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METAL-GILL SURFACE INTERACTIONS IN RAINBOW TROUT

ABSTRACT

Experiments were conducted on rainbow trout to analyze: 1) the impact on gill function of cadmium (Cd^{++}) as compared to the effects of copper (Cu^{++}) and low pH (H^+), 2) the metal binding characteristics of the external gill surface, and 3) modifications in metal binding activity of the gills associated with chronic exposure to low Ca^{++} water or aluminum (Al).

Short-term exposure (24 h) to equimolar (6.5 $\mu mol \cdot L^{-1}$) Cd^{++}, Cu^{++}, or H^+ (pH 4.8) resulted in the disruption of transepithelial ion exchange in trout, confirming the surface activity of Cu^{++} and H^+, and characterizing the impact of Cd^{++} on gill function. The effects of each metal were different from one another with respect to specific site of action, rapidity of action, and persistence of the disturbance. Furthermore, Cu^{++} and H^+ had only minor effects on Ca^{++} balance and major effects on Na^+ balance whereas the reverse was the case for Cd^{++}, with water hardness ($[Ca^{++}]$) having a fundamentally different role to play in the toxicity of Cd^{++} compared to Cu^{++}.

Analysis of metal-gill surface interactions, using an *in vitro* methodology, revealed that metal binding kinetics were related predominantly to the charge of the metal. The gills had the highest affinity for lanthanum (La^{+++}) and Al, intermediate affinity for Ca^{++} and Cd^{++}, and the lowest for Cu^{++} and H^+. All metals, with the exception of Cu^{++}, would inhibit the binding of Ca^{++} to the gills. These metals were ranked, in decreasing ability to act as Ca^{++}-antagonists at the gill surface, as follows: Al, La^{+++}, Cd^{++} and H^+ then Cu^{++}. Although the binding affinity, and, to some extent, the

competitiveness of these metals was correlated with charge, metal toxicity was correlated with neither the metal charge nor the affinity of the metal-gill surface interaction.

The gill micro-environment was found to be capable of responding in an adaptive fashion to environmental stressors (low Ca^{++} water or elevated Al). Modifications of the gill, in response to the reduction of water Ca^{++}, resulted in a significant increase in the Ca^{++} binding affinity and capacity of the external gill surface. Furthermore, chronic exposure to elevated Al induced a prophylactic response by the gill micro-environment, resulting in a significant suppression of the surface activity of Al manifested as an enhanced resistance to the ionoregulatory and hematological impact of Al exposure.

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

CHAPTER 1

GENERAL INTRODUCTION

Most trace metals are toxic to freshwater fish, but not equally toxic. Indeed, acute metal toxicity ranges over at least 3 orders of magnitude, from as little as 0.3 $\mu M.L^{-1}$ (copper, 96 hr LC_{50}; Howarth and Sprague 1978) to as much as 338 $\mu M \cdot L^{-1}$ (nickel, 48 hr LC_{50}; England and Cummings 1971). Furthermore, the toxicity of any one metal can greatly vary. The toxicity of cadmium to rainbow trout fry increases by over 40 times with a 16-fold reduction in water hardness (Calamari et al 1980). There also can be large interspecific differences, as illustrated by aluminum sensitivity differences between rainbow trout (*Oncorhynchus mykiss*), brook trout (*Salvelinus fontinalis*) and smallmouth bass (*Micropterus dolomieui* L.; McDonald, D.G., C.M. Wood and B.P. Simons, unpublished data) and low pH sensitivity differences between yellow perch (*Perca flavescens*), common shiner (*Notropis cornutus*) and rainbow trout (Freda and McDonald 1988). The toxic effect of metals usually involves an interaction between the free metal ion and its biological target (Klaassen 1986). Interspecific differences may eventually be explained by differences in the chemical composition of the target tissue or binding surface. Thus, metal toxicity may be viewed as being dependent on metal chemistry, while modified by water chemistry.

Metal Toxicology

With the multitude of factors capable of modifying metal bioavailability and metal interactions, attempting to identify general principles governing the mechanisms of metal toxicity is clearly a complex problem. However, such studies often lead to the development or improvement of models of metal toxicity. Such models may prove

invaluable in predicting the consequences of exposure of fish to either metal mixtures or to individual metals of unknown toxicity. In addition, an understanding of the underlying mechanisms of toxic action also contributes to the knowledge of basic physiology, pharmacology, cell biology and biochemistry.

Classically, mechanistic studies of metal toxicity have been done predominantly on mammals for reasons related to human health concerns. In mammals, routes of entry are the gastrointestinal tract (ingestion), lungs (inhalation), and skin (topical). These absorptive surfaces tend to be isolated from the environment or possess elaborate defense mechanisms to deal with environmental stressors. For example, the mucous blanket of the ciliated nasal surface carries with it insoluble particles as it is propelled by beating of the cilia. These particles as well as particles inhaled through the mouth are cleared within minutes by expectorating, sneezing or swallowing. The mammalian approach stresses the internal activity of chemical agents, including metals. Klaassen (1986), for example, states that:

> "...adverse or toxic effects in a biological system are not produced by a chemical agent unless that agent or its biotransformation products reach appropriate sites in the body at a concentration and for a length of time sufficient to produce toxic manifestations."

By implication, if the agent (metal) is not absorbed, then it is not toxic.

Issues which arise when studying internal toxicity of metals include aspects of metal transport (multi-compartment distribution analysis), sites of accumulation (organ and tissue metal loads) and clearance or storage mechanisms (inducible metal-binding proteins). Largely ignored in this mammalian approach is the initial interactions between toxic agents and the external surface through which they must pass (surface activity), since it is assumed that this initial stage of absorption has little effect on metal toxicity. The same assumption can not be made for fish, particularly if the gills are the main route of metal uptake. The surface activity of metals may be of key importance to

metal toxicity. In contrast to internal metal activity, the issues concerning surface activity are: the relationship between concentration and the metal binding characteristics (surface binding capacity and affinity), modifying factors of binding (metal chemistry and metal competition), effects on surface or membrane function (barrier integrity), and effects of water chemistry (pH, hardness, metal complexing capacity) and other modifiers (i.e. adaptation responses) on toxicity.

The Primary Locus of Metal Accumulation in Fish

The focus of this thesis is on the activity of metals on the surface of the gills of the rainbow trout, *Oncorhynchus mykiss*. Gas exchange, as well as, electrolyte and acid-base homeostasis, regulated at the gills, require an organ with a large, permeable, surface area and architecturally complex design. The gills of a 250 gram adult rainbow trout, for example, represent 60% of the total surface area exposed to the environment which is irrigated, in a one hour period, with over 48 litres of water. The gill surface is covered with a layer of mucus, comprising of a mixture of glycoproteins, mucopolysaccharides, low molecular weight compounds and water (Wold and Selset 1977, Fletcher et al 1977, Van der Winkel et al 1986). The mucus is thought to aid in electrolyte transport capacity of the gills (Kirschner 1978, Marshall 1978). Layers analogous to that of gill mucus, such as the bacterial cell wall, have been shown to attract cations due to an abundance of surface components which are negatively charged at environmentally relevant pH's (Beveridge and Murray 1980, Doyle et al 1980). Therefore, the gills are likely the target for water-borne metals due to their chemical composition and their intimate contact with the environment.

Metal Surface Activity

Surface activity of metals is best identified in fish by the disruption of normal gill function. Both ionoregulation and gas exchange can be used as indicators of surface activity. Exposure of trout to either zinc (Spry and Wood 1985) or aluminum (Booth et al 1988) results in impairment of both gill functions. However, both are not necessarily reciprocally dependent. Copper, for example, appears to predominantly affect ionoregulation. Lauren and McDonald (1985) detailed the disruption of sodium transport in adult trout exposed for 24 hours to 12.5 μg $Cu \cdot L^{-1}$, while changes in pO_2 do not develop until levels reach 129 μg $Cu \cdot L^{-1}$ (Sellers et al 1975). The relative sensitivity of ion transport and gas transport to metals has not yet been established. However, branchial ionregulation is likely more sensitive to surface active metals than is gas exchange, due to the chemical composition of the gills and the complex nature of the cellular processes involved in electrolyte translocation (see McDonald et al 1988). The measurement of unidirectional flux rates is the most direct measure of branchial ionoregulation. Changes in internal (whole body and/or plasma) electrolyte levels can also be measures of gill function, but are less sensitive. Electrolyte loss can develop without measurable changes in the internal concentration if large electrolyte spaces, or pools, are involved.

To study the activity of metals on the gill surface, a range of metals of known or suspected surface activity was selected in an attempt to identify key properties of metals to explain variations in metal toxicity. Characteristics to consider would be size, charge and affinities for various biological ligands. The metals chosen were lanthanum (La^{+++}), copper (Cu^{++}), aluminum (Al), cadmium (Cd^{++}) and hydrogen ion (H^+, considered an 'honorary metal' as discussed subsequently). The first three (La^{+++}, Cu^{++}, Al) disturb electrolyte balance in trout through the inhibition of active uptake and stimulation of passive loss (La^{+++}, Freda and McDonald 1988, Perry and Flik 1988; Cu^{++}, Lauren and

McDonald 1985; Al, Booth et al, Wood et al 1988a, McDonald and Milligan 1988). Lanthanum has similar chemical properties to calcium (Nieboer and Richardson 1980) and is solely surface active since it fails to penetrate cell membranes (Weiss 1974). Accumulation of Al on the external gill surface results in net losses of internal Na^+ and Cl^- through the ability of Al to inhibit processes involved in both uptake (McDonald and Milligan 1988) and loss (Booth et al 1988, Wood et al 1988a). Copper has been shown to specifically inhibit Na^+ influx in trout, and at higher thresholds appears also to stimulate electrolyte efflux (Lauren and McDonald 1985).

Cadmium was included because of the suspected surface activity of this metal. The classical response to Cd^{++} exposure in trout is a reduction in plasma Ca^{++} concentration (Roch and Maly 1979). Giles (1984) reported no significant change in renal electrolyte handling during exposure to sublethal Cd^{++} levels for 178 days. This suggests that Cd^{++} disturbs the ionoregulatory abilities of the gill and is, therefore, surface active. Of further interest is the chemical similarities between Cd^{++} and Ca^{++} which indicates possible competitive interactions between these metals at the gill surface.

The influence of hydrogen ion (pH) on metal toxicity is rather complex in that it can influence both metal speciation and solubility. The general relationship between pH and metal speciation is that as the pH decreases, the greater the proportion of free cation rather than the other dissolved species (Campbell and Stokes 1985). Furthermore, hydrogen ions, themselves, have been shown to be surface active. Exposure of trout to acidified waters results in the inhibition of sodium and chloride uptake and the stimulation of the passive loss of these ions (McDonald 1983). During acute, lethal exposures, stimulation of the ion efflux rates makes the quantitatively greater contribution to net ion losses. The effects of hydrogen ions on gill function are thought to involve the displacement of membrane bound calcium through the titration of the surface negative charges. The

impairment of gill function by hydrogen ions is similar to that of other toxic metals with known surface activity. Therefore, hydrogen ion could be classified as an honorary metal. However, the surface activity of this 'metal' may significantly alter other toxic metal-gill surface interactions. For example, although at low pH, when both the solubility of copper and the proportion of the free cation (Cu^{++}) are increased, the toxicity of copper to trout is decreased (Howarth and Sprague 1978, Miller and MacKay 1980, Cusimano et al 1986). It was suggested that increased competition between hydrogen ions and copper for metal binding sites at the gill surface resulted in the decrease in the surface toxicity of copper. Similar relationships between pH and metal toxicity have been described for zinc and cadmium (Cusimano et al 1986).

Calcium and the Gill Surface

Environmental Ca^{++} acts to increase membrane stability and reduce electrolyte permeability by cross-linking ligands at tight junctions (Oschman 1978), ion channels (Hille et al 1975) and the membrane itself (Steen and Stray-Pederson 1975). In addition, the presence of elevated concentrations of Ca^{++} reduces trace metal toxicity (Miller and MacKay 1980) due to competition between Ca^{++} and trace metals for key gill surface binding sites (Zitko and Carson 1976, Pagenkopf 1983). Magnesium also protects fish from the toxic effects of trace metals (Zitko and Carson 1976, Miller and MacKay 1980). However, Ca^{++} is the more effective protective agent of the two (Potts and Fleming 1971), which is likely due to the more adaptable coordination chemistry of Ca^{++} (Williams 1974). Variations in the protective effects of Ca^{++}, as observed by Brown (1968), could be explained on the basis of the degree of binding site competition between Ca^{++} and toxic trace metals at the gill surface.

Prolonged exposure of fish to low environmental stressor levels could influence the surface activity of metals. For example, fish exposed to low Ca^{++} (i.e. soft

water) respond with a number of physiological adjustments to compensate for the change in their environment. Some of these adjustments appear to involve prolactin, recognized as the hormone responsible for adapting freshwater fish to ion-poor water (Chan et al 1968, Ogawa 1968, Pang et al 1973, Pang et al 1979, Wendelaar Bonga 1982). Prolactin has been shown to stimulate mucocyte proliferation and epithelial growth, resulting in a thicker mucus barrier and an overall reduction in permeability (Olivereau and Olivereau 1971, Wendelaar Bonga 1978) The increase in mucus thickness would increase the number of potential calcium binding sites on the gill surface. Furthermore, the stimulation of mucocytes occurs concurrent with an increase in the concentration of calmodulin, a high affinity calcium binding protein, in the mucus (Flik et al 1984). Therefore, as suggested by McWilliams (1982), the gills of trout adapted to soft waters may have a higher binding affinity and greater capacity for Ca^{++}, than fish from hard waters.

Paradoxically, however, the hypothetical modifications in Ca^{++} binding activity associated with acclimation to soft water, would likely also increase surface accumulation of trace metals, provided that the trace metals bind as effectively as Ca^{++} to the gill surface. In fact, this has been confirmed, at least indirectly, since Cd^{++} and other trace metals, have been shown to have higher binding affinities for calmodulin than Ca^{++} (Locke et al 1984, Richardt et al 1986, Suzuki et al 1985). Therefore, modification of the external gill surface in response to environmental stress may increase trace metal toxicity in conjunction with the reduction in competitive hardness cations. On the other hand, the acclimation of fish to sublethal levels of trace metals could result in physiological adjustments which alter metal gill surface activity in adaptive fashion, depending on the specificity of the stressor-induced response of the gills.

Modelling Metal Surface Activity

Several models have been proposed to explain both variations in surface activity of any one metal and differences in the surface activity between trace metals. Pagenkopf (1983) developed the Gill Surface Interaction Model (G.S.I.M.) to account for the variability in toxicities of trace metals (Cu^{++}, Zn^{++}, Cd^{++}) to fishes as a function of water chemistry (alkalinity, hardness, pH). The GSIM was based on the premise that trace metal species bound to the gill surface cause impairment of physiological functions and that the amount of trace metal bound is affected by the chemical composition of the test water. Specifically, hardness metals (Ca^{++} and Mg^{++}) reduce the surface bioavailability of toxic metal species through competition for interaction sites on the gill. Zitko and Carson (1976) correlated the modification of heavy metal (Cu^{++}, Zn^{++}, Cd^{++}) lethality by Ca^{++} and Mg^{++} with the cation glycine binding constants. They also proposed that the degree of lethality modification was based on the specificity and strength of binding to active sites in fish tissues.

Although these models are useful for explaining variations in metal toxicity due to the effects of water chemistry, both are limited by the range in toxic metals studied and the extrapolation of binding competition from lethality data. In an attempt to associate some biologically and chemically significant meaning to metal classification, Nieboer and Richardson (1980) detailed the ionic and covalent tendencies of metals according to their electronegativity, ionic radius, formal charge and binding ligand preference. Using this theoretical classification scheme, Nieboer and Richardson (1980) were able to interpret the biochemical basis of differences in metal toxicity, and also predict the outcome of metal competition for biologically significant binding sites. My study of the activity of toxic metals on the surface of gills will incorporate these experimentally and theoretically derived ideas, and provide a more complete understanding of gill surface-

metal interaction and the role of metal chemistry and water chemistry in variations of metal toxicity to fish

Research Objectives

The objectives of this thesis are:

1) To determine the surface activity of Cd^{++}, the degree of which will be assessed relative to Cu^{++} and H^{+}. As mentioned previously, these metals are potential Ca^{++}-antagonists, yet the sensitivity of branchial ionoregulation to Cd^{++} had not been previously described.

2) To characterize the metal binding properties of the gill surface. *In vitro* analysis of the metal binding activity of the gills provided relative estimates of metal binding affinity (K_D), half-saturation time ($t_{1/2}$), maximal amounts of metal that could be bound by the gill (B_{max}) and binding cooperativity (H_n). These values were used in an attempt to identify key determinants of gill-metal interactions. Further characterization of the metal binding activity of the gills was achieved through an additional series of *in vitro* competition experiments and the calculation of IC_{50} values, the IC_{50} being the concentration of a metal which results in a 50% reduction in maximal Ca^{++} binding. The competitive inhibition experiments allowed for the determination of the role of Ca^{++}-displacement as a mechanism of, as well as a modifier of, metal surface activity.

3) To evaluate the effects of acclimation of fish to prolonged, low levels of environmental stressors on the metal binding activity of the gills. These experiments tested the hypothesis that alterations in the structure/composition of the gill surface associated with acclimation to environmental stressors directly results in the modification of metal-gill surface interactions. In one series of experiments the Ca^{++} binding kinetics of the gills were determined prior to and following acclimation of two life stages of hardwater-acclimated trout to soft water, while in an additional series of

experiments, a variety of metal-gill surface interactions and physiological parameters were monitored during acclimation of juvenile trout to chronic low level aluminum exposure.

CHAPTER 2

THE GILL SURFACE ACTIVITY OF CADMIUM IN RELATION TO COPPER AND LOW pH

INTRODUCTION

Acidification of aquatic environments is often accompanied by elevation of several toxic trace metals. This occurs either because of atmospheric input in the proximity of industrial operations, or as a result of dissolution and mobilization from soils and sediments (Beamish and Van Loon 1977). To understand the various interactions of these contaminants investigators have conducted lethality studies employing mixtures of metals at reduced pH (Brown 1968; Hutchinson and Sprague 1981; Wong et al 1981). While this approach can be very productive, particularly in providing regulatory information, it is simply not feasible to test all possible combinations for their interactions and toxicity.

Another approach is to examine the mechanism of action of individual toxicants with the aim of eventually predicting how various components of a toxic mixture might interact. Thus, the main purpose of the present study is to contribute to this knowledge by examining how copper and cadmium, arguably the most toxic of the trace metals present in acidified softwaters, affect branchial ion regulation in the rainbow trout. The focus here is on the gills, because they are likely the primary initial target of metal/H^+ toxicity, and upon ionoregulation because this is likely the most sensitive of the physiological functions performed by the gills (c.f. McDonald, Reader and Dalziel 1986). In the case of ionoregulation, flux rates of Ca^{++} and Na^+ were studied rather than their respective balances, since disturbances to flux rates are easier to detect than disturbances to body levels, particularly in the case of calcium where the skeleton represents a

considerable Ca^{++} reserve (Simmons 1971, Dacke 1979, Hobe et al 1984, Glowacki et al 1986).

Metal exposures were conducted for 24 hours only. This avoided possible complications arising from more prolonged exposures such as metal accumulation in various internal organs or induction of metal-binding proteins. Also, to increase the likelihood of obtaining measurable disturbances, relatively high concentrations of Cu and Cd were employed; 6.5 $\mu mol \cdot L^{-1}$. This concentration is about 20 fold higher than the 96 h LC_{50} to rainbow trout in softwater for each (Howarth and Sprague 1978, copper; Cusimano et al 1986, cadmium).

Cadmium and copper were examined in relation to three variables: water pH, age of the test animals, and water [Ca^{++}]. The low pH exposure was pH 4.8 or 15.8 $\mu mol \cdot L^{-1}$ H^+. This is substantially below the 96h LC_{50} for rainbow trout in softwater (pH 4.0 - Graham and Wood 1981), but is a realistic level for natural waters undergoing acidification. Both juvenile and adult rainbow trout were employed as a recent study (Lauren and McDonald 1986) has shown that electrolyte homeostasis of juveniles is more sensitive than that of adults to disturbance by metal and acid exposure. Furthermore, many of the physiological studies of toxicants have used adults, whereas, lethality studies have been, for the most part, conducted on juveniles. The water calcium variable (i.e. water hardness) was examined for three reasons: acidification is a problem mainly in softwater environments where variations in water calcium can be as important in determining fisheries status as variations in pH (Wright and Snevik 1978); second, calcium has a well established protective effect against metal and H^+ toxicity (Alabaster and Lloyd 1980, Brown 1982); and third, calcium is not only one of the major electrolytes transported by the gills (Perry and Wood 1985), but is also important in the maintenance of the integrity and stability of the branchial epithelium (Potts and Fleming 1971, Cuthbert

and Maetz 1972, Chase 1984, McDonald and Rogano 1986). The calcium levels employed were 1.00 and 0.04 mmol·L^{-1}. These are representative of moderately hard waters such as the Great Lakes and extremely softwaters, respectively (NRCC 1981).

METHODS AND MATERIALS

Experimental Animals

Adult (170-300 g) and juvenile (1.5-2.5 g) rainbow trout, *Oncorhynchus mykiss*, were obtained from Spring Valley Trout Farm, Petersburg, Ontario and Aquafarms of Feversham, Ontario, respectively. The trout were held in large polyethylene tanks supplied with aerated, dechlorinated Hamilton tap water at 11-15^{0}C (Ca^{++} ~1 mmol·L^{-1}, NaCl ~0.6 mmol·L^{-1}). Trout were fed regularly with commercial trout pellets. For trout studied in hard water, acclimation commenced 9-12 days prior to experimentation, to water of temperature (14±1^{0}C) and composition of that employed in the experiment. Soft water acclimation was accomplished by holding trout in a 400 liter temperature-controlled tank, while every other day, the ionic composition was reduced approximately 50% by dilution with deionized tap water. Acclimation of trout to soft water was accomplished over a 30 day period to reduce the stress associated with direct transfer from hard to soft water (McDonald and Rogano 1986). Acclimation water was changed periodically to maintain the ionic composition of the water and reduce the build-up of waste products. Feeding was stopped 4-5 days prior to experimentation.

Test Conditions

Water for acclimation and experimentation was prepared from distilled tap water with appropriate additions of NaCl and $Ca(NO_3)_2$ to reach the desired ionic

composition. Water pH was reduced by titration with H_2SO_4. Water chemistry was monitored throughout the exposure periods and is presented in Table 2.1.

Test Chambers

Individual adult rainbow trout were contained within acrylic flux chambers as described by McDonald and Rogano (1986). These rectangular flux chambers (total volume = 5.6 liters, flux volume = 2.8 liters, internal dimensions (in cm) = 17H X 9W X 37L) were constructed of black acrylic plastic. An inner acrylic chamber (ID = 7H X 5.5W X 34L), mounted 1 cm off the bottom of the chamber and fitted with a removable lid, served to prevent the fish from turning or rotating in the flux chamber but did not physically restrain it. A multi-perforated air line was mounted on the outside of the inner chamber to aerate and mix chamber contents.

Juvenile trout were contained in polyethylene containers (total volume = 3.8 liters, dimensions = 15.5H X 10.5W X 10.5 L). The containers were cut from sheets of black polyethylene and heat-sealed to provide the final shape. Flux chamber temperature was regulated at 14±1°C. Each container held 5 fish in 2 L of water and was fitted with an air line for aeration and mixing.

Experimental Protocol

The first series of experiments examined the effects of metals and H^+ on calcium influx and net sodium flux in both juveniles and adults. Trout (4 fish per treatment, adults; 3 groups of 5 fish, juveniles) were allowed to acclimate to the test chambers for 24 hours prior to the beginning of an experiment. Experiments were initiated with the addition of ^{45}Ca (6.9-20.6 $KBq \cdot L^{-1}$) to all chambers while cadmium (as 8.9 mM cadmium acetate) or copper (as 15.7 mM copper sulfate) were added as appropriate. Actual

mean exposure concentrations are listed in Table 2.1. For low pH exposures, acid (as 0.1 N H_2SO_4) was added to reduce the test chamber water to pH 4.8 ([H+] = 15.8 $\mu mol \cdot L^{-1}$). After the initial acidification of the water, pH was monitored and readjusted every 15 minutes for the first 4 hours and every 30 minutes for the remainder of the experiments. Water samples were taken at 0, 2, 4, 8, 12, and 24 h for the determination of net and unidirectional ion fluxes and metal concentrations. Condition of animals was also noted at these times with any dead trout being removed from their flux chambers to avoid post-mortem alteration of water chemistry. All experiments were repeated at least once.

Upon completion of exposure, all fish were killed and weighed. Blood samples were obtained from about one half of the fish. Blood of adult trout was sampled by caudal puncture using a 1 ml syringe and a 22 ga needle. Juvenile trout were sampled from the caudal peduncle using 10 µl micropipets. The remainder of the fish were rinsed, frozen and stored for later analysis of Ca^{++} uptake (see below). Although all exposures were 24 h in duration, the adult trout were exposed for two periods of 12 h. At the end of the initial 12 h period, the chambers were flushed with fresh water and the second exposure started. However, adult trout exposed to cadmium during the initial 12 h flux period were not re-exposed during the subsequent flux period. The purpose of this was to evaluate the persistence of any ionoregulatory disturbance resulting from the initial 12 h of cadmium exposure.

A second series of experiments was designed specifically to examine the effect of cadmium on sodium influx in juvenile trout in relation to water pH and Ca^{++}. In these experiments groups of 10 to 15 fish were exposed to a matrix of [Cd] (0, 0.4. 0.8 or 1.6 $\mu mol \cdot L^{-1}$) by pH (7.6 or 5.0) by [Ca^{++}] (1.0, 0.1 or 0.001 $mmol \cdot L^{-1}$) for 24 h. The experiment was started by the addition of cadmium (as $CdCl_2$), 0.1N H_2SO_4 (where appropriate) and 3.7

$KBq \cdot L^{-1}$ of $^{22}Na^{+}$. At the end of 24 h the animals were collected, killed, weighed and placed into a sealed 15 ml gamma tube for whole body counting.

Analytical Techniques

i) Ion and Radiotracer analysis:

Concentrations of Na^{+}, K^{+}, Ca^{++}, Cd^{++} and Cu^{++} in plasma and water were measured, after appropriate dilution, by flame atomic absorption spectrophotometry (Varian AA-1275). Cl^{-} levels were determined by titration with a Buchler-Cotlove chloridometer. Ammonia was measured by a modification of the colorimetric method of Verdouw et al (1978). Radio-calcium activity was detected using liquid scintillation techniques (Beckman LS 230 and a LKB Rackbeta). All activity was corrected for quench based on quench correction factors generated automatically on the LKB Rackbeta. Radio-sodium in tissues and water was measured with a Nuclear Chicago (Model 1085) well-type gamma counter.

ii) Flux measurements:

Calcium and sodium influx rates ($J_{in}Ca^{++}$ and $J_{in}Na^{+}$) were calculated based on the accumulation of radio-activity in the body of the fish using the equation:

$$J_{in} = C_{wb} * (SA_{wa} * W * t)^{-1} \qquad 2.1)$$

where C_{wb} represents the whole body radio-activity (dpm), SA_{wa} was the radio-specific activity of the water ($dpm \cdot \mu mol^{-1}$), W was the weight of the fish (kg or g) while t was the total time (h) of exposure to the radiotracer. Backflux correction was determined to be unnecessary as the internal specific activity of the animals was never greater than 3% of SA_{wa}. Water specific activity was virtually constant throughout the exposures.

Whole body $^{45}Ca^{++}$ activity of the adult trout was determined using the "in vivo" Ca^{++} uptake method described by Perry and Wood (1985). Briefly, the method entailed rinsing trout carcasses for 2 minutes in flowing well water (10 mmol·L^{-1}) to displace adsorbed $^{45}Ca^{++}$ from the body surface. The carcasses were weighed and homogenized with an additional 100 ml of distilled water in a Waring blender. Quadruplicate aliquots (0.4 - 0.8 g) of the homogenate from each fish were digested overnight at 50°C in tissue solubilizer (NCS, Amersham). The digests were then neutralized with acetic acid and diluted with 10 ml of scintillation fluid (OCS, Amersham) prior to counting. A similar approach was used to determine the juvenile trout $^{45}Ca^{++}$ C_{wb}. Juvenile trout were digested for 2-3 days in 5 ml of 10N KOH. Five microlitres of digest was diluted to 5 ml with deionized water followed by the addition of 300 µl of glacial acetic acid, 1 ml of triton X-114 (BDH Chemical) and 10 ml of ACS (Amersham Corporation). Quadruplicate samples were prepared for each fish. Whole body $^{22}Na^{+}$ activity of juvenile trout was determined by counting the whole fish in the gamma counter.

Unidirectional Ca^{++} flux rates used to assess the time course of the metal/H^{+} effects were calculated based on the reduction in the water $^{45}Ca^{++}$ activity according to the equation:

$$J_{in} = (C_i - C_f) * (SA_{wa} * W * t)^{-1} \qquad 2.2)$$

where C represents the total $^{45}Ca^{++}$ activity in the water at the beginning (i) and end (f) of a flux period.

Net fluxes (J_{net}) of Na^{+} and Ca^{++}, in µmol·kg^{-1}·h^{-1}, were calculated from changes in their water concentrations. In the case of the juveniles exposure experiments, where net flux rates were based on groups of fish, rather than individuals, the group net flux rate data were corrected for the effect of mortality by calculating a time-weighted

group body weight. Calcium efflux rates (J_{out}) were calculated from the difference between J_{net} and J_{in}.

Statistics

All data presented are means ± 1 standard error about the mean (SEM). Statistical differences between treatments were analyzed by ANOVA and a Student-Newman-Keuls ranking test for means of equal or unequal sample size (Rosner 1982). Significance was set at a 95% level of confidence.

RESULTS

Exposures

Despite the relatively high loading densities in these experiments (adults = 80 $g \cdot L^{-1}$, juveniles = 5 $g \cdot L^{-1}$) compared to those recommended for static toxicity bioassays (1 $g \cdot L^{-1}$, Sprague 1969), metal concentrations remained stable throughout the exposures (means in Table 2.1) and ammonia levels rose to no more than 390 $\mu mol \cdot L^{-1}$. The latter were unlikely to have had toxic effects as the final values represented less than 20% of the 96 h LC_{50} of ammonia for rainbow trout (0.53 $mg.L^{-1}$, Arthur et al 1987) at the water pH's and temperature employed (Emerson et al 1975). Equally important was the lack of any significant change in Na^+ influx or efflux in control animals, over the course of the exposures, which suggests that ammonia accumulation was below the threshold to cause ionoregulatory disturbances.

Mortality

In terms of mortality, adult trout were more resistant than juveniles. No adults died during either holding, soft water acclimation, or metal/H^+ exposure. In juveniles, there was less than 4% mortality during holding and acclimation, but significant

Table 2.1. The composition (means, SEM in brackets) and pH (means, range in brackets) of the test water. Water samples were taken throughout the exposure period for determination of water chemistry. N = 6. Temperature = 14 ± 1°C. All ions in $\mu mol \cdot L^{-1}$.

