ZINC UPTAKE IN THE RAINBOW TROUT, *SALMO GAIRDNERI* (RICHARDSON), AS AFFECTED BY DIETARY AND WATERBORNE ZINC, AND WATERBORNE CALCIUM

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ABSTRACT

Rainbow trout took up Zn from both the diet and the water. Trout on a low Zn diet became Zn-deficient, based upon mortality, lack of growth, and depressed plasma protein and hematocrit. Both plasma and whole body [Zn] were also depressed. Elevated [Zn], in either diet or water, reversed all symptoms of Zn deficiency. At high [Zn], Zn was taken up in excess of requirements, but in an attenuated fashion. These results support a relative homeostasis for Zn for both plasma and whole body, but also indicated that Zn uptake from water and diet were partially independent. There was no overt toxicity. Metal-binding proteins, important in mineral metabolism, were induced by this exposure in gill and intestine but not in liver.

The gill was identified as the major site of Zn uptake from the water by the use of an *in vitro* preparation which directly measured flux rates. Pre-exposure of trout to soft water for 5 days gave considerably higher influx rates than occurred in tapwater-acclimated trout, probably by stimulating proliferation of gill chloride cells. Zn influx in soft water, studied from 0.4 to 7.5 mg Zn/L followed saturable, first-order kinetics,

suggesting a restricting pore or carrier mediation. Influx was studied in vivo for 24 h using a novel technique which overcame the limitations of non-specific adsorption found with traditional methods. Influx rates measured with this technique confirmed the saturable nature of influx, but were much higher. Waterborne Ca interacted with Zn influx in two separate ways. Acute increases in [Ca] up to 5 fold showed a competitive interaction, suggesting that Zn and Ca compete for the same mechanism of branchial entry. Acute removal of Ca however revealed in addition, a large influx with increasing [Zn], indicative of opening of a paracellular leak pathway across the gill.

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TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

LIST OF ABBREVIATIONS

ABS	butyl styrene drain pipe
ANOVA	analysis of variance
AV	arterio-venous
AST	acid-soluble thiols
ASW	artificial soft water
ATPase	adenosine triphosphatase
cpm	counts per minute radioactivity
C_{Pr}	plasma protein
C_{ss}	concentration at steady state
DA	dorsal aorta
DTNB	dithionitrobenzoic acid, a sulphydryl reagent
FVS	filamental venous sinus
Hb	hemoglobin
hct	hematocrit
HDS	heat-denatured supernatant
HW	hard water
ID	inside diameter
IPHP	isolated, perfused head preparation
iu	international units
J_{in}	influx rate
J_{max}	maximum flux rate
K	condition factor
k	rate constant
K_i	inhibitor constant

K_m	Michaelis constant
M_r	relative molecular mass (replaces mw)
MT	metallothionein, usually containing Zn and Cd
MS-222	tricaine methane sulphonate, fish anesthetic
n	number of determinations or samples
$P_{a_{O_2}}$	partial pressure of oxygen in arterial blood
pH	negative log of the hydrogen ion concentration
P_{in}	input perfusion pressure
P_{O_2}	partial pressure of oxygen
PVC	polyvinyl chloride
$\dot{Q}$	flow rate
Q	total mass of element
Q*	total radioactivity (cpm)
R_g	branchial resistance to flow
SD	standard deviation
SDS-PAGE	sodium dodecyl sulphate polyacrylamide gel electrophoresis
SE	standard error of the mean
TEP	transepithelial electrical potential
V_d	volume of distribution of a substance in the body
V_e	elution volume, in gel filtration
V_0	void volume, in gel filtration
w	as a subscript, denotes "waterborne"
W	body weight of fish

[ ]	denotes concentration
units	SI units are the prefered units. However, in most of the toxicological and nutritional literature metric units of mass are most common. To maintain clarity, these latter units are used for waterborne concentrations. Both units are given where necessary.

CHAPTER 1

GENERAL INTRODUCTION

This thesis is concerned with two aspects of Zn in the physiology of rainbow trout. The first is the relative importance of waterborne versus dietary Zn as a source of Zn. Second, is the actual sites and rates of Zn influx across the gill. This introductory chapter draws together diverse areas which are necessary to the understanding of the physiology of Zn in trout. Some of these set boundary conditions such as toxicity at high waterborne Zn exposures, and overt deficiency at low dietary levels. Other areas provide conceptual models which are directed at an understanding of the mechanisms by which Zn uptake might occur.

SOURCES OF WATERBORNE ZINC

Zinc concentrations found in fresh water vary widely, from levels which are very low (< 1 ug/L) up to levels which are acutely toxic to fish (several mg/L and higher) and other aquatic organisms (Nriagu, 1980; Spear, 1981). Moreover, the distribution of these levels in the field tends to be highly skewed, with most values very low, but a few very high.

These high levels may arise from weathering and runoff from natural ore bodies (see Taylor et al., 1982), but much is due to anthropogenic enrichment from smelter leachate, industrial spills, wet and dry atmospheric deposition and industrial sewage (Weatherly et al., 1980). The progressive acidification of many freshwater lakes is also associated with increases in waterborne zinc concentration ([Zn]), and an additional source here may direct dissolution of sediment-bound Zn as water pH declines (Baker, 1982).

CHEMISTRY

Zinc is a metallic element with an atomic weight of 65.38. It is the lightest member of group IIB of the periodic table, which also includes Cd and Hg. As such, it has filled 3d electron shell (and is therefore not a transition element), only one oxidation state (2+), and does not participate in electron transfer reactions. Metallic and metalloid elements have been divided into three classes based upon their affinity for either oxygen (class A) or nitrogen/sulphur centers (class B), and those of intermediate ("borderline") character (Nieboer and Richardson, 1980). The thermodynamic properties upon which this classification is based determine the co-ordination (chelation) chemistry which is of prime importance in their biological reactivities. It is interesting that all the macro-nutrient metals (e.g. Na, K, Ca, Mg) are in class A, whereas the micro-nutrient metals

(i.e. Cu, Zn, Fe) are classed as borderline. Zn is thus borderline, but has considerable class A character. It is probably this ability to combine with a wide variety of ligands which accounts for Zn's diverse role in over 200 structural and functional proteins (Hambidge et al., 1986).

The aquatic chemistry of Zn, although more straightforward than that of elements such as Al, Hg, Pb and Cu, is still complex, and not completely understood. It depends to a great degree upon other ions present in the water column with which Zn may complex. The free ion (or its aquo ion $Zn(H_2O)_6^{2+}$) appears to be the most biologically available and toxic (see Spear, 1981). Other cations such as Ca^{2+}, Mg^{2+}, and even H^+ may compete with Zn directly for biological ligands, whereas inorganic anions (such as CO_3^{2-}, OH^-) or organic anions (such as humates and fulvates) may complex Zn and decrease its activity. In soft, low alkalinity waters typical of the Canadian Shield, Zn remains largely free at low or circumneutral pH (see Campbell and Stokes, 1985). As hardness, alkalinity and pH increase, Zn speciation changes and Zn becomes complexed to carbonates, hydroxides and chlorides. Nevertheless, under commonly encountered conditions of hardness, pH, alkalinity, and low concentrations of organic anions (and similar to those reported in this thesis), the free ion predominates.

TOXICITY

Zn toxicity to fish is widely documented (Skidmore, 1964; Spear, 1981) and water quality guidelines have been established for acceptable levels (NAS/NAE, 1972; EIFAC, 1973; IJC, 1976; USEPA, 1977; McKim et al., 1979). Upon acute exposure, zinc is less toxic to fish than many other trace metals. For stickleback (Gasterosteus aculeatus L.), Jones (1939) gave the following series: Ag> Hg > Cu> Pb> Cd > Au > Al > Zn > H > Ni > Cr> Co > Mn> K> Ba > Mg > Sr > Ca > Na. For salmonids, the acutely toxic range is from 0.2-8.0 mg/L as a log function of total hardness (mainly waterborne Ca) over the range of 5-500 mg/L hardness (for reference, Lake Ontario is 140 mg/L, while Canadian Shield lakes are 5-10% of this level). The toxic mechanism under acute exposure is well-known, and consists primarily of decreased oxygen transfer across the gill (Sellers et al., 1975; Spry and Wood, 1984). This arises from an inflammatory edema (Skidmore and Tovell, 1972) which dramatically increases the diffusion distance for oxygen. Chronic, or delayed mortality is rare. A variety of sublethal effects on growth, reproduction, behaviour and enzyme activities have been documented (see Spear, 1981). The significance of these changes is not often clear. One such change however, the induction of metallothioneins (or metal-binding proteins), may be the mechanism underlying the acclimation to otherwise toxic level of waterborne Zn (Bradley et al., 1985). Changes in

branchial ion fluxes, especially inhibition of Ca have been noted (Appendix 1).

ZINC METABOLISM IN MAMMALS

There has been something of a dichotomy in the study of Zn between mammals and fish. In natural fish populations, there is ample evidence of toxicity, but nutritional deficiency, although it has occurred in hatcheries, has never been identified in wild fish. In contrast, toxicity is rare in mammals (and man), resulting mainly from industrial exposure or accidental poisoning. Deficiency is much more common, and ranges from slight to lethal.

The body of mammalian literature on Zn metabolism is extensive and has been thoroughly reviewed (Prasad, 1977;, 1979; Bettger and O'Dell, 1981; Hambidge et al., 1986; Cousins, 1985, 1986). It will be briefly and selectively summarized here to provide a practical framework for the studies on fish.

Although there are well over 200 structural and functional Zn-requiring proteins (Hambidge et al., 1986), and the pathology associated with Zn-deficiency is well-described, the critical role which Zn plays is still unknown. It may lie in membrane stabilization (Bettger and O'Dell, 1981), or as a cytosolic protective/trigger agent (Williams, 1985). There is thus a nutritional requirement for Zn, and the concensus is that Zn uptake is homeostatically controlled.

Some tissues however are insensitive to either Zn excess, or depletion and total [Zn] remains unchanged (e.g. brain, lung, muscle or heart), whereas in other tissues, [Zn] falls in deficiency, or rises under conditions of excess (e.g. blood, hair, bone, testes and liver, see Hambidge et al., 1986).

Not surprisingly, diet is the sole source of Zn input in mammals. Once in the digestive tract, there is little absorption from either the stomach, or the colon. Most Zn is absorbed from the small intestine, although the relative importance of individual segments may vary with animal species.

Zinc is transported throughout the body via the blood. Mammalian total blood Zn is 0.12-0.21 umol/g, of which 75-88% is in the red blood cell. Normal plasma levels range from 0.010-0.015 umol/mL. Of this, approximately 18% is tightly bound to alpha$_2$- macroglobulin, 80% is loosely bound to albumin, and the remaining 2% is bound to very low molecular weight species, such as citrate and the amino acids histidine and cysteine. Free [Zn] is <1% of total plasma Zn. Liver is the primary organ responsible for the metabolism of Zn. About 40% of the Zn entering the liver via the hepatic portal plasma is removed, processed and resecreted into the hepatic venous blood. From there it is distributed to various tissues where it performs a wide variety of metabolic functions.

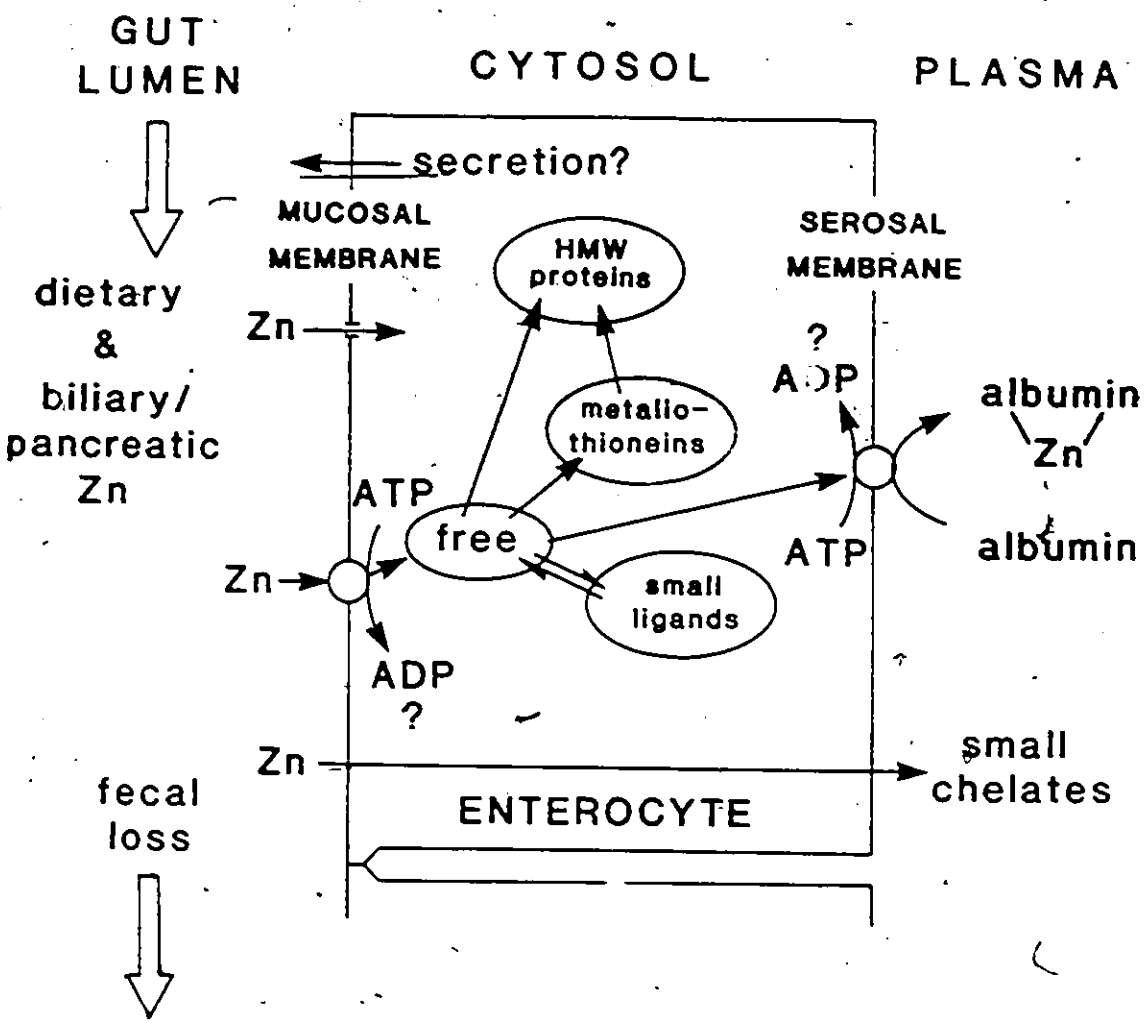
Excretion of Zn is primarily by the fecal route, with a small amount lost in the urine and sweat. Zinc can enter

the gut lumen through the diet, pancreatic and biliary secretions, and a small amount of back secretion from the enterocytes themselves (see Cousins, 1985). Net excretion is thus the difference between what enters the lumen and what is absorbed. There is thus considerable recirculation of Zn within the body, and at any one time, Zn in the intestinal mucosa may have arisen from either uptake from the gut, or secretion from the blood.

CELLULAR METABOLISM IN MAMMALS

Considerable effort has been expended to characterize the mechanism of entry of Zn into cells. Very recently, model systems used to study this aspect of Zn metabolism have employed enterocytes from the intestinal mucosa (the primary site of absorption into the body), or hepatocytes (the cell type most prominent in Zn metabolism). The mechanism by which Zn crosses enterocytes from mucosa to serosa and enters the blood has been intensively investigated but is still subject to speculation. Cousins (1985, 1986) provides the basis for the model given in Fig 1.1. Some of the Zn in the gut lumen is free, while most exists as chelates. At low-to-physiological luminal [Zn], uptake is saturable, whereas at high luminal [Zn], there is a significant non-saturable uptake component. This may be an artifact reflecting a leakage pathway opened by membrane damage. Chelates may pass through the cell intact (based on studies with EDTA). Entry of free Zn across the membrane may be

Figure 1.1: A model for the uptake of zinc by mammalian intestinal mucosa (based on a figure and data reviewed by Cousins, 1985, 1986). See text for details.


GUT LUMEN
CYTOSOL
PLASMA
secretion?
MUCOSAL MEMBRANE
SEROSAL MEMBRANE
dietary & biliary/ pancreatic Zn
Zn
HMW proteins
metallo-thioneins
free
small ligands
ATP
ADP ?
? ADP
ATP
albumin
Zn
albumin
Zn
small chelates
fecal loss
ENTEROCYTE

energy requiring, although studies using brush border vesicles suggest otherwise. Once inside the cell, a small pool of free Zn interacts with small ligands, metallothionein (which probably acts as a Zn buffer) and the high molecular weight enzyme pool. Transport at the serosal membrane is the putative rate limiting step in Zn absorption; energy appears to be required here based on studies with vesicles, using metabolic inhibitors. Oddly though, albumin is required in the portal plasma (in perfused intestinal segment experiments) in order for Zn to pass into the plasma. In this model paracellular pathways are not specifically discussed, a seeming short-coming in view of the leaky nature of intestinal epithelium compared with the tight nature of the gill. Some degree of regulation appears to exist in the system. Brush border vesicles from Zn-deficient rats exhibit increased rates of uptake and also an increase in the concentration of a 45000 M_r protein, the putative carrier. Influx is inversely related to the concentration of metallothionein, and hormones such as glucagon and glucocorticoids stimulate influx. This has lead to the concensus that absorption is homeostatically controlled.

Metallothionein is presently thought to play a central role in the cellular metabolism of Zn (and also Cu). This protein, of which there may be several isoforms, is strongly induced by Zn, and also Cu and Cd. While it has no enzymic function, it presumably plays a role in metal

homeostasis perhaps acting as a metal buffer. It also binds and detoxifies excesses of essential Zn and Cu as well as other non-essential trace metals (Brady, 1982; Webb and Cain, 1982; Cherian and Goyer, 1978).

ZINC METABOLISM IN FISH

Less is known about the metabolism of Zn by fish than by mammals. Existing data reveal some similarities, and some differences. Most work has been aimed at preventing dietary deficiency (e.g. establishing dietary [Zn] requirements and studying interactions with dietary components), rather than mechanisms of Zn metabolism per se. It is thus not clear to what extent whole body [Zn] may be regulated.

A dietary requirement has been established for fish. Diets low in Zn, or containing dietary substances which reduced the bioavailability of Zn have resulted in poor growth, high mortality, erosion of fins and cataracts of the eye (Ogino and Yang, 1978, Wekell et al., 1983; Ketola, 1979; Hardy and Shearer, 1985; Richardson et al., 1985; Jeng and Sun, 1981). High levels of dietary Zn, in contrast to waterborne Zn, do not appear to be toxic (Wekell et al., 1983).

Blood Zn studies are scarce, but a few measurements exist for both sea water and fresh water teleosts. Plasma levels were considerably higher than the 0.01 umol/mL normally seen in either birds or mammals (see Hambidge et al., 1986). During seawater to freshwater migration, plasma

levels in sockeye salmon Oncorhynchus nerka, fell from a high of 0.43 mmol/L to ~0.06 mmol/L (Fletcher et al., 1975). In winter flounder (Pseudopleuronectes americanus) plasma [Zn] was 0.29 mmol/L (Fletcher and Fletcher, 1978) and nearly all of this was bound to protein. A later study identified albumin (76,000 M_r) as the protein which bound 95% of total plasma Zn in flounder, with 5% bound by ceruloplasmin in males, and ceruloplasmin and vitellogenin in females (Fletcher and Fletcher, 1980). There was no high affinity binding similar to that of alpha$_2$ - macroglobulin in mammals. Bettger et al. (1987) also found plasma [Zn] ranging from 0.14 to 0.23 mmol/L in four species of freshwater fish. These data clearly show that plasma [Zn] is very much higher than in mammals, and suggest that plasma Zn may be handled differently in fish, both in terms of its physical transport and its physiological role.

