

# Zinc Influx Across the Isolated, Perfused Head Preparation of the Rainbow Trout (*Salmo gairdneri*) in Hard and Soft Water

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At a waterborne [Zn] of 1.9 mg·L<sup>-1</sup> in hard water (~1 mmol Ca·L<sup>-1</sup>), Zn influx across an isolated, saline-perfused head preparation of rainbow trout (*Salmo gairdneri*) was about 1.5 nmol·kg<sup>-1</sup>·h<sup>-1</sup> through the lamellar pathway and about 1 nmol·kg<sup>-1</sup>·h<sup>-1</sup> through the filamental route. Flux rates came rapidly to steady state in both pathways. Trout preexposed to artificial soft water (~0.05 mmol Ca·L<sup>-1</sup>) for 5 d showed differential stimulation of flux rates to about 42 and 5 nmol Zn·kg<sup>-1</sup>·h<sup>-1</sup> through the lamellar and filamental pathways, respectively. Under these conditions, steady-state fluxes across the lamellae did not occur until 15–20 min after the start of perfusion. Preparations from hardwater-acclimated trout tested in soft water gave typical hardwater fluxes showing that these changes in influx were not simply due to acute exposure of the gill surface to low waterborne [Ca]. Influxes in softwater trout, studied over [Zn] from 0.4 to 7.5 mg Zn·L<sup>-1</sup>, revealed a saturable, first-order uptake with apparent  $J_{\max}$  and  $K_m$  of 150 nequiv·kg<sup>-1</sup>·h<sup>-1</sup> and 1.5 mg Zn·L<sup>-1</sup> (23 μmol·L<sup>-1</sup>), respectively. Because the apparent  $K_m$  is in the toxic range, Zn is clearly not the primary substrate. Scanning electron micrography revealed hypertrophy and increased apical exposure of chloride cells; this stimulation, coupled with the increase in Zn influx, suggests that chloride cells may be the site of entry of Zn across the gill.

À une concentration de zinc dans l'eau de 1,9 mg·L<sup>-1</sup> dans l'eau dure (environ 1 mmol Ca·L<sup>-1</sup>), l'influx du Zn à travers une préparation de têtes de truites arc-en-ciel (*Salmo gairdneri*) isolées, après perfusion en solution saline, était d'environ 1,5 nmol·kg<sup>-1</sup>·h<sup>-1</sup> dans la voie lamellaire, et d'environ 1 nmol·kg<sup>-1</sup>·h<sup>-1</sup> à travers la voie filamentale. Les taux d'influx des deux voies sont rapidement arrivés à l'état d'équilibre. Les truites pré-exposées à de l'eau douce artificielle (environ 0,05 mmol Ca·L<sup>-1</sup>) pendant 5 d présentaient une stimulation différentielle des valeurs d'influx d'environ 42 et 5 nmol Zn·kg<sup>-1</sup>·h<sup>-1</sup> par les voies lamellaire et filamentale, respectivement. Dans ces conditions, des influx à l'équilibre à travers les lamelles n'ont pas été observés avant 15–20 min après le début de la perfusion. Des préparations provenant de truites acclimatées à l'eau calcaire essayées dans de l'eau douce ont donné des influx typiques des eaux calcaires, ce qui montre que ces changements d'influx n'étaient pas simplement dus à une réponse aiguë de la surface des branchies à de faibles concentrations de Ca dans l'eau. Les influx dans la truite d'eau douce, observés à des concentrations de 0,4 à 7,5 mg Zn·L<sup>-1</sup>, ont mis en évidence une réaction d'absorption saturable du premier ordre avec des valeurs  $J_{\max}$  et  $K_m$  apparentes de 150 nequiv·kg<sup>-1</sup>·h<sup>-1</sup> et de 1,5 mg Zn·L<sup>-1</sup> (23 μmol·L<sup>-1</sup>), respectivement. Parce que la valeur du  $K_m$  apparent est dans la plage toxique, le Zn ne semble pas être le substrat primaire. Par micrographie à balayage électronique, on a mis en évidence l'hypertrophie et une exposition apicale accrue des cellules à chlorure; cette stimulation, combinée à l'augmentation de l'influx du Zn, suggère que les cellules à chlorure pourraient être le point d'entrée du Zn à travers les branchies.

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**Z**inc (Zn) can be taken up from the water by fish. Although dietary sources may be more important when [Zn] is low in both seawater (Pentreath 1973, 1976; Willis and Sunda 1984) and freshwater (Spry et al. 1988), as waterborne [Zn] increases, so does the importance of this source (Milner 1982; Spry et al. 1988). Despite this, little is understood of the mechanism by which divalent metals traverse the gill. In contrast with monovalent ions, even flux measurements are rare. Branchial calcium (Ca) transport is a recent exception. There are several estimates for unidirectional fluxes, and an understanding of transport mechanisms is beginning to emerge (Höbe et al. 1984; Flik et al. 1985; Perry and Wood 1985).

Flux rates for trace metals, which are important both nutritionally and as toxic contaminants, have scarcely been examined. Cadmium (Cd) influx rates, studied using a perfused head technique, were extremely low compared with those of monovalent ions and Ca (Pärt and Svanberg 1981). Chelation of the free Cd either decreased or increased influx, depending on the polarity of the resulting chelate (Pärt and Svanberg 1981; Block and Pärt 1986). Copper (Cu) uptake rates in intact rainbow trout (*Salmo gairdneri*) also appeared to be very low, although absolute rates were confounded by adsorption (Laurén and McDonald 1986). Few other metals of environmental significance have been similarly studied.

The mechanism of entry of metals across the gill has generally been assumed to be passive diffusion driven by gradient from water to blood (Pentreath 1973; Rankin et al. 1982). While

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this may be true for metals other than Ca, there are at present few data. Ca, in contrast, appears to be taken up by active mechanisms. Flik et al. (1985) suggested that Ca is actively pumped against a concentration gradient via high-affinity Ca-ATPase in the branchial chloride cell. In accord with enzymatic mediation, this transport was inducible both by exposure to low environmental Ca and cortisol injection and showed saturable kinetics (Perry and Wood 1985). Thus, at least theoretically, two distinctly different mechanisms exist by which metals may traverse the gill.

Our aims were to see if Zn influx across the gill could be detected, and if so, to measure the rate of influx and compare it with those rates which exist for other divalent metals. Second, we wished to gain some insight into the mechanism by which Zn might traverse the gill and to see whether water hardness (i.e. Ca) had any influence on the flux rates. We suspected that there might be some interaction between Zn and Ca, first, because of the well-known action of Ca on permeability, and second, because we have shown that waterborne Zn blocks net Ca uptake and interferes with the plasma Ca regulation in rainbow trout (Spry and Wood 1984, 1985). Third, increased waterborne Ca or Mg decreased Cd transfer across the perfused trout head (Pärt et al. 1985).

