MECHANISMS OF COPPER TOXICITY AND ACCLIMATION TO COPPER IN RAINBOW TROUT (*Salmo gairdneri* R.)

by

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ABSTRACT

Experiments were conducted on rainbow trout to analyse 1) the physiological mechanisms of short-term copper toxicity, 2) the effects of water hardness, pH, and alkalinity on copper toxicity, and 3) the physiological and biochemical mechanisms of acclimation to copper toxicity. Unidirectional and net sodium fluxes were the basic parameters measured.

Disruption of the ionoregulatory functions of the gill accounted for short-term (24 h) and long-term (28 day) copper toxicity. Copper inhibited sodium uptake at concentrations as low as 12.5 µg/L, and stimulated sodium efflux at copper concentrations greater than 100 µg/L. High alkalinity water (i.e., 1000 µM $Ca(CO)_3$) significantly reduced copper effects on both sodium uptake and sodium efflux. Although water hardness (i.e., 25 vs. 1000 µM $Ca(NO_3)_2$) had no effect on sodium uptake (at either pH 7.8 or pH 5.0), hardwater fish were better able to reduce sodium efflux than softwater fish. At pH 5.0 (in both hard and softwater), a significant additional inhibition of sodium uptake was found at low levels of copper but not at higher levels. Juvenile trout were about twice as sensitive to copper as adult trout.

Juvenile trout were able to acclimate to 55 µg/L copper. Acclimation was defined as the return of sodium uptake and whole body sodium to control levels during continuous exposure to copper. Sodium uptake kinetics and whole body sodium

concentration were analysed weekly during 4 weeks of exposure. The recovery of sodium uptake took about 3 weeks to complete, but whole body sodium recovered within 1 week. The time necessary for the recovery of influx was provided by a reduction in efflux. The inhibition and recovery of sodium influx was correlated with the inhibition and recovery of the Na^+-K^+-ATPase transport pool of the gill. Metal binding proteins played no apparent protective role in the recovery of Na^+-K^+-ATPase. Metal binding proteins were induced in the liver, but a significant portion of the whole body copper burden was accumulated by other tissues.

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TABLE OF CONTENTS

LIST OF FIGURES

Chapter 2

Chapter 3

LIST OF TABLES

CHAPTER 1

GENERAL INTRODUCTION AND METHODS

GENERAL INTRODUCTION

Copper: Sources and Basic Chemistry

Copper, an essential micronutrient with an atomic weight of 63.54, belongs to the first series of the transition metals. Its position in subgroup 1B of the periodic table is enclosed on either side by nickel and zinc, elements which are also micronutrients, but, because of its outer electron configuration, copper is placed directly over silver, a metal without known biological function, and one of the most toxic of the transition metals.

Copper can be acutely toxic to fish in the 10 to 1000 µg/L range (Spear and Pierce, 1979). Toxic concentrations occur in nature mainly through such processes as smelting, the combustion of fossil fuels, the dissolution of metal tailings from mining dumpsites, and the dissolution of copper from bedrock and soil due to acid precipitation (Hodson et al., 1979; Spry et al., 1981). Of these potential sources of copper, the atmospheric emission of particulate copper comprises the largest portion of the total, global copper budget. In fact, Nriagu (1979) has estimated that about 80% of the total copper ever mined has been mined during the 20th century, and that about 30% of the all-time atmospheric emmisions of copper will occur in the decade 1980 to 1990. In natural surface waters, copper concentrations rarely exceed 5 µg/L. However, copper has been reported at greater than 92 µg/L in an acidified lake in Ontario (Spry et al., 1981), at 200 µg/L at the drainage of a mine

tailings dump site in British Columbia (Roch et al., 1985), and at 4 mg/L in snow melt water near the copper smelter at Copper Cliff, Ontario (Freedman, 1978). Jones (1939) has shown that the toxicity of high levels of metals to fish follows the order: silver> mercury> copper> lead> cadmium> aluminum> zinc> nickel> cobalt. Since silver and mercury are relatively rare metals, copper is the most toxic metal commonly found in the aquatic environment.

The coordination chemistry of copper governs its interactions with inorganic ligands in natural waters (Sillén and Martell, 1964; Stumm and Morgan, 1970; Leckie and Davis, 1979). Metallic copper has an outer electron configuration of $3d^{10}s^{1}$, but the oxidation state of copper in the aquatic environment is usually 2^{+}, with the outer shell configuration being $3d^{9}$. Elements with partially filled 3d shells have a large tendency to form complex ions, and exist in various oxidation states. In the aquatic environment, copper ions coordinate with six water molecules and form the aquo ion, $[Cu(H_2O)_6]^{2+}$. Cupric copper is classified as an intermediate between hard and soft electron acceptors and forms strong complexes with electron donors such as oxygen, nitrogen and sulfur. Thus, in natural waters, stable complexes are formed by the replacement of H_2O by hard bases such as CO_3^{2-}, SO_4^{2-}, OH^{-}, and NH_3, and negatively charged residues of organic ligands such as humic acid. In freshwater, pH and alkalinity are the major inorganic factors modifying copper speciation. At pHs greater than 5.0, the aquo ion is predominant, while at neutral pH, copper carbonates such as $CuCO_3$ and $Cu(CO_3)_2^{2-}$, and copper hydroxides such as $CuOH^{+}$ predominate.

The coordination chemistry of copper also controls copper toxicity. The $3d^9$ configuration of coppers' outer electron shell gives copper the same electron withdrawing capacity as silver and mercury. It is well known that copper has a high affinity for sulphydryl residues of proteins and forms both square planar and tetrahedral covalent bonds in natural copper-dependent enzymes such as cytochrome c oxidase, and superoxide dismutase. In this respect, it is interesting to note that the administration of exogenous sulfhydryl compounds such as BAL (British anti-lewisite) and DTT (dithiothreitol), has been shown to be effective in the clearance of the body of toxic metals and the reversal of the effects of these metals. Thus, although ubiquitous in biological systems, the sulfhydryl group is thought to play a central role in metal toxicity in general, and copper toxicity, specifically.

However, non-sulfur ligands may substitute for one or more sulfur residues because copper also binds to oxygen (RPO_4^{2-}) and nitrogen (R_2NH) residues. Oxygen centers are usually occupied by calcium, and the displacement of calcium is likely to account for at least some of the toxic action of copper in the aquatic environment. Furthermore, copper has the potential for reacting with a wide variety of biological ligands and forms more stable complexes with oxygen, nitrogen, and sulfur than most other transition metals (Leckie and Davis, 1979). Because of this broad spectrum of possible ligands, copper toxicity may be due to the blocking of essential functional groups of biomolecules, the modification of the tertiary structure of a biomolecule, or the displacement of essential metal ions in a biomolecule (Ochiai, 1977).

Since copper can accept electrons from anions or donor atoms, e.g. $:NH_2-R$, it may also enter into oxidation-reduction cycles which form highly cytotoxic compounds such as peroxides and free radicals (Hochstein et al., 1980).

Copper Toxicity to Salmonids

The toxicity literature for copper on aquatic organisms is voluminous, and has been frequently reviewed (Hodson et al., 1979; Spear and Pierce, 1979; Alabaster and Lloyd, 1980). In general, Cu^{2+} is thought to be the most toxic species, but, except in acidic waters, Cu^{2+} is also a very small component of the total copper concentration. On the other hand, the copper carbonates are thought to be the least toxic species, and predominate at neutral pH in natural, alkaline water. Therefore, copper toxicity is enhanced by low pH, and reduced by high hardness/alkalinity waters (Mount and Stephan, 1969; Shaw and Brown, 1974; Howarth and Sprague, 1978; Chakoumakos et al., 1979; Miller and Mackay, 1980).

Most toxicity studies have considered water hardness and alkalinity as synonomous, and no previous studies have varied hardness, alkalinity, and NaCl independently (i.e., NaCl always varied with either hardness or alkalinity). However, Chakoumakos et al. (1979) tested the independent effects of hardness and alkalinity on cutthroat trout (Salmo clarki) and found that both hardness and alkalinity contributed to the 96 h LC50. Although carbonate complexation was used to explain the ameliorative effect of alkalinity on toxicity, no explanation of the

ameliorative effect of hardness was given. Miller and Mackay (1980) also tested the independent effects of hardness, alkalinity and pH on the lethality of copper to rainbow trout (Salmo gairdneri). They reported that while carbonate alkalinity can protect against copper toxicity in hard water, calcium hardness is more important than carbonate complexation in ameliorating lethality. Reducing pH increased copper toxicity down to pH 5.4; however, at lower pH, copper and pH were antagonistic. Similiar results at low pH were presented by Howarth and Sprague (1978). These results were explained on the basis of the high copper binding capacity of mucus, and the stimulation of mucus secretion by low pH (Miller and Mackay, 1980; 1982).

Very few experiments have examined the physiological or biochemical effects of copper on fish. Lorz and McPherson (1976) exposed yearling coho salmon to 5 to 30 µg/L copper and measured gill ATPase activity and the ability of the fish to acclimate to seawater (smoltification). Feeding was inhibited, and both survivorship of downstream migrants and ATPase activity decreased in proportion to the level of copper exposure. However, a marked recovery of the ability to survive downstream migration was found in fish chronically exposed at the highest level of copper. These fish became copper-resistant and resumed feeding.

Sellers et al. (1975) exposed adult rainbow trout (Salmo gairdneri) to copper concentrations up to 129 µg/L for 24 h, and analysed blood pH and pO_2. Copper had no effect on blood pO_2 within 24 h, but caused a transient rise in blood pH. However, it was noted that

blood pO_2 decreased from about 120 mm Hg under control conditions to about 75 mm Hg after 86 h of exposure to 129 µg/L copper. These authors also found an increased variation in ventilatory activity with the onset of copper exposure, suggesting that the fish were able to recognize the presence of the toxicant. In this respect, it is interesting to note that Donaldson and Dye (1975) showed that copper elicits a cortisol response in sockeye salmon (Oncorhynchus nerka) within 2 h of exposure. Thus, the increase in ventilatory variability may have been part of a generalized response to the presence of a stressor (Mazeaud et al., 1977).

Schreck and Lorz (1978) showed that in coho salmon (Oncorhynchus kisutch) exposure to 140 µg/L copper elicits both a decrease in plasma chloride, and an increase in plasma cortisol titer. No change in plasma osmolarity was found despite the fact that plasma chloride had decreased by about 30%. Schreck and Lorz (1978) suggested that the increase in cortisol either led to the decrease in plasma chloride, or was responsible for its correction since cortisol has been implicated in the maintenance of electrolyte balance and gill ATPase activity. However, several studies have shown that cortisol has no effect on ATPase activity in freshwater fish (Langdon et al., 1984; Eib and Hossner, 1985), and there is no evidence that cortisol stimulates ion loss in freshwater. This again suggests that the rise in cortisol in copper exposed fish is a generalized stress response, the main function of which is to increase resistance, possibly through gluconeogenesis (Freeman and Idler, 1973). An increase in plasma glucose may also

explain the discrepancy between plasma chloride and osmolarity found by Schreck and Lorz (1978).

McKim et al. (1970) found that exposure of yearling brook trout (Salvelinus fontinalis) to 38 and 68 µg/L copper for 21 days, led to decreases in serum osmolarity and chloride, and increases in hematocrit, red cell count, and plasma glutamic oxalacetic transaminase (PGOT). No significant effects were found at 23 µg/L. All the fish in the 68 µg/L group, and 57% of the 38 µg/L fish died before 337 days, but neither serum chloride, osmolarity, red cell count, or hematocrit were significantly different from control plasma values in the survivors at 38 µg/L after 337 days. PGOT, an indicator of tissue damage, actually decreased below control values. These results suggest that copper disrupts ion balance, but that below a certain level, adult fish can acclimate to these effects. Nevertheless, McKim et al. (1970) predicted that only copper concentrations of less than 23 µg/L would be safe for adult trout. Indeed, in a continuation of the same study, McKim and Benoit (1971) found that 33 µg/L copper reduced growth in adults, the number of viable eggs produced, and egg hatchability. Furthermore, the surviving alevins and juveniles from this group were no less sensitive to subsequent exposures to copper than those whose parents had never been exposed to copper. It may be concluded that although measurements of blood parameters may be a sensitive indicator of short-term detrimental effects of copper, they are of little value during long-term exposures unless monitored continuously, i.e., although apparent acclimation was found for blood parameters, the cost of acclimation was

taken from growth and reproductive success. Furthermore, acclimation of adults did not result in acclimation of their progeny, and juvenile trout are more sensitive to copper than adults (McKim and Benoit, 1971).

Lett et al. (1976) exposed juvenile rainbow trout for 40 days to copper concentrations ranging from 75 to 225 µg/L in very hard, high alkalinity water and determined the effects of food ration on growth. They found an initial cessation of feeding and a decrease in growth rate. After 40 days of exposure at the highest concentration, growth rate was only slightly lower than controls. These authors concluded that trout could adapt to copper concentrations up to 50% of the 96h LC50 value. However, the recovery of growth may not have been an adequate indicator for complete acclimation, and the results of McKim et al. (1970) and McKim and Benoit (1971) suggest that longer term studies (i.e., several generations) may be necessary to resolve this question.

More recently, Dixon and Sprague (1981a, b) and McCarter et al. (1982) and Buckley et al. (1982) have shown that exposing fish to sublethal levels of copper for several days increases the lethal resistance time (i.e., 96 h LC50). Both groups have also shown an increase in the metal-binding protein, metallothionein, in the liver, and suggested that the induction of metallothionein is responsible for the increase in lethal resistance time. However, McCarter and Roch (1983) showed that the peak period of increased resistance preceeded the peak of metallothionein induction by 3 weeks, and at 50 µg/L copper, there was no apparent induction of metallothionein at all during the peak of lethal resistance. The poor correlation between resistance and

metallothionein suggests that metallothionein may be a secondary, rather than a primary response.

Research Objectives: Mechanisms of Toxicity

Taken in concert, these physiological and biochemical studies strongly suggested that at least a major target of copper toxicity in fish is the mechanism for maintaining electrolyte balance. No previous study has specifically examined the mechanisms of ionic regulation in copper exposed fish, but simil ar effects on ionoregulation have been described for fish exposed to low pH (Packer and Dunson, 1970; Leivestad and Muniz, 1976). It seemed pertinent, therefore, to examine the effects of copper on fish by measuring unidirectional and net ion fluxes, methods which have previously been shown to be very sensitive indicators of ionoregulatory disturbance in fish (McDonald et al., 1980; McDonald and Wood, 1981; Milligan and Wood, 1982; Wood and McDonald, 1982; McDonald, 1983a, b; McDonald et al., 1983). Copper exposures were limited to 24 h in an attempt to reduce the masking of the primary effects of copper by secondary responses of the fish. Furthermore, fish were exposed to copper in artificial media which allowed the effects of water hardness, pH, and alkalinity to be examined independently of each other, but at a constant [NaCl].

The objectives of this phase of research were: 1) to establish the short-term physiological mechanisms of copper toxicity to fish at the tissue level, 2) to establish the modulatory effects of water hardness, pH, and alkalinity on the short-term mechanisms of copper

toxicity, 3) to establish the de novo uptake of copper as a function of copper concentration, and 4) to establish indices of stress as a function of copper concentration. The results of this phase of research are summarized in the Chapter 2 and 3 of this thesis. A modified version of Chapter 2 has previously been published in the Journal of Comparative Physiology, and a modified version of Chapter 3 is currently in press in the Canadian Journal of Fisheries and Aquatic Sciences.

Mechanisms of Acclimation

Once these objectives were met, it was decided to investigate the physiological effects of long-term exposure to sublethal copper. Fish are found in nature in copper polluted waters, suggesting that they are able to adapt to the presence of copper in concentrations which have been shown to be toxic in laboratory studies (Grande, 1967; Van Loon and Beamish, 1977; Franzin and McFarlane, 1980; Alabaster and Lloyd, 1980; Black et al. 1982; Roch et al., 1985). Furthermore, current literature suggested that prior exposure of fish to sublethal concentrations leads to an increase in lethal resistance (96 h LC50) during subsequent exposure. However, the physiological basis for this increase in lethal resistance has not previously been investigated.

In Chapter 2 and 3, it will be shown that at least a major mechanism of sublethal copper toxicity is the disruption of branchial ionoregulation. Therefore, it was reasoned that true acclimation to copper must be accompanied by recovery of both whole body sodium balance and sodium uptake. Kinetic analysis of sodium uptake is a very

sensitive indicator of both uptake velocity and affinity of the sodium uptake mechanism. Therefore, juvenile rainbow trout were exposed to a sublethal copper concentration for 28 days, and whole body sodium as well as sodium uptake kinetics were measured at weekly intervals. Because previous authors had indicated metallothionein and branchial ATPases are important factors in the development of copper resistance in fish, these biochemical parameters were also measured.

The objectives of this phase of research were 1) to establish if physiological acclimation to a sublethal, environmentally relevant, copper exposure occurs, 2) to determine the tissue distribution of copper and evaluate the possibility that the accumulation of copper may lead to hepatic failure, 3) to evaluate the biochemical basis of acclimation to copper. The results of this phase of research are summarized in Chapter 4 and 5 of this thesis, both of which have been submitted for publication in the Canadian Journal of Fisheries and Aquatic Science.

GENERAL METHODS AND MATERIALS

Rainbow trout, Salmo gairdneri, obtained from Spring Valley Trout Farm, Petersberg, Ontario, were used in all experiments. Sodium and chloride regulation were the basic functions studied, and only non-invasive methods were used. Unidirectional fluxes of sodium and chloride were measured using the radioisotopes, ^{22}Na and ^{36}Cl, as markers. Net fluxes of sodium, chloride, potassium, and ammonia were measured by changes in concentrations in the flux media. All flux measurements were conducted in aerated, static systems, using artificial media consisting of NaCl, and either $Ca(NO_3)_2$ or $CaCO_3$ salts. Copper was added as $Cu(NO_3)_2$, and pH was adjusted with HNO_3, H_2SO_4, or KOH. All fish were acclimated to the test media for 10 to 14 days before exposure to copper or to low pH. All experiments as well as acclimation were conducted at about $15 \pm 1^{\circ}C$.

Statistical Analysis

All data presented are means $\pm$ 1 standard error of the mean. Statistical significance was determined by Student's two tailed t-test for unpaired treatments, and the level of significance taken as $P < 0.05$. Interactions between the independent variables, pH, hardness, and alkalinity, and the dependent variables, sodium uptake, net sodium balance, and sodium efflux, were tested by multiple linear regression using SPSS (Stastical Package for the Social Sciences).

CHAPTER 2

EFFECTS OF COPPER ON BRANCHIAL IONOREGULATION IN ADULT RAINBOW TROUT:

Modulation by water hardness and pH.

INTRODUCTION

There are many reports that copper is more toxic to fish in soft- than in hardwater at neutral pH (Lloyd and Herbert, 1962; Mount and Stephan, 1969), and more toxic still at low pH (Howarth and Sprague, 1978; Chakoumakos et al., 1979). However, very little is known about the mechanism of copper toxicity, or the manner by which water hardness and pH modify this mechanism.

Previous studies have shown decreased plasma osmolality and $[Cl^-]$, and increased hematocrit and cortisol titer in copper exposed fish (McKim et al., 1970; Lewis and Lewis, 1971; Christensen et al., 1972; Schreck and Lorz, 1978). Simil ar effects have been reported by McDonald and Wood (1981) for fish exposed to low pH, and McDonald et al. (1983) found that these changes could be attributed to the effects of H^+ on the branchial ionoregulatory apparatus of fish. Thus, it seemed likely that copper and H^+ might share a common toxic mechanism. The following experiments were designed to 1) Define the toxic mechanism of copper, and 2) Determine the basis for the modifying effects of both water hardness and pH on this mechanism.

METHODS AND MATERIALS

Animals

Adult rainbow trout (mean wet weight = 202±28 g; n = 140) were held in large, opaque, polyethylene (food grade) tanks supplied with

flow-through dechlorinated Hamilton tap water. The fish were acclimated for 10 days in recirculating water of the same ionic composition subsequently employed in the flux experiments (Table 2.1). During this time the trout were not fed, and the water was changed daily.

Exposure Media

Water for acclimation and experimentation was prepared by either reverse osmosis of tap dechlorinated water, or from in-house tap distilled water. NaCl and $Ca(NO_3)_2$ were then added to the appropriate level (Table 2.1), and pH was adjusted to pH 7.8 by addition of KOH, or to pH 5.0 by the addition of H_2SO_4. In all experiments with adult trout, the fish were contained with acrylic flux chambers as described by McDonald (1983a).

Experimental Protocol

In order to avoid stress, beyond that produced by ion loss and confinement, no indwelling catheters were used in any of the following experiments. Trout were placed within the flux chambers in a recirculating system 24 h before the beginning of an experiment, and the water was changed daily to maintain ion levels. For flux measurements, the flux chambers were isolated from the recirculating system, and the volume adjusted to about 2.7 to 6.9 L. Temperature was maintained by submersion of the chambers in a cooled water bath. Resting rates of ion flux were measured during a 2 h period of ^{22}Na and ^{36}Cl fluxes prior to the addition of copper. Water samples were collected for the measurement of net and unidirectional fluxes at -2, 0, +2, +4, +8, and +12 h from the addition of copper. Copper exposures were begun by the

	Hardwater pH 7.8	Softwater pH 7.8	Hardwater pH 5.0	Softwater pH 5.0
Na^+	226.2±13.7	219.4± 4.5	247.9± 1.8	205.6± 1.8
Cl^-	218.1± 6.1	217.9± 3.3	231.6± 3.3	182.5± 2.1
K^+	35.5± 1.9	53.6± 1.7	31.1± 4.3	14.1± 2.0
Ca^{2+}	1,035±17.0	27.0± 2.8	993± 2.0	29.5± 3.5
Ammonia	51.9± 4.2	66.4± 5.7	67.9± 4.4	29.2± 1.3
pH	7.86	7.89	4.98	4.97
	7.4- 7.8	7.4- 8.0	4.4- 5.3	4.6- 5.6

Table 2.1. The composition (means ± 1 SEM) and pH (means, range) of test water. Temperature= 15 ± 1°C. All ions in μM.

addition of a $Cu(NO_3)_2$ solution. Nominal copper concentrations were 25, 50, 100, 200, and 400 µg/L total copper. To enable the fast and accurate measurement of copper concentration, ^{64}copper, prepared by the McMaster University Nuclear Reactor, was added to a known specific activity, and water samples counted on either a NaI gamma counter (Nuclear Chicago model 1085), or a liquid scintillation beta counter (Beckman model LS 230). At the end of 12 h, the water was quickly changed, and a second 12 h period of copper exposure begun.

In order to assess the effects of hard- and softwater acclimation, and the effects of neutral pH and pH 5.0 exposure, control groups were run at the same time as copper exposures. Prior to the beginning of a flux period, fresh water of the appropriate composition was pumped into each flux chamber, and the chamber drained down to the fish's dorsal fin. This was repeated twice more to flush the box thoroughly and to minimize ammonia accumulation and fluctuations in ion levels between individual fish. At the end of the experiment the fish were stunned by a blow to the head, and a blood sample removed by caudal puncture.

Analytical Techniques

Na^+, K^+, and Ca^{2+} were measured by flame photometry (EEL mark II for Na^+ and K^+; Coleman 20 for Ca^{2+}). Chloride levels were determined by coulometric titration (Radiometer CMT-10, or Buchler-Cotlove Chloridometer). Ammonia was measured by a modification of the colorometric method of Verdouw et al. (1978). Glucose was measured by

the o-toluidine method of Hyvarinen and Nikkila (1962), using Sigma reagents (Sigma Chemical Co. St. Louis, Mo.).

Water copper was measured by atomic absorption spectroscopy (Jarrel Ash 800) after first chelating the copper with ammonium pyrollidine dithiocarbamate (APDC), and extracting the copper-chelate with methyl isobutyl ketone (MIBK; Brooks et al., 1967). This was followed by back extraction into concentrated HNO_3, and direct aspiration into the flame. Standards were treated in the same manner and analysed simultaneously. No significant differences were found between this method and the ^{64}copper method.

Flux Measurements

Net fluxes (J_{net}) of Na^+, K^+, Cl^-, and ammonia were calculated from changes in their concentrations in the water. Na^+ and Cl^- influxes (J_{in}) were determined from the disappearance of ^{22}Na (26 KBq/L) and ^{36}Cl (26 KBq/L) from the water according to the equation given by McDonald et al (1983):

$$J_{in} = \frac{(\ln Q^*_o - \ln Q^*_t) \times (Q_{out})}{t \times W} , \quad (2.1)$$

where Q^*_o and Q^*_t are the counts per minute in the flux chambers at the beginning and end of the flux period; Q_{out} is the average amount of Na^+ or Cl^- in the medium over the flux period; t the time in hours; and W the weight of the fish in kg, to the nearest g. J_{out} was calculated by subtracting J_{net} from J_{in}. Fluxes were expressed in μM/kg/h.

RESULTS

Evaluation of Copper Exposure Method

In each experiment, regardless of water hardness or pH, the total copper concentration decreased with time in a bimodal fashion, the decrease being initially very fast and then slowing to a more gradual rate (Fig. 2.1). This occurred whether or not there was a fish present, thus indicating that there was a significant absorption of copper by the acrylic material of the flux chambers. Fortunately, the proportion of copper lost from solution was independent of the initial copper concentration, and was consistently reproducible, so that for each 12 h period, the mean exposure concentration (i.e., $[Copper]_{\bar{x}}$) could be accurately described by the following relationship:

$$[Copper]_{\bar{x}} = 0.54 \times [Copper]_{initial} \qquad (2.2)$$

An initial concern was whether a declining copper concentration produced the same effects as a constant concentration. This was tested in an 8 h experiment where trout were exposed to an average copper concentration of 100 µg/L produced in two different ways; by a single initial addition of copper at 200 µg/L (n=4), and by maintaining a relatively constant 100 µg/L by periodic addition (n=4). After 8 h both groups had lost similar amounts of Na^+ (Fig. 2.2). Furthermore, these losses arose in a similar manner; by an 83% inhibition of influx (J_{in}) and by a 50% stimulation of efflux (J_{out}), relative to controls. However, there was an initial rapid decline in ion loss rate with time in the single dose experiments (Fig. 2.1) that was not observed in the

Fig. 2.1. Effect of copper on J_{net} sodium in adult rainbow trout over 12 h of exposure at an initial copper concentration of 400 µg/L in low alkalinity hardwater. Data are means ± 1 SEM, n=6.