	Soft Water neutral pH	Hard Water neutral pH	Soft Water pH 4.8	Hard Water pH 4.8
Calcium	30.7 (2.4)	915.0 (16.5)	46.7 (4.4)	1075.0 (14.5)
Sodium	245.0 (8.0)	255.0 (7.0)	276.0 (12.0)	270.0 (6.0)
Chloride	274.0 (20.0)	268.0 (12.0)	242.0 (5.0)	268.0 (12.0)
Potassium	5.1 (1.5)	8.3 (1.0)	6.9 (2.8)	12.1 (0.4)
Cadmium	6.8 (0.6)	6.5 (0.6)	6.7 (0.7)	6.5 (0.6)
Copper	6.7 (0.7)	6.2 (0.6)	6.9 (0.7)	5.9 (0.6)
pH	7.82 (7.5-8.0)	7.78 (7.4-7.9)	4.77 (4.6-4.9)	4.84 (4.7-5.0)

mortality occurred with both Cd and Cu exposure with the latter being more toxic (Table 2.2). Reduction of Ca^{++} in the water tended to increase metal toxicity. However, in both hard and soft water, the addition of H^+ appeared to reduce metal toxicity.

Changes in Plasma Electrolytes

Measurements of both [Ca^{++}] and [Na^+] were made on plasma of adult trout (Figs 2.1 and 2.2), but on juveniles, only the former were made because of the small sample volumes (2 - 5 µl) obtained. Furthermore, the small sample volume probably contributed to the variability of those data (Fig 2.1). For example, one soft water-acclimated juvenile exposed to copper (Fig 2.1B) had a plasma calcium level of 12.6 $mmol \cdot L^{-1}$. This could be real and due, for example, to hemoconcentration secondary to NaCl loss (McDonald 1983a) but a value this high is more likely a sampling artifact.

Despite these variations in the data it is apparent that 24 h metal / H^+ exposures had relatively little effect upon plasma electrolytes. The only major disturbance was a reduction in plasma [Na^+] in adults exposed to copper (Fig 2.2). Other than these data, no significant effect remained of water Ca^{++} level, water pH or cadmium on plasma [Na^+] in adults or plasma [Ca^{++}] in both adults and juveniles. Furthermore, plasma [Ca^{++}] was virtually identical in adults and juveniles. Plasma [Na^+] averaged 126.7 ± 0.37 $mmol \cdot L^{-1}$ (N = 48, hard and soft water adults combined) and plasma [Ca^{++}] averaged 2.48 ± 0.066 $mmol \cdot L^{-1}$ (N = 156, hard and soft water adults and juveniles combined).

Calcium Influx

In control animals (neutral pH, no metals) the rate of calcium uptake ($J_{in}Ca^{++}$) was relatively unaffected by either age or ambient [Ca^{++}]. In low Ca^{++} ([Ca^{++}] = 0.037 $mmol \cdot L^{-1}$ for 30 days) $J_{in}Ca^{++}$ was 6.4 ± 0.56 $nmol \cdot g^{-1} \cdot h^{-1}$, in juveniles and 6.4 ±

Table 2.2. Percent mortality in juvenile rainbow trout (1.5 - 2.5 g) exposed to Cd, Cu or H^+ alone or in metal/H^+ combinations for 24h. Concentrations of metals were 6 $\mu mol \cdot L^{-1}$. Water pH in H^+ was 4.8 (control pH = 7.8). Sample size for each treatment at start of exposure was 20 - 21 trout.

	Control	H^+	Cd	Cd/H^+	Cu	Cu/H^+
Hard water (Ca^{++} = 1 $mmol \cdot L^{-1}$)	0.0	0.0	28.6	0.0	71.4	40.9
Soft water (Ca^{++} = 0.04 $mmol \cdot L^{-1}$)	0.0	0.0	34.8	19.0	82.6	52.4

Figure 2.1 Plasma Ca^{++} concentrations of rainbow trout following 24 h exposure to 6.5 $\mu mol \cdot L^{-1}$ cadmium or copper at circumneutral pH (pH 7.6) or pH 4.8. Animals were acclimated and exposed in either hard water ($[Ca^{++}]$ = 1 $mmol \cdot L^{-1}$) or soft water ($[Ca^{++}]$ = 0.03 $mmol \cdot L^{-1}$). Data are presented as means ± 1 SEM. Statistical comparisons, using ANOVA and a Student-Newman-Keuls ranking test at a 95% level of confidence, have been depicted to illustrate significant differences due to: 1) metal treatment, 2) water Ca^{++} content, or 3) water pH. Significantly different data are indicated with matched pairs of symbols.

A. Plasma levels of adult trout (150 - 300 g). N = 8 for each treatment.

B. Plasma concentrations of juveniles (1.5 - 2.5 g). N = 5 for each treatment.

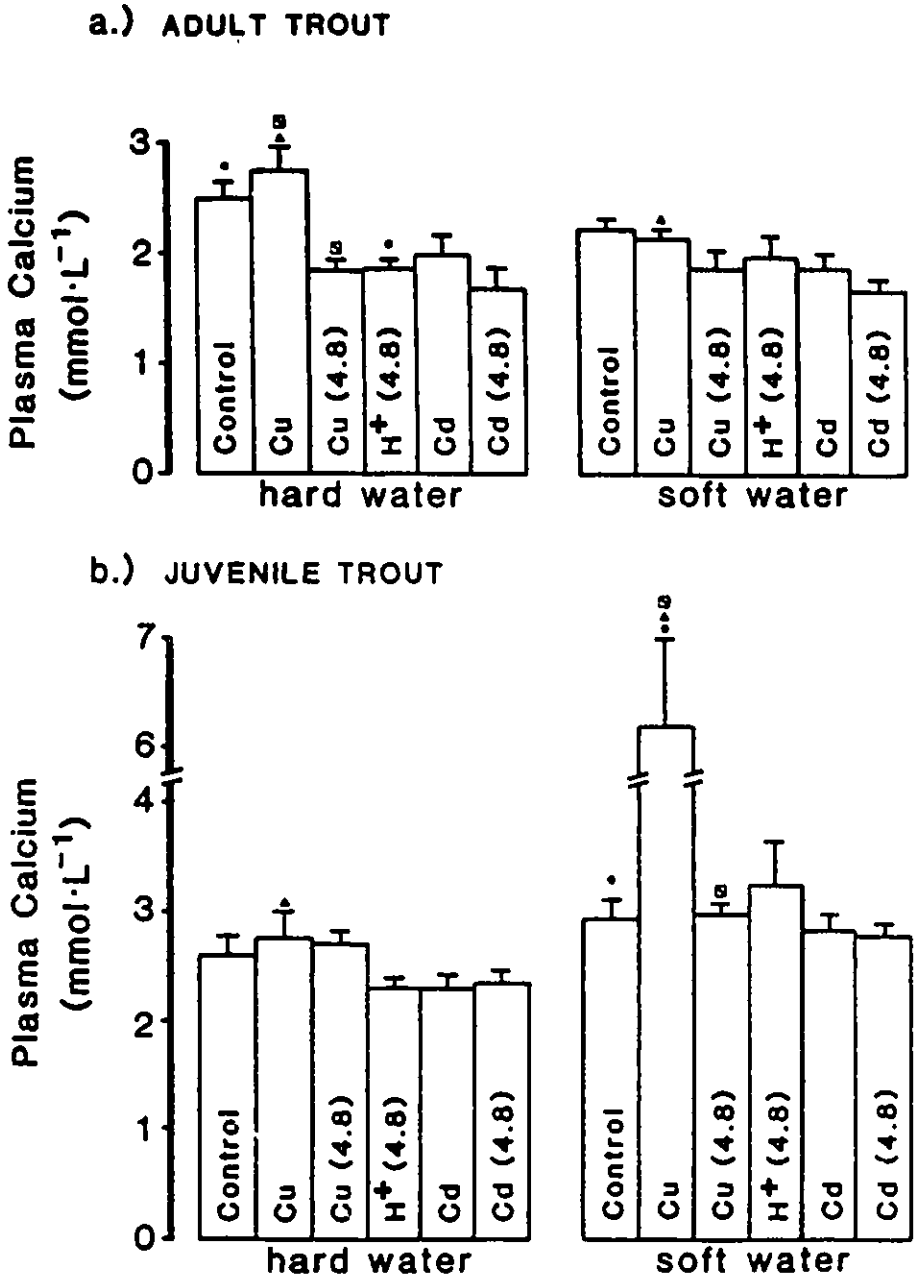
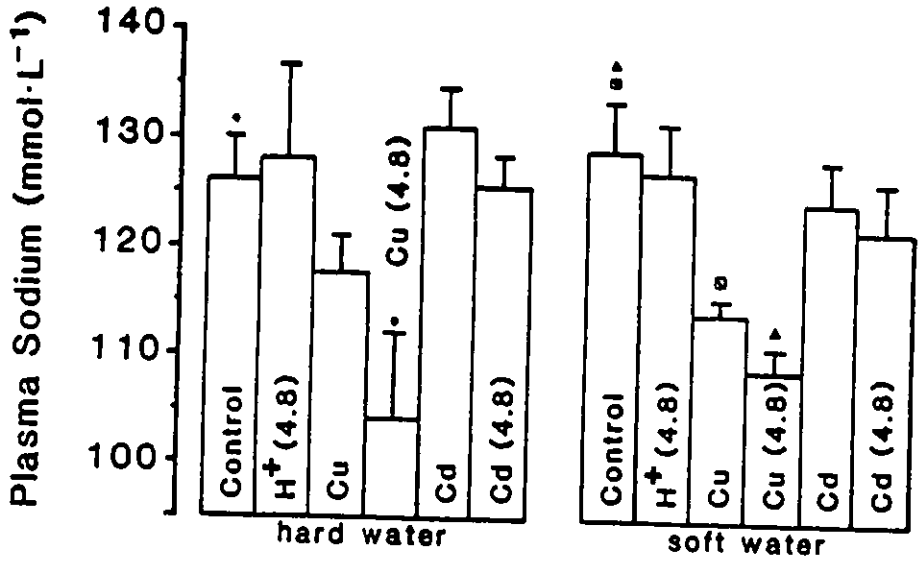

a.) ADULT TROUT
Plasma Calcium (mmol·L⁻¹)
3
2
1
0
Control
Cu
Cu (4.8)
H+ (4.8)
Cd
Cd (4.8)
hard water
soft water
b.) JUVENILE TROUT
7
6
4
3
2
1
0

Figure 2.2. Plasma Na^+ concentrations of adult rainbow trout following 24 h exposure to 6.5 $\mu mol \cdot L^{-1}$ cadmium or copper at circumneutral pH (pH 7.6) or pH 4.8. Animals were acclimated and exposed in either hard water ($[Ca^{++}]$ = 1 $mmol \cdot L^{-1}$) or soft water ($[Ca^{++}]$ = 0.03 $mmol \cdot L^{-1}$). Data presentation and statistical comparisons as detailed for Fig 2.1. N = 6 for each all treatments.


Plasma Sodium (mmol·L⁻¹)
140
130
120
110
100
Control
H+ (4.8)
Cu
Cu (4.8)
Cd
Cd (4.8)
hard water
Control
H+ (4.8)
Cu
Cu (4.8)
Cd
Cd (4.8)
soft water

ADULT TROUT

1.24 $\mu mol \cdot kg^{-1} \cdot h^{-1}$ in adults. In high Ca^{++} (1 $mmol \cdot L^{-1}$ for >30 days) $J_{in}Ca^{++}$ was 7.1 ± 0.69 $nmol \cdot g^{-1} \cdot h^{-1}$ in juveniles ε .4 ± 0.44 $\mu mol \cdot kg^{-1} \cdot h^{-1}$ in adults.

With a few exceptions noted below, all metal and H^+ exposures caused a significant inhibition of $J_{in}Ca^{++}$ in adults and juveniles relative to controls. In Figs 2.3 and 2.4, the average influx values over the 24 h exposure period are shown ranked according to the degree of inhibition. Regardless of age of animal or Ca^{++} acclimation, Cd was more inhibitory than either Cu or low pH. Indeed, Cu exposure had no effect on $J_{in}Ca^{++}$ in hard water-acclimated juveniles (Fig 2.4A).

In both adults and juveniles, water Ca^{++} provided significant protection against the effects of Cd, Cu and H^+. The degree of inhibition of $J_{in}Ca^{++}$ relative to controls was much greater in soft water-acclimated animals than in hard water animals (Fig 2.3A vs 2.3B; Fig 2.4A vs 2.4B).

The effects of low pH exposure were somewhat more complicated than those of either Cu or Cd. By itself, low pH exposure had a relatively small effect, particularly in hard water (Fig 2.3A, 2.4A). Where inhibition by H^+ did occur (Fig 2.3B, 2.4B) it was similar in magnitude to that of Cu alone. Consequently, when the two were combined, the effect was additive although the degree of inhibition was less than the sum of the two individual effects. In contrast, low pH had very little further effect when combined with Cd and in one case (hard water-acclimated juveniles; Fig 2.4A) appeared to be protective.

Calcium Efflux

The maintenance of electrolyte balance depends not only on the rate of influx but also upon the rate of loss, or efflux. Fig 2.5 shows efflux data calculated from net flux and influx values for adult trout. These values were quite variable, particularly in hard water where small changes in calcium concentration were being measured against a

Figure 2.3. Mean Ca^{++} influxes ($J_{in}Ca^{++}$) of adult rainbow trout over 12 h of exposure to 6 $\mu mol \cdot L^{-1}$ cadmium or copper at circumneutral pH (pH 7.6) or pH 4.8. Data are presented as means ± 1 SEM. N = 8 for each treatment. The horizontal lines connect bars which are not significantly different from one another at a confidence limit of 95% (ANOVA).

A. Exposures of hardwater-acclimated animals in hard water ($[Ca^{++}]$ = 1 $mmol \cdot L^{-1}$).

B. Softwater-acclimated animals exposed in soft water ($[Ca^{++}]$ = 0.03 $mmol \cdot L^{-1}$).

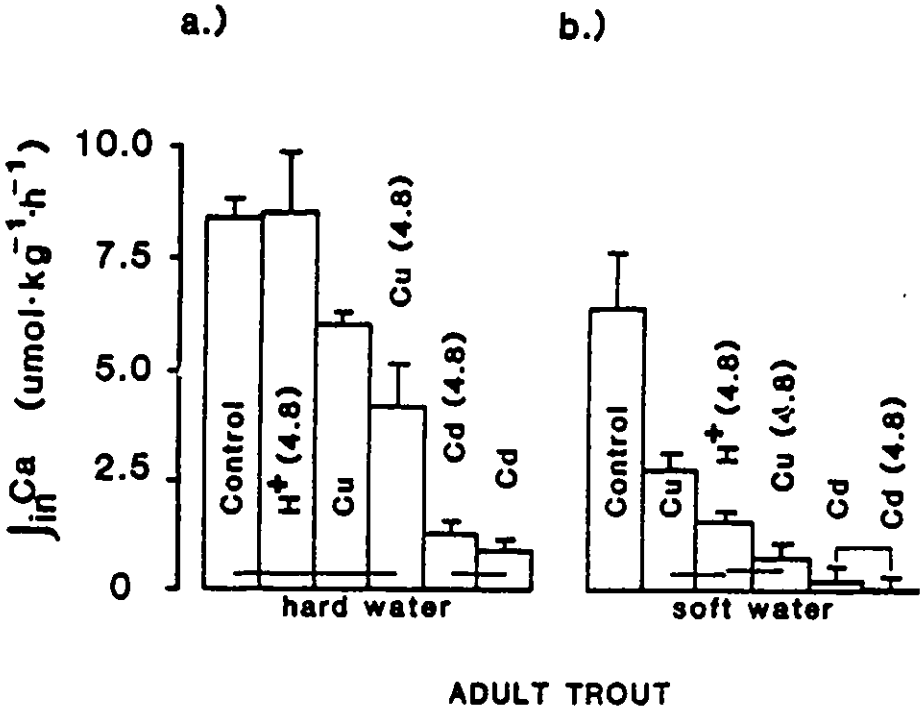
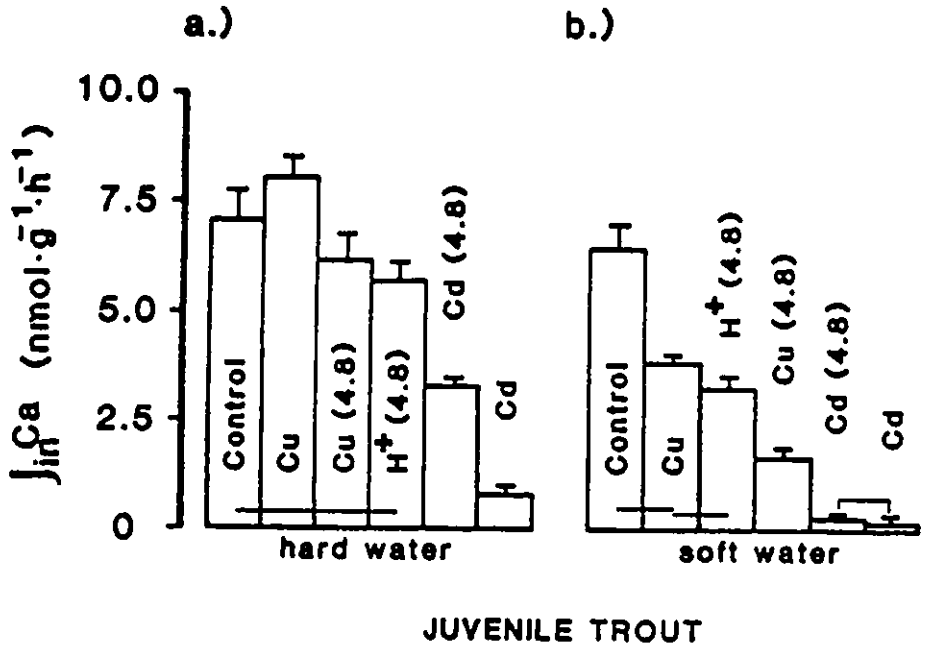

a.)
b.)
J_{in}^{Ca} (umol·kg^{-1}·h^{-1})
10.0
7.5
5.0
2.5
0
Control
H^+ (4.8)
Cu
Cu (4.8)
Cd (4.8)
Cd
hard water
Control
Cu
H^+ (4.8)
Cu (4.8)
Cd
Cd (4.8)
soft water
ADULT TROUT

Figure 2.4. Mean Ca^{++} influxes ($J_{in}Ca^{++}$) of juvenile rainbow trout over 24 h exposure to 6.5 $\mu mol \cdot L^{-1}$ cadmium or copper at circumneutral pH (pH 7.6) or pH 4.8. Data presentation and statistical comparisons as detailed for 2.3.

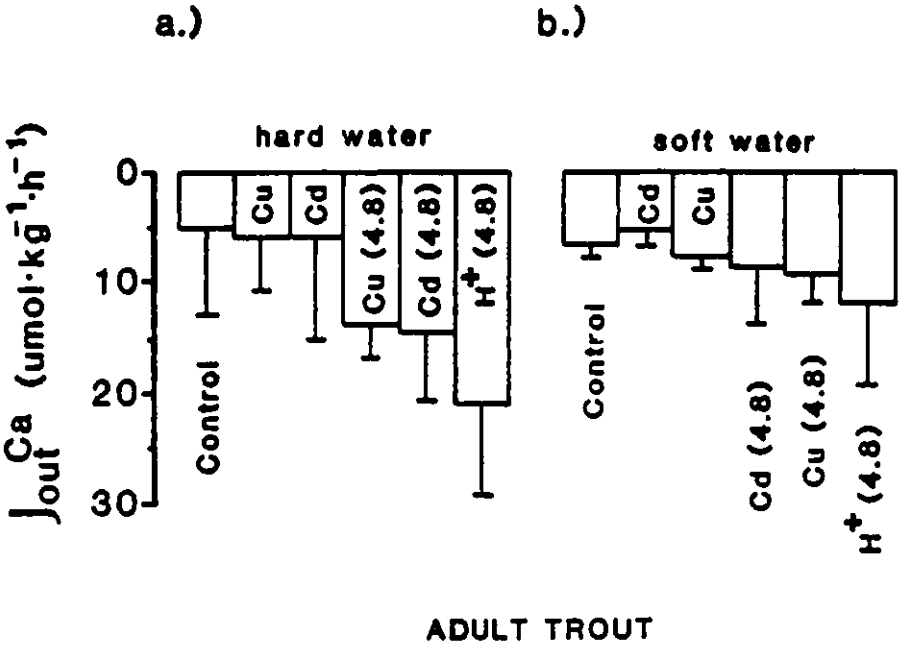
A. Hardwater-acclimated animals exposed in hard water ($[Ca^{++}]$ = 1 $mmol \cdot L^{-1}$).

B. Softwater-acclimated animals exposed in soft water ($[Ca^{++}]$ = 0.003 $mmol \cdot L^{-1}$).



JUVENILE TROUT

Figure 2.5. Mean Ca^{++} effluxes ($J_{out}Ca^{++}$) of adult rainbow trout over 24 h of exposure to 6.5 $\mu mol \cdot L^{-1}$ cadmium or copper at circumneutral pH (pH 7.6) or pH 4.8. Data presented as means ± 1 SEM. N = 6 for each treatment.


a.)
b.)
hard water
soft water
J_{out}^{Ca} (umol·kg^{-1}·h^{-1})
0
10
20
30
Control
Cu
Cd
Cu (4.8)
Cd (4.8)
H+ (4.8)
Control
Cd
Cu
Cd (4.8)
Cu (4.8)
H+ (4.8)

ADULT TROUT

large background. As a result, there were no statistically significant differences amongst the various treatments. Nonetheless, the data suggest that of the various exposures, only the low pH treatment stimulated Ca^{++} efflux. When low pH was combined with metals, the stimulation of Ca^{++} efflux by H^+ was slightly reduced. Alone, neither metal exposure, nor changes in water Ca^{++} appeared to alter $J_{out}Ca^{++}$ which overall averaged 5.9 ± 0.9 $\mu mol \cdot kg^{-1} \cdot h^{-1}$.

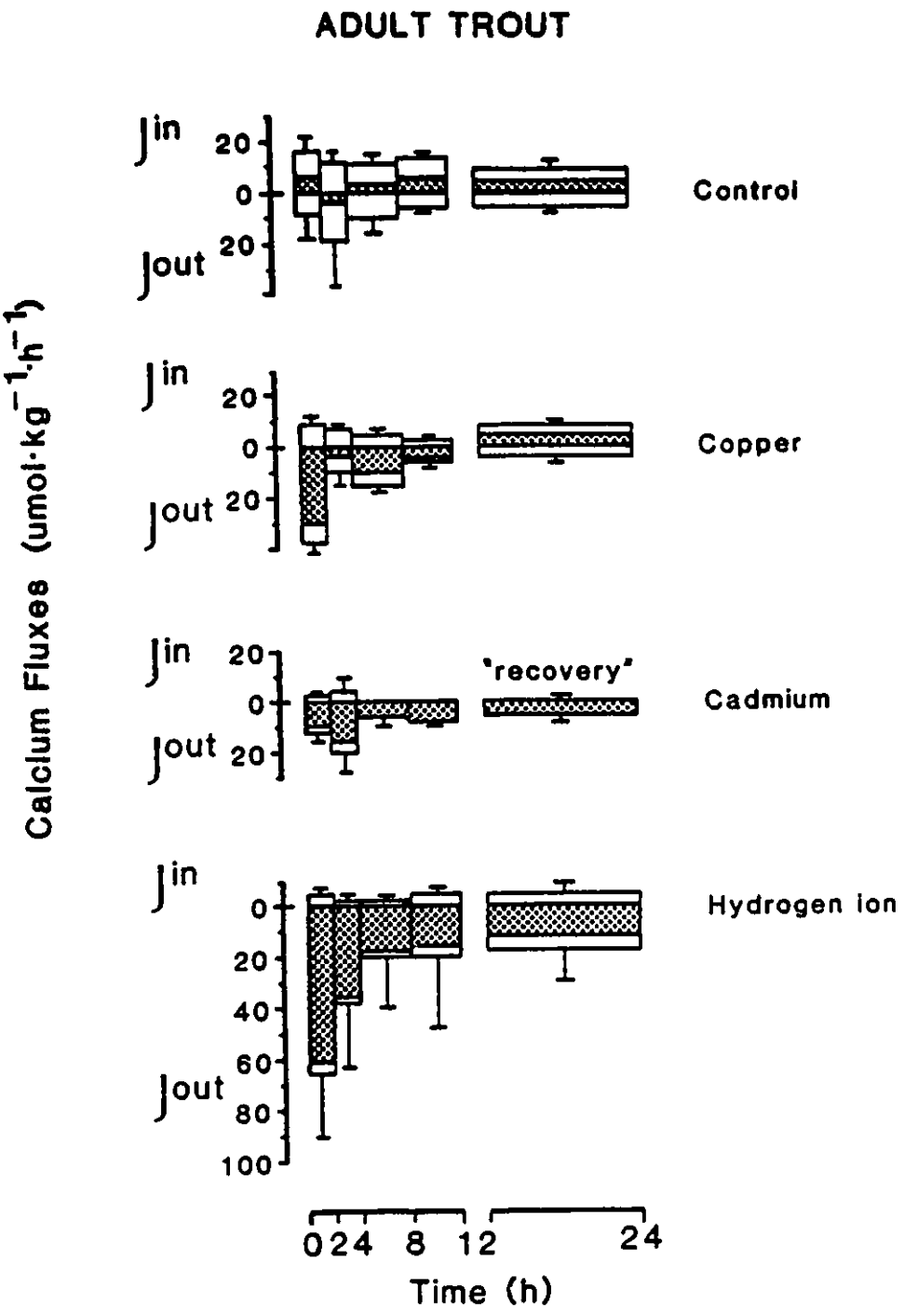
Persistence of the Disturbance

The 24 h averages for $J_{in}Ca^{++}$ and $J_{out}Ca^{++}$ (Figs 2.3-2.5) reveal marked differences amongst the various treatments. Even further differences are revealed when the development of the metal/H+ effects over time are examined. These data are shown in Fig 2.6 for adult trout acclimated to soft water. Similar data were obtained for hard water animals, but the effects were generally less (see Fig 2.3) and somewhat more variable as discussed above. As Fig 2.6 illustrates, most of the variation in time with unidirectional Ca^{++} flux rates can be attributed to the metal / H^+ exposures since J_{in} and J_{out} in controls changed little during the 24 h exposure period.

When fish were Cu-exposed, $J_{in}Ca^{++}$ gradually declined over the initial 12 hours of the experiment. $J_{out}Ca^{++}$ was initially stimulated but this response diminished over time and the average $J_{out}Ca^{++}$ was not significantly different from controls. Upon re-exposure, the trout were able to re-establish a positive $J_{net}Ca^{++}$ identical to that of the controls even though the metal was still present.

When fish were exposed to low pH, $J_{in}Ca^{++}$ was immediately inhibited and $J_{out}Ca^{++}$ stimulated leading to net Ca^{++} losses. Initially, $J_{out}Ca^{++}$ increased 13-fold but gradually decreased during the first 12 hours until stabilizing (at +4h) at a level about 2X

Figure 2.6. Unidirectional Ca^{++} fluxes (J_{in} and J_{out}, J_{net} indicated by stippled areas) of softwater-acclimated adult trout over the two 12 h exposure periods (controls, pH 7.8; copper, 6 $\mu mol \cdot L^{-1}$; cadmium, 6.5 $\mu mol \cdot L^{-1}$; hydrogen ion, pH 4.8). Data are means ± 1 SEM. N = 6 for all treatments. "Recovery" indicates that during this period, Cd^{++} was not re-introduced into the flux chambers; i.e. fish were allowed to recover from the initial 12 h of Cd^{++} exposure.


ADULT TROUT
Calcium Fluxes (umol·kg⁻¹·h⁻¹)
Jin
Jout
20
0
20
Control
Copper
"recovery"
Cadmium
Hydrogen ion
0
20
40
60
80
100
0 2 4 8 12 24
Time (h)

the control rate. During the final 12 hours of exposure, the H^+-exposed fish continued to lose calcium due to a slight reduction in influx and a stimulation of efflux.

Cadmium did not initially disrupt $J_{in}Ca^{++}$, rather, it took approximately 4 hours before significant inhibition was evident. Furthermore, cadmium had no significant effect upon $J_{out}Ca^{++}$. During the final 12 hour flux period, Cd was not re-introduced into the flux chambers. Although the Cd concentration was reduced to 9% of the initial exposure concentration (0.54 ± 0.066 $\mu mol \cdot L^{-1}$) by flushing with Cd-free water, $J_{in}Ca^{++}$ remained significantly inhibited.

Sodium fluxes

The effects of Cd, Cu and low pH on $J_{net}Na^+$ over 24 are shown in Figs 2.7 and 2.8. Control fish from both hard and soft water were found to be in Na^+ balance with net fluxes not significantly different from zero. Exposure to Cd alone, had little effect on Na^+ balance in adults (Fig 2.7) or juveniles (Fig 2.8). In both hard and soft water, H^+ exposure led to net losses of Na^+. These losses, although significantly different from zero, were not significantly different from controls. In contrast, Cu caused significant losses of Na^+ which were similar in hard and soft water. In adults these losses were 420 ± 65 and 465 ± 75 $\mu mol \cdot kg^{-1} \cdot h^{-1}$, respectively and in juveniles were found to be approximately twice as great: 760 ± 120 and 1080 ± 140 $\mu mol \cdot kg^{-1} \cdot h^{-1}$. Combining the metals with low pH did not significantly increase net Na^+ losses beyond those caused by exposure to the metals alone.

The effects of Cd and low pH on Na^+ influx ($J_{in}Na^+$) in juveniles are shown in Fig 2.9. In this experiment a range of Cd concentrations up to 1.6 μM was used, the maximum being about 5X the LC_{50}. Despite this, there were no mortalities over the 24 h exposure. At circumneutral pH, $J_{in}Na^+$ averaged 712 ± 26 $nmol \cdot g^{-1} \cdot h^{-1}$ and was not

Figure 2.7. Mean net Na^+ fluxes ($J_{net}Na^+$) of adult rainbow trout over 24 h of exposure to 6.5 $\mu mol \cdot L^{-1}$ cadmium or copper at circumneutral pH (pH 7.6) or pH 4.8. Data presented as means ± 1 SEM. N = 8 for each treatment. The horizontal lines connect bars which are not significantly different from one another at a confidence limit of 95% (ANOVA).

