl^- and Na^+. Assuming these losses constitute 14.6% and 10.5% decreases in the animals' total exchangeable Cl^- pool (32.9 mmol kg^{-1}) and Na^+ pool (42.05 mmol kg^{-1}), respectively (Wood 1988), plasma Cl^- and Na^+ concentrations would have been ca. 116.7 and 134.6 mmol L^{-1} after 24 h. These predicted concentrations are in the range of previous measurements made in rainbow trout after 24 h at high pH in moderately hard water (e.g., Wilkie and Wood 1991, 1995; Yesaki and Iwama 1992) but are slightly lower than the plasma Cl^- and Na^+ concentrations measured in the present study (Table 1).

Complete restoration of branchial Cl^- influx to control rates, after 48 h at pH 9.5, likely accounted for the reestablishment of Cl^- balance previously reported in rainbow trout at high pH (Wilkie and Wood 1991, 1995; Yesaki and Iwama 1992). However, recovery of Na^+ balance ($J_{net}^{Na} \simeq 0$) was caused by reduced Na^+ outflux, which counterbalanced chronically lowered J_{in}^{Na} by 48–72 h. Similar reductions in Na^+ outflux help to restore Na^+ balance in acid-exposed trout (McDonald et al. 1983; Audet et al. 1988).

Although rainbow trout readily survived in alkaline water, it is noteworthy that water Ca^{++} levels were relatively high (0.4 to 0.6 mmol L^{-1}), and this likely prevented more severe ionic disturbances. As in acidic water (Reid 1995), water Ca^{++} concentration (hardness) is important for maintaining internal electrolyte balance at high pH (Yesaki and Iwama 1992). In soft alkaline water ($[Ca^{++}] = 0.03$ mmol L^{-1}; pH = 10.1), chronic declines in plasma Na^+ and Cl^- are associated with low rainbow trout survival, but at higher Ca^{++} concentrations ion losses are less and survival enhanced (Yesaki and Iwama 1992).

The significant decrease in J_{max}^{Cl} after 10 h at pH 9.5 suggests initial reductions in J_{in}^{Cl} were caused by decreases in Cl^- transport site number. The return of J_{max}^{Cl} to control values by 3 d demonstrated that reduced Cl^- transport capacity was transient, however, and likely accounted for the complete recovery of J_{in}^{Cl} observed at 72 h (Fig. 1*A*). The stable K_m^{Cl} indicates there was no change in transporter affinity for Cl^-, precluding competitive inhibition of Cl^- transport by HCO_3^- or the inherently higher OH^- ion concentrations at pH 9.5.

The greatest reduction in J_{max}^{Na} occurred at 10 h of pH 9.5 exposure, which corresponds with the time of lowest Na^+ influx in Part 1 (Fig. 1*B*). Although J_{max}^{Na} gradually recovered, a marked fourfold increase in K_m^{Na} was observed after 3 d at pH 9.5. Thus, reduced Na^+ influx at high pH initially resulted from a rapid decrease in Na^+ transport capacity, but reduced transporter affinity for Na^+ after 3 d prevented complete recovery of Na^+ influx, despite the partial recovery of maximal Na^+ transport capacity. Overall, these observations support the hypothesis that alkaline water causes transient decreases in ion transport system capacity by directly acting on the gill's respective Cl^- and Na^+ transport sites.

Internal acid-base disturbances at high pH could also influence Na^+ and Cl^- uptake rates. Specifically, a lack of internal counterions for the respective Cl^-/base (Cl^-/HCO_3^- exchange) and the Na^+/acid (Na^+ influx coupled to H^+-ATPase H^+ excretion) transport systems could directly reduce Cl^- and Na^+ influx rates (Goss and Wood 1991). Indeed, using a very similar experimental protocol, we have demonstrated that a respiratory alkalosis, characterized by 70% decreases in arterial blood P_{CO_2}, takes place in rainbow trout (Table 2; Wilkie and Wood 1991). Further, a simultaneous metabolic acidosis (production of metabolic protons) exacerbates the decline in plasma $[HCO_3^-]$ but helps stabilize blood pH as P_{CO_2} decreases (Table 2; Wilkie and Wood 1991). Since these observed changes in plasma $[H^+]$ and $[HCO_3^-]$ (Table 2) likely extend to the intracellular space of the gills (Goss and Wood 1991), inward Na^+ and Cl^- movement would be curtailed under these conditions.