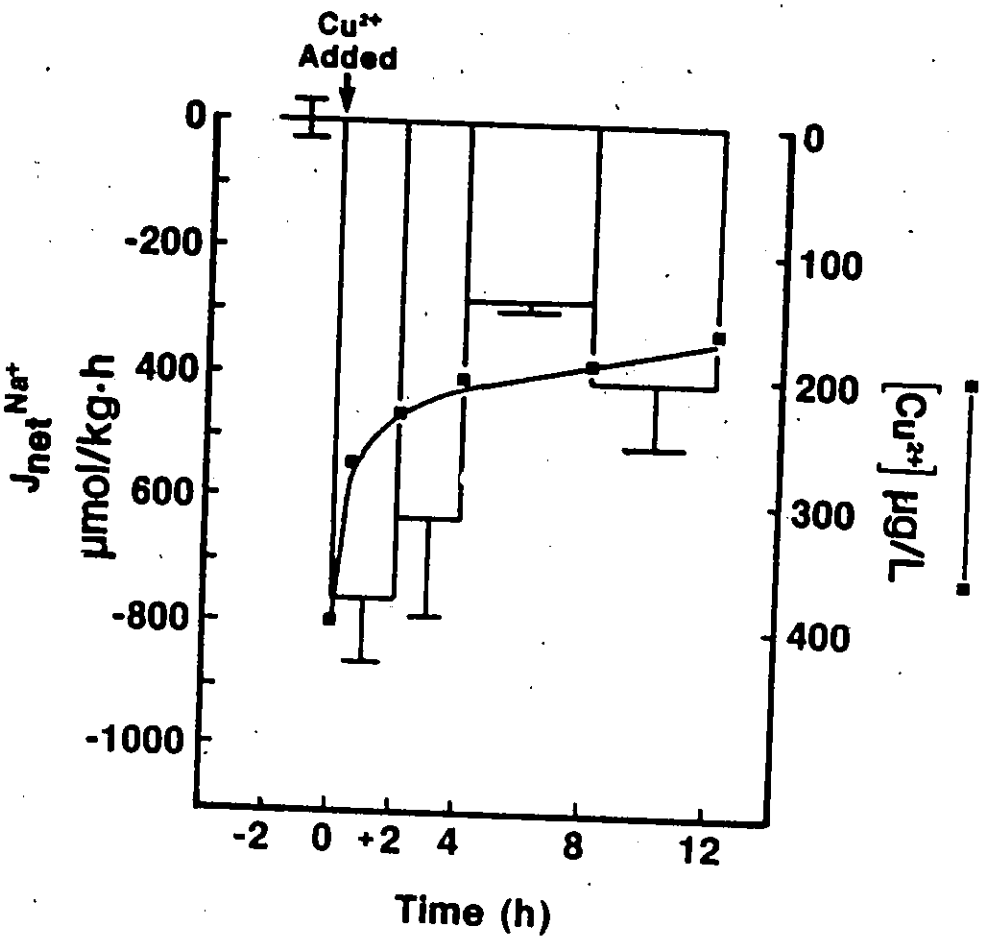
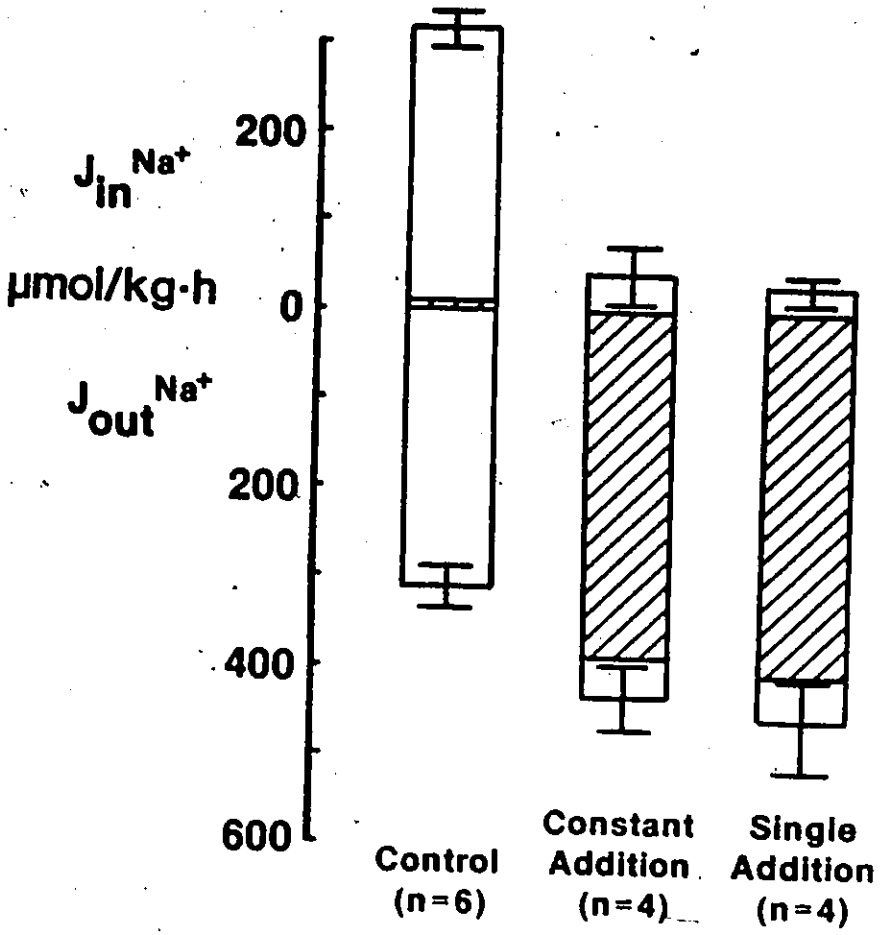

Cu²⁺
Added
0
-200
-400
-600
-800
-1000
$J_{net}^{Na^+}$
µmol/kg·h
0
100
200
300
400
[Cu²⁺] µg/L
-2
0
+2
4
8
12
Time (h)

Fig. 2.2 Unidirectional sodium fluxes following exposure to a single addition of 200 µg/L copper, and a constant exposure to 100 µg/L copper for 8 h in low alkalinity hardwater at neutral pH. Means ± 1 SEM. Sample size is indicated in brackets. Hashed area is the net flux.


$J_{in}^{Na^+}$
µmol/kg·h
$J_{out}^{Na^+}$
200
0
200
400
600
Control
(n=6)
Constant
Addition
(n=4)
Single
Addition
(n=4)

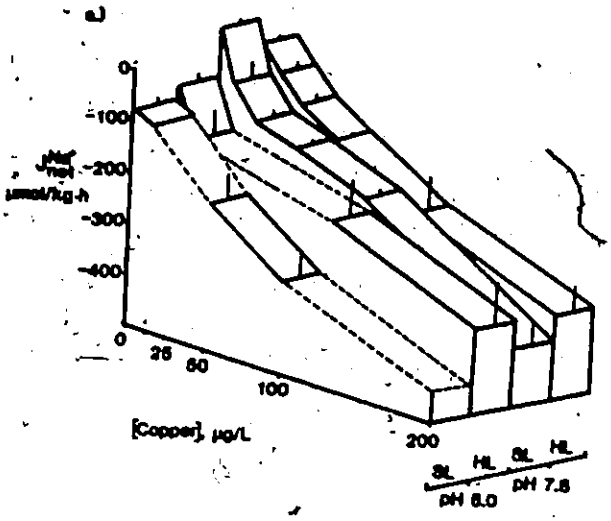
constant-dosing treatment. This is most likely attributable to the declining copper concentration in the former. This was judged not to be a serious problem since the period of rapid copper concentration change (Fig. 2.1) would be a relatively small proportion of the total exposure period of 24 h. Thus, in subsequent experiments a single initial addition of copper to a flux chamber was employed, and all exposure copper concentrations have been expressed according to Eq. 2.2.

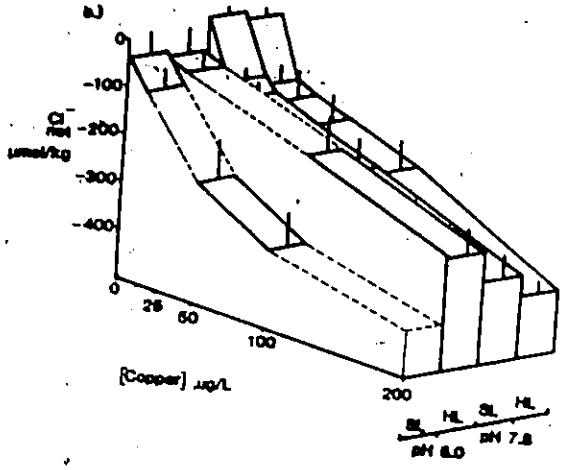
Ion Regulation in Control Fish

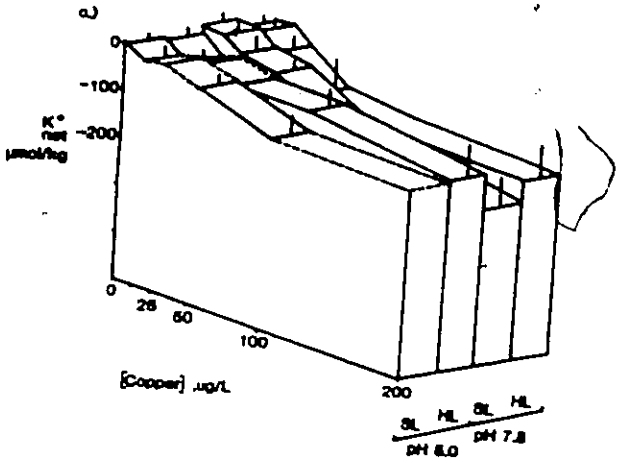
In circumneutral pH in hardwater (1000 μM Ca^{2+}; Table 2.1), net Na^+ and Cl^- fluxes were not significantly different from zero (Fig. 2.3). J_{net} K^+ was negligible (Fig. 2.3) and ammonia efflux (Fig. 2.4) was comparable to previously published values for rainbow trout (McDonald et al., 1983). Similarly, plasma Na^+, Cl^- (Fig. 2.5), K^+, glucose, and ammonia (Fig. 2.6) concentrations were within the range previously reported for this species (McDonald, 1983a; Hille, 1982).

Following 10 days exposure to softwater (25 μM Ca^{2+}, Table 2.1) there was a slight, but not statistically significant, net uptake of both Na^+ and Cl^- of about 25 μmol/kg.h (Fig. 2.3). Net K^+ and ammonia fluxes were not significantly different from hardwater values (Figs. 2.3, 2.4) nor were plasma Na^+, Cl^- or glucose concentrations (Figs. 2.5, 2.6). Plasma ammonia (Fig. 2.6) was, however, significantly greater than in hardwater, although these values still fell within the normal range previously reported (Hille, 1982). Thus, control fish in softwater at neutral pH had a similar ionic status to controls from hardwater. No mortalities were found in either control group;

Fig. 2.3. Mean net a) sodium, b) chloride, and c) potassium fluxes over 24 h of copper exposure in low alkalinity hard (HL) and softwater (SL) at neutral pH, and pH 5.0. Dashed lines indicate that only 12 h data are available. Means $\pm$ 1 SEM. Sample size as in Fig. 2.4.


a)
0
-100
-200
-300
-400
0
25
50
100
200
[Copper] µg/L
St
HL
St
HL
pH 6.0
pH 7.8


a)
0
-100
-200
-300
-400
0
25
50
100
200
[Copper] µg/L
St
HL
St
HL
pH 6.0
pH 7.8


a)
0
-100
-200
0
25
50
100
200
[Copper] µg/L
St
HL
St
HL
pH 6.0
pH 7.8

nevertheless, softwater acclimated fish were in general, more easily excited and responded more vigorously to external stimuli than hardwater fish. In addition, examination of the gills of softwater fish by both scanning and transmission electron microscopy, revealed the proliferation of chloride cells on the lamellar epithelium, which may have been responsible for the slight net positive NaCl balance observed.

In low pH experiments, fish were first acclimated for 10 days to either artificial hard- or softwater at neutral pH, and then acutely exposed to pH 5.0 for two consecutive 12 h periods. In both hard- and softwater, exposure to pH 5.0 without copper led to net NaCl losses (Fig. 2.3) with a significant reduction in plasma NaCl levels (Fig. 2.5) and to an increase in ammonia efflux (Fig. 2.4) in hardwater fish. However, no increases in K^+ loss (Fig. 2.3) or in plasma glucose or ammonia levels (Fig. 2.6) were found.

In general, NaCl turnover rates (i.e., J_{in} plus J_{out}) were lower in soft- than in hardwater at pH 5.0 (Fig. 2.7C vs. D, G vs H). As a result, net NaCl losses were about 50% lower in softwater during the 24 h exposure (Fig. 2.3). However, net losses were reduced in hardwater in the second 12 h period, while losses continued at the same level during both periods in softwater. This was attributable to a reduction in J_{out} in hardwater (Fig. 2.8, hashed area).

Effects of Copper Exposure

Ion losses by rainbow trout were strongly dependent upon copper concentration over the entire 24 h of exposure (Fig. 2.3). For

Fig. 2.4 Net ammonia fluxes during the first (open bars) and second (hashed bars) 12 h of exposure to copper. Means ± 1 SEM. Sample size indicated within each bar.

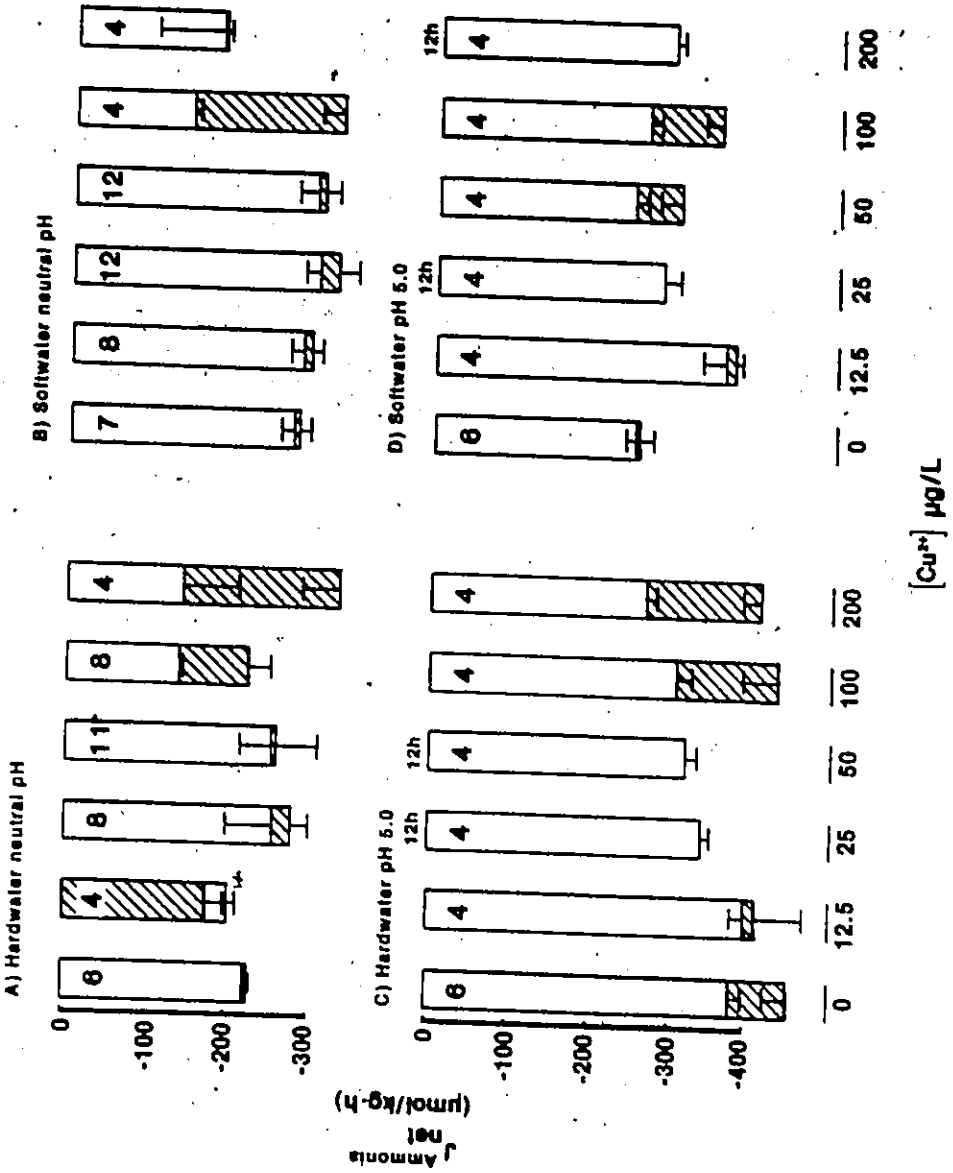

A) Hardwater neutral pH
B) Softwater neutral pH
C) Hardwater pH 5.0
D) Softwater pH 5.0
12h
0
-100
-200
-300
-400
0
12.5
25
50
100
200
J_{net} Ammonia (µmol/kg·h)
$[Cu^{2+}]$ µg/L

Fig. 2.5 Plasma [Na^+] (●) and [Cl^-] (▲) after 24 h exposure to copper in A) hardwater at neutral pH, B) softwater at neutral pH, C) hardwater at pH 5.0, and D) softwater at pH 5.0. Means $\pm$ 1 SEM. Sample size as in Fig. 2.4. Lines fitted by least squares regression.

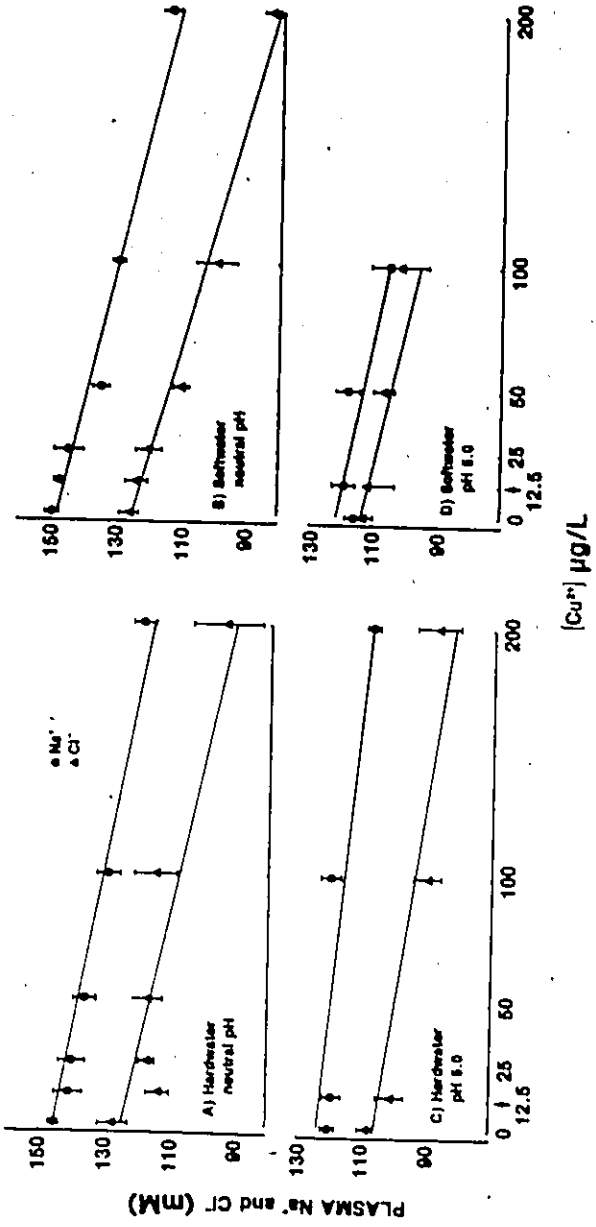
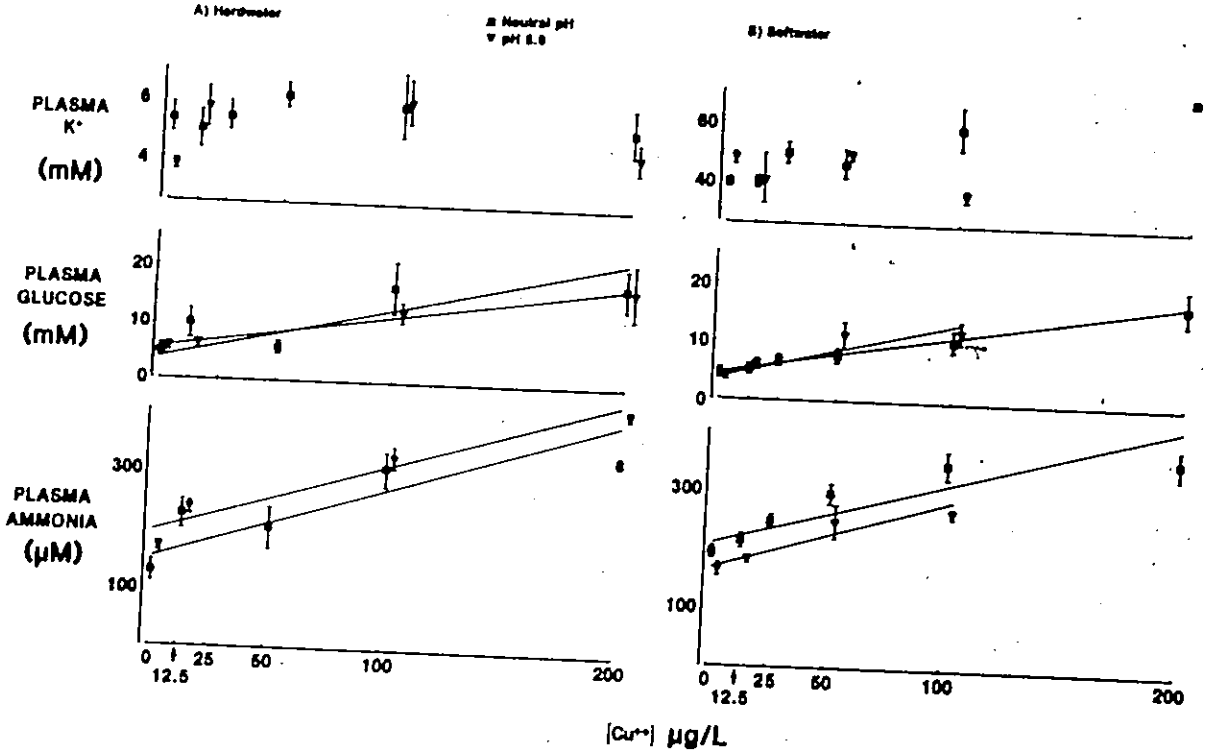

PLASMA Na+ and Cl- (mM)
[Cu2+] µg/L
A) Hardwater neutral pH
B) Softwater neutral pH
C) Hardwater pH 6.0
D) Softwater pH 6.0
Na+
Cl-
150
130
110
90
0
12.5
25
50
100
200

Fig. 2.6 Plasma K^+, glucose, and ammonia after 24 h exposure to copper in A) hardwater, and B) softwater at neutral pH (■) and pH 5.0 (▼). Means ± 1 SEM. Lines fitted by least squares regression.

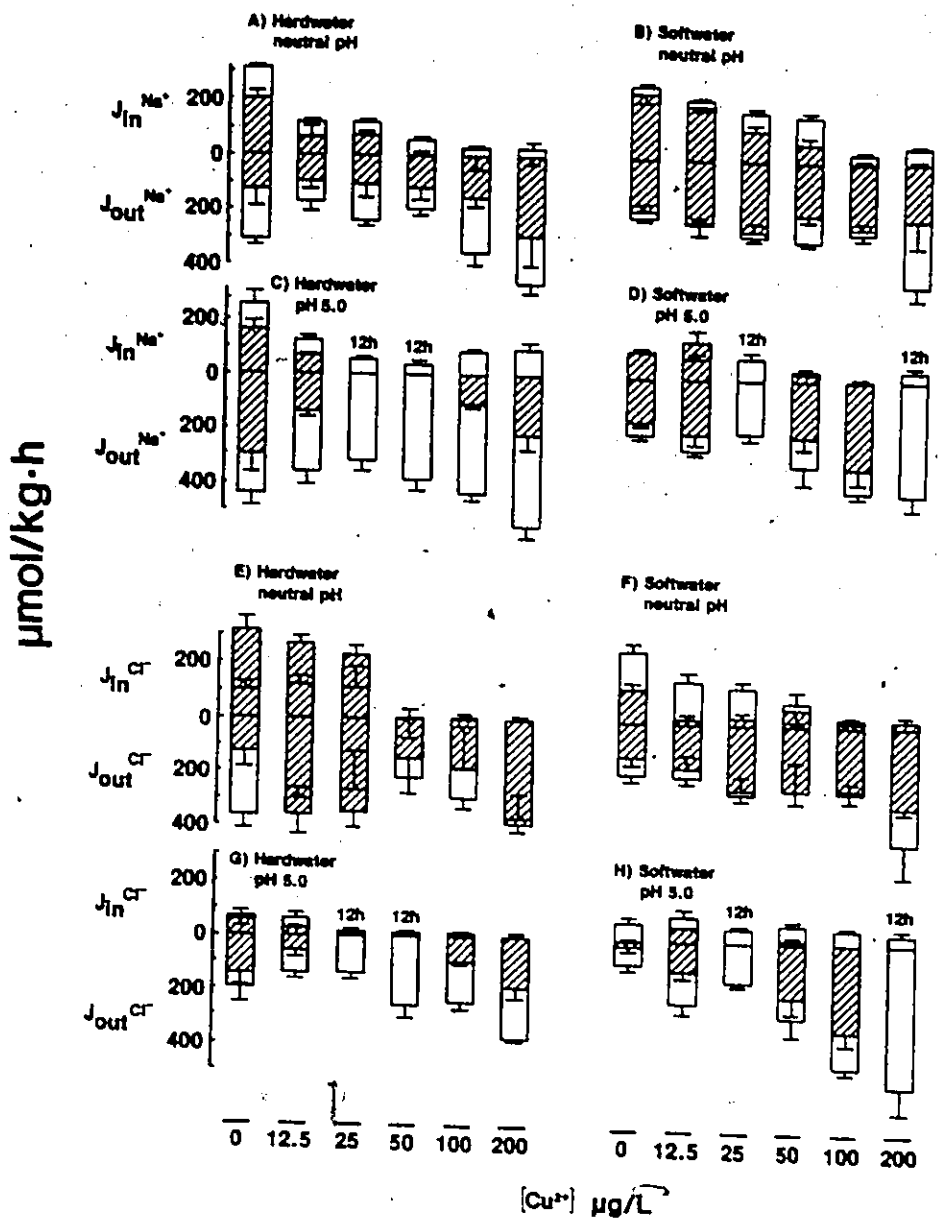

A) Hardwater
B) Softwater
PLASMA K+ (mM)
PLASMA GLUCOSE (mM)
PLASMA AMMONIA (µM)
6
4
60
40
20
10
0
300
100
0
12.5
25
50
100
200
[Cu++] µg/L

each of Na^+, K^+, and Cl^-, the losses were linearly related to the external copper concentration in the range of 12.5 to 200 µg/L at neutral pH, and 0 to 200 µg/L at pH 5.0. However, the very marked stimulation of ion losses at neutral pH and 12.5 µg/L copper suggests that the threshold for copper effects upon the gills may occur at much lower copper concentrations than those tested in the present study.

Na^+ losses were, for the most part, similar in magnitude to the Cl^- losses, and were about 2.7 fold greater than K^+ losses. After 24 h, NaCl losses were of sufficient magnitude at all copper concentrations to have caused significant reductions in plasma ion levels (Fig. 2.5). Plasma K^+ values (Fig. 2.6) were somewhat unreliable because of hemolysis in some terminal blood samples; nevertheless, plasma K^+ values remained within the normal range reported by Hille (1982). Accompanying the plasma ionic disturbances were significant increases in both glucose and ammonia concentrations in plasma (Fig. 2.6). Again, these showed a linear proportionality with external copper concentration. Ammonia excretion, on the other hand, was constant up to 50 µg/L of copper, but at 100 and 200 µg/L, it was significantly inhibited in the first 12 h period (Fig. 2.4). However, except at 200 µg/L in softwater, ammonia excretion returned to normal during the second 12 h period (Fig. 2.4, hashed area).

In general, the net NaCl losses arose because of a copper concentration-dependent inhibition of J_{in}, and at 200 µg/L, a stimulation of J_{out} (Fig. 2.7).

Fig. 2.7 Unidirectional Na^+ (A-D) and Cl^- (E-H) fluxes during the first (open bars) and second (hashed bars) 12 h periods of copper exposure. A) hardwater, neutral pH, B) softwater, neutral pH, C) hardwater, pH 5.0, D) softwater, pH 5.0. Means $\pm$ 1 SEM. Sample size as in Fig. 2.4.


A) Hardwater neutral pH
B) Softwater neutral pH
C) Hardwater pH 5.0
D) Softwater pH 5.0
E) Hardwater neutral pH
F) Softwater neutral pH
G) Hardwater pH 5.0
H) Softwater pH 5.0
µmol/kg·h
$J_{in}^{Na^+}$
$J_{out}^{Na^+}$
$J_{in}^{Cl^-}$
$J_{out}^{Cl^-}$
200
0
200
400
12h
0 12.5 25 50 100 200
[Cu²⁺] µg/L

Effects of Water Hardness

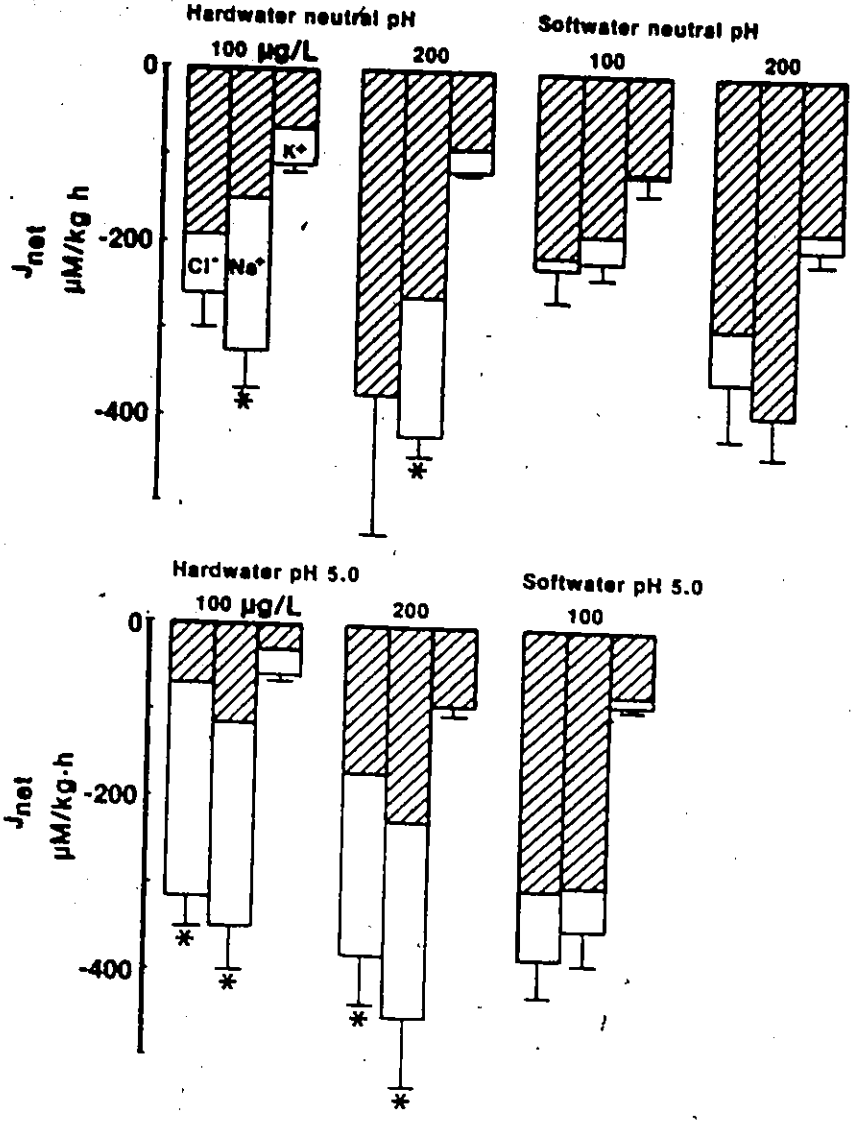
At neutral pH, calcium concentration had no effect on the net NaCl or K^+ losses caused by copper exposure (Fig. 2.3A vs. B), even though the calcium concentration of the artificial hardwater was 40 times that of the softwater (Table 2.1). Nevertheless, there were differences between hard- and softwater fish similar to those noted in control fish at pH 5.0. In the second 12 h period of exposure in hardwater, there were significant reductions in NaCl losses that were not found in softwater fish (Fig. 2.8, hashed area).