In contrast to mammals, diet is not the only potential source of Zn. The gills of fish are thin membranes, specialized for the diffusive exchange of gases, and are constantly bathed by water. Considerable transbranchial fluxes of water and ions have been measured. These include macronutrients such as Na and Cl (Maetz, 1971; Girard and Payan, 1980; Payan et al., 1984), K (Eddy, 1985) and Ca (Berg, 1968; Fleming, 1968; Mugiya and Ichii, 1981; Payan et al., 1981; Hōbe et al., 1984; Perry and Wood, 1985), all of which are class A elements. In freshwater, Na and Cl

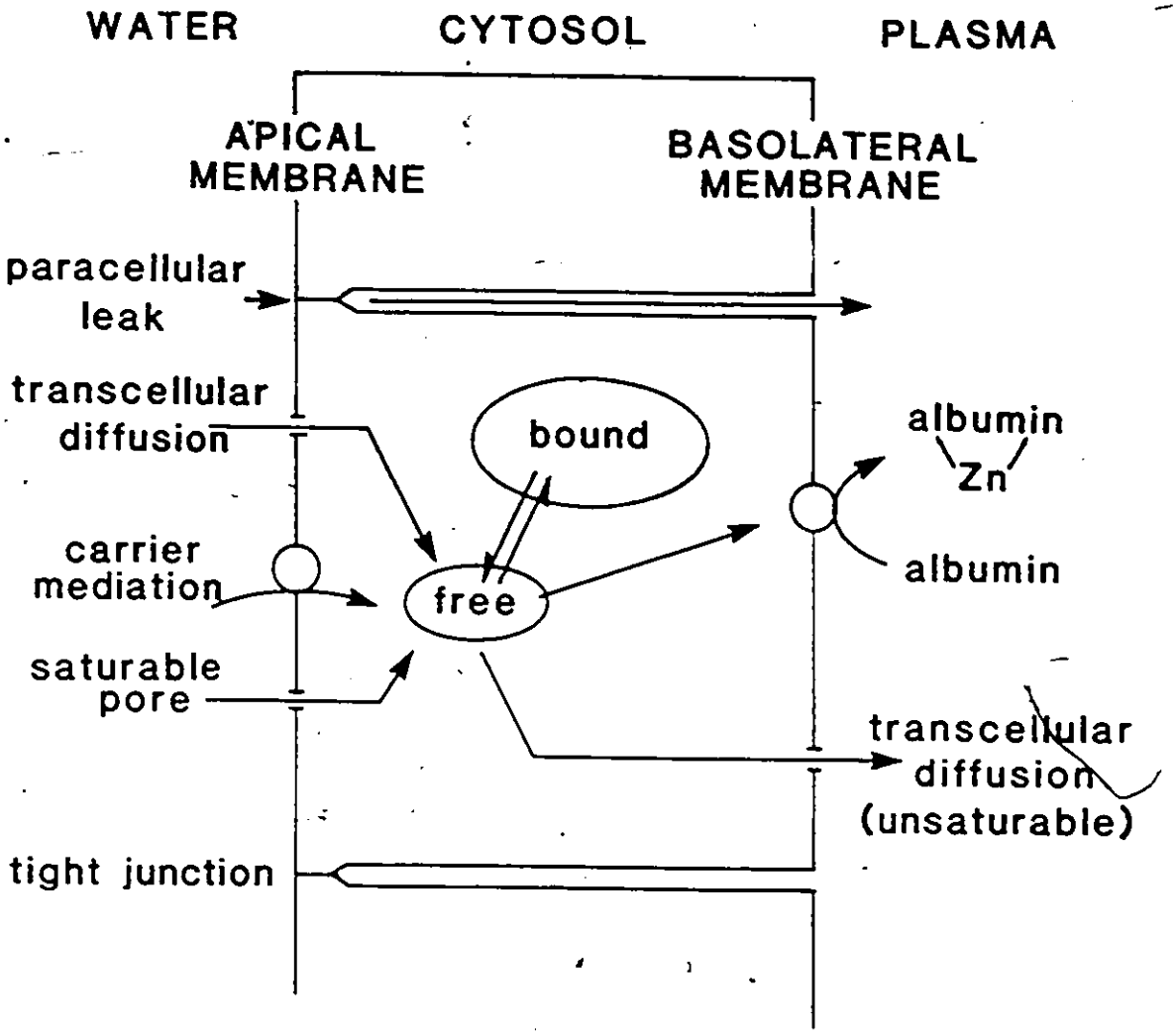
are actively taken up by coupled, electroneutral exchanges for H^+/NH_4^+ and HCO_3^- respectively. These exchanges probably occur through the "chloride" cells. Current theory holds that the energy required is generated by a Na/K-ATPase pump on the basolateral border. Calcium also appears to be taken up by active mechanisms, though it is unknown whether this transport is electroneutral or electrogenic, or whether Ca^{2+} is exchanged for another cation or cotransported with an anion. Flik et al. (1985) suggested that Ca is actively pumped against a concentration gradient via a high affinity Ca-ATPase in the branchial chloride cell. In accord with enzymatic mediation, this transport was inducible both by exposure to low environmental Ca and cortisol injection, and showed saturable kinetics (Perry and Wood, 1985).

At least some "borderline" elements, also appear to cross the gill. Data for this contention have for the most part been indirect, based upon body burdens with varied exposure e.g. for Zn (Holcombe et al., 1979; Farmer et al., 1979; Mattheissen and Brafield, 1977), Pb (Hodson et al., 1979) and Cu (Laurén and McDonald, 1986). Direct branchial transfer has only been measured for Cd, using an isolated, perfused trout head preparation (Pärt and Svanberg, 1981; Pärt et al., 1985; Block and Pärt, 1985). Pärt (1983) proposed that Cd as the free, or aquo ion passed through the gill cell itself (transcellular) whereas Cd chelated with EDTA crossed the gill through the intercellular tight

junctions (paracellular entry). There are thus several mechanisms by which metal ions might traverse the gill. These are detailed in Fig 1.2. Since the intracellular [Zn] is probably very small, Zn might enter the cell passively down its electrochemical gradient via a carrier or through an aqueous pore. Passage across the basolateral border might be passive or active, carrier-mediated or not, depending on the direction of the electrochemical gradient. Mammalian models have documented a requirement for albumin in the plasma as a carrier ligand. This might be true in trout as well. Involvement of a carrier or a pore at either the apical or the basolateral surface would probably result in saturable influx, whereas paracellular leak pathways would probably show a linear uptake with increased waterborne [Zn]. At toxic levels of waterborne Zn, Zn entry would either increase or decrease, depending on the whether leak pathways were opened, or diffusion distances increased due to inflammation and edema. Since Zn and other borderline elements have variable degrees of class A character, and since they are divalent cations, they might cross the gill by using either the putative Ca mechanism or something similar to it.

Relative contributions of dietary versus waterborne Zn to Zn uptake have rarely been assessed. In marine fish, when waterborne [Zn] was low (background), diet provided most of the Zn (Pentreath, 1973, 1976; Willis and Sunda, 1984) but fell to about 50% at elevated waterborne [Zn] (Milner, 1982).

Figure 1.2: A model showing possible sites of Zn entry across the gill. See text for details.


WATER
CYTOSOL
PLASMA
APICAL MEMBRANE
BASOLATERAL MEMBRANE
paracellular leak
transcellular diffusion
carrier mediation
saturable pore
tight junction
bound
free
albumin
Zn
albumin
transcellular diffusion (unsaturable)

In freshwater fish, studies have again been limited but suggested that diet is the more important source (Stary et al., 1982; Merlini et al., 1976), although uptake from water occurs (Holcombe et al., 1979; Farmer et al., 1979; Lovegrove and Eddy, 1982). None of these studies have varied both dietary and waterborne [Zn] in a single experiment.

A potentially-important, major difference between marine and freshwater fish is the drinking rate. This varies from nil in freshwater to as much as 13% of body weight per day in seawater (Smith, 1930; Shehadeh and Gordon, 1969). Much of the waterborne contribution in marine fish may be due to drinking, although extraintestinal (e.g. branchial) uptake has been suggested (Pentreath, 1973).

Excretion of Zn by fish is quite slow. Half times for retention were greater than 250 d (Willis and Jones, 1977; Pentreath, 1973). Detailed excretion studies in fish are lacking. There have been no balance studies, nor mechanisms proposed. Given this slow rate of excretion, rates of uptake and their homeostatic regulation may be the most important factor in determining long term whole body concentrations. The responsiveness of excretion to increased loading is however, unknown. Branchial excretion of metals has been suggested (Oronsaye and Brafield, 1984; Shephard, 1981), but the evidence is not compelling.

CELLULAR METABOLISM IN FISH

Actual studies of cellular metabolism similar to those done with mammalian enterocytes and hepatocytes are rare. Saltman and Boroughs (1960) found that liver slices from the puffer fish (Tetraodon hispidus) could accumulate Zn against a seven-fold concentration gradient, apparently without an energy requirement. A comprehensive scheme similar to Fig 1.1 for mammals has not however, been proposed for fish.

Metallothionein (MT), an important factor in cellular metabolism of Zn and Cu, has been widely studied in fish however. This interest has resulted from its apparent ability to detoxify such pollutant metals as Cd, and excess Cu and Zn. Dixon and Sprague (1981) and Bradley et al. (1985) have commented on its possible role in acclimation to elevated levels of Zn and Cu by rainbow trout. Although fish might be exposed either via the diet or the water, studies to date have concentrated on the waterborne component.

Variation in waterborne calcium is an important factor which may alter Zn metabolism. The ameliorating effect of waterborne Ca on toxicity is well-known (see Spear, 1981). While Ca may exert this effect on the external surface of the gill (Pagenkopf, 1983), it is not clear if Ca alters entry of Zn into or across the gill.

RESEARCH OBJECTIVES

Overall, the work addresses whole body Zn homestasis,

and the role which elevated dietary or waterborne Zn might play. This research was also undertaken to shed light on the routes and rates of Zn uptake, and to place these in perspective with other ions which traverse the gill. An understanding of the mechanisms would further our knowledge about the ionoregulatory function of the gill, particularly with respect to its selectivity - that is, whether the gill is ion-specific, or more general in its ion transfer functions.

I. Relative importance of dietary vs waterborne Zn in freshwater

Dietary deficiency has previously been induced in trout (Ogino and Yang, 1978; Richardson et al., 1985), but combined exposures to dietary and waterborne Zn in freshwater have not been reported. Therefore, three hypotheses were tested: i) exposure to low dietary and waterborne [Zn] would cause a deficiency in growing trout, whereas exposure to high [Zn] would result in toxicity (e.g. slow growth or altered metabolism of other minerals such as Ca)., ii) at higher waterborne [Zn], accumulation from the water would prevent the deficiency, and iii) given the tendency toward homeostasis in mammals, that in Zn-adequate trout, uptake from one source would influence uptake from the other resulting in more or less stable values for whole body and plasma [Zn].

II. Involvement of metal-binding proteins in tissues subject to different Zn loading

As an extension of the third hypothesis above, and given the importance of MT and possibly other metal-binding proteins in mineral metabolism, the major hypothesis tested here was that metal-binding proteins would be induced in trout exposed to elevated levels of Zn, and that individual tissues would reflect the sites of exposure. Specifically, gill levels would correlate with waterborne [Zn], intestine levels would correlate with dietary [Zn], while the liver would indicate the sum of the exposure.

In order to test these hypotheses it was first necessary to have an assay for MT, of which there are several. Direct, specific assays in the form of radioimmunoassay and other molecular probes have been developed for mammals, but they are not widely available, require considerable expertise, and cross-reactivity to fish proteins is not known. Indirect assays based on the high cysteine sulphur content of the molecule or its metal-binding properties have been widely employed. Pulse polarography (Olafson and Sim, 1979) has been used for fish tissues (Roch et al., 1982), but instrumentation is scarce and difficult to use. Another thiol measurement is acid-soluble thiols (AST) which utilizes the acid solubility of MT (Wofford and Thomas, 1984). Metal-binding assays make use of the inherent ability of the protein to bind metals with varying affinities; a radiolabel

is used to displace bound metals (Onosaka et al., 1978; Eaton and Toal, 1982). This assay has not previously reported for fish tissue.

It was therefore necessary to critically evaluate the Cd-binding assay (Onosaka et al., 1978; Eaton and Toal, 1982) for use in trout tissue. Recent reports indicate that the specificity of metal-binding assays may be complicated by the presence in fish of non-MT low molecular weight proteins which also bind metals (Thomas et al., 1983a, b; Pierson, 1985a, b; Kay et al., 1986; Price-Haughey et al., 1986). Nevertheless, as such proteins may also function in metal metabolism or detoxification, the inclusion of these in the measurement may be of value when a general index of metal exposure is required. For this reason, the results of the Cd-binding assay were compared with acid-soluble thiols in the various treatments and tissues.

III. Rates and Mechanisms of Transbranchial Zn Influx

Of the divalent cations, Ca appears to be actively transported, but little information exists for others such as Zn. Passive diffusion has been suggested (Pentreath, 1973; Rankin et al., 1982), with carrier-mediation being a possibility (Bryan, 1979).

The specific hypotheses tested in this section were i) that Zn would enter trout from the water in measureable quantities by traversing the gill, ii) that it would do so as

a linear function of the waterborne [Zn], and iii) that waterborne Ca would interfere with Zn transport. It was suspected that there might be some interaction between Zn and Ca, first because of calcium's well-known effect on branchial permeability (Eddy, 1975; McWilliams, 1983), and second because we have shown that waterborne Zn blocks net Ca uptake and interferes with the plasma Ca regulation in rainbow trout (Spry and Wood, 1984; an extension of this work was performed as part of the initial work on this thesis project, was subsequently published, and is presented here as Appendix 1). Third, increased waterborne Ca or Mg decreased Cd transfer across the perfused trout head (Pärt et al., 1985).

i) influx in vitro - the isolated, perfused trout head preparation

The isolated, perfused head preparation (IPHP; Payan and Matty, 1975) has found great utility in the measurement of flux rates of many substances across fish gills (see Girard and Payan, 1980; Payan et al., 1984). Although it has been used extensively for flux measurements of bulk ions, it is particularly suited to cases where the ions of interest adsorb strongly to the apparatus and the fish. This has occurred for Ca (Höbe et al., 1984; Perry and Wood, 1985), Cu (Laurén and McDonald, 1986), Zn (see Chapter 5) and is probably true for other trace metals as well. Under these conditions, classical flux studies using two compartment analysis would overestimate

rates of flux (see ii below). The IPHP overcomes this objection by measuring only metal which actually entered the fish. This section of the thesis was undertaken to measure directly, rates of Zn flux across the gill as a function of waterborne [Zn]. In accord with the hypothesis of linear influx with increasing waterborne [Zn], a positive result would indicate simple, passive influx. A saturating rate on the other hand would imply a more complex phenomenon which might be active, or at least mediated by a specific carrier or channel. Secondly, the effects of removal of waterborne [Ca], both acutely and as a pre-treatment were studied.

ii) influx in vivo

To investigate the rate of Zn influx in vivo, two existing techniques were evaluated; disappearance of ^{65}Zn from the water, and appearance of ^{65}Zn in the fish as detected by whole body counting. Neither proved satisfactory, so a new kinetic method was devised. The aims in these experiments were first, to develop an accurate method to measure Zn influx in rainbow trout in vivo, and to compare this with the in vitro estimates above. Secondly, the effect of changes in waterborne [Zn] on these rates was studied. Thirdly, the effect of acute changes, both increases and decreases, in waterborne [Ca], on Zn influx was studied. A significant Ca effect might signal competition for sites of entry, or be the underlying cause for the ameliorating effect

of Ca on the acute toxicity of many metals in general (Pagenkopf, 1983), and Zn in particular (Spear, 1981).

CHAPTER 2

RELATIVE CONTRIBUTIONS OF DIETARY AND WATERBORNE ZINC IN THE RAINBOW TROUT

INTRODUCTION

A dietary requirement for zinc has been documented in rainbow trout based upon low growth, high mortality, cataracts and fin erosion induced in trout fed a diet containing 1-4 ug/g (Ogino and Yang, 1978; Wekell et al., 1983). Supplementing the diet to 15-30 ug/g alleviated these symptoms. Diets containing high levels of calcium (and phosphate), and/or phytate (myoinositol hexaphosphate) increased the requirement for dietary zinc in freshwater salmonids, since they reduced zinc bioavailability (Ketola, 1979; Hardy and Shearer, 1985; Richardson et al., 1985). Poor growth, cataracts and high mortality were reported, together with low plasma and whole body [Zn]. In contrast, high levels of dietary zinc appear fairly benign. Thus Wekell et al. (1983) found no growth inhibition in fingerling trout over a dietary range of 440-1700 ug/g. Liver and whole blood [Zn] increased significantly over this range but in an attenuated fashion, whereas gill [Zn] increased almost linearly. No mortality or toxic symptoms occurred.

While dietary concentrations up to 1700 ug/g are well-tolerated, waterborne concentrations of 1 ug/mL (i.e. 1000 ug/L) are well into the toxic range for rainbow trout (Spear, 1981). Detrimental effects on ionoregulation, acid-base balance and gas exchange have been documented (Skidmore, 1970; Lewis and Lewis, 1971; Sellers et al., 1975; Spry and Wood, 1984, Appendix 1). The maximum acceptable toxicant concentration based upon fry mortality for rainbow trout in hard water was 320-640 ug/L (Sipley et al., 1974). Even below this range, elevated waterborne [Zn] depressed growth in several species due to appetite suppression and/or decreased conversion (Bengtsson, 1974; Watson and McKeown, 1976; Farmer et al., 1979).

Zinc can be accumulated directly from the water. Hodson (1975) found uptake of ^{65}Zn in gill tissues of trout increased with time over a 10 h period, and increased with increasing temperature. It was not clear how much was due to adsorption, and how much was absorbed. Holcombe et al. (1979) exposed brook trout (Salvelinus fontinalis) for 24 weeks to a waterborne [Zn] of 534 ug/L and found 3-fold increases above controls in gill, kidney and opercular bone [Zn], whereas brain and muscle were unaffected. Manipulation of the ration (amount fed per day) was used by Farmer et al. (1979) to study its effect upon Zn uptake from water. Freshwater-adapted Atlantic salmon (Salmo salar) fed a practical diet to satiation (dietary [Zn] unspecified),

reached steady-state whole body concentrations after 50 d. These steady-state whole body [Zn] reflected waterborne [Zn] in a concentration-dependent fashion. Restriction of the ration to 2% or 3.5% body weight per day resulted in continual accumulation over the 80 d exposure. The rate of accumulation was directly proportional to the waterborne [Zn].

Relative contributions of dietary versus waterborne zinc to zinc uptake have been assessed in marine fish. In plaice (Pleuronectes platessa) food was the major source for larvae and adults when waterborne [Zn] was low (15 ug/L), based upon ^{65}Zn accumulation (Pentreath, 1973, 1976). However, in the same species, water contributed 10% at 100 ug/L but increased to 50% at 600 ug/L (Milner, 1982) indicating that waterborne input can be significant. Willis and Sunda (1984) used a model food chain (Chlamydomonas → Artemia → fish) and a single waterborne [Zn] (0.21 ug/L free Zn) to estimate relative contributions in mosquito fish (Gambusia affinis) and spot (Leiostomus xanthurus). Food supplied 77% of the isotope load in mosquito fish after 120 d, and 82% in spot after 30 d. Marine fish drink considerable amounts of the medium (~4-13% body weight/d, Smith 1930; Shehadeh and Gordon, 1969), and this could contribute half or more of the waterborne input. The remaining input from the water was suggested to be passive, being driven by the large gradient formed by the adsorption of zinc on the gill (Pentreath, 1973).