The two-substrate model developed in our lab by Goss and Wood (1991), using rainbow trout of the same genetic stock under similar control conditions, can be used to illustrate how changes in internal substrate influence Cl^- and Na^+ influx. Goss and Wood (1991) plotted changes in $1/J_{max}^{Cl}$ against 1/plasma $[HCO_3^-]$, and $1/J_{max}^{Na}$ against 1/plasma $[H^+]$ when trout were subjected to a variety of acid-base disturbances in order to determine the true J_{max}^{ION} values, or transporting capacities, for the Na^+ or Cl^- uptake systems (see Goss and Wood 1991 for detailed analysis and calculations). These "true" J_{max}^{ION} estimates are the *y*-intercepts of the corresponding regression lines and incorporate both the internal (HCO_3^-, H^+) and external substrate concentrations (Na^+, Cl^-) that influence ion transport

Table 2: Arterial blood acid-base status of rainbow trout at high pH (pH 9.5)

	Blood Acid-Base Status[a]			
Water pH	Blood pH	$[H^+]$ (nmol L^{-1})	PCO_2 (Torr)	$[HCO_3^-]$ (mmol L^{-1})
pH 8.0	7.83 ± .01	14.8 ± .3	3.07 ± .09	7.50 ± .30
pH 9.5:				
8 h	7.97 ± .01[b]	10.7 ± .3[b]	1.43 ± .08[b]	5.04 ± .34[b]
24 h	7.99 ± .01[b]	10.2 ± .2[b]	1.12 ± .14[b]	4.08 ± .40[b]
72 h	7.98 ± .03[b]	10.5 ± .7[b]	.68 ± .07[b]	2.58 ± .37[b]

Note. $N = 42$ at pH 8.0 and after 8 h at pH 9.5; $N = 28$ and $N = 9$ after 24 h and 72 h at pH 9.5, respectively.

[a] Data taken from Wilkie and Wood (1991).

[b] Significantly lower than corresponding values measured at pH 8.0 ($P < 0.05$).

rates. The present graphical analyses use the regression lines constructed from the earlier work of Goss and Wood (Fig. 5), making it possible to qualitatively separate altered internal counterion availability from changes in transport site density on the gills of trout at high pH. Note that external substrate (Na^+, Cl^-) availability is a constant in these experiments. Altered internal counterion availability only, without changes in transport site numbers, lead to shifts along the regression lines. Decreases in transport site number alone lead to upward vertical deviations away from the regression line and vice versa (i.e., altered *y*-intercepts).

In the present two-substrate kinetic analysis, we used the arterial plasma acid-base data from the earlier, virtually identical high pH exposure experiments by Wilkie and Wood (1991; Table 2) and the present kinetic data (Figs. 3, 4) to plot $1/J_{max}^{Cl}$ against 1/plasma $[HCO_3^-]$, and $1/J_{max}^{Na}$ against 1/plasma $[H^+]$. These data points were then compared with the regression lines originally developed and calibrated by Goss and Wood (1991) in Figure 5. The present control points agreed closely with the control data of Goss and Wood (1991). The upward shift of the 10 h data point away form the regression lines for both the Cl^- and Na^+ transport systems indicates alkaline water lead to decreased ion transport capacity. The pronounced rightward shift of the Cl^- uptake point in parallel to the regression line (Fig. 5*A*) also illustrates that internal HCO_3^- supply to the Cl^-/HCO_3^- transporter was limiting after 10 h. The further shift to the right suggests HCO_3^- supply became increasingly limiting after 1 d. Thus, the continual drops in plasma P_{CO_2} and the simultaneous metabolic acidosis at high pH (Table 2) likely led to steady decreases in intracellular HCO_3^- in the gill and thereby limited Cl^-/HCO_3^- exchange. The marked vertical displacement of the points down below the regression line by 3 d, however, suggests complete recovery of Cl^- influx after 72 h was caused by greater transporter number.

Greater branchial chloride cell surface area at pH 9.5 would provide an increased abundance of Cl^-/HCO_3^- exchangers, thereby countering decreased internal HCO_3^- supply. Indeed, chloride cell fractional surface area increased fourfold in rainbow trout held under identical pH 9.5 conditions for 72 h (Wilkie and Wood 1994). The Lahontan cutthroat trout also responds to the alkaline waters (pH 9.4) of Pyramid Lake, Nevada, by greatly elevating chloride cell surface area (Wilkie et al. 1994). The convincing evidence that chloride cells are the site of Cl^- uptake in freshwater teleosts (Sullivan et al. 1996) further supports this hypothesis.

The transport capacity of the Na^+/acid system was markedly reduced in the initial stages (10 h) of high pH exposure, as illustrated using the two-substrate analysis where the data point representing Na^+ influx shifted up and away from the regression line (Fig. 5*B*). The influence of limited internal H^+ supply was slight, however (Fig. 5*B*), as initial H^+ reductions were relatively small and then stabilized (Table 2). The slight rightward shift along the regression line illustrates this point (Fig. 5*B*). Thus, decreased H^+-ATPase and/or Na^+ channel populations likely accounted for most of the initial reductions in Na^+ influx at high pH.