More dramatic effects of water hardness were seen upon the unidirectional NaCl fluxes. In particular, there was significantly less inhibition of J_{in} in softwater compared to hardwater (Fig. 2.7A vs B). However, J_{out} was not stimulated to the same extent in hardwater as in softwater during the first 12 h period of exposure, and was further reduced during the second period (Fig. 2.7).

Effects of Water pH

In hard- and softwater at pH 5.0, net NaCl losses at 12.5 to 50 µg/L copper were 30 to 60% greater than could be attributed to the effects of copper alone (Fig. 2.3C vs A, D vs B). Furthermore, although neither 12.5 µg/L copper, nor pH 5.0 alone had an effect on J_{net} K^+, the combination of the two led to a significant increase in net K^+ loss. Nevertheless, net Na^+, Cl^-, and K^+ losses at 100 and 200 µg/L at both neutral pH and pH 5.0 suggests that a maximal effect may have been approached. As might have been predicted then, at 100 and 200 µg/L, there were no differences in plasma Na^+, Cl^-, K^+, glucose or ammonia

Fig. 2.8 Effects of water hardness on J_{net} Cl^+, Na^+, and K^+ during the first (open bars) and second (hashed bars) 12 h periods of copper exposure. Means $\pm$ 1 SEM. Sample size as in Fig. 2.4. Asterisks (*) indicate significant reduction over 12 h value.


Hardwater neutral pH
100 µg/L
200
Softwater neutral pH
100
200
0
-200
-400
J_{net}
µM/kg h
K+
Cl⁻
Na+
*
Hardwater pH 5.0
100 µg/L
200
Softwater pH 5.0
100
0
-200
-400
J_{net}
µM/kg·h
*

at pH 5.0 compared to neutral pH, but both Na^+ and Cl^- were lower at pH 5.0 from 0 to 50 µg/L.

The causes for net losses at pH 5.0 were the same as at neutral pH , i.e., the inhibition of J_{in} from 12.5 and 25 µg/L, and the stimulation of J_{out} from 50 to 200 µg/L (Fig. 2.7C and D vs A and B, G and H vs E and F). However, both the inhibition of J_{in} Cl^- and the stimulation of J_{out} Na^+ were more severe at pH 5.0. As at neutral pH, there was a significant reduction of net NaCl loss from the first to the second 12 h period of copper exposure in hardwater (Fig. 2.8) which resulted entirely from a decrease in J_{out} NaCl (Fig. 2.7, hashed area). Again, this decrease in net loss was not observed in softwater (Fig. 2.8).

DISCUSSION

Most toxicity studies are run for at least 96 h and provide no information about sublethal effects or the mechanisms of toxicity. In contrast, the present physiological study has shown that sublethal, environmentally relevant levels of copper (Chapter 1) cause serious, concentration-dependent disruptions of branchial ionoregulatory functions in adult rainbow trout within 2 h. Although Lorz and McPherson (1976) showed that copper inhibits branchial Na^+-K^+-ATPase in vivo, this is the first demonstration that copper also inhibits ion uptake. Similarly, numerous investigators have shown that copper exposure leads to decreases in plasma Cl^- in freshwater fish (McKim et al., 1970; Lewis and Lewis, 1971; Christensen et al., 1972; Schreck and Lorz, 1978), but this is the first demonstration that copper exposure

leads to the stimulation of Na^+, K^+, and Cl^- efflux. It has also been shown that water hardness has no significant effect on net ion losses within the first 24 h of exposure, and that the effects of low pH are not simply additive with those of copper.

Based upon previous studies of juvenile trout, it was expected that softwater acclimated fish would be more sensitive to copper than hardwater fish. This was not found to be the case in the present study largely because J_{in} was more resistant to copper inhibition in softwater than in hardwater. The origin of this resistance is not known, but may be related to the mechanisms of compensation for ions lost during adaptation to softwater (Olivereau et al., 1980; McDonald and Rogano, 1986)? possibly by the proliferation of chloride cells noted in the present study, and discussed in detail by Laurent et al. (1985). Softwater acclimated fish may therefore be pre-adapted to resist ion losses, especially when those ion losses arise from the inhibition of J_{in}. On this basis alone, net ion losses in hardwater should have exceeded those found in softwater. However, hardwater fish also made a compensatory response to ion loss by significantly reducing the magnitude of the efflux component of the net loss. The mechanism by which this is accomplished is also unknown, but similiar progressive reductions of ion loss have been reported in fish during exposures to La^{3+} (Eddy and Bath, 1979), H^+ (McDonald et al., 1983), and poly-L-lysine (Greenwald and Kirschner, 1976).

The net effect of resistance of J_{in} to inhibition in softwater and reduction of J_{out} in hardwater was that the two adaptations had the effect of cancelling each other out, and equal losses were found by the

end of 24 h of exposure. However, the inhibition of J_{in} was progressive with time even in softwater, so that this adaptation would be of only short-term benefit. Furthermore, McDonald et al. (1983) showed that the inhibition of J_{in} in trout exposed to pH 4.0, was eventually more marked in softwater than in hardwater. Thus, the progressive reductions of J_{out} in hardwater may become more important to survival as exposure time increases.

Mechanisms of Copper Toxicity

The mechanism for normal Na^+ uptake is believed to depend upon active transport via Na^+-K^+-ATPase located in the basolateral membranes of the branchial epithelium. The inhibition of J_{in} Na^+ and the inhibition of this enzyme by copper in vivo (Lorz and McPherson, 1976), suggests that the primary site of copper action is branchial Na^+-K^+-ATPase (see Chapter 5). This further implies that copper must enter the branchial epithelium, presumably across the apical cell membrane.

The inhibition of J_{in} Na^+ was accompanied by an inhibition of ammonia excretion of up to 40%. This observation provides further evidence for the existence of Na^+/NH_4^+ exchange at the gills as first proposed by Krough (1939), and currently the subject of much controversy (Kormanik and Cameron, 1981). However, the fact that the inhibition of ammonia excretion was much less than the inhibition of Na^+ uptake and, also, that ammonia excretion returned to control levels while J_{in} Na^+ remained inhibited, indicates that such exchange is not obligatory.

The large stimulation of passive NaCl efflux suggests that one of the important sites of copper action is the paracellular tight junctions of the branchial epithelium, an effect similar to that found by McDonald (1983b) for H^+. Since tight junction permeability appears to be controlled to a large extent by calcium bound to anionic residues of the intercellular "cement" (Oschman, 1978), McDonald (1983a) proposed that H^+ acts at these junctions by displacing calcium. It is well known that copper can also displace calcium from biological ligands (Nieboer and Richardson, 1980) and that copper likely binds these sites with greater affinity than either calcium or H^+. Thus, it would seem that H^+ and copper may compete for the same binding sites in the paracellular spaces, and these sites are normally occupied by calcium.

Another important site of copper action is suggested by the stimulation of K^+ losses. Copper-dependent K^+ losses were more than 10 times greater than those predicted simply from the ratio of plasma K^+/Na^+ (i.e., 5/155= 0.03) whereas in control fish (neutral pH and pH 5.0), the predicted K^+ losses closely approached this predicted ratio. Based solely on the relative concentrations of K^+ in the extracellular and intracellular compartments, this suggests that copper is able to increase the permeability of the apical cell membrane, thus allowing intracellular K^+ to diffuse down its concentration gradient. A similar effect has previously been shown for the in vitro frog skin (Ferreira, 1978). Interestingly, although neither 12.5 µg/L copper nor pH 5.0 led to net K^+ loss, the combination of the two led to a large increase in K^+ loss.

Copper appeared to be much more effective at disrupting the branchial epithelium than H^+ since 1.6 µequiv/L copper (50 µg/L) produced the same net Na^+ losses as 10 µequiv/L H^+ (pH 5.0). The relative effectiveness of copper was even more dramatic on the apical surface when only 0.8 µequiv/L copper (25 µg/L) was required to produce K^+ losses of the same order of magnitude as those previously found at 100 µequiv/L H^+ (pH 4.0; D.G. McDonald, unpublished).

Other important effects of copper exposure were the elevation of plasma glucose and ammonia levels. Such increases are typical of cortisol and catecholamine-mediated stress responses (Mazeaud et al., 1977) which are characterized by a stimulation of both protein catabolism and gluconeogenesis (Freeman and Idler, 1973; Chan and Woo, 1978). Plasma cortisol levels have often been suggested as a good measure of degree of stress in fish (Donaldson, 1981), but Schreck and Lorz (1978) showed that cortisol levels, although elevated, are not strongly correlated with copper concentration. Furthermore, cortisol levels tend to decrease with continued exposure. Since both plasma glucose and ammonia levels showed a strong correlation with copper concentration even after 24 h of copper exposure, this suggests that either measurement may be a better indicator of stress than cortisol.

CHAPTER 3

INFLUENCE OF WATER HARDNESS, pH, AND ALKALINITY ON THE MECHANISMS OF COPPER TOXICITY IN JUVENILE TROUT

INTRODUCTION

It has been shown that the basic mechanism of copper toxicity in adult rainbow trout is the disruption of branchial ionoregulatory function (Chapter 2). However, that study was conducted in only low alkalinity water, and no significant effects of either water hardness (within the range of 25 to 1000 μM), or pH (within the range of pH 7.8 to pH 5.0) were found within 24 h (Chapter 2). Since previous experiments with juvenile trout have shown significant effects of water hardness, pH, and alkalinity on the lethality of copper, i.e., 96 h LC50 (Shaw and Brown, 1974; Zitko and Carson, 1976; Howarth and Sprague, 1978; Chakoumakos et al., 1979; Miller and Mackay, 1980), it seemed that juvenile trout might be more sensitive than adults. Furthermore, the effects of high alkalinity were not assayed in the previous study (Chapter 2). Therefore, the objectives of this study were 1) to examine the relative role of alkalinity, as well as that of hardness and pH, in modulating the effects of copper on juvenile as opposed to adult trout, and 2) to compare these results with those found in Chapter 2 with adult trout exposed under similar conditions.

METHODS AND MATERIALS

Animals

Juvenile rainbow trout (Mean wet weight = 2.46±0.02 g; n = 495), were maintained in flowing tap-dechlorinated water. The fish were fed

every other day, and mortalities were less than about 1% per week. The $[Na^+]$ and $[Ca^{2+}]$ of the holding system were about 600, and 1000 µM, respectively, and temperature was maintained at 15±2°C.

Exposure Media

All flux media were made from distilled water with salts added. To facilitate net Na^+ measurements, and to more closely represent the $[Na^+]$ of those waters which are most likely to suffer from elevated copper concentrations due to acid precipitation, fish were acclimated for 14 days to about 200 µM $[Na^+]$ (Table 3.1). For those experiments conducted in hardwater of low alkalinity (HL), the $[Ca^{2+}]$ of the acclimation media was about 1000 µM, and for softwater, low alkalinity experiments (SL), the $[Ca^{2+}]$ was about 25 µM (Table 3.1). Calcium was added as $Ca(NO_3)_2$. For experiments conducted at pH 5.0, 0.1 M HNO_3 was used to titrate the water, and the pH was monitored and adjusted every 15-30 min. Artificial hard, high alkalinity water (HH) was prepared by adding 1000 µM $CaCO_3$ and 200 µM Na^+ to distilled water. The $CaCO_3$ was dissolved by bubbling with CO_2, and the pCO_2 reduced to normal levels by bubbling with air for 24 h. Fish were acclimated for 14 days to about 200 µM Na^+ and 1000 µM $Ca(NO_3)_2$ as in the other experiments using artificial hardwater. Alkalinity was measured by titrating a known volume to pH 4.0 with 0.02 M HCl. All experiments were conducted at 15±1°C.

Treatment	pH	$[Na^+]$	$[Cl^-]$	$[Ca^{2+}]$	$[K^+]$	[Ammonia]
HL	7.8	237± 6	192± 5	1064±20	6.4±1.1	8.6±0.5
SL	7.8	223± 2	187± 1	27± 4	3.9±0.4	10.6±0.4
HL	5.0	257± 3	234± 4	1047±11	15.3±0.6	126 ±1.5
SL	5.0	241± 3	228± 7	31± 6	11.1±0.6	110 ±3.8
HH	8.1	219± 3	207±14	1103±28	-----	12.7±1.6

Table 3.1. Ionic composition of high Ca^{2+} (HL), low Ca^{2+} (SL), low alkalinity, and high Ca^{2+} (HH), high alkalinity test media at the beginning of flux periods for all experiments. Values are means ± 1 SEM in µM.

Ion Flux Measurements

Fish were exposed to copper in 2 liter square bags constructed from polyethylene sheeting, and sealed along the seams with a commercial bag sealer. Polyethylene was chosen because copper uptake into the plastic was less than 5% over a 24 h period at neutral pH, and even lower at pH 5.0. Groups of 5 fish were exposed for 24 h to 25, 50, 100, 200, or 400 µg/L copper in 1 liter of the appropriate medium (Table 3.1), so as to form a matrix of high and low hardness, alkalinity, and pH. To reduce the stress of confinement, the fish were first transferred to an identical flux bag for 12 hours. The flux measurements were started by transferring the fish to bags containing the appropriate $[Cu^{2}+]$ and ^{22}Na at 37 KBq/L. Water samples were collected at 4 h intervals. Sodium uptake (J_{in}) was determined after 24 h, or at the time of death, by counting the whole fish in a Nuclear Chicago gamma counter, as:

$$J_{in} = \frac{WBA}{SA \times W \times t}, \qquad (3.1)$$

where WBA is the whole body activity in counts per minute, SA is the mean specific activity (counts per minute/ µM), W is the weight to the nearest 0.01 g, and t is the time of death in hours.

Whole body copper uptake was measured simultaneously with ^{22}Na uptake, using ^{64}copper (3.1 MBq/L) and counting whole fish. For this purpose we used the same formula as for J_{in} (after first

correcting for the decay of ^{64}copper, $t_{1/2}$=768 min), but not dividing by t.

After allowing sufficient time for the complete decay of ^{64}copper (e.g., about 10 half-lives or 5 days), and recounting of the fish for ^{22}Na uptake alone, each fish was digested in 5 mL of concentrated nitric acid, and whole body total [Na^+] was measured with a Varian 1275 atomic absorption spectrophotometer. From this information, it was possible to calculate net losses of whole body [Na^+] in individual fish as:

$$J_{net} = \frac{[Na^+]_C - [Na^+]_T}{gxt} , \quad (3.2)$$

where $[Na^+]_C$ is the whole body [Na^+] for control fish (n=20); $[Na]_T$ is the whole body total [Na^+] for the particular treatment in μmol/g wet weight; and g and t are the wet weight and the time to death; respectively.

Sodium efflux (J_{out}) was calculated as:

$$J_{out} = J_{in} - J_{net} , \quad (3.3)$$

To assess the allometric differences in the effect of copper on juvenile and adult trout, it was necessary to compare changes in whole body exchangeable sodium. Whole body exchangeable [Na^+] of control fish ($[Na]_{exC}$) was determined as:

$$[Na]_{exC} = {}^{22}Na \text{ space} \times [Na^+]_{plasma} , \quad (3.4)$$

Plasma was collected by caudal transection, and $[Na^+]_{plasma}$ was measured by atomic absorption. Mayer and Nibelle (1969) showed that the sodium space of eels is uniformly labelled after about 10 h of exposure in ^{22}Na labelled water. To ensure uniform labelling of the sodium space in trout, we exposed juvenile rainbow trout (n=33) for 24 h in low alkalinity hardwater (HL) labelled with ^{22}Na at 148 KBq/L, and calculated the ^{22}Na space as:

$$^{22}Na \text{ space} = (\text{cpm/g wet weight})/(\text{cpm/mL plasma}), \quad (3.5)$$

Whole fish were counted for the determination of cpm/g wet weight, and 5 µL aliquots of plasma were collected after caudal transection and counted for the determination of cpm/mL plasma. $[Na^+]_{plasma}$ and cpm/mL plasma were measured on the same samples.

Because whole body chloride cannot be measured in acid digests, and whole body potassium was variable, fluxes of chloride and potassium were calculated from changes in their concentrations in the media. Ammonia fluxes were also calculated from changes in the [Ammonia] in the media. Sodium and potassium were measured with a Varian 1275 atomic absorption spectrophotometer. Chloride was measured with a Cotlove chloride titrator, and ammonia was measured with a modification of the method of Verdouw et al. (1978).

Estimation of 24 h LC50

Mortalities were recorded at hourly intervals and the time to 50% mortality estimated by the method of Litchfield (1949). The 24 h LC50 was estimated by the method of Litchfield and Wilcoxon (1949).

Copper Uptake vs. Sodium Uptake

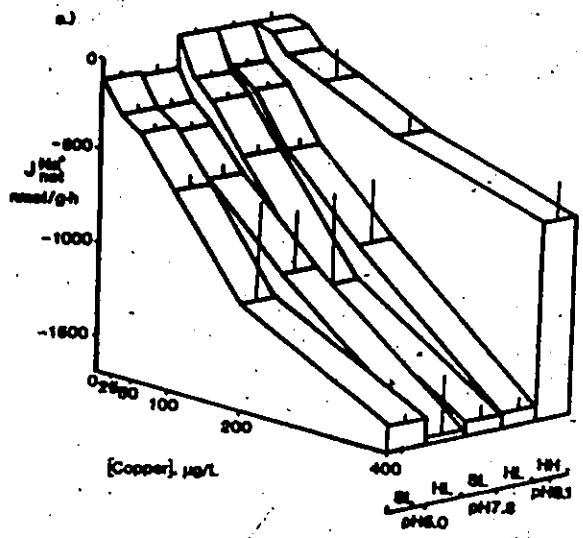
In a separate experiment designed to examine the correlation between the accumulation of copper and the inhibition of sodium uptake, fish were exposed to 50 µg/L copper in low alkalinity hardwater (HL), labelled with 1.5 MBq/L ^{64}copper and 37 KBq/L ^{22}Na. Groups of 5 fish were removed at regular intervals for up to 24 h. Whole fish were counted, and sodium and copper uptake calculated as described by equation 3.1, above.

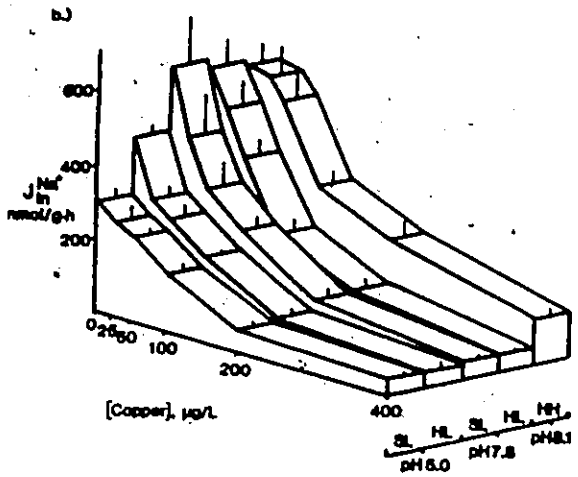
RESULTS

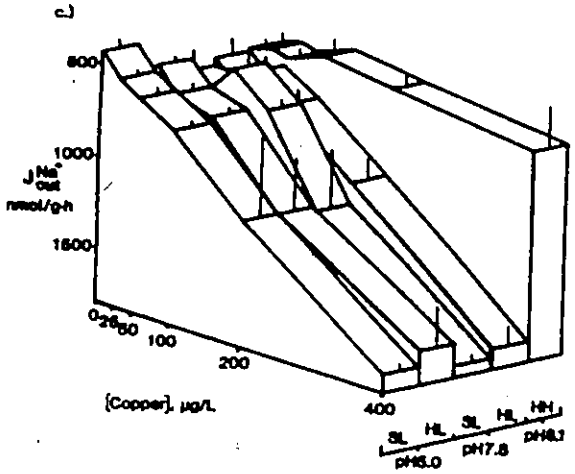
Control Fish

No mortalities were found among control animals at neutral pH, in either hard (HL), or soft, low alkalinity (SL) water, or hard, high alkalinity (HH) water. Whole body [Na^+] was 54.8±3.0, and 54.4±2.1 µmol/g in HL and SL fish, respectively. Whole body [Na^+] for HH fish was 65.0±1.4 µmol/g. This change occurred over the 4 month period between the two groups of experiments. Radiosodium space was determined to be 27±4%, and plasma [Na^+], 152±5 mM (n=33); therefore, the exchangeable Na^+ pool at neutral pH was determined to be about 75% of the total whole body [Na^+]. For control fish, net sodium fluxes could not be reliably calculated from changes in whole body [Na^+] because the changes were so small. Therefore, they were calculated from changes in the [Na^+] of the flux media. Although control fish initially lost ions immediately after being transferred from the acclimation bath to the flux bath, net Na+ fluxes were +51±30, +35±53, and +25±20 nmol/g·h for

Fig. 3.1 Sodium balance. a.) Net sodium loss rate of juvenile rainbow trout exposed to copper for 24 h, or until death. Means ± 1 SEM. For high (HL) and low (SL) Ca^{2+}, low alkalinity water at neutral pH, n=15, for high (HL) and low (SL) Ca^{2+}, low alkalinity water at pH 5.0, n=10, and for high (HH) Ca^{2+}, high alkalinity water at neutral pH, n=20. b.) Sodium influx rate. Sample size as in figure 2.1a, except that n=10 in high (HH) Ca^{2+}, high alkalinity water. c.) Sodium efflux rate. Sample size as in figure 2.1b.


a)
0
-800
-1600
0
25
50
100
200
400
[Copper], µg/L
SL
HL
SL
HL
HH
pH6.0
pH7.8
pH8.1


b)
800
400
200
0
25
50
100
200
400
[Copper], µg/L
SL
HL
SL
HL
HH
pH6.0
pH7.8
pH8.1


c)
800
1000
1600
0
25
50
100
200
400
[Copper], µg/L
SL
HL
SL
HL
HH
pH6.0
pH7.8
pH8.1

Fluxes	pH	[Copper], µg/L 0	25	50	100	200	400
J_{net} Cl^-							
HL	7.8	+70±180	−304±82	−346±119	−632±102*	−1087±522*	−1632±404*
SL	7.8	+110±77	−282±180	−363±67*	−527±120*	−1340±126*	−2211±787*
HL	5.0	−138±108	−338±116	−394±177	−621±147*	−1002±55*	−1024±153*
SL	5.0	−60±53	−301±106*	−365±77*	−499±87*	−1068±208*	−1132±141*
HH	8.1	+66±44	−11±34	−112±17	−225±54*	−420±102*	−740±176*
J_{net} K^+							
HL	7.8	−54±11	−92±5	−122±12*	−137±36*	−253±32*	−352±48*
SL	7.8	−62±9	−112±25	−125±18*	−136±28*	−260±40*	−328±64*
HL	5.0	−110±55	−112±16	−133±28*	−139±33*	−195±50*	−223±39*
SL	5.0	−64±8	−109±18	−130±25*	−142±28*	−185±51*	−234±18*
HH	8.1	−48±15	−86±22	−117±10*	−136±10*	−200±38*	−244±33*
J_{net} Ammonia							
HL	7.8	−1016±181	−1175±188	−959±168	−880±178	−897±228	−637±186*
SL	7.8	−947±56	−876±107	−907±107	−1213±288	−566±42*	−543±207*
HL	5.0	−907±52	−826±46	−961±52	−931±64	−1160±194	−1013±173
SL	5.0	−1003±52	−1041±81	−869±123	−893±62	−893±80	−629±96*
HH	8.1	−1010±59	−987±23	−1021±143	−976±58	−963±102	−852±97

Table 3.2. Net Cl^-, K^+, and ammonia fluxes in juvenile rainbow trout exposed for 24 h to copper at neutral pH, and pH 5.0, in high Ca^{2+} (HL), and low Ca^{2+} (SL), low alkalinity, and high Ca^{2+} (HH), high alkalinity water. For neutral pH (HL and SL), n=15, for pH 5.0 (HL and Sl), n=10, and for HH, n=20. Values are means ± 1 SEM, in nmol/g·h. Asterisks denote significant differences from control values ($P<.05$).

HL, SL, and HH fish, respectively (Fig 3.1a). Control fish also lost K^+, and Cl^- immediately after transfer, but again, these losses were negligible by the end of the 24 h flux period (Table 3.2). Ammonia efflux was about -1000 nmol/g·h in HL, SL, and HH water (Table 3.2).

Sodium uptake (J_{in}) was about 600 nmol/g·h, and there was no significant difference between HL, SL, and HH water (Fig 3.1b). Because the fish were in sodium balance for the 24 h flux period, sodium efflux was also about 600 nmol/g·h in all control conditions (Fig 3.1c).

Effects of Exposure to pH 5.0

Control fish exposed to pH 5.0 without copper lost between 7 to 10% of their exchangeable Na^+ within 24 h, but no mortalities occured (Table 3.3). Net K^+ losses were also found, but these were not significantly different from those at neutral pH (Table 3.1b). Net chloride losses were found (Table 3.2), but were always lower than Na^+ losses (Fig. 3.1a). There was no significant effect of pH on ammonia efflux (Table 3.2). Sodium uptake was inhibited by about 25% in HL, and by about 50% in SL at pH 5.0 (Fig 3.1b). This was the only significant effect of water hardness (Appendix A, p. 154). Sodium efflux (J_{out}) was about the same in HL at pH 5.0, as at neutral pH, but was about 28% lower in SL at pH 5.0 than at neutral pH (Fig 3.1c).

Effects of Copper Exposure on Net Sodium Balance

Exposure to 25 ug/L copper led to net Na^+, K^+, and Cl^- losses within 24 h (Fig 3.1a; Table 3.2), but there was no significant effect of water hardness within this time. The net ion loss rate increased

linearly with copper concentration (Fig 3.1a; Table 3.2). A significant inhibition of ammonia efflux was found at 400 µg/L copper at neutral pH in both HL and SL (Table 3.2).

Fish exposed to 25 and 50 ug/L copper at pH 5.0 experienced larger net Na^+ losses than those exposed at neutral pH (pH accounted for about 19% of the combined effects of pH and copper), but there was no significant effect of either pH, or hardness from 100 to 400 µg/L (Fig 3.1a), so that copper alone accounted for about 80% of the losses found over the entire range of copper concentrations tested (Appendix A). There was no significant effect of pH on either K^+, or Cl^- net losses at any copper concentration (Table 3.2). Ammonia efflux was significantly inhibited at 200 and 400 µg/L copper in SL, but not in HL at pH 5.0 (Table 3.2).

Exposure of fish to copper in high alkalinity, hardwater (HH), significantly reduced the effect of copper on net Na^+, and Cl^- losses, but had no effect on net K^+ losses (Fig 3.1a; Table 3.2). Thus, HH fish suffered overall lower ion loss rates than either HL, or SL fish (Fig 3.1a; Table 3.2), and, furthermore, no net Na^+ or Cl^- losses were found at 25 µg/L. Ion losses increased as copper concentration increased from 50 to 400 µg/L, but the ameliorative effect of HH was also more apparent as the copper concentration increased (Fig 3.1a; Table 3.2). Alkalinity had no significant effect on ammonia efflux (Table 3.2).