A. Hardwater-acclimated animals exposed in hard water ($[Ca^{++}]$ = 1 $mmol \cdot L^{-1}$).

B. Softwater-acclimated animals exposed in soft water ($[Ca^{++}$ = 0.03 $mmol \cdot L^{-1}$).

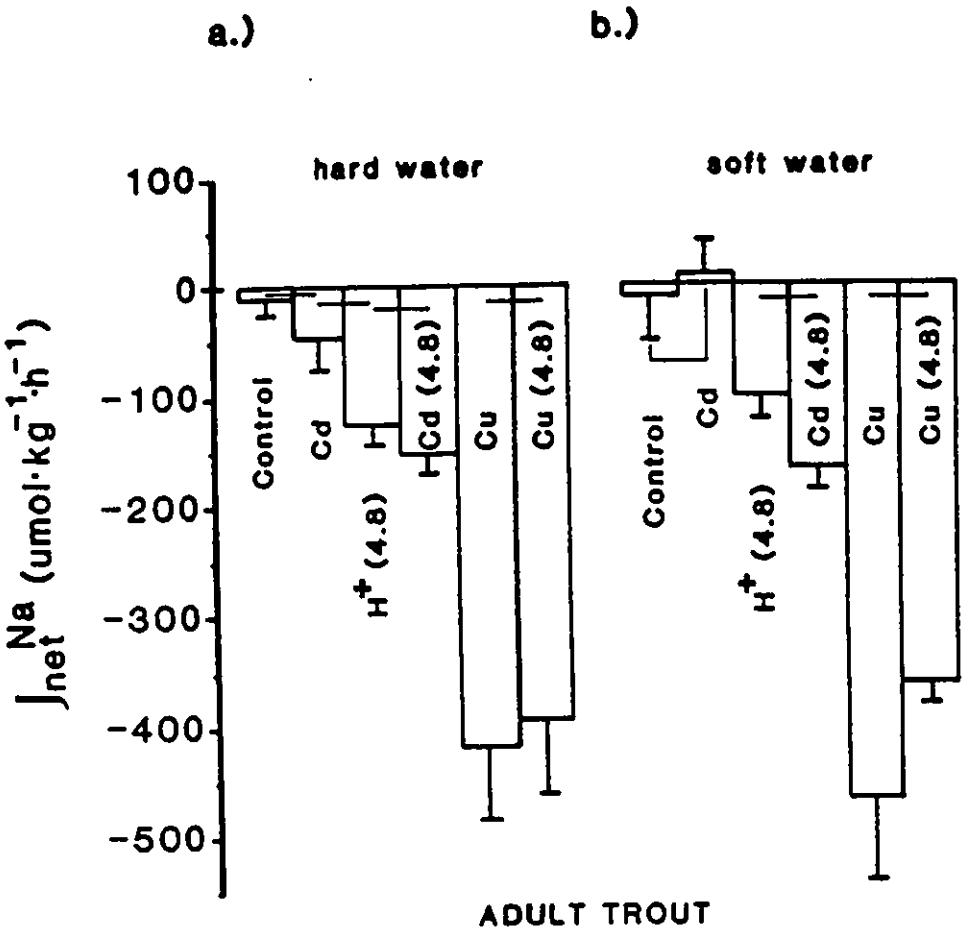

a.)
b.)
hard water
soft water
100
0
-100
-200
-300
-400
-500
J_{net}^{Na} (umol·kg^{-1}·h^{-1})
Control
Cd
H+ (4.8)
Cd (4.8)
Cu
Cu (4.8)
ADULT TROUT

Figure 2.8. Mean net Na^+ fluxes ($J_{net}Na^+$) of juvenile rainbow trout over 24 h exposure to 6.5 $\mu mol \cdot L^{-1}$ cadmium or copper at circumneutral pH (pH 7.6) or pH 4.8. Data presentation and statistical comparisons as detailed for Fig. 2.1. N = 10 for each treatment.

A. Exposures of hardwater-acclimated animals in hard water ($[Ca^{++}]$ = 1 $mmol \cdot L^{-1}$).

B. Softwater-acclimated animals exposed in soft water ($[Ca^{++}]$ = 0.03 $mmol \cdot L^{-1}$).

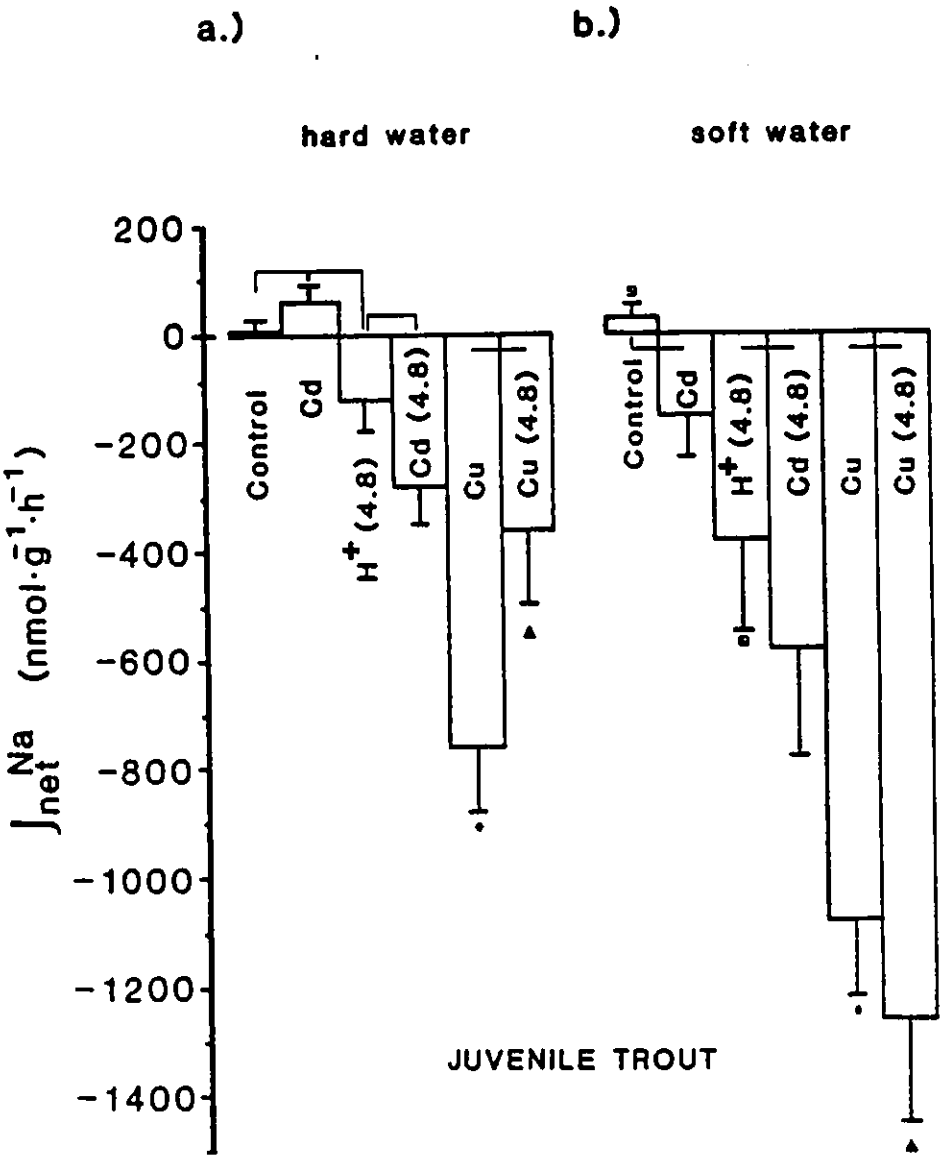
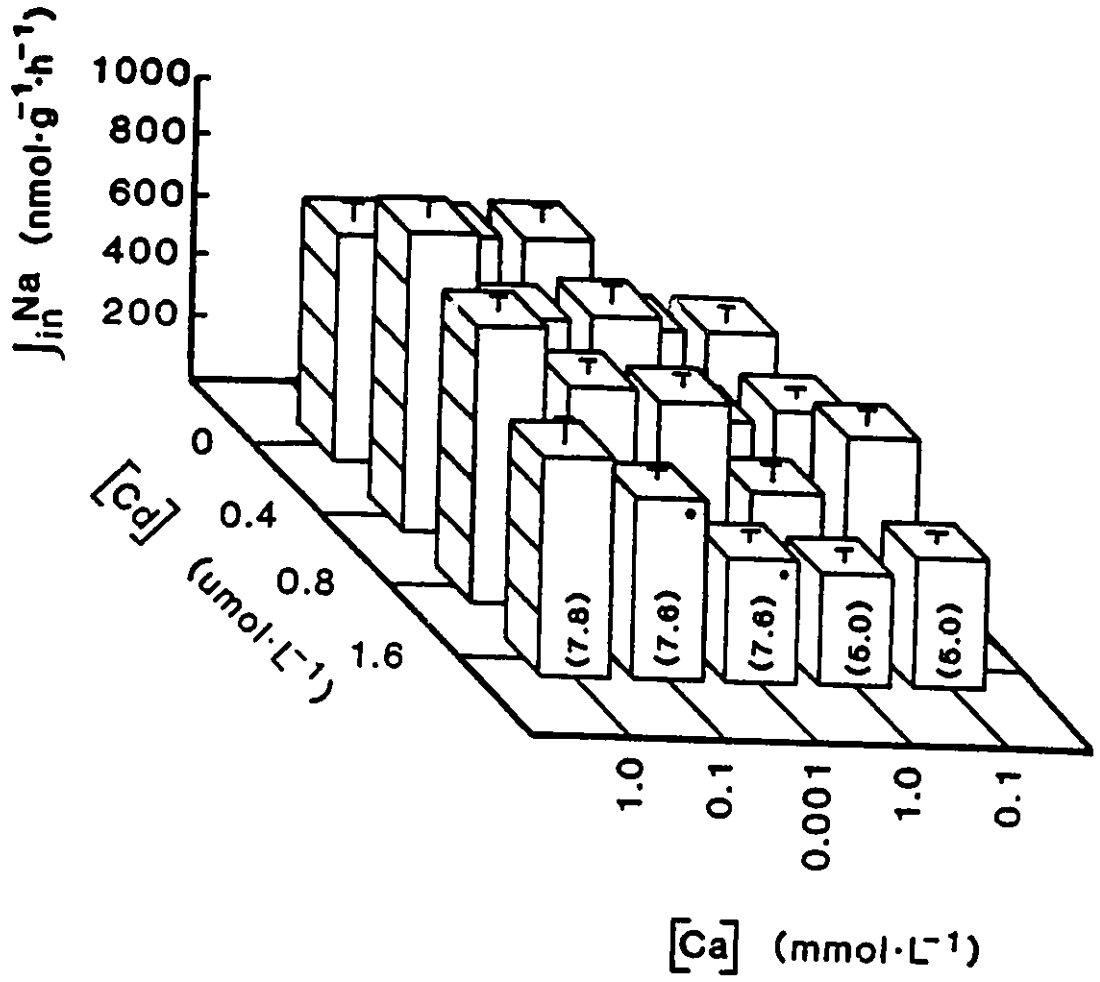

a.)
b.)
hard water
soft water
200
0
-200
-400
-600
-800
-1000
-1200
-1400
J_{net}^{Na} (nmol·g^{-1}·h^{-1})
Control
Cd
H+ (4.8)
Cd (4.8)
Cu
Cu (4.8)
JUVENILE TROUT

Figure 2.9. Effect of cadmium on Na^+ influx ($J_{in}Na^+$) in juvenile rainbow trout at different pH and water [Ca^{++}]. Data are presented as means ± 1 SEM. N = 10 to 14. Asterisks indicate mean significantly different from those animals at the same pH and [Ca^{++}] which were exposed to cadmium.


J_{in}^{Na} (nmol·g⁻¹·h⁻¹)
1000
800
600
400
200
[Cd]
(umol·L⁻¹)
0
0.4
0.8
1.6
(7.8)
(7.8)
(7.8)
(6.0)
(6.0)
1.0
0.1
0.001
1.0
0.1
[Ca] (mmol·L⁻¹)

influenced by external Ca^{++}. At pH 5.0, $J_{in}Na^{+}$ was reduced by about 55% and averaged 319 ± 26 nmol·g^{-1}·h^{-1} and again no influence of external Ca^{++} was apparent.

Cadmium had no effect on $J_{in}Na^{+}$ except at the highest concentration tested (1.6 μmol·L^{-1}) at pH 7.6. Here, water calcium had a protective effect; that is, the inhibition by 1.6 μmol·L^{-1} Cd was progressively greater with declining calcium concentration. At pH 5.0 and 1.6 μmol·L^{-1} cadmium, no significant inhibition was observed beyond that caused by pH 5.0 alone.

DISCUSSION

Although the characteristics of sodium and calcium regulation have been studied in the rainbow trout (e.g. Kerstetter et al 1970; Wood and Randall 1973; Perry and Wood 1985; McDonald and Rogano 1986) this is the first study to examine Na^{+} and Ca^{++} fluxes in both adults and juveniles. As a result we are able to offer some new insights into developmental changes in ion regulation. Table 2.3 lists a comparison of influx rates for both ions together with turnover rates calculated using whole body ion contents (after Shearer 1984). This analysis reveals two important points. Firstly, Na^{+} turnover (as a percent of whole body Na^{+}) is about 100 fold higher than Ca^{++} turnover in both juveniles and adults, thus indicating the much greater exchangeability of the Na^{+} pool. Secondly, turnover rates for both ions decrease with age. The drop is greater for Na^{+} than Ca^{++} and is nearly 50% in the former. This may be the result of a decrease in branchial ion permeability or may simply reflect a reduction in the weight specific surface area of the gills with growth (Morgan 1971). In either case, the phenomenon of decreasing ion turnover may at least partially explain the decreased sensitivity to toxicants with age. In the present study this was evident both as smaller net ion losses for the same severity of exposure (Fig 2.7 vs Fig 2.8) and in a much reduced mortality (Table 2.2). However, the important point is that the differences were mainly of a quantitative nature, i.e. the same

Table 2.3. Comparison of ion flux and turnover rates for rainbow trout. Data are from present study except where noted. References: (a) Shearer 1984, (b) Lauren and McDonald 1985, (c) Hughes and Morgan 1973.

	Juveniles	Adults
Weight (g)	1.5 - 2.5	170 - 300
$J_{in}Ca^{++}$ ($nmol \cdot g^{-1} \cdot h^{-1}$)	6.8	7.8
Whole body Ca^{++} ($\mu mol \cdot g^{-1}$)	86[a]	125[a]
Daily turnover (%)	0.19	0.15
$J_{in}Na^{+}$ ($nmol \cdot g^{-1} \cdot h^{-1}$)	712	320[b]
Whole body Na^{+} ($\mu mol.g-1$)	67[a]	56[a]
Daily turnover (%)	25.5	15.4
Gill surface area ($cm^2 \cdot g^{-1}$)	2.73[c]	1.99[c]

basic physiological responses were seen in juveniles and adults. This means that the toxic mechanisms of metal/H^+ may well be independent of growth at least over the size range 1.5 to 300 grams.

This study has clearly shown that cadmium, copper and hydrogen ions can each disrupt transepithelial ion exchange in the rainbow trout. However, the more important observation is that the effects of each were different from one another with respect to specific site of action, rapidity of action and persistence of the disturbance. Furthermore, copper and H^+ had only minor effects on Ca^{++} balance and major effects on Na^+ balance whereas the reverse was the case for cadmium.

In the ensuing discussion, we have assumed that the primary site of action of the metals is the gills, at least over the short term of exposure employed here. For the disturbances to Na^+ balance, this can be accepted with little question, since it is well established that the gills are the primary route for both uptake and loss of this ion (McDonald 1983a). This is perhaps less certain for calcium. Potential extra-branchial sites of calcium exchange include the intestine, the general body surface (scales), and the kidneys. Calcium absorption by the body surface has been shown to exist (Simmons 1971, Dacke 1979, Perry and Wood 1985), but the exact mechanism(s) and relative importance in calcium balance have not been determined. Intestinal uptake of calcium has also been shown to exist (Dacke 1979) but this may be relatively unimportant since non-feeding fish can satisfy their calcium requirements by direct branchial uptake (Ichii and Mugiya 1983) and even when feeding, only 35-50% of the total absorbed calcium is of dietary origin (McCay et al 1936, Berg 1968, Ichii and Mugiya 1983). The precise importance of renal calcium loss in calcium homeostasis has also yet to be determined. Nonetheless, renal

losses are likely second only in magnitude to losses across the gills. Under normal circumstances, renal calcium loss can amount to 50% of the total whole body efflux (Hobe et al 1984). However, Giles (1984) reported no significant change in renal electrolyte handling during 178 days of exposure to sublethal cadmium levels. This tends to suggest that while renal calcium losses may be significant, their contribution to the total loss is unlikely to increase in the face of metal/H^+ exposures.

The gill surfaces of fish, by analogy with other transport epithelia, are likely to contain a variety of complex organic anionic ligands which can probably act as cationic binding sites. Consequently much of the toxic action of metals and H^+ can likely be interpreted in terms of gill surface interactions (Pagenkopf 1983, McDonald et al 1986). Factors important for such interactions could then be any one or more of the following: the metal's formal charge, ionic radius, donor ligand preference, tendency to form covalent complexes and binding affinity. If these factors are considered then it is possible to explain why the branchial effects of H^+, copper and cadmium are different from one another.

Action of Cadmium

Cadmium appears to be much more specific in its action than either H^+ or copper, inhibiting calcium uptake in a persistent fashion (Fig 2.6) while having little effect on Ca^{++} efflux (Fig 2.5), net Na^+ flux (Fig 2.8) or Na^+ influx (Fig 2.9). In a variety of circumstances, cadmium has been shown to be a highly specific inhibitor of calcium-binding proteins. It is a blocker of Ca^{++} channels in neurons (Giles et al 1983) and has a higher binding affinity than calcium for calmodulin (Lock et al 1984, Suzuki et al 1985, Richardt et al 1986). Calmodulin has been found in the cell fraction of the gills containing the high affinity Ca^{++}-ATPase thought to be responsible for calcium influx (Flik et al

1985). Therefore, if the branchial uptake of calcium is calmodulin-dependent, then its blockade could easily lead to a disruption of calcium transport.

The specific biochemical nature of cadmium's action on the gills has not been resolved. Studies by Bansal et al (1985) showed that cadmium inhibited a Ca^{++}-ATPase isolated from the gills and heart of the freshwater teleost, *Saccobrancus fossilis*, but only at concentrations greater than 500 µM; at lower concentrations, cadmium was stimulatory. This is a somewhat contradictory finding since we show that cadmium at 6 µM inhibits calcium uptake *in vivo*. One possible explanation may be the particular ATPase examined. As Flik et al (1985) point out, there are both high- and low-affinity ATPase's present in the gills, and only the former is thought to play a role in calcium transport. However, Verbost et al (1988), also found an apparent stimulation of Ca^{++}-dependent enzymes in basolateral membrane vesicles prepared from rainbow trout branchial epithelium by Cd^{++} at concentrations between 0 and 100 µM. At greater concentrations, Verbost et al (1988,) demonstrated *in vitro* that Ca^{++} binding sites on active Ca^{++} transport systems have a high affinity for Cd^{++}. Furthermore, although the Ca^{++} pump appeared to be calmodulin dependent, Verbost et al (1988) concluded that the inhibition by Cd^{++} occurred directly on the Ca^{++}-binding site of the transporting ATPase and not via binding to calmodulin based on observed alterations in Ca^{++}-ATPase K_m and V_{max}.

Action of Copper

Copper had very little effect on calcium transport relative to cadmium. This is somewhat surprising since both are similar in toxicity (96h LC_{50} = 0.3 µM, c.f. McDonald et al 1986), were used in equimolar concentrations (6 $\mu mol \cdot L^{-1}$) and both have similar chemistries with regard to their tendency to form covalent complexes or ion pairs with ligands (Nieboer and Richardson 1980). Although perhaps oversimplified, their respective

ionic radii may be the key to the difference in their effects. Cadmium has a very similar ionic radius to that of calcium (0.097 vs 0.099 nm) whereas copper is much smaller (0.072 nm). Binding ligands that are highly specific for calcium are therefore much more likely to bind cadmium than copper.

Copper, on the other hand, is highly toxic to sodium transport. For example, Cu at 1.6 μM caused 72% inhibition of $J_{in}Na^+$ in juvenile rainbow trout (Lauren and McDonald 1986) whereas Cd at 1.6 $\mu mol \cdot L^{-1}$ (Fig 2.9) caused only 20% (at $[Ca^{++}] = 0.1$ $mmol \cdot L^{-1}$) or only 3% inhibition (at $[Ca^{++}] = 1.0$ $mmol \cdot L^{-1}$). Indeed, copper concentrations as low as 0.2 $\mu mol \cdot L^{-1}$ have been shown to inhibit sodium influx in rainbow trout (Lauren and McDonald 1985), an effect which has been shown both *in vivo* and *in vitro* to be due to the inhibition of branchial Na^+ / K^+-ATPase (*in vitro*, Lorz and McPherson 1976, Riedel and Christensen 1979; *in vivo*, Lauren and McDonald 1987b). Based on kinetic measurements, Lauren and McDonald (1987a) argued that the inhibition was of a non-competitive nature, due, perhaps, to damage of the ATPase (e.g catalysis of sulphydryl bridge formation) rather than to competitive Cu binding to the Na^+ / K^+ binding site.

Copper also has a much greater effect on the ionic permeability of the gills than cadmium. This is seen as the greatly increased net sodium loss relative to that induced by cadmium (Figs 2.7 and 2.8). This stimulation of efflux is thought to be the result of the weakening of the intercellular tight junctions brought on by the displacement of membrane bound Ca^{++} (McWilliams 1983, McDonald 1983b, Lauren and McDonald 1985). Since the intercellular (paracellular) pathway is not thought to be particularly ion specific (Marshall 1985), one might expect that copper should also increase $J_{out}Ca^{++}$. The absence of this occurrence (Fig 2.5) might be explained if Ca^{++} and Na^+ diffuse by a different route across the gills, but the simplest explanation may be the marked difference between Ca^{++}

and Na^+ in their respective transbranchial diffusion gradients. The ratio of blood to water concentrations for Na^+ in the present study was greater than 250:1 while for Ca^{++} it was only about 40:1 in softwater and 1:1 in hardwater (calculated assuming that 57% of blood Ca^{++} is ionic, Andreasen 1985).

If copper increases ionic permeability because of the displacement of calcium from surface ligands then one might expect cadmium to have a similar effect. Since it did not (Fig 2.5A) might be interpreted as meaning that Ca^{++} plays less of a role in gill membrane stability than has been hypothesized. Alternatively, cadmium may act in some respects as a calcium agonist, replacing calcium but having similar stabilizing effects on membranes.

Action of Hydrogen Ions

The present study shows that the action of H^+ is intermediate in many respects to that of copper and cadmium. Hydrogen ions have a somewhat greater effect than copper on calcium influx and efflux, and a smaller effect on sodium fluxes. In this regard, one can conclude that hydrogen ions are relatively weaker and less specific in their action upon the gills than either copper or cadmium. In fact, the effects of H^+ on the gills are potentially quite numerous and include: non-specific binding to surface ligands, competitive interactions with Ca^{++}, Na^+ and metals for surface ligands (Nieboer and Richardson 1980) and alteration of metal speciation (Campbell and Stokes 1985). This competition is evident in the case of cadmium where the inhibition of $J_{in}Ca^{++}$ (Fig 2.4A) and $J_{in}Na^+$ (Fig 2.9) was substantially less at pH 5.0 than at pH 7.6. No such effect was seen in the case of copper, instead H^+ and Cu were seen to be less than fully additive in their effects. The absence of a full additive interaction could be because copper has a much higher binding affinity than H^+ for various ligands or because of speciation changes in copper at pH 7.6 to pH 5.0. At pH > 7.0, a significant fraction of the total soluble copper will

be in the form of the first hydrolysis species of copper ($CuOH^+$) is presumably less surface-active, whereas at pH 5.0 or less, copper will be entirely in the form of the free ion, Cu^{++}.

Nonetheless, the competitive interactions of H^+ are important with regard to both copper and cadmium. Lowering pH has been shown to both reduce the toxicity of both copper and cadmium to rainbow trout (Table 2.2; also Cusimano et al 1986) and also to reduce the uptake of both metals (McDonald et al 1986, Lauren and McDonald 1986).

Conclusions

On the basis of this study, one can now draw three conclusions which should be useful in predicting toxic metal interactions in low pH environments:

1. Overall, cadmium appears to have what smaller impact on branchial ionoregulation than copper, at least over the 24 h period examined here. Nonetheless, these metals have similar acute toxicities (i.e. 96h LC_{50}'s). This apparent discrepancy may either be attributable to either greater persistence of cadmium's action relative to copper (i.e. branchial effects of cadmium become more severe with time), or that cadmium exerts a greater proportion of its toxic action internally (e.g. via neural or renal function).

2. Water calcium (i.e. water hardness) has a fundamentally different role to play in the toxicity of cadmium compared to copper. Calcium and cadmium are antagonistic whereas calcium has really little influence on copper's toxic action.

3. Reduction of water pH modifies the toxic effects of copper and cadmium in a largely protective fashion. However, this protective is illusory because it is the acidification process which is primarily responsible for increased metal levels in the first place.

CHAPTER 3

METAL BINDING ACTIVITY OF THE GILLS OF RAINBOW TROUT

INTRODUCTION

The gill is physiologically complex and structurally delicate, and consequently is the initial target of waterborne toxicant accumulation. This is illustrated by the observation that under conditions of acute exposure, the gill is the primary site of toxicity of several metals (Cu^{++}, Lorz and McPherson 1976; Lauren and McDonald 1985; H^{+}, McDonald 1983; Cd^{++}, Verbost et al 1987, Verbost et al 1988; Chapter 2; Zn^{++}, Spry and Wood 1985; Al Booth et al 1988)

In Chapter 2, it was shown that Cd^{++}, Cu^{++} and H^{+} disrupt gill function in rainbow trout by impairing transepithelial ion exchange. However, the more important observation was the qualitative differences amongst their effects. Since Cd^{++}, Cu^{++} and H^{+} undoubtedly react with biological ligands in fundamentally different ways, the differences in toxic effects could to related to any one or more of the following: charge, ionic radius, ligand binding preference (e.g. oxygen vs nitrogen or sulfur centers) and binding affinity (Nieboer and Richardson 1980).

Therefore, it is the goal of this chapter to characterize the metal binding activity of the external gill surface in an attempt to relate metal chemistry, gill surface binding activity and possibly, metal toxicity.

To accomplish this goal, a pharmacological approach to the binding of metals to the gill surface was used. With this approach, the gill surface is viewed as a tissue containing metal-binding ligands (metal "receptors"). The properties of the "receptors" were characterized by radiotracer binding techniques (i.e. *in vitro* titration of isolated

gills with metal radioisotopes) in association with the determination of key binding parameters, namely: the apparent dissociation constant (K_D), capacity (B_{max}), time to half-saturation ($t_{1/2}$) and the mode (cooperativity, H_n) of binding.

MATERIALS AND METHODS

Animals

Adult rainbow trout, *Oncorhynchus mykiss* (weight range 235 - 287 g), were obtained from a commercial hatchery (Silver Springs Trout Farm, Petersburg, Ont.). The fish were held in a 400-L polyethylene tank continuously supplied with aerated, dechlorinated Hamilton tap water at 13 to 16°C (hard water: ~1 mmol $Ca^{++} \cdot L^{-1}$, ~0.6 mmol $Na^{+} \cdot L^{-1}$). Trout were acclimated to the holding tank water chemistry and temperature for at least 2 weeks prior to experimentation. Fish were fed daily to satiation with commercial trout pellets and the tank was siphoned regularly to prevent the build-up of organic debris.

Experimental Protocol

Fish were killed with a single blow to the head. Individual gill arches were carefully removed from the animals and placed immediately in a 0.9% NaCl solution (15°C) until all arches from one animal were removed and the radiometal exposures could be initiated (2-3 min). Only the 3 most anterior gill arches from each side of the branchial basket were utilized from each fish in an attempt to maintain a standard gill weight (weight range 352 to 456 mg).

The exposure solutions (50 ml), containing radioisotopes of the appropriate metal, were held in 125 ml sample cups. The metals used in this study included: lanthanum (as $LaCl_3$), cadmium (as $CdCl_2$), calcium (as $CaNO_3$), and copper (as $Cu(NO_3)_2$). The radioisotopes of Ca (^{45}Ca) and Cd (^{109}Cd) were purchased from New

England Nuclear (NEN), while those of La (^{140}La) and Cu (^{64}Cu) were manufactured at the McMaster University Reactor. Each sample cup was fitted with an individual air line, to provide constant mixing, and were placed in a temperature controlled water bath. Water bath temperature (15 ± 1°C) was achieved using a Haake (model D1) heater/recirculator. Assay solution pH was adjusted to pH 6.7 (range 6.53 - 6.80). Four 5 ml samples of assay solution were taken, 2 prior to gill exposure, and 2 immediately after, for the determination of radioactivity and metal concentration.

Individual arches were removed from the saline and rinsed briefly in deionized water (15 sec) to clean the external surface of the isolated gills of debris associated with removal from the animal. This was followed by a subsequent 15 sec rinse in 5 mM EDTA (ethylenediaminetetra acetic acid, BDH Chemicals) following a procedure developed by McWilliams (1983). The EDTA was prepared as a potassium salt and neutralized to pH 7.7 with 0.1N KOH and was utilized to remove Ca^{++} and Mg^{++} for the gills prior to the *in vitro* metal exposures. Preliminary experiments determined that 5 mM EDTA was optimal for obtaining maximal and reproducible metal binding. Excess EDTA was then removed by quickly rinsing the tissue in another deionized water bath for 15 sec.

Once cleaned, the arches were used in one of two separately conducted sets of metal binding activity experiments to: i) characterize the time course of metal saturation of the external gill surface through the determination of the half-saturation time ($t_{1/2}$) or ii) characterize the binding kinetics of the metals to the gill surface through the determination of apparent dissociation constants (K_D), binding capacities (B_{max}) and Hill coefficients (H_n) of the various metal-gill surface interactions. In both sets of experiments, which were repeated at least twice, only one isolated gill was exposed to a radiometal solution per sample cup, with a total of 6 sample cups in use at any one time.

i) $t_{1/2}$

Isolated gills were exposed to 10^{-2} M radioisotope (10^{-3} M in the case of Cu^{++}) for either 0, 15, 30, 60, 90, 150, 300, 600, 900, 1500 or 1800 sec (N = 4 at each time). The specific activities of La^{+++}, Ca^{++}, Cu^{++}, and Cd^{++} were 18.0, 54.0, 11.5 and 54.0 kBq·mole^{-1} respectively.

ii) K_D, B_{max} and Hill coefficient

Additional isolated gills were exposed to one of 9 radioisotope concentrations ranging from 10^{-7} to 10^{-2} M (up to 10^{-3} M for Cu^{++}) for exactly 300 sec. The specific activities of La^{+++}, Ca^{++}, Cu^{++}, and Cd^{++} were kept constant at approximately 35.5, 106.7, 23.4, and 105.1 kBq·mole^{-1}, respectively, over the range of concentrations.

Following the radioisotope exposure, the isolated gill was immediately rinsed (15 sec) in deionized water to remove any metal loosely bound to the gill. For all metals, this brief rinse removed over 80% of the rapidly removable metal with only an additional 12% removed with a further 270 sec of rinsing. Excess rinse water was then gently shaken off the gill, the gill tissue was removed from the cartilaginous gill arch, and the tissue placed in a pre-weighed 20 ml scintillation vial (Fischer Scientific). Gill tissue was then digested at room temperature for 7 days in 3 mls of 3 N nitric acid (Fischer Scientific). Gill tissue digests were vortexed several times daily to ensure complete tissue digestion.

Analytical Methodology

Total gill-bound metals was determined from the tissue digests using either liquid or solid scintillation techniques, depending on the radioisotope used. One millilitre

aliquots of tissue digests, prepared in triplicate, from gills exposed to either ^{140}La, ^{64}Cu or ^{109}Cd were placed in 15x110 mm gamma counting tubes (John's Scientific) and counted on an Nuclear Chicago (model 1085) automatic gamma counting system with a 3x3 inch NaI(TI) crystal. Counts from samples containing ^{140}La (half-life = 40.9 h) or ^{64}Cu (half-life = 12.9 h) were corrected for decay.

Gill calcium (^{45}Ca) content was determined by mixing 100 µl of digest plus 4.9 ml of water with 10 ml of scintillation cocktail (ACS Amersham). Liquid scintillation samples, prepared in triplicate, were counted on an LKB Rackbeta and automatically corrected for quench against standards of known radioactivity and variable concentrations of tissue digest.

In all cases, bound metal was calculated on a per gram gill tissue wet weight basis according to the following equation:

$$\text{Bound Metal (M.g-1)} = \frac{\text{DPM x DF x SAw}}{\text{tissue wet weight}} \qquad 3.1)$$

where DPM is the background and quench corrected radioactivity of the tissue digest, DF is a correction factor to account for the dilution of the tissue digest, and SA_W representing the specific activity of the assay solution.

All assay solutions were prepared by serial dilution of concentrated (1 M) stock solutions. Metal concentrations were verified using atomic absorption flame photometry (Ca^{++}, Cd^{++}, Na^{+}, Cu^{++}) and flame emission photometry (La^{+++}) on a Varian AA (Model 1275) against known standards.