Traditional measurements of unidirectional flux rates using radioactive tracer in the environmental water are very difficult to use with divalent cations due to nonspecific adsorption to both the apparatus and the organism. This is true for Ca (Höbe et al. 1984; Perry and Wood 1985), Cu (Laurén and McDonald 1986), Zn (D. J. Spry and C. M. Wood, unpubl. obs.), and probably other trace metals as well. To circumvent this difficulty, an isolated, perfused rainbow trout head preparation was used. In addition to measuring only that Zn which traverses the gill, use of this preparation allows estimates of lamellar versus filamental contributions to the influx (see Girard and Payan 1980; Payan et al. 1984). Initially, influx measurements were made in hard water, but trout were also preexposed to soft water, since this treatment is reported to induce branchial chloride cell proliferation on the lamellae (Laurent et al. 1985) which appears to be correlated with increased Ca influx (Perry and Wood 1985). The ability to separate lamellar versus filamental routes of uptake allowed us to assess the importance of this proliferation in altering Zn influx.

In the present report, we employ the isolated, perfused head preparation to characterize the uptake of Zn under hard- and softwater conditions and make some assessment of the possible mechanism by which Zn crosses the gill. In the future, this preparation has the potential to elucidate further the mechanism(s) by which Zn traverses the branchial membrane and to determine which waterborne Zn species are available for uptake across the gill.

## Materials and Methods

### Experimental Animals

Underyearling rainbow trout ( $297 \pm 14$  (24) g) were obtained from Spring Valley Trout Farm, Petersburg, Ontario. They were held at McMaster University in flowing, charcoal-dechlorinated, Hamilton city tap water (hardwater (HW): [Ca] 0.9, [Na] 0.6, [Cl] 0.8 mmol·L<sup>-1</sup>, pH 8.1) at ambient temperature for several months before use and fed a pelleted commercial diet (Martin Feed Mills, Elmira, Ontario). Artificial soft water (ASW) used in some of the experiments was produced

by a 15-fold dilution of tap water with either distilled water or water from a reverse osmosis unit. In some experiments, trout were exposed to this medium for 5–7 d prior to experimentation. All trout, regardless of the medium, were temperature acclimated to 15°C at an overall rate not exceeding 1°C·d<sup>-1</sup>. The trout were acclimated in batches of 10–20 in 500-L polyethylene tanks and not fed under these conditions; water was changed on alternate days.

The morphological response of the gill appears to vary with the type of soft water used (Laurent et al. 1985; Perry and Wood 1985). To assess the changes in the surficial tissues of the gill during exposure to the particular soft water used in our experiments, tissue samples were taken from control trout, and trout exposed to ASW for either 1 or 7 d. The second right gill arch was excised and fixed in 2% glutaraldehyde in Cortland saline (Wolf 1963) for 2 h at 4°C, followed by postfixation for 1 h in 1% OsO<sub>4</sub> also in saline. Tissues were dehydrated through an ethanol series, critical point dried out of CO<sub>2</sub>, mounted on aluminum stubs, and sputter-coated with gold. Samples were then examined in a Philips 501 scanning electron microscope at 15 keV.

### Perfused Head Preparation

Zn influx was measured using the isolated, perfused trout head preparation of Payan and Matty (1975) with various modifications (Perry et al. 1984a; Perry et al. 1985a; Perry and Wood 1985). Briefly, trout were heparinized (2500 USP units i.v.) prior to decapitation. The head was irrigated on an operating table while the gills were cleared of blood with perfusion saline (composition as in Perry and Wood 1985, with 10<sup>-6</sup> M l-epinephrine bitartrate, 40 g 40 000 molecular weight polyvinylpyrrolidone·L<sup>-1</sup>, 2 g bovine serum albumin·L<sup>-1</sup>, fraction V, all from Sigma). A latex diaphragm (condom) was fitted and the head transferred to the flux chamber. The gills were irrigated with 200 mL of either HW or ASW at 200 mL·min<sup>-1</sup>. Water was aerated and temperature ranged from 14 to 18°C (change <1° during any one experiment). In most experiments, perfusion saline was used. This was gassed with 0.35% CO<sub>2</sub>, 4% O<sub>2</sub>, balance N<sub>2</sub> to closely approximate in vivo venous gas tensions. Where either whole blood or plasma was required for perfusion, blood was collected from large rainbow trout fitted with indwelling dorsal aortic catheters (Soivio et al. 1972), plugged with stainless steel pins. Blood was drawn as required, pooled, heparinized at a ratio of 200 USP units·mL<sup>-1</sup>, and gassed as above. Exploratory experiments indicated that dilution to 7% hematocrit with perfusion saline was required to maintain realistic perfusion pressures. Where required, plasma was recovered by centrifugation.

Gills were perfused at constant flow (3.5 mL·min<sup>-1</sup>, 10 cm H<sub>2</sub>O pulse pressure) against a 10 cm H<sub>2</sub>O dorsal aortic back-pressure, using a Harvard 1405 cardiac pump fitted with a small volume remote head (Davie and Daxboeck 1983). Weight ranges were very similar in all treatment groups, so differences in weight-specific flow were not a complicating factor. Input pressure was monitored continuously with a Hewlett-Packard 267BC transducer, and baseline pressure due to the resistance of tubing and ligatures was subtracted to give the true input perfusion pressure ( $P_{in}$ ). Data from preparations whose true input pressure did not quickly stabilize at <50 cm H<sub>2</sub>O, or which were less than 90% cleared of blood after the experiment, were discarded.

TABLE 1. Types of experiments performed and values for water variables under test conditions; means  $\pm$  SE.

Series	Perfusate	Exposure	Test	<i>n</i>	Temp. (°C)	pH <sub>w</sub>	Ca (mmol·L <sup>-1</sup> )	Na (mmol·L <sup>-1</sup> )	Zn (mg·L <sup>-1</sup> )
1	Saline	HW	HW	7	15.8 $\pm 0.3$	7.72 $\pm 0.09$	0.98 $\pm 0.02$	0.88 $\pm 0.06$	1.89 $\pm 0.12$
2	Saline	ASW	ASW	20	15.8 $\pm 0.6$	7.26 $\pm 0.02$	0.05 $\pm 0.02$	0.21 $\pm 0.04$	Various
3	Saline	ASW	HW	7	14.2 $\pm 0.1$	7.78 $\pm 0.50$	0.07 $\pm 0.01$	0.79 $\pm 0.04$	1.99 $\pm 0.05$
4	Blood	ASW	ASW	4	14.5 $\pm 0.1$	7.33 $\pm 0.02$	0.04 $\pm 0.02$	0.23 $\pm 0.04$	0.61 $\pm 0.14$
5	Plasma	ASW	ASW	4	17.0 $\pm 0.6$	7.16 $\pm 0.04$	0.05 $\pm 0.02$	0.23 $\pm 0.04$	1.25 $\pm 0.20$

TABLE 2. Characteristics of perfusion medium and hemodynamic characteristics of perfused trout heads.