Effects of Copper on Sodium Influx

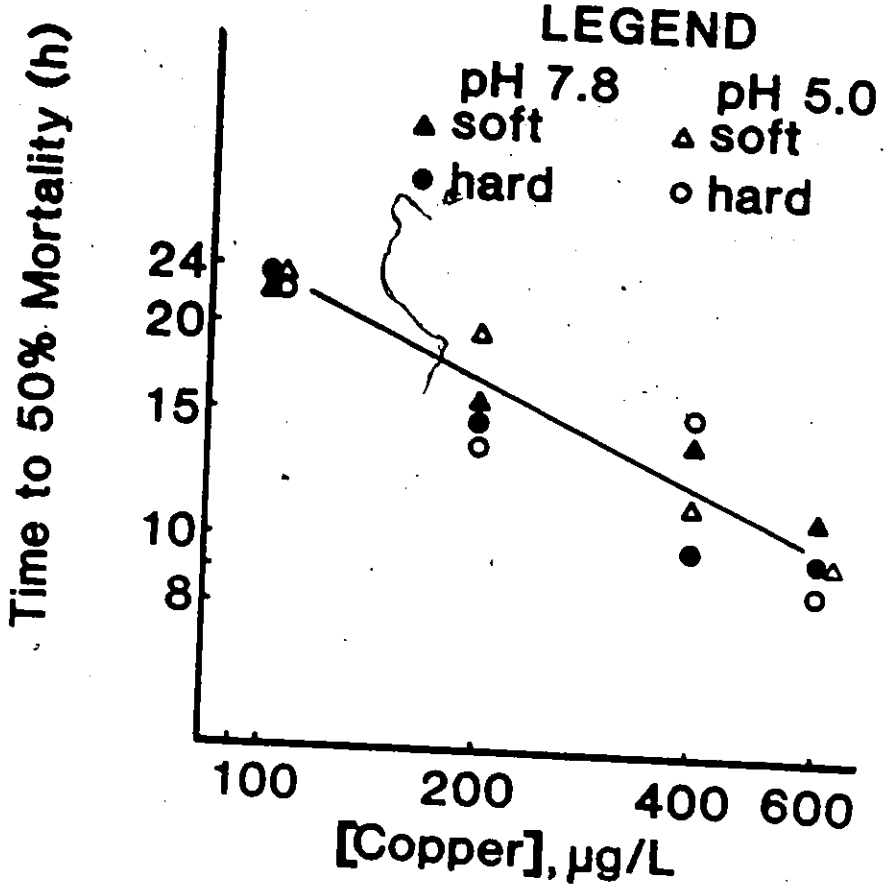
Exposure of juvenile trout to copper resulted in a concentration-dependent inhibition of sodium influx (J_{in}). However, there was no significant effect of water hardness on the inhibition of J_{in} by copper (Fig 3.1b; Appendix A). At neutral pH the inhibition of J_{in} appeared to be logarithmic with copper concentration, with most of the inhibition of J_{in} occurring between 25 and 100 µg/L, (Fig 3.1b). At 100 µg/L, J_{in} was inhibited by about 65% (Fig 3.1b). At 25 and 50 µg/L copper, exposure to pH 5.0 led to a significantly greater inhibition of J_{in} than was found for copper exposure alone (Fig 3.1b). At 100 µg/L, J_{in} was inhibited by about 72% (Fig. 3.1b). However, there was no effect of pH on J_{in} above 100 µg/L copper (Fig. 3.1b; Appendix A), and the effect of pH was not strictly additive with that of copper even at the lower copper concentrations (Fig 3.1b).

The inhibitory effect of copper on J_{in} was significantly reduced by exposure in HH water (Fig. 3.1b). There was no significant effect of copper at 25 ug/L, and copper was about 20 to 30% less inhibitory at all copper concentrations tested (Fig. 3.1b). As in HL and SL at neutral pH, most of the inhibition of J_{in} occured between 25 and 100 µg/L copper (Fig. 3.1b).

Effects of Copper on Sodium Efflux

There was no significant effect of copper on sodium efflux (J_{out}) from 25 to 50 µg/L copper, in HL or SL at either neutral pH, or pH 5.0

Fig. 3.2 Estimation of 24 h LC50 for copper exposed juvenile rainbow trout. For neutral pH, high and low Ca^{2+}, low alkalinity water, n=15, for pH 5.0, high and low Ca^{2+} water, n=10. Line fitted by eye.


LEGEND
pH 7.8
pH 5.0
soft
hard
soft
hard
Time to 50% Mortality (h)
24
20
15
10
8
100
200
400
600
[Copper], µg/L

Treatment	pH	Copper, µg/L					
		Control	25	50	100	200	400
HL	7.8						
juveniles		+ 0.3	- 10.8	- 14.5	- 32.3	- 54.2	- 65.6
adults		- 0.4	- 6.1	- 9.2	- 15.3	- 21.6	N.D.
SL	7.8						
juveniles		- 0.5	- 11.5	- 16.2	- 30.6	- 63.9	- 60.8
adults		+ 2.8	- 7.2	- 8.6	- 12.8	- 24.3	N.D.
HL	5.0						
juveniles		-10.1	- 17.9	- 23.2	- 36.2	- 56.9	- 53.2
adults		- 9.2	N.D.	N.D.	- 14.8	- 21.7	N.D.
SL	5.0						
juveniles		- 7.4	- 17.1	- 20.1	- 34.7	- 65.6	- 60.8
adults		- 4.7	N.D.	- 13.6	- 20.5	N.D.	N.D.
HH	8.1						
juveniles		- 0.4	- 0.5	- 1.0	- 2.7	- 11.5	- 39.5
adults		N.D.	N.D.	N.D.	N.D.	N.D.	N.D.

Table 3.3. Change in mean percent whole body exchangeable sodium in juvenile and adult rainbow trout exposed to copper for 24 h. Adult data from Lauren and McDonald (1985). N.D.= not determined.

(Fig. 3.1c; Appendix A). However, a significant increase in J_{out} was found from 100 to 200 µg/L in Hl and Sl at both neutral pH, and pH 5.0 (Fig. 3.1c; Appendix A).

In contrast to HL and SL, no stimulation of J_{out} was found in HH until between 200 and 400 µg/L copper (Fig. 3.1c).

Mortalities

Fish died when they had lost about 55% of their whole body exchangeable Na^+. This occured at 200 and 400 µg/L copper in HL and SL fish at neutral pH and pH 5.0, but only at 400 µg/L in HH (Table 3.2). There was no significant effect of either water hardness or pH on mortality during the 24 h of copper exposure, and the predicted 24 h LC50 for both HL and SL fish at neutral pH and pH 5.0 was about 90 µg/L (Fig. 3.2). The slopes of the time to death vs. copper concentration for HL, SL, at neutral pH, and pH 5.0, were parallel, indicating no differences in the mechanism of toxicity (Fig. 3.2). Insufficient mortalities occurred in HH for the accurate estimation of a 24 h LC50, but the few mortalities which did occur, suggest that this concentration is in excess of 400 µg/L copper.

Copper Uptake

Copper uptake among fish which survived for 24 h was linear with copper concentration up to about 100 µg/L, and then leveled off (Fig. 3.3). There was no significant effect of water hardness on copper uptake by juvenile trout at neutral pH (Fig. 3.3). However, there was a significantly higher copper uptake among fish which died before 24 h.

At 200 and 400 µg/L, copper uptake in fish which died was 441±69 and 1210±111 ng/g in HL, and 594±71 and 1359±186 ng/g in SL, whereas copper uptake in survivors reached a maximum of about 360±50 ng/g.

There was a significant inhibition of copper uptake at pH 5.0 (Fig. 3.3). Again, there was no significant effect of water hardness, and copper uptake leveled off at about 100 µg/L (Fig. 3.3). Copper uptake was also significantly greater among fish which died before 24 h than those which survived for 24 h. Thus, at 200 and 400 µg/L, copper uptake for fish which died was 534±86 and 816±90 ng/g in HL, and 497±63 and 1221±166 ng/g in SL at pH 5.0. Thus, there was less effect of pH on copper uptake among fish which died before 24 h than among survivors (Fig. 3.3).

High alkalinity hardwater (HH) did not significantly affect copper uptake relative to low alkalinity hard or softwater (Fig. 3.3).

Copper Uptake vs. Sodium Uptake

This experiment revealed that, at least at 50 µg/L copper in low alkalinity hardwater at neutral pH, copper uptake was not constant with time, and the rate of sodium uptake was progressively reduced (Fig. 3.4a). The copper uptake rate during the first hour was 88.42±11.69 ng/g·h; this accounted for about 50% of the total copper accumulated during 24 H (Fig. 3.4a). The time weighted mean uptake rate from 2 to 24 h was only 4.17±1.42 ng/g·h (Fig. 3.4b). No inhibition of J_{in} was found during the first 2 h of copper exposure (Fig. 3.4a). From 2 to 4

Fig. 3.3. Copper uptake of juvenile rainbow trout which survived exposure to copper for 24 h. Means $\pm$ 1 SEM. For neutral pH, high and low Ca^{2+}, low alkalinity water, n=15, for pH 5.0, high Ca^{2+} water, n=10, and for high Ca^{2+}, high alkalinity water at neutral pH, n=10.

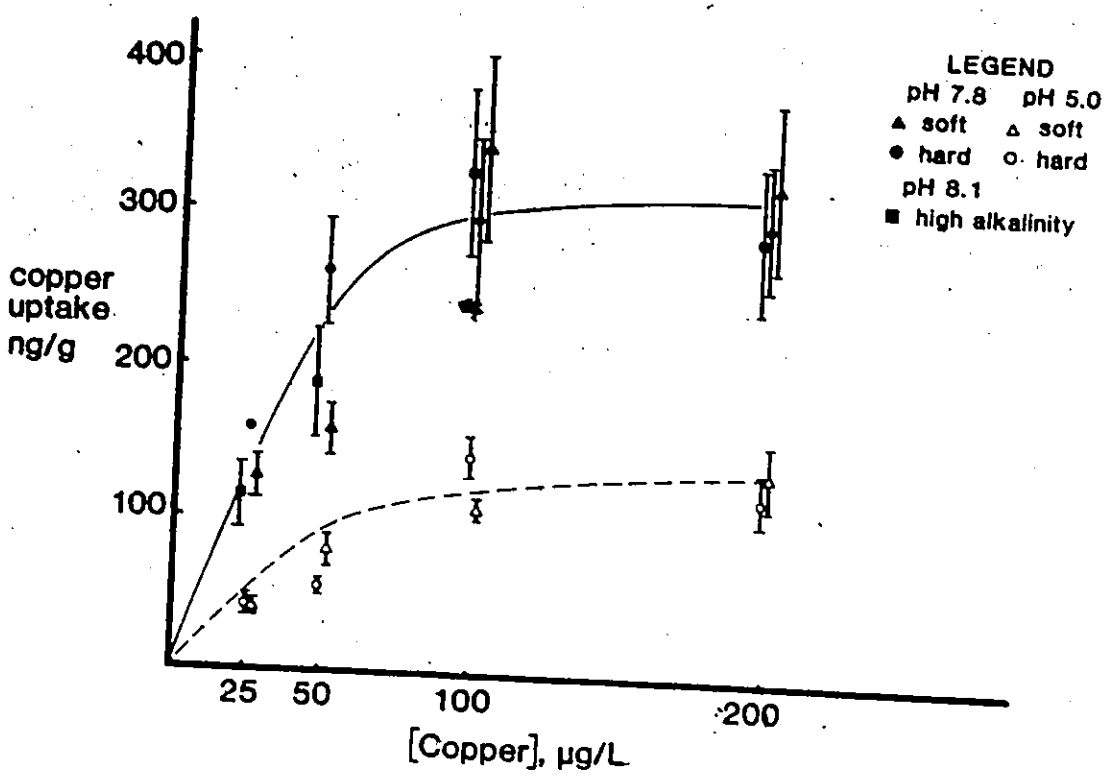
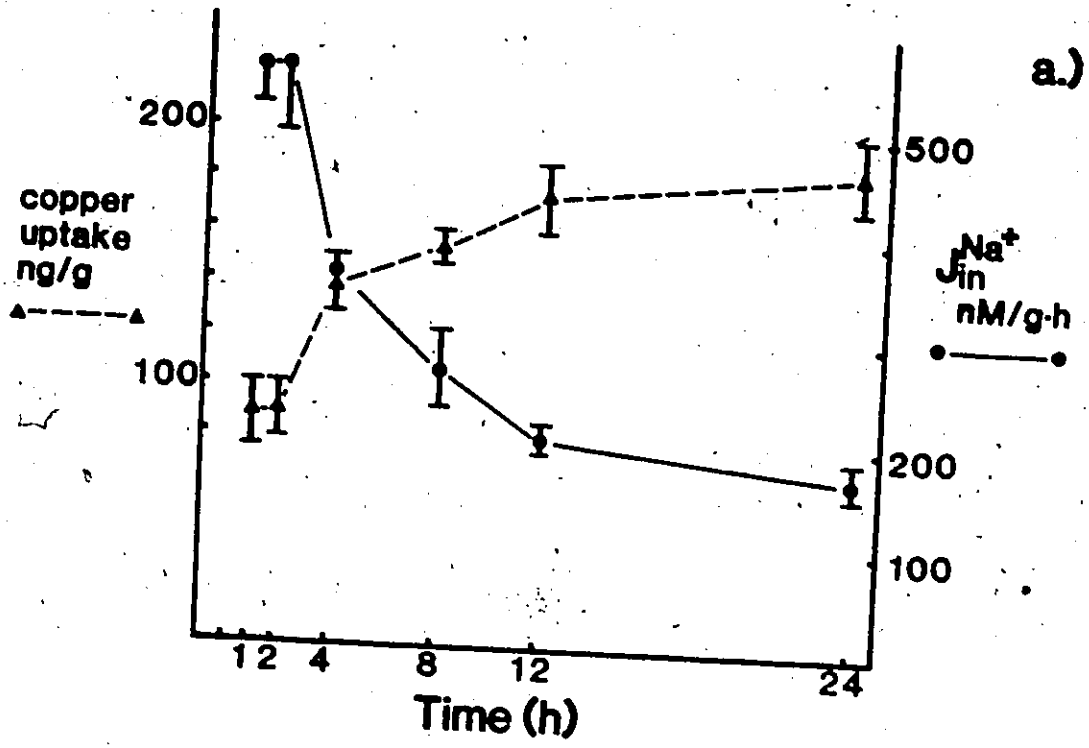
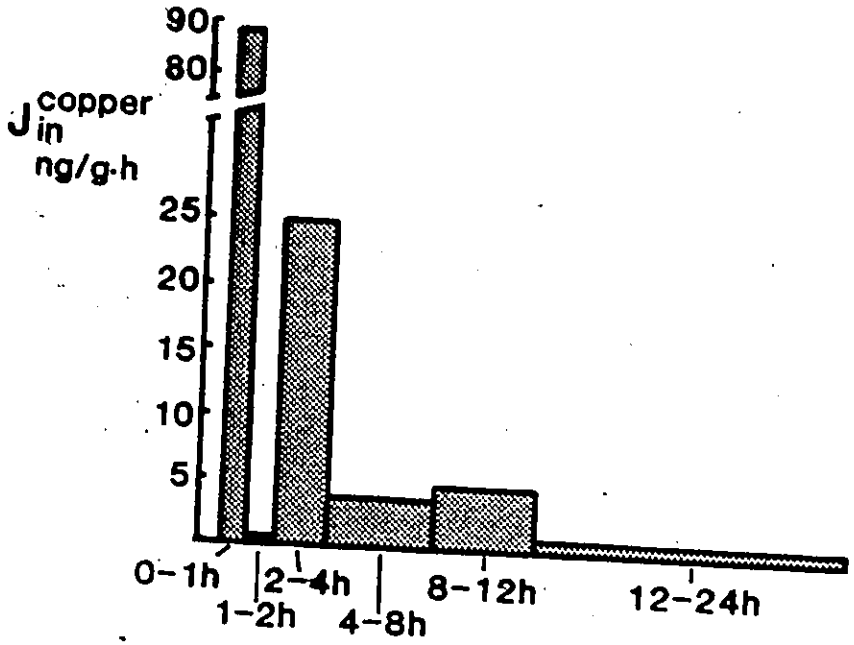

400
300
200
100
copper
uptake
ng/g
25
50
100
200
[Copper], µg/L
LEGEND
pH 7.8
▲ soft
● hard
pH 5.0
△ soft
○ hard
pH 8.1
■ high alkalinity

Fig. 3.4. a.) Copper uptake (▲), and sodium uptake rate (●) of juvenile rainbow trout exposed to 50 µg/L copper in low alkalinity hardwater at neutral pH, for varying periods of time. Means ± 1 SEM, n=5. b.) Copper uptake rate. Means, n=5. These values were calculated by subtracting the copper uptake (ng/g) during the previous exposure period, from the copper uptake (ng/g) from time 0 to time t, and dividing the difference by t.


a.)
200
100
copper
uptake
ng/g
500
200
100
$J_{in}^{Na^+}$
nM/g·h
1 2 4 8 12 24
Time (h)


b.)
90
80
25
20
15
10
5
J_{in}^{copper}
ng/g·h
0–1h
1–2h
2–4h
4–8h
8–12h
12–24h

h. the copper uptake rate had increased above the 2 h value (Fig. 3.4b), and J_{in} was significantly inhibited (Fig. 3.4a). Copper continued to accumulate at a slower rate, and J_{in} continued to decrease for the remainder of the exposure (Fig. 3.4a).

DISCUSSION

Effects of Alkalinity on Copper Toxicity

By varying only alkalinity, while maintaining hardness, $[Na^+]$, and pH constant, it has been shown that alkalinity, not hardness, is the major modulator of short-term copper toxicity to rainbow trout. Furthermore, it has been shown that the physiological basis for the ameliorative effect of increased alkalinity lies in the reduced effect of copper on both sodium influx and efflux. This confirms the hypothesis of Stiff (1971) that differences in copper toxicity between hard and softwaters are dependent upon carbonate complexation capacity rather than hardness. Similar conclusions were reached by Andrew (1976) for fathead minnows (Pimephales promelas), by Shaw and Brown (1974) for rainbow trout, and by Zitko and Carson (1976) for Atlantic salmon (Salmo salar).

Previous studies have shown that increases in alkalinity reduce copper toxicity to trout, but these studies used lethality as the only indicator of the effects of water chemistry on copper toxicity (Shaw and Brown, 1974; Zitko and Carson, 1976; Howarth and Sprague, 1978; Chakoumakos et al., 1979; Miller and Mackay, 1980). In the present

study, it has been shown that the physiological basis for these ameliorative effects lies in the reduction of the effects of copper on branchial ionoregulatory function. This protective effect is also fast-acting (i.e., found within 24 h), and apparent at sublethal levels of copper.

Alkalinity affects the physical form of the toxicant (Stiff, 1971) by way of the equilibrium between the toxic species, Cu^{2+}, $CuOH^{+}$, and $CuOH_2^{0}$, and the non-toxic carbonate species, $CuHCO_3^{+}$, $CuCO_3^{0}$, and $Cu(CO_3)_2^{-2}$ (Chakoumakos et al., 1979). Thus, in natural low alkalinity waters, regardless of pH, non-carbonate species of copper would predominate (e.g., from about 99% at pH 5.0 to about 65% at pH 7.6). In the present study, carbonate species were kept low by the use of only calcium nitrate, as opposed to calcium carbonate salts. Nitrate salts were chosen because nitrates are non-toxic in themselves, and because copper nitrate dissociates completely in water (Sillén and Martell, 1964). In contrast, in high alkalinity experiments, non-toxic copper carbonate species would predominate as they would in natural high alkalinity waters (e.g., about 67% at pH 7.4; Chakoumakos et al., 1979; Howarth and Sprague, 1978).

Effects of Hardness on Copper Toxicity

It was also confirmed that water $[Ca^{2+}]$ plays no significant role in short-term copper toxicity (Chapter 2). Zitko and Carson (1976) also found no effect of water hardness on LT50 toxicity tests of Atlantic salmon, exposed to copper. Nevertheless, Chakoumakos et al. (1979) and

Miller and Mackay (1980) have shown a significant effect of water hardness on the 96 h LC50 and 15 day ILC50, respectively. These differences in the effect of calcium may be resolved in at least two ways. First, the fish used by Chakoumakos et al. (1979) and Miller and Mackay (1980) were acclimated to softwater for only 2 to 3 days. Such short acclimation times would increase the apparent effect of calcium by decreasing the fitness of the softwater fish (i.e., incomplete acclimation; see Mechanisms of Toxicity). Secondly, Lloyd (1965) suggested that hardwater fish must lose calcium before they become as sensitive to toxic metals as softwater fish. At low pH, McDonald (1983a) suggested that H^+ displaces calcium from the paracellular tight junctions of the branchial epithelium, leading to the stimulation of ion loss. However, no effect of hardness on low pH stressed rainbow trout was found until after 40 h of exposure to pH 4.3 (McDonald et al. 1980). Thus, even in fully acclimated softwater fish, the displacement of calcium takes longer than 24 h to be expressed in a measurable physiological response.

Calcium is known to exert considerable control over the permeability of the gill itself (Potts and Fleming, 1971; Cuthbert and Maetz, 1972; Oduleye, 1975; Eddy, 1975). Removal of calcium from the medium stimulates ion loss and water uptake, and initiates a suite of physiological responses, which over the course of several weeks leads to an increase in the thickness of the mucus coat (Wendelaar Bonga, 1978), and increased levels of the calcium-binding protein, calmodulin (Flik et al., 1984). The objective of these adjustments seems to be the

maintainance of a high calcium micro-environment around the branchial epithelium, a conclusion supported by the observations of McWilliams (1983) on higher calcium binding on the gills of softwater acclimated fish. Thus, sub-lethal, or chronically toxic levels of copper may only slowly displace calcium from the gills, and this slow displacement may allow additional adaptive responses such as the proliferation of chloride cells (Chapter 1; Laurent et al., 1985), and the stimulation of calcium uptake (Perry and Wood, 1985), which further act to retain calcium. Nevertheless, beyond about 40 h, softwater acclimated fish are still less resistant to low pH than hardwater fish (McDonald et al., 1980), and the same would be expected for fish exposed to copper.

Thus, it seems likely that alkalinity, as the most important determinant of copper speciation, is the major short-term determinant of copper toxicity, but that mucus-, or membrane-bound calcium, as the major determinant of gill permeability, plays a significant longer-term role in modulating the biological response to this toxicant.

Effects of pH on Copper Toxicity

It was also confirmed that exposure to copper at pH 5.0 does not significantly increase the toxicity of copper concentrations above 100 µg/L, but that the net Na^+ losses found from 0 to 100 µg/L at pH 5.0 were the result of the independent effects of copper and H^+. A similar situation was found with adult trout exposed to copper at pH 5.0, and suggested that this phenomenon may be explained by the ability of copper to compete directly with H^+ for the same site of toxic action, and

therefore exert a pure copper effect at high copper concentrations (Chapter 2). Since ionic copper, Cu^{2+}, is much more abundant at pH 5.0 than at neutral pH, and since $Cu(OH)_2$ are the major non-carbonate species expected at neutral pH in our system, the failure to find a difference in toxicity between neutral pH and pH 5.0, suggests these are both toxic forms of copper.

H^+ alone causes significantly greater inhibition of J_{in} in SL than HL juvenile trout. This effect of water hardness is the same as reported by McDonald et al. (1983) for adult trout exposed to pH 4.3, but is the opposite of what was found with adult trout at pH 5.0 (Chapter 2). This is probably due to the greater sensitivity of the juvenile fish used in the present experiments, and the large difference in $[H^+]$ between pH 5.0 and pH 4.3.

Mechanisms of Toxicity

As with adult fish (Chapter 2), J_{in} was more sensitive to copper than J_{out}. However, the stimulation of efflux was found at 100 µg/L in juvenile fish, as opposed to 200 µg/L in adults.

Juvenile rainbow trout die when they lose about 50 to 55% of their whole body exchangeable Na^+. This occured in juveniles at about 200 µg/L copper, a concentration which did not cause death within 24 h in adults, and resulted in only about a 23% reduction in exchangeable Na^+ in adults (Table 3.3). Thus, copper causes about twice the net ion loss in juveniles as in adults (Table 3.3). The basis for this phenomenon lies in the 2 fold increase in sodium efflux rate between adult and

juvenile trout. Thus, a given percentage of inhibition of J_{in} Na^+ would lead to twice as large a net loss of Na^+ in juveniles as in adults. This may be explained by the nearly 2 fold increase in the weight specific surface area of the gill in juveniles compared to adults (Morgan, 1971).

A 24 h LC50 of 90 µg/L was calculated for juvenile rainbow trout in low alkalinity water, regardless of the hardness and pH. As the slopes of the mortality lines were not significantly different, there is no suggestion that the mechanism of toxicity changes with either hardness or pH. Howarth (1976) also found 24 h LC50 values of 75-125 ug/L in low alkalinity water. In the high alkalinity experiments, too few fish died within 24 h for the accurate estimation of the 24 h LC50. However, the few deaths that did occur, suggest that this figure would be in excess of 400 µg/L. Thus, the effects of high alkalinity on sodium balance are well correlated with the amelioration of lethality.

Several authors have suggested that organic complexation, like carbonate complexation, may explain the wide range of 96 h LC50's found in different studies of copper toxicity (Spear and Pierce, 1979; Alalbaster and Lloyd, 1980). By measuring whole body ions, yet another possible source for these discrepancies has been identified. When trout are transferred to media of a lower $[Na^+]$, or $[Ca^{2+}]$, they lose Na^+ until they reduce J_{out} Na^+, and increase J_{in} Na^+. These losses are somewhat additive, such that fish exposed to both a reduced $[Na^+]$ and $[Ca^{2+}]$, lose more Na^+ than those exposed to only a reduced $[Na^+]$ or a reduced $[Ca^{2+}]$. Thus, unless the fish are first acclimated to their

respective assay media for at least 2 weeks prior to the beginning of an experiment, the fish would likely exhibit lower whole body [Na^+] before the addition of toxicant. Published acclimation times for 96 h LC50 determinations range from 2 to 3 days (Chakoumakos et al., 1979; Miller and Mackay, 1980) to 13 days (Howarth and Sprague, 1978). Furthermore, none of the previous studies (Howarth and Sprague, 1978; Chakoumakos et al., 1979; Miller and Mackay, 1980), varied hardness, alkalinity and [Na^+] independently. When alkalinity is adjusted with $NaHCO_3$ (Chakoumakos et al., 1979; Miller and Mackay, 1980), or by diluting natural hardwater with distilled water (Shaw and Brown, 1974; Howarth and Sprague, 1978), up to 40-fold changes (e.g., 25 to 1000 μM as $CaCO_3$) in [Na^+] occur. In this respect, it is interesting to note that Brown (1981) found that increasing the water [Na^+] increases the survival of trout exposed to low pH. Thus, diluting natural hardwater to produce softwater would be expected to decrease survival because the [Na^+], as well as hardness and alkalinity, are reduced.

Copper Uptake vs. Toxicity

In 24 h experiments, apparent copper uptake was not well correlated with the physiological effects of copper. In high alkalinity water, copper uptake was the same as in low alkalinity water, but toxicity was reduced, while at pH 5.0, copper uptake was only about 50% that found at neutral pH, but the toxicity was the same. One possible reason for these discrepancies is that a large portion of the calculated whole body copper uptake may not have entered the body at all, but was

apparently due to the binding of copper to the body surface alone, and that this high degree of surface binding may have masked small, but significant, differences in the amount of copper which actually penetrated the gill. At 50 µg/L copper in low alkalinity hardwater at neutral pH, copper uptake was very high for the first hour of exposure, but decreased rapidly with time. Such a change in the rate of copper uptake may be interpreted as the rapid loading of a small volume pool, such as the body surface, followed by a much slower rate of transfer to the internal body tissues. Copper uptake during this period of rapid uptake accounted for about 50% of the total copper accumulated during 24 h.

The slower phase of copper uptake is apparently the only component of the total, short-term uptake which is important to toxicity, since the sodium uptake rate was progressively inhibited during the slower period of copper accumulation, 2 to 24 h, but no inhibition of the sodium uptake rate was found during the period of highest copper uptake rate. Despite the fact that the copper accumulated during this slower phase of copper uptake accounted for about 50% of the total copper accumulated during 24 h, it will be shown that more than 95% of the copper accumulated in juvenile trout exposed to copper for 28 days, was found in tissues other than the gills (Chapter 4). Therefore, only a small portion of the copper accumulated during this slower phase of uptake, actually stays in the gills.

It is hypothesized that toxicity should be correlated with the actual amount of copper which enters the fish, rather than the apparent

uptake which includes surface binding. If this is true, then the reduction of toxicity in high alkalinity water must have been due to an increase in the portion of the whole body uptake attributable to surface binding, i.e., a reduction in uptake. Furthermore, the reduction in apparent uptake at pH 5.0, with no reduction in toxicity, must have been due to a decrease in the portion of the whole body uptake attributable to surface binding , i.e., an increase in actual uptake. In this respect, Miller and Mackay (1982) have shown that the copper binding capacity of mucus from rainbow trout is progressively reduced as pH is decreased, and it might be expected that mucus binding would be a major component of surface binding in fish. The other possible explanation for the apparent lack of correlation between uptake and toxicity is that the more toxic copper species, Cu^{2+}, was taken up at pH 5.0, while the non-toxic copper carbonates were taken up in high alkalinity hardwater. Longer term experiments would be required to resolve these questions.

CHAPTER 4

ACCLIMATION TO COPPER BY JUVENILE TROUT: PHYSIOLOGICAL MECHANISMS

INTRODUCTION

Although copper, zinc, cadmium, and mercury can be acutely toxic to fish, numerous laboratory studies have shown that prior sublethal exposure to metals can result in enhanced lethal resistance (Chapman, 1978; Bouquegneau, 1979; Pascoe and Beattie, 1979; Dixon and Sprague, 1981a; Buckley et al., 1982; Duncan and Klaverkamp, 1983). Fewer studies have demonstrated true acclimation of fish to metals, especially if one accepts that acclimation involves the return of normal rate functions to the pre-exposure steady state, during the continued presence of the stressor (Prosser, 1973). Apparent acclimation to copper has been shown by McKim et al. (1970), and Lett et al. (1976), but very little is known about the physiological basis for this process. Thus, the objectives of the present study were 1) to determine if true acclimation to copper occurs in the laboratory, and 2) to describe some of the physiological changes which occur during acclimation.