In agreement with the one-substrate analysis, two-substrate analysis also indicated a substantial recovery of Na^+ transport capacity by 3 d at pH 9.5 (Fig. 5*B*). This recovery may have been related to changes in H^+-ATPase activity, Na^+ channel numbers, or both. The partial recovery of Na^+ transport capacity was counteracted by the fourfold higher K_m^{Na} (Fig. 4*B*), however, which explains the chronic reduction of J_{in}^{Na} seen in Part 1. Such chronically lowered Na^+ influx at high pH might also benefit the trout, however, by permitting it to retain more H^+, which would help offset the chronic respiratory alkalosis.

In conclusion, disturbances to internal electrolyte balance in rainbow trout at high pH primarily resulted from reduced Na^+ and Cl^- influx caused by initial reductions in gill ion-transporting capacity. In addition to direct effects on transport sites, high-pH-induced reductions in ion influx were probably caused by lowered P_{CO_2} in the blood and gills, which limited HCO_3^- supply to apical Cl^-/HCO_3^- exchangers. Similarly, H^+

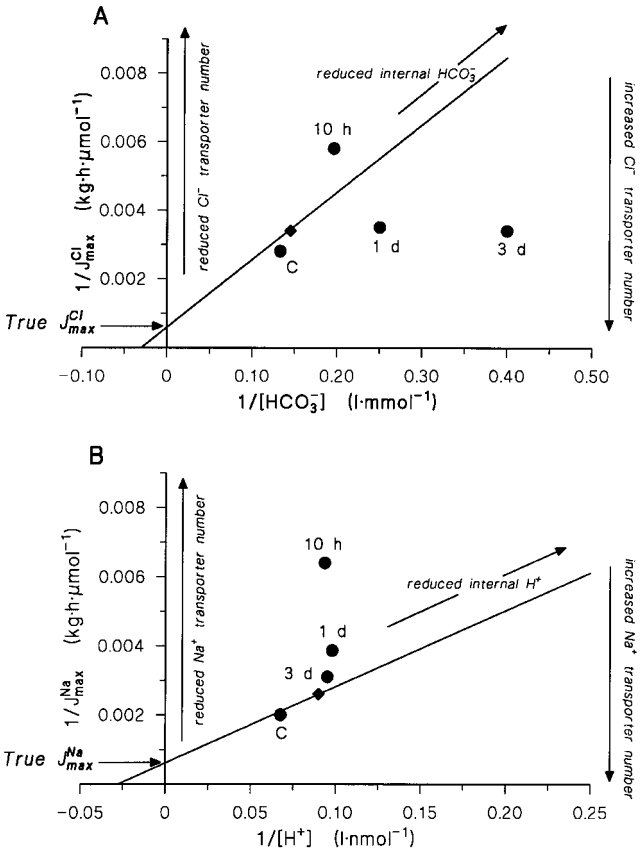

Figure 5. Two-substrate kinetic analysis of ion uptake across rainbow trout gills at pH 8.0 or pH 9.5, employing the framework of Goss and Wood (1991). The analyses demonstrate the relative roles that internal substrate (counterion) availability and transporter number play in altering the respective apparent J_{max}^{Cl} and apparent J_{max}^{Na} for (*A*) Cl^- and (*B*) Na^+ uptake by rainbow trout. Respective arterial plasma HCO_3^- ($[HCO_3^-]_a$) and H^+ ($[H^+]_a$) concentrations were taken from trout exposed to identical conditions in a previous study (Wilkie and Wood 1991). The regression lines represent data collected by Goss and Wood (1991) following imposition of various internal acid-base disturbances to rainbow trout in Hamilton tap water. The control data points of Goss and Wood (1991; *diamonds*) are indicated. Circles are the inverse of the apparent J_{max}^{Cl} and J_{max}^{Na} estimates presented in Figures 3 and 4, plotted against corresponding inverse measurements of $[HCO_3^-]_a$ and $[H^+]_a$. Upward or downward deviations (*vertical arrows*) away from the regression line represent true changes in transporter number, while changes in internal substrate availability are reflected by movements along or in parallel to the regression line (*diagonal arrows*).

supply to H^+-ATPases was also likely lower and, therefore, limited Na^+ influx. Greater apical exposure of branchial chloride cells (cf. Wilkie and Wood 1994) likely led to restoration of Cl^- influx at high pH, while net Na^+ losses were mitigated by reduced Na^+ outflux.

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