Series	Perfusate	hct (%)	Differential pressure $P_{in} - 10$ (cm H <sub>2</sub> O)	Perfusate flow $Q_{tot}$ (mL·min <sup>-1</sup> )	Branchial resistance $R_g$ (cm H <sub>2</sub> O·min·kg·mL <sup>-1</sup> )
1	Saline	0	33.4 $\pm 2.8$ (6)	3.21 $\pm 0.05$ (6)	2.46 $\pm 0.25$ (6)
2	Saline	0	40.4 $\pm 3.5$ (9)	3.24 $\pm 0.09$ (9)	2.88 $\pm 0.46$ (9)
3	Saline	0	29.0 $\pm 1.9$ (8)	3.11 $\pm 0.19$ (7)	1.68 $\pm 0.22$ (7)
4	Blood	7.1 $\pm 1.5$ (4)	70.9 $\pm 8.8$ (4)	3.21 $\pm 0.17$ (4)	4.95 $\pm 0.82$ (4)
5	Plasma	0	66.2 $\pm 10.2$ (4)	2.76 $\pm 0.16$ (4)	4.74 $\pm 1.22$ (4)

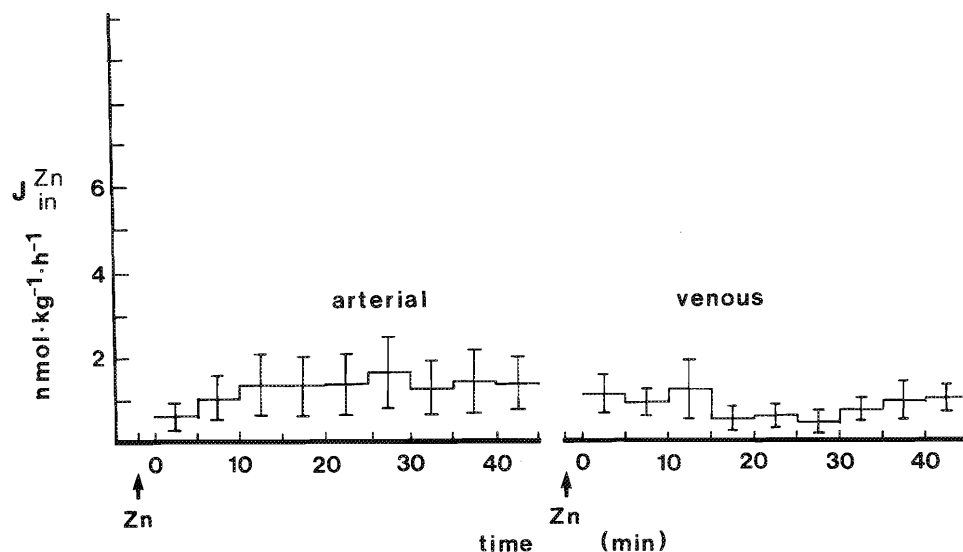


FIG. 1. Zinc influx across the perfused trout head over time. Fish were acclimated to hard water and tested in hard water. Arrows indicate the time of Zn addition. Values are means  $\pm$  SE,  $n = 7$ . Mean external [Zn] was  $1.89 \pm 0.12$  mg·L<sup>-1</sup>.

When pressure had stabilized (5–10 min), various amounts of Zn (as sulphate) and  $^{65}\text{Zn}$  tracer (100  $\mu\text{Ci}$  per experiment) were added to the irrigation water and allowed to mix for 2 min before collection of the perfusate began. Perfusate flows from the head preparation were collected in two different streams, the rationale for which was outlined in the introduction. The arterio-arterial flow, passing only through the lamellae, was collected as it dripped from the dorsal aortic cannula whereas the arterio-venous flow, passing through the filaments after the lamellae, was collected from the cut surface of the preparation. Since even small leaks of the irrigation water would contaminate the arterio-venous samples with radioactivity, the refractive index of the arterio-arterial and arterio-venous effluents was carefully monitored with a refractometer (American Optical TS meter). This method was sufficient to detect a leakage constituting only 1% of the arterio-venous flow (Perry and Wood 1985).

Experiments lasted 45–60 min, since this is the optimal life of the preparation. All of the perfusate was collected, in 5-min aliquots, and weighed for flow rate calculations. Water samples (3 mL) were taken concurrently, and replaced with 3 mL of fresh water, to maintain the level in the head chamber. Samples were immediately acidified to 0.2% (water) or 0.4% (perfusate) with high-purity  $\text{HNO}_3$  (BDH Aristar). Total [Zn] by atomic absorption spectrophotometry (Varian AA 1275) and  $^{65}\text{Zn}$  gamma activity (Chicago-Nuclear well-type counter model 1084) were measured on these water samples whereas perfusate was counted only for gamma activity. For  $^{65}\text{Zn}$  activity, samples were adjusted for sample geometry, and since samples were counted within days of the experiments, no decay correction was required.

### Experimental Protocol

Five series of experiments were performed (Table 1), all of which examined the time course and rate of influx of Zn into the perfusate. The first series, which utilized saline as the per-

fusate, assessed influx in trout which were acclimated to the moderately hard Hamilton city tap water (HW). A second series used trout which were preexposed to ASW, with flux rates measured in ASW. Data from this series were used to assess both the time course of uptake, and the effect of varying waterborne [Zn] on influx. Transepithelial potential (TEP) was also measured in these fish using Narco Ag–AgCl electrodes and a Radiometer PHM82 high-impedance voltmeter, as described by Perry et al. (1985b) and Perry and Wood (1985). TEP was used both as a general index of stability of the preparation and to determine whether waterborne Zn affected the electrical gradient for Zn movement across the gills. In the third series, flux measurements were made in ASW, but using HW-acclimated trout. Two other series measured the effect of perfusion with either blood or plasma to see if either of these media would increase Zn influx. In these latter procedures, perfusion commenced with saline and when pressures had stabilized, the perfusate was switched either to plasma or blood. These were performed with fish preexposed and tested in ASW.