There have been no directly comparable studies in freshwater, where drinking rates are known to be negligible (Shehadeh and Gordon, 1969; Oduleye, 1975). Stary et al. (1982) exposed guppies (Lebistes reticulata) to ^{65}Zn in either the water or in the diet. They concluded that diet was the more important factor. Pumpkinseed sunfish, (Lepomis gibbosus), exposed to ^{65}Zn in either the diet or the water, accumulated ^{65}Zn faster from the diet, whether the diet was natural (snail) or purified (Merlini et al., 1976). In contrast, waterborne Zn was the main source for goldfish (Carassius auratus), (no detail, Berg and Brasselli, cited by Merlini et al., 1976).

It thus appears that in both fresh- and seawater fish there is a capacity to accumulate zinc through both routes, but the dietary source is usually the more important. However, the interactions between the two pathways appear highly complex, and dependent upon the zinc concentrations in the two sources. It remains unclear whether the waterborne route can replace the dietary route.

MATERIALS AND METHODS

Experimental Design

Rainbow trout (Salmo gairdneri) fingerlings (3-4 g) were purchased from Spring Valley Trout Farm, Petersburg, Ontario. They were moved directly to the Canada Center for Inland Waters where the study was performed. There they were held in flowing Burlington tapwater dechlorinated by

carbon filtration and sulphite addition. The photoperiod was 18L:6D, and ambient [Zn] was 7 ± 8 ug/L (mean ± SD). Fish were held for 2 weeks in a large holding tank and gradually changed from a commercial diet (200 ug Zn/g, Martin Feed Mills, Elmira, Ontario) to the semi-purified diet described below, and the temperature was raised from 11°C to 15°C. After this initial acclimation, fish were transferred to the experimental battery which consisted of a grid of 30 tanks. Each tank was approximately a 30 cm cube holding 20-23 L of water. Water flowed continuously to the tanks from mixing chambers at 300 mL/min/tank, giving a 95% particle replacement time of 3-4 h (Sprague, 1969). Tanks were gently aerated to ensure >90% air saturation.

The experimental design was a complete block factorial consisting of 3 waterborne [Zn] levels (ambient plus 2 treatments) x 3 dietary [Zn] levels x 3 replicates (blocks) for a total of 27 tanks. To determine the effect of an even greater waterborne [Zn] on the experimental variables, particularly whether or not toxicity would occur, the remaining 3 tanks were used for 3 replicates of a further increase in the waterborne [Zn] to 529 ug/L at the highest dietary [Zn] (590 ug/g). The experiment ran for 16 weeks. The waterborne Zn (as $ZnSO_4.7H_2O$) was metered by a peristaltic pump (Gilson Minipulse) into the mixing cells of the diluter from 3 stock bottles. Daily measurements of waterborne [Zn] gave means ± SD of 7 ± 8 for ambient, and

39 ± 14, 148 ± 35 and 529 ± 85 ug/L for the three elevated levels. Temperature was 15.3 ± 1.3°C. Weekly measurements of pH, dissolved oxygen and conductivity were 8.16 ± 0.23, 8.8 ± 1.0 mg/L and 281 ± 10 uS. Previously reported hardness and alkalinity values (as $CaCO_3$) were 135 ± 2 and 90 ± 4 mg/L; Hodson et al., 1978).

On the basis of past experiments under the same conditions, some aggression and subsequent mortality was expected (Hodson et al., 1978). Small lengths of 2.5 cm. ID PVC pipe were added to provide shelter, and the tank lids were covered with black plastic on week 2 to minimize this effect, but aggression persisted. Typically, fish showing severe signs of aggression did not feed. Opercles and fins became eroded until the fish was unable to propel itself or remain upright, and fungal infestation was common. Fish which rolled over were considered moribund and were recorded and sacrificed. The same criteria were used for removal of diseased fish (see Results).

Diet

The semi-purified diet formulated by Dr. J.W. Hilton (University of Guelph, Table 2.1), was calculated to provide all essential nutrients. Dietary [Zn] was nominally 0, 100 (practical level) and 700 ug/g. This was confirmed by dry ashing at 400°C and atomic absorption spectophotometry, and independently by neutron activation analysis, yielding measured [Zn]=1, 90, 590 ug/g. The proximate composition was

Table 2.1: Formulation of basal diet fed to rainbow trout.

Ingredient	Concentration %
Egg albumin	40
Gelatin	10
alpha-starch	10
Cerelose	10
Cellulose	5
Vitamin premix	2
Mineral premix	8
Fish oil	15

The mineral premix provided the following (g/kg dry diet):

$CaHPO_4.2H_20$, 30; $CaCO_3$, 3; NaCl 15; K_2SO_4, 20; $MgSO_4$,10; $FeSO_4.7H_2O$, 0.7; $MnSO_4.H_20$, 0.3; $CuSO_4.5H_20$, 0.16; KI 0.015.

The vitamin premix provided the following (mg/kg dry diet, unless noted otherwise): thiamine, 10; riboflavin, 10; pantothenic acid, 10; niacin, 20; pyridoxine, 40; biotin, 0.5; folic acid, 20; vitamin B12, 0.2; inositol, 500; ascorbic acid, 1000; choline chloride, 5500; vitamin A, 7000 iu, vitamin D, 3000 iu; vitamin E, 200 iu, vitamin K, 50; butylated hydroxytoluene (BHT), 25.

protein 40%, lipid 15%, ash 8%, moisture 9%. All diets were steam-pelleted and crumbled to sizes readily acceptable to the fish over the course of the experiment. All fish were conditioned to the 90 ug/g diet for one week prior to their placement in the test battery.

To begin the experiment, the rainbow trout fingerlings were added to the tanks in a stratified hierarchical fashion, 33 per tank. Small adjustments were necessary in a few tanks to standardize the initial starting weights. Trout were fed to satiation (cessation of feeding) 3-4 times daily, decreasing to 2-3 times daily after six weeks. Food consumption was monitored by feeding fish from preweighed aliquots of the diet. All diets were stored at -20°C when not in use. Fish weight was measured by batch weighing (netting all fish in the tank into a tared, water-filled bucket) once a week for the first 4 wk then every other week for the remainder of the experiment. When weighing coincided with sampling, the sample was taken first, then the remaining fish in the tank were weighed and the weight of fish sampled was added to the tank weight. Mean fish weight was calculated as the total fish weight divided by the number of fish per tank.

Sampling

For each sample day (weeks 0, 1, 2, 4, 8 and 16), ninety fish were sampled in random order, three from each tank. Fish were netted indiscriminately from the tank and blood samples collected in ammonium heparinized glass

hematocrit tubes from the severed caudal peduncle. Hematocrit tubes were stored briefly on ice, then centrifuged. Hematocrit was read, and plasma recovered from the tubes for later analysis for Na, Ca, Mg, Zn (all by atomic absorption on a Varian AA-1275) and for week 16, total protein by refractometry on an American Optical TS meter (Alexander and Bell, 1980). Fish weight and standard length were recorded, and the general appearance of the fish noted for overt pathology, particularly cataracts and fin erosion, as these are symptomatic of zinc deficiency in trout (Ogino and Yang, 1978). Fish were individually bagged and frozen at -20°C for whole body ion analysis. Fish from the final sample day were frozen in liquid nitrogen and stored at -70°C for later analysis of metal binding protein (Chapter 3).

Neutron Activation Analysis

Whole body element concentrations were determined by exposure of fish samples to thermal neutrons in the McMaster University reactor. Fish from the 16 week sample were slit longitudinally to expose the gut and then freeze-dried to determine water content. The intestines were then examined, and any food or fecal matter removed; the entire fish was sealed in 7 g polyethylene vials. For short-lived isotopes (I, Br, Mg, Cu, Na, V, K, Cl, Mn, Ca), the sample was irradiated in a neutron flux of $5 \times 10^{12} n/cm^2.s$ for a 10 s irradiation. After a delay time of 120 s the samples were

counted for 600 s using an APTEC hyper-pure germanium detector (22% efficient with a 1.9 kev resolution at the 1332 kev ^{60}Co peak). Counts were accumulated on a Canberra multi-channel analyzer Model 40 or 90 equipped with internal live time correction and pile up rejection unit. Citrus leaf (NBS 1572) was used as the standard. For long-lived isotopes (Fe, Zn), the samples were loaded directly into the core (flux intensity 7.5×10^{12} $n/cm^2.s$) for 5-6 h. After a delay time of about 3 week during which time the short- and medium-lived isotopes decayed, the samples were counted for a period of 900 s. Here the standard used was lobster hepatopancreas (TORT-1; National Research Council of Canada, Ottawa, Ontario). Different sample groups were standardized for variations in flux intensity by the use of flux wires.

Calculations and Statistics

Since the number of fish in each tank decreased due to sampling, mortality was expressed as a function of the total number of fish days accumulated per tank. Food conversion was the wet weight gain divided by the wet weight of feed consumed per tank. Food intake (% body weight/d) was calculated from the wet weight of feed consumed over the interval between fish weighings, the average of the weights at the two times, and the elapsed time. The apparent zinc retention (%) was calculated from the difference between the initial (week 0) and final (week 16) body burden (ug Zn/fish)

divided by the dietary [Zn] times the amount of food consumed by the average fish in that tank, and expressed as a percentage. Estimates of the relative contribution of waterborne zinc to the final body Zn burden were arrived at by assuming that at ambient waterborne [Zn], uptake from the water was negligible, and that the entire Zn load was from the diet. For each water level above ambient, the difference between the initial and final body burden (ug Zn/fish), minus the same difference at ambient [Zn] for that dietary level was considered the absolute amount contributed by the water. In turn, this was expressed as a percentage of the total accumulated burden over the 16 weeks for that dietary treatment.

Analysis of the Data

Results are expressed as means ± SE (n), where SE is the standard error of the mean, and n the number of observations. The balanced part of the experiment (i.e. all the treatments exclusive of the high water x high diet) was analyzed by two-way ANOVA having 3 dietary x 3 waterborne x 3 replicates, with 3 observations per cell for a total of 81 observations. Interaction terms between replicates and the two other factors were significant in only two instances. In all other cases therefore, the pooled interaction variance was used to test the main effects. Where there were significant treatment effects, individual means were tested for significant differences by a multiple comparison test

Peritz' F test (Harper, 1984), with the experiment-wise confidence level specified as 95%.

RESULTS

Growth and Survival

Although fish were sampled at weeks 0, 1, 2, 4, 8 and 16, the trends shown over the course of the experiment were borne out by the results at week 16. Thus, except in rare instances, only the week 0 (hereafter called the initial values) and week 16 values will be discussed.

The fish fed actively, but growth was less than on practical diets, or even casein-based semi-purified diets (e.g. Hodson et al., 1980). Aggression may have been a factor here. There was no significant growth over the first four weeks, and no difference in growth among treatments over the first six weeks. Thereafter a gradual separation occurred (Fig 2.1), most clearly seen in the fish on the lowest dietary [Zn] (1 ug/g) at the lowest waterborne [Zn] (7 ug/L). These fish (hereafter called the deficient fish) had noticeably slower growth by week 8. For simplicity, Figure 2.1 shows only the extremes for the other 9 treatments, but terminal weights for all treatments at week 16 are shown in Figure 2.2.

The deficient fish continued to grow until week 12, but the mean weight per tank had less than doubled from 3.20 ± 0.03 (3) to 5.49 ± 0.28 (3) g. No further growth

Figure 2.1: Growth of rainbow trout with varied dietary or waterborne Zn. Upper two lines give extremes of growth exhibited by all treatments except the deficient dietary [Zn] of 1 ug/g at ambient waterborne [Zn] (7 ug/L). All unplotted treatments lie between these extremes. The lowest line gives the growth response for the deficient treatment. Values are means ± SE of 3 replicates.

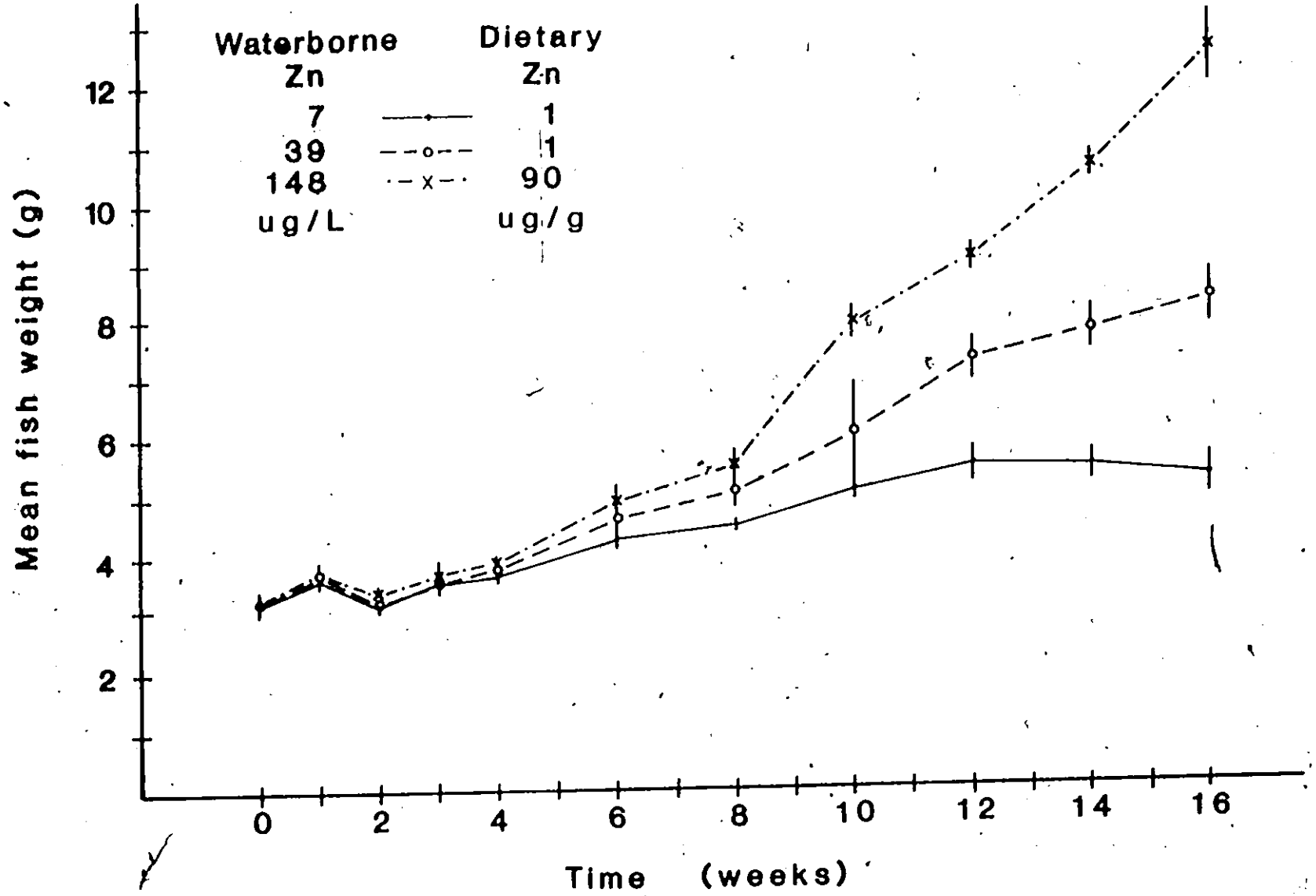
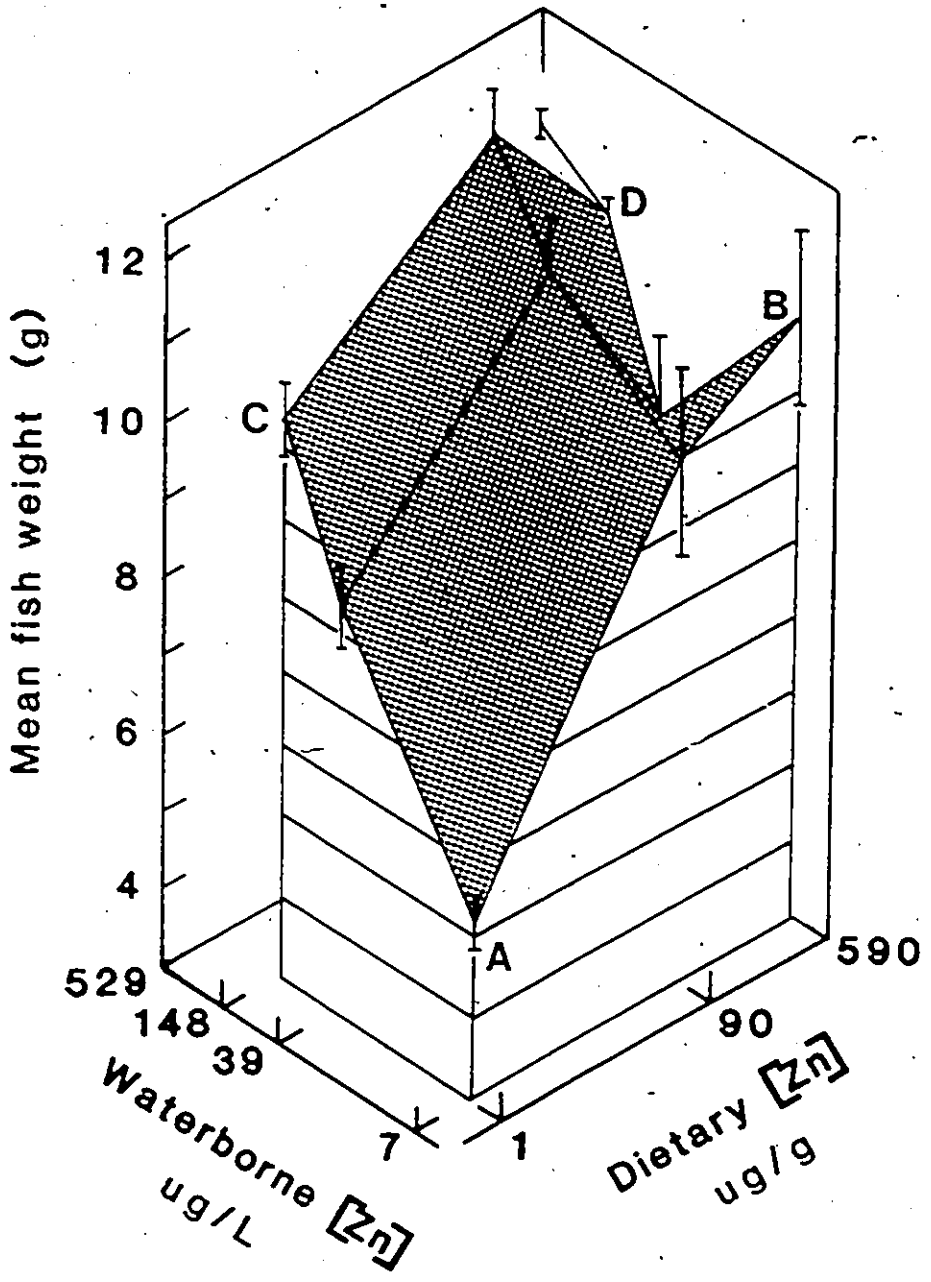

Waterborne Zn
7
39
148
ug/L
Dietary Zn
1
1
90
ug/g
Mean fish weight (g)
2
4
6
8
10
12
0
2
4
6
8
10
12
14
16
Time (weeks)

Figure 2.2: Mean fish weight at the-end of the experiment, as a function of waterborne and dietary inputs. Values are means ± SE (9, i.e. 3 fish from 3 different replicates). Letters ABCD are simply for orientation; see text for details.