In Chapters 2 and 3, it was demonstrated that the primary site of sub-lethal copper toxicity to rainbow trout is the sodium transport mechanism of the gills. Many authors have shown that sodium uptake may be described by Michaelis-Menten kinetics (Kerstetter et al., 1970; Maetz, 1972; DeRenzis and Maetz, 1973), and thus may be analysed in terms of saturability (J_{max}), and affinity (K_m) for substrate. In addition, the nature of the inhibition of ion uptake (e.g., competitive, non-competitive) may be determined (DeRenzis, 1975). If trout are able

to acclimate to copper, this acclimation should occur at the primary site of toxic insult, the gills, and should be apparent in terms of velocity and/or affinity of sodium uptake. In order to test this hypothesis, juvenile rainbow trout were exposed to a sublethal concentration of copper for 28 days and the kinetics of sodium uptake were analysed at regular intervals.

METHODS and MATERIALS

Two different series of experiments were conducted. The first type assessed the effects of long-term copper exposure on sodium uptake kinetics and whole body sodium concentration. In order to test for reproducibility, this experiment was repeated. The second series of experiments examined the effects of short-term copper exposures on sodium uptake and whole body sodium concentration.

Animals

Juvenile rainbow trout (mean= 2.77 g wet wt $\pm$ 0.22 (SEM), range= 1.05 - 5.71 g; n=490) were maintained in flowing dechlorinated natural hardwater (pH= 7.8$\pm$0.1, $[Na^+]$= 595$\pm$25, $[Cl^-]$= 890$\pm$34, $[Ca^{2+}]$= 1040$\pm$51, [ammonia]= 28$\pm$7, and [titratable alkalinity]= 875$\pm$25 μM (fixed endpoint titration to pH 4.0), n=8). In the first type of experiment the fish were exposed to copper in a flow-through system for 28 days, and allowed to recover in the absence of copper for 7 days. The fish were fed daily for the entire period of exposure and recovery, and no fish died during either exposure or recovery. The experiments were conducted at 15$\pm$1^{o}C with a daily photoperiod of 14 h light, and 10 h darkness. Copper stock

(as $Cu(NO_3)_2$) was metered by a peristaltic pump into diluent water in a head tank. This tank, in turn, fed 8 separate 80 liter tanks at a rate of 75 mL/min each. Each tank was aerated and contained 40 fish. Under these conditions each tank received about twice the minimum daily volume replacement recommended by Craig and Beggs (1979) for copper. The copper concentrations in the fish tanks were measured daily and averaged 54.9±1.7 µg/L (n=28).

Sodium Uptake Kinetics

At weekly intervals, a subsample of fish from the flow-through copper exposure was transferred to individual flux chambers. In order to measure Na^+ uptake kinetics the fish were exposed for 2 h to approximately 25, 50, 100, 200, 400, 600, or 800 µM Na^+, labelled with 148 KBq/L ^{22}Na. Five fish were exposed at each of the sodium concentrations and copper (55 µg/L) and calcium (1000 µM) were held constant. On day 0, before the exposure began, and on day 1 and 7 following the end of copper exposure, fish were exposed for 2 h to the same conditions as above, but without copper. Unidirectional sodium flux measurements were conducted in covered, aerated, 500 mL polyethylene containers each holding a single fish, and filled with 250 ml of medium. Sodium influx (J_{in}) was calculated from measurements of whole body ^{22}Na according to equation 3.1, except that t was 2 h. Lineweaver-Burk transformed data (i.e., $1000/J_{in}$ vs. $1000/[Na^+]$) were evaluated by least squares linear regression and the resulting equations

used to estimate the kinetic parameters, J_{max} and K_m, where J_{max} = the x-intercept, and $1/K_m$ = the y-intercept.

Prior to flux measurements, each fish was transferred to and held for 30 min in a 250 mL rinse bath, and then transferred to the flux chamber. This allowed the fish to adjust to confinement in the flux chamber, and also reduced the carry-over of ions from the exposure system. The rinse water was identical in composition to that of the flux chamber water. During flux measurements, water samples were collected at time 0 (prior to the addition of fish), at 60, and at 120 min. The fish were then killed by cervical dislocation, weighed, and counted in a gamma counter.

Whole Body and Water Ion Measurements

Following gamma counting, the fish were digested in 5 mL of concentrated nitric acid. These digests, as well as the water samples collected during the experiment, were analysed for Na^+, K^+, Ca^{2+}, and Cu^{2+}, with a Varian 1275 atomic absorption spectrophotometer. Lanthanum chloride was added to Ca^{2+} samples to reduce the interference due to the high Na^+ levels.

Short-Term Copper Exposures

In this series, juvenile trout were first exposed to 100 µg/L copper for 24 h in artificial hardwater (200 µM NaCl, and 1000 µM $Ca(NO_3)_2$, pH 7.8), and then exposed for an additional 24 h to 0, 50, 100, or 200 µg/L copper. At the end of this period the fish were killed and analysed for whole body Na^+ as described above. The values so

obtained were compared to values previously obtained for fish exposed to 0, 50, 100, and 200 µg/L copper for a single 24 h period (i.e., no prior copper exposure; Chapter 3). In addition, J_{in} Na^+ was also measured as described above. For both groups of fish, sodium efflux constants were calculated using the exponential decay equation:

$$[Na]_{t24}=[Na]_{t0} \times e^{-dt}, \quad (4.1)$$

which can be rearranged as:

$$d= \frac{(\ln[Na]_{t0})-(\ln[Na]_{t24})}{t}, \quad (4.2)$$

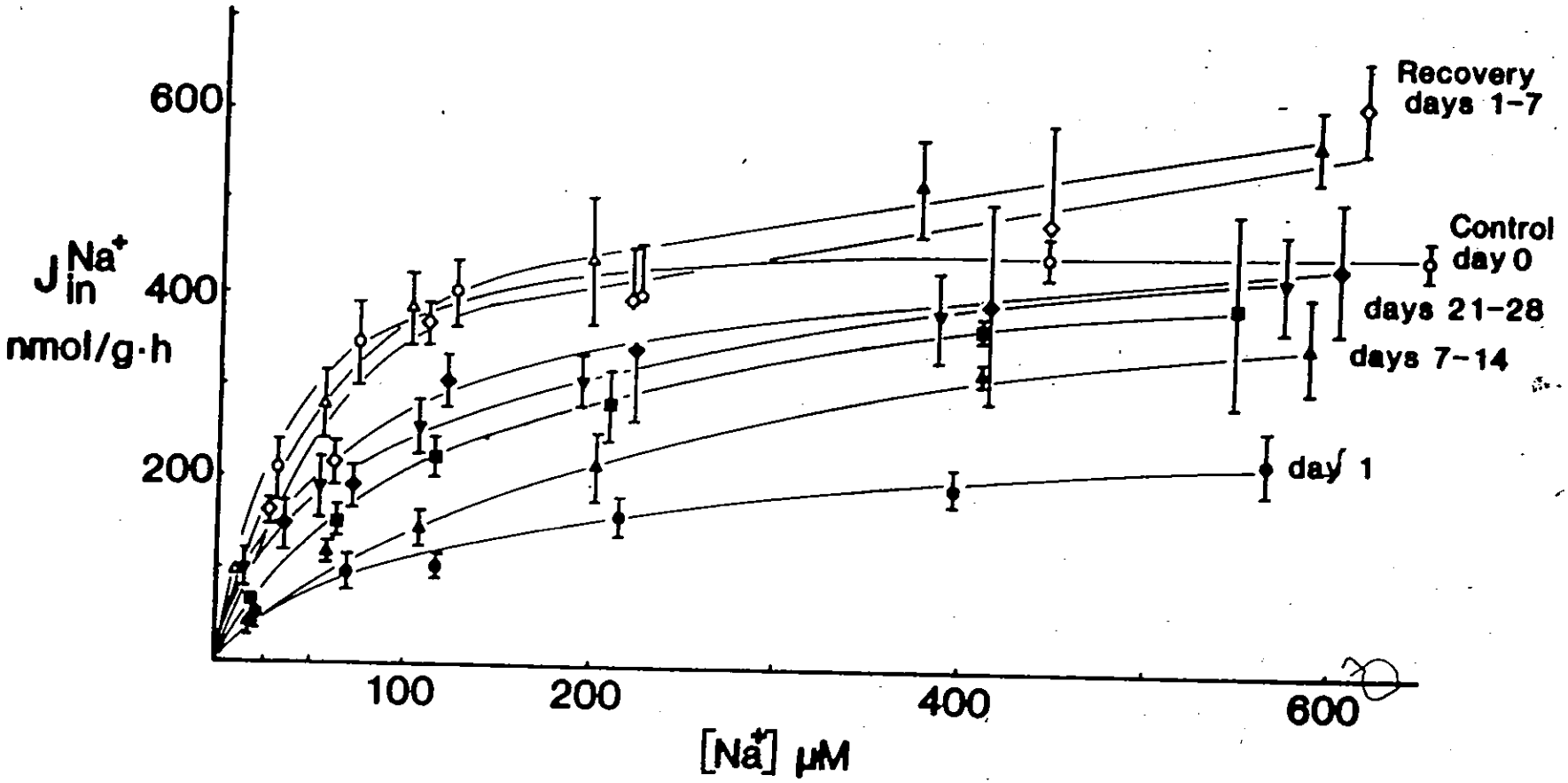
where $[Na]_{to}$ is the mean whole body $[Na^+]$ per unit weight at the beginning of a 24 h period of exposure; $[Na]_{t24}$ is the mean whole body $[Na^+]$ at the end of a 24 h period; t is 24 h; and d is the sodium efflux constant. This is a dimensionless value which estimates diffusional efflux, independent of the Na^+ gradient, providing certain conditions are met (see Results, below).

RESULTS

Reproducibility of Results

In order to test the reproducibility of the results, the uptake kinetics experiment was run on two separate occasions. The first experiment was begun on February 20, 1985, and the replicate experiment was begun on April 16, 1985. There were no significant differences

Fig. 4.1. Inhibition and recovery of sodium uptake in juvenile rainbow trout exposed to 55 µg/L copper in natural hardwater at neutral pH. Means ± 1 SEM, n=10. Symbols are as follows: Pre-exposure controls (○); day 1 of copper exposure (●); day 7 (▲); day 14 (■); day 21 (▼); day 28 (◆); recovery day 1 (△); and recovery day 7 (◇).


600
400
200
$J_{in}^{Na^+}$
nmol/g·h
Recovery
days 1-7
Control
day 0
days 21-28
days 7-14
day 1
100
200
400
600
[Na⁺] μM

between the results of the two experiments in regard to water copper concentration, sodium uptake, or whole body ions. Therefore the data were pooled for analysis.

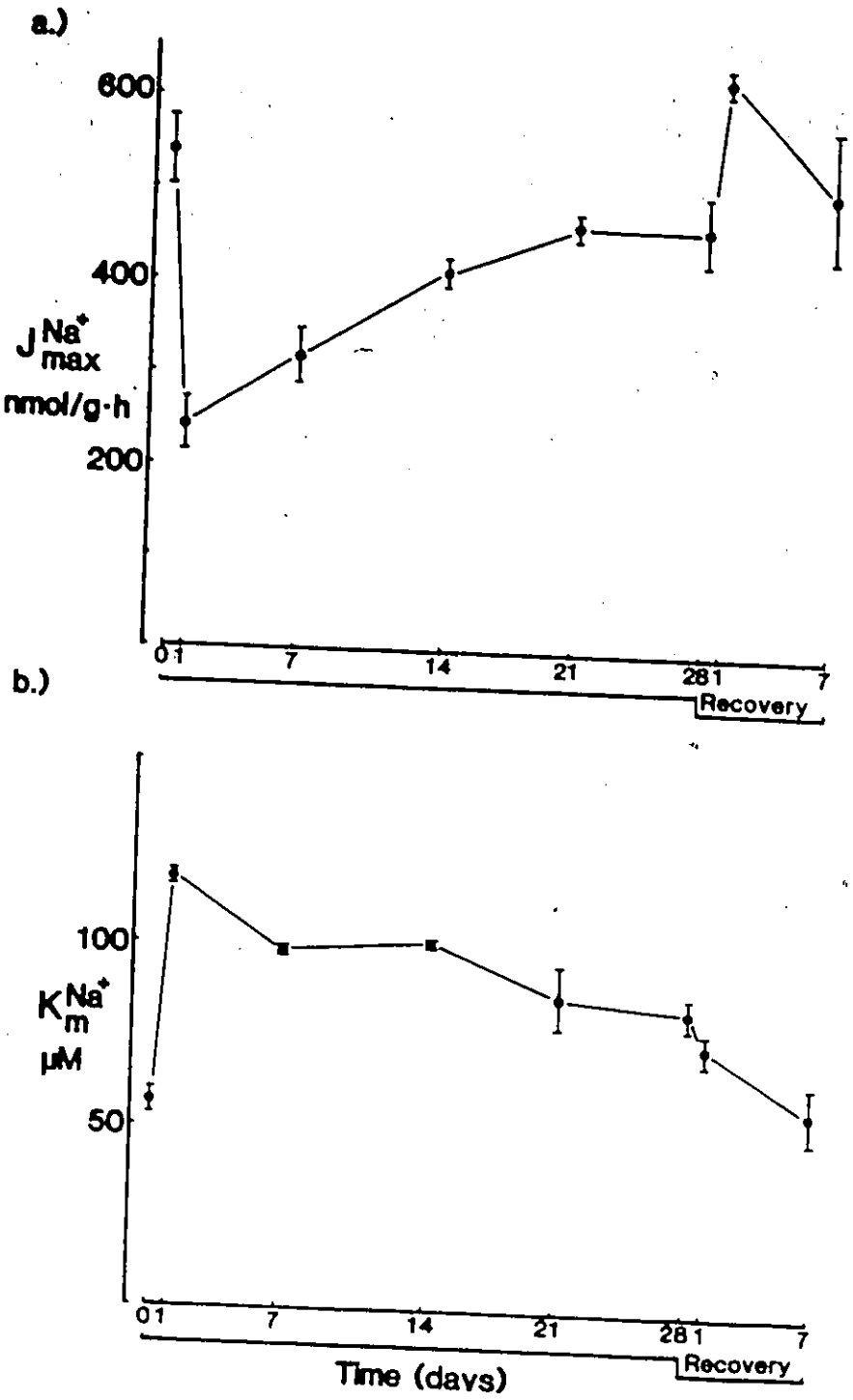
Effects of Copper on Sodium Influx

In control fish, sodium influx (J_{in}) exhibited apparent saturation kinetics (day 0, Fig. 4.1). Although the gill is a complex organ, and the nature and location of the sodium transport mechanism is not known, J_{in} Na^+ could be effectively described using the Lineweaver-Burk procedure for estimating kinetic parameters (i.e., J_{max} (Fig. 4.2a) and K_m (Fig. 4.2b). For control fish, this yielded a maximal uptake velocity (J_{max}) of 540±37 nmol/g·h, and an apparent affinity for substrate (K_m) of 57±4 µM.

After 24 h of exposure to 55 µg/L copper, there was significant inhibition of J_{in} at all Na^+ concentrations relative to control values (day 1, Fig. 4.1). The apparent J_{max} and K_m were calculated as 244±28 nmol/g·h, and 118 ± 2 µM, respectively (Fig. 4.2a, b), representing a 55% reduction in maximal velocity, and a 49% reduction in apparent affinity.

Following 7 days of copper exposure, J_{max} had recovered relative to day 1, but was still only about 59% that of control values (Fig. 4.2a). After 14 days, J_{max} was about 77% that of controls, a significant increase over the day 7 value (Fig. 4.2a). The apparent affinity of the carrier was estimated to be 98±0.1 µM on day 7, and

Fig. 4.2 a.) Maximal velocity (J_{max}) calculated by linear regression of Lineweaver-Burk transformed data in the form $1000/J_{in}$ vs. $1000/[Na^+]$. Means $\pm$ 1 SEM, n=60. b.) Apparent affinity (K_m) calculated as in 4.2a.


a.)
600
400
$J^{Na^+}_{max}$
nmol/g·h
200
0 1
7
14
21
28 1
7
Recovery
b.)
100
$K^{Na^+}_m$
μM
50
0 1
7
14
21
28 1
7
Recovery
Time (days)

101±0.7 μM Na^+ on day 14 (Fig. 4.2b). Thus, K_m was still inhibited by about 42% relative to controls.

A general trend for the continuing recovery of J_{in} was found from days 14 to 28, but most of this was significant only at about 25 μM Na^+ on day 21, and at 25 and 100 μM Na^+ on day 28 (Fig. 4.1). No significant changes were found between days 21 and 28. On day 28 J_{max} was still inhibited by about 14% (Fig. 4.2a), but this was not significantly different from control values, whereas Km was still significantly inhibited by about 31% relative to control values (Fig. 4.2b).

After 28 days the fish were transferred to natural tap dechlorinated hardwater where the ambient copper concentration was less than 5 μg/L. Within 24 h of recovery, no significant differences from control values of J_{in} Na^+ were found except at the 600 μM (R1, Fig. 4.1) where J_{in} was significantly higher than found under control conditions. J_{max} was calculated to be 630±15 nmol/g·h (Fig. 4.2a), but K_m was still inhibited by about 23% (Fig. 4.2b). After 7 days recovery in copper-free water, J_{in} at 600 uM Na^+ was still significantly greater than under pre-exposure control conditions (R7, Fig. 4.1). Despite this difference, J_{max} was 510±71 nmol/g·h, and K_m was 57±8 μM. These values are nearly identical to those of controls found on day 0 (i.e., 540±37 nmol/g·h, and 57±4 μM; Fig. 4.2a, b).

Effects of Copper on Whole Body Ions

In control fish in natural dechlorinated hardwater, whole body Na^+, K^+, and Ca^{2+} concentrations were 56.9±1.2, 98.9±2.8, and 78.5±2.2 µmol/g wet wt. (Table 4.1).

Within 24 h of exposure to copper, whole body Na^+, K^+, and Ca^{2+} concentrations decreased significantly, to 49.8±0.6, 93.9±0.7, and 74.8±1.1 µM/g, respectively (Table 4.1). For Na^+, this amounts to an average net loss rate of about 295 nM/g·h, or about 12.5% of the whole body Na^+ concentration.

After 7 days of copper exposure, whole body Na^+, K^+, and Ca^{2+} concentrations had recovered completely (Table 4.2), and these values were maintained for the duration of the experiment. Whole body K^+ increased significantly over the control value by day 14, and continued to increase as body weight increased. Body weight and K^+ concentration were found to be significantly correlated (r^2 = .8813).

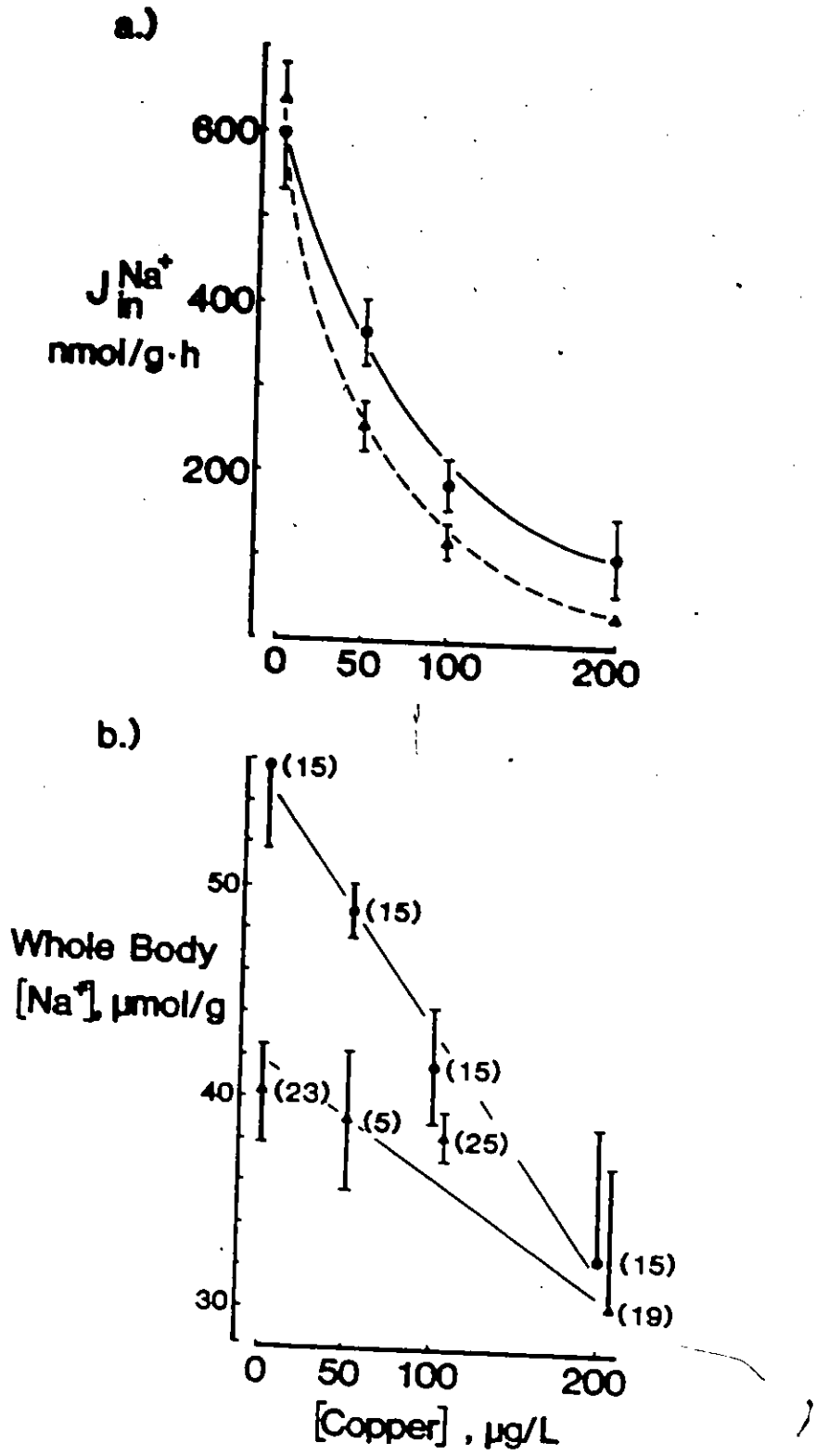
Effects of Short-Term Copper Exposure

In Chapter 3 it was shown that copper exposure leads to concentration-dependent decreases in both J_{in} Na and whole body Na^+ in juvenile trout (upper lines, Fig. 4.3a and b; Chapter 3). In the present study, it has been shown that trout pre-exposed to 100 µg/L copper for 24 h, and then returned to copper-free water, suffered comparable losses of whole body Na^+, although J_{in} recovered (0 µg/L, lower lines, Fig. 4.3a and b). When pre-exposed fish were re-exposed to 50 to 200 µg/L copper for a second 24 h they suffered losses of whole

Days of Exposure	Wet Weight (g)	$[Na^+]$ µmol/g	$[K^+]$ µmol/g	$[Ca^{2+}]$ µmol/g
Day 0	2.42±0.12	56.9±1.2	98.9±2.8	78.5±2.3
Day 1	2.22±0.09	49.8±0.6 *	93.9±0.7 *	74.8±1.1*
Day 7	2.38±0.11	56.6±1.6	102.0±5.1	79.5±2.8
Day 14	2.64±0.11	57.7±0.9	104.4±4.0	81.1±1.4
Day 21	2.82±0.13	58.3±0.7	106.8±4.3	79.2±2.7
Day 28	3.34±0.12	56.4±0.7	107.9±4.7	79.2±1.5
Days of Recovery				
Day 1	3.31±0.11	57.5±0.8	111.5±5.7	79.9±0.9
Day 7	3.78±0.16	57.5±0.6	113.8±7.5	79.3±1.1

Table 4.1. Whole body ions in juvenile rainbow trout exposed to 55 µg/L copper for 28 days, and 7 days of recovery in copper-free water. For the exposure period each value is the mean ± 1 SEM of 60 fish; for the recovery period each value is the mean ± 1 SEM of 30 fish. Asterisks indicate a significant ($P \leq 0.05$) decrease from control (day 0) values.

Fig. 4.3 a.) The effects of copper on sodium uptake (J_{in}) in juvenile rainbow trout exposed for 24 h (●, data from Chapter 3), or pre-exposed to 100 ug/L copper for 24 h, and then exposed for an additional 24 h (▲). Means ± 1 SEM. b.) The effects of copper on losses of whole body Na^+ in the same fish as in Fig. 4.3a. Sample size for both a and b is indicated in parentheses. Means ± 1 SEM.


a.)
600
400
200
$J_{in}^{Na^+}$
nmol/g·h
0
50
100
200
b.)
(15)
(15)
(15)
(23)
(5)
(25)
(15)
(19)
50
40
30
Whole Body
[Na⁺], µmol/g
0
50
100
200
[Copper] , µg/L

body Na^+, but at a lower rate than fish exposed for the first time (Fig. 4.3b). However, much of this decrease in loss rate was likely due to the reduction in the Na^+ diffusion gradient arising from copper pre-exposure. To account for this factor, the exponential decay equation was used to calculate Na^+ efflux constants, independent of the Na^+ gradient (see Methods, above). The use of this equation is only justified when J_{in} approaches zero (i.e., J_{net} is entirely due to J_{out}). In the present series of experiments, this condition was only met in those fish exposed to 200 µg/L copper (Fig. 4.3a). The efflux constant calculated for fish exposed for a single 24 h period was 0.0325, while that calculated for the pre-exposed group was 0.0154. This is a reduction in the Na^+ constant of 53%, and suggests a major reduction in Na^+ permeability.

DISCUSSION

The objective of the present study was to examine the physiological basis for acclimation of fish to sublethal levels of copper. In previous studies with rainbow trout, it was shown that one of the major mechanisms of copper toxicity to fish is the disruption of normal gill ionoregulatory function. Copper concentrations as low as 12.5 µg/L inhibit sodium uptake and disrupt sodium balance, with death ensuing within 24 h at copper concentrations greater than 100 µg/L (Chapter 2, 3). In the present study, it was confirmed that 55 µg/L copper inhibits sodium uptake and reduces whole body sodium concentration. In addition however, it has now been shown that both of

these parameters can recover completely within 21 days. Furthermore, since naturally occurring copper concentrations generally range from about 5 to 100 µg/L copper (Spry et al., 1981), the present results suggest these same mechanisms may be responsible for acclimation to copper in the wild.

In the present study, whole body Na^+ had returned to control levels before complete recovery in J_{in} was found (Table 4.1; Fig. 4.1). Since it has also been shown that the inhibition of J_{in} (at least at 100 µg/L) is, in fact, greater at 48 h than at 24 h (Fig. 4.3a), the recovery of whole body Na^+ is best explained by a decrease in sodium efflux (J_{out}) to levels below that found in normal control fish. Reductions in J_{out} have been previously shown to play an important role in ameliorating the effects of low pH (McDonald, 1983), lanthanum (Eddy and Bath, 1979), and copper (Chapter 2). At a J_{in} Na^+ of 244 nM/g.h (ie., J_{in} after 24 h), the net loss of whole body Na^+ found by 24 h could be fully compensated within 7 days if J_{out} Na^+ were reduced to about 40% of control values. In fact, it was shown in Chapter 2 that reductions in J_{out} of up to 50% occur within 12 h in adult trout exposed to copper (Fig. 2.8).

J_{out} may be reduced by either a decrease in the Na^+ gradient due to ion losses, or by a reduction in epithelial permeability. By using the exponential decay equation, we have shown a gradient-independent reduction in Na^+ efflux within 48 h of exposure to copper. The nature of this reduction in permeability is unknown and could be specific Na^+

alone, or could be part a more general reduction in the permeability of the branchial epithelium to both cations and anions.

In general, the mechanism by which epithelial permeability is reduced is not presently understood. However, it has been shown that the administration of exogenous mammalian prolactin reduces gill permeability in several species of fish (Dharmamba and Maetz, 1972; Wendelaar Bonga and Van der Meij, 1981), and acid exposure has been shown to increase the metabolic activity of prolactin cells in the brook trout (Notter et al., 1976). This suggests that the reduction of epithelial permeability may be under endocrine control and may be an essential part of the acclimation of fish to general ionoregulatory disturbances.

According to classical Michaelis-Menten kinetics, a reduction of J_{max} is indicative of non-competitive inhibition, while an increase in K_m is indicative of competitive inhibition. Thus, copper affects J_{in} Na^+ by mixed-type inhibition. Copper has a high affinity for sulfhydryl groups (Nieboer and Richardson, 1980), and may inhibit enzymes either by forming mercaptides or by catalysing the formation of disulfide bonds (Rothstein, 1959). In this context, it is interesting to note that the copper-dependent formation of disulfide bonds between the subunits of Na^+-K^+-ATPase results in cross-linking and complete denaturation (i.e., non-competitive inhibition; Huang and Askari, 1979), but copper also inhibits the Mg^{2+}-dependent hydrolysis of ATP in Na^+-K^+-ATPase (i.e., competitive inhibition; Shuurmans Stekhoven and Bonting, 1981). Since this enzyme is generally thought to be a major component of the

biochemical mechanism for Na^+ transport by the fish gill, it is apparent that these two inhibitory actions could be responsible for the mixed-type inhibition of J_{in} found in the present study. Furthermore, the replacement of such damaged enzyme subunits could result in the recovery of both J_{max} and K_m.