### Calculations and Statistics

Zn influx rates (nanomoles per kilogram per hour) were calculated using the following equations:

$$(1) \quad J_{\text{in}a} = \frac{\text{cpm} \cdot \text{mL}^{-1} \text{ DA} \times Q_{\text{tot}} \times 60}{\text{water specific activity} \times \text{fish weight}}$$

$$(2) \quad J_{\text{in}v} = \frac{(\text{cpm} \cdot \text{mL}^{-1} \text{ AV} - \text{cpm} \cdot \text{mL}^{-1} \text{ DA}) \times Q_{\text{AV}} \times 60}{\text{water specific activity} \times \text{fish weight}}$$

where DA and AV refer to the dorsal aortic (arterio-arterial) and arterio-venous effluents, respectively.  $Q_{\text{tot}}$  is the total perfusion flow (millilitres per minute),  $Q_{\text{AV}}$  is the arterio-venous perfusion flow (millilitres per minute), water specific activity is in counts per minute per nanomole Zn, and fish weight is in kilograms.

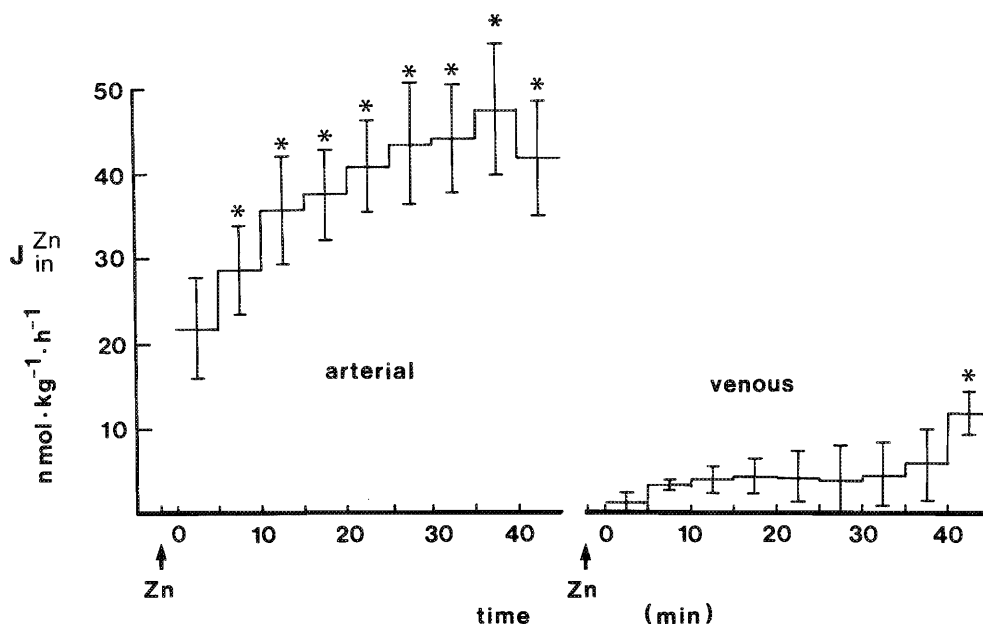


FIG. 2. Zinc influx across the perfused trout head over time. Fish were preexposed and tested in artificial soft water. Values are means  $\pm$  SE,  $n = 6$ . Mean external [Zn] was  $1.61 \pm 0.10 \text{ mg} \cdot \text{L}^{-1}$ . Asterisks denote means which were significantly different from the first flux period by paired  $t$ -test. ( $P < 0.05$ ).

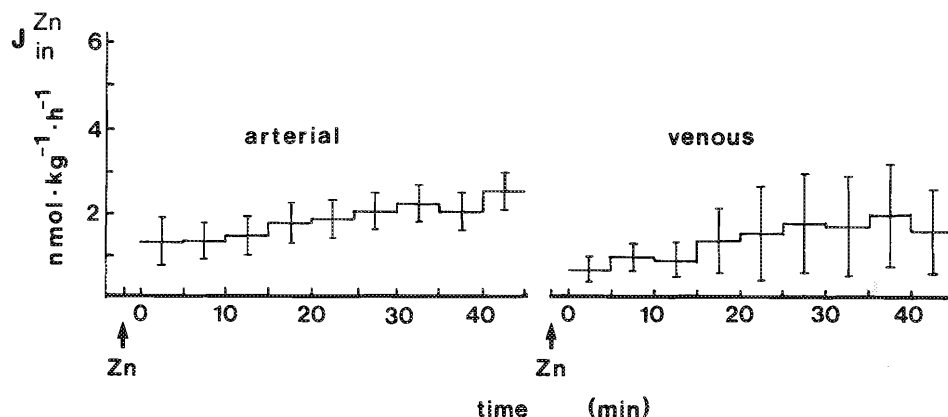


FIG. 3. Zinc influx across the perfused trout head over time. Fish were acclimated to hard water and tested in artificial soft water. Values are means  $\pm$  SE,  $n = 7$ . Mean external  $[Zn]$  was  $1.99 \pm 0.05$   $mg \cdot L^{-1}$ . There were no significant differences.

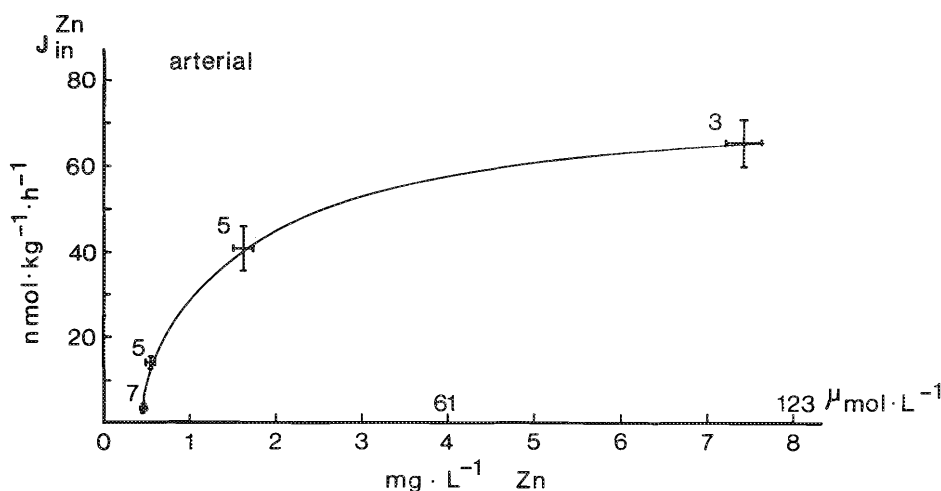


FIG. 4. Zinc influx across the arterio-arterial pathway of the perfused trout head as a function of waterborne  $[Zn]$ . Numbers beside each mean denote  $n$ .