Mean fish weight (g)
12
10
8
6
4
C
D
B
A
529
148
39
1
Waterborne [Zn]
ug/L
1
90
590
Dietary [Zn]
ug/g

occurred. An increase in Zn either from the diet alone (Fig 2.2, edge AB) or from the water alone (Fig 2.2, edge AC) provided for continued growth in a concentration-dependent fashion. By the end of the experiment, fish at the lowest treatment combination were less than half the size of the fish in the other treatments (Fig 2.1, 2.2) illustrating significant effects of both the water and dietary treatments. Based on growth and mortality, there were no toxic effects of the high levels of either dietary or waterborne [Zn]. In fact, fish vigour appeared to increase with increasing waterborne [Zn].

Fish consumed about 2.4-4.0% of their body weight/d. Intake tended to be lower at the highest dietary [Zn] (590 ug/g), perhaps reflecting a palatability effect. Over the last 2 weeks of the experiment, deficient fish ate more than any other treatment, and although the food conversion values were low for all treatments, fish in the deficient diet had a conversion of zero (Table 2.2). Despite the fact that fish in some treatments did not grow after 12 weeks, there was no significant difference in condition factor among treatments (Table 2.3), an effect which may have been masked by an apparent but non-significant increase in % water in the deficient treatment (Table 2.3).

Some disease and mortality occurred. Mortality from other than accidental causes was 7% (73/1008, Table 2.4). Of this, 49% was due to nodular gill disease (Daoust and

Table 2.2: Food intake [a] (% body weight per day) and food conversion [b] (%) for the last 2 week interval. Values are means ± SE of 3 replicate tanks except for the initial values.

Waterborne Zn (ug/L)		Dietary [Zn] (ug/g) 1	90	590
7	intake	3.97 ± 0.16	3.83 ± 0.65	2.62 ± 0.24
	conversion	-7 ± 11	23 ± 2	30 ± 1
39	intake	2.53 ± 0.27	2.60 ± 0.18	2.55 ± 0.15
	conversion	19 ± 5	38 ± 6	27 ± 7
148	intake	2.62 ± 0.24	2.98 ± 0.21	2.61 ± 0.06
	conversion	36 ± 9	41 ± 9	29 ± 1
529	intake	-	-	2.49 ± 0.04
	conversion	-	-	33 ± 6
initial values (wk 0 - wk 1)	intake	2.79 ± 0.04 (30)		
(all tanks)	conversion	55 ± 4 (30)		

[a] food intake calculated as the weight of food fed to the fish in the tank over the interval, divided by the midpoint bulk weight of the fish, divided by the days in the interval.

[b] food conversion is the wet weight gain divided by food intake multiplied by 100.

Table 2.3: Condition factor [a] (K) and water content [b] (%) at week 16. Values are means ± SE (9), 3 fish taken from 3 replicate tanks, except for initial values.

Waterborne Zn (ug/L)		Dietary [Zn] (ug/g) 1	90	590
7	K	1.30 ± 0.04	1.35 ± 0.03	1.35 ± 0.03
	% water	73.4 ± 0.5	72.1 ± 0.6	71.0 ± 0.8
39	K	1.31 ± 0.03	1.34 ± 0.03	1.30 ± 0.02
	% water	71.2 ± 0.6	71.6 ± 0.6	72.1 ± 0.7
148	K	1.32 ± 0.02	1.35 ± 0.03	1.33 ± 0.03
	% water	71.2 ± 0.07	70.0 ± 0.07	71.1 ± 0.07
529	K	-	-	1.35 ± 0.03
	% water	-	--	71.5 ± 0.7
initial values	K	1.27 ± 0.01 (90)		
	% water	73.2 ± 0.2 (30)		

[a] Condition factor = $\frac{\text{weight (g)}}{\text{standard length (cm)}^3} \times 100$

[b] Water content (%) = $1 - \frac{\text{dry weight}}{\text{wet weight}} \times 100$

Table 2.4: Number dying at the end of the experiment (per thousand fish days). Values are means ± SE (3).

Waterborne Zn (ug/L)	Dietary [Zn] (ug/g) 1	90	590
7	3.86 ± 0.82	4.72 ± 2.32	0.91 ± 0.29
39	0.28 ± 0.14	0.35 ± 0.35	0.63 ± 0.32
148	0.42 ± 0.25	1.65 ± 0.54	0.14 ± 0.14
529	-	-	0.13 ± 0.13

Ferguson, 1985), aggression (26%), indeterminate causes (18%) and fungal infestation (7%). Disease mortality persisted despite formalin treatments (5 min immersions in 1:8000 dilution) in weeks 8 and 10. ANOVA indicated that disease was treatment-related. There was a highly significant decrease in mortality with increasing waterborne [Zn]. Zinc-deficient fish seemed particularly susceptible to both nodular gill disease (most prevalent) and fungal infestation. There were only two incidents of gross cataracts, both in zinc-deficient fish. At the end of the experiment, all fish were examined for the presence or absence of nodules on the gill. There was clear evidence of the disease at all dietary treatments in fish at the lowest (i.e. ambient) waterborne [Zn]. At increased waterborne [Zn] however, very little evidence of the disease was found.

Blood Variables

Hematocrits at week 16 were similar to the week 0 value, except for the fish in the deficient treatment (Table 2.5) which were slightly anemic (about a 20% relative reduction) due to a significant diet effect. Plasma protein was also depressed in the deficient treatment by 40-50% (Table 2.5). There was a significant diet effect and a water-diet interaction.

There were no significant treatment differences for the plasma ion concentrations of Na, Ca, or Mg (Table 2.6) at week 16 or at any time throughout the experiment (data not

Table 2.5: Hematocrit (hct; %) and plasma protein (C_{Pr}; g/100 mL) at week 16. Values are means ± SE (9).

Waterborne Zn (ug/L)		Dietary [Zn] (ug/g) 1	90	590
7	hct	25.9 ± 2.1	36.1 ± 2.0	32.3 ± 1.8
	C_{Pr}	2.10 ± 1.00	3.81 ± 0.82	3.82 ± 1.02
39	hct	30.2 ± 1.3	34.4 ± 2.2	33.4 ± 1.3
	C_{Pr}	3.51 ± 0.54	4.03 ± 0.75	3.36 ± 0.71
148	hct	31.5 ± 2.5	31.9 ± 1.8	31.5 ± 1.7
	C_{Pr}	3.56 ± 1.07	3.99 ± 0.72	3.48 ± 0.82
529	hct	-	-	33.4 ± 1.6
	C_{Pr}	-	-	3.63 ± 0.55
initial values	hct	33.1 ± 0.3 (87)		
	C_{Pr}	nd[a]		

[a]- not determined

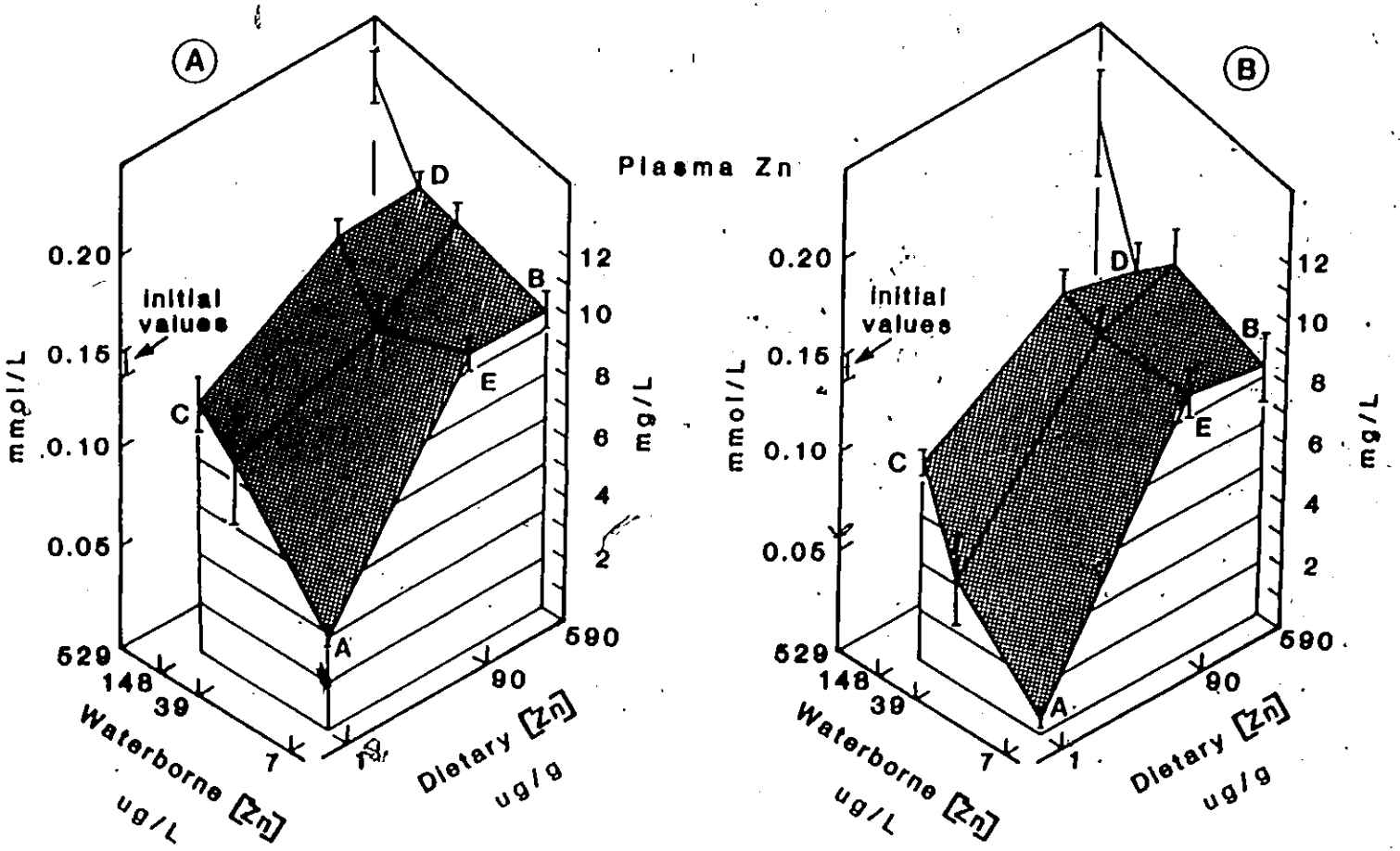
Table 2.6: Plasma ions at 16 weeks. Values are mequiv/L ± SE (9), unless otherwise noted.

Waterborne Zn (ug/L)		Dietary [Zn] (ug/g) 1	90	590
7	Na	148 ± 2 (7)	155 ± 4 (8)	156 ± 4
	Ca	4.54 ± 0.06	4.99 ± 0.13	4.93 ± 0.14
	Mg	2.14 ± 0.11	2.07 ± 0.07	2.10 ± 0.05
39	Na	157 ± 3	150 ± 3	152 ± 1
	Ca	4.76 ± 0.09	5.24 ± 0.19	4.83 ± 0.19
	Mg	2.07 ± 0.04	2.16 ± 0.05	2.12 ± 0.04
148	Na	155 ± 4	158 ± 2	151 ± 3
	Ca	4.84 ± 0.12	4.95 ± 0.55	4.31 ± 0.55
	Mg	2.02 ± 0.04	2.09 ± 0.04	1.88 ± 0.17
529	Na	-	-	154 ± 4
	Ca	-	-	4.93 ± 0.16
	Mg	-	-	1.97 ± 0.14
initial values	Na	146 ± 2 (61)		
	Ca	5.18 ± 0.07 (84)		
	Mg	1.82 ± 0.03 (83)		

shown). There was a significant water-diet interaction for plasma Na at week 16, but given the lack of significant differences or apparent trends, interpretation is difficult.

In contrast, the effect of treatment upon plasma [Zn] was striking. There were significant water and diet effects, but no interaction. Initial values were 0.147 ± 0.004 (88) mmol/L (9.57 ± 0.23 mg/L). After only one week of treatment, all of the fish on the deficient diet (Fig 2.3A, edge AC) had significantly lower plasma [Zn]. Increasing the waterborne [Zn] increased the plasma [Zn], but still not to initial levels. Those on the intermediate diet were not different from initial values regardless of the waterborne concentrations. Fish on the high [Zn] diet were no different from the initial values at ambient waterborne concentrations but as waterborne [Zn] increased, so did the plasma [Zn]. The lack of an interaction term between the diet and waterborne [Zn], indicated that uptake via one route was independent of uptake via the other. The results at week 16 (Fig 2.3b) were similar, except that at ambient waterborne [Zn] the plasma [Zn] from fish on the deficient diet had fallen to the point where it was scarcely detectable. Again, increases in waterborne [Zn] did permit some increases in plasma [Zn], but still not to initial levels. At higher dietary [Zn], there was generally a plateau around initial levels. Only at the highest waterborne and dietary level was there a significant increase above this plateau. Assuming the normal condition to be the

Figure 2.3: Plasma [Zn] in mmol/L: (a) after 1 week of treatment, (b) after 16 weeks of exposure to various waterborne and dietary Zn input. Other details as in Figure 2.2. Initial values (week 0) were 0.142 ± 0.004 (88) mmol/L.


Plasma Zn
A
B
initial values
initial values
mmol/L
mg/L
0.20
0.15
0.10
0.05
12
10
8
6
4
2
C
D
E
A
B
529
148
39
1
90
590
Waterborne [Zn] ug/L
Dietary [Zn] ug/g

medium diet and the ambient waterborne exposure, a decrement in dietary [Zn] from 90 to 1 ug/g under these water exposure conditions had a drastic effect on the plasma Zn status of the fish. Increasing the dietary [Zn] from 90 to 590 ug/g caused no increase in plasma [Zn] (Figs 2.3a, b edge EB). Increasing the waterborne [Zn] from 7 to 148 ug/L had no effect on plasma [Zn] (Fig 2.3a, b, edge BD). This is indicative of plasma homeostasis over a broad range of dietary and waterborne [Zn].

Whole Body Elemental Analysis

Most of the whole body elements which were readily detectable using neutron activation analysis (Table 2.7, Figs 2.4, 2.5) showed significant treatment effects. All the results are expressed on a dry weight basis. Since the % water was not significantly different across treatments (Table 2.3), expressing results on a wet weight basis gave identical trends.

High waterborne [Zn] exposures had no effect on whole body elemental composition (other than Zn), further supporting the lack of any toxic effect of Zn on ion regulation, as seen previously with the plasma data. With the exception of whole body [Zn] and [Cu], treatment differences were restricted to the deficient fish, and all showed virtually the same pattern of response to treatment, although statistical significance varied somewhat (Fig 2.4, Table 2.7).

Whole body [Ca] (Fig 2.4) showed the treatment response most clearly. Elemental concentrations rose

Figure 2.4: Whole body [Ca] in umol/g dry weight after 16 weeks of treatment. Values are means ± SE (9). Initial values (week 0) were 460 ± 11 (15) umol/g dry weight.

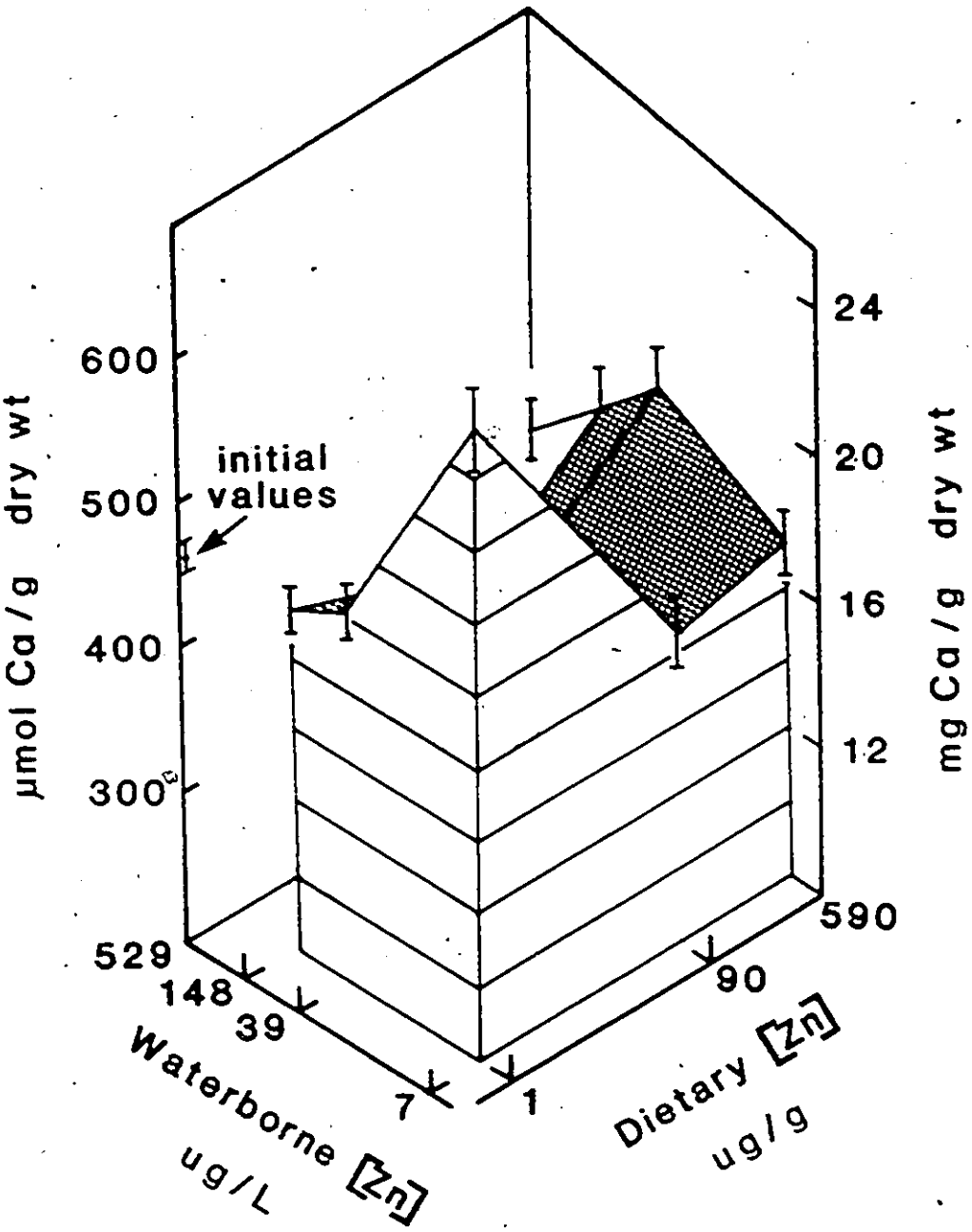

µmol Ca / g dry wt
600
500
400
300
initial values
mg Ca / g dry wt
24
20
16
12
529
148
39
7
Waterborne [Zn] ug/L
1
90
590
Dietary [Zn] ug/g

Table 2.7: Whole body element concentrations at week 16. Values are umol/g dry weight, means ± SE (9).

Water [Zn] mg/L	Diet [Zn] mg/kg	Na	Mg	Cl	K	Ca
7	1	176 ± 7	48 ± 2	150 ± 8	253 ± 8	685 ± 29
	90	140 ± 9	40 ± 2	127 ± 10	225 ± 9	458 ± 25
	590	139 ± 7	39 ± 2	127 ± 7	236 ± 10	473 ± 21
39	1	148 ± 5	39 ± 2	133 ± 6	232 ± 10	502 ± 19
	90	147 ± 8	40 ± 2	137 ± 10	238 ± 10	465 ± 28
	590	153 ± 7	41 ± 2	140 ± 6	264 ± 11	525 ± 26
148	1	142 ± 5	39 ± 2	127 ± 7	225 ± 9	481 ± 16
	90	134 ± 6	38 ± 2	126 ± 6	222 ± 10	423 ± 19
	590	145 ± 7	37 ± 2	134 ± 7	233 ± 11	490 ± 30
529	590	139 ± 7	36 ± 2	133 ± 7	237 ± 11	450 ± 19
Initial values (n=15)		180 ± 5	59 ± 3	145 ± 5	337 ± 7	462 ± 11

... continued

Table 2.7 (Continued): Whole body element concentrations at week 16. Values are nmol/g dry weight, means ± SE (9).