CHAPTER 5

ACCLIMATION TO COPPER BY JUVENILE TROUT: BIOCHEMICAL MECHANISMS

INTRODUCTION

It has been shown that lethality due to copper exposure in fish may be accounted for by the disruption of gill ionoregulatory function (Chapter 2, 3). Furthermore, it has been shown that acclimation occurs by the reduction of sodium efflux and the recovery of sodium influx (Chapter 4). Evidence has been presented that sodium efflux is reduced as a result of a decrease in the permeability of the branchial epithelium, but the biochemical mechanisms which control permeability are unknown. However, the biochemical basis for the recovery of sodium influx may be an increase in Na^+-K^+-ATPase activity due to an increase in the number of enzyme units, as has been shown in metal-stressed fish by several authors (Bouquegneau, 1977; Shephard and Simkiss, 1978; Watson and Beamish, 1980; Stagg and Shuttleworth, 1982), or may be due to the protection of the transport mechanism by the binding of copper by metallothionein, as proposed by Bouquegneau et al. (1975). In order to distinguish between these two possibilities, branchial Na^+-K^+-ATPase activity and branchial metal-binding ligand concentrations were measured in fish exposed to 55 µg/L copper for 28 days. Since previous authors have suggested a major role for the liver in the detoxification of copper, the concentration of hepatic metal-binding ligands was also measured, and copper uptake in liver, gills, and whole body was compared.

METHODS and MATERIALS

Animals

The fish used in these experiments were maintained and treated in an identical manner to those used in Chapter 4. Subsamples of fish were removed at regular intervals for analyses.

Copper Uptake

At weekly intervals, the net whole body accumulation of copper was determined by atomic absorption (Varian 1275) using the same acid digests used for whole body Na^+ measurements (Chapter 4).

The size of the exchangeable copper pool of the gills, liver, and carcass (i.e., whole body minus gills and liver), was also measured. A separate subsample of 10 fish was removed from the continuous exposure system at weekly intervals and exposed for 24 h to 55 µg/L copper, labelled with ^{64}copper (22 MBq/uM). Sodium (200 µM) and calcium (1000 µM) were held constant. Radioactivity of water, gills, liver, and carcass, was measured in a Nuclear Chicago well-type gamma counter. After counting, these tissues were digested in concentrated nitric acid and analysed as above for copper concentration. The size of the exchangeable copper pool was then estimated as:

$$\frac{^{64}\text{copper} \times (\text{S.A.})}{\text{Total tissue copper}} \times 100 , \qquad (5.1)$$

where ^{64}copper is the counts per minute of radiocopper per gram wet wt taken up by the tissue during 24 h; S.A. is the specific activity of the exposure water (in ug/cpm); and total tissue copper (as µg/g wet wt) was

determined by atomic absorption spectrophotometry. Control copper values for gill, liver, and carcass, were collected from a separate group of 10 fish that had never been exposed to copper above ambient levels (less than 5 µg/L).

Protocol for Biochemical Analyses

At weekly intervals, 12 fish were removed from the continuous exposure system, and the gills and livers were quickly removed. The gill basket was trimmed to reduce the amount of extraneous cartilage and divided into 2 approximately equal sized aliquots. Approximately half of the whole gill basket was immersed in 0.9% NaCl, then frozen in liquid nitrogen and stored at -60°C for assay of total ATPase, and ouabain inhibitable, Na^+-K^+-dependent ATPase activity. The remaining half of the gill basket and the entire liver were frozen in liquid nitrogen until analysis for metal-binding proteins.

Branchial ATPases

The gills from individual fish (52.0±2.2 mg wet weight; n=96) were homogenized in 5 mL of an ice cold solution (Hendler et al., 1972) containing 0.25 M sucrose, 6mM sodium EDTA, 20 mM imidazole, and 0.1% sodium deoxycholate (to make leaky vesicles). Homogenization was accomplished by 12 complete strokes of a motor-driven (500 rpm) Potter-Elvehjem grinder equipped with a teflon pestle. The cartilaginous gill arches and cell debris were removed by centrifuging for 10 min at 900 rpm. The microsomal fraction was concentrated by differential centrifugation for 30 min at 9.5K rpm in a Sorvall RC2B

equipped with an SS-34 rotor, followed by centrifugation of the supernatant for 60 min at 29K rpm in a Beckman L8-78 ultracentrifuge equipped with an SW 50.1 rotor. The temperature was 4°C in all centrifugations. The resulting microsomal pellet was resuspended in 5 times the tissue weight of homogenization solution minus sodium deoxycholate and frozen in liquid nitrogen until not more than 5 days later. On the day of the assay, the gills from 6 fish, which had never been exposed to copper, were prepared in an identical manner and assayed to check for loss of activity during storage of the microsomal suspension. No significant changes in activity were found between freshly prepared and frozen enzyme preparations. There was no detectable activity in the 29K rpm supernatant. Total ATPase activity, and ouabain-inhibitable Na^+-K^+-ATPase activity were assayed by the method of Towle et al. (1976). The assay medium consisted of: 20 mM imidazole (Sigma), pH 7.8, 100 mM NaCl, 10 mM KCl, 5 mM $MgCl_2$, 1.25 g% phosphoenolpyruvate (Sigma), and 5 mM Na_2ATP (Sigma). To 1.7 mL of assay medium, the following were added: 200 µL 1 mM NADH (Sigma, grade II in imidazole buffer, pH 7.4), 20 µL pyruvate kinase/lactate dehydrogenase (Sigma), and 100 µL water (total assay), or 100 µL 20 mM ouabain (total ATPase minus Na^+-K^+-ATPase). The enzyme activity was assessed at 37°C by monitoring the disappearance of NADH at 340 nM for 5 min on a Perkin-Elmer Lambda 3 spectrophotometer. Since the hydrolysis of ATP was coupled to the oxidation of NADH, the enzyme activity was expressed as µmol inorganic phosphate per mg microsomal protein per h. Protein was measured by the Coomassie Blue method of Bradford (1976).

Metal Binding Ligands

The concentrations of metal-binding ligands were assessed by two different techniques; 1) the acid-soluble thiol assay of Ellman (1959), and 2) the ^{109}cadmium-binding assay of Eaton and Toal (1982).

1. Acid-Soluble Thiols

Separate assays were conducted for each fish (n=96). The other half of the whole gill basket and the entire liver were thawed and homogenized in 5 volumes of ice cold 0.9% NaCl. At this point a 50 µL aliquot of this homogenate was removed for use in the ^{109}cadmium-binding assay (see below). The remaining aliquot of tissue homogenate was combined 1:1 with 10% TCA, and the precipitated protein removed by centrifugation in a Beckman Microfuge for 5 min. Total acid-soluble thiols in this supernatant were measured by the DTNB (5,5'-dithiobis(2-nitrobenzoic acid)) method of Ellman (1959) as modified by Moron et al. (1979). DTNB reacts with reduced thiols to form thionitrobenzoic acid, a compound which absorbs light at 412 nm. This compound was measured with a Perkin Elmer lambda 3 spectrophotometer, using reduced glutathione as the standard. Cysteine was measured by the colorometric method of Gaitonde (1967), using acid ninhydrin. Glutathione (both oxidized and reduced) was measured by the enzymatic method of Owens and Belcher (1965), using glutathione reductase, NADH, and DTNB and measuring the change in absorbance at 412 nm. All values were expressed as µM of sulfhydryl groups/g wet tissue weight. Metal-binding ligand concentration was calculated, in terms of

sulfhydryl groups/g wet tissue wt, as the total acid-soluble thiol minus the sum of glutathione plus cysteine (Wofford and Thomas, 1984).

2. ^{109}Cadmium Binding

The twelve 50 µL aliqouts removed after homogenization in 0.9% NaCl (see above) were pooled for each sample period and combined 1:1 with buffer (40 mM Tris -HCL, pH 7.4) containing 5 mM dithiothreitol, a sulfhydryl reducing agent. These homogenates were heated for 10 min at 85°C, and the denatured proteins removed by spinning for 5 min in a Beckman microfuge at room temperature. ^{109}Cd was added to the heat-denatured supernatant, according to the method of Eaton and Toal (1982). The heat-stable, cadmium-binding ligands in 500 µL subsamples were fractionated by Sephadex G-75 in a 2x30 cm column eluted at 1 mL/min with 25 mM Tris-HCl, pH 7.4. Blue dextran, rabbit liver Cd/Zn metallothionein (types I and II, Sigma), and reduced glutathione (Sigma) were used to calibrate the column. Fractions of 1 mL each were collected and counted in a Nuclear Chicago gamma counter.

RESULTS

Effects of Copper Exposure on Copper Uptake

In control fish, whole body copper concentration (measured by atomic absorption) was 1.23±0.06 µg/g (n=60; Fig. 5.1a), liver copper was 23.2±3.4 µg/g (n=10; Fig. 5.1b), and gill copper was 1.3±0.1 µg/g wet weight (n=10; Fig. 5.1c). In control fish, about 24% of the whole body copper was found in the liver, with gills accounting for only 4%

and the carcass, ie., whole body minus gills and liver, accounting for the remaining 69% (Fig. 5.2).

During the first 48 h of copper exposure, the liver accumulated copper at a much higher rate than either gills or carcass (Fig. 5.1b vs. a, c), and accounted for about 40% of the whole body copper burden at 48 h (Fig. 5.2). However, by day 7 the accumulation rate of the liver had slowed (Fig. 5.1b). After 14 days, the liver accounted for only about 21% of the whole body copper burden, while the carcass now accounted for about 78% (Fig. 5.2). Thus, while the contribution of the liver returned to pre-exposure levels, that of the carcass increased. Whole body copper accumulated at a mean rate of about 33 ng/g·h during the first 24 h of copper exposure, but decreased to an overall mean rate of about 8 ng/g·h (n=150) for the entire 28 day period (i.e., after 28 days of exposure whole body copper reached 6.79±1.8 µg/g (n=30; Fig. 5.1a). Liver copper increased by about 91 ng/g·h during the first 48 h of exposure, and by about 74 ng/g·h thereafter, and reached 113.0±18.0 µg/g wet wt by day 28 (n=30; Fig 5.1b). On day 28, the liver accounted for only about 17% of the whole body copper, while the carcass accounted for about 82% of the total (Fig. 5.2). Gill copper never accounted for more than about 6% of the whole body copper burden (Fig. 5.2). Gill copper increased by about 25% after 7 days of copper exposure (i.e., 1.72±0.1 µg/g), but then decreased to below control levels on day 21 (Fig. 5.1c). After 28 days, gill copper was 1.56±0.1 µg/g wet wt.

In the gills, the exchangeable copper pool was estimated at about 25% after 24 h of copper exposure, while the exchangeable pool of the

Fig. 5.1. a.) Uptake rate of copper in whole body and carcass, b.) liver, and c.) gills of juvenile rainbow trout exposed to 55 µg/L copper for 28 days, and then transferred to clean water for 7 days. Means ± 1 SEM, n=60 for whole body, and n=10 for liver and gills.

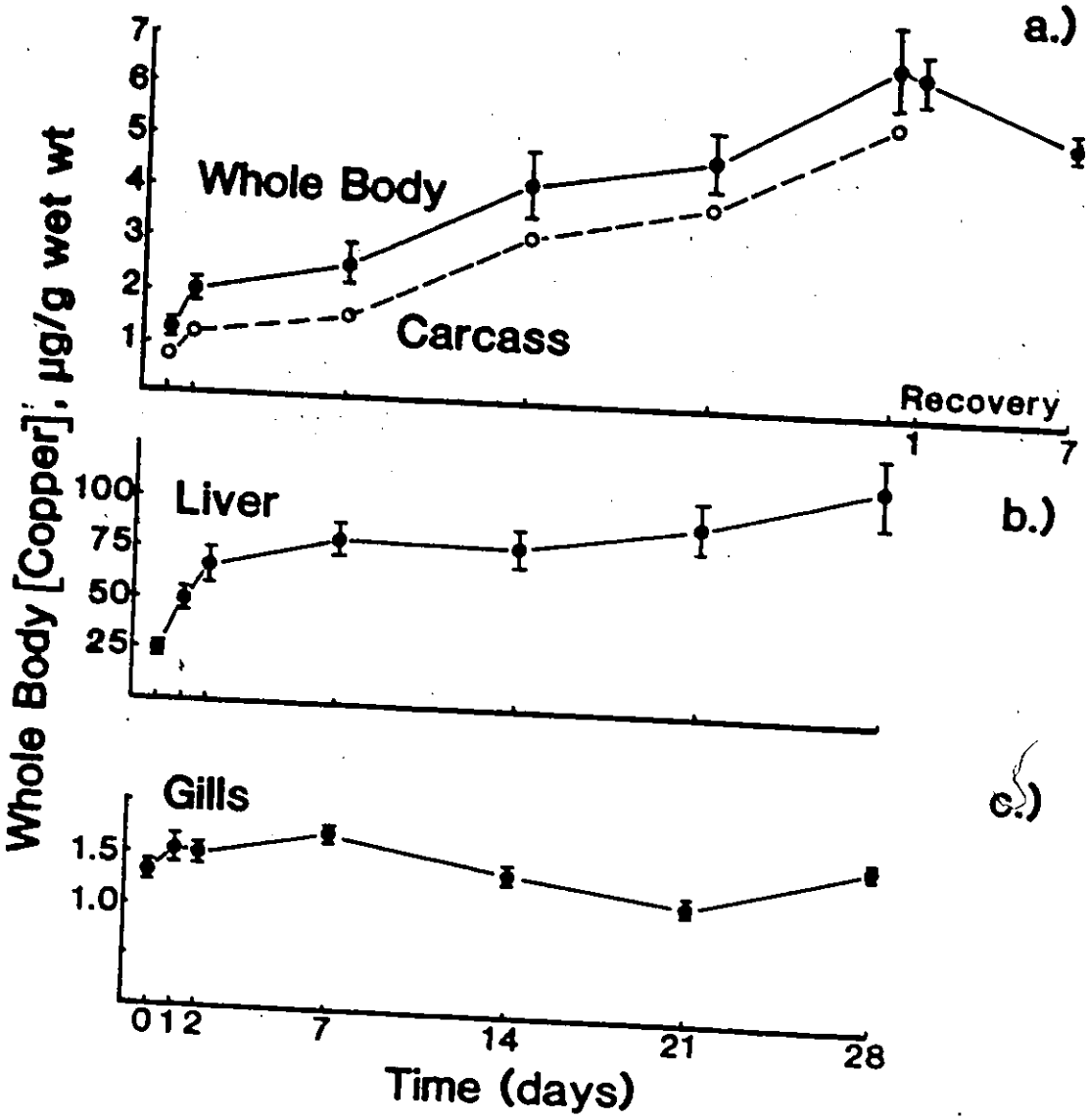
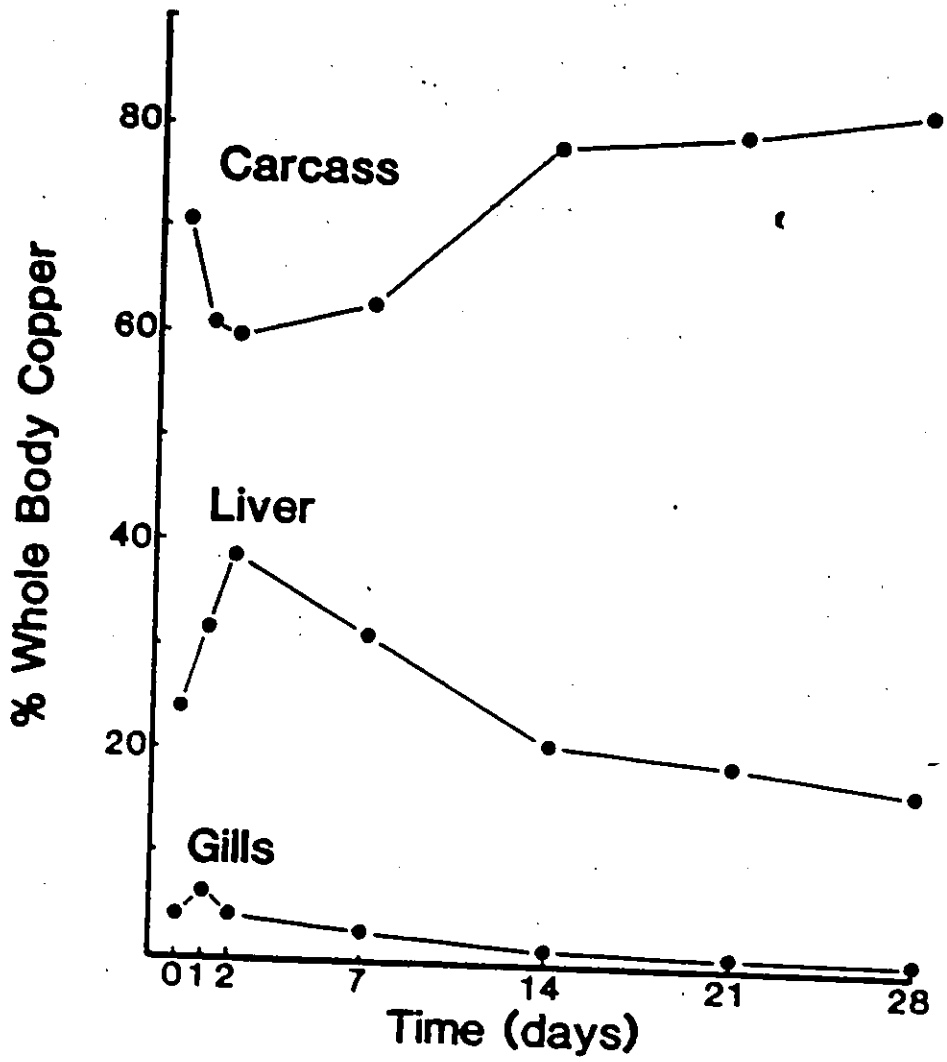

a.)
Whole Body
Carcass
Recovery
1
7
b.)
Liver
c.)
Gills
Whole Body [Copper], µg/g wet wt
Time (days)
0 1 2 7 14 21 28

Fig. 5.2. Percent of whole body copper found in carcass, liver, and gills of fish exposed to 55 µg/L for 28 days. Means, n=10.


80
60
40
20
% Whole Body Copper
Carcass
Liver
Gills
0 1 2
7
14
21
28
Time (days)

liver was only about 4% and that of the carcass, about 10%. In the gill, the size of this pool increased to 54% after 7 days of exposure, and remained stable at 52.8±1.3% until the end of the copper exposure. The exchangeable pool size of the liver decreased to about 2% on day 7, rose slightly on days 14 and 21, and decreased again on day 28 to 1.4%. The exchangeable pool size of the carcass also decreased gradually from about 8% on day 7 to 2.5% on day 28.

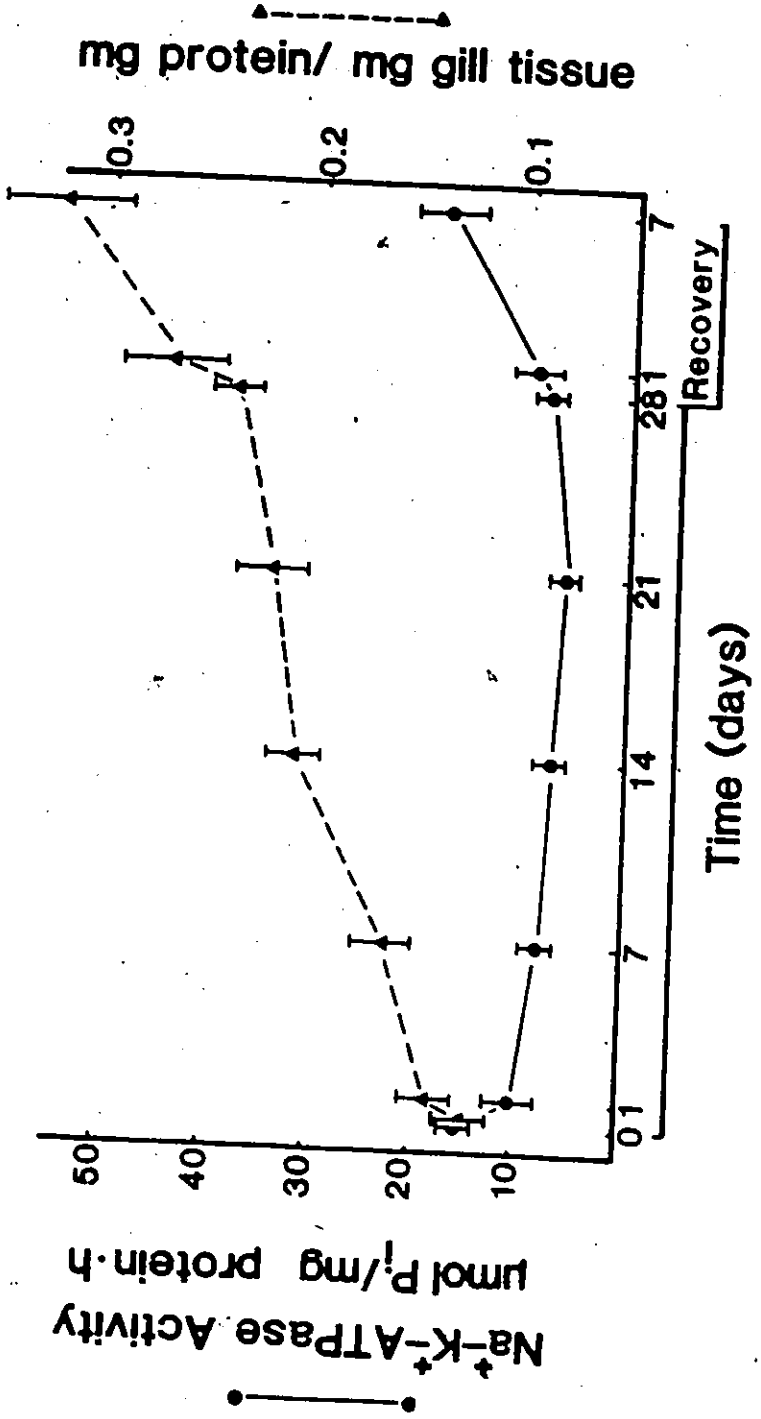
Following transfer to copper-free water, trout lost whole body copper at a rate of about 7.21±0.32 ng/g·h (n=10; Fig 5.1a).

Effects of Copper Exposure on Branchial ATPase Activity

The Mg^{2+}-ATPase and Na^{+}-K^{+}-ATPase specific activities in control fish were 31.6±1.7, and 15.7±1.8 µmol Pi/mg microsomal protein·h at 37°C. After 24 h exposure to 55 µg/L copper, both of these values were reduced by about 33%. Mg^{2+}-ATPase was not significantly different from control values after 7 days of exposure, but specific activity decreased from day 7 to 28. Na^{+}-K^{+}-ATPase specific activity remained significantly depressed and relatively constant from 24 h to 28 days of exposure (Fig 5.3). After 24 h in clean water, both Mg^{2+}-, and Na^{+}-K^{+}-ATPase specific activities increased, but the change was not significant. However, after 7 days of recovery, there was no significant difference in the specific activity of either enzyme from control values (Fig 5.3).

Microsomal protein was also measured as a function of whole gill wet weight (Fig 5.3). After 24 h of copper exposure, the amount of

Fig. 5.3. Branchial, microsomal Na^+-K^+-ATPase specific activity (●) and total microsomal protein (▲) in juvenile rainbow trout exposed to 55 µg/L copper for 28 days, and then transferred to clean water for 7 days. Means ± 1 SEM, n=12.


mg protein/ mg gill tissue
0.3
0.2
0.1
Recovery
7
28
21
14
7
0
Time (days)
50
40
30
20
10
Na+-K+-ATPase Activity
µmol Pi/mg protein·h

microsomal protein per mg gill tissue increased, and this trend became significant after 7 days, and continued for the 28 days of exposure (Fig 5.3). After 24 h recovery in clean water, the microsomal protein/mg gill tissue again increased, and was significantly higher than control values by day 7 of recovery (Fig 5.3).

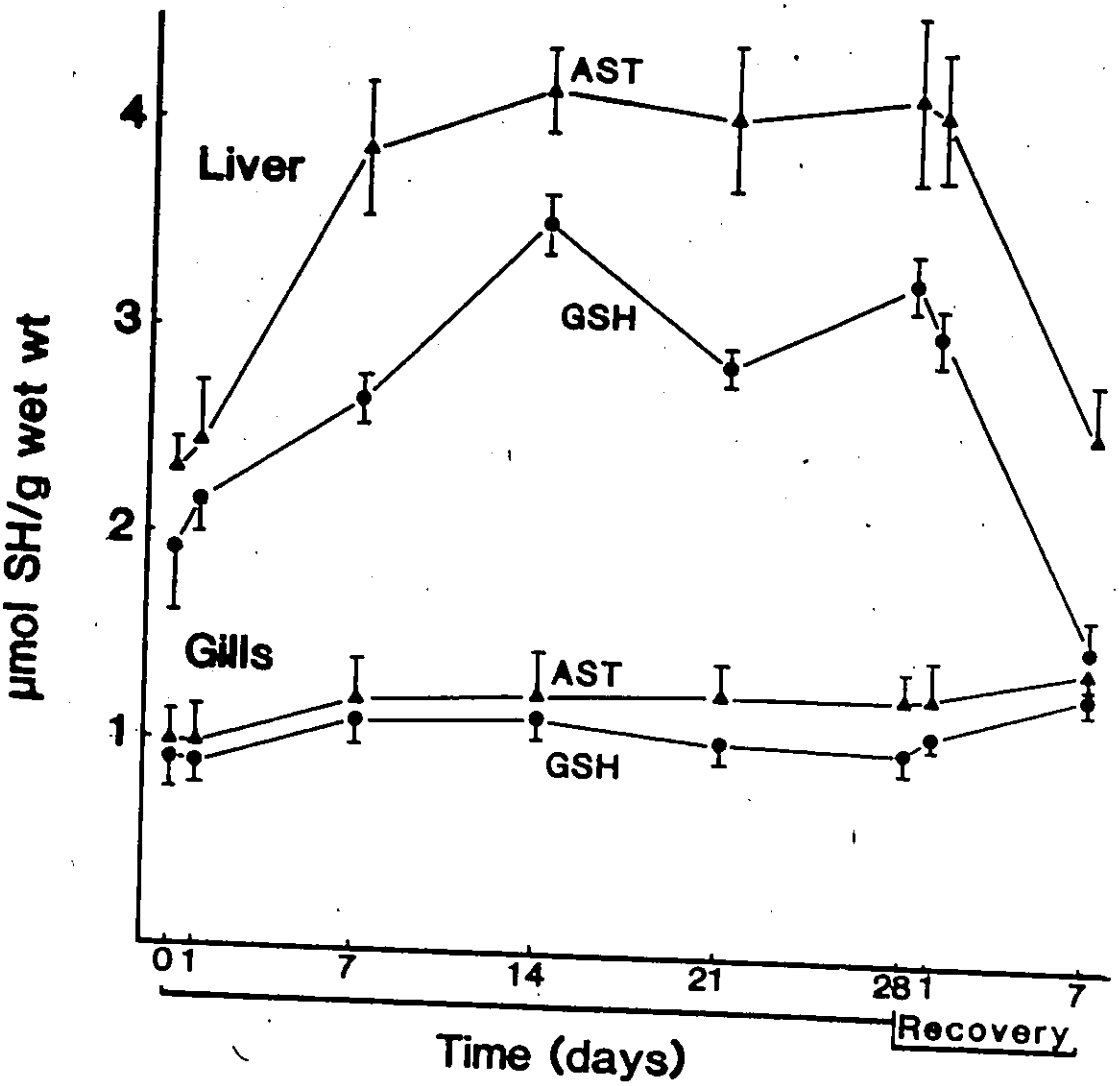
The mean total gill Na^+-K^+-ATPase activity (ie., Na^+-K^+-ATPase specific activity times total microsomal protein/ mg gill wet wt.) was 2009±382 µmol P_i/h under control conditions. After 24 h of copper exposure, this total gill activity was reduced by about 30% to about 1363±313 µmol P_i/h, but had recovered to 2166±448 µmol P_i/h by day 14. After 7 days of recovery, the total gill activity was about 4815±1118 µmol P_i/h; or 2.5 times that found under control conditions.

Effects of Copper Exposure on Gill and Liver Acid-Soluble Thiols

In control fish, acid-soluble thiol (AST) concentrations were 0.98±0.06 µmol sulfhydryl/g wet wt. in gills, and 2.28±0.12 µmol/g in liver (n=12, Fig 5.4). Glutathione (Glut) accounted for nearly all of this total thiol, with concentrations of 0.91±0.04, and 1.87±0.13 µmol/g for gill and liver, respectively. Gill cysteine was 0.08 ±0.004 nmol/g, and liver cysteine was 0.07±0.004 nmol/g wet wt, and therefore did not make a significant contribution to the total AST. Glut accounted for about 85% of liver AST and about 98% of gill AST under control conditions.