Data Analysis

Measurements of gill metal binding were analyzed using nonlinear regression (Duggleby 1981, Johnston 1985) and Scatchard plot techniques (Scatchard 1949).

Half-saturation time ($t_{1/2}$) was calculated from the time-saturation data using nonlinear regression (Duggleby 1981). Since the concentration-saturation curves appeared to possess a non-saturable binding component, two methods of obtaining K_D and B_{max} estimates of the saturable component were employed. In the first method, estimates of K_D and B_{max} were obtained directly from the original (total) saturation curves using nonlinear regression which included a linear term according to the model of Johnston (1985):

$$\text{Total Bound} = \frac{B_{max} \times [Me]}{K_D + [Me]} + (m \times [Me]) \qquad 3.2)$$

where m is the slope of the linear component which passes through the origin and [Me] represents the metal concentration. The second method consisted of a computer-based iterative curve-stripping technique where a linear component was removed according to the best fit of a Scatchard plot. A Scatchard plot is the graph generated by the following equation:

$$\frac{MeG}{Me} = -\frac{MeG}{K_D} + \frac{B_{max}}{K_D} \qquad 3.3)$$

where, for our application, Me is the free metal concentration (Free), MeG is amount of metal bound (Bound) to the gill (G), K_D is the apparent dissociation constant the gill for a metal , and B_{max} represents total binding capacity. By varying the amount of calcium in the assay solution and plotting Bound/Free against the amount Bound, the K_D and B_{max} are determined from the slope and x-intercept of the Scatchard plot, respectively. Good agreement was achieved between both methods. The latter method was used for all data analysis following the evaluation of both methods, as it proved to be a less time consuming method of analysis. Hill analysis (Hill 1910) was also employed to describe the nature of the metal binding according to the following equation:

$$\log [\%\ \text{total bound} \times (1 - \%\ \text{total bound})^{-1}] = Hn \times \log (Me) \qquad 3.4)$$

where [% total bound x (1- % total bound)$^{-1}$] is the "receptor" fractional occupancy and H_n is the Hill coefficient obtained from the slope of the linear regression of log (Me) vs log (fractional occupancy). An H_n equal to one indicates independent binding of a ligand to a site or sites with one apparent affinity, while an H_n significantly greater than one indicates positive cooperativity among binding sites and an H_n significantly less than one indicates negative cooperativity or multiple binding sites.

Statistics

Data are expressed as means ± 1 SEM (N). Comparisons between specific binding characteristics ($t_{1/2}$, K_D, and H_n) were made using a two tailed, unpaired Student's t-test at a 95% level of confidence. Regression lines (Scatchard and Hill plots) were fitted by the method of least squares, and the significance of the correlation coefficient (r) assessed. The coefficient of determination (R^2) was used as a measure of the amount of variation in the data fitted by both regression models. Lack of Fit tests were performed on an HP-3000 (Hewlett Packard) using MINITAB (Minitab Inc.) to determine possible curvature in the data, possible lack of fit at outer X-values and overall lack of fit of the model, with a level of confidence, in all cases, of 95%.

RESULTS

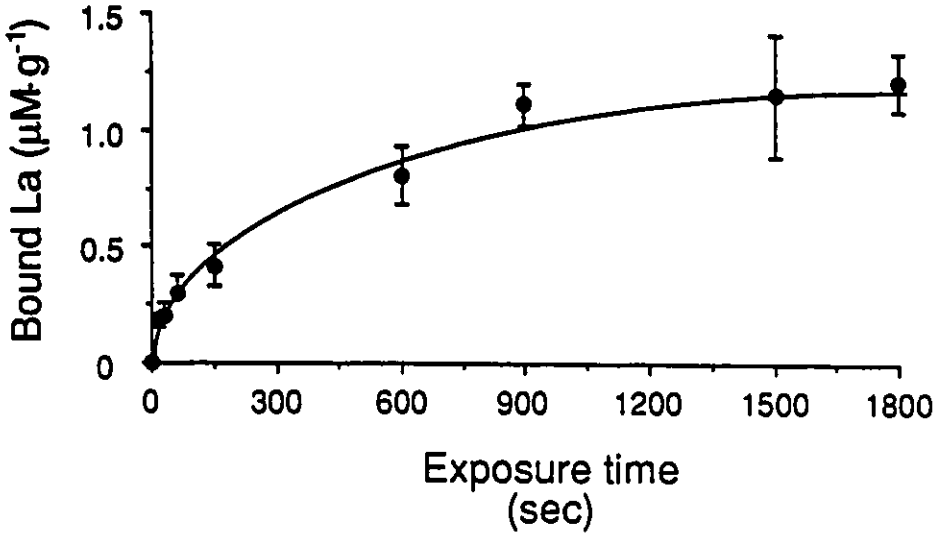
Initial experiments were performed to determine the time course of metal saturation of isolated gills. Initial experiments were conducted using La^{+++}, a metal previously shown to bind to the gill surface (McWilliams 1983) without penetrating the membrane surface (Weiss 1974, Perry and Flik 1988). Saturation of the gill with 10^{-2} M La^{+++} was found to occur rapidly (Fig 3.1) with a mean $t_{1/2}$ of 182 ± 11.5 sec (N=3, Fig 3.3A). Binding of La^{+++} to the gill surface was determined over a range of La^{+++}

Figure 3.1. Kinetics of gill La^{+++} binding. Data presented as means ± 1 SEM.

A. Time course for La^{+++} saturation of the gill micro-environment. Gills from hardwater-acclimated adults (n = 4) were removed, rinsed and exposed to 10^{-2} M La^{+++} (See Chapter 3 Methods for details). The characteristic $t_{1/2}$ was determined from this curve using non-linear regression analysis.

B. A La^{+++} saturation curve. Data was generated by exposing isolated gills to a range of La^{+++} concentrations for exactly 300 sec each (See Chapter 3 Methods for details). Saturable (closed circles) and non-saturable (open triangles) La^{+++} binding was calculated from total bound (open circles) using an iterative curve-stripping technique.

A


Bound La (μM·g⁻¹)
1.5
1.0
0.5
0
0
300
600
900
1200
1500
1800
Exposure time
(sec)

B

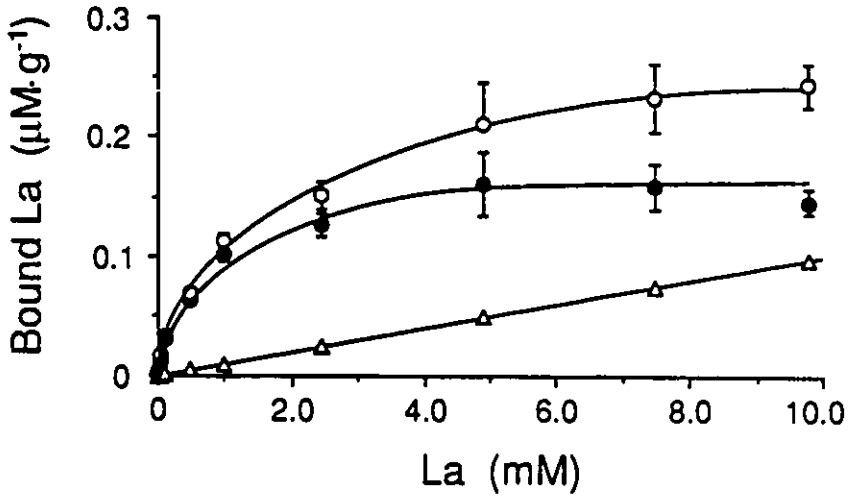
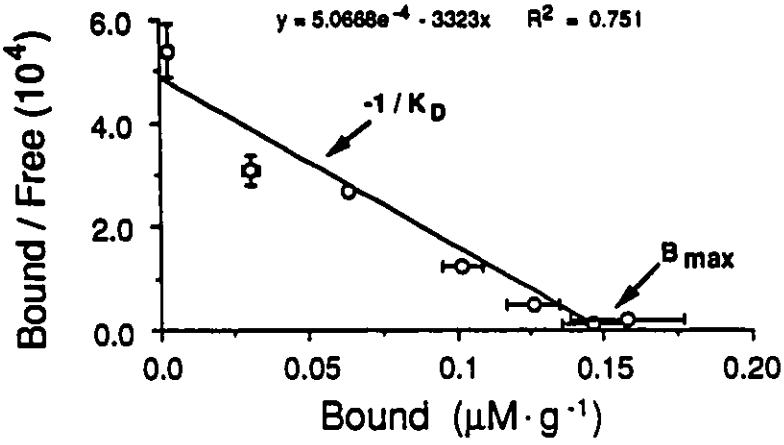

Bound La (μM·g⁻¹)
0.3
0.2
0.1
0
0
2.0
4.0
6.0
8.0
10.0
La (mM)

Figure 3.2. Characteristic parameters of the binding of La^{+++} to isolated gills. The specific component of the La^{+++} saturation curve (Fig. 3.1B) was used to calculate the apparent dissociation constant (K_D), capacity (B_{max}) and binding cooperativity (H_n) for gill La^{+++} binding. Data presented as means ± 1 SEM (N = 4).

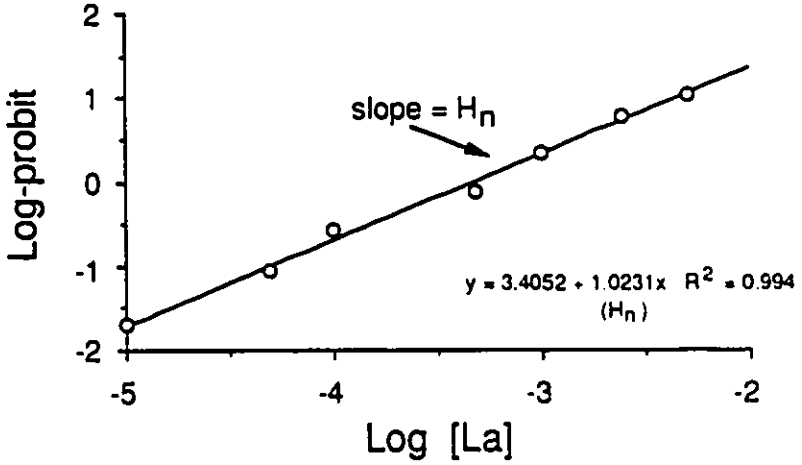
A. A La^{+++} Scatchard plot. The saturable component of the La^{+++} saturation curve (Fig. 3.1B) was transformed according to equation 3.2 for the determination of the K_D, and B_{max} of gills for La^{+++}. The equation of the Scatchard plot was calculated using least squares linear regression. The K_D and B_{max} are determined from the slope and x-intercept of this plot, as indicated on the figure.

B. A La^{+++} Hill plot. The specific component of a La^{+++} saturation curve (Fig. 3.1B) was transformed according to equation 3.3 for the determination of H_n. The equation of the Hill plot was calculated using least squares linear regression. The H_n represents the slope of the Hill plot, as indicated on the figure.

A


y = 5.0668e-4 - 3323x R2 = 0.751
-1 / KD
Bmax
Bound / Free (10^4)
6.0
4.0
2.0
0.0
0.0
0.05
0.1
0.15
0.20
Bound (μM · g-1)

B


slope = Hn
y = 3.4052 + 1.0231x R2 = 0.994
(Hn)
Log-probit
2
1
0
-1
-2
-5
-4
-3
-2
Log [La]

concentrations and appeared to have both saturable and non-saturable components, both of which were derived from total La^{+++} binding using the iterative curve stripping technique (Fig 3.1B). The saturable or specific La^{+++} binding data were used to generate Scatchard plots from which both the apparent dissociation constant (K_D) and binding capacity (B_{max}) of the gills for La^{+++} was determined (Fig 3.2A). The x-intercept of this Scatchard plot yields an estimate of the maximum amount of metal that can be bound by the gill micro-environment under present conditions (B_{max}), while the slope of the line is the negative inverse of the apparent dissociation constant (K_D). The La^{+++} saturation kinetic experiments were done a total of three times, yielding total, specific and non-specific La^{+++} binding curves that were reproducible. Based on this analysis, the gill was found to bind La^{+++} with a K_D of 0.31 ± 0.02 mM and B_{max} of 0.14 ± 0.001 $\mu mole \cdot g^{-1}$ (N=3, Figs 3.3B and 3.3C). The Hill plot (Fig 3.2B) was not significantly different from unity at 1.04 ± 0.04 (N=3, Fig 3.3D) indicating no cooperativity amongst binding sites or only one binding site on the gill.

The exact procedure by which characterization of gill La^{+++} binding activity was completed was then repeated using Cd^{++}, Ca^{++} and Cu^{++}. The findings of these experiments have been summarized in Fig 3.3. The $t_{1/2}$ estimates varied from 182 ± 11.5 sec for La^{+++} binding to 148 ± 6.7 sec for Cu binding, but were not significantly different from one another (Fig 3.3A). Similarly, no discernible differences between H_n for La^{+++}, Ca^{++}, Cd^{++} and Cu^{++}-binding were found although they ranged from 1.09 ± 0.04 for La^{+++} to 0.87 ± 0.09 for Cd^{++} (Fig 3.3D). However, marked differences between the K_D and B_{max} values for each metal were found.

The K_D for gill metal binding ranged from a low of 0.31 ± 0.02 mM for La^{+++} to a high of 4.3 ± 0.04 mM for Cu^{++} (Fig 3.3B). As affinity is inversely proportional to K_D, La^{+++} was, therefore, bound by the gills with the highest affinity. The affinity of Ca^{++}

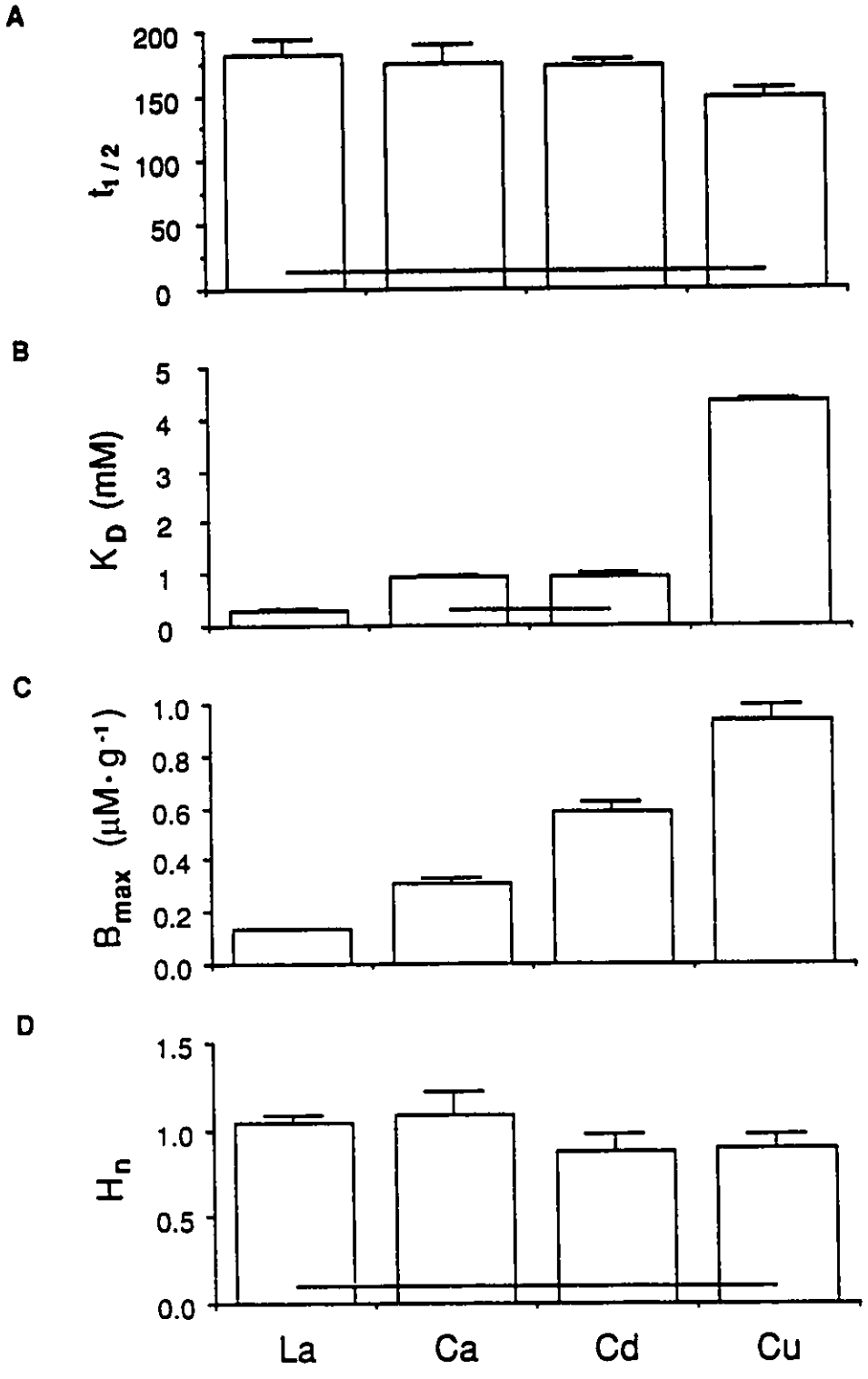
Figure 3.3. Metal binding activity of the gill micro-environment. This histogram represents a comparison of the specific binding characteristics for La^{+++}, Ca^{++}, Cd^{++} and Cu^{++}. These data are presented as means of the three replicates for each binding characteristic ± 1 SEM (N = 3). The horizontal lines connect bars which are not significantly different from one another at a confidence limit of 95%.

A. Half-saturation times ($t_{1/2}$).

B. Apparent dissociation constants (K_D).

C. Binding capacity (B_{max}).

D. Binding cooperativity or mode (H_n).


A
200
150
100
50
0
$t_{1/2}$
B
5
4
3
2
1
0
K_D (mM)
C
1.0
0.8
0.6
0.4
0.2
0.0
B_{max} ($\mu M \cdot g^{-1}$)
D
1.5
1.0
0.5
0.0
H_n
La
Ca
Cd
Cu

binding was found to be 3 fold lower than that for La^{+++}. However, the gills had nearly identical affinities for both Cd^{++} and Ca^{++}. The gills had the lowest affinity for Cu^{++}, which was found to be 4.6 times lower than the affinity of Cd^{++} or Ca^{++} binding, while being almost 14 times lower than that for La^{+++}.

There appeared to be a inverse relationship between the affinity (K_D^{-1}) and the B_{max}; the higher the affinity, the lower the binding capacity for that metal (Fig 3.3C). The binding capacity of the gills for La^{+++} was found to be significantly lower than for any other metal, while nearly 7 times as much Cu^{++} was accumulated by the gills compared to La^{+++}. The Ca^{++} and Cd^{++} B_{max} estimates were intermediate.

DISCUSSION

Methodology

In defining the binding kinetics of the surface of the gills of trout, a method of titrating entire isolated gills with radioisotopes of various metals was developed. McWilliams (1983) used a similar experimental protocol to investigate the effect of low pH on the rate of loss of surface-bound Ca^{++} in trout, as did Freda and McDonald (1988) in their investigation into physiological correlates of interspecific variation in acid tolerance in fish. The resultant binding data was characterized according to models used in radioreceptor assays. However, in contrast to classic receptor-ligand binding, it is highly unlikely that the binding of metals to the gills involves specific "receptors", although specific cation binding proteins have been shown to be present in the mucus of fish (calmodulin, Flik et al 1983).

The gill surface was found to saturate rapidly when exposed to La^{+++} ($t_{1/2}$ = 182.1 ± 11.5 sec, Table 3.6A) with the simple curvilinear nature of the binding curve suggesting titration of a single compartment. Exposure durations of about twice the $t_{1/2}$

(i.e. 300 sec) were chosen for the saturation kinetics experiments. The rationale for this choice was that the isolation of the gill surface as a site of metal binding was possible only with relatively brief exposure times, particularly in the case of certain metals which bind to a greater variety of sites than just on the gill surface (i.e. metals other than La^{+++}). In addition, brief exposure times reduced probable morphological alteration of the isolated gills and any modifications of cation binding over the duration of the exposure. One consequence of the brief exposure times, however, is the inability to determine absolute binding characteristics. In radioreceptor assays, exposure times are usually set at longer than one hour (12 times the $t_{1/2}$ of this study) since an equilibrium between the agonist and the receptor is generally reached within this time (Titeler 1981). Although this has not been done in the present study, the lack of a discernible difference in the $t_{1/2}$ estimates amongst the metals (Fig 3.3A) means that relative differences in the binding characteristics of the metals can be assessed.

The Micro-environment of the Gills

For the purposes of this study, it was assumed that the gill micro-environment consists of at least two compartments: the outer mucous layer covering the gill epithelium and the apical epithelial surface. There is, perhaps a third compartment, less well defined, which represents an unknown amount of sub-surface metal accumulation. Although the role of the third compartment in the characterization of the metal binding kinetics is unknown, its participation has been minimized with the use of brief exposure times, as previously mentioned.

The complete chemical composition of the gill micro-environment has yet to be established, however, many of the constituents of this region are known. The epithelial membrane, composed primarily of phospholipids (phosphatidylcholine,

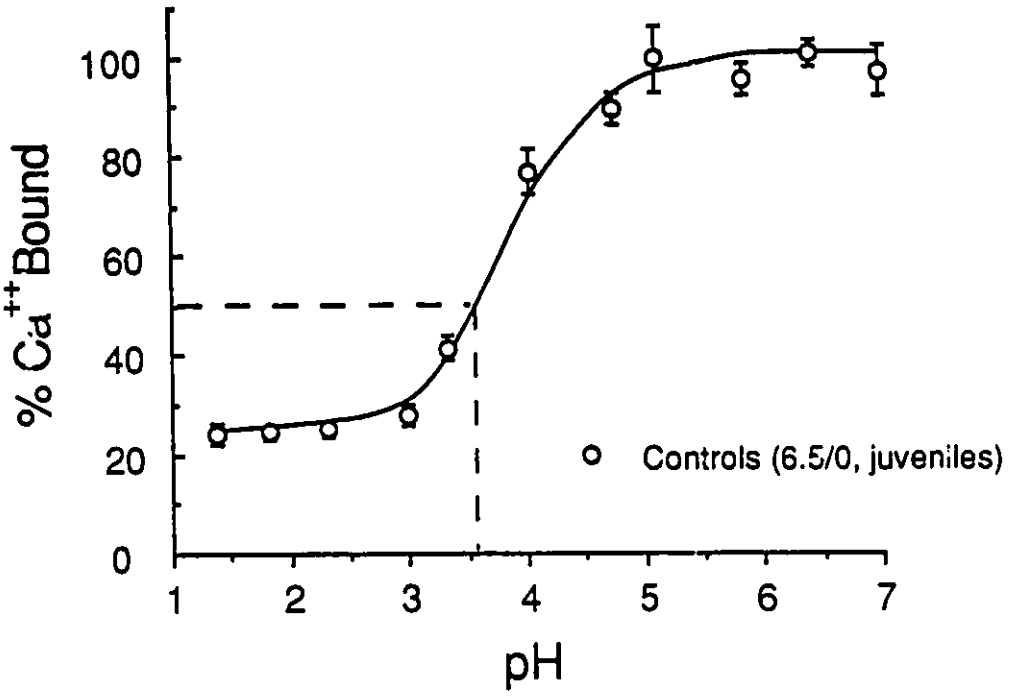
phosphatidylethanolamine, phosphatidylserine; Bolis et al 1984), is covered with a layer of mucus, a watery mixture of glycosaminoglycans (hyaluronic acid, chondroitin sulfate; Fletcher et al 1977, Wold and Selset 1977, Van de Winkel et al 1986). Of direct importance to the metal binding activity of the gills is the fact that the phosphate, carboxylate and sulfate groups of the chemical constituents of the gill surface have pK's ranging from 1.4 to 4.0 (Seimiya and Ohki 1973, Dawson et al 1986). Furthermore, the gill micro-environnment was found to have a measured pK of 3.6 (Fig 3.4). Therefore, at environmentally relevant pH's (pH >5), these groups will be fully ionized, resulting in a negatively charged gill surface and potential sites for metal-gill interactions (i.e. metal-"receptors").

Gill-Metal Binding

This study clearly demonstrates that, the binding activity of the gills for various metals can be characterized and that the gills exhibit relative differences in both the binding affinity and the binding capacity for various metals.

According to Nieboer and Richardson (1980), the relative binding affinity of a metal for biological ligands will be a function of the tendency of the metal to form ionic versus covalent bonds, and the particular chemistry of the ligand. This concept of metal-biological ligand interactions is the cumulation of several theories (Arhland et al 1958, Pearson 1968a, Pearson 1968b, Klopman 1968 and Klopman 1974), all of which are based on very similar principles thought to control cation-anion interactions. The classification scheme of Nieboer and Richardson is best summarized and visualized by the two parameters which they call the ionic index and the covalent index. The ionic index of Nieboer and Richardson (1980) provides an indication of the tendency of metals to form ionic bonds with biological ligands and takes into account both the formal charge and the

Figure 3.4. The pK of the gill micro-environment. Data replotted from Fig 6.7A to show the pK of the ionizable groups of the gill surface. The pK of the surface was taken as the pH at which the gill micro-environment was half-saturated with Ca^{++}. Curves through the data were fitted by eye with the data presented as means ± 1 SEM. N = 4.


% Ca++ Bound
100
80
60
40
20
0
1
2
3
4
5
6
7
pH
Controls (6.5/0, juveniles)

ionic radius. Metals with a strong tendency for ionic bond formation (class A metals; La^{+++}, Ca^{++}) tend to bind to oxygen-containing ligands (i.e. carboxylate, carbonyl, alcohol, phosphate or phosphodiester groups). The covalent index, on the other hand, is a measure of covalent bond formation, and is the product of the metal-ion electronegativity and the ionic radius. Metals with strong tendencies towards covalent interactions (class B metals) typically bind to nitrogen- or sulfur-rich ligands (i.e. sulphydryl, disulphide, thioether, heterocyclic nitrogen or amino groups). In addition, there is a class of metals (borderline metals; Cd^{++}, Cu^{++}) that exhibit both class A and class B characteristics.

The strength of this classification scheme lies in the biological perspective taken by Nieboer and Richardson (1980). In comparison with earlier models, Nieboer and Richardson (1980) most clearly addresses the biological significance of their model by detailing how their covalent and ionic indices can be used to predict or interpret the activity of metals at biological surfaces. It is for this reason that the model of Nieboer and Richardson (1980) has been used to aid in the interpretation of the metal-gill surface interactions.

To this point it has been assumed that the interaction on metals with the gill surface involves direct physical interactions; the forming of complexes at the gill surface (binding). However, there is an alternative interaction possible between cations and negatively charged surfaces which should be addressed. According to the Gouy-Chapman-Stern theory (Grahame 1947) or the diffuse double layer theory, cations in close proximity to a negatively charged surface, can hide or 'screen' the anionic groups from the bulk solution. This 'screening' results in an overall reduction in the negativity of the surface and, in the case of excitable cells, a subsequent depolarization of the membrane (Hille et al 1975). In the context of this study, 'screening' would result in a reduction of B_{max} and possible alterations in K_D. However, according to the double diffusion layer hypothesis,

there is no physical interaction (binding) between the anionic groups and the cation involved. This would imply that there would be no measurable apparent dissociation constant, yet the metal would still interact with the charged surface. 'Screening' is an unsatisfactory interpretation of the interaction of metals with the gill micro-environment for the following two reasons: 1) based on 'screening', it is difficult to explain the exclusion of one metal by another at the gill surface (Chapter 4) and 2) it has been demonstrated in this Chapter that the dissociation constants for the interaction (binding) of metals and the gill surface can be measured. These suggest that the results of this and subsequent Chapters are due to the direct physical interaction of metals with specific ligands. It is possible that 'screening' and binding represent the extremes of the way in which cations interact with negatively charged surfaces, with 'screening' the dominant event when the external cation concentration is very low, while binding becomes the dominant cation-surface interaction at greater cation concentrations.

The rank order of binding according to decreasing affinity as measured in this study (Table 3.1) are as follows:

$$La^{+++} > Ca^{++} = Cd^{++} > Cu \text{ (measured).}$$

The ranking of the these metals by way of decreasing ionic indices would be:

$$La^{+++} >> Cu^{++} > Cd^{++} = Ca^{++} > Cu^{+} \text{ (predicted)}$$

whereas the ranking of these metals by decreasing covalent indices would be:

$$Cu^{+} > Cu^{++} > Cd^{++} >> La^{+++} > Ca^{++} \text{ (predicted).}$$

The close agreement between the ranking of the binding affinities of these metals to the gill surface and their respective ionic indices (except Cu^{++}; see below) suggests that the metal-gill surface binding is according to ionic, not covalent, interactions (Table 3.1). Furthermore, since ionic interactions are more characteristic of oxygen-rich ligands (Nieboer and Richardson 1980), these data also suggest that the dominant metal-

METAL:	KD (mM)	B_{max} (µmol/g)	FORMAL CHARGE	IONIC[a] RADIUS (nm)	ATOMIC[a] NUMBER	96h LC50 (µmol/L)	IONIC[b] INDEX (Z^2/r)	COVALENT[b] INDEX ($X^2_m r$)
La	0.31	0.14	3+	0.106	57	------	8.5	1.3
Ca	0.93	0.31	2+	0.099	20	------	4.0	1.0
Cd	0.94	0.58	2+	0.097	48	0.3 [c]	4.1	2.9
Cu	4.31	0.93	2+	0.076	29	0.3 [d]	5.4	3.0

Table 3.1. Metal chemistry and toxicity summary

a. Weast et al (1984)
b. Nieboer and Richardson (1980)
c. Cusimano et al (1986)
d. Howarth and Sprague (1978)

"receptors" of the gill micro-environment consist of oxygen-rich centers (carboxylate, sulphate, phosphate) as opposed to nitrogen or sulphur centers (amino nitrogen, sulphydryl groups).

In support of the hypothesis that metal-gill interactions are predominantly ionic in nature, are the findings of Doyle et al (1980), who, using similar methods of data analysis, also found differences in the affinity of the cell wall of *Bacillus subtilis* for various metals (Na^{+}, Ca^{++}, Mn^{++}, Ni^{++}, Sr^{++}, Zn^{++} and Mg^{++}). The bacterial cell wall and the gill surface can be considered analogous since the bacterial cell wall is also thought to be negatively charged and accumulate metals due to an abundance of ionized carboxylate and phosphate groups (Beveridge and Murray 1980, Doyle et al 1980). Using the binding affinities for these seven metals of Doyle et al (1980) and ranking them in accordance with either their covalent or ionic indices, one finds that the affinity of binding is best correlated with the ionic tendencies of the metals (ionic: $r = 0.85$ vs covalent: $r = 0.34$, $n = 7$ for both).

Hille et al (1975) provide further evidence that the tendency of metals to form ionic bonds with biological ligands (strong ionic index) can be used to predict the physiological impact of those metals. Hille et al (1975) investigated the influence of several divalent metals (and hydrogen ions) on the sodium activation of frog myelinated nerve fibres. The presence of the metals in the regions of the fixed negative charges near the sodium channels resulted in shifts in the voltage dependence of sodium channel openings. Hille et al (1975) ranked the cations in the following sequence for positive shifts with equimolar exposure:

$$Zn^{++} > Ni^{++} > Mn^{++} \sim Co^{++} > Ca^{++} > Mg^{++} > Sr^{++} \sim Ba^{++}$$

Ranking these metals, as was done with the findings of Doyle et al 1980) in accordance with either their covalent or ionic indices, similarly, one finds that the effectiveness of the

metals is best correlated with the ionic tendencies of the metals (ionic: r = 0.72 vs covalent: r = 0.00, n = 8 for both). Nearly identical results are obtained using the data of Baustein and Goldman (1968; ionic: r = 0.74 vs covalent: r = 0.00, n = 8 for both). The objective of the Blaustein and Goldman (1968) study was similar to that of Hille et al (1975) with only the source of the axon (lobster) and assortment of cations (La^{+++}, Ni^{++}, Zn^{++}, Co^{++}, Cd^{++}, Ba^{++}, Ca^{++},. Mg^{++}) differing between studies.