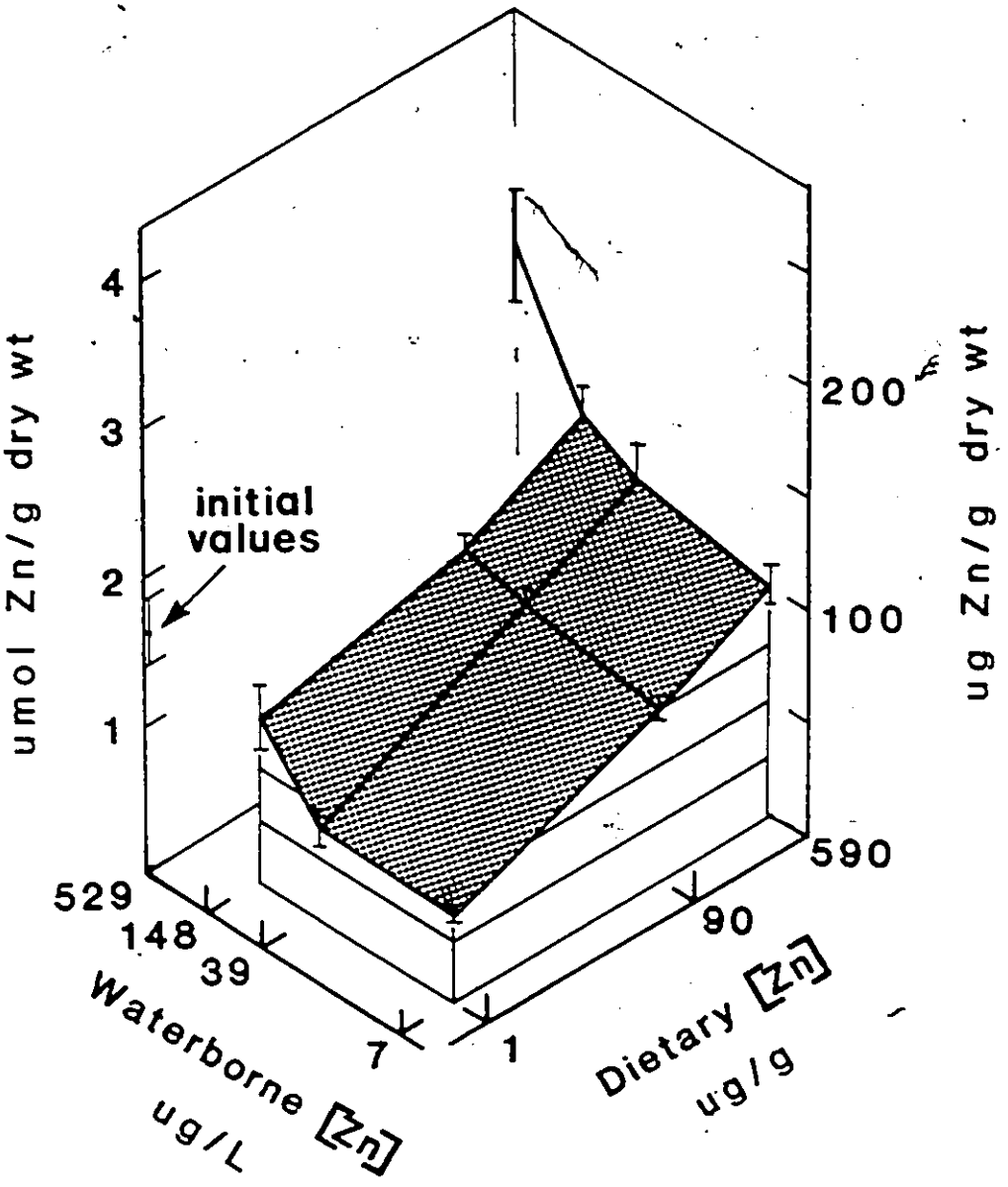
Water [Zn] mg/L	Diet [Zn] mg/kg	Zn	Fe	Cu	Mn	V	Br
7	1	586 ± 20	956 ± 75	26 ± 13	233 ± 14	29 ± 3	85 ± 8
	90	1163 ± 44	546 ± 33	64 ± 12	158 ± 22	17 ± 2	56 ± 5
	590	1526 ± 75	561 ± 42	39 ± 9	116 ± 9	15 ± 3	56 ± 5
39	1	592 ± 68	625 ± 39	50 ± 11	168 ± 16	18 ± 3	57 ± 3
	90	1293 ± 41	609 ± 63	70 ± 18	146 ± 15	16 ± 2	56 ± 7
	590	1709 ± 127	548 ± 46	61 ± 20	107 ± 12	18 ± 4	61 ± 9
148	1	1050 ± 121	555 ± 32	42 ± 7	153 ± 24	18 ± 3	56 ± 2
	90	1398 ± 44	540 ± 16	52 ± 9	135 ± 14	12 ± 2	50 ± 8
	590	1840 ± 96	665 ± 103	39 ± 11	141 ± 13	20 ± 2	57 ± 4
529	590	2737 ± 219	568 ± 35	24 ± 9	139 ± 7	16 ± 2	58 ± 4
Initial values (n=15)		1409 ± 48	781 ± 91	114 ± 20	148 ± 7	6 ± 2	109 ± 9

significantly, but only in the deficient fish. In contrast, the other treatments constituted a plateau which showed neither trends nor significant differences. This single treatment caused significant effects not only for dietary and waterborne [Zn], but for the interaction as well. Similar significant responses were seen for Na, Mg, Br and Fe (Table 2.7). The elements V, K and Cl showed similar elevations in deficient fish, but these were not significant. Whole body [Mn] was slightly different, because in addition to elevations in whole body concentration seen in deficient fish, only the diet had a highly significant effect showing a decreasing whole body concentration as dietary [Zn] fell. Whole body [Cu] showed no treatment effects at all.

The generally higher elemental concentration in the deficient fish, compared with the other treatments at week 16, may have been an effect of fish size, since a scatter plot of whole body [Ca] vs fish weight irrespective of treatment (not shown) revealed an exponential decline with increasing size up to about 15g. In fact, compared with initial values (Table 2.7), deficient fish had similar or lower concentrations for 8 of 11 elements. This again suggests a size effect. Only for Ca, Mn, and V were concentrations elevated significantly above initial values.

In contrast, whole body [Zn] (Fig 2.5) reflected the loading to which the fish were subjected. Both diet and water were highly significant factors, and showed clear

Figure 2.5: Whole body [Zn] in umol/g dry weight after 16 weeks of treatment. Values are means ± SE (9). Initial values (week 0) were 1.41 ± 0.05 (15) umol/g dry weight.


umol Zn/g dry wt
4
3
2
1
initial values
ug Zn/g dry wt
200
100
590
529
148
39
7
Waterborne [Zn]
ug/L
1
90
Dietary [Zn]
ug/g

trends. There was no interaction between them, again indicating that Zn uptake by one route was independent of uptake via the other. The diet had by far the largest F value and was the major determinant of whole body [Zn] (Fig 2.5). Fish on the deficient diet at ambient waterborne [Zn] (7 ug/L) had the lowest whole body [Zn]. This did not increase when waterborne [Zn] was raised to 39 ug/L, despite the fact that plasma [Zn] was higher (Fig 2.3). Thus plasma [Zn] under these conditions was more sensitive to loading than was whole body concentration. Only at a waterborne [Zn] = 148 ug/L was there an increased whole body concentration. At the other two dietary levels there were modest increases in the [Zn] as the waterborne [Zn] increased. Finally, at the highest dietary and the highest waterborne [Zn] there was a significant increase in whole body [Zn] to nearly double that of the week 0 value (week 0 = 1.41 ± 0.05 (15)) umol/g. The deficient fish in contrast, had whole body [Zn] of half the initial value. Unlike plasma [Zn], (Fig 2.3b), whole body [Zn] (Fig 2.5) showed no clear plateau region where its level was independent of dietary and waterborne [Zn].

DISCUSSION

Effects on Variables other than Zinc

Growth on the zinc-adequate diets was less than expected, even for albumin-based diets (Ogino and Yang, 1978;

Wekell et al., 1983). However, all plasma variables were in the reported range for the species (Hille, 1982). As well, all eleven measured whole body element concentrations fell within the ranges given by Shearer (1984) for the corresponding fish size.

There were clearly no toxic effects of exposure to high dietary and/or waterborne [Zn] based upon growth, mortality, major plasma ions, hematocrit or plasma protein. Similarly, Wekell et al. (1983) reported that dietary [Zn] as high as 1700 ug/g had no toxic effect on growth and mortality. No effect of high [Zn] upon whole body element concentrations was ever found in the present study, despite indications that high dietary [Zn] interfered with accumulation of Cu in the liver of rainbow trout (Knox et al., 1984), and waterborne [Zn]=800 ug/L blocked net Ca uptake in soft water (Appendix 1).

Dietary restriction of zinc (1 ug/g), on the other hand, rendered trout zinc-deficient, using the criteria of cessation of growth, significant mortality, and decreased plasma and whole body [Zn]. Decreased plasma protein concentration and low hematocrit in zinc-deficient fish have not previously been reported, and may signify reduced protein synthetic ability. Cataract formation seen in earlier studies of zinc deficiency (Ogino and Yang, 1978; Ketola, 1979) occurred in only two zinc-deficient fish in the present study, and was not seen in the study of Wekell et al. (1983)

using a similar diet. Additional dietary components such as high [Ca] and/or phytate which reduce the bioavailability of dietary zinc may be necessary for significant cataract formation (Richardson et al., 1985).

High mortality is always associated with zinc deficiency (Ogino and Yang, 1978; Wekell et al., 1983) although Richardson et al. (1985) were the only ones to categorically state that this occurred in the absence of any identifiable pathogen. In my study, mortality was generally restricted to deficient fish and was invariably associated with pathology such as fin erosion, fungal infection and nodular gill disease. In part, this might be due to reduced immune function, which is common in zinc-deficient mammals (see Fraker et al., 1986). On the other hand, nodular gill disease was, after 16 weeks, most prevalent in trout at ambient waterborne [Zn] regardless of dietary [Zn]. This suggests that elevated waterborne [Zn] itself may have had a prophylactic effect independent of the nutritional status of the fish, and warrants further study.

Concentration of all whole body elements (with the exception of Cu) was elevated in deficient fish (Fig 2.4, Table 2.7). As noted earlier, this may have been a size effect, since these fish were significantly smaller than in other treatments, and negative correlations with size have been reported in wild fish (Wiener and Giesy, 1979). Shearer (1984) however found positive correlations with size

for most elements in laboratory-reared rainbow trout. Alternatively, a change in proximate body concentration may have accounted for the observed increases. Zinc-deficient fish had slightly higher ash weight, and less lipid (Ogino and Yang, 1978). Lipid has very low mineral concentrations, and high levels would tend to dilute elemental concentration (Shearer, 1984).

Effects on Plasma and Whole Body [Zn]

There are relatively few measurements of plasma [Zn] in trout. Although mammalian values are about 0.01-0.02 mmol/L (Underwood, 1977) plasma [Zn] in fish is, for reasons which are unclear, frequently 10-fold higher (Bettger et al., 1987) and shows a higher range; 0.18-0.37 (Knox et al., 1984), 0.28 (Zeitoun et al., 1977) falling to 0.06 ± 0.03 mmol/L in rainbow trout fasted for 7d (Spry and Wood, 1984). Migratory (non-feeding) salmon (Oncorhynchus nerka) had initial values of ~0.37 mmol/L which fell 50% over a month during spawning (Fletcher et al., 1975). In the present study, both the initial value (week 0, 0.15 mmol/L) and all treatments at week 16 (with the exception of deficient fish), compared well with literature values for fish.

Plasma [Zn] after only one week was a sensitive indicator of zinc restriction (Fig 2.3a), and was depressed to quite low levels well before there was a treatment effect on growth (Fig 2.1), or diseases appeared. By the end of the experiment, it was obvious that in the deficient fish,

plasma [Zn] was extremely low (Fig 2.3b) and growth had ceased. In fact, plasma [Zn] was a good indicator of growth, since it approximately mirrored the mean fish weight at the end of the experiment. These results indicated a broad plateau of zinc input (waterborne [Zn] 7-148 ug/L, dietary [Zn] 90-590 ug/g) over which plasma [Zn] remained unaltered, suggesting considerable homeostasis which was only overridden at extremes of very high or very low input.

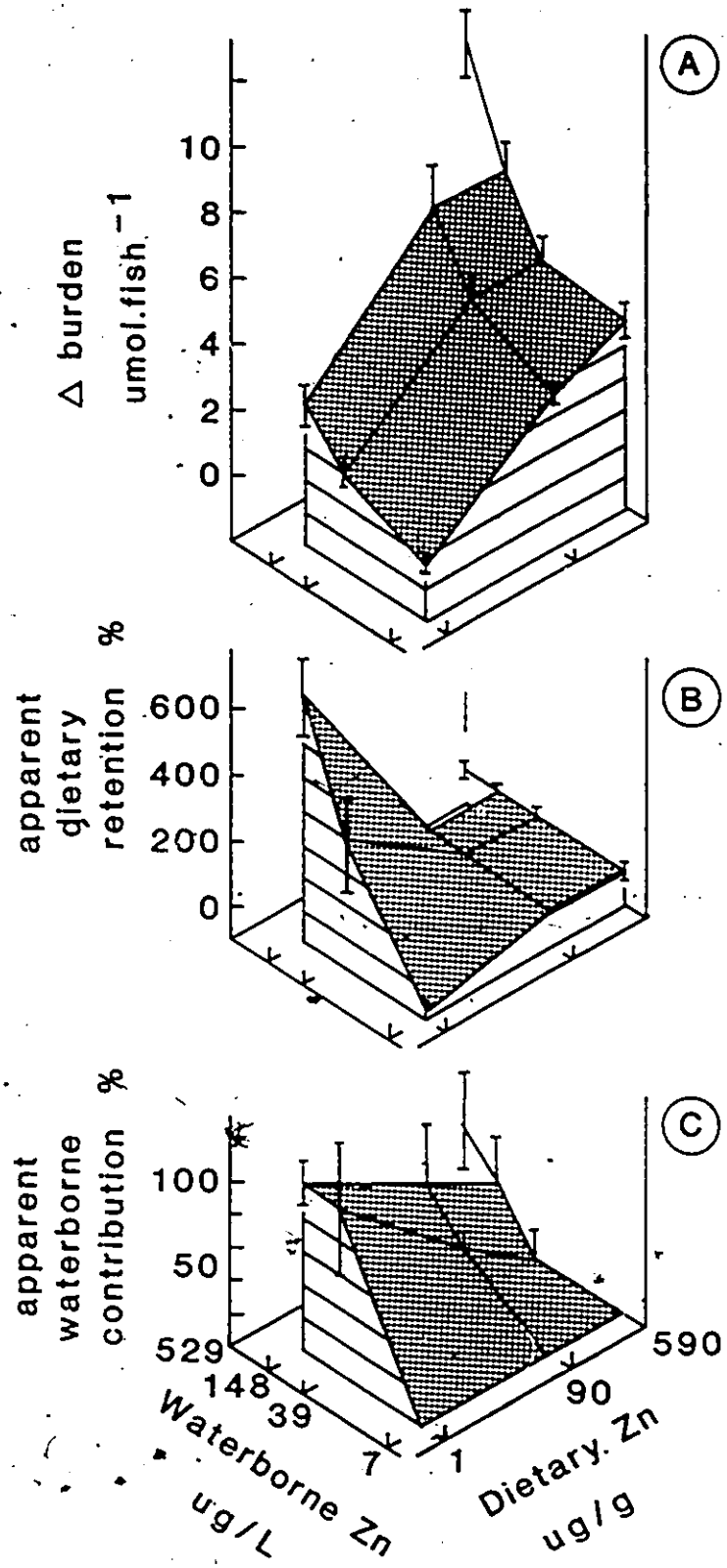
Whole body [Zn] was less responsive than plasma [Zn] to changes in waterborne and dietary levels (compare Figs 2.5 and 2.3b), though it was clearly affected by both. In the most deficient fish (waterborne [Zn] = 7 ug/L, dietary [Zn] = 1 ug/g) which had stopped growing, whole body [Zn] was ~0.6 umol/g dry weight. A similar concentration, but greater absolute load, was seen at the next higher waterborne [Zn] (39 ug/L) where the fish grew nearly twice as large (Figs 2.2, 2.5). This concentration may therefore represent a critical whole body level below which fish will not grow. Interestingly, Ogino and Yang (1978) found 0.43 umol Zn/g dry weight in zinc-deficient trout.

As dietary [Zn] increased, whole body [Zn] also increased, but in a log-linear fashion, with the result that fish retained much less Zn than they were being fed (see below). For example, at ambient waterborne [Zn], a nearly 7 fold increase in dietary [Zn] only increased whole body [Zn] by ~31%. The interesting fact here again is that when a

waterborne Zn concentration was imposed in addition to diet, there were smaller, but nearly linear increases in whole body [Zn]. Some isolation thus exists between the two routes of uptake.

Calculations of dietary Zn retention (Fig 2.6) indicated four major points. First, deficient fish had negative retention, indicating that these fish actually had lower Zn body burdens at week 16 than they did at week 0. This probably occurred because intestinal loss of Zn, which is large in mammals (Spencer et al., 1980; Weigand and Kirchgessner, 1980), exceeded absorptive capacity. Absorptive capacity in flounder was only about 20% of various $ZnCl_2$ loads (Shears and Fletcher, 1983). Second, trout on the deficient diet accumulated much larger loads than could be accounted for by diet alone, even assuming complete absorption, thereby yielding apparent retention figures well over 100% (i.e. 292, 665%). Nevertheless, the absolute increase in Zn load in the fish was less than that seen at any of the higher dietary [Zn]'s. A stimulatory effect of waterborne [Zn] on apparent Zn retention was also seen at the higher dietary levels, but to a much lesser extent (values in 2-18% range). Third, as dietary [Zn] increased, trout retained much less on a percentage basis, until at dietary [Zn] = 590 ug/g, apparent retention from the diet was only about 2%. Fourth, at every dietary level, increases in waterborne [Zn] were associated with increased contributions from the waterborne source, with

Figure 2.6: (a) Change in body burden in umol Zn/fish calculated as the difference in the means between the initial body burden (=0.99 umol/fish at week 0; n=15) and that for each treatment mean at 16 weeks; (b) apparent dietary Zn retention (%); (c) apparent contribution by waterborne Zn to the total body burden. See text for details.


A
Δ burden
umol.fish⁻¹
10
8
6
4
2
0
B
apparent
dietary
retention
%
600
400
200
0
C
apparent
waterborne
contribution
%
100
50
529
148
39
7
Waterborne Zn
ug/L
1
90
590
Dietary. Zn
ug/g

many values in the 31-57% range, even when dietary [Zn] was normal (90 ug/g) or higher, and values of 100% when dietary [Zn] was deficient (1 ug/g).

In view of this importance of zinc uptake from the water, by what route does it occur? One possibility is drinking of the medium, which might be especially important in fish on a low zinc diet. Estimates of drinking rates for rainbow trout in fresh water range from nil (Shehadeh and Gordon, 1969) to 1.43 uL/g.h (Lovegrove and Eddy, 1982). Using the higher estimate, the highest waterborne [Zn] tested in fish on the deficient diet, 148 ug Zn/L, would provide only 0.005 ug Zn/g.d. Food consumption of 3% body weight/d, at dietary [Zn] = 1, 90, and 590 ug/g, would give dietary inputs of 0.03, 2.7, 17.7 ug Zn/d. Even at the lowest dietary [Zn], the maximum input from imbibed water would only be 17% of the available dietary intake.