Mean input perfusion pressure ( $P_{in}$ , centimetres  $H_2O$ ) was calculated as the diastolic pressure plus one-third the pulse pressure. Then:

$$(3) R_g = (P_{in} - 10)/Q_{tot}$$

where  $R_g$  is the branchial resistance to flow (centimetres  $H_2O$  per minute per kilogram per millilitre), and the dorsal aortic pressure (10 cm  $H_2O$ ) is subtracted.  $Q_{tot}$  here is in millilitres per minute per kilogram.

Mean values  $\pm$  SE ( $n$ ) were calculated for each interval for any particular  $Zn$  concentration. Statistical significance was calculated by comparing the first interval with subsequent intervals using a paired  $t$ -test at the 95% level.

## Results

Table 1 summarizes the types of experiments performed and gives the water variables measured. The hemodynamic characteristics for the perfusions are given in Table 2. Ratios of arterial to venous flows (mean  $2.8 \pm 0.2$ ,  $n = 21$ ) were within the published range for the preparation (Perry and Wood 1985; Perry et al. 1985a). Perfusate recovery was always  $>90\%$ . The preparation showed good hemodynamic stability and did not show the rapid deterioration seen in some of the preparations

used by earlier investigators. In general, with saline perfusion,  $P_{in}$  started high, but stabilized quickly at values comparable with those in vivo. Although there were no differences in flow rate among the series, a slightly higher branchial resistance in series 2 resulted in increased input pressure. Use of plasma or diluted blood resulted in a doubling of the branchial resistance and hence  $P_{in}$ .

$Zn$  adsorbed readily to the experimental apparatus, such that the external waterborne  $[Zn]$  decreased about 25% during the course of a single experiment. The median  $[Zn]$  was used as the average value for the experiment, since it gave the same value as the area under the time-concentration curve. Despite this decrease, the specific activity remained constant, so that no correction was required.

In the first series of experiments (HW acclimation, tested in HW, with an external  $[Zn]$  of  $1.89$   $mg \cdot L^{-1}$ ), significant influx occurred during the first 5 min of exposure to  $Zn$ , in both the arterio-arterial (arterial) and arterio-venous (venous) compartments (Fig. 1). This indicated rapid entry of  $Zn$  across the gill. Influx appeared to rise slightly, but not significantly, on the arterial side during the next 5-min interval. Thereafter, influx remained constant. A slight drop in venous influx halfway through the experiment was likewise insignificant. Mean values were about  $1.5$   $nmol \cdot kg^{-1} \cdot h^{-1}$  on the arterial side and about 1

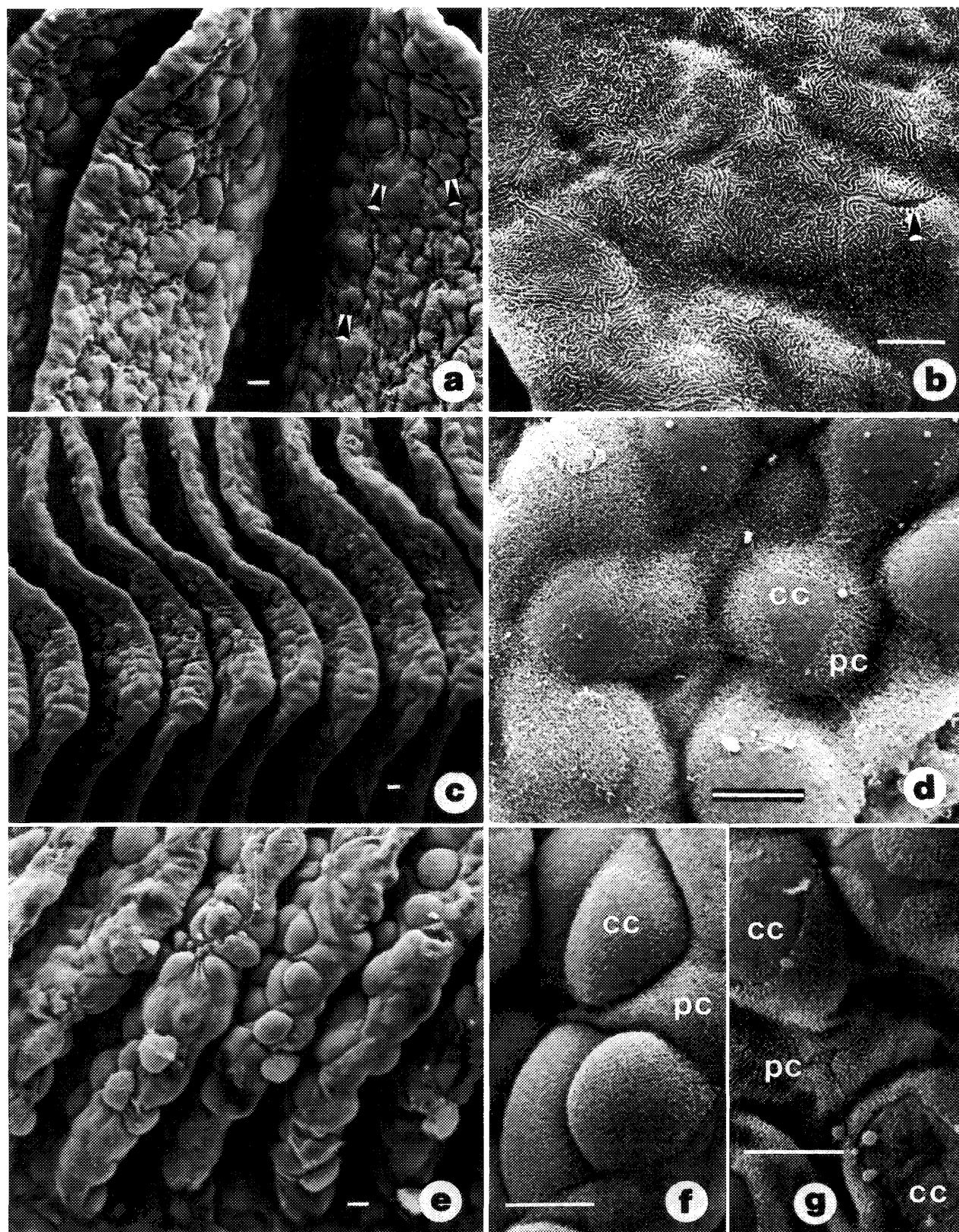


FIG. 5. Scanning electron micrographs of the lamellar surfaces of the gills of rainbow trout. (a) Hardwater control; chloride cells are evident as large bumps. Most are completely overlain by pavement cells; others (arrows) show lenticular-shaped apical exposure. (b) Hardwater control; arrow denotes chloride cell exposure. (c) 1 d of exposure to artificial soft water (ASW). (d) 1 d of exposure to ASW showing pavement cells (pc) and triangular apical exposure of chloride cells (cc) showing some developing microvilli. (e) 7 d of exposure to ASW. (f) 7 d of exposure to ASW showing completely erupted chloride cells. (g) 7 d of exposure to ASW showing overgrowth of chloride cells by pavement cells (upper) and a crenated, degenerating chloride cell (lower right). Scale bars are 10  $\mu$ m.