The observation that Cu^{++} binding is not predicted by its ionic index suggests either that Cu^{++} reacts at the gill surface in a form other than Cu^{++}, or that the ionic index may not be entirely appropriate for predicting all metal-gill surface interactions. Although Cu has more than one form (the two oxidation states, Cu^{++} and Cu^{+}, and the first hydrolysis species of Cu^{++}: $CuOH^{+}$), there are two reasons why the kinetics of gill surface Cu^{++} binding can not be explained on the basis of any other Cu form than Cu^{++}. Firstly, Cu^{++} is the predominant aqueous metal species at the pH of the *in vitro* exposures (pH 6.5). Cu^{++} would make up approximately 70% of the total with the remainder being $CuOH^{+}$ (Campbell and Stokes 1983, Cusimano et al 1986). Secondly, Cu^{+} is 6 orders of magnitude less stable in aqueous environments than Cu^{++} and has a tendency to auto-oxidize to Cu^{++} (Buckingham 1973). Therefore it is unlikely that Cu^{+} would be present at the gill surface.

Thus, the question is: What other properties of Cu^{++} might explain its weaker-than-expected binding affinity? The answer may well lie with the somewhat unique coordination chemistry of Cu^{++}. Coordination chemistry describes the specific bonding patterns of metals in metal-ligand (Me.L) complexes (Mortimer 1979). A Me.L complex typically consists of a central metal cation to which several ligands (anions and/or uncharged molecules) are bonded. The number of atoms directly bonded to the central metal ion is referred to as the coordination number. Multidentate ligands or chelates, are ligands capable of occupying more than one coordination position of the metal ion.

Cu^{++} typically has a coordination number of 4 which results in a square-planar coordination geometry, i.e. 4 donor atoms (preferably nitrogen and/or sulfur), are arranged around Cu^{++} such that the bonds are located on the same plane in space. In contrast, all of the other metals of this study have coordination numbers ranging from 6 to 9 and consequently more complex coordination geometries (i.e. tetrahedral, octahedral). The weak binding of Cu^{++} suggests that within the gill micro-environment, the preferred coordination geometry of Cu^{++}, involving the available ligands, is not easily established. Consequently, one can conclude that the metal-"receptors" at the gill surface are more likely multidentate ligands, with greater than 4 donor atoms (from carboxylate, sulfate and phosphate groups) involved in the metal coordination. Since the gill micro-environment is thought to be a collection of flexible polyanions in an aqueous environment, the coordination of metals by multiple donor atoms is not an unreasonable interpretation.

The apparent relationship between B_{max} and K_D (i.e. that B_{max} increases as the binding affinity decreases, Table 3.1) is surprising as one would expect the number of binding sites to remain constant even though binding affinities may vary. This may very well be the case for the binding of La^{+++} and Ca^{++} but may not be an appropriate assumption for Cd^{++} or Cu^{++}-binding. The differences in the B_{max} of the latter two might simply be an indication that these metals bind to different anionic groups or "receptors" and that the concentration of these "receptors" are not the same as those that bind either La^{+++} or Ca^{++}. In terms of ligand preference, both La^{+++} and Ca^{++} specifically bind through oxygen. If one assumes that there is a fixed number of oxygen atoms available, then it would be expected that the binding capacity for La^{+++} would be approximately 33% lower than for Ca^{++}. This calculation is based on the premise that one atom of La^{+++} would neutralize three anionic groups, compared to two by Ca^{++}. In contrast, since Cd^{++}

and Cu^{++} are borderline metals, they share a tendency to bind to a more diverse selection of ligands than La^{+++} and Ca^{++} (which bind exclusively to oxygen-rich ligands). Therefore, it would be expected that the gill binding capacity for Cd^{++} and Cu^{++} would be greater than for La^{+++} and Ca^{++}, as the borderline metals bind to more sites on the gill, independent of binding affinity. Cu^{++}, having the greater tendency to interact with non-oxygen donor atoms of the two borderline metals, would likely bind to more sites than Cd^{++}, as was observed (Fig 3.3C). A variety of binding sites may not necessarily be identified using Scatchard or Hill plots if the affinities of all sites are not significantly different and the additional binding sites are only a minor component of the total sites available for metal-surface interactions.

Mechanisms of Toxicity

The question posed now, is: In what way do the binding characteristics of the metals correlate with their toxicity? A low affinity of the gill surface for a metal can be expected to be more permissive of entry to the intracellular compartment. Once there, a variety of more complex binding sites are present, i.e. calmodulin, glutathione, metallothionein and ATPases. Binding to these ligands could then result in any one, or a combination of the following 3 types of toxic mechanisms: 1) blocking of the essential biological functional groups of biomolecules, 2) displacing the essential metal ion in molecules, and 3) modifying the active conformation of biomolecules (c.f. Ochiai 1977). These mechanisms of metal toxicity can be used to explain the specific inhibition of active ion transport resulting from both Cd^{++} and Cu^{++} exposure (Chapter 2; Verbost et al 1987, 1988; Lauren and McDonald 1987). Both are thought to bind to specific basolateral ATPases (Verbost et al 1987, Verbost et al 1988, Lauren and McDonald 1987). Cu^{++} is more disruptive to gill function than Cd^{++} (Chapter 2) likely because it has a stronger tendency

ATPase itself (Lauren 1988). In addition, the affinity of the gill surface for Cu^{++} is lower than for Cd^{++} (Fig 3.3C) which may indicate that the effective dose of Cu^{++} in Chapter 2, was in fact, greater than Cd^{++} even though the concentrations were equimolar. This would result, if, as shown by Part and Locke (1976), that a greater surface binding affinity more effectively retards metal penetration than lower affinity binding. This interpretation is independent of the Cu^{++} species present since both Cu^{++} and $CuOH^{+}$ are toxic (Lauren and McDonald 1985). Thus, Pagenkopf's (1983) contention that the greater the binding affinity, the greater the toxicity, may only be correct for the interaction of a metal with the specific site at which the metal manifests its toxic effect, and not with the overall surface.

On the other hand, metals have also been shown to directly influence the ionic permeability of the branchial membrane, promoting substantial net ion loss which can lead to fish mortality (McDonald 1983, McDonald et al 1988). This impact of metals on gill permeability has been attributed, in large part, to displacement of surface bound Ca^{++} (McWilliams 1983, Lauren and McDonald 1985, Booth et al 1988, Freda and McDonald 1988, McDonald 1988, McDonald et al 1988). The development of this particular toxic mechanism can be associated with the fact that Ca^{++} stabilizes the epithelial membrane, consequently reducing ionic permeability (Hille et al 1975, Steen and Stray-Pederson 1975, Oschman 1978). The displacement of Ca^{++} and subsequent increase in permeability would occur only if a metal had an equal or greater relative affinity for the Ca^{++}-"receptors", and in addition, did not assume the membrane stablizing function of Ca^{++}. The phenomenon of direct competition between toxic trace metals and Ca^{++} for gill binding sites is the subject of the next chapter.

CHAPTER 4

COMPETITIVE INHIBITION OF GILL CALCIUM BINDING

INTRODUCTION

One of the central principles in the mechanisms of action of surface active toxicants to fish is competition between hardness cations (i.e. Ca^{++} and Mg^{++}) and toxicants (H^+, trace metals) for gill surface ligands. This notion arises from two separate themes in the fish toxicology literature. Firstly, starting from the early work of Brown (1968), a number of studies have now clearly shown that hardness cations, particularly Ca^{++}, are effective in reducing toxicity of a number of metals. Secondly, a number of other studies also suggest that it is, in fact, the displacement of Ca^{++} from the gill surface that is responsible for a significant fraction of the toxicity of metals and H^+ (Cd^{++}, Chapter 2; Verbost et al 1987, Verbost et al 1988; Zn^{++}, Spry and Wood 1985; Cu^{++}, Lauren and McDonald 1985; Al, Booth et al 1988; H^+, McDonald 1983). Competition between hardness cations and toxicants for gill surface ligands is of prime importance to the models of both Zitko and Carson (1976) and Pagenkopf (1983). As discussed in the preceding chapter, these models attempt to account for the variability in toxicities of trace metals (e.g. Zn^{++}, Cu^{++} and Cd^{++}) to fishes and relating these toxicities to variations in alkalinity, pH (Pagenkopf 1983) and hardness (Pagenkopf 1983, Zitko and Carson 1976).

Calcium displacement is considered deleterious to proper gill function, as Ca^{++} has the ability to cross-link ligands (Williams 1974), which stabilizes the gill membrane and reduces gill electrolyte permeability (Potts and Fleming 1971, Cuthbert and Maetz 1972, Steen and Stray-Pederson 1975, Oscham 1978, McDonald and Rogano 1986). For metals and H^+ to displace Ca^{++} implies that they bind to the same sites as Ca^{++}. Since, it was previously concluded (Chapter 3) that the ionic index of a metal greatly influences its

affinity for the gill, one might predict that metals with ionic indices greater than or equal to that of Ca^{++}, would compete with Ca^{++} for sites on the gill surface.

Therefore, the objective of the present study was to determine if, and in what manner, trace metals and H^+ compete with Ca^{++} for binding sites on the gills. To address this issue, a pharmacological approach to metal-gill interactions has again been used. With this approach, competition for sites normally occupied by Ca^{++} was quantified with the determination of IC_{50}, i.e. the concentration of antagonist, either metal or H^+, which resulted in a 50% reduction in agonist (Ca^{++}) binding.

MATERIALS AND METHODS

Animals

Adult rainbow trout, *Oncorhynchus mykiss* (245-297 g), were obtained from a commercial hatchery (Silver Springs Trout Farm, Petersburg, Ont.). The fish were held in a 400 L tank and acclimated for at least 2 weeks to dechlorinated Hamilton tap water containing ~1 $mmol \cdot L^{-1}$ calcium and ~0.6 $mmol \cdot L^{-1}$ sodium. The holding tank was continously supplied with water varying in temperature from 13 to 15 °C. Fish were fed daily to satiation and the tank was syphoned regularly to prevent build-up of organic debris.

Experimental Protocol

Fish were killed with a single blow to the head. Complete gill arches (0.4 - 0.9 g) were carefully removed from the animals and placed immediately in a 0.9% saline solution. Only the 3 most anterior gill arches from each side of the branchial basket were used from each animal. All gill arches were cleaned prior to exposure in distilled water, 5 mM EDTA, which was followed by fresh distilled water as outlined in Chapter 3 methods.

Gill arches were exposed, individually, to 50 ml of assay solution contained in 125 ml sample cups for 300 seconds. Sample cups were fitted with small air lines to provide vigorous mixing of the exposure solution. Assay temperature was maintained at 15 ± 0.5°C by placing the sample cups in a constant-temperature water table (Haake heater/recirculator model D1).

The assay solutions for the competitive inhibition experiments contained a constant concentration of Ca^{++} and ^{45}Ca (specific activity: 1.33 mM, 370 kBq $^{45}Ca \cdot L^{-1}$; New England Nuclear) with concentrations of either lanthanum (La^{+++}), cadmium (Cd^{++}), or hydrogen ion (H^{+}) ranging from approximately 10^{-7} to 10^{-2} M. The sensitivity of Ca^{++}-binding to copper (Cu^{++}) was also determined, however, the Cu^{++} concentrations ranged from 10^{-7} to only 10^{-3} M due to the solubility of Cu^{++} under experimental conditions. The pH of these solutions was adjusted to 6.56 (range: 6.49 - 6.7) except in the case of the H^{+} inhibition experiments (range: 6.98 - 1.21).

Following exposure of the isolated gills to the assay solution (300 sec), the gill arches were briefly rinsed in distilled water (15 sec) and excess water shaken from the arch. Gill tissue was then removed from the arch, weighed and digested in 3.5 ml of 3N nitric acid overnight at room temperature.

Analytical Methods

Total bound calcium was determined from the amount of bound radiocalcium according to equation 3.1, with radioactivity measured using liquid scintillation counting techniques. A 100 µl aliquot of tissue digest and 4.0 ml of deionized water was added to 10 ml of scintillation fluor (ACS Amersham), mixed thoroughly using a vortex, then placed in a darkened area for 12 hours to reduce chemiluminescence. All samples were prepared in triplicate. Samples of the exposure solutions, taken pre- and post-gill exposures, were

similarly prepared in duplicate for the determination of the assay solution specific activity. Radiocalcium activity was detected on a LKB Rackbeta with a quench correction package. A quench curve was generated with counting samples containing a known amount of ^{45}Ca activity and a range of dilutions of gill tissue digests.

The solutions of Cd^{++} (as $CdCl_2$), Cu^{++} (as $Cu(NO_3)_2$), and Ca^{++} (as $Ca(NO_3)_2$) were prepared by serial dilution of concentrated stock solutions. Dilutions and concentrations were verified using atomic absorption flame photometry (Varian AA-1275). La^{+++} (as $LaCl_3$) exposure solutions were similarly prepared. However, flame emission photometry (Varian AA-1275) was used to confirm expected concentrations of La^{+++}.

Data Analysis

In direct competitive inhibition experiments, the concentration of $^{45}Ca^{++}$ and the amount of tissue are kept constant, while the competing metals concentration (La^{+++}, Cd^{++}, Cu^{++}, or H^{+}) is varied. If the metal binds to the same sites a Ca^{++}, it will interfere with the binding of $^{45}Ca^{++}$. The equation describing this type of experiment is as follows:

$$\frac{CaG}{B_{max}} = \frac{1}{(1 + K_D^*/[Ca])(1 + [Me]/K_{Da})} \qquad 4.1)$$

where CaG = the amount of Ca^{++} (radioactive) bound to the gill micro-environment, B_{max} = the binding capacity of the gill micro-environment, K_D^* = the apparent dissociation constant of the gill micro-environment for Ca^{++}, Me = the Ca^{++} antagonist (La^{+++}, Cd^{++}, Cu^{++} or H^{+}) and K_{Da} = the apparent dissociation constant of Me for sites normally occupied by Ca^{++}.

At low concentrations of competing ligand, Me/K_{Da} approaches zero, and little or no competition is observed. At high concentrations of competing ligand, $Me/K_{Da} >> 1$,

and the per cent Ca^{++} bound (CaG/B_{max}) is reduced. Competition curves are generated when the percentage of total Ca^{++} bound is plotted against the log antagonist concentration.

The IC_{50} is the concentration of metal at which one-half of the binding sites, originally occupied by Ca^{++}, are occupied by the antagonistic metal. The IC_{50} is obtained following linearization of the competitive inhibition curve using the ln-logit transformation according to Sandor et al 1984:

$$\text{Ln-logit} = \ln (CaG \times (B_{max} - CaG)^{-1}) \qquad 4.2)$$

The IC_{50} values are taken as the antagonist concentration at a ln-logit value of zero when the ln-logit values are plotted against the log inhibitor concentration.

Statistics

Data are presented as means ± 1 SEM. Regression lines were fitted to the ln-logit transformed data by the method of least squares, and the significance of the coefficient of determination (R^2) and correlation coefficient (r) assessed. Comparisons between the estimated IC_{50}'s were made using a two-tailed, unpaired Student's t-test at a 95% level of confidence. Standard errors of the mean IC_{50} estimates were calculated based on the individual estimates of IC_{50} obtained from the competitive inhibition experiments completed in triplicate.

RESULTS

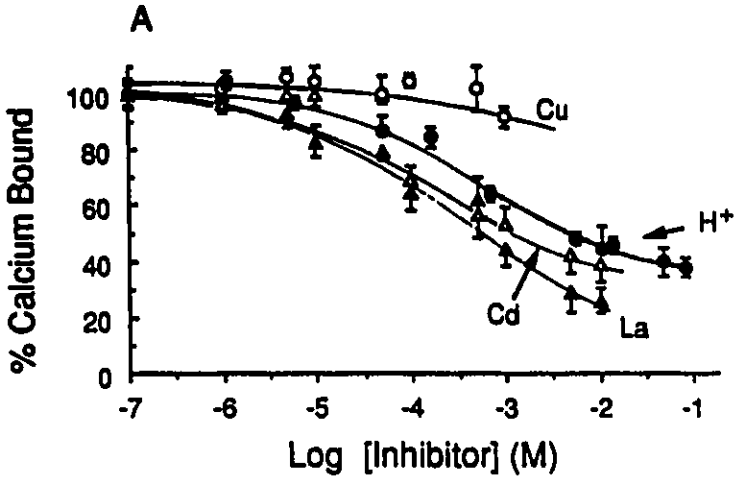
Of the metals tested, three of the 4 (La^{+++}, Cd^{++} and H^{+}) showed a sigmoidal relationship between their log concentration and the amount of Ca^{++} bound to the gills (Fig 4.1A) suggesting direct competition between these metals and Ca^{++} for sites normally occupied by Ca^{++}. While these metals resulted in maximal reductions in Ca^{++} binding of 60-80%, Cu^{++} resulted in only a minor inhibition of Ca^{++} binding. Concentrations of Cu^{++}

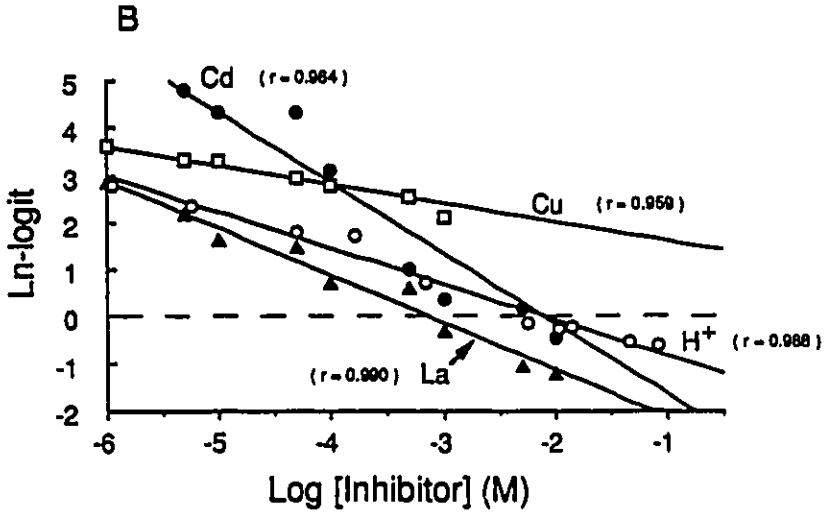
Figure 4.1. Competitive inhibition of gill Ca^{++} binding.

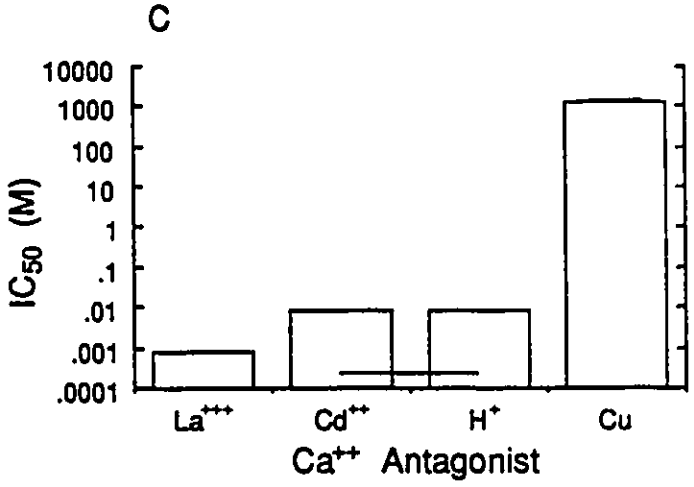
A. The competitive inhibition curves. Gills isolated from hard water-acclimated adult trout were exposed, for exactly 300 sec, to one of several concentrations of a particular metal (inhibitors: Cu, open circles; Cd, open triangles; H^+, closed circles; La, closed triangles) at a constant Ca^{++} concentration (1.33 mM) to determine the ability of the metals to compete with Ca^{++} for gill surface Ca^{++}-binding sites (See Chapter 4 Methods for details). The competitive inhibition curves were fitted to the data by eye. Data presented as means ± 1 SEM (N = 4).

B. Ln-logit transformation of the competitive inhibition curves for the determination of the IC_{50}. The competitive inhibition curves were linearized according to equation 4.2 to calculate the inhibitor concentration resulting in a 50% reduction in maximal Ca^{++}-binding (IC_{50}). Lines fitted to the data (Cd, closed circles; Cu, open squares; H^+ open circles; La, closed triangles) using least squares linear regression analysis. The IC_{50}'s, calculated from the obtained linear regression, represent the intersection of the linear regression with ln-logit = 0 (dashed line).

C. The inhibition of Ca^{++} binding by La^{+++}, Cd^{++}, H^+ and Cu^{++} within the gill micro-environment of adult, hard water-acclimated trout. IC_{50}'s were calculated as illustrated in Fig. 4.1B and plotted on a log scale. Data based on separate estimates of the IC_{50}'s of the preceding metals and are presented as means ± 1 SEM (N = 3).


A
% Calcium Bound
100
80
60
40
20
0
-7
-6
-5
-4
-3
-2
-1
Log [Inhibitor] (M)
Cu
H+
Cd
La


B
Ln-logit
5
4
3
2
1
0
-1
-2
-6
-5
-4
-3
-2
-1
Log [Inhibitor] (M)
Cd (r=0.964)
Cu (r=0.959)
H+ (r=0.988)
(r=0.990) La


C
IC50 (M)
10000
1000
100
10
1
.1
.01
.001
.0001
La+++
Cd++
H+
Cu
Ca++ Antagonist

from 0.001-1.0 mM failed to result in any more than approximately a 10% reduction in gill Ca^{++} binding.

The transformation of these data for the calculation of the IC_{50} values of these metals is shown in Fig 4.1B. Based on this analysis, using the data presented in Fig 4.1A, the IC_{50}'s for La^{+++}, Cd^{++}, H^{+} and Cu^{++} were found to be 0.73 mM, 8.1 mM, 8.1 mM, and 978 M (by extrapolation), respectively.

The competitive inhibition experiments were repeated twice and the mean IC_{50}'s for La^{+++}, Cd^{++}, H^{+} and Cu^{++} from the three independently completed experiments are presented in Fig 4.1C. La^{+++} was found to be the most potent inhibitor of gill Ca^{++} binding, while approximately 10-fold greater concentrations of Cd^{++} or H^{+} were required to achieve equivalent inhibition. No significant difference between the inhibitory abilities of Cd^{++} and H^{+} were found. Copper was found to be 5 orders of magnitude less effective as a Ca^{++} antagonist than was La^{+++}.

DISCUSSION

This is the first study to show direct competitive inhibition of gill Ca^{++} binding by trace metals and H^{+}. In addition, these findings strengthen the hypothesis that the displacement of Ca^{++} at the gill surface is a potential mechanism of metal surface activity. Also, this study supports the notion that the ionic index of a metal may be a major predictor of its competitiveness for Ca^{++} binding sites on the gills as discussed below.

Calcium/Lanthanum Interactions

Lanthanum proved to be the strongest Ca^{++} antagonist of the metals used in this study with an IC_{50} of only 0.75 ± 0.03 mM (Fig 4.3). It is likely that this ability to inhibit Ca^{++} binding is a reflection of the formal charge of the La^{+++} relative to Ca^{++} because, in terms of ionic radius, covalent index (Chapter 3, Table 3.1) and tendency to bind

exclusively to oxygen-rich ligands (Nieboer and Richardson 1980), both metals are nearly identical. These data suggest that La^{+++} binds almost exclusively to sites normally occupied by Ca^{++}. These findings confirm the usefulness of La^{+++} as a probe of Ca^{++} binding, as suggested by Freda and McDonald (1988) in their investigation into physiological correlates of interspecific variation in acid tolerance in fish.

Calcium/Cadmium Interactions

The fact that this study found that Cd^{++} binds to sites normally occupied by Ca^{++} was not unexpected since the toxic mechanism of Cd^{++} is thought to involve direct Ca^{++} antagonism at specific sites of Ca^{++} binding (Zitko and Carson 1976, Calamari et al 1979, Verbost et al 1987, Verbost et al 1988, Chapter 2). The ability of Cd^{++} to bind to sites normally occupied by Ca^{++} is likely due to the near identical ionic radii and formal charge (Chapter 3, Table 3.1) However, unlike La^{+++} and Ca^{++} (class A metals), Cd^{++} is considered either a borderline (Nieboer and Richardson 1980) or class A (Pearson 1968) metal due to the tendency of Cd^{++} to covalently interact with nitrogen and sulfur-rich ligands. This tendency of Cd^{++} to interact with non-oxygen binding centers could be the basis for the persistence of the disruptive effect of Cd^{++} on branchial Ca^{++} transport (Chapter 2, Fig 2.6). Cadmium was, however, a significantly weaker Ca^{++} antagonist compared to La^{+++}, with an IC_{50} of 8.05 ± 0.56 mM (Fig 4.3). The lower Ca^{++} inhibitory effectiveness (IC_{50}) of Cd^{++} can best be related to the differences in the formal charge (2^+ vs 3^+), which was also suggested to reduce the gill surface affinity for Cd^{++} (Chapter 3, Fig 3.4B).

Calcium/Hydrogen ion Interactions

Hydrogen ions were found to be as effective as Cd^{++} at disrupting Ca^{++} binding with an IC_{50} of 8.17 ± 0.39 mM. The ionic index of H^+ is unknown since the ionic radius of

H^+ cannot be determined. In spite of this, Nieboer and Richardson (1980) regard H^+ as a borderline cation citing the chemistry and chemical reactivity calculations of Klopman (1968). However, their classification of H^+ is in contrast to the traditional view, that H^+ is a pure class A cation (Pearson 1968). as are La^{+++} and Ca^{++}. The findings of this study concur with the position taken by Nieboer and Richardson (1980), based on the similarity between Cd^{++} (borderline metal) and H^+, in their effectiveness as Ca^{++} (class A) inhibitors. If H^+ were a strong class A cation, as proposed by Pearson (1968), the calculated IC_{50} would have been expected to be closer to that of La^{+++} (class A) than Cd^{++}.

In the Gouy-Chapman-Stern theory applied to surfaces with ionizable acid groups (briefly mentioned in Chapter 3, Grahame 1947), surface potentials become less negative as the pH of the surrounding environment is lowered and fewer acid groups remain dissociated. Thus, H^+ acts to reduce the charge distribution and would reduce the overall number of sites available for Ca^{++} binding (Fig 6.6A). However, not as evident is the likelihood that hydrogen ion alters the affinity of the surface for Ca^{++}. The relative affinities of a site for divalent cations depends critically upon intersite spacing (Diamond and Wright 1969). Closely spaced sites (strong sites) prefer smaller, nonhydrated, cations and the selectivity sequence is $Mg^{++} > Ca^{++} > Sr^{++} > Ba^{++}$ (similar to sequence of Hille et al 1975), while the selectivity sequence completely reversed at weaker sites (more widely spaced). The selectivity pattern for divalent cations shifts with decreasing pH in the direction from the strong-field sequence to the weak-field sequence. In the context of this Chapter, a shift in the selectivity sequence due to a reduction in pH would be observed as a shift in the affinity of the gill micro-environment for Ca^{++} (Fig 6.7C)

Calcium/Copper Interactions

Unless copper is present in impossibly elevated levels (IC_{50} of 1318 M, N =3), this study indicates that Cu^{++} fails to act as a Ca^{++} antagonist. In other words, Cu^{++} and

Ca^{++} do not bind to the same sites on the gill surface. The absence of any inhibitory influence by Cu^{++} on Ca^{++} binding at the gills is consistent with the notion that the major modulator of short-term Cu^{++} toxicity to rainbow trout is water alkalinity (carbonates), not water hardness (Ca^{++}) (Lauren and McDonald 1985, Lauren and McDonald 1986). In addition, these findings confirm previous suppositions (Chapter 3), that due to the somewhat unique coordination chemistry of Cu^{++}, Cu^{++} fails to bind at the same sites on the gills as Ca^{++}, and binds to the gill surface with low affinity. Of the metals tested, Cu^{++} has the strongest class B tendencies (i.e. covalent bond formation with nitrogen- or sulfur-rich ligands, Nieboer and Richardson 1980).

The fact that Cu^{++} fails to displace Ca^{++}, by no means implies that Cu^{++} is non-toxic. As clearly shown in Chapter 2 (Fig 2.9) and by Lauren and McDonald (1985), Cu^{++} inhibits Na^{+} uptake at extremely low concentrations (0.20 $\mu mol \cdot L^{-1}$, Lauren and McDonald 1985). Na^{+}, like Ca^{++}, is a class A metal which, unlike Cu^{++}, show a preference for oxygen binding environments. Based on these differences between Na^{+} and Cu^{++}, one could conclude that, in contrast to Ca^{++}-Cd^{++} competition, direct competition between Na^{+} and Cu^{++} for specific Na^{+} binding sites probably does not occur. Rather, the impairment of branchial Na^{+} uptake must occur through non-competitive inhibition of Na^{+}/K^{+}-ATPase activity, likely through an interaction between Cu^{++} and the conformation stabilizing sulfphydryl groups.

Conclusion

Metals with an ionic index greater than or equal to that of Ca^{++} would be expected to act as Ca^{++}-antagonists at the gills. For example, Al^{+++}, according to the ideas formulated to this point, should bind with high affinity and more importantly, should act as a strong Ca^{++}-antagonist at the gills. The affinity of the gills for Al^{+++} should be the strongest of any metal-gill interaction, as Al^{+++} is a class A metal (preferring oxygen-

rich ligands, Nieboer and Richardson 1980) having the greatest ionic index (twice that of La^{+++}; Chapter 3, Table 3.1). These characteristics, in turn, suggest that Al^{+++} would be even more effective at displacing Ca^{++} from the gills than La^{+++} (Figs 4.1A and 4.2). One of the objectives of Chapter 6 is to quantify the ability of Al to act as a Ca^{++} antagonist within the gill micro-environment.

CHAPTER 5

MODIFICATION OF GILL CALCIUM BINDING ACTIVITY: ACCLIMATION TO SOFT WATER

INTRODUCTION

It is now well established that maintaining freshwater fish in water of reduced Ca^{++} concentration for prolonged periods (>1 week) invokes hormonally driven compensatory responses which counteract changes in ion gradients and gill permeability, e.g. increased transport activity for Na^+, Cl^- (Maetz 1974) and Ca^{++} (Perry and Wood 1985), increased epidermal thickness and density of mucocytes (Wendelaar Bonga 1978), and proliferation of chloride cells (Laurent et al 1985).

One of the mechanisms by which permeability is thought to be reduced is by an increase in the Ca^{++} binding activity by the gill surface (McDonald and Rogano 1986, McWilliams 1983). Flik et al (1984) found that calmodulin, a highly specific Ca^{++}-binding protein, was present in the epidermal mucus of trout and that calmodulin concentrations increased 2.3-fold following one month of exposure to reduced Ca^{++} water (0.2 vs 0.8 mM). Similarly, Van de Winkel et al (1983), using an identical treatment regime, have shown a comparable increase in glycoproteins (chondroitin sulfate and hyaluronic acid) of epidermal mucus. In previous Chapters (3 and 4) it has been argued that these glycoproteins are key components of the metal binding ability of the gill surface. Finally, McWilliams (1983) showed that, *in vitro*, the rate of loss of Ca^{++} bound to gills obtained from a softwater strain of brown trout (*Salmo trutta*) was significantly less at pH 7.0 and pH 3.0 than in a hard water strain.