The other, far more likely, possibility is uptake across the gills. In acute exposures, the gill is always a site of high Zn concentration (Joyner, 1961; Hodson, 1976; Bradley et al., 1985). Joyner's study further showed that esophageal wax plugs in brown bullheads (Ictalurus nebulosus) did not prevent accumulation of ^{65}Zn by several internal organs (Joyner, 1961). Uptake by the intact trout was also unaffected by esophageal ligation (Chapter 4).

Our results clearly support homeostasis of internal zinc levels in the face of dietary and waterborne variation.

For plasma, there was a broad region of regulation regardless of input, which was only depressed during extreme deprivation, and only elevated at very high dietary and waterborne input (Fig 2.3a, b). Whole body [Zn] exhibited a rather less tightly controlled homeostasis in which there was no plateau region where concentration was independent of exposure. Perhaps "excess" whole body Zn was stored in a "sink" of lesser physiological importance (e.g. scales, Sauer and Watabe, 1984; or bone). Nevertheless, over the same range where plasma showed a perfect plateau (Fig 2.3b), whole body [Zn] exhibited less then 2-fold variation in the face of 7- to 21-fold variation in the dietary and waterborne levels respectively (Fig 2.5). Indeed, on a whole body basis, apparent dietary retention was reduced to as low as 2% at high dietary [Zn] (Fig 2.6). The true dietary retention would be even lower, since zinc was also entering from the water. This reduction may have been accomplished by decreased intestinal absorption, increased excretion, or both.

Studies on zinc accumulation in field studies (see Bryan, 1979; Giesy and Wiener, 1977; Wiener and Giesy, 1979; Roch et al., 1982; Saltes and Bailey, 1984) support the concept of zinc homeostasis in fish, as has been well-documented in mammals (e.g. Cousins, 1985). In laboratory exposures, whole body and tissue levels have risen, but only slightly (2-3 fold), when waterborne [Zn] has increased several hundred fold (Farmer et al., 1979; Holcombe et al.,

1979). Steady-state concentrations which have been observed with time, may be due to growth dilution (Spehar, 1976; Farmer et al., 1979), although active excretion has been proposed (Pierson, 1981). It is still not clear how closely whole body zinc is regulated, or whether this is effected by active or passive mechanisms which maximize net Zn uptake in deficiency, and limit net uptake when excess Zn is present in the water or diet.

In summary, a deficiency was clearly induced in trout fed a diet deficient in zinc for 16 weeks at ambient waterborne [Zn]. Elevations of waterborne [Zn] to 39 and 148 ug/L partially corrected the deficiency but did not restore either plasma or whole body [Zn] to levels seen either initially, or in fish raised for 16 weeks on a Zn-adequate diet. At elevated waterborne [Zn], zinc was taken up from the water regardless of the dietary load. Waterborne contribution was up to 57% of the increased burden on zinc-adequate diets, despite the fact that dietary zinc was three orders of magnitude higher. At waterborne [Zn] most commonly encountered in the wild (< 10 ug Zn/L), waterborne contributions to whole body [Zn] are likely to be insignificant. Non-lethal waterborne [Zn] which may be encountered as a result of pollution (e.g. 39 - 529 ug/L), may cause considerable uptake from the water. Elevation of waterborne [Zn] to even 500 ug/L was not stressful to fingerling trout based upon growth and mortality, and may

have been prophylactic against waterborne pathogens. Plasma [Zn] was relatively constant over most of the range of zinc input, whereas whole body [Zn] reflected input from both sources, but in an attenuated fashion. Metabolism of other elements did not appear to be directly affected by zinc treatments, and there were no toxic effects.

CHAPTER 3

THE INFLUENCE OF DIETARY AND WATERBORNE Zn ON HEAT-STABLE METAL LIGANDS IN RAINBOW TROUT: QUANTITATION BY ^{109}Cd RADIOASSAY, AND EVALUATION OF THE ASSAY.

INTRODUCTION

Exposure of many organisms to elevated levels of group IB and IIB metals leads to the induction of metal-binding proteins. The most studied class are the metallothioneins (MT). These proteins have no enzymic function but presumably play a role in metabolism and homeostasis of micronutrient metals as well as detoxification of toxic metals and excess micronutrients (Brady, 1982; Webb and Cain, 1982; Cherian and Goyer, 1978).

The structure of mammalian MT is well-described (Furey et al., 1986; Otvos et al., 1985). The proteins seem conserved across major taxa, though MT from fish may differ somewhat from mammalian MT in primary structure (see Klaverkamp et al., 1984). In general, the proteins have the following characteristics which may be exploited in their identification and quantitation. They are cytosolic, heat/acid stable, low molecular weight (M_r ~6,000 by amino acid analysis, ~11,000 by gel filtration) compounds low in

aromatic and basic amino acids, resulting in high 250/280 nm absorbance ratios, but high in thiols (cysteine 20-35%). This high thiol content provides the sulphur centers for which group B and borderline metals have high affinity (Nieboer and Richardson, 1981). As many as three isoforms having the same weight but differing in isoelectric point may exist in fish (see Klaverkamp et al., 1984).

Although direct MT assays exist in the form of radioimmunoassay and other molecular probes, they are not widely available and require considerable expertise. Indirect assays based on the high cysteine sulphur content of the molecule or its metal-binding properties have been widely employed. For example, Wofford and Thomas (1984) have recently measured an increase in acid-soluble thiols (AST) in Cd-exposed mullet, which was attributed equally to glutathione and putative MT. Metal-binding assays make use of the high affinity of the protein for certain metals and use Hg or Cd, either radioalabelled or not, to displace the other metals. Excess metal is then removed with a low affinity agent. The Cd-binding assay of Onosaka et al. (1978) as modified by Eaton and Toal (1982) has found wide acceptance in mammalian toxicology and compares well with radioimmunoassay although it consistently yields 1.3 to 1.5 fold higher absolute values (Waalkes et al., 1985). This assay has not been previously reported for fish tissue.

Sources of metal exposure in fish may be either via

the water or the diet. Several freshwater species have shown increased levels of MT when exposed to elevated waterborne concentrations of metals, which in most studies have been Cd, Cu, or Hg (see Hamilton and Mehrle, 1986). However, Kito et al. (1982) demonstrated MT in carp exposed to 10 mg Zn/L for 14 d, and Bradley et al. (1985) found a doubling of heat-stable, sulphydryl-rich protein in rainbow trout exposed to 2100 ug Zn/L (0.3 of the LC50) for 5-20 d. There appear to have been no studies to date on MT induction through dietary exposure to Zn in fish.

The results of Chapter 2 indicated that juvenile rainbow trout may accumulate Zn either from the water or from the diet, and that uptake from either source was essentially independent. Furthermore, Zn uptake from the water source was sufficient to support normal growth when dietary Zn was deficient. Zn accumulation was less then proportional to concentration through either route, and there were no toxic symptoms at high concentrations. These observations raise the prospect that induction of metal-binding proteins may have occurred, and played an important role in the regulation of Zn uptake and/or detoxification during the exposures, as has been suggested for mammalian systems (Cousins, 1985).

This section of the thesis was undertaken to evaluate the ^{109}Cd-binding assay for use with fish tissues, and to apply this to tissues whose content of MT or other metal binding proteins might reflect Zn exposure (e.g. gill, liver

and intestine). This would test the hypothesis that gill and intestine would reflect waterborne and dietary concentrations respectively, whereas liver concentration would reflect the combined exposure.

MATERIALS AND METHODS

Experimental treatments

Rainbow trout were obtained from Spring Valley Trout Farm, Petersburg, Ontario. Underyearling trout (120-340 g) used for injection studies were held at McMaster University in flowing, charcoal-dechlorinated, Hamilton city tapwater ([Ca] 0.9, [Na] 0.6, [Cl] 0.8 mmol/L, pH 8.1) at ambient temperature for several months before use and fed a pelleted commercial diet (Martin Feed Mills, Elmira, Ontario).

For the injection studies, underyearling trout, acclimated for 7 days to 6°C, were injected intraperitoneally with either Cd (1 mg/kg body weight, as acetate) or Zn (10 mg/kg body weight, as sulphate) in Cortland saline (Wolf, 1963). The fish were given a second injection 24 hours later and killed at 48h by a blow on the head. Tissues were treated as described below.

Fish from the combined diet/waterborne study were exposed for 16 weeks as detailed in Chapter 2. At 16 weeks, 3-9 fish were sampled from each treatment, killed with MS-222, quick frozen in liquid nitrogen and stored at -70°C until dissected. Due to treatment effects from the

experiment, only one fish was available at dietary [Zn] = 90 mg/kg, waterborne [Zn] = 7 ug/L.

Trout, either fresh or frozen, were dissected for gill, liver, or intestine. Gills were trimmed from the arches, livers were removed whole and kept free from bile. The intestine from the pyloric sphincter to the beginning of the large intestine was gently purged with ice-cold saline to remove gut contents, and external fat was removed. All tissues were homogenized in 4 ml/g of ice-cold buffer (30 mM Tris, pH 8.0, 150 mM KCl) with a tissuemizer (Tissue-Tek) for 30 s at medium speed. After centrifugation at 4,500 rpm for 5 min to remove debris, a sample of supernatant was removed and preserved for measurement of AST (see below). The remaining supernatant was heated in a boiling water bath for 2 min, then the tubes were cooled on ice and centrifuged at 8,000 g for 5 min. The heat-denatured supernatant (HDS) was transferred to clean centrifuge tubes and stored at -20°C for up to 2 wk until required for either the ^{109}Cd radioassay, or gel chromatography.

Gel chromatography

For gel chromatography, elution buffer (20mM Tris, pH 8.0, 150 mM KCl, 0.02% sodium azide) was pumped through a 1.5 x 28 cm Sephadex G-75 column at 15 ml/h and 2.5 or 5 ml fractions were collected. The column was calibrated with blue dextran (2,000,000 M_r), ovalbumin (43,000 M_r), chymotrypsinogen A (25,000 M_r), ribonuclease A (13,700 M_r),

or cytochrome C (12,300 M_r). Rabbit Cd-Zn MT (Sigma M7641) and Cd (as $CdCl_2$) were also put through the column. Initially, elutions were done at 4°C, but later at room temperature as this did not affect the performance of the HDS. Fractions were variously assayed for Zn or Cd by flame atomic absorption spectrophotometry, ^{109}Cd by gamma activity (Chicago-Nuclear well-type counter, model 1084), or absorbance at 250 and 280 nm (Perkin-Elmer Lambda 3). Absorbance at 280 nm is due to the presence of tyrosine and tryptophan. These are present in most proteins, but not in MT. MT does however absorb strongly in the 250 nm region due to the presence of the metal-thiolate bond. Although this property is quantitative with purified MT, it is non-specific in semi-purified extracts.

^{109}Cd-binding assay

The assay was based on that of Onosaka et al. (1978) as modified by Eaton and Toal (1982). The principle of the assay relies upon displacement of Zn from MT by an excess of $^{109}Cd/Cd$. Any unbound Cd is removed by the addition and subsequent heat precipitation of hemoglobin. The supernatant, now containing MT saturated with $^{109}Cd/Cd$, is counted for radioactivity, and total Cd binding calculated from the known specific activity.

In the assay, 400 ul of HDS was added to 400 ul of homogenization buffer. One hundred ul of the Cd solution (1 uCi/mL, 2.22 nmol Cd/mL) was added, mixed, and the mixture

incubated at room temperature for 30 min. Then, 100 ul of 2% bovine hemoglobin (Hb, Type II, Sigma) was added, mixed, and the mixture heated for 2 min in a boiling water bath. After cooling on ice, the tubes were centrifuged at 8,000 g for 5 min. The Hb addition, heating, cooling and centrifugation was repeated once. A 900 uL aliquot of the supernatant was counted for gamma activity. The Cd-binding activity was then calculated as:

$$\frac{\text{nmoles Cd bound}}{\text{g wet tissue}} = \text{cpm} \times \frac{1}{\text{SA}} \times \frac{\text{total assay volume}}{\text{sample volume}} \times \text{tissue dilution} \quad 3.1)$$

where SA was the specific activity in cpm/nmol Cd.

The linearity of the assay was evaluated with a dilution series of rabbit Cd-Zn MT (Sigma M7641: 0.59 umol Cd, 0.05 umol Zn/g solid). The recovery of Cd from the added Cd-Zn MT was measured by atomic absorption analysis of the same dilution series i) placed directly in the buffer alone, ii) after the addition of Hb plus heating and centrifugation, and iii) after the added MT was processed through the complete assay, which included the addition of ^{109}Cd. These latter three steps assessed whether error occurred either due to stripping of Cd from the MT by Hb, or due to incomplete removal of added free Cd by the Hb. The effect of changes in sample size was evaluated using HDS from gill and liver of Zn-injected fish. Sample sizes of HDS were varied from 50 to 800 ul, and by varying the amount of buffer, the total assay volume was kept constant. Gel chromatography of selected

assay supernatants was performed to estimate molecular weight range of ligands for ^{109}Cd.

As a check on day-to-day variability in the assay, a "reference" HDS was constructed based on pooled supernatant from the Zn-injected fish, and stored in small batches at -20°C. On each day of the assay, both this reference material and rabbit Cd-Zn MT standards were included in the measurements.

Acid-soluble thiols

For the measurement of acid-soluble thiols a 100 uL aliquot of the supernatant from the crude homogenate was added to an equal volume of 10% trichloroacetic acid and the precipitate pelleted by centrifugation at 8,000 g for 5 min. This was then stored at -20°C until analysed for thiols by the dithionitrobenzoic acid (DTNB) method of Ellman (1959).

Analysis of the Data

Data have been expressed as means ± 1 SE (n) where n represents the number of fish in a treatment. Simple comparisons used Student's two-tailed t test (unpaired) at $p<0.05$. Multiple comparison tests were by Peritz' F test (Harper, 1984), with the experiment-wise confidence level maintained at 95%.

RESULTS

Evaluation of the Assay

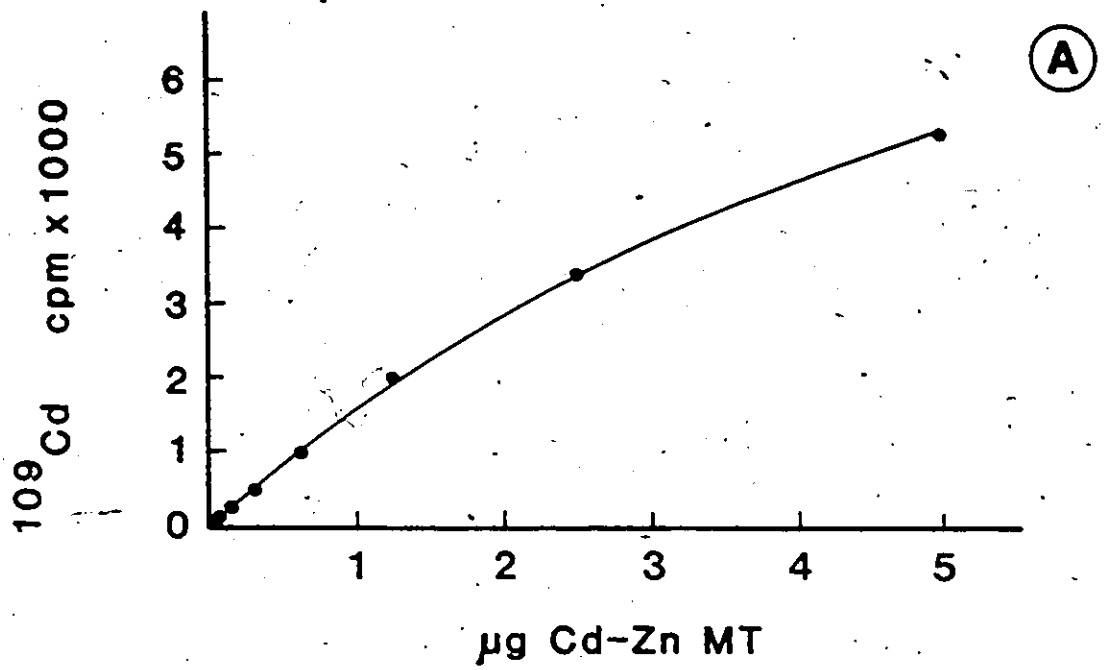
The standard curve for the Cd-binding assay (Fig 3.1a) was linear up to 1.25 ug of Cd-Zn MT, which corresponds to

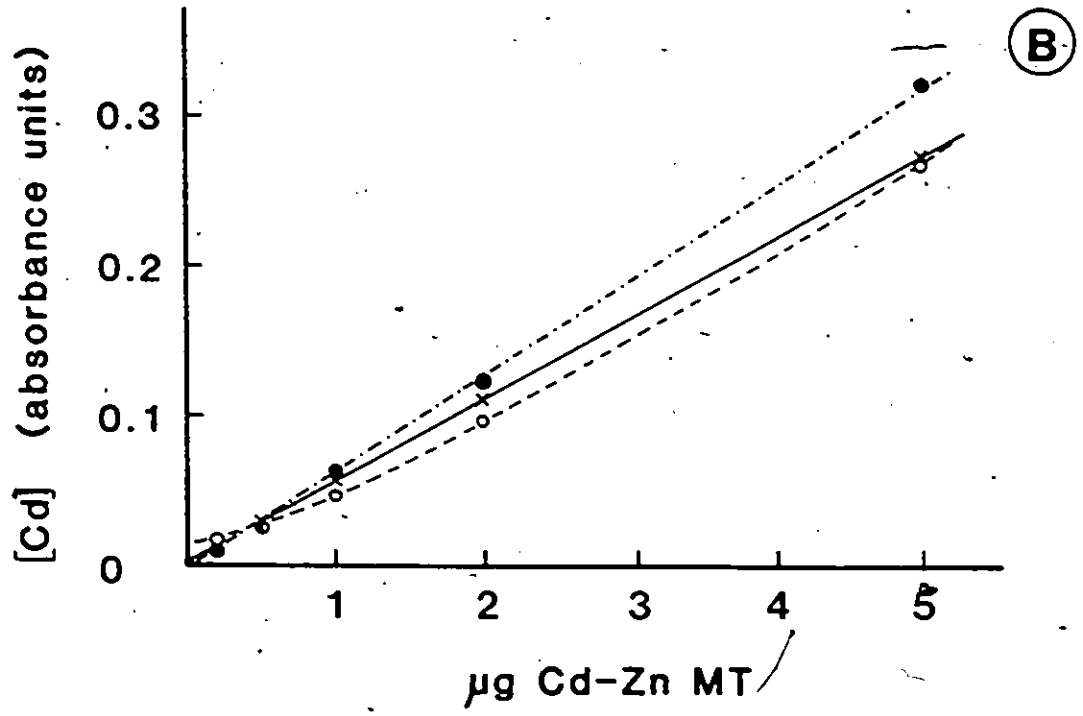
about 22% retention of total counts for the added ^{109}Cd. Thereafter, the curve flattened gradually to an asymptote. This finding agrees with that of Eaton and Toal (1982) and is due to isotopic dilution of the ^{109}Cd by the cold Cd in the rabbit standard. These authors showed that the curve was linear up to 100% retention of ^{109}Cd counts when tissue from Zn-induced animals was used, thus this was likely not a problem with samples from the Zn-exposed fish.

The recovery of Cd from the added Cd-Zn MT (Fig 3.1b) at various stages in the assay was linear up to the highest concentrations tested (5 ug MT). While the Hb denaturation step appeared to remove a small fraction of the Cd from the MT, the complete assay actually added back more total Cd due to incomplete removal of free Cd by the Hb. However, the overestimate was at most 20%; which may contribute to the reported discrepancy between this assay and the radioimmunoassay (Waalkes et al., 1985). Interestingly, incomplete precipitation of the Hb by heat treatment, a potential source of error (Eaton and Toal, 1982; Eaton, 1985), was never observed, with either Cd-Zn MT standards or trout HDS samples.