$\text{nmol}\cdot\text{kg}^{-1}\cdot\text{h}^{-1}$  on the venous side. Thus in HW, the uptake across the lamellae was only slightly greater than across the filament.

Preexposure and testing in ASW greatly stimulated influx through both arterial and venous routes (Fig. 2). This stimulation was by far more significant on the arterial side. Under these circumstances,  $J_{\text{in}}$  did not stabilize immediately, but continued to increase, only reaching equilibrium values after 15–20 min. Venous  $J_{\text{in}}$  stabilized quickly, at much lower levels. Here,  $J_{\text{in}}$  was significantly increased only during the last interval. It is therefore not clear whether this single high value was anomalous or was the start of an upward trend. Stable values in preparations from ASW-exposed fish were about  $42 \text{ nmol}\cdot\text{kg}^{-1}\cdot\text{h}^{-1}$  on the arterial side and  $5 \text{ nmol}\cdot\text{kg}^{-1}\cdot\text{h}^{-1}$  on the venous side. In contrast with the HW-acclimated fish, where the arterial contribution was only slightly higher than the venous side, ASW exposure resulted in the arterial side contributing nearly 9 times more Zn than the venous side. Overall, compared with HW acclimation, ASW exposure resulted in 28-fold stimulation in  $J_{\text{in}}$  on the arterial side and fourfold stimulation on the venous side (compare Fig. 1 and 2).

To shed some light on whether this increase was due simply to the removal of Ca from the gill surface, which is likely to occur during ASW exposure (see below), or to the actual changes in the gill surface which arise from ASW preexposure, the third series of experiments examined flux rates using HW-acclimated fish tested in ASW. The response (Fig. 3) was basically that of the HW trout tested in HW (see Fig. 1).  $J_{\text{in}}$  tended to rise with time in both arterial and venous pathways, although not significantly, and there were no significant differences between the data sets of Fig. 1 and 3.

The kinetics of branchial Zn influx were measured in ASW-exposed trout by using waterborne [Zn] values ranging from  $0.4$  to  $7.5 \text{ mg}\cdot\text{L}^{-1}$  (Fig. 4). Since 15–20 min was required to reach equilibrium flux rates in ASW (Fig. 2), kinetics could not be done using several [Zn] values with a single head, as was done previously with Ca (Perry and Wood 1985). Only a single [Zn] could be tested with each head preparation.  $J_{\text{in}}$  at  $0.4 \text{ mg}\cdot\text{Zn}\cdot\text{L}^{-1}$  was very low, but thereafter rose rapidly, showing typical first-order saturation kinetics. In fact, there did not appear to be any significant unsaturable influx. It was significant that there was an apparent threshold of  $0.4 \text{ mg}\cdot\text{Zn}\cdot\text{L}^{-1}$ , below which there was no measurable uptake. This was a departure from first-order reactions, which should pass through the origin. The estimated kinetic constants using arterial data gave an apparent  $J_{\text{max}}$  of  $75 \text{ nmol}\cdot\text{kg}^{-1}\cdot\text{h}^{-1}$  and a  $K_m$  of  $1.5 \text{ mg}\cdot\text{Zn}\cdot\text{L}^{-1}$  ( $23 \text{ }\mu\text{mol}\cdot\text{L}^{-1}$ ,  $n = 20$ ). Since the venous fluxes were small under these conditions, and followed similar kinetics with increasing [Zn], estimations of  $K_m$  and  $J_{\text{max}}$  using total flux gave nearly identical values.

TEP's were stable with time, varying only 1–2 mV during the course of any one experiment. The total range observed was from  $-8$  to  $-23 \text{ mV}$ , inside negative. TEP was little affected by [Zn]. At the lowest waterborne [Zn] ( $0.4 \pm 0.04 \text{ mg}\cdot\text{L}^{-1}$ ,  $n = 7$ ), the mean TEP was  $-16.5 \pm 1.6$  (8). Raising the [Zn] to  $7.42 \pm 0.21$  (3) gave a mean TEP of  $-10.3 \pm 1.2$  (3), a change which was not significant.

Perfusion with either plasma or diluted whole blood was done to determine if these natural fluids provided additional substances (ligands) which would enhance  $J_{\text{in}}$ . Despite the presence of red blood cells and/or increased plasma protein (as determined by refractive index), influx was actually less than that found with perfusion saline, although individual values were

quite variable. For blood diluted with perfusion saline to a hematocrit of 7% (Table 2), arterial  $J_{\text{in}}$  was  $2.4 \pm 0.8$  (4)  $\text{nmol}\cdot\text{kg}^{-1}\cdot\text{h}^{-1}$  at a waterborne [Zn] of  $0.61 \pm 0.14 \text{ mg}\cdot\text{Zn}\cdot\text{L}^{-1}$ . For undiluted plasma, arterial  $J_{\text{in}}$  was  $19.7 \pm 4.2$  ( $n = 4$ ) at a waterborne [Zn] of  $1.25 \pm 0.2 \text{ mg}\cdot\text{Zn}\cdot\text{L}^{-1}$ . Comparable values for saline perfusion from the influx curve of Fig. 4 would be about  $18 \text{ nmol}\cdot\text{kg}^{-1}\cdot\text{h}^{-1}$  at  $0.6 \text{ mg}\cdot\text{Zn}\cdot\text{L}^{-1}$  and  $35 \text{ nmol}\cdot\text{kg}^{-1}\cdot\text{h}^{-1}$  at  $1.25 \text{ mg}\cdot\text{Zn}\cdot\text{L}^{-1}$ .