Therefore, the main objective of this study was to determine whether the biochemical responses to soft water acclimation described in Chapter 1, lead to changes in the Ca^{++} binding activity of the gills. A secondary objective of this study was to investigate whether or not modifications of the gill surface Ca^{++} binding activity are age-dependent (i.e. juvenile vs adults). For example, Zuchelkowski et al (1985) showed that the chemical modification of skin mucocyte content, evident in adult catfish (*Ictalurus mebulosus* LeSeur) in response to low pH exposure (pH 4.0 and 4.8 vs 6.8), was age-dependent since the shift in production from sialo- to sulfomucin was not observed in younger fish (< 1 year old).

MATERIALS AND METHODS

Animals

Adult (180-270 g) rainbow trout were obtained from a hard water source (Spring Valley Trout Farm, Petersberg, Ont.) and were held in large polyethylene tanks supplied with aerated, dechlorinated Hamilton tap water at 11-15°C (Ca ~ 1.0 $mmol \cdot L^{-1}$, NaCl ~ 0.6 $mmol \cdot L^{-1}$). Trout were fed regularly with commercial trout pellets. Forty of these fish were acclimated for approximately 5 months to artificial soft water (Ca ~ 50 $\mu mol \cdot L^{-1}$, Na ~ 50 $\mu mol \cdot L^{-1}$, pH 6.5). Soft water, supplied to the acclimation tank (400 L) at a rate of 670 $ml \cdot min^{-1}$ (90% replacement in 24 h), was produced from tap declorinated water passing through a reverse osmosis system (Culligan Aqua-Clear MP1000). The final water composition was accomplished with the addition of $CaCl_2$ and NaCl stock solutions to the reverse osmosis product via a peristaltic pump.

Juvenile (8-15 g) rainbow trout were also obtained from a hard water source (Rainbow Springs Hatchery, Thamesford, Ont.) and were fed and held under conditions identical to the holding conditions of the adult, hardwater acclimated fish. Two hundred

and ten (210) of these fish were acclimated for approximately 4 months to artificial soft water (Ca ~ 30 $\mu mol \cdot L^{-1}$, NaCl ~ 40 $\mu mol \cdot L^{-1}$, pH 6.5) in a large 450 L tank. Every other day, the soft water was changed to maintain the ionic composition of the water and reduce the build-up of waste products. Soft water was generated from in-house tap distilled water with the final water composition accomplished with the addition of $CaCl_2$ and NaCl stock solutions which was mixed prior to the changing of the tank water. The tank was well aerated and the water temperature was a constant 14 - 15°C.

Calcium Binding Activity

Separate experiments were performed to determine: i) the half-saturation time ($t_{1/2}$) of gill Ca^{++} binding and ii) the apparent dissociation constant (K_D), maximum capacity (B_{max}) and cooperativity of Ca^{++} binding (H_n) to the gill micro-environments of two life-stages of rainbow trout acclimated to both soft and hard water. These experiments were completed in triplicate essentially as outlined in Chapter 3. However, these assays were performed using entire gill baskets due to the size and difficulty in handling juvenile gills.

Following removal and rinsing of the isolated gill baskets/arches, the gill baskets/arches were exposed immediately to radiocalcium (^{45}Ca) solutions, for various lengths of time dependent on the parameter of interest. All binding assays were performed on individual gill baskets/arches in 50 ml assay solution in a 125 ml sample cup. Each sample cup was fitted with air lines to provide aeration and mixing, and placed in a 15 ± 0.5 °C water bath.

i) $t_{1/2}$

Isolated gill baskets/arches were exposed to 10^{-2} M ^{45}Ca (New England Nuclear) for one of 9 durations ranging from 15 to 1800 sec (N = 4). The specific activity of the ^{45}Ca solution was approximately 31.6 kBq·µmole^{-1}.

ii) K_D, B_{max} and H_n

Gill baskets/arches were exposed to one of 9 ^{45}Ca solution concentrations ranging from 10^{-7} to 10^{-2} M Ca^{++} for exactly 300 sec. Over the range of Ca^{++} concentrations, the specific activity was kept approximately constant at 53.4 kBq·µmole^{-1}.

At the end of the binding activity exposures, individual gill arches and branchial baskets were rinsed, weighed and digested for further analysis. Duplicate 5 ml samples of assay solution were taken following exposure for the determination of both ^{45}Ca radioactivity and Ca^{++} concentration, as were duplicate samples taken just prior to the gill basket/arch exposures.

Net Na^+ fluxes

The net exchange of Na^+ between the fish and the water was measured in black acrylic flux boxes (adults, n = 4) and black linear polyethylene flux bags (juveniles, n = 3; 3 bags containing 5 fish per bag). Methods have been described in detail in Chapter 2. Fish were placed in the appropriate flux chamber containing water to which the fish were acclimated. Animals were given 24 hours to acclimate to the flux chambers prior to the determination of the net Na^+ flux rate. During this acclimation period, adults were held under flow-through water conditions, while water in the flux bags (juveniles) was replaced

after 12 h of acclimation. The concentration of Na^+ in the water was taken prior to and following an 8 hour flux period.

Analytical Methodology

Radiocalcium activity was determined on 100 µl of gill digest and on 5 ml volume of assay solution taken immediately before and after the gill exposures. Each sample was added to 10 ml of scintillation fluor (ACS, Amersham), with 4.9 ml of water added to the gill digests, and each was counted for at least 20 min on a liquid scintillation counter (LKB Rackbeta). Each sample was prepared in triplicate and all samples were automatically corrected for quench according to a quench curve generated with counting samples containing a known amount of radioactivity and varied dilutions of gill digest.

Water Na^+ and Ca^{++}, as well as the assay solution Ca^{++} concentrations were measured, after appropriate dilution, by flame atomic absorption spectrophotometry (Varian 1275) against known standards.

Data and Statistical Analysis

Calcium binding data generated through the exposure of gills from juvenile and adult trout acclimated to low and high calcium waters were analyzed as detailed in the Methods of Chapter 3 (nonlinear regression analysis and/or Scatchard analysis; Duggleby 1981, Johnston 1985, Scatchard 1949). Net fluxes (Jnet) of Na^+, in $nmol \cdot g^{-1} \cdot h^{-1}$ were calculated from changes in the water Na^+. Data are expressed as means ± 1 SEM (n). Comparisons of specific binding characteristics (K_D, B_{max}, H_n) and Jnet rates between hard and soft water-acclimated animals, were made using a Student's unpaired t-test (two tailed) with significance set at a 95% level of confidence.

RESULTS

Fish acclimated to soft water, and those maintained in hard water, were found to be in net electrolyte balance. All treatment groups had net Na^+ flux rates not significantly different from zero (Fig 5.1).

The acclimation of hard water animals to soft water did not influence either the $t_{1/2}$ or H_n of gill Ca^{++} binding. The $t_{1/2}$ of adult and juvenile fish were found to be 164 ± 11.4 and 177 ± 9.6 sec, respectively. The H_n for the binding of Ca^{++} to the gills were calculated to be 0.99 ± 0.057 for adult fish and 0.97 ± 0.04 for juvenile fish, with neither significantly different from unity. There was, in other words, no evidence of binding cooperativity or for more than one type of Ca^{++} binding site.

Soft water acclimation (>4 months) did, however, result in significant 22.4% and 29.4% reductions in the K_D (increased affinity) for Ca^{++} binding in both adult and juvenile trout, respectively (Fig 5.2A). Furthermore, soft water acclimation lead to changes in the Ca^{++}-binding capacity (B_{max}) in both adult and juvenile rainbow trout (Fig 5.2B). Although the 10.4% increase in B_{max} of the adult fish was not statistically significant, juvenile trout acclimated to soft water were found to have 64.5% greater capacity for Ca^{++} compared to those fish maintained in hard water.

DISCUSSION

This study confirms and extends the suggestion by McWilliams (1983) that the gills of fish acclimated to low Ca^{++} environments bind Ca^{++} more tightly than fish living in high Ca^{++} conditions. McWilliams (1983), in his study on the effects of low pH on the rate of loss of surficially bound Ca^{++}, provided the first evidence of a fundamental difference in the Ca^{++} binding activity of gills from fish from environments of different necessarily involved.

Figure 5.1. Mean net Na^+ fluxes ($J_{net}Na^+$) of adult and juvenile trout. Na^+ fluxes were measured over an 8 hour exposure period with fish held in either hard water (H.W.: $[Ca^{++}] = 1.0$ mmol·L^{-1}, $[NaCl] = 0.6$ mmol·L^{-1}) or soft water (S..W. juvenile: $[Ca^{++}] = 0.03$ mmol·L^{-1}, $[NaCl] = 0.04$ mmol·L^{-1}; S.W. adults: $[Ca^{++}] = 0.04$ mmol·L^{-1}, $[NaCl] = 0.05$ mmol·L^{-1}) to which all fish had been acclimated to for over 4 months. Data are presented as means ± 1 SEM. N = 4 in the adult exposures and N = 3 in the juvenile exposures. Statistical significance was examined using a two-tailed, Student's t-test with a level of confidence of 95%.

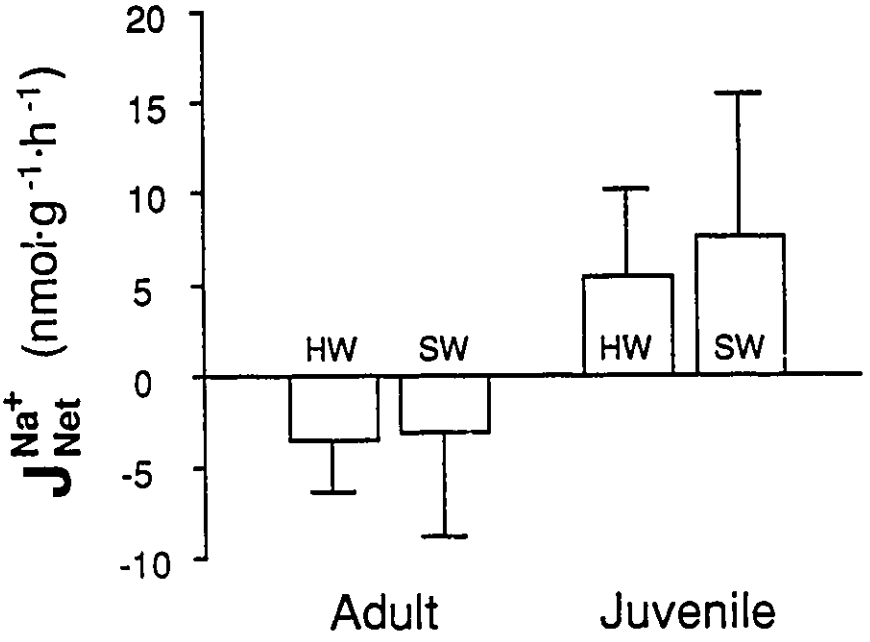
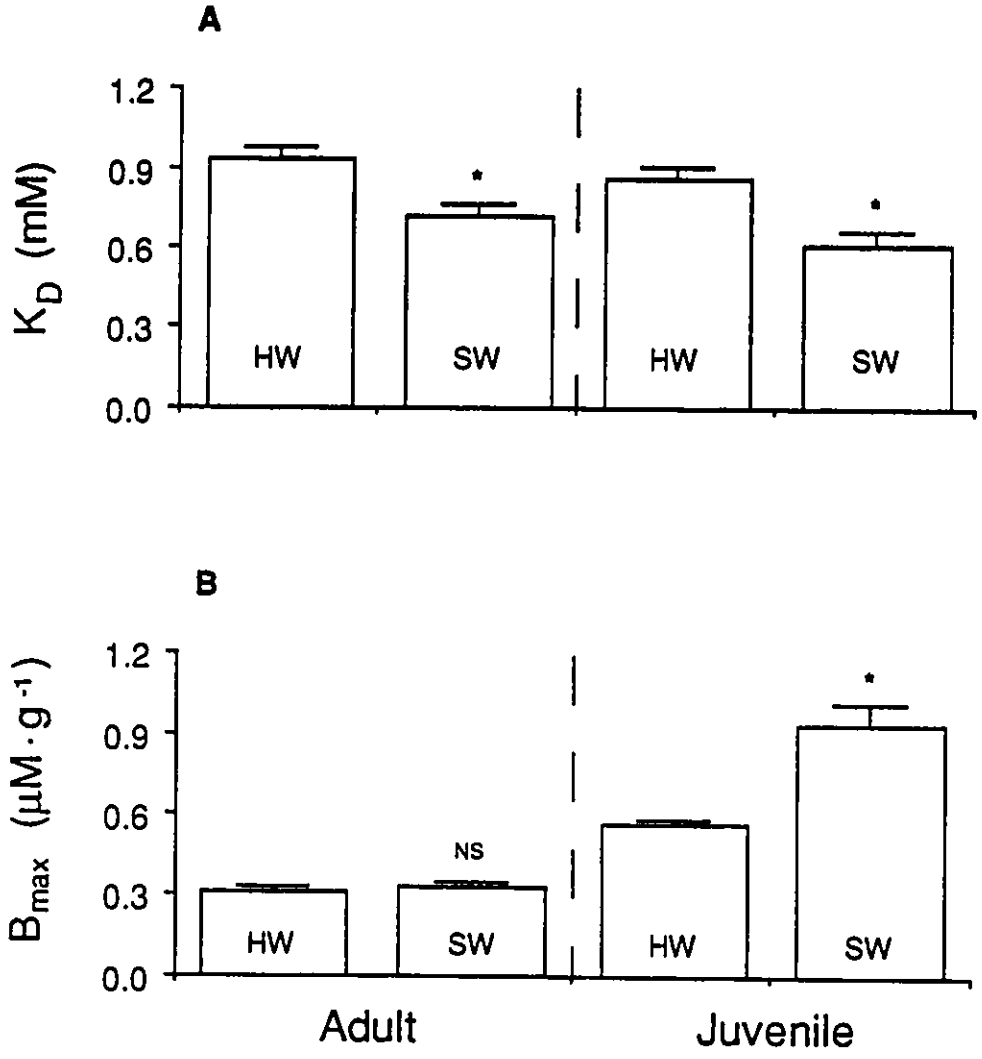

20
15
10
5
0
-5
-10
$J^{Na^+}_{Net}$ (nmol·g^{-1}·h^{-1})
HW
SW
HW
SW
Adult
Juvenile

Figure 5.2. Modification in the Ca^{++} binding activity of the gills associated with soft water acclimation in trout. This histogram represents a comparison of the specific Ca^{++} binding characteristics for adult and juvenile trout prior to and following over 4 months exposure to soft water. These data are presented as means ± 1 SEM from three replicates of the saturation experiments (N = 3). Differences in Ca^{++} binding activity, within age groups, were tested using a two-tailed Student's t-test with a confidence limit of 95%.

A. The effect of acclimation on the apparent dissociation constant (K_D) of Ca^{++} binding.

B. The effect of acclimation on the Ca^{++} binding capacity (B_{max}).


A
1.2
0.9
0.6
0.3
0.0
K_D (mM)
HW
SW
HW
SW
*
*
B
1.2
0.9
0.6
0.3
0.0
B_{max} ($\mu M \cdot g^{-1}$)
NS
*
HW
SW
HW
SW
Adult
Juvenile

The present study is methodologically similar to that of McWilliams (1983). The only difference is that he used the dissociation rate (k_1) of Ca^{++} as an estimate of the affinity of the gill surface for Ca^{++}. In contrast, this study determined the apparent dissociation constant for Ca^{++} binding (K_D) which was similarly used as an indicator of binding affinity. The K_D and k_1 are related as the K_D is equal to the ratio of the dissociation (k_1) to association (k_2) rates (i.e. $K_D = k_1/k_2$, Titeler 1981). Both are direct measures of the nature of the metal-gill interactions, however, the methodology utilized by McWilliams (1983) does not allow the determination of receptor cooperativity, the time course of saturation or binding capacity. Therefore, this study provides a more detailed analysis, than that of McWilliams (1983), of the changes in Ca^{++} binding activity of the gills in response to soft water.

The nature of the changes in Ca^{++} binding activity suggests that both the character and the number of the binding sites available to Ca^{++} are significantly increased by soft water acclimation (Fig 5.2), independent of genotype. These changes could be explained by increases in either calmodulin and/or glycoprotein (Flik et al 1984, Van de Winkel et al 1983 respectively) although the former is unlikely for several reasons. Firstly, the K_D of calmodulin, calculated by Richardt et al (1986) using flow dialysis, was 4 μM, an estimate over 2 orders of magnitude lower than the Ca^{++} K_D of the gills of fish acclimated to either soft or hard water (Fig 5.2A). In other words, calmodulin has over 100-fold greater affinity for Ca^{++} than the affinity of the gills for Ca^{++}. Secondly, Flik et al (1984) collected mucus from the body surface, not the gills, and there may well be a difference in the chemical composition and cation binding activity between these two mucous types. Thirdly, although Flik et al (1984) studied mucus from 3 species of freshwater fish (tilapia, *Saratharedon mossambicus*; catfish, *Clarias lazera*; trout, *Salmo gairdneri*) they fail to provide evidence for the presence and alterations in mucous

calmodulin concentration in either catfish or trout. Therefore the presence of calmodulin in the mucus of rainbow trout can be questioned.

Increases in mucous and surface glycoproteins is a more likely explanation for the increase in B_{max}. However, it is less likely to have resulted in the shift in binding affinity (Fig 4.2), unless the increased concentration of oxygen-rich ligands translates to a more favourable environment for the binding of Ca^{++}. It may be more likely, that the chemical composition of the mucus is altered as part of the overall mechanism of soft water acclimation. Since Ca^{++} binds almost exclusively to oxygen-rich ligands (Nieboer and Richardson 1980, Williams 1974), by reducing the concentration of non-oxygen donor ligands, higher affinity class A metal binding could be achieved (Nieboer and Richardson 1980). Compositional modifications in mucus in response to changes in water chemistry have, in fact been observed (low pH, Zuchelkowski et al 1984).

Although the method of analysis for these two life-stages were not identical, and thus not truly comparable, these data do suggest that the general response of the gill micro-environment to the reduction in environmental Ca^{++} was independent of age. In contrast, Zuchelkowski et al (1985) has shown, based on histochemical and autoradiographic analysis of skin mucocytes of catfish (*Ictalurus mebulosus* LeSeur), that the chemical modification of mucus of skin mucocytes in response to low pH exposure was age-dependent since the shift in production form sialo- to sulfomucin was not observed in younger fish (< 1 year old).

This study provides evidence to support the notion that the gills can dynamically respond to an environmental stressor (soft water) with changes in their surface. The goal of this response of the gills appears to be a reduction in ionic permeability by the creation of a "hard water environment" at the gill surface. Thus, this

is yet another response to add to the repertoire of adaptive gill responses to low environmental Ca^{++} detailed in the Introduction of this chapter.

CHAPTER 6

MODIFICATIONS OF METAL-GILL SURFACE INTERACTIONS OF JUVENILE TROUT:

ACCLIMATION TO SUBLETHAL ALUMINUM

INTRODUCTION

Water in regions impacted by acidic precipitation often exhibit elevated aluminum concentrations as a result of aluminum mobilization from soils and sediments (Wright and Gjessing 1976). Loss of fish populations, often attributed directly to the acidification of lakes and rivers (Harvey 1982), have been primarily ascribed to elevated aluminum levels (Schofield and Trojnar 1980) in some instances. The mechanism of aluminum toxicity to fish is thought to involve either ionoregulatory or respiratory disturbances or both. The severity and relative contribution of each appears to be critically dependent upon the water pH and the aluminum concentration (Neville 1985, Booth et al 1988, Wood et al 1988a, 1988b, 1988c). Since significant accumulation of aluminum on the gills has been observed (Youson and Neville 1987), with no increase in plasma aluminum levels (Booth et al 1988), it has been proposed that aluminum is toxic to fish primarily because of its action on the gill (Schofield and Trojnar 1980, Wood et al 1988c). One postulated toxic mechanism of aluminum is displacement of calcium from membrane stabilizing sites on the gill (Booth et al 1988, Wood et al 1988a). Thus, exposure to aluminum in concert with reduced water pH, is thought to lead to an impairment of ionoregulation, as a result of a decrease in gill membrane integrity. Hydrogen ion is thought to operate by a similar fashion (McDonald 1983).

One characteristic of prolonged sublethal exposure to aluminum is that fish develop a resistance to aluminum (i.e. "acclimate" to aluminum). Toxicant tolerance is typically assessed by performance of the animals in LC_{50} trials. The LC_{50} is the concentration of toxicant resulting in 50% mortality within a specific time. Orr et al (1986) demonstrated that a 1 or 2 week exposure to sublethal aluminum provides increased aluminum resistance in juvenile rainbow trout based on a near doubling of the 120 h LC_{50}. Increased resistance of fish to other metals (Cu, Cd, Zn) following exposure to sublethal concentrations, has also been reported and typically results in a 1.5 to 2.7 fold increase in the observed LC_{50} (see review by Hodson 1988). In some instances, the increase in metal resistance was linked to the induction of intracellular metal-binding proteins, including cysteine-rich metallothionein. However, at least in the case of copper, increased resistances, as indicated by the recovery of branchial Na^+ exchange in the presence of copper, occurred without the induction of metal-binding proteins in the gills (Lauren and McDonald 1987a, 1987b).

Since aluminum causes ionoregulatory disturbances via its action on the gill, it is reasonable to assume that the mechanism of acclimation invokes modifications in the gill. In the brook trout, this gill modification appears to result in the recovery of lost electrolytes (Wood et al 1988b), both from a recovery of sodium transport activity (McDonald and Milligan 1988) and a reduction in passive permeability during aluminum/acid challenge (Booth et al 1988). Modification in the interaction of aluminum and calcium at the level of the gill may lead to improved epithelial integrity, due to the role Ca^{++} in maintaining membrane stability.

Therefore, the objectives of this Chapter were: 1) to document physiological disturbances in rainbow trout caused by sublethal aluminum exposure, 2) to investigate the time course and nature of physiological resistance (i.e. acclimation) to aluminum and, 3)

to study some of the quantitative modifications of the gills that may be associated with aluminum acclimation: gill sialic acid (an indicator of gill mucus content), gill aluminum accumulation in relation to chronic sublethal and acutely lethal aluminum levels, and gill calcium binding activity as assessed by the *in vitro* methodology detailed in Chapters 3 and 4.

MATERIALS AND METHODS

Animals

Experiments were conducted at the Fish Physiology and Toxicology Laboratory, University of Wyoming. Juvenile rainbow trout, *Oncorhynchus mykiss* (weight range 10-25 g), were obtained from Cline's Trout Farm (Boulder, Colorado) and were maintained prior to experimentation for at least 6 weeks in a 600 L fiberglass holding tank continuously supplied with very soft water (Table 6.1) at a temperature of 15 ± 0.5 °C. Fish were fed 1% of body weight per day commercial trout chow and tanks were siphoned daily to remove organic debris.

Experimental Protocol

The basic experimental approach was to acclimate juvenile trout to one of three conditions (control, low pH, low pH and aluminum) for 21 days. During the acclimation period blood chemistry, gill tissue composition, and gill calcium binding activity were monitored. After 21 days, fish from all treatments were challenged with an acutely lethal concentration of aluminum. Increased aluminum resistance was assessed on the basis of the challenge estimated times to 50% mortality (ET_{50}'s) for the three acclimation groups.

Aluminum exposure levels for acclimation (30 $\mu g \cdot L^{-1}$, pH 5.2) and lethal challenge (100 $\mu g \cdot L^{-1}$, pH 5.2) were chosen on the basis of preliminary trials with various

combinations of aluminum and/or low pH. Actual mean concentrations of aluminum were respectively 27 and 118 $\mu g \cdot L^{-1}$ (Table 6.1). These low pH and aluminum concentrations are comparable with levels measured in Canadian Shield and Adirondack lakes undergoing acidification (Schofield and Trojnar 1980, Chevalier et al 1985). The original stock of fish maintained under the holding conditions are referred to as the 6.5/0 (pH = 6.5/Al = 0) or control fish. Those animals exposed to aluminum and low pH during the acclimation period are referred to as the 5.2/27 or aluminum fish, while fish that were exposed to low pH alone are referred to as the low pH or 5.2/0 fish.

Acclimation studies

At the start (Day 0), 165 fish were removed from the control tank. Each was killed with a single, sharp blow to the head. From 5 fish, blood samples were taken by caudal puncture with a heparinized syringe and processed immediately for hematocrit, plasma chloride and plasma protein.

The branchial baskets of these fish were carefully removed, weighed and digested in 3.5 volumes of 0.1 N nitric acid for later determination of gill aluminum and sialic acid content. From the remaining 160 fish, whole branchial baskets were removed, rinsed for 15 sec in each of distilled water, 5.0 mM EDTA, and distilled water, and then used immediately for the determination of the half-saturation time ($t_{1/2}$, N = 80), or gill binding capacity (B_{max}) and apparent dissociation constant (K_D, N = 80).

Approximately 1300 6.5/0 fish were then transferred and maintained at either pH 5.2 alone or pH 5.2 and 27 $\mu g \cdot L^{-1}$ aluminum for 21 days while an additional 700 fish remained in the 6.5/0 tank. Two 350 L tanks were used for the 5.2/0 and 5.2/27 treatment conditions with the fish divided equally amongst the four tanks. Each tank was supplied continuously with soft water of the appropriate water chemistry (Table 6.1) at a temperature

Water chemistry during holding, 21 days of exposure and aluminum challenge.

	pH	Ca++ (µequiv.L-1)	Total Al (µg.L-1)	Na+ (µequiv.L-1)	Mg++ (µequiv.L-1)	K+ (µequiv.L-1)	F- (µequiv.L-1)	Cl- (µequiv.L-1)	NO3- (µequiv.L-1)	SO42- (µequiv.L-1)
Control (6.5/0)	6.48 (0.04)	32.6 (1.78)	0.3 (0.16)	43.3 (1.79)	21.2 (16.4)	77.5 (18.8)	1.0 (0.09)	44.3 (0.78)	1.4 (0.14)	33 (16.4)
Low pH (5.2/0)	5.19 (0.06)	34.0 (1.02)	0.2 (0.09)	45.2 (0.85)	21.6 (0.36)	77.9 (18.8)	1.2 (0.08)	46.4 (1.08)	1.4 (0.20)	65.7 (23.6)
Low pH/Al (5.2/27)	5.12 (0.06)	27.4 (0.84)	26.6 (3.12)	44.6 (1.01)	20.8 (0.38)	78.3 (18.4)	1.0 (0.09)	48.6 (1.39)	1.2 (0.18)	126.8 (20.8)
Al Challenge	5.17 (0.06)	30.2 (0.44)	117.7 (3.30)	43.0 (0.91)	21.6 (0.28)	3.3 (0.39)	3.0 (0.10)	54.5 (0.38)	2.2 (0.32)	57.8 (0.02)

Table 1. Composition and pH (means, SEM in parenthesis) of the Test water. Water samples were taken throughout the exposure period for determination of water chemistry. All ions in µequiv/l, except for total Al (µg/g). N = 12; temperature = 15 + 0.5 C

of 15 ± 0.5 °C. Feeding of fish continued during the 21 day exposures at 1% of body weight per day using commercial trout chow. Tanks were siphoned daily to remove organic debris.

At Days 8 and 21, 165 fish were again removed from the three exposure conditions (6.5/0, 5.2/0, 5.2/27). Blood and branchial baskets were sampled from 5 fish in each group and the remainder were utilized in the measurements of gill calcium binding activity. Also on Day 21, the effect of hydrogen ion or of aluminum (at pH 5.2) on gill calcium binding was determined on an additional 160 fish from each group. Half of these fish (N = 80) were used to determine the effect of aluminum (pH 5.2) on calcium binding activity (Al IC_{50}),while the remainder (N = 80) were used to determine the effect of hydrogen ion on calcium binding activity (H^+ IC_{50}).

Lethal aluminum challenge

On day 21, fish from each acclimation condition (6.5/0, 5.2/0, 5.2/27, N = 20 from each) were transferred to separate challenge tanks. Each tank was supplied with soft water at pH 5.2 containing 118 $\mu g \cdot L^{-1}$ aluminum at a temperature of 15 ± 0.5 °C (Table 6.1). Mortality in these tanks was monitored approximately every 2 hours for 70 hours, with dead fish being removed immediately upon discovery. After 40 hours, 5 survivors form each treatment groups were removed from the challenge tanks and from these fish were measured: blood hematocrit, plasma Cl^-, plasma protein and gill aluminum.

Analytical Methodology

Blood

Hematocrit was read directly from heparinized microhematocrit tubes spun for 3 min at 9000 X g. Protein concentration in 8 µl of plasma was determined with an

American Optical Goldberg refractometer (Alexander and Ingram 1980). Plasma chloride was determined on a 20 µl sample by coulometric titration using a Radiometer CMT10.

Gill Sialic Acid Content

Mucus secreted by the gills of trout is rich in sialoglycoprotein (Fletcher et al 1976). Therefore, we used a method of analyzing the sialic acid content of the gill as an indicator of the gill mucus content. Gill sialic acid content was assayed on 25 µl samples of gill tissue digest according to the thiobarbituric acid assay of Warren (1959, 1963). Sialic acid samples were prepared in triplicate and measured against known standards (Sialic acid = N-acetylneuraminic acid, Sigma A2751) on a UV/VIS spectrophotometer (Philips PU 8600).

Gill Aluminum

Gill aluminum was assayed in triplicate on 50 µl of gill tissue digest added to 3.5 ml of 0.1 N HCl using a modified version of the pyrocatechol violet method of Dougan and Wilson (1974). To account for colourimetric interference from the tissue digest itself, 50 µl of tissue digest from 6.5/0 fish was added to all standards and blanks. Absolute gill content of aluminum may be underestimated by the accumulation of trace aluminum in the 6.5/0 water, and thus, the measurements are considered relative to controls (6.5/0).

Calcium Binding Activity

Separate experiments were performed to determine : i) the half-saturation time ($t_{1/2}$) of gill calcium binding, ii) the apparent dissociation constant (K_D) and maximum capacity (B_{max}) of gill calcium binding, and iii) the ability of aluminum (at pH 5.2) or of hydrogen ion to interfere with gill calcium binding (IC_{50}) according to the methodologies outlined in Chapters 2 and 3.

Following removal and rinsing of the isolated branchial baskets, the branchial baskets were exposed immediately to radiocalcium (^{45}Ca) solutions, for various lengths of time dependent on the parameter of interest. All binding assays were performed on individual branchial baskets in 50 ml assay solution in a 125 ml sample cup. Each sample cup was fitted with air line to provide aeration and mixing, and placed in a 15 ± 0.5 °C water bath.

i) $t_{1/2}$

Isolated branchial baskets were exposed to 10^{-2} M radiocalcium (^{45}Ca, New England Nuclear) for one of 9 durations ranging from 15 to 1800 sec (N = 4). The specific activity of the ^{45}Ca solution was approximately 18 kBq·µmole^{-1}.

ii) K_D and B_{max}

Branchial baskets were exposed to one of 9 radiocalcium concentrations ranging from 10^{-7} to 10^{-2} M Ca^{++} exactly 300 sec. Over the range of calcium concentrations, the specific activity was kept constant at 18.5 kBq·µmole^{-1}.

iii) IC_{50}

Branchial baskets were exposed to one of 9 solutions of constant calcium concentration (0.5 mM) and inhibitor concentrations ranging from 10^{-6} to 10^{-2} M hydrogen ion or 3.7×10^{-6} to 3.7×10^{-5} M (100 to 1000 µg·L^{-1}) aluminum (pH 5.2) for 300 sec. The specific activity of the ^{45}Ca was kept constant at approximately 18.3 kBq·µmole^{-1}.