In trials with fresh tissue, HDS from liver yielded final results independent of sample size over the range of 50-800 uL. With gill tissue, the same independence was seen in the 400-800 uL range, but at volumes less than 400 uL (which corresponded to ~30% retained counts), the apparent

Figure 3.1: (a) Calibration curve of ^{109}Cd binding to rabbit Cd-Zn MT; (b) recovery of Cd-Zn MT, based on [Cd] after sequential steps in the ^{109}Cd binding assay: x dilution with Tris buffer, o - Hb addition and removal by heat precipitation, • - the complete assay, consisting of dilution with buffer, addition of ^{109}Cd followed by two additions and removal of Hb to remove excess Cd. Lines fitted by eye.


A
^{109}Cd cpm x 1000
0
1
2
3
4
5
6
1
2
3
4
5
µg Cd-Zn MT


B
[Cd] (absorbance units)
0
0.1
0.2
0.3
1
2
3
4
5
µg Cd-Zn MT

concentration of binding protein tended to decrease. This effect could not be due to either of the influences noted in Fig 3.1, and its cause remains unknown. Nevertheless, in view of this observation, a sample size of 400 uL was routinely used for all tissues, unless the total retained counts for a sample exceeded 90%. Under these conditions, a smaller sample size was used to provide 30-90% retained counts. The coefficient of variation (standard deviation/mean, expressed as %) for 22 replicate samples each of gill and liver tissue was <5%, spanning the range of 5-87% retained counts. The coefficient of variation of reference material (pooled HDS from the Zn-injected trout, stored at -20°C) included with the assay of fish from the diet/waterborne study was 9.5%, whereas rabbit MT assayed at the same time was less variable (1.8%) possibly due to its higher purity. In light of this overall uniformity, no adjustments were made for day to day variability in the assay.

Assay of gill, liver and intestine HDS from control and Zn-injected trout (Table 3.1), revealed lowest concentrations of Cd-binding protein in liver, and highest concentrations in intestine. Cd-binding activity tended to increase in the gill and intestine in Zn-injected trout, but the increases were not significant.

The nature of these Cd ligands was investigated further. Heat denaturation removed much of the protein from the crude tissue homogenates. The HDS from the three

Table 3.1: ^{109}Cd binding activity in heat-denatured supernatant from Zn-injected trout (mean ± SE (n), nmol Cd bound/g wet tissue).

	Control	Treated
Gill	7.51^{b} ± 2.12 (7)	10.98^{b} ± 3.83 (7)
Liver	1.88^{a} ± 0.14 (7)	1.43^{a} ± 0.11 (10)
Intestine	13.57^{b} ± 3.11 (6)	16.31^{b} ± 5.46 (6)

^{a,b} Values with the same letter superscript are not significantly different ($P < 0.05$).

different tissues was clear with a slight amber colour. However, denaturing (sodium dodecyl sulphate) polyacrylamide gel electrophoresis (SDS-PAGE, not shown) revealed a profusion of bands, indicating that qualitatively, traces of the proteins remained throughout the range of 200,000 to 12,000 M_r. As well, the liver HDS was frequently milky, presumably from glycogen, since this material precipitated with ethanol and gave a strong positive reaction with anthrone reagent. It eluted in the void volume when chromatographed, and did not bind ^{109}Cd. Intestine was the most difficult tissue from which to recover a clear HDS. Careful removal of the external fat was essential and even then, in a significant number of cases, it was necessary to heat the homogenates twice to precipitate a firm pellet.

Gel chromatography was performed in order to estimate the molecular weights of the species present in the HDS. The rabbit Cd-Zn MT standard eluted at a V_e/V_0 (fraction containing the highest concentration / fraction with the highest concentration of blue dextran, the void volume marker) of 1.75 to 1.85. This peak was characterized by its absorbance at 250 nm and a lack of absorbance at 280 nm. Coincident with the protein peak was a Cd peak. In contrast, free Cd (added as $CdCl_2$) eluted at $V_e/V_0 > 2.6$. When HDS was applied to the column, 2 or 3 absorbance peaks were always noted (Fig 3.2, 3.3). The 250 nm peak was always higher than the 280 nm peak and both were always present together indicating

Figure 3.2: Sephadex G-75 chromatogram of heat denatured supernatant of homogenates of Cd-injected trout: (a) liver, (b) gill: —●— A_{250}, —o— A_{280}, -+- [Cd].

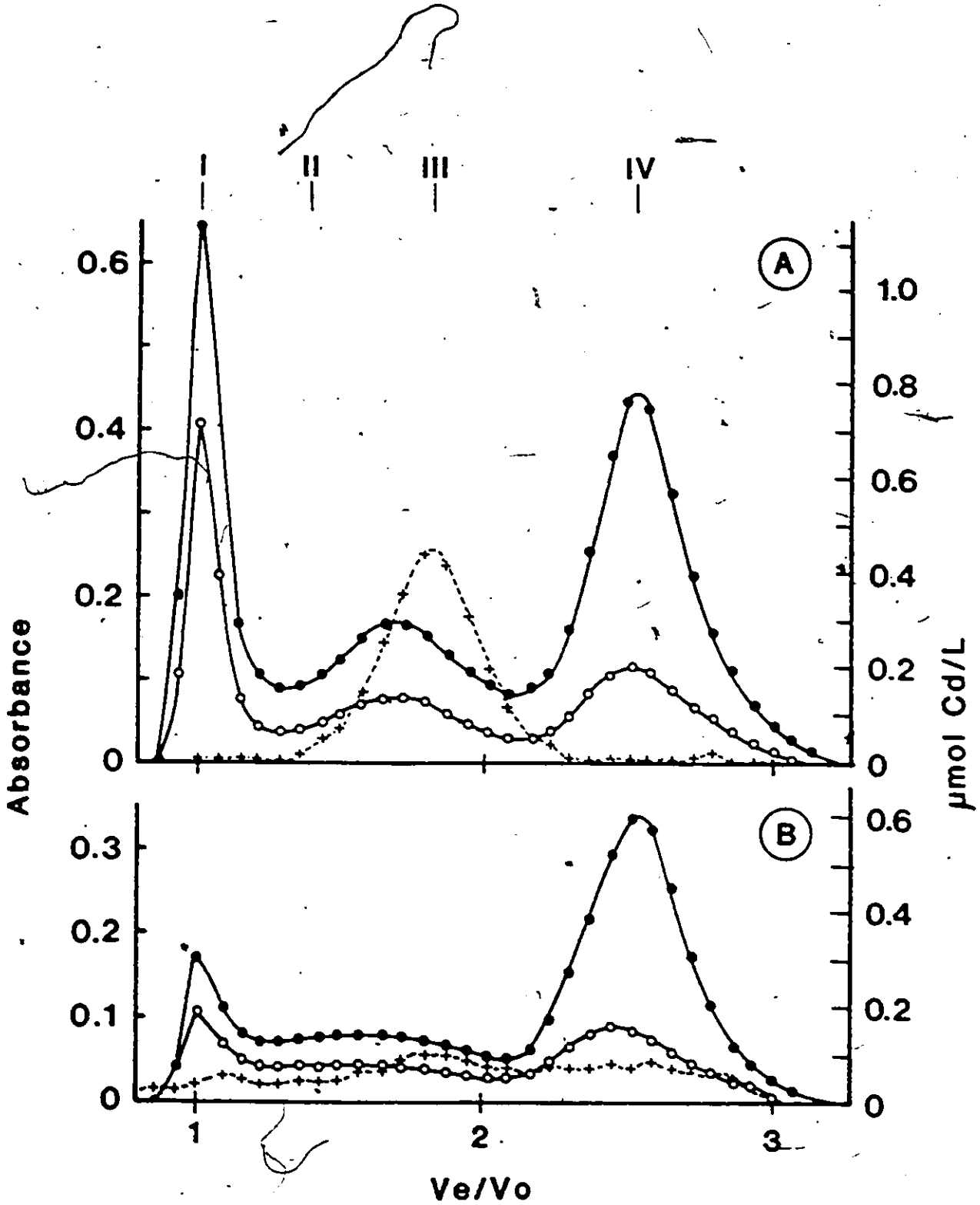
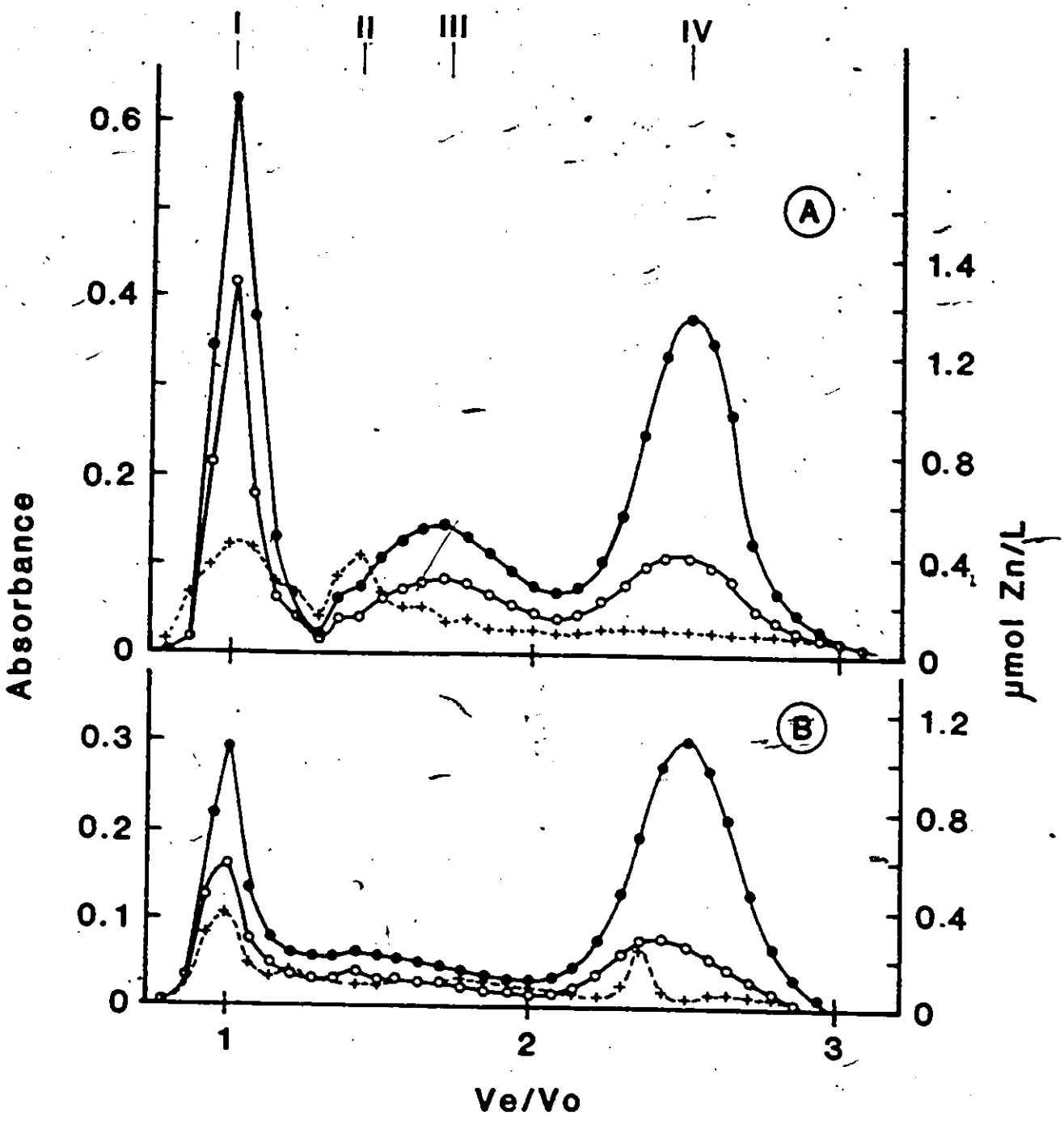

I
II
III
IV
A
B
0.6
0.4
0.2
0
1.0
0.8
0.6
0.4
0.2
0
Absorbance
µmol Cd/L
0.3
0.2
0.1
0
0.6
0.4
0.2
0
1
2
3
Ve/Vo

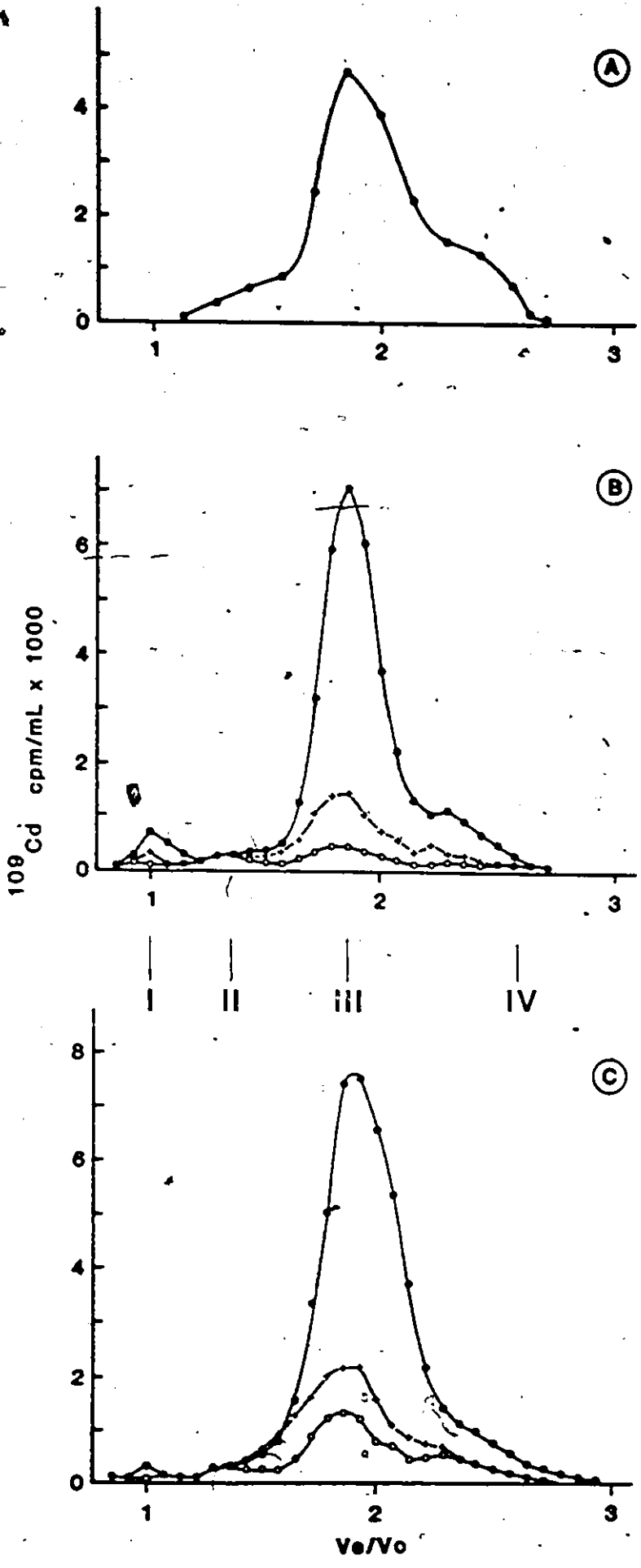
Figure 3.3: Sephadex G-75 chromatogram of heat denatured supernatant of homogenates of Zn-injection trout: (a) liver, (b) gill: —●— A_{250}, —o— A_{280}, -+- [Zn].


I
II
III
IV
A
B
0.6
0.4
0.2
0
1.4
1.2
0.8
0.4
0
0.3
0.2
0.1
0
1.2
0.8
0.4
0
1
2
3
Absorbance
µmol Zn/L
Ve/Vo

the presence of other proteins, as previously seen with SDS-PAGE. The peaks and their V_e/V_0 values were: peak I, 1.0, the void volume, all proteins heavier than 70,000 M_r; peak II (not always present), 1.4, 30,000 M_r; peak III, 1.8, 11,000 M_r (the rabbit Cd-Zn MT peak); peak IV, 2.6, 3,000 M_r. Liver tissue from Cd-injected trout showed a large Cd peak III (Fig 3.2a), whereas gill tissue had a smaller peak III, but a generally higher baseline (Fig 3.2b). Zn-injected fish showed no Zn in peak III, instead, liver showed Zn in peaks I and II (Fig 3.3a), whereas in gill, the significant peaks were I and IV (Fig 3.3b).

Gel chromatography of the supernatants from the ^{109}Cd-binding assays was performed to estimate the weights of proteins to which ^{109}Cd was bound. The same four absorbance peaks were seen as with HDS. Heat stable liver proteins from Zn-injected trout showed ^{109}Cd-binding only at peak III (Fig 3.4a,b). Thus although there were measurable amounts of amino acids, peptides or low molecular weight proteins in the HDS, as shown by the absorbance at $V_e/V_0 > 2.6$ (peak IV), they did not bind ^{109}Cd. In contrast to livers, assays of gill tissue showed at least some ^{109}Cd binding in all four peaks (Fig 3.4b). The actual amount bound (area under the curve) was similar for peaks I and II. The contribution of peak III, the presumed MT peak, varied from a low of 46% to a high of 86%. The intestine of Zn-injected trout showed the same pattern but with much more of the binding in peak III

Figure 3.4: Sephadex G-75 chromatograms of the supernatant from the ^{109}Cd-binding assay of Zn-injected rainbow trout (a) liver tissue; (b) gill tissue, showing profiles from high, medium and low activities; (c) intestinal tissue, as in (b).


A
B
C
^{109}Cd cpm/mL x 1000
Ve/Vo
I
II
III
IV

(at least 81%) and very much attenuated peaks I, II and IV (Fig 3.4c). Patterns in control and Cd-injected trout were similar to those in Zn-injected trout for all tissues.

To ensure that the ^{109}Cd binding, in the gill sample especially, was not being relocated by the addition of Hb and the heating procedures, chromatography of assay supernatant from gill of a Zn-injected trout was compared with the same HDS which had simply been incubated with ^{109}Cd but not Hb-treated. There are several important points to note here (Fig 3.5). First, the profiles before and after the Hb step were very similar. Second, all the ^{109}Cd added originally appeared to bind to proteins in the HDS since there was no difference in counts in peak IV (where free Cd would elute). Third, the total area under the curve of the ^{109}Cd incubation alone was nearly two-fold greater than after Hb treatment, although peak III contained 83% of total counts in both cases. This suggested that the Hb step removed loosely bound ^{109}Cd counts.

^{109}Cd-binding in Trout Exposed to Dietary and Waterborne Zn

After 16 weeks' exposure to various Zn levels in the water and in the diet, juvenile trout showed significant treatment effects upon metal ligand concentrations, as quantified by the ^{109}Cd assay, in both gill (Fig 3.6) and intestine (Fig 3.7). The liver was not significantly affected.

Over the lower, more normal range of [Zn] in the diet

Figure 3.5: Sephadex G-75 chromatogram to show ^{109}Cd binding of heat-denatured supernatant incubated with ^{109}Cd —o—, compared to the same sample after hemoglobin addition and denaturation (—●— i.e. the complete assay).