Examination of the gill tissues by scanning electron micrography revealed that under control conditions (Fig. 5a, 5b), chloride cells were clearly visible on the lamellae as large bumps which tended to be more numerous toward the trailing (downstream) edge. The cells were completely overlain by pavement cells, or nearly so, with only occasional lenticular exposure of the apical surface (Fig. 5b). Under these conditions, the apical surfaces of the chloride cells did not show the microvillar and microridge structure which was typical of pavement cells. After 1 d of exposure to ASW, little difference was seen at low magnification (Fig. 5c). At higher magnification, however, exposed apical surfaces were triangular in shape and clearly larger in area (Fig. 5d). Surfaces of chloride cells often appeared stippled with microvilli. When exposure to ASW extended to 7 d, profound changes occurred in lamellar chloride cells which were obvious even at lower magnification (Fig. 5e). Chloride cells were much more numerous and showed three distinct types representing a progression of stages. The most common chloride cells were quite rounded, and erupted from the lamellar surface. The apical surfaces frequently had well-developed microvilli and microridges with little, if any, pavement cell encroachment (Fig. 5f). Other chloride cells showed varying degrees of overgrowth by pavement cells, and in a few instances the chloride cells themselves appeared degenerate, having shrunken apical surfaces (Fig. 5g). Complete coverage by pavement cells at any stage was rare.

## Discussion

Zn influx was successfully measured under all conditions of acclimation and testing using the perfused head preparation. In addition, further information was obtained concerning the contribution by arterial and venous pathways. The rationale for the partitioning of perfusate flow through the head was described by Girard and Payan (1976, 1980) and Payan et al. (1984). The arterial flow is quite straightforward, being that perfusate which is collected from the dorsal aorta, and is considered to represent flux through the lamellae. However, assumptions underlying the venous flow and its representation of filamental fluxes are somewhat more complex. Venous flows in the gill are considered to arise from oxygenated blood which has already transited the lamellae and entered the filamental venous sinus (FVS) where it is in close contact with the filamental epithelium. The FVS then drains via the branchial veins back to the ventricle. Discrete sampling of this drainage would indeed give a measure of filamental input, simply by subtracting the arterial component. However, other fluids may also find their way into this pool. There is venous drainage which was never in contact with the FVS, namely venous drainage arising from systemic arterial flow to the brain and musculature of the head. There is also the possibility of arterial flow from arterioles at the cut surface. This extraneous drainage into the arterio-venous collection does not alter the calculation of filamental uptake, as long as no Zn is lost in the systemic circulation prior to collection. However, Zn is cleared quite quickly to the tissues from the systemic

circulation in vivo (D. J. Spry and C. M. Wood, unpubl. data) although extent of "first pass" clearance (similar to the situation with the perfused head) is unknown. Thus the venous values could underestimate filamental Zn influx. A second possible cause of underestimation of the venous influx might arise from lack of true venous steady state. This was suggested, but not proven, by Fig. 2. Similarly, Gardaire et al. (1985) have discussed the circuitousness of the venous circulation and the time lag for isotopic steady state between the arterial and venous circulation with regard to Na flux. The opposite possibility, that of overestimates of venous flux due to contamination from the irrigation water, was described by Perry and Wood (1985). Large leaks, mainly through the esophageal ligature, were detected readily by refractometry in the present study and either rectified or the data discarded. Small leaks (<1%) cannot be completely ruled out, and given the high specific activity of the water, these would contribute significantly to the venous flux. However, the constancy of the fluxes over time (Fig. 1-3) and the rapid saturation with increasing [Zn] (not shown, but similar to the arterial data of Fig. 4) argue against even small leaks. On balance we conclude that the arterial (lamellar) Zn uptake estimates in our experiments are valid, while the venous (filamental) uptake estimates are either valid or underestimates.

Zn fluxes in HW-acclimated and tested trout came quickly to steady state with minimal time lag and were the same for both arterial and venous fluxes. The flux rates, in the low nanomoles per kilogram per hour range, were comparable with those of Cd at similar exposure concentrations (Pärt and Svanberg 1981). However, they were small compared with flux rates for Ca which have ranged from 2 to 50  $\mu\text{mol}\cdot\text{kg}^{-1}\cdot\text{h}^{-1}$  in vivo (Berg 1968; Pang et al. 1980; Mayer-Gostan et al. 1983; Höbe et al. 1984; Perry and Wood 1985) and using perfused gill preparations (Payan et al. 1981; Perry and Wood 1985). At least in HW, Zn did not exhibit the disparity between arterial (90-95% of total) and venous fluxes reported by Perry and Wood (1985) for Ca. The similarity of arterial and venous Zn fluxes suggests that Zn uptake per unit area (specific uptake) was greater through the filament than through the lamella, since lamellar area in rainbow trout is about 27-fold higher than filamental area (Girard and Payan 1980). Chloride cell density is much higher in the filament, especially in hard water (Laurent and Dunel 1980; D. J. Spry, unpubl. obs.), which may account for the higher apparent specific uptake (see below).

Zn fluxes in fish preexposed and tested in ASW were stimulated more than 20-fold above HW rates. This increased uptake may at least partially explain the well-known fact that Zn toxicity increases with decreasing water hardness (Spear 1981). Furthermore, the Zn uptake in ASW differed in two other ways from that in HW. First, steady-state flux rates did not occur in the arterial perfusate until 15-20 min after addition of the isotope. Second, the venous influx, although stimulated, was considerably less than the arterial influx. Unless the relative surface areas of the lamellae and the filament changed drastically, this would suggest that the Zn uptake per unit area across the lamellae increased much more than through the filament. However, the increase in venous influx during the last flux period (Fig. 2) may again indicate a long time lag in Zn flux through this compartment.

The TEP's measured under these conditions were similar to those measured in other freshwater-adapted teleosts (see Potts 1984; Perry et al. 1985b; Perry and Wood 1985). Since this electrical potential is, in freshwater fish, largely the result of  $\text{Na}^+$  and  $\text{Cl}^-$  diffusion, the stability of the TEP both over time

and across [Zn] indicated relative permeabilities for  $\text{Na}^+$  and  $\text{Cl}^-$  which were also stable. This agrees with earlier in vivo work of Spry and Wood (1985) which indicated that Zn effects upon  $\text{Na}^+$  and  $\text{Cl}^-$  were minimal compared with other toxicants such as acid or Cu (McDonald 1983; Laurén and McDonald 1986).

The stimulation of Zn influx by exposure to ASW and the preferential effect on the lamellar pathway were clearly due to more than the removal of Ca from the water (and gill surface), as was demonstrated by the experiments of series 3. These results strongly suggest that actual morphological and/or functional changes occurred in the gill, which were responsible for this increased flux. Recent work has demonstrated that many stresses, and in particular, exposure to water low in Ca (Perry and Wood 1985) or NaCl (Laurent et al. 1985) induced changes in the gill epithelium. Not only did the number of chloride cells on the lamellae increase, but the overlying pavement cells receded, so that more of the cell was exposed to the environment (Perry and Wood 1985). We also found (Fig. 5) that simultaneous dilution of both NaCl and Ca induced similar changes in branchial chloride cells. In addition, there was dramatic development of microvilli and microridges. While this undoubtedly further increased surface area, the functional significance is less clear. Although the changes in chloride cells in terms of number, distribution, exposure, and surface structure do not show causation, the correlation strongly suggests a role for the chloride cell in the uptake of Zn from the environment. In contrast, Pentreath (1973) failed to find any localization of  $^{65}\text{Zn}$  in autoradiographs of chloride cells from seawater plaice (*Pleuronectes platessa*), but the Zn concentration was much lower ( $0.015\text{ mg}\cdot\text{L}^{-1}$ ).