At the end of the binding activity exposures, branchial baskets were rinsed in distilled water for 15 sec to remove excess assay solution, placed in pre-weighed 20 ml scintillation vials, weighed, and digested in 3.5 vols of 0.1 N nitric acid overnight.

Radiocalcium activity was determined on 100 µl of gill digest and on 5 ml volume of assay solution taken immediately before and after the gill exposures. Each

sample was added to 10 ml of scintillation fluor (ACS, Amersham), with 4.9 ml of water added to the gill digests, and each was counted for at least 20 min on a liquid scintillation counter (Beckman LS 9000). Each sample was prepared in triplicate and all samples were corrected for quench according to a quench curve generated with counting samples containing a known amount of radioactivity and varied quantities of gill digest.

Measurements of gill calcium binding were analyzed using nonlinear regression (Johnston 1985) and Scatchard plot techniques (Scatchard 1949) as described in detail in Chapters 3 and 4.

Apparent dissociation constants (K_D) for hydrogen ion and aluminum binding were determined indirectly according to the equation of Cheng and Prusoff (1973):

$$K_D = IC_{50} \cdot (1 + (D^*/K_{D^*}))^{-1} \qquad 6.1)$$

using the estimated IC_{50} for the appropriate antagonist, the apparent calcium dissociation constant of the specific treatment group (K_{D^*}), and the concentration of radiocalcium in the assay solution (D^*).

Calcium and Aluminum Concentrations

Calcium concentration in acclimation tanks, challenge tanks and the calcium binding activity assay solutions was measured in duplicate against known standards by atomic absorption flame spectrophotometry (Perkin-Elmer Model 2380). Aluminum content of the calcium/aluminum assay solutions used in the competitive inhibition assays were measured in triplicate samples of 3.5 ml using a modified version of the pyrocatechol violet method of Dougan and Wilson (1974). Acclimation and challenge tank aluminum levels were measured on the Perkin-Elmer A.A. with an associated high-temperature graphite furnace (Perkin-Elmer Model HGA-400).

Statistics

Data reported as means ± 1 SEM. Differences between means and specific binding characteristics were determined using a two-tailed, unpaired, Student's t-test with significance set at a 95% level of confidence. Survival curves were compared using standard log/probit analysis and nomographic methods to determine median survival times (ET_{50}), their 95% confidence limits, slope functions, and the significance of differences between treatments (Litchfield 1949, Litchfield and Wilcoxin 1949).

RESULTS

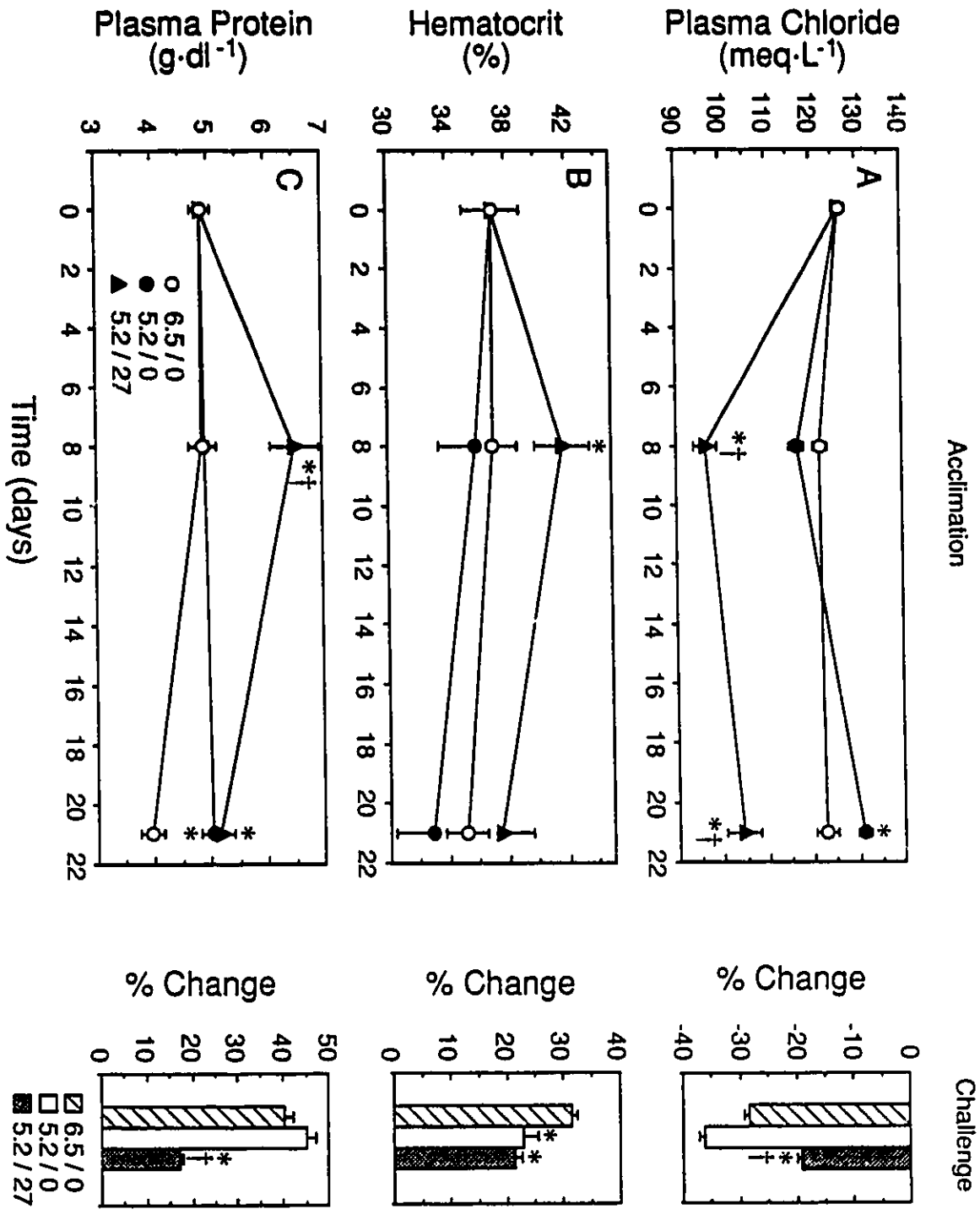
Aluminum Acclimation

Blood chemistry

Plasma Cl^- in fish held at 6.5/0 (controls) did not change significantly from an overall mean concentration of 123.6 ± 1.41 mequiv·L^{-1} (Fig 6.1A). Exposure to pH 5.2 soft water (5.2/0) resulted in only minor changes in Cl^-, with only the increase at Day 21 being statistically significant. A severe reduction in Cl^- resulted from the exposure of juvenile trout to aluminum and low pH (5.2/27). A 24% decline in Cl^- was measured after 8 days exposure to 27 μg·L^{-1} aluminum at pH 5.2 (5.2/27) and Cl^- remained significantly lower than both control and low pH only levels, although some recovery of Cl^- was noted at Day 21.

No significant changes in hematocrit or protein concentration in the 6.5/0 group were noted, though both tended to decline over the 21 day period (Fig 6.1B and 6.1C). As was the case for Cl^-, only minor differences between the 6.5/0 and 5.2/0 estimates of protein and hematocrit were evident (Fig 6.1C). In contrast, protein and hematocrit were elevated 34.2 and 12.5%, respectively, in the 5.2/27 group at Day 8 compared to controls. Increased hematocrit and plasma protein is usually taken as evidence of

Figure 6.1. Plasma chloride (A), plasma protein (B) and hematocrit (C) of juvenile rainbow trout during a 21 day acclimation exposure to soft water at pH 6.5 (6.5/0, open circles), at ph 5.2 (5.2/0, closed circles), or 27 $\mu g \cdot L^{-1}$ Al at pH 5.2 (5.2/27, closed triangles). Panels to the right depict the percent change in the Day 21 values after 40 hours of the challenge exposure of 118 $\mu g \cdot L^{-1}$ Al at pH 5.2. Data presented as means ± 1 SEM; N = 5 for each treatment. Significantly different data, as determined using a two-tailed Student's t-test at a 95% level of confidence, are indicated by (*) or (†) for comparisons with control (6.5/0) or low pH only (5.2/0) respectively.


Plasma Chloride (meq·L-1)
Hematocrit (%)
Plasma Protein (g·dl-1)
A
B
C
Acclimation
Challenge
Time (days)
o 6.5/0
● 5.2/0
▲ 5.2/27
% Change
% Change
% Change
6.5/0
5.2/0
5.2/27

hemoconcentration resulting from loss of electrolytes (Milligan and Wood 1982). By Day 21, some recovery was evident as hematocrit was no longer significantly different from either 6.5/0 or 5.2/0 levels. In addition, the protein concentration of the 5.2/27 fish was the same as the 6.5/0 and 5.2/27 protein levels was reduced.

Gills

Aluminum exposure (27 $\mu g \cdot L^{-1}$) resulted in a significant accumulation of aluminum. By Day 8, gills of the 5.2/27 fish contained 25.6 ± 1.47 $\mu g \cdot g^{-1}$ with a significant reduction in gill aluminum occurring on Day 21 (Fig 6.2A). In contrast, gill sialic acid content remained virtually constant in all 3 groups (Fig 6.2B).

Aluminum Challenge

Mortality

Prior exposure to aluminum (27 $\mu g \cdot l^{-1}$, pH 5.2) but not to pH 5.2 alone, significantly reduced mortality to the 118 $\mu g \cdot L^{-1}$ (pH 5.2) aluminum challenge (Fig 6.3). The ET_{50}'s for the 6.5/0 and 5.2/0 fish were 36.0 and 31.5 hours, respectively, and not significantly different from one another. At this time (30-40 h) the mortality of the 5.2/27 fish was only 5%.

Blood chemistry

Little difference was noted in the blood chemistry between the 6.5/0 and 5.2/0 fish when challenged with 118 $\mu g \cdot L^{-1}$ aluminum at pH 5.2 for 40 hours (Fig 6.1). Both groups experienced significant reductions in Cl^- (Fig 6.1A) and elevations in both hematocrit (Fig 6.1B) and plasma protein (Fig 6.1C), although the rise in 5.2/0 hematocrit was significantly lower than in the controls. The increased survival of the 5.2/27 trout was

Figure 6.2. Gill aluminum (A) and sialic acid (B) content of juvenile rainbow trout during a 21 day acclimation exposure to soft water at pH 6.5 (6.5/0, open circles), at pH 5.2 (5.2/0, closed circles) or 27 $\mu g \cdot L^{-1}$ Al at pH 5.2 (5.2/27, closed triangles). The panel to the right depicts the change in the Day 21 values after 40 hours of the challenge exposure of 118 $\mu g \cdot L^{-1}$ Al at pH 5.2. Data presented as means ± 1 SEM; N = 5 for each treatment. Significantly different data, as determined using a two-tailed Student's t-test at a 95% level of confidence, are indicated by an (*) or (†) for comparisons with control (6.5/0) or low pH only (5.2/0) respectively.

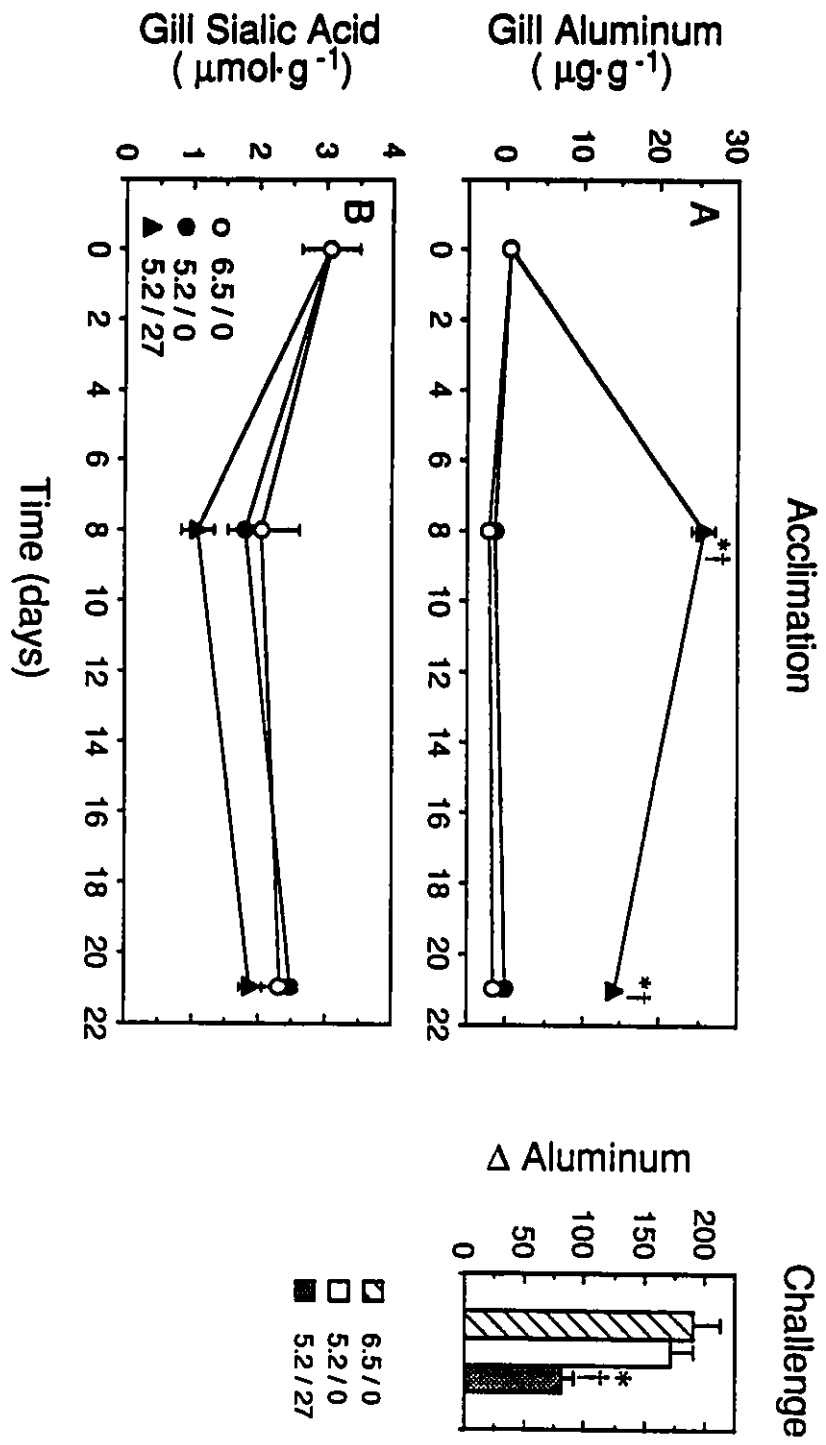
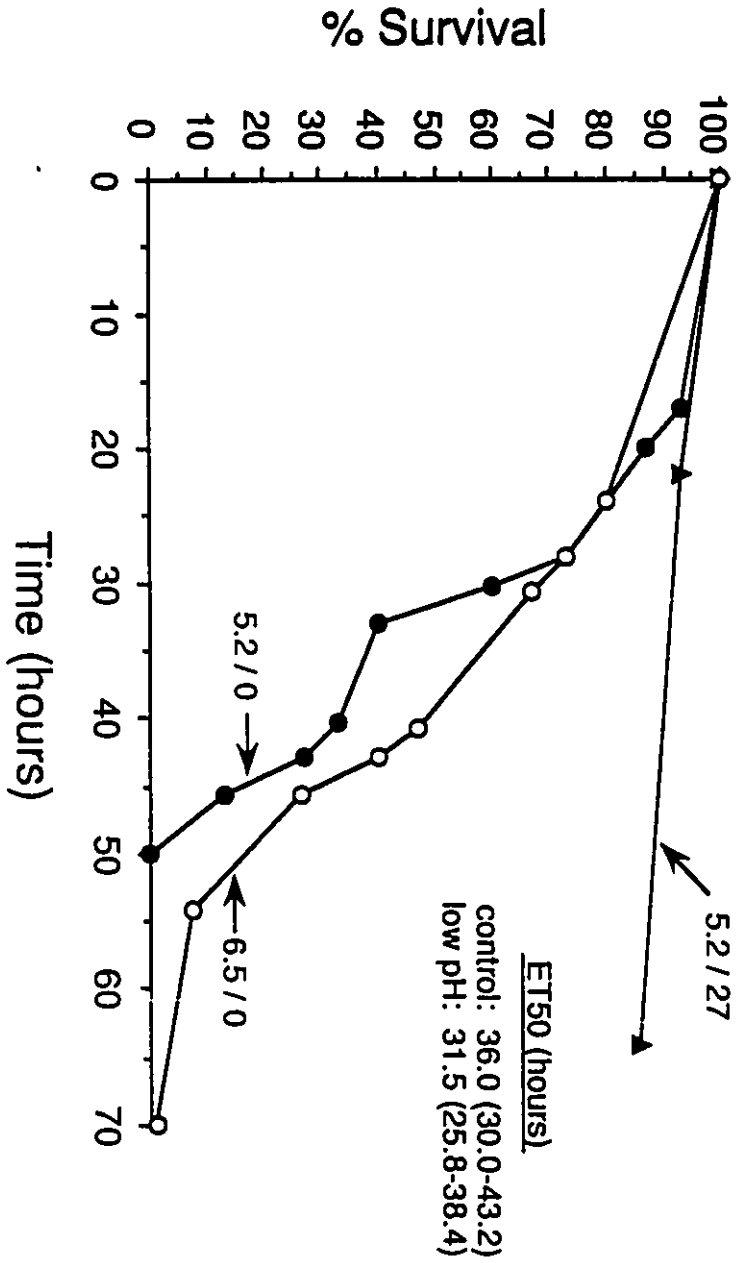

Acclimation
Gill Aluminum
(µg·g -1)
A
Gill Sialic Acid
(µmol·g -1)
B
o 6.5 / 0
● 5.2 / 0
▲ 5.2 / 27
Time (days)
Challenge
Δ Aluminum
6.5 / 0
5.2 / 0
5.2 / 27

Figure 6.3. Percent survival of fish from all three treatment groups (6.5/0, 5.2/0, 5.2/27) during a 118 $\mu g \cdot L^{-1}$ Al (pH 5.2) challenge following the 21 day acclimation exposures. Initial sample size was 20 for all treatment groups. ET_{50}'s (range) were calculated and compared using standard log/probit analysis.


% Survival
100
90
80
70
60
50
40
30
20
10
0
0
10
20
30
40
50
60
70
Time (hours)
5.2 / 0
6.5 / 0
5.2 / 27
ET50 (hours)
control: 36.0 (30.0-43.2)
low pH: 31.5 (25.8-38.4)

reflected in a significantly lower plasma Cl^- loss and a correspondingly smaller hematological disturbance, compared to controls (Fig 6.1).

Gills

The gills of fish from all treatment groups (6.5/0, 5.2/0, 5.2/27) accumulated aluminum (Fig 6.2A). In the 6..5/0 and 5.2/0 groups this amounted to 190 ± 24.0 and 172 ± 18.0 $\mu g \cdot g^{-1}$ respectively, whereas the additional Al accumulation of the 5.2/27 fish was approximately 50% less (81.7 ± 9.1 $\mu g \cdot g^{-1}$).

Calcium Binding Activity

Calcium saturation kinetics

The half-saturation time ($t_{1/2}$) for Ca^{++} binding of the 6.5/0 gill tissue was 179 ± 11.5 seconds (Fig 6.4) and did not vary significantly with either time or treatment (i.e. 5.2/0, 5.2/27). K_D of controls was 0.66 ± 0.058 mM with a Bmax of 0.93 ± 0.082 $\mu mol \cdot g^{-1}$ on Day 0 also did not change significantly over the course of the experiment (Fig 6.5). The Ca^{++} binding activity of these soft water acclimated fish was not significantly different from that measured using similarly acclimated juvenile rainbow trout completed at McMaster University prior to this study (Chapter 5, Fig 5.2).

B_{max} of the 5.2/0 gills declined with time and was found to be significantly lower than the controls by Day 21 (Fig 6.6A). B_{max} of the 5.2/27 gills declined more rapidly but by Day 21 had recovered to control levels (Fig 6.6A). Exposure to both 5.2/0 and 5.2/27 led to a significant increase in the Ca^{++} binding affinity (reduction in K_D) when compared to controls (Fig 6.6B) on Day 8. Further exposure had no further effect on the affinity of the 5.2/0 fish, whereas in the 5.2/27 fish the affinity had decreased to 1.4 times control by Day 21.

Figure 6.4. A time saturation curve of gill baskets of control (6.5/0) trout. Gill baskets were removed, cleaned, then exposed to 10^{-2} M Ca^{++} for one of several periods of time (See Chapter 6 Methods for details). The curve was fitted to the data by eye. Data presented as means ± 1 SEM. N = 4.

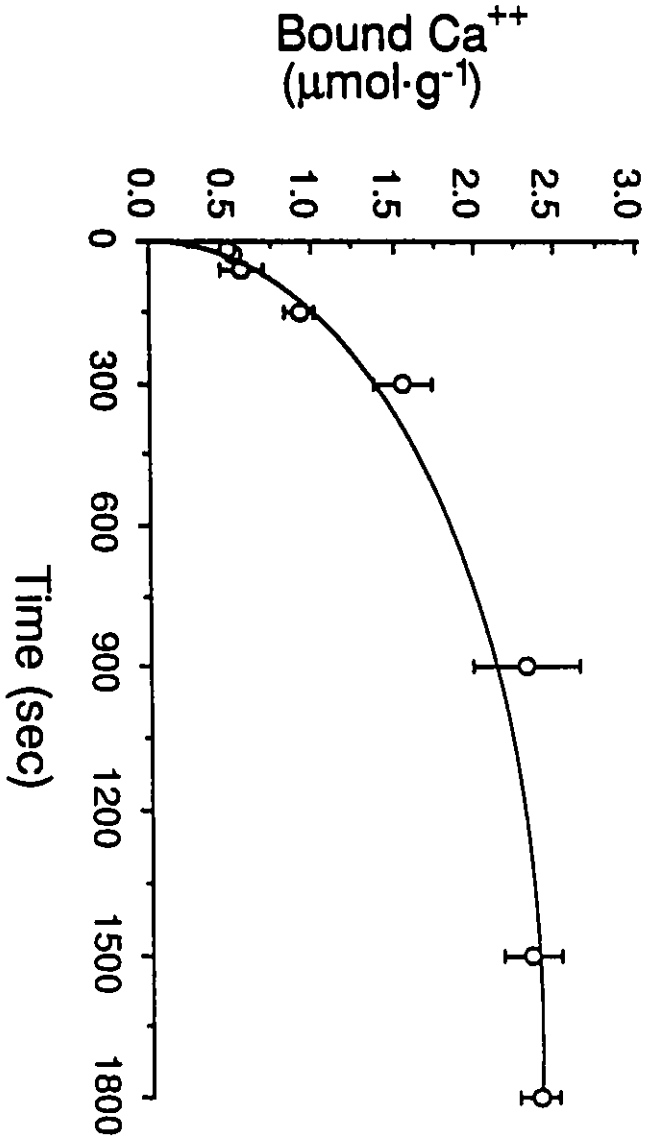

Bound Ca++
(µmol·g-1)
0.0
0.5
1.0
1.5
2.0
2.5
3.0
0
300
600
900
1200
1500
1800
Time (sec)

Figure 6.5. A concentration saturation curve for isolated gills of the 6.5/0 fish. Gill baskets were removed, cleaned, then exposed to a range of Ca^{++} concentrations for exactly 300 sec (See Chapter 6 Methods for details). The curve was fitted to the data by eye. Data presented as means ± 1 SEM. N = 4.

INSERT: The Scatchard plot generated from this saturation curve from which estimates of the Ca^{++} apparent dissociation constant (K_D) and the Ca^{++} binding capacity (B_{max}) were determined. The line was fitted to the Scatchard plot by linear regression. Data presented as means ± 1 SEM. (N = 4).

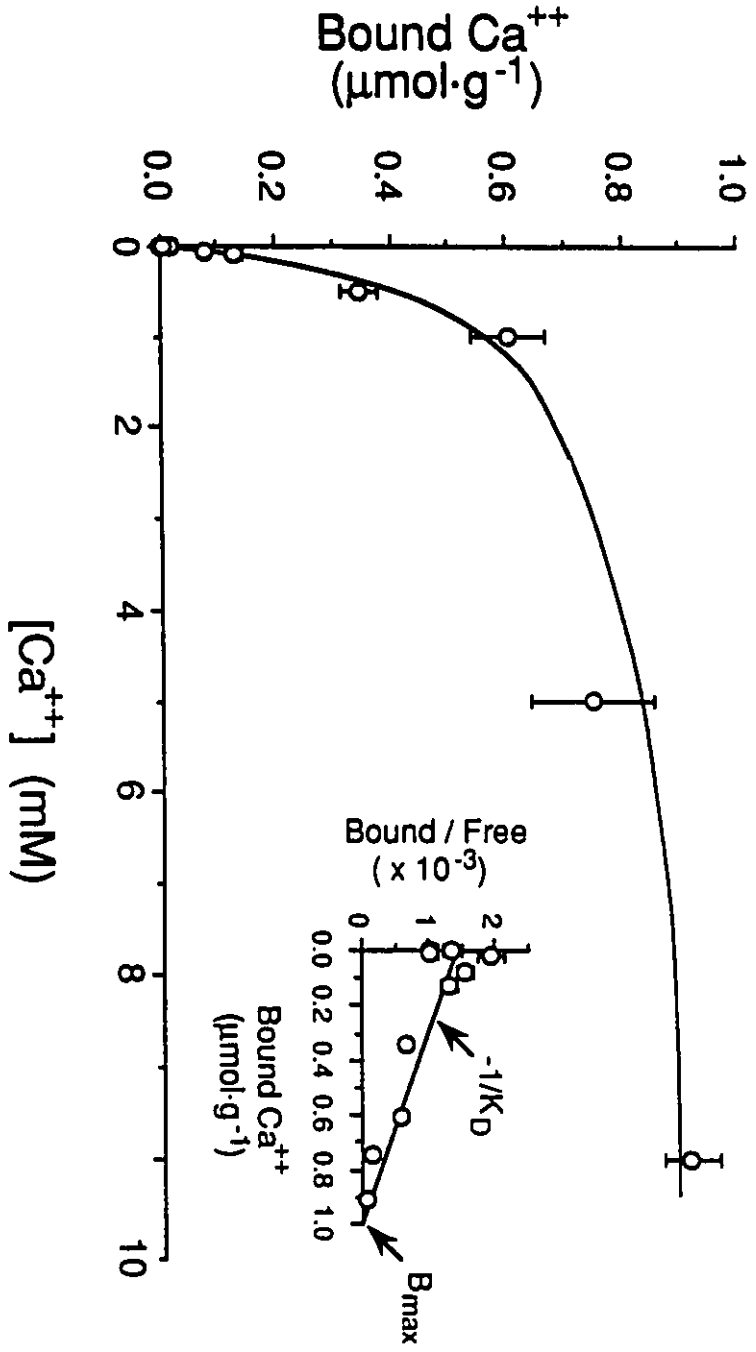
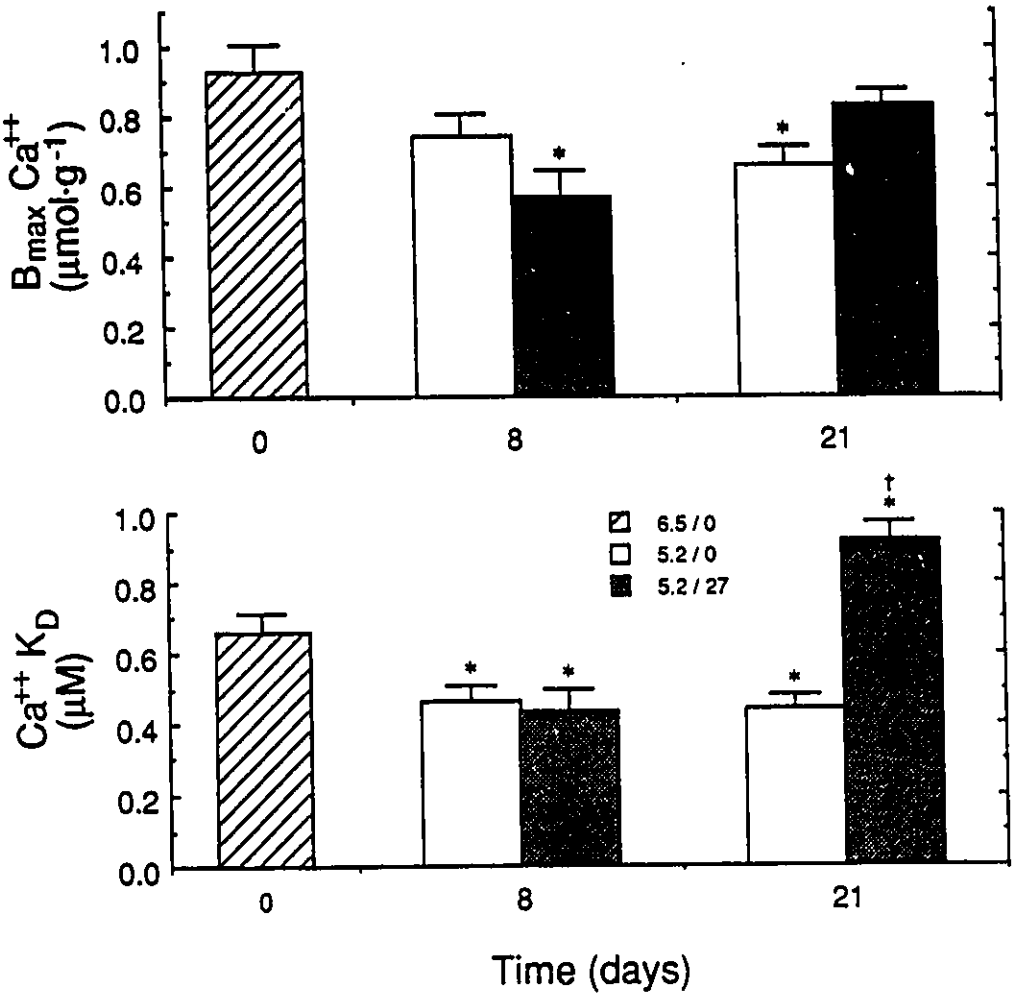

Bound Ca++
(µmol·g-1)
0.0
0.2
0.4
0.6
0.8
1.0
0
2
4
6
8
10
[Ca++] (mM)
Bound / Free
(x 10-3)
0
1
2
0.0 0.2 0.4 0.6 0.8 1.0
Bound Ca++
(µmol·g-1)
-1/KD
Bmax

Figure 6.6. Time course of changes in Ca^{++} binding activity. Changes in the Ca^{++} binding characteristics of isolated gill baskets were measured in the three treatment groups (6.5/0, 5.2/0, 5.2/27) during the 21 day acclimation period. B_{max} (A) and K_D (B), derived from the concentration saturation curves at Day 0 (control), Day 8 and Day 21 of exposure, are presented as means ± 1 SEM. N = 2. Significantly different data, as determined using a two-tailed Student's t-test at a 95% level of confidence, are indicated by an (*) or (†) for comparisons with control (6.5/0) or low pH only (5.2/0) respectively.