Gill AST and Glut did not change significantly during the entire 28 day period of copper exposure (Fig 5.4). However, by day 7, liver

Fig. 5.4. Branchial and hepatic acid-soluble thiol (AST, ▲) and glutathione (GSH, ●) in juvenile rainbow trout exposed to 55 µg/L copper for 28 days, and then transferred to clean water for 7 days. Means ± 1 SEM in µmol -SH/g wet weight, n=12.


µmol SH/g wet wt
4
3
2
1
Liver
AST
GSH
Gills
AST
GSH
0 1
7
14
21
28 1
7
Recovery
Time (days)

AST had about doubled and the non-Glut, non-cysteine component of hepatic AST had increased about 2.8 times that found under control conditions (Fig 5.4). Liver Glut and cysteine accounted for about 73% of the total AST on day 28.

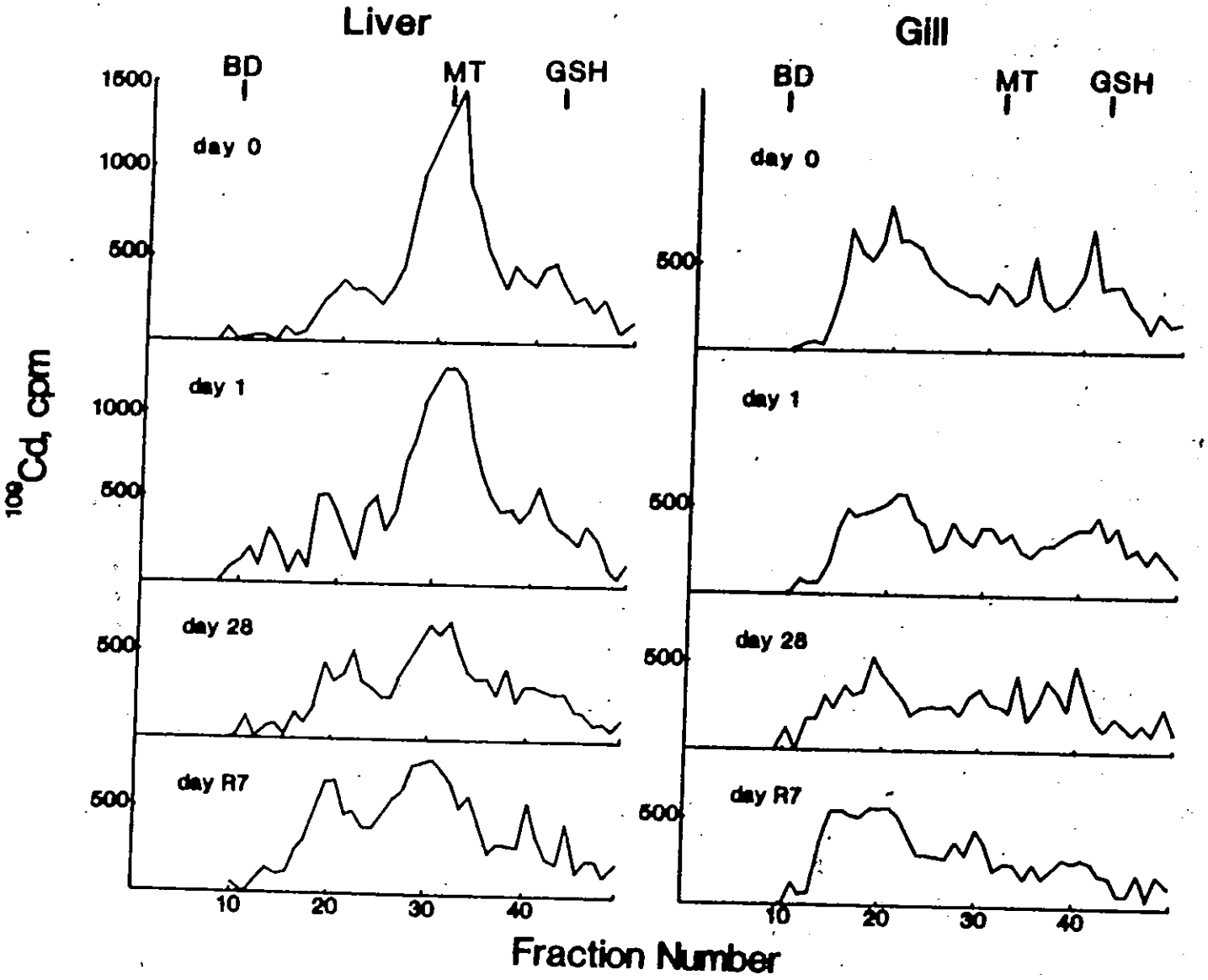
After 7 days of recovery in copper-free water, gill AST and Glut were unchanged (Fig. 5.4), but large decreases in both AST and Glut were found in the liver. In the liver, however, the non-Glut, non-cysteine component of AST did not decrease significantly.

Effects of Copper Exposure on Metal-Binding Proteins in the Gill and Liver

Standard Cd/Zn metallothionein eluted from the Sephadex G-75 column between fractions 25 and 35, with a peak at fraction 31. Therefore, arbitrary designations were asssigned to fractions 1 through 24 as high molecular weight (HMW), fractions 25 to 35, as medium molecular weight (MMW), and fractions 36 to 60 as low molecular weight (LMW) fractions. A distinct protein peak was found in trout liver, which has the same approximate molecular weight as our Cd/Zn metallothionein standard. This protein is also heat-stable, and has a high affinity for cadmium.

In control fish most of the ^{109}Cd bound to gill supernatants was found in the HMW fractions, and no peak was found in the region where standard metallothionein eluted; this continued for the duration of the copper exposure and recovery period (Fig. 5.5). On the other hand, in

Fig. 5.5. Sephadex G-75 separation of in vitro hepatic and branchial ^{109}cadmium binding in pooled, heat-denatured supernatants from juvenile rainbow trout exposed to 55 µg/L copper for 28 days and then transferred to clean water for 7 days. BD, blue dextran; MT, Cd/Zn metallothionein; GSH, glutathione.


Liver
Gill
BD
MT
GSH
day 0
day 1
day 28
day R7
1500
1000
500
10
20
30
40
^{109}Cd, cpm
Fraction Number

control liver, about 80% of the ^{109}Cd binding was associated with proteins of about 10,000 daltons, with relatively little binding to HMW and LMW proteins (Fig. 5.5). There was no significant change in ^{109}Cd binding in liver after 24 h, but ^{109}Cd binding decreased progressively from day 7 to 28, so that at day 28, ^{109}Cd binding was only about 50% that of control livers (Fig. 5.5). No change was found after 7 days of recovery in copper-free water (Fig 5.5).

DISCUSSION

Copper Toxicity

The inhibition of Na^+-K^+-ATPase is the most likely biochemical explanation for the physiological inhibition of sodium uptake (Chapter 2, 3, 4). Previous studies have shown that copper inhibits Na^+-K^+-ATPase (Lorz and McPherson, 1976; Stagg and Shuttleworth, 1982), but this is the first study to attempt to correlate the specific activity of the enzyme in vitro with the uptake of sodium and copper in vivo. The specific activity of the branchial Na^+-K^+-ATPase was inhibited by about 33% but sodium uptake was inhibited by about 55% (Chapter 4). This suggests, as might be expected, that in vitro assays do not necessarily reflect in vivo conditions and/or that copper has effects on other components of the sodium uptake mechanism such as sodium channels, mitochondria, membrane fluidity, etc.

Copper was not significantly elevated in the gills, even at the point of greatest inhibition of sodium uptake and Na^+-K^+-ATPase

inhibition. One possible explanation for this observation is that very little copper may be needed to inhibit this enzyme. Reidel and Christensen (1980) reported that copper inhibits Na^+-K^+-ATPase in vitro at about a 1:1 molar ratio. Under control conditions we measured about 130 µg microsomal protein/ mg gill tissue. For our average gill weight of about 104 mg, this is 13.5 mg of microsomal protein. At an estimated molecular weight of 250,000 daltons for Na^+-K^+-ATPase (using Coomassie Blue; Shuurmans Stekhoven and Bonting, 1981), and assuming that all of the microsomal protein was Na^+-K^+-ATPase this is only about 54 nmol of Na^+-K^+-ATPase; such a small change in tissue copper would have been below our limits of detection. Furthermore, copper can enter into oxidation-reduction cycles with sulfhydryl groups to form disulfide bonds (Rothstein, 1959), and/or can catalyse the formation of the cross-linking agent malonyldialdehyde from membrane phospholipids (Hochstein et al. 1980).

The Response of the Gills to Copper Exposure

The induction of additional Na^+-K^+-ATPase subunits is the most likely biochemical exlanation for the recovery of sodium uptake (Chapter 4). Although Na^+-K^+-ATPase specific activity was significantly inhibited within 24 h of exposure to copper, there was a significant increase in microsomal protein from 7 to 28 days of exposure, and the total transport capacity (i.e., specific activity times total microsomal protein) returned to control values by day 14. However, sodium uptake did not recover until day 21 (Chapter 4). This again suggests that

either in vitro assays do not reflect in vivo conditions perfectly, or that copper has effects on other cellular components of sodium uptake than Na^+-K^+-ATPase alone. The enzyme specific activity did not continue to decrease during continuous copper exposure, so that the turnover rate of enzyme subunits must have equaled the rate of inhibition.

Copper accumulation in the gills was low and variable (Fig 5.1c), and acid-soluble thiols (AST; Fig 5.4), glutathione (Glut), cysteine, and ^{109}cadmium binding proteins (Fig 5.5) were not induced by copper exposure. Furthermore, if copper were tightly bound by the gill, one would expect the exchangeable copper pool to decrease, rather than increase or remain stable, as was found in the present experiment. Thus, the combined results of exchangeable copper space, tissue accumulation, AST status, and gel filtration suggest that the gill does not have a high copper binding capacity, and that neither metal-binding proteins, nor glutathione, are readily induced in this tissue by exposure to 55 µg/L copper. Nöel-Lambot et al. (1978) reached the same conclusion in regard to the gills of eels (Anguilla anguilla) exposed to 13 mg/L cadmium in seawater for 14 days.

The Response of the Liver to Copper Exposure

Copper exposure led to copper accumulation by the liver, and increases in hepatic AST and glutathione. Wofford and Thomas (1984) have shown that in the striped mullet (Mugil cephalus) AST includes oxidized and reduced glutathione (Glut), cysteine, and a cadmium-inducible, sulfhydryl-rich, metal-binding protein of about the

same molecular weight as metallothionein. In the present study, it was also shown that the sum of Glut and cysteine does not equal the total AST, and that an acid-soluble, sulfhydryl-rich, cadmium-binding protein with a molecular weight of about 10,000 daltons is present in the livers of control trout. On these criteria, it would seem that this protein is also metallothionein. This protein was induced in the liver of trout after 7 days exposure to copper, but then remained at a relatively constant high level. The size of the exchangeable copper pool of the liver also decreased between day 1 and 7, after which it remained relatively constant. This might be expected if metallothionein was induced and copper was being bound more firmly.

On the other hand, ^{109}Cd binding in the liver decreased as copper exposure continued. The obvious interpretation of these results is that the concentration of metallothionein decreased during copper exposure. However, this is not a satisfactory explanation because it has also been demonstrated that metallothionein, as determined by AST minus Glut, increased by day 7 and remained at this level for at least 28 days. The alternative and more reasonable explanation is based on the relative binding strengths of copper, zinc, and cadmium for metallothionein. All metallothionein contains zinc, and both copper and cadmium are incorporated into metallothionein by displacing zinc (Cousins, 1985; Day et al., 1984; Noel-Lambot et al. 1978). However, cadmium cannot displace copper from metallothionein (Nöel-Lambot et al., 1978; Scheuhammer and Cherian, 1986). Therefore, during copper exposure when the ratio of copper to zinc in metallothionein increases, the cadmium

binding capacity of metallothionein decreases. It is apparent then that hepatic detoxification of copper may act in two distinct ways: 1) by the induction of metallothionein, and 2) by increasing the percentage of metal binding sites occupied by copper. In this respect it is important to recognize that Goering and Klassen (1984) reported that the increase in lethal resistance found when rats are pretreated with cadmium, is dependent on presynthesized metallothionein, rather than an increase in the rate of metallothionein production. Thus, measurement of metallothionein by such indirect assays as ^{109}cadmium-binding, pulse polarography, or radioimmunoassay may seriously misrepresent the true detoxifying potential of a given tissue.

Hepatic metallothionein may be induced by a variety of toxic metals, as well as a variety of non-specific stresses and the glucocorticoid hormones (Cousins, 1985). The induction of hepatic metallothionein has been considered the most satisfactory explanation of the acquired resistance of animals to metal toxicity (Dixon and Sprague, 1981b; Brown and Parsons, 1978; Webb and Cain, 1982; Cousins, 1985). However, McCarter and Roch (1983) showed that although metallothionein was induced by copper exposure in coho salmon (Oncorhynchus kisutch), the increase in resistance actually preceeded the increase in metallothionein. Furthermore, it has been shown that the gill is the primary target of copper in fish (Chapters 2, 3, and 4) and there is no evidence for copper-induced liver dysfunction in fish exposed to environmentally relevant levels of copper (Baker, 1969; Gardner and LaRoche, 1973). Thus, at least for copper, the increase in hepatic

metallothionein may be a secondary, rather than primary response. In this context it must also be pointed out that little is known about the sites and mechanisms of the toxicity of other metals to fish, and there is clear evidence that zinc, mercury and cadmium act at the gills as well as internal organs of fish (Spry and Wood, 1984, 1985; Renfro et al., 1974; Bouquegneau, 1975). Therefore, to the extent that these metals affect ionoregulatory function, the classical mammalian model for hepatic detoxification of metals may not hold true.

The Uptake and Distribution of Copper

Previous studies have only examined liver metallothionein levels (Dixon and Sprague, 1981b; McCarter and Roch, 1983; Roch and McCarter, 1984). The present study shows the importance of examining both the distribution of copper, and the metal-binding capacity of other tissues, especially when the site of toxicity is not the liver. The copper-binding capacity of the liver reached an apparent plateau by about day 7 and further copper uptake was distributed to extra-hepatic tissues. Under control conditions, the liver accounted for about 24% of the whole body copper content. This increased to about 40% on day 2, but decreased steadily thereafter to only about 17% on day 28. On the other hand, the contribution of the carcass increased from about 69% under control conditions to about 82% on day 28. Since white muscle makes up about 66% of the whole body wet weight of rainbow trout (Stevens, 1968), it is likely that this tissue accounts for most of this extra-hepatic copper. This may possibly help to account for the

decrease in critical swimming performance of copper-exposed rainbow trout reported by Waiwood and Beamish (1978). However, it must be cautioned that the carcass, as defined in the present study, includes both the gut and kidney, tissues which have been shown to play important roles in metal metabolism in mammals (Cousins, 1985).

Whole body copper uptake continued to increase and did not reach equilibrium during the 28 days of exposure. Interestingly, Dixon and Sprague (1981a) found that fish exposed to 30, 131, and 194 µg/L reached apparent equilibrium, but fish exposed to intermediate levels, 58 and 94 µg/L, did not reach equilibrium within 21 days. The fish exposed to 30 and 58 µg/L were less tolerant, while those exposed at the higher levels were more tolerant during subsequent lethality trials. Thus, the relationship between copper uptake, equilibrium, and resistance is not clear at the present time.

The loss of whole body copper at approximately the same rate that copper was accumulated suggests that despite the high affinity of copper for biological ligands (Nieboer and Richardson, 1981), and the apparent incorporation of copper into non-exchangeable or slowly exchangeable pools, the whole body copper pool is quite labile once copper exposure has ceased. It has been shown that a large portion of the apparent copper uptake during short-term experiments is due to surface binding (Chapter 3), and this may explain a portion of the loss of whole body copper following transfer to clean water, but the magnitude of the losses found in this study suggest that internalized copper was the predominant source.

CHAPTER 6

GENERAL DISCUSSION

GENERAL DISCUSSION

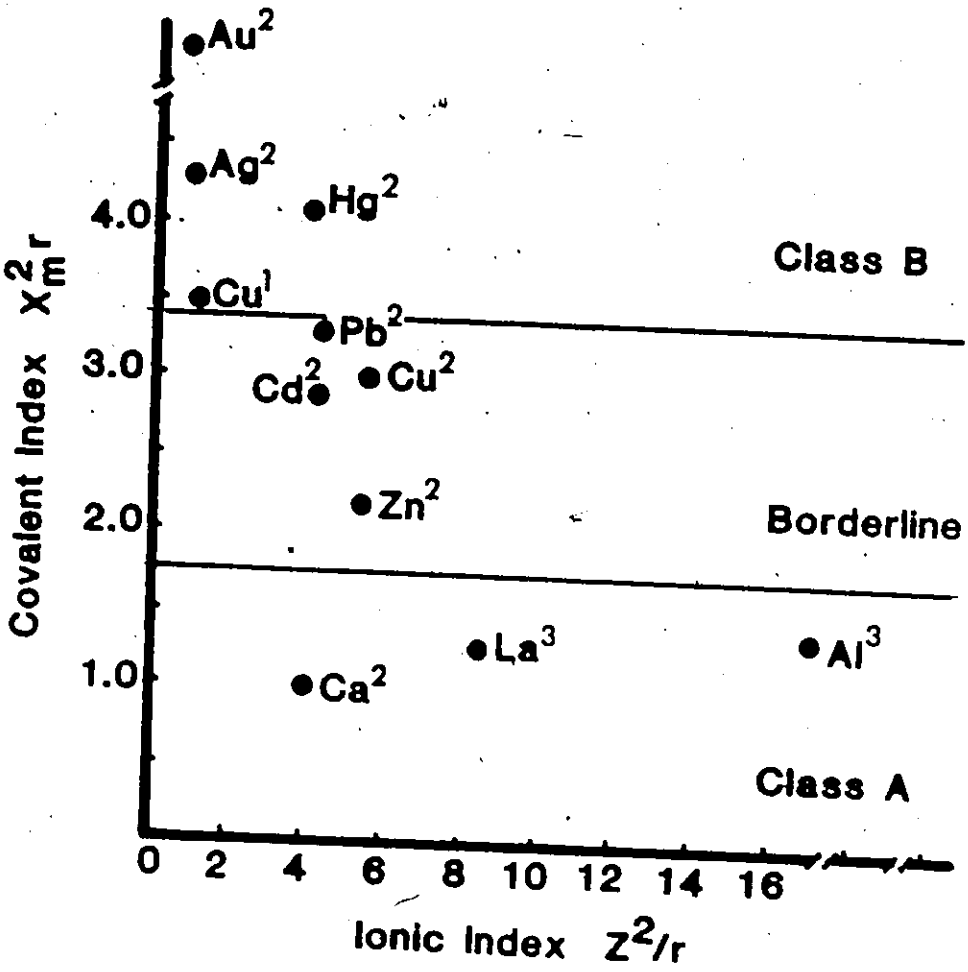
Mechanisms of Toxicity

Rothstein (1959) has suggested that the most useful toxicity studies are those in which a correlation can be made between a chemical reaction and a known physiological effect. Furthermore, he suggested that short-term experiments are preferred because the longer the chain of events between the chemical insult and the measured response, the more difficult and complicated is the reconstruction of the mechanism of toxicity. Although long-term studies reveal important aspects of metal toxicity not found in short-term assays (e.g. reproductive effects, McKim and Benoit, 1971), they have the potential to mask primary effects with secondary responses. Most previous studies of the effects of copper on fish have lasted at least 96 h (Shaw and Brown, 1974; Howarth and Sprague, 1978; Chakoumakos et al. 1979; Miller and Mackay, 1980), and although several authors have found evidence of ionoregulatory disturbance (McKim et al., 1970; Christensen et al., 1971; Schreck and Lorz, 1978) the origin of this disturbance was not assessed. By using unidirectional sodium fluxes as a sensitive indicator of ionoregulatory disturbance, and sampling at frequent intervals during short-term assays, it was shown that the most important effect of copper on fish is the disruption of gill ionoregulatory function. Furthermore, this disturbance in ionic balance could fully account for the acute lethality due to copper exposure. By making some rather basic assumptions, it was

also possible to suggest that copper has physiological effects on at least two different sites of action on the branchial epithelium (Chapter 2).

Physiological effects of toxic substances ultimately arise from chemical reactions with biochemical ligands in or on cells. Nieboer and Richardson (1980) have suggested that the toxicity of transition metals may be explained in terms of the tendency to form covalent, rather than ionic bonds, with biological ligands. This characteristic is determined by the size to charge ratio, and the electron accepting affinity of the metal ion (Z^2/r, and $\phi X_m^2 r$; Fig. 6.1). By these criteria, the important toxic metals in the aquatic environment are, in descending order of tendency to form covalent bonds: mercury, lead, tin, copper, cadmium, chromium, and zinc. Of these, only mercury is classified as a pure class "B" metal, having thermodynamic preferences for forming covalent bonds with donor atoms of biological molecules in the order, S>N>O. The remaining common toxic metals are largely classified as borderline metals, having an increasing tendency to form class "A" type (i.e., ionic) bonds in the order O>N>S. In a physiological context, it is important to note that calcium is a pure class "A" metal, and that, because of the greater elecronegativity of class "B" metals, these can all displace calcium from its normal binding sites. Copper is unique in this sequence because it is the only micronutrient which exists as a pure class "B" metal, as Cu^+, and also exists as a borderline metal as Cu^{2+}. This also means that copper is the most electronegative metal commonly found in the aquatic environment.

Fig. 6.1. Separation of metals into class "A" and class "B" according to tendency to form covalent or ionic bonds (adapted from Nieboer and Richardson, 1980). The class "B" index $X_m^2 r$ is plotted against the class "A" index Z^2/r. X_m is the metal-ion electronegativity, r is the ionic radius (in angstroms), and Z is the charge.


Au2
Ag2
Hg2
Cu1
Pb2
Cd2
Cu2
Zn2
La3
Al3
Ca2
Class B
Borderline
Class A
4.0
3.0
2.0
1.0
0
2
4
6
8
10
12
14
16
Covalent Index $X_m^2 r$
Ionic Index Z^2/r

The results of many studies support this approach to understanding the toxicity of metal ions, both to whole animals in vivo, and to enzyme preparations in vitro (Table 6.1). The concentrations at which all of these studies caused either 50% lethality of the test animals or 50% inhibition of enzyme activity, are remarkably constant (Table 6.1), and (with the exception of monovalent Ag) the sequence of toxicity follows the order of binding constants of metals for glycine and glutathione (Sillen and Martell, 1964).

It is the intention of this final thesis chapter to explain the known physiological effects of copper on fish in terms of the affinities copper has for known biochemical ligands, and to use the same kinds of arguments to draw some basic conclusions about the mechanisms of action of some other toxic metals.

Copper Effects on Sodium Influx

At low, more environmentally relevant copper concentrations, only sodium influx was affected (Chapter 2, 3, 4). Sodium influx may be reduced by a decrease in the fluidity of the apical membrane of the transporting cell, by directly blocking the sodium channels of the apical cell membrane, by inhibiting oxidative phosphorylation and reducing the energy available to drive the active transport of sodium, or by inhibiting the transport protein Na^+-K^+-ATPase, directly.

In Chapter 3, it was shown that sodium influx is not reduced until about 2 h after the beginning of copper exposure. Metal binding to cell surfaces should be complete within minutes (Wedemeyer, 1968), so if

	Ag	Hg	Cu	Pb	Cd	Al	Zn	Ca
a)								
Jones (1939)	2.8×10^{-8}	4×10^{-8}	2.4×10^{-7}	4.8×10^{-7}	1.78×10^{-6}	2.6×10^{-6}	4.6×10^{-6}	2×10^{-2}
Sam and Grushkin (1959)	$1 \times 10^{-7.2}$	1×10^{-7}	$1 \times 10^{-6.8}$	1×10^{-6}	$1 \times 10^{-6.5}$	N.A.	$1 \times 10^{-5.5}$	1×10^{-3}
Reidel and Christensen (1979)	8×10^{-8}	2.5×10^{-7}	4.8×10^{-7}	10^{-7}	10^{-6}	N.A.	10^{-5}	N.A.
b)								
Glycine, log K_1	3.73	10.3	8.19	5.39	4.47	N.A.	5.18	1.39
Glutathione, log K_1	N.A.	N.A.	N.A.	10.6	10.5	N.A.	8.3	N.A.
OH^-, log K_1	4.42	- 2.4	6.6	7.51	5.52	- 4.12	8.84	1.07
CO_3^{3-}, log K_1	N.A.	N.A.	6.3	11.89	N.A.	N.A.	10.89	N.A.
c)								
OH^-, log K_{so}	-7.65	-25.82	-19.0	-14.93	-13.65	N.A.	-13.15	- 5.26
CO_3^{2-}, log K_{so}	N.A.	-16.05	- 9.86	-13.48	-10.84	N.A.	- 9.06	- 8.55

Table 6.1. a) Metal concentrations which cause 50% mortality of fish within 96 h, or 50% inhibition of Na^+-K^+-ATPase within 45 min. Data from Shaw and Grushkin (1959) estimated from graph. b) Metal binding constants of some organic and inorganic ligands. c) Solubility constants for some metal-complexes. All data expressed in M. Binding and solubility constants from Sillen and Martell, 1964.

copper were having major effects at the apical cell membrane, these effects should have been expressed before 2 h. In Chapter 3 it was also shown that the inhibition of sodium uptake was correlated with the slow phase of whole body copper uptake/binding, and copper uptake and the inhibition of sodium uptake were nearly mirror images of each other. Taken in concert, this data suggests that the major site of copper toxicity is not at the branchial cell surface, and that copper must penetrate the gill to inhibit sodium uptake.

Within the gill, copper may have access to a large number of physiologically important enzymes, very few of which have been studied with regard to metal toxicity. The trout gill uses glucose and lactate as its primary fuel sources (Mommesen, 1984). Bilinski and Jonas (1973) exposed rainbow trout to 10^{-6} M copper (64 µg/L) for 24 h and found no significant inhibition in the ability of isolated gill filaments to metabolize lactate to CO_2. The complete metabolism of lactate involves the complete oxidation of its carbon skeleton to pyruvate and acetyl coenzyme A, followed by the oxidation of the latter in the mitochondria by enzymes of the tricarboxylic acid cycle. This suggests that mitochondrial function and oxidative phosphorylation would not have been inhibited in the present, 24 h exposures of trout to 1.97×10^{-7} to 8.66×10^{-7} M copper (12.5 to 55 µg/L). However, copper effects on the initial, cytosolic steps of glycolysis (ie., from glucose to lactate) in fish gill have not apparently been tested. Heath (1984) found no decrease in adenylate energy charge (a measure of oxidative metabolism) in bluegill (Lepomis macrochirus) liver and muscle following 24 h

exposure to 3.14×10^{-5} M copper (2000 ug/L), but, unfortunately, gills were not assayed and it might be expected that they would be more sensitive than either the liver or muscle. This is clearly an area of research which deserves further investigation.

On the other hand, several studies have shown that copper inhibits the activity of branchial Na^+-K^+-ATPase (Lorz and McPherson, 1976; Stagg and Shuttleworth, 1982). In the present study, a similiar effect was found (Chapter 5), but the degree of inhibition found in the in vitro assay accounted for only about 60% of the in vivo inhibition of sodium uptake (Chapter 4). This difference could merely be the result of the different physiological conditions of the enzyme in vivo and in vitro, or could be the result of copper effects on other, unidentified biochemical components.

Metal ions, in general, are very non-specific toxicants. This results largely from the ubiquitous presence of sulfhydryl groups on enzymes and structural proteins, and the great affinity of the class "B" metals and borderline metals such as copper, for such sulfur centers (Fig. 6.1). Sulfhydryl groups are of great importance to enzyme function because they play a major role in determining the conformation of the catalytic sites. The two catalytic alpha subunits of Na^+-K^+-ATPase have 18 sulfhydryl groups per molecule (Shuurmans Stekhoven and Bonting, 1981), and copper inhibits this enzyme with a molar ratio of about 1 to 1 in vitro (Reidel and Christensen, 1979). Because Na^+-K^+-ATPase is a membrane bound enzyme, which depends partially upon the fluidity of its phospholipid domain for activity,

inhibition can occur directly by the formation of copper mercaptides, S-Cu-S, between adjacent alpha subunits, or indirectly by catalysing the formation of disulfide bonds,

$$\begin{matrix} 2GSH \\ GSSG \end{matrix} \rightleftharpoons \begin{matrix} \text{enzyme S-S} \\ \text{enzyme -SH} \end{matrix} \rightleftharpoons \begin{matrix} Cu^{+} \\ Cu^{2+} \end{matrix} \rightleftharpoons \begin{matrix} O_2 \\ O_2^{-} \end{matrix},$$

and/or the peroxidation of membrane phospholipids,

$$2O_2^{-} + 2H^{+} \longrightarrow H_2O_2 + O_2,$$

to form malonyldialdehyde,

$$H_2O_2 + \text{phospholipid} \longrightarrow \text{peroxidized phospholipid} \longrightarrow \text{malonyldialdehyde},$$

which also cross-links proteins.