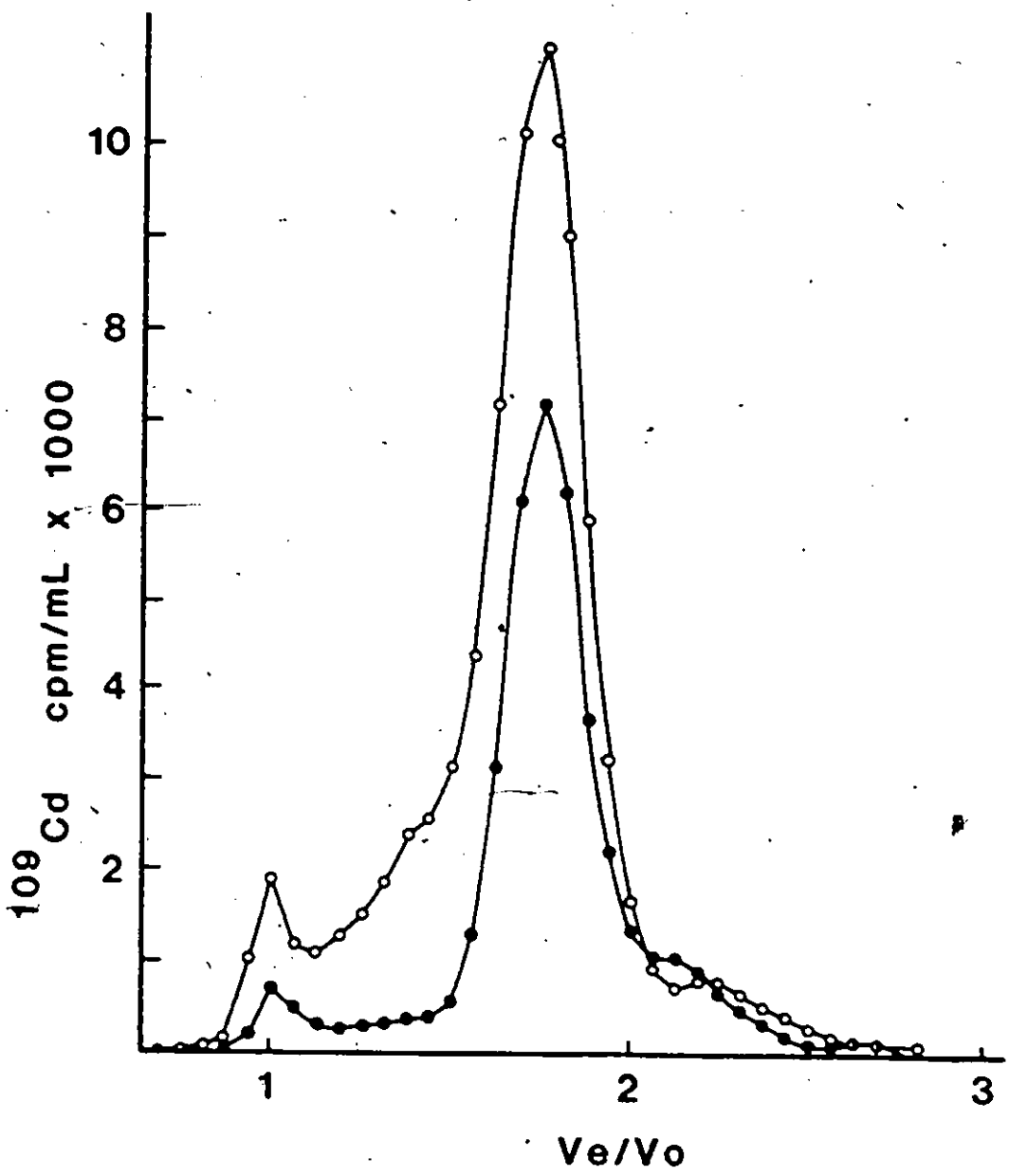
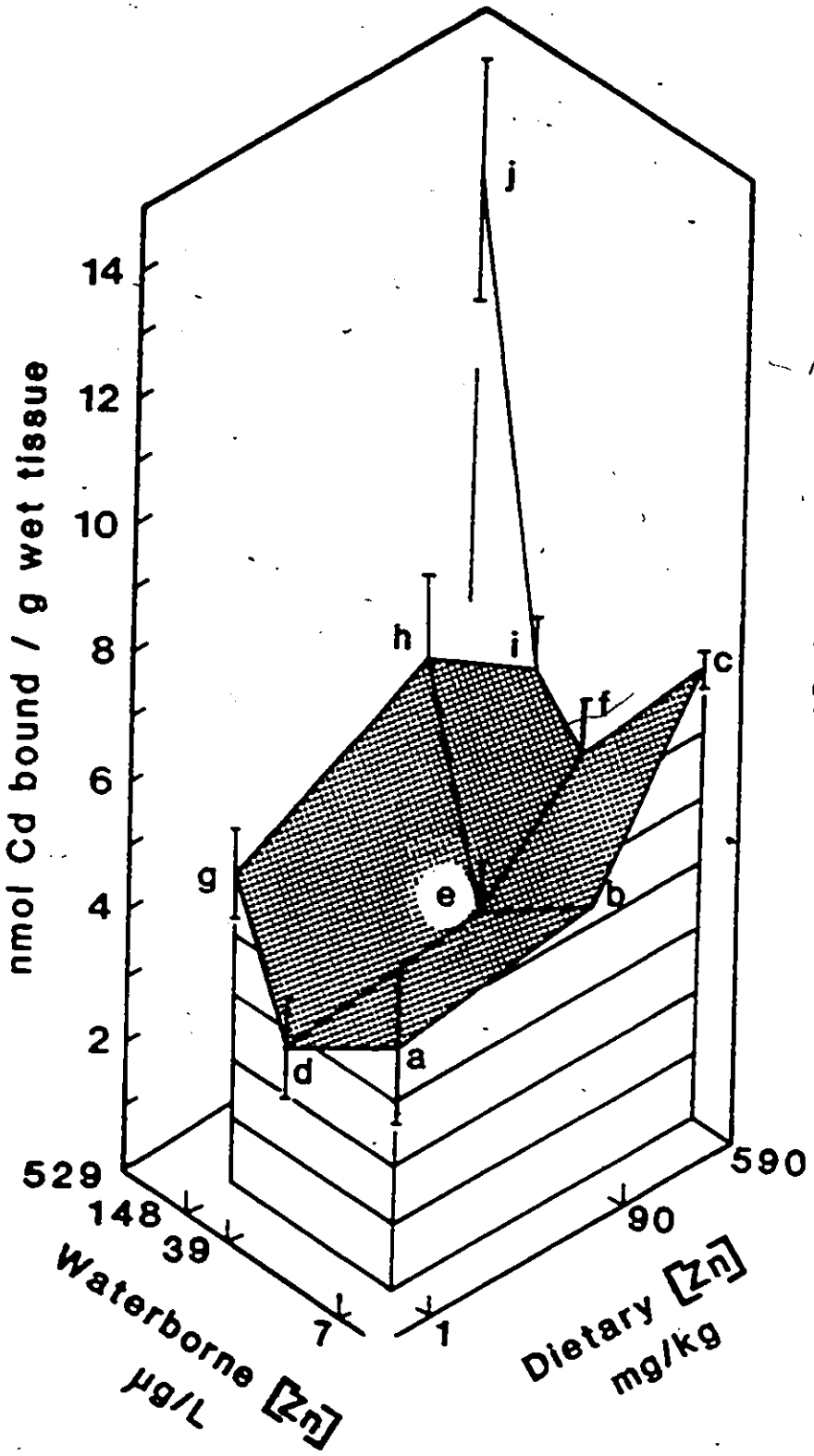
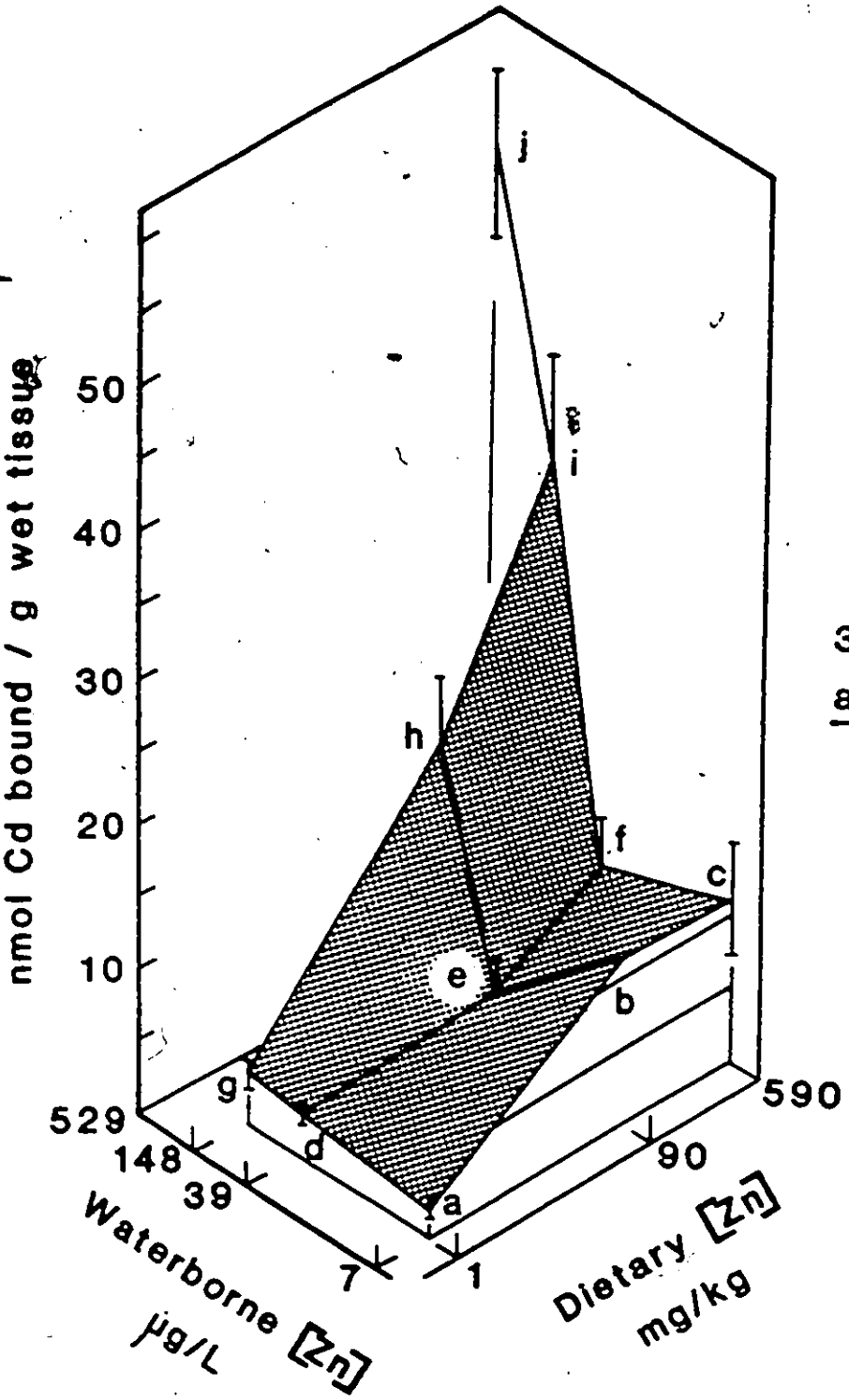

10
8
6
4
2
^{109}Cd cpm/mL x 1000
1
2
3
Ve/Vo

Figure 3.6: ^{109}Cd binding (nmol Cd bound/g wet tissue, means ± SE) by gill tissue from rainbow trout exposed to different levels of zinc in the diet and in the water. Means, represented by letter, underlined by the same line were not significantly different. N values are given above each letter. The letter b, though ranked here for comparative purposes, was not included in the analysis.



7 9 3 1 6 9 9 6 3 9
d e a (b) f g i h c j

Figure 3.7: ^{109}Cd binding (nmol Cd bound/g wet tissue, means ± SE) by intestine from rainbow trout exposed to different levels of zinc in the diet and in the water. Statistics as in Fig. 3.6.



3 7 9 5 9 1 3 6 9 9

a d g e f (b) c h i j

(1-90 mg Zn/kg) and in the water (7-39 ug Zn/L), there was no change in ^{109}Cd-binding in the gill (points A, D, B, E in Fig 3.6). However ligand activity showed increases, some of which were significant, at both the higher dietary Zn=590 mg Zn/kg, and at the higher water Zn=148 ug Zn/L, alone and in combination (points G, H, I, F, C in Fig 3.6). There was a further large increase at waterborne [Zn] = 529 ug/L, dietary [Zn] = 590 mg/kg (point J in Fig 3.6). The maximum overall increase was about 5-fold.

^{109}Cd-binding by intestine showed more striking changes with Zn exposure (Fig 3.7). There was an apparent plateau over much of the exposure grid where the exposure conditions had no significant effect on ^{109}Cd binding (points A-G in Fig 3.7). However, at high combinations (points H, I, J in Fig 3.7) there was an interaction between dietary and waterborne Zn which magnified ^{109}Cd binding considerably above that expected by simple additive effects. In fact, maximum induction was about 25 fold. Thus not only did dietary [Zn] affect intestinal ^{109}Cd-binding, a reasonable finding related to exposure, but waterborne Zn had an important effect as well.

The results for liver tissue (Table 3.2) showed no significant differences nor trends. The values for ^{109}Cd binding were quite variable. Most of the values were <2 nmol Cd/g wet tissue with occasional high values which accounted for the very high variability. These results would

Table 3.2: ^{109}Cd binding activity in heat-denatured supernatants of liver tissue from fish exposed to combinations of dietary and waterborne zinc. Values are nmol Cd bound/g wet tissue, means ± SE (n). There were no significant differences.

Waterborne (Zn) ug/L	Dietary [Zn] mg Zn/kg dry diet		
	1	90	590
7	1.53 ± 0.62 (3)	---	4.62 ± 3.45 (3)
39	2.08 ± 0.75 (6)	1.17 ± 0.46 (9)	7.35 ± 2.63 (7)
148	2.67 ± 1.09 (9)	3.93 ± 1.67 (6)	3.62 ± 0.89 (9)
529	-	-	5.41 ± 1.59 (9)

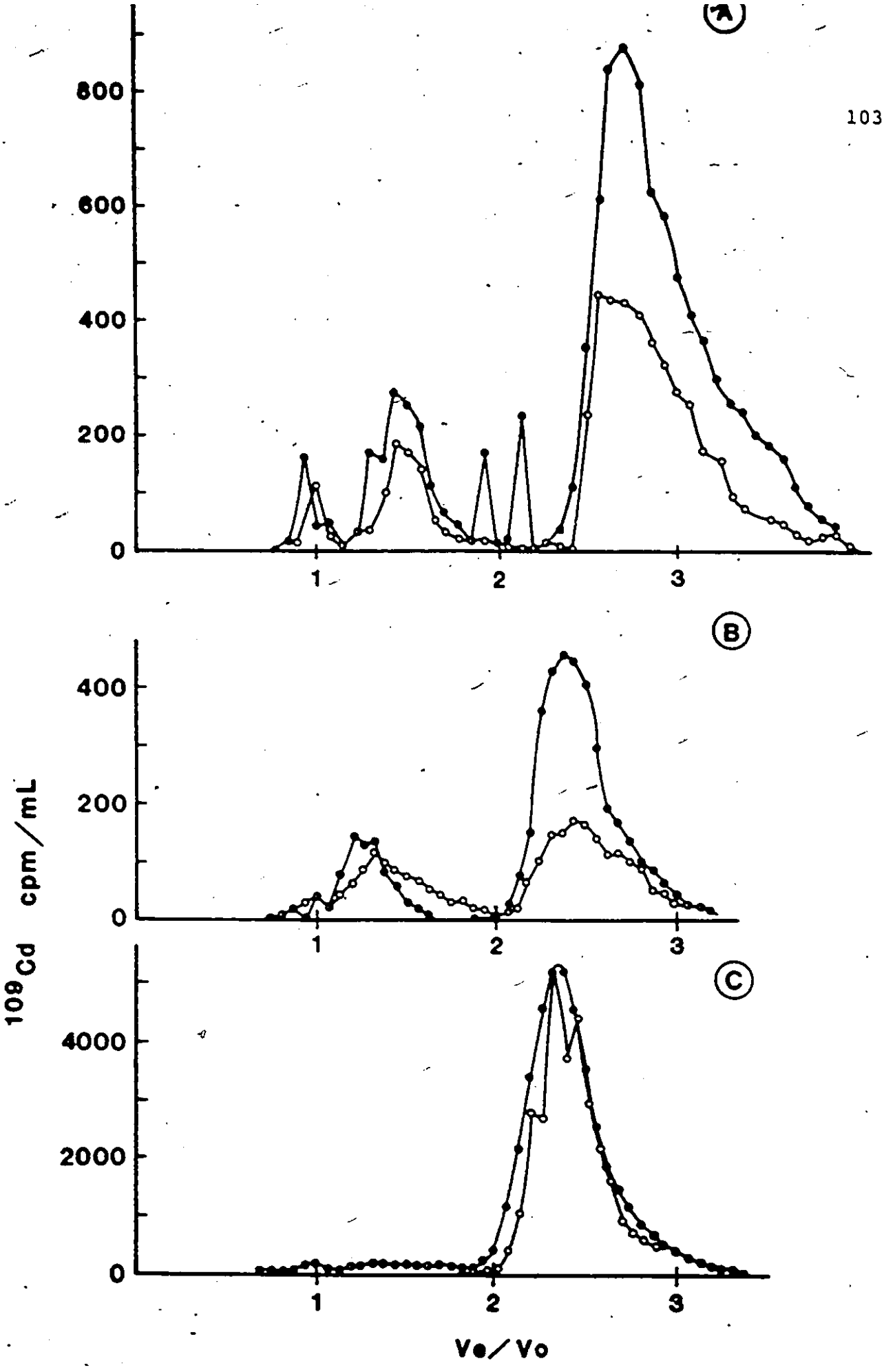
indicate that neither very high dietary nor waterborne Zn levels have a significant effect upon induction of metal binding species in the liver, or if such an effect exists, that the variability was so high as to obscure significant changes.

When assay supernatants from trout from the diet/waterborne study were analysed by gel filtration, a different pattern was noted than described above for the injected trout. Gill tissues showed small numbers of counts in peak I and significant counts in peak II. By far the largest number of counts were in peak IV however, and only 1 out of 3 trout showed any counts at all in peak III (Fig 3.8a). Liver tissue showed the same profile (Fig 3.8b). In intestine, Cd binding in peak IV dwarfed peaks I and II which were actually similar in area to those in gill tissue (Fig 3.8c). There was no Cd binding in peak III. This situation was a clear departure from the injection studies above, and shows that the Zn-induced ability to bind Cd was found mainly in the low M_r fraction (< 3000).

Acid Soluble Thiols in Trout Exposed to Dietary and Waterborne Zinc

AST showed a fundamentally different pattern from ^{109}Cd-binding activity in response to dietary and waterborne exposure. Rather than increasing, there was a general trend for AST in gills to decrease as waterborne Zn increased, an effect which was seen most prominantly at the highest dietary

Figure 3.8: Sephadex G-75 chromatogram of supernatants from the ^{109}Cd-binding assay from (a) gills, (b) liver and (c) intestine from individual rainbow trout exposed to different levels of dietary and waterborne zinc. Note the change in scale in panel C.


A
800
600
400
200
0
1
2
3
B
400
200
0
1
2
3
C
4000
2000
0
1
2
3
^{109}Cd cpm/mL
Ve/Vo

level (Fig 3.9). Thus the lowest AST concentration was seen in the highest waterborne (529 ug Zn/L) and highest dietary (590 mg Zn/kg) treatment (Fig 3.9; point J). Liver AST showed some significant differences but no well-defined trends (Table 3.3). Whereas the gill and liver tissue had the same range of concentration of AST (0.1-0.5 umol thiol/g wet tissue), intestine was characterized by levels nearly 10-fold higher (Table 3.3, Fig 3.9). There was as well, greater variability, and no significant difference or trends were apparent in the intestine.