The reason for the long time lag to steady-state Zn flux in ASW-exposed trout is unclear. Pärt and Svanberg (1981) reported similar time lags to steady state for Cd, even though their work was done in hard water. Perhaps unstirred layers on the gill increased the diffusion distance sufficiently to slow the rate of arrival of metal at the site of entry on the gill. These layers may have arisen from increased mucus production as a result of ASW exposure (in our experiments) or from Cd exposure (in Pärt and Svanberg's experiments). Mucus also bound Cd and Hg, and while it did not impede the movement of Ca, diffusion of Cd and Hg was significantly slower (Pärt and Lock 1983). If Zn were trapped by anionic sites in mucus and the gill surface, this might result in the observed time lag. This phenomenon might also explain the apparent threshold of  $\sim 0.4\text{ mg Zn}\cdot\text{L}^{-1}$  below which there appeared to be no influx. Longer exposure times might, however, result in detectable influx.

The experiments with plasma and blood perfusion were performed to test whether Zn uptake by the perfused head was limited by the Zn binding capacity of the perfusate, i.e. whether the provision of organic ligands in plasma protein and red blood cells would stimulate influx. The results showed that this was clearly not the case. Influx in fact, was considerably lower, probably due to increases in the branchial resistance (Table 2) indicative of altered hemodynamics with blood and plasma.

We initially proposed two possible mechanisms by which Zn might traverse the gill: simple passive diffusion or active uptake. The electrochemical gradient probably favours the passive entry of Zn into the fish. This is so first, because the inside of the preparation had negative potential (TEP  $-8$  to  $-23\text{ mV}$  inside negative in ASW). Second, at this water pH, most of the Zn exists as hydrated free ion in the water (Campbell and Stokes 1985) whereas in blood and other biological fluids, Zn is tightly



bound, with very little existing as free ion (Hambidge et al. 1986). The kinetic data suggest that passive influx, in which flux would be a simple linear function of external concentration, was of very minor importance if it occurred at all. The curvilinear nature of the influx-concentration relationship supports a saturable uptake mechanism, although the mechanism is not necessarily active or even carrier mediated.

One mechanism which might give the appearance of saturable influx is passive diffusion being reduced by an increase in diffusion distance with increasing waterborne [Zn]. High waterborne concentrations of Zn cause inflammation and edema of gill tissue in vivo (Skidmore and Tovell 1972; Tuurala and Soivio 1982; Tuurala 1983). Could edema result in a concentration-dependent fashion with increased waterborne [Zn], and could this masquerade as a saturable phenomenon? First, the preparation itself is free of any significant edema (Perry et al. 1984a, 1984b) using current methodology. The edema associated with waterborne Zn exposure, on the other hand, resulted from its corrosive action as an external irritant. This took 2 h of exposure at a very high level (40 mg Zn·L<sup>-1</sup>; Skidmore and Tovell 1972) and 96 h of exposure to 1.25 mg·L<sup>-1</sup> (Tuurala and Soivio 1982) for pathology to occur. Decreases in Pa<sub>O<sub>2</sub></sub> indicative of increased diffusion distance, occurred after 6 h of exposure to ~1.5 mg Zn·L<sup>-1</sup> (Sellers et al. 1975; Spry and Wood 1984). Tuurala (1983) correlated increased difference between inspired water P<sub>O<sub>2</sub></sub> and Pa<sub>O<sub>2</sub></sub> with increased diffusion distance after 17–20 h of exposure to 1 mg Zn·L<sup>-1</sup>. The brief period of perfusion in the present study, and the lower [Zn] used, precludes this as a significant factor. Further evidence against edema is that there was never a decrease in influx rate over time, as would be expected if edema were creating a diffusion limitation. The saturability of influx must thus be due to other factors, the most likely of which is a saturable carrier, or a selective pore. It is not possible to distinguish between these possibilities based on the present data. What is readily apparent from the kinetic constants, however, is that the *K<sub>m</sub>* is well into the toxic range for trout (2.5–3 mg·L<sup>-1</sup>; Holcombe and Andrew 1978) and thus Zn transport is coincidental. Such "accidental" uptake by a Ca mechanism was suggested to occur in freshwater amphipods (Wright 1980).

Our results have clearly shown that significant uptake of Zn occurred from the water and indicate a mechanism by which this transfer is accomplished. Actual transfer rates for Zn may be considerably higher in vivo based on two facts. First, uptake from moderately hard water, when dietary Zn was deficient, was about 120 nequiv·kg<sup>-1</sup>·h<sup>-1</sup> (Spry et al. 1988), and whole-body influx based on <sup>65</sup>Zn kinetics in vivo (D. J. Spry and C. M. Wood, unpubl. data) was 200–400 nequiv·kg<sup>-1</sup>·h<sup>-1</sup>. Nevertheless, this preparation has yielded important qualitative understanding of the mechanism involved in transbranchial Zn transfer. Even these higher rates are small (1%) compared with those of other ions such as Na, Cl, and Ca. Excretion of Zn, however, is also very low (Pentreath 1973, 1976; Willis and Jones 1977). Estimates for elimination half-times were 235 d for the largest of three compartments (91%) in *Gambusia* (Willis and Jones 1977) and about 300 d for plaice (Pentreath 1973), both in seawater. Coupled with the fact that Zn was taken up independently from both the water and the diet (Spry et al. 1988), elevated waterborne concentrations could cause significant branchial uptake and retention.

In conclusion, the saline-perfused head was a useful model to demonstrate entry of Zn across the branchial epithelium of trout and gain some insight into relative importance of lamellar

and filamental pathways at different hardness. Use of this method permitted measurements of influx which were relatively free from the confounding effects of metal adsorption. Zn uptake rates were extremely low (0.01–1% of Na, Cl, and Ca influx rates). Use of blood or plasma as perfusates did not augment Zn uptake. ASW exposure greatly stimulated Zn influx, especially through the lamellae, and this effect likely arose from morphological changes in the gill. Changes in chloride cell number and distribution were the most likely explanation.

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