Other metals and H^{+} also inhibit Na^{+}-K^{+}-ATPase, as well as other enzymes in vitro, and the sequence of concentrations necessary to inhibit this enzyme are well correlated with the strength of the class "B" nature of the particular metal ion (Reidel and Christensen, 1979; Shaw and Grushkin, 1957; Pfeiler and Kirschner, 1972; Fig. 6.1). Saunders et al. (1983) also showed that in vivo exposure of Atlantic salmon (Salmo salar) to pH 4.2 to 4.7 led to the in vitro inhibition of Na^{+}-K^{+}-ATPase activity. However, Lorz and McPherson (1976) found that in vivo exposure of coho salmon to 3.06×10^{-5} M zinc had no effect on in vitro Na^{+}-K^{+}-ATPase activity or survival in seawater, as might be expected from its low class "B" value (Fig. 6.1).

Fewer metals have been shown to inhibit sodium uptake in vivo, and the construction of an inhibition sequence is hampered because only partial data sets are available. Renfro et al. (1973) reported that sodium uptake in sodium-depleted Fundulus heteroclitus was completely

inhibited following 24 h exposure to 4.6×10^{-6} M mercuric chloride (903 µg/L), but no lower concentrations were tested. For rainbow trout, it has been shown that exposure to 6.56×10^{-6} M cadmium (737 µg/L; S.D. Reid, McMaster Univ., personnel communication) inhibits sodium uptake by about the same amount as 7.87×10^{-7} M copper (50 µg/L), and 10^{-5} M H^+ (pH 5.0) inhibits sodium uptake less than 1.97×10^{-7} M copper (12.5 µg/L; Chapter 2). In the only other comparable study, Spry and Wood (1985) found that 1.22×10^{-5} M zinc (800 µg/L) actually stimulated sodium uptake. Thus, the sequence of metal effects on sodium uptake in rainbow trout is: copper> cadmium> H^+> zinc, with mercury probably falling between copper and cadmium.

The sequence of threshold concentrations for the effects of metal ions on sodium uptake in vivo and Na^+-K^+-ATPase in vitro, is nearly the same as the sequence of the strengths of the binding constants for these metals to glutathione, and (to a lesser extent) glycine (Table 6.1).

Copper Effects on Sodium Efflux

Due to the large sodium gradient between the fish and the freshwater environment, the stimulation of efflux may lead to a much more rapid decrease in the exchangeable sodium pool than the complete inhibition of sodium uptake. However, sodium efflux was only stimulated at about 1.57×10^{-6} M copper (100 µg/L), a level of copper in excess of that normally found in all but the most polluted environments. This high threshold for the effects of copper on efflux may be explained in terms of the chemical ligands which control permeability in the gill.

There are 2 possible pathways for ion loss across the branchial epithelium, a transcellular route, requiring the crossing of both the basolateral and apical cell membranes, and the paracellular route, requiring the crossing of the junctions which connect adjacent cells together at the apical membrane surface. Because of the complex morphology of the fish gill, there is little direct evidence for the pathway of ion loss across the gill, but electron microscopic examination of the gills of freshwater fish shows that epithelial cells are connected by tight junctions. The opercular membrane of the freshwater trout is a tight epithelium in which the pathways of ion loss can be easily assessed, and Marshall (1985) has shown that sodium losses across this membrane follow the same pathway as mannitol. Since mannitol does not cross cell membranes, this is good evidence that sodium losses normally occur across the paracellular tight junctions between the cells of the branchial epithelium. When this model tight epithelium is exposed to H^+, these losses increase in a similar manner to that found in the gills of trout exposed to low pH or copper in vivo (McDonald, 1983b; Chapter 2, 3).

Oschman (1978) has shown that tight junctions are held together by ionic bonds between calcium ions and the anionic residues of the adjacent cell membranes. Since calcium is a pure class "A" ion, it may be displaced by any class "B", borderline, or class "A" metal with a higher covalent or ionic index ($X_m^2 r$ or Z^2/r; Fig. 6.1). It has been suggested that it is the labile nature of calcium binding which allows calcium to play such an important role in biological systems, and that

the occupation of binding sites alone (e.g. by another metal) does not convey biological activity (Horrocks, 1981). Copper, having both higher class "B" and class "A" values than calcium (Fig. 6.1) also has the potential to occupy calcium binding sites and to form much less labile bonds than calcium.

Both cadmium and H^+ also stimulate sodium efflux. In softwater, sodium efflux was stimulated to about the same extent by 6.56×10^{-6} M cadmium (737 µg/L; S.D. Reid, personnel communication) as was caused by 1.57×10^{-6} M copper (100 µg/; Chapter 3). Although pH 5.0 inhibits sodium uptake, significant stimulation of efflux was not found at this pH (Chapter 2, 3). This suggests, as does the copper data, that efflux is less sensitive than influx, despite the fact that efflux appears to be stimulated by action at the cell surface. This may be because H^+ and copper have lower binding constants for oxygen centers than for nitrogen or sulfur centers, or simply because of the quantity of calcium that must be displaced before efflux is stimulated, or because the binding sites of the tight junctions are not readily accessible. Although zinc stimulates sodium efflux, it stimulates sodium influx to the same degree, so that no net losses are found (Spry and Wood, 1985). Thus, the sequence for effects of metals on sodium efflux is the same as that found for sodium influx.

Effects of Water pH, Hardness, and Alkalinity

pH

pH had no significant effect on the way in which copper affected either sodium influx or sodium efflux. If copper speciation were an important factor in toxicity, then it would have been expected that the effects of pH 5.0 and copper would have been more than additive, since there is a major shift to Cu^{2+} at about pH 5.0; instead, the combined effects were only additive or less than additive. Some toxicity data suggests that not only are the effects of pH 5.0 and copper not additive, but that H^+ has a protective effect, perhaps through the stimulation of mucus secretion (Miller and Mackay, 1980), or by competition between H^+ and copper for binding sites on the gill (Cuismano et al., 1986). In Chapter 3 evidence for the latter possibility was presented, but it was not possible to separate uptake from adsorption and no amelioration of toxicity was found in these short-term experiments. Long-term metal exposures combined with measurements of mucus secretion and metal uptake in gills and other tissues could possibly resolve this question.

No comparable data are available for other metals, but Reid (unpublished) found a reduction in net sodium losses and mortality in juvenile trout exposed to 6.56×10^{-6} M cadmium for 24 h at 10^{-5} M H^+ (pH 5.0) compared to circumneutral pH. Campbell and Stokes (1985) have recently reported that pH does not cause major shifts in the speciation of cadmium, cobalt, nickel, silver, or zinc, so that any reduction of

metal effects at low pH must be due to competition by H^+, or the stimulation of mucus secretion, and any increase in toxicity must be due to the effects of H^+, itself. However, lowering pH has major effects on aluminum and mercury, and to a lesser extent, on lead (and copper) speciation (Campbell and Stokes, 1985), so that the effects of pH on the toxicity of these metals will be more difficult to assess.

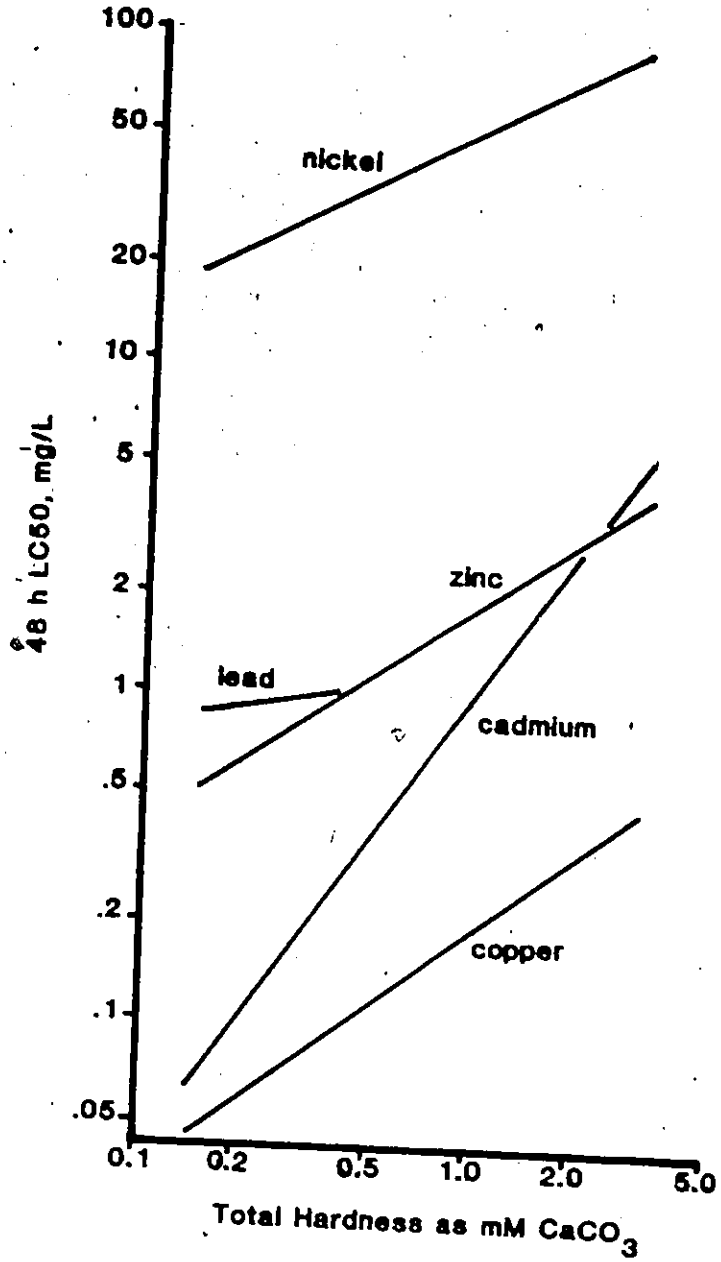
Hardness

Water calcium, within the range of 25 to 1000 μM, did not have significant effects of the way copper inhibited sodium influx or stimulated efflux within 24 h. Likewise, McDonald et al. (1983) found no effect of water hardness (30 to 2850 μM $Ca(NO_3)_2$) on trout exposed to pH 4.0 for 4 h. Both of these studies encompassed a greater range of water calcium concentrations than that found in acidified lakes in Ontario (NRCC, 1981). One possible reason for the failure to find an effect of water calcium on toxicity in these instances is that H^+ and copper simply overwhelm any protective effect of calcium on the gills. Since copper and H^+ have very high electronegativity values of 1.9 and 2.1 respectively, and calcium has a much lower value of 1.0, both copper and H^+ have the potential to displace calcium from its normal binding sites. As discussed above, calcium is important in maintaining membrane stability and has significant effects on gill permeability (McDonald and Rogano, 1986), but these effects are relatively small compared to the effects of either H^+ or copper. For instance, the mean net sodium loss produced by acute transfer of adult trout from 1000 μM calcium to 100 μM

calcium for 24 h (McDonald and Rogano, 1986) was only about 30% that found when fish were exposed to 1.97×10^{-7} M copper (12.5 µg/L), or about 50% that found in fish exposed to 10^{-5} M H^+ (pH 5.0, Chapter 2). An alternative explanation is that longer term experiments are required to reveal differences between hard and softwater fish. This interpretation is supported by the fact that many 96 h lethality tests have shown apparent hardness effects on copper toxicity (Howarth and Sprague, 1978; Chakoumakos et al., 1979; Miller and Mackay, 1980), and McDonald et al. (1983) found a significantly greater effect of pH 4.0 in softwater compared to hardwater, after about 40 h of continuous exposure.

Water hardness apparently has significant effects on short-term cadmium toxicity. Sodium influx was reduced by exposure to 6.56×10^{-6} M cadmium to a greater extent in softwater than in hardwater at neutral pH, and although sodium efflux was stimulated in softwater, no stimulation of efflux was found in hardwater (Reid, unpublished). Brown (1968) also showed a more pronounced effect of calcium on the 48 h LC50 values of cadmium than of either copper, zinc, lead or nickel (Fig. 6.2). This may possibly be explained by the fact that cadmium has an electronegativity value of only 1.7, so that calcium is more likely to effectively compete with cadmium for binding sites than with either copper or H^+. If this is the reason for the effect of calcium on cadmium toxicity, then significant ameliorative effects of hardness would also be expected for zinc and aluminum toxicity as well, since these elements have electronegativity values of 1.6 and 1.5, respectively. Competitive binding studies between ^{45}calcium and

Fig. 6.2 Correlation between hardness and the 48 h LC50 for rainbow trout exposed to copper, cadmium, zinc, and nickel (from Brown, 1968).


100
50
20
10
5
2
1
.5
.2
.1
.05
48 h LC50, mg/L
nickel
zinc
lead
cadmium
copper
0.1
0.2
0.5
1.0
2.0
5.0
Total Hardness as mM $CaCO_3$

cadmium, copper or H^+, or between ^{109}cadmium or ^{64}copper and calcium may help to explain some of these intriguing differences.

Alkalinity

Raising carbonate alkalinity reduced the effects of copper on both sodium influx and efflux. High alkalinity had a greater effect on efflux than on influx because copper carbonates reduce the amount of soluble copper available to the gills, and the threshold for the stimulation of efflux is higher than that for the inhibition of influx.

Lead is the only other metal commonly found in the polluted environment which forms significant carbonate complexes (Stumm and Morgan, 1970; Campbell and Stokes, 1985), and whose toxicity should, therefore, be limited by water alkalinity.

Physiological and Biochemical Mechanisms of Acclimation

In the natural environment, there are several possible consequences of copper exposure to fish. In the very broadest of terms, these include 1) detection and avoidance, 2) acclimation, and/or 3) death. The mechanisms of toxicity and lethality have been reviewed in preceeding sections. In this section, it is my purpose to briefly review some of the possible mechanisms fish might employ to avoid lethality due to ionoregulatory toxicants in general, and to suggest possible experiments which might lead to a better understanding of these mechanisms. Since the exterior surface of fish is the first site of

action of toxicants, this discussion will proceed from the outside to the inside compartments.

In certain instances it may be possible for a fish to avoid the onset of toxicity if it is able to detect the toxicant before stressful conditions are reached. In this context, it is important to note that Atlantic salmon (Salmo salar) avoided 6.9×10^{-8} M copper (4.4 µg/L; Sprague, 1964), a level of copper which is lower than that reported for many un-polluted freshwater lakes and streams (Spry et al., 1981). However, the secretion of catecholamines and cortisol are classical indicators of stress in animals (Selye, 1950) and Donaldson and Dye (1975) have shown that in sockeye salmon (Oncorhynchus nerka) plasma cortisol is elevated within 2 to 4 h of exposure to only 10^{-7} M copper (6.4 µg/L). Thus, copper may not only be the most toxic metal commonly found in the aquatic environment, but, given the problems of measuring such low concentrations of copper, it may also be stressful at levels which are not avoided by fish.

In the event that fish cannot avoid stressful conditions, the action of either cortisol or the catecholamines produces a suite of biochemical and physiological responses the purpose of which seems to be the defense of homeostasis. These responses include increased mucus secretion, efflux reduction, influx recovery, and increased metal detoxification.

The gill and body surface of fish is covered with a thin layer of mucus. In mammals, catecholamines have been shown to control the secretion of mucus, and mucus secretion in fish has been shown to be

stimulated by exposure to Hg, Zn, Cd, Pb, and H^+ (Lock and Overbeeke, 1981; Miller and Mackay,. 1982; Eddy and Fraser, 1982). Since the sloughing off of metal laden mucus has been suggested to ameliorate metal and acid toxicity (Part and Lock, 1983; Miller and Mackay, 1982), this may mean that the secretion of catecholamines plays an important role in the amelioration of metal toxicity. Furthermore, Zuchelkowski et al. (1985) showed that the composition of mucus in acid stressed fish shifted from predominately sialomicins to predominately sulfomucins. The increased presence of sulfur centers could be expected to increase the binding of class "B" metal ions, and possibly provide added protection.

In the present study, the most important adaptive responses found were the reduction of sodium efflux (Chapter 2, 3) and the recovery of sodium influx (Chapter 4). Of these, the reduction of efflux is probably the most important, since sodium efflux rates can be much greater than influx rates under stressful conditions. Furthermore, the reduction of efflux takes place within hours of exposure (Chapter 2), while the recovery of influx takes days to weeks (Chapter 4). Although cortisol is generally thought to modulate the uptake of sodium in fish, there is some evidence that it may also be important to the reduction of sodium efflux. When brook trout (Salvelinus fontinalis) were exposed to low pH, plasma sodium decreased more rapidly in fish in which cortisol secretion was inhibited (with metyrapone, an 11β-hydroxylase inhibitor) than in the acid-exposed control group (Ashcom, 1979). Many more studies suggest that sodium efflux may be controlled by the secretion of

the peptide hormone, prolactin, but this line of research has been hampered, until lately, by the lack of an antibody specific for salmonid prolactin. Prolactin secretion in fish can be inhibited by administration of levadopa and stimulated by L-tryptophan, so that plasma prolactin may be experimentally manipulated (James and Wigham, 1984). The role of prolactin in the reduction of efflux under stressful conditions may now be tested by combining plasma measurements of prolactin (Prunet et al., 1985) with measurements of sodium efflux rates. Clearly, understanding these mechanisms has implications beyond problems in aquatic toxicology, such as the effects of exercise, handling stress, smoltification, etc., and is an important area for future research.

A second line of defense is probably the induction of biochemical pathways which attempt to compensate directly for the toxic action. Branchial transport ATPases are inhibited in vivo by exposure to H^+, copper, mercury, and aluminum (McKeown et al., 1985; Shephard and Simkiss, 1978; Stagg and Shuttleworth, 1982; Bouquegneau, 1977; Staurnes et al., 1983; Chapter 5), but compensation for this inhibition has only been reported for H^+ and copper (Parker et al. 1985; Shepard and Simkiss, 1978; Stagg and Shuttleworth, 1982; Chapter 5). ATPase activity is easy to measure and should be included in the analysis of the mechanism of action of any suspected ionoregulatory toxicant.

Numerous authors have also reported the proliferation of chloride cells during exposure to metals and low pH (Calamari et al., 1980; Crespo et al. 1981; Leino and McCormick, 1984; Chevalier et al., 1985;

Mallatt, 1985; Karlsson-Norrgren et al., 1985; Karlsson-Norrgren et al., 1986a, b). Since the chloride cell is the only cell in the branchial epithelium which has the ultrastructural characteristics of ion transporting tissues (Berridge and Oschman, 1972; Laurent et al., 1985), it is probable that the increases in ATPase activity cited above coincide with the proliferation of chloride cells. Perry and Wood (1984) found that calcium uptake and chloride cell proliferation were correlated with both cortisol injection and softwater acclimation in rainbow trout, and the present study showed the recovery of sodium uptake with an increase in the size of the ATPase transport pool. Unfortunately, no study of which I am aware, has combined physiological, biochemical, and anatomical analyses. Such studies will be necessary before a complete understanding of acclimation to ionoregulatory stressful agents is possible.

Exposure to environmental stressors may also result in physiological or biochemical changes which are not directly related to the toxic insult, but which are, nevertheless, important to adaptation. Several authors have shown that prior exposure to sub-lethal metal concentrations leads to enhanced lethal resistance times (Dixon and Sprague, 1981b; McCarter et al. 1982; Duncan and Klaverkamp, 1983; Pascoe and Beattie, 1979; Bradley et al., 1985), suggesting that some mechanism of adaptation has been 'primed' by the pre-exposure. These authors have shown a correlation between hepatic metal-binding proteins and resistance to copper and other metals, but since metallothioneins are also induced by catecholamines and cortisol in mammals (Cousins,

1985), this may only be a secondary stress response. Lethal resistance and branchial metal-binding ligands have also been shown to be correlated in zinc (Bradley et al., 1985) and cadmium (Benson and Birge, 1985) exposed fish, but, except for the studies of Bouquegneau et al. (1975) and Bouquegneau (1977) with eels exposed to high levels of mercury, there is no evidence that metallothioneins protect gill function from toxic metals (Chapter 5; Nöel-Lambot et al., 1978). The uncertainity about the role of metallothioneins results, in part, from the fact that the mechanism of toxicity to fish at normal environmental levels of zinc and cadmium is not known, so that it is not yet possible to measure the effects of metal exposure on the appropriate physiological or biochemical function. This is the first question which must be addressed in studies of acclimation to metals by fish. It is also important to note that although zinc and cadmium have the potential to induce branchial metallothionein, no study has demonstrated induction at the levels of these metals which are found in the polluted environment. Furthermore, fish can adapt to elevated levels of H^+ (McWilliams, 1980; Lacroix et al., 1985; C. Audet, personal communication), an ionoregulatory toxicant which is not bound by metallothionein, so that there is no a priori reason to expect that acclimation to copper should be dependent on the induction of metallothionein. Thus, the recovery of electrolyte homeostasis shown in this and other studies would appear to be a more reasonable explanation for acclimation to ionoregulatory toxicants in general. This could be further tested by comparing the 96 h LC50 of fish which have never been

exposed to copper with fish which have been injected with copper. Since gill metallothionein is not induced in injected fish (Sabourin et al., 1982), any difference in toxicity would be due to hepatic or renal metallothionein.

Since metallothionein has a high affinity for copper, it would not be surprizing if metallothionein plays an important secondary role during chronic low level exposure by restricting metal accumulation largely to the liver. The liver is the site of ceruloplasmin synthesis as well as copper excretion through biliary clearance (Owen, 1965). However, since copper can displace cadmium and zinc from metallothionein, exposure to mixtures of these metals may increase toxicity above that expected from simple additive effects, by forcing the spill-over of cadmium and zinc into the high molecular weight enzyme pool. Indeed, Roch et al. (1985) reported that although wild trout were exposed to zinc, copper, and cadmium in the ratio of about 400:20:1, only the ratio of copper to zinc increased in the metallothionein peak from the livers of these fish. However, the liver was the only tissue analysed so that it is not known if zinc was redistributed within the fish or excreted. Copper, zinc, and cadmium also appeared in the high molecular weight pool (Roch et al., 1982), suggesting that, like the gill (Chapter 5), the compensation for the effects of these metals on liver function may also be afforded by the increased turnover of metal-inhibited enzymes. Clearly, these considerations are very important in the environment and should be considered in the interpretation of the results of mixed-metal studies.

Costs of Acclimation

Precht (1958) defined a gradient of physiological responses to stressors beginning with no compensation and proceeding to complete compensation, and over-compensation. During exposure to copper, acclimation of sodium influx was completely compensated by day 21, but over-compensation was found during recovery (Chapter 4). However, the costs of acclimation to a toxicant are different from those to such stresses as temperature or salinity for which this system of analysis was originally designed. Specifically, while changes in temperature and salinity are compensated for largely by changes in metabolic products such as the phospholipid composition of membranes, or by the induction of alternative isozymes of important enzymes (Hochachka and Somero, 1973), these changes probably do not add to the long-term cost of survival. On the other hand, the types of changes required by toxicants are likely to be long-term commitments of energy in addition to the costs of survival. In the present study, this would include the costs of increased turnover of both Na^+-K^+-ATPase as well as metallothionein molecules (Chapter 5), and these costs must come from the budget available for growth and reproduction. Indeed, McKim and Benoit (1971) showed that even though plasma chloride and osmolarity returned to normal in trout exposed to about 5.2×10^{-7} M copper (33 µg/L) for 337 days, the growth rate and fecundity of these fish was reduced. Furthermore, the second generation of these trout were no more tolerant of copper than their parents. Thus, apparent acclimation to copper does not ensure the long-term survival of the species. This suggests that

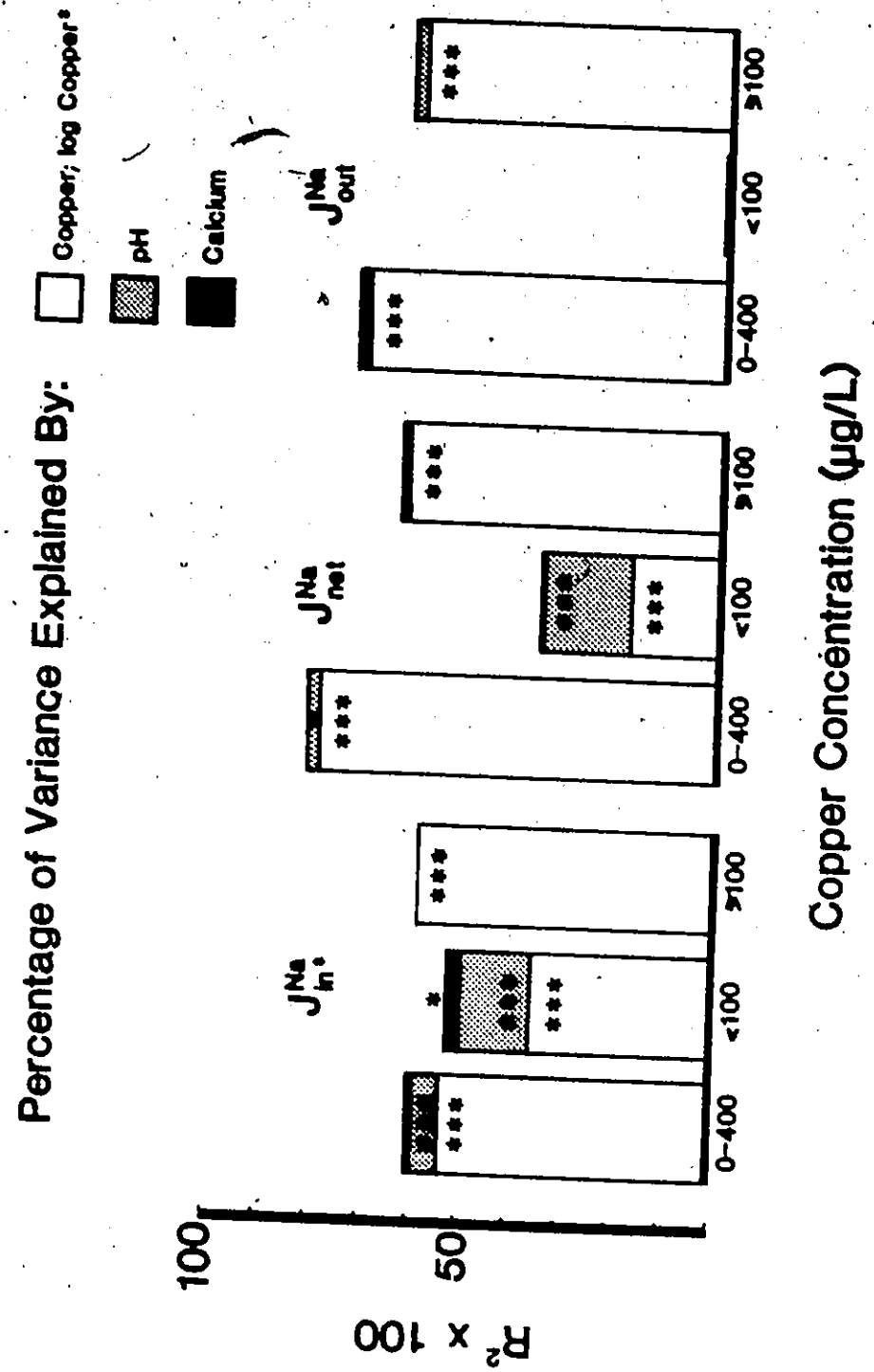
copper may also act as an internal toxicant in chronic low-level exposures such as are found in the natural environment. Since the whole body copper burden of fish probably increases with age, this suggests that complete acclimation to toxic metals may not exist in the wild, but that species longevity may only be assured by natural selection for resistant genotypes.

Summary and Conclusions

Copper is an important environmental toxicant and has been the subject of many lethality studies, but the present study is the first to examine the physiological mechanisms of copper toxicity. These studies are easy to set-up, run, and analyse, and yield more information than the traditional 96 h lethality test. By applying standard physiological proceedures for the measurement of ion fluxes, it has been shown that copper, at normal levels for the polluted environment, inhibits sodium uptake, probably by inhibiting branchial Na^+-K^+-ATPase. At levels of copper found only in the most polluted environments, sodium efflux is also stimulated. Lethality is probably caused by the loss of exchangeable sodium. Carbonate alkalinity significantly reduces copper toxicity, but hardness does not affect toxicity in the short-term. Exposure to pH 5.0 increases the toxicity of copper at low concentrations but has no additive effect at high copper concentrations. Trout are able to compensate the inhibition of sodium uptake by reducing sodium efflux and by producing more Na^+-K^+-ATPase transport proteins. The success of this approach to understanding the mechanisms of action

of copper in rainbow trout should be generally applicable both to other aquatic organisms, and to other pollutants.

Appendix A. Percentage of variance of sodium fluxes in juvenile rainbow trout as analysed by multiple linear regression, using water copper concentration, pH, and calcium as the independent variables. Because of the biphasic nature of the effects of pH and copper (see Chapters 2 and 3), the data have been analysed at both low (0-100 µg/L) and high (100-400 µg/L) copper concentrations, as well as the entire range (0-400 µg/L). Data presented for J_{in} are based on log transformations of copper concentrations since this gave a better coefficient of determination (R^2) than copper. The significant effect of calcium on J_{in} at copper concentrations below 100 µg/L was not found when control values were omitted. Percentage variance calculated as R^2 x 100. Asterisks indicate significance, where P> 0.05 = *, and P> 0.005 = ***.


Percentage of Variance Explained By:
Copper; log Copper²
pH
Calcium
J^{Na}_{in}
J^{Na}_{net}
J^{Na}_{out}
R^2 x 100
100
50
0-400
<100
≥100
Copper Concentration (µg/L)

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