B_{max} Ca^{++} (μmol·g^{-1})
1.0
0.8
0.6
0.4
0.2
0.0
0
8
21
Ca^{++} K_D (μM)
6.5 / 0
5.2 / 0
5.2 / 27
Time (days)

Competitive inhibition experiments

At Day 21, increasing the concentration of H^+ or Al in the presence of constant Ca^{++}, resulted in a decrease in gill surface Ca^{++} binding (Fig 6.7A and 6..7B) in all 3 treatment groups. The IC_{50}'s for H^+ of fish with prior low pH exposure (5.2/0, 5.2/27) were significantly lower than controls, although not significantly lower than each other, indicating an increase in the ability of hydrogen ion to inhibit gill Ca^{++} binding. The Al IC_{50}'s were up to an order of magnitude lower than the IC_{50}'s for H^+ (Fig 6.7D), suggesting greater inhibition of calcium binding by aluminum than H^+. Twenty-one days exposure to both 5.2/0 and 5.2/27 resulted in significant increase in the Al IC_{50} compared to controls, indicating a decrease in the ability of Al to inhibit gill Ca^{++} binding. The effectiveness of Al as a Ca^{++} antagonist in the 5.2/0 group was reduced 13% and reduced further, almost 80%, in the 5.2/27 fish.

The calculation of the K_D's for Al and H^+ (Fig 6.8) show that the gill surface has the highest affinity for Al, then H^+, followed by Ca^{++}, in the ratio of 46:3:1 (based on the K_D's for controls). This ranking did not change with treatment, however the relative ratios did vary, resulting in ratios of 28:8:1 for the low pH (5.2/0) fish and 16:10:1 for the aluminum (5.2/27) fish. The relative affinity ratios suggest that chronic exposure to low pH results in an increase in the gill's affinity for H^+ with respect to Ca^{++} and Al, whereas chronic exposure to low pH and Al results in a decrease in the affinity for Al relative to Ca^{++}.

DISCUSSION

This study shows that exposure of rainbow trout to aluminum and low pH results in disturbances in branchial ionoregulation similar to those recently described in detail for brook trout (*Salvelinus fontinalis*; Booth et al 1988, McDonald and Milligan 1988, Wood

Figure 6.7. Competitive inhibition of Ca^{++}-binding by H^+ (A) and Al (B) prior to (6.5/0) and following chronic exposure to low pH (5.2/0) or Al in combination with low pH (5.2/27). Curves through the data were fitted by eye with the data presented as means ± 1 SEM. N = 4. Competitive inhibition curves were linearized to calculated the H^+ (C) and Al (D) IC_{50}'s for Ca^{++}-binding of the three treatment groups according to equation 6.2. Lines fitted to the linearized inhibition curves were fitted using least squares linear regression. The IC_{50}'s, calculated from the obtained linear regression, are presented as means ± 1 SEM from the inhibition experiments run in duplicate (N = 2). Significantly different data, as determined using a two-tailed Student's t-test at a 95% level of confidence, are indicated by an (*) or (†) for comparisons with control (6.5/0) or low pH only (5.2/0) respectively

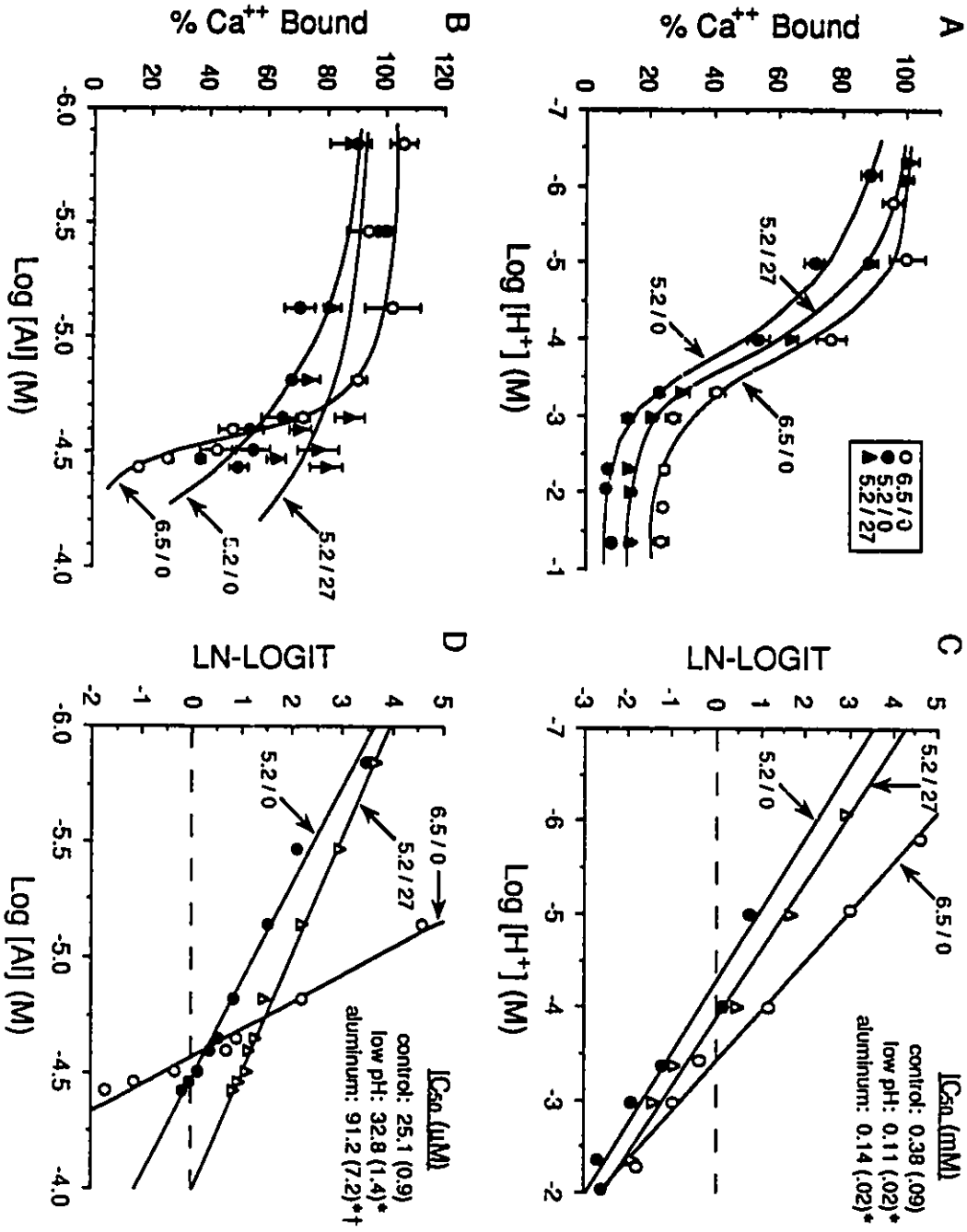
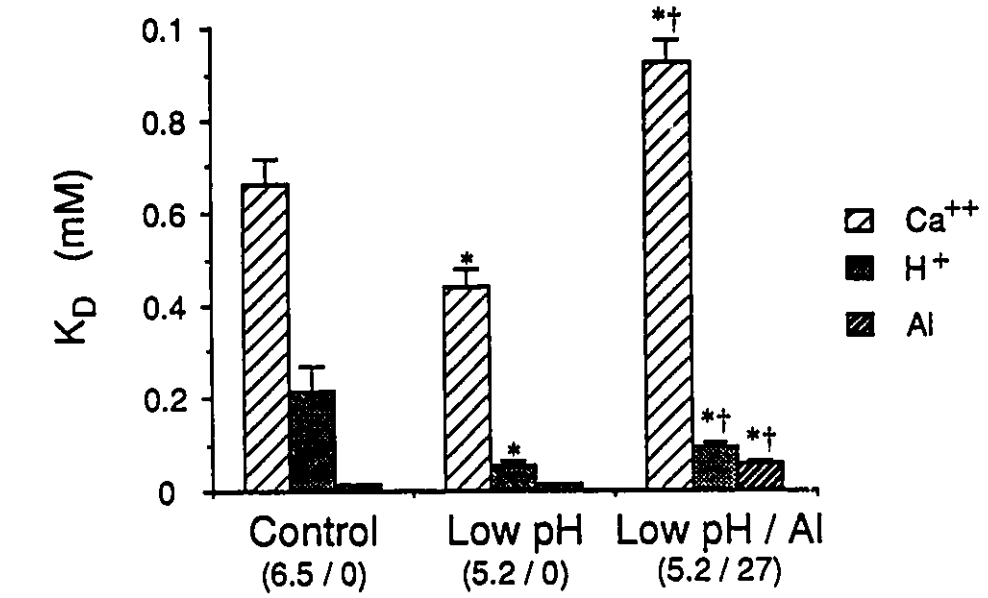

A
% Ca++ Bound
Log [H+] (M)
6.5/0
5.2/0
5.2/27
B
% Ca++ Bound
Log [Al] (M)
C
LN-LOGIT
Log [H+] (M)
IC50 (mM)
control: 0.38 (.09)
low pH: 0.11 (.02)*
aluminum: 0.14 (.02)*
D
LN-LOGIT
Log [Al] (M)
IC50 (μM)
control: 25.1 (0.9)
low pH: 32.8 (1.4)*
aluminum: 91.2 (7.2)*†

Figure 6.8. Apparent dissociation constants for the binding of Ca^{++}, H^+ and Al to the gill micro-environment. The K_D's for H^+ and Al were calculated according to equation 6.1 of the Methods. Affinity ratio was calculated using the inverse of the calculated K_D estimates of the gills for the appropriate cation. Data are presented as means ± 1 SEM. Significantly different data, as determined using a two-tailed Student's t-test at a 95% level of confidence, are indicated by an (*) or (†) for comparisons with control (6.5/0) or low pH only (5.2/0) respectively.


0.1
0.8
0.6
0.4
0.2
0
K_D (mM)
*†
*
*
*†
*†
Ca^{++}
H^+
Al
Control
(6.5 / 0)
Low pH
(5.2 / 0)
Low pH / Al
(5.2 / 27)
Affinity Ratio
(Al : H^+ : Ca^{++})
46:3:1
28:8:1
16:10:1

et al 1988a, McDonald et al 1989). However, rainbow trout are clearly more sensitive to aluminum and low pH than brook trout. For example, no aluminum-dependent ionoregulatory disturbances developed in brook trout exposed to 75 $\mu g \cdot L^{-1}$ aluminum at pH 5.2 (McDonald et al 1989), while in this study, under comparable conditions, significant aluminum-dependent physiological disturbances were observed in rainbow trout at only 27 $\mu g \cdot L^{-1}$ aluminum. In addition, rainbow trout at 27 $\mu g \cdot L^{-1}$ aluminum accumulate as much gill aluminum as do brook trout at an almost 3-fold greater aluminum concentration (McDonald et al 1989).

This study also confirmed that juvenile rainbow trout develop increased aluminum resistance specifically due to sublethal aluminum exposure (Orr et al 1986), and not due to the accompanying low pH (Audet and Wood 1988). The 5.2/27 fish performed well in the aluminum challenge (118 $\mu g \cdot L^{-1}$ Al), while both the 5.2/0 and 6.5/0 fish were equally adversely affected, exhibiting 50% mortality within 35 hours. Finally, characterization of the gill micro-environment according to its cation binding properties, has contributed new information to the understanding of the specific mechanism of Al toxicity and can now provide some of the physiological/biochemical basis for the development of Al resistance.

The Gill Micro-environment and Cation Binding

In this study, and in previous Chapters (namely, 3,4 and 5), it was shown that the gill micro-environment, which has been defined as the apical epithelial surface and its external mucous layer. has quantifiable and reproducible cation binding characteristics (Fig 6.5). The binding properties of this region are probably due, in large part, to sulphate and carboxylate groups of acid sugars (Wold and Selset 1977, Van de Winkel et al 1983) with pK's ranging form 2.6 to 4.0 (Dawson et al 1986). Essential cations, such as Ca^{++},

Na^+ and K^+ (Marshall 1878, McWilliams 1983) and trace metals (e.g Hg^{++} and Cd^{++}, Part and Lock 1983), have been shown to bind to these anionic groups. Pagenkopf (1983), in developing his Gill Surface Interaction Model (GSIM), recognized the cation-binding properties of the gill as a mechanism for explaining variations in trace metal toxicity in fish. In his view, toxicity resulted from the binding of a metal to anionic groups on the gills, with toxicity being dependent not only on metal-ligand chemistry, but also on competition from hardness cations (Ca^{++} and Mg^{++}). Thus, Ca^{++} has a critical role to play in gill physiology as it would act to limit trace metal binding. In addition, many studies suggest that Ca^{++} acts to stabilize membranes through cross-binding of intercellular ligands (Williams 1979) thereby limiting overall electrolyte and non-electrolyte permeability (Cuthbert and Maetz 1972, Eddy 1975, Ogasawara and Hirano 1984).

Toxic Mechanism of Al/H^+

Exposure of trout to H^+ or Al results in an increase in the passive electrolyte loss across the gill (McDonald 1983, Booth et al 1988). The increase in electrolyte permeability is thought to occur, at least in part, by the displacement of membrane-bound Ca^{++} by H^+ and Al (McDonald 1983, Booth et al 1988). While this hypothesis is not yet proven, we can now at least confirm that Ca^{++} binding sites are highly sensitive to titration by Al, having a 46 fold higher affinity for Al than Ca^{++} at pH 5.2 (Fig 6.8). At 27 $\mu g \cdot L^{-1}$ Al (the acclimation Al level) the Ca^{++}/Al concentration ratio would be only 14:1 (Fig 6.8), therefore one can expect significant displacement of Ca^{++} from binding sites. This is supported by the finding of significant initial accumulation of Al on the gills, which represents a 948 fold concentration factor over Al in the aqueous phase (25.6 $\mu g \cdot g^{-1}$ vs 0.027 $\mu g \cdot ml^{-1}$). In addition to Al/Ca^{++} interactions, H^+/Ca^{++} interactions also occur (Fig 6.7).

The H^+ IC_{50} for the Ca^{++}-binding groups was 3.4, i.e. an external pH of 3.4 would be required for 50% displacement of Ca^{++}. This suggests that at the moderately acid pH of 5.2 only minor physiological impact would be expected since little or no Ca^{++}/H^+ competition would occur at this pH. Indeed, only a minor physiological disturbance was noted in fish maintained at 5.2/0 (Fig 6.1).

Mechanism of Al Acclimation

These results suggest that the mechanism of Al acclimation involves specific changes in gill cation binding sites. By Day 21, the Al:Ca^{++} affinity ratio had declined from 46:1 to only 16:1 (Fig 6.8). With the actual Ca^{++}:Al ratio in the water remaining at 14:1, Ca^{++} and Al would now be competing on an equal basis for gill binding sites. Correlated with the decrease in Al affinity was a 44% decrease in gill Al content (Fig 6..3). Furthermore, when acclimated rainbow trout were challenged with 118 $\mu g \cdot L^{-1}$ Al (pH 5.2), the 5.2/27 fish accumulated 50% less Al than the other two treatment groups (6.5/0, 5.2/0; Fig 6.3). The increased Al IC_{50} associated with increased Al resistance (Fig 6.7D) means that a much higher Al concentration would now be required to displace Ca^{++} from the gills. Consequently, elevated Al should result in a reduced electrolyte disturbance if Ca^{++} displacement is key to the effect. This was confirmed, at least indirectly, since significantly lower plasma Cl^- losses and correspondingly smaller hematological disturbances were apparent in the 5.2/27 fish when Al challenged (Fig 6.1).

Specific Alterations in the Gill Micro-environment

Quite clearly, Al acclimation results in the reduction of the surface activity of Al. At least 2 distinct mechanisms can be suggested to explain this phenomenon: i) an

increase in mucous layer thickness on the gills, or ii) biochemical modifications to the binding ligands.

Mucification of the external gill surface has often been shown to occur in response to environmental toxicants (Mallatt 1985) and, at least in some cases, specifically to Al (Muniz and Levestad 1980, Schofield and Trojnar 1980). Part and Lock (1983) have argued that mucus plays a critical role in protecting the gills from the effects of toxic trace metals and supported this view by showing that mucous solutions, obtained from rainbow trout, would significantly reduce the diffusion of trace metals (Cd^{++} and Hg^{++}) *in vitro* when compared with rates measured in buffer solutions. However, the weight of evidence tends to argue against this simplistic view, at least in the case of Al. Firstly, Al exposure is not always accompanied by a mucous response. For example, Karlsson-Norrgren et al (1986) found abnormal accumulations of mucus on the surface of gills only occasionally in one of two groups of farmed brown trout (*Salmo trutta*) from acid-susceptible lakes with elevated concentrations of Al (200-300 $\mu g \cdot L^{-1}$). In addition, Chevalier et al (1984) showed that the mucocytes, in gills of brook trout inhabiting acidified lakes, exhibited no abnormal morphology, while a whole spectrum of morphological modifications were evident in a large number of chloride cells. Secondly, one would expect that an increase in the mucous layer should be accompanied by an increase in cation binding capacity, in gill sialic acid content and in Al content with time. The present study, in contrast, shows no further increase in B_{max} in the 5.2/27 fish beyond that of the controls (Fig 6.6), no change in the gill sialic acid content during the acclimation exposures (Fig 6.3B), and, in fact, a significant decrease in the gill Al content of the 5.2/27 fish following the initial accumulation (Fig 6..3A). Finally, it is unclear as to how a thicker mucous layer, simply containing more anionic groups with the same chemical characteristics, could result in differential changes in Al and Ca^{++} binding activity. Therefore, qualitative changes in

the mucus and membrane constituents responsible for cation binding is the more probable mechanism.

A number of studies have now shown that the external epithelium of fish can respond to environmental change with qualitative alterations in ligand chemistry. Flik et al (1984) showed that skin mucus of tilapia responded to prolonged low Ca^{++} exposure (1 month at 0.2 mM Ca^{++}) with a 2.3 fold increase in calmodulin, a Ca^{++}-specific protein, over fish maintained in hard water (0.8 mM Ca^{++}). Furthermore, Van de Winkel et al (1985) reported, with an identical treatment regime, a 3.2 fold increase in the glycoproteins, chondroitin sulfate and hyaluronic acid, in mucus removed from the body surface of rainbow trout. In addition, Zuchelkowski et al (1985) found, based on histochemical and autoradiographic analyses of skin mucocytes of catfish (*Ictalurus mebulosus* LeSueur), an apparent shift towards increased sulfomucin production in fish exposed to pH 4.8 and 4.0 compared to controls (pH 6.8). In a previous Chapter (Chapter 5), with techniques identical to those of the present study, it was demonstrated that low [Ca^{++}] acclimation in rainbow trout is also accompanied by significant alterations in cation binding activity of the gills (Fig 5.2). After 8 weeks at 0.02 mM external [Ca^{++}], gills of juvenile trout showed a 1.64 fold increase in B_{max} and a 1.4 fold increase in Ca^{++} binding affinity compared to gills of trout in hardwater (0.9 mM Ca^{++}).

So clearly there is evidence that fish can dynamically respond to environmental stress with changes in the surface chemistry of the gills. This Chapter shows a response somewhat different from that accompanying soft water acclimation. Cation binding capacity did not increase, instead it decreased slightly by Day 8 and then recovered to no greater than control levels by Day 21 (Fig 6.6). This suggests that rather than an increase in the quantity of various anionic groups per se, there was a change in the chemical characteristics of those groups. These changes could have occurred in the mucus

layer or at the external membrane. Arguably, changes in the latter would be more important for it is metal interactions here which have direct toxic impact. Biochemically, the gill micro-environment is very poorly characterized but it is obviously highly complex. The types of changes that could occur could include any one or more of the following: i) chemical changes in membrane lipids, phospholipids, glycolipids. proteins and glycoproteins, ii) increased rates of membrane turnover, and iii) incorporation or exclusion of membrane components. Quite clearly, a detailed biochemical analysis of the gills of fish, similar to that of Bolis et al (1984), who studied gill membrane lipids of brown trout during acute acid exposure, would be a fruitful way of more accurately defining the exact character of the response underlying the acclimation of the gills to environmental stress.

CHAPTER 7

GENERAL DISCUSSION

Several key observations regarding metal-gill interactions can be summarized according to the following general headings: i) The gill micro-environment, ii) Metal surface activity and iii) Modifications in the gill micro environment.

i) The Gill Micro-environment

The *in vitro* analysis of Ca^{++} binding activity (Fig 3.6B) suggests that, under normal conditions (i.e. a hard water trout living in hard water), approximately 50% of the gill Ca^{++}binding sites (Ca^{++}-"receptors") are normally occupied by Ca^{++}. The number of unoccupied "receptors" increases significantly in low Ca^{++} environments (Fig 5.2), despite modifications in "receptor" number and affinity in response to this environmental stress. However, it must be realized that the gill surface is normally a very dynamic region. Thus the *in vitro* analysis provides only a 'snapshot' of the metal binding characteristics of the gill micro-environment. Essentially, the *in vitro* analyses of gill-metal interactions were performed essentially on static tissue preparation. Yet the gill mucous layer is constantly renewed, with *in situ* rates of production ranging from as high as 8.23 $mg \cdot hr^{-1} \cdot g$ body $weight^{-1}$ (Eddy and Fraser 1982) to as low as 0.54 $mg \cdot hr^{-1} \cdot g$ body $weight^{-1}$ (Lock and Overbeeke 1981; calculated using the estimate of 0.184 μM NANA per g of mucus, Eddy and Fraser 1982). In addition, Conte and Lin (1967) have shown that the rate of cellular renewal in the gill filament of coho salmon (Oncorhynchus kisutch) is 15.8 ± 1 days, thereby providing a second continuous source of new metal binding sites within the gill micro-environment. Therefore, although much of the metal-binding character of trout

gills has been quantified, the full potential of the gill micro-environment as a metal-binding surface has not yet been realized.

ii) Modification of the Gill Micro-environment

This thesis demonstrates that the gills change in response to environmental stressors (low Ca^{++}, sublethal Al). The changes in the the gill surface are clearly adaptive. Increased Ca^{++} affinity, in response to low Ca^{++}, increases the ability of the gill to accumulate Ca^{++}. Decreased Al affinity in response to Al reduces the ability of Al to accumulate on the gills and thus reduces competition between Ca^{++} and Al at the gill surface.

The exact nature of the differences or modifications in the chemical constituents and relationships between binding at the gill and metal toxicity, proposed within this thesis are not purely speculative, but do lack an extensive literature from which support could be drawn. Therefore, more direct investigations similar to that of Bolis et al(1984), who characterized the phospholipid content of the gill membrane as a function of environmental pH, in conjunction with carbohydrate or glycosaminoglycan analysis of the gill micro-environment, should be initiated in the near future. Such thorough analysis of the gill surface, would not only lead to further our understanding of the basic composition, and proposed modifications in composition, of the gill micro-environment, but also would allow for more direct testing of some the general hypotheses of metal-gill interactions proposed within this work.

iii) Metal Surface Activity

The experiments detailed in Chapter 2 were important in demonstrating that metals have separate and specific disruptive effects on gill function despite sharing some

chemical properties. For example, Cd^{++} was extremely effective in inhibiting branchial Ca^{++} influx but had no effect on Na^{+} influx, while Cu^{++} specifically inhibited branchial Na^{+} influx (Figs 2.4 vs 2.7). In other words, the metals acted differently despite both being divalent and having similar tendencies to form ionic bonds with biological ligands (Table 7.1). These data suggest that there is more than one mechanism of toxicity at the level of the gill, and that the chemistry of the metal determines the mechanism. One mechanism which has received considerable attention within this thesis is the displacement of surficially-bound Ca^{++}. The binding of trace metals or H^{+} at sites on the gill normally occupied by Ca^{++} compromises membrane integrity, which can lead to a stimulation of electrolyte loss. The other general mechanism of metal toxicity involves subsurface competitive or non-competitive inhibition of specific enzymes. It has been argued that class A metals, with a greater ionic index than that of Ca^{++}, are toxic because they bind more tightly to gill Ca^{++}-"receptors" (e.g. Al). In contrast, metals with strong tendencies to form covalent bonds (such as Cu^{++}), are more likely toxic because of their ability to pass relatively freely through the gill surface to a subsurface region of more preferred ligands.

In an attempt to analyze these relationships in greater detail, the chemistry and binding activity of the metals (and H^{+}) used in this study have been summarized in Table 7.1. The metals are ranked top to bottom in order of decreasing affinity (increasing K_D) for the gills. In all cases (except Al and H^{+}), affinity was taken from the direct measurement of metal binding activity (see Figs 3.1 and 3.2). Estimates of the K_D of Al and H^{+} were calculated according to equation 6.3 which takes into account the Ca^{++} K_D, the Al or H^{+} IC_{50}, and the concentration of Ca^{++} in the assay solutions used to determine IC_{50}. In contrast to direct measurements of metal binding activity, estimates of metal capacity (B_{max}) are not obtainable with this calculation. Furthermore, the affinity estimate for Al is unique in that the it was made on gills of soft water-acclimated juvenile trout (Chapter 6).

METAL:	K_D (mM)	B_{max} (μmol/g)	FORMAL CHARGE	IONIC[a] RADIUS (nm)	ATOMIC[a] NUMBER	96h LC50 (μmol/L)	IONIC[b] INDEX (Z^2/r)	COVALENT[b] INDEX ($X^2_m r$)
Als	0.014	-----	3+	0.053	54	7.3[f]	17	0.9
Hs	0.22	-----	1+	-----	1	100[e]	-----	-----
La	0.31	0.14	3+	0.106	57	440[g]	8.5	1.3
Cas	0.65	0.45	2+	0.099	20	(>100) non-toxic	4.0	1.0
Cah	0.93	0.31	2+	0.099	20	non-toxic	4.0	1.0
Cd	0.94	0.58	2+	0.097	48	0.3 [c]	4.1	2.9
Hh	3.34	-----	1+	-----	1	100[e]	-----	-----
Cu	4.31	0.93	2+	0.076	29	0.3 [d]	5.4	3.0

s - soft water trout h - hard water trout

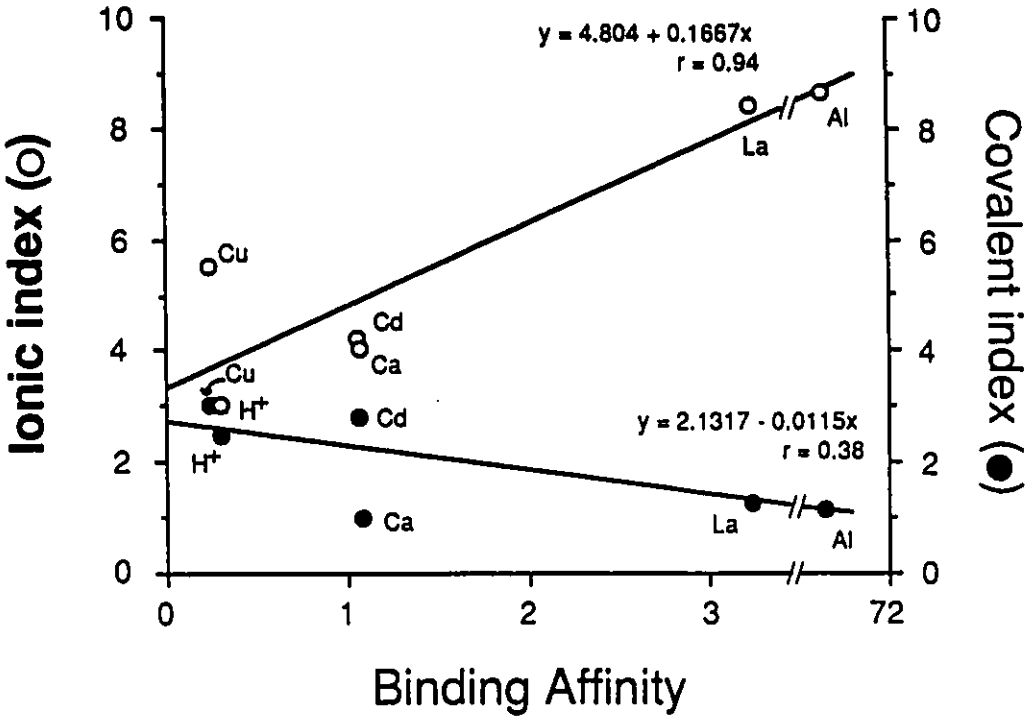
Table 7.1 Metal chemistry and toxicity summary

a. Weast et al (1984)
b. Nieboer and Richardson (1980)
c. Cusimano et al (1986)
d. Howarth and Sprague (1978)
e. Graham and Wood (1981)
f. Orr et al (1986)
g. Freda and McDonald (1988)

All other metal binding parameters were made on isolated gills from hard water-acclimated, adult trout. Prolonged soft water exposure was shown to significantly increase the affinity and capacity of the gill surface for Ca^{++} (Fig 5.2) and would be expected to similarly increase the affinity and capacity of all metals which binds to the same sites at the gill as does Ca^{++}. The affinity of the gill for Ca^{++} increased by 24 percent with acclimation to soft water, yet does not influence the ranking of Ca^{++}. Similarly, the ranking of Al relative to La^{+++} is not altered by acclimation condition, assuming that the gill affinity for Al is modified to a comparable extent as that for Ca^{++}. In contrast, the affinity for H^{+} was altered significantly as a result of acclimation condition, thus both estimates of the H^{+} affinity (as calculated according to equation 6.1 using data presented in Chapter 4) were included in the listing of metals.

There are two conclusions which can be drawn from Table 7.1. Firstly, the affinity of the gills for a particular metal is positively correlated to its ionic index (Fig 7.1). There also appears to be an inverse relationship between gill affinity and the covalent index, however this is not as clear cut as the link between the ionic index and binding affinity. Neither charge nor ionic radius, alone, seem to be directly related to the characteristic binding activity of these metals. However, this is not to imply that these properties are without influence since both are, in fact, components of both the ionic index and covalent indices. Secondly, there appears to be no clear relationship between either the binding affinity or binding capacity of the gills for these metals, and their respective toxicities. However, it could be suggested that metal toxicity is directly related to the covalent index, while inversely related to the ionic index. Consequently, it is not the ability of metals to bind to the gill surface, but the ability of metals to diffuse beyond the external gill surface, which determines the relative toxicity of various metals. The generality of this statement, could, however, be challenged simply on the basis of the

Figure 7.1. The affinity of the gill micro-environment for various metals as a function of both the covalent and ionic indices of the metals. Data were replotted from that presented in Table 7.1. Binding affinities were calculated as the inverse of the apparent dissociation constant .


y = 4.804 + 0.1667x
r = 0.94
y = 2.1317 - 0.0115x
r = 0.38
Ionic index (○)
Covalent index (●)
Binding Affinity
Cu
Cd
Ca
H+
La
Al
0
1
2
3
72
0
2
4
6
8
10

selection of metals presented here. Even though the covalent index for Al is lower than that of La^{+++}, the toxicity of Al is approximately 6 times greater than that of La^{+++}. The 96 h LC_{50} for La^{+++} was estimated relative to that of H^+ based on the magnitude of the Na^+ efflux rates from trout exposed to equivalent concentrations of either La^{+++} or H^+. When Freda and McDonald (1988), exposed juvenile rainbow trout (5-15 g) to pH 3.0, low Ca^{++} water (0.025 mM), the Na^+ efflux rate was measured to be 7.25 $mM \cdot kg^{-1} \cdot h^{-1}$. As part of the same study (Freda and McDonald 1988), the Na^+ efflux rate of trout exposed to 1 mM La^{+++} (equimolar with pH 3.0) was approximately 1.2 $mM \cdot kg^{-1} h^{-1}$ in the absence of Ca^{++}. The difference in the toxicities of La^{+++} and Al is likely a reflection of the 2-fold greater ionic index of Al relative to La^{+++}, since there is little difference in their covalent indices. Therefore, although a higher ionic index tends to indicate reduced toxicity, this may not always be so.

Obviously, this type of metal binding analysis needs to be continued to characterize the binding behavour of a wider variety of metals to the gill microenvironment. These data, combined with a detailed analysis of the chemistry of the external gill surface, will allow for a thorough testing of the proposed relationships between binding activity at the gill, metal chemistry, surface chemistry and metal toxicity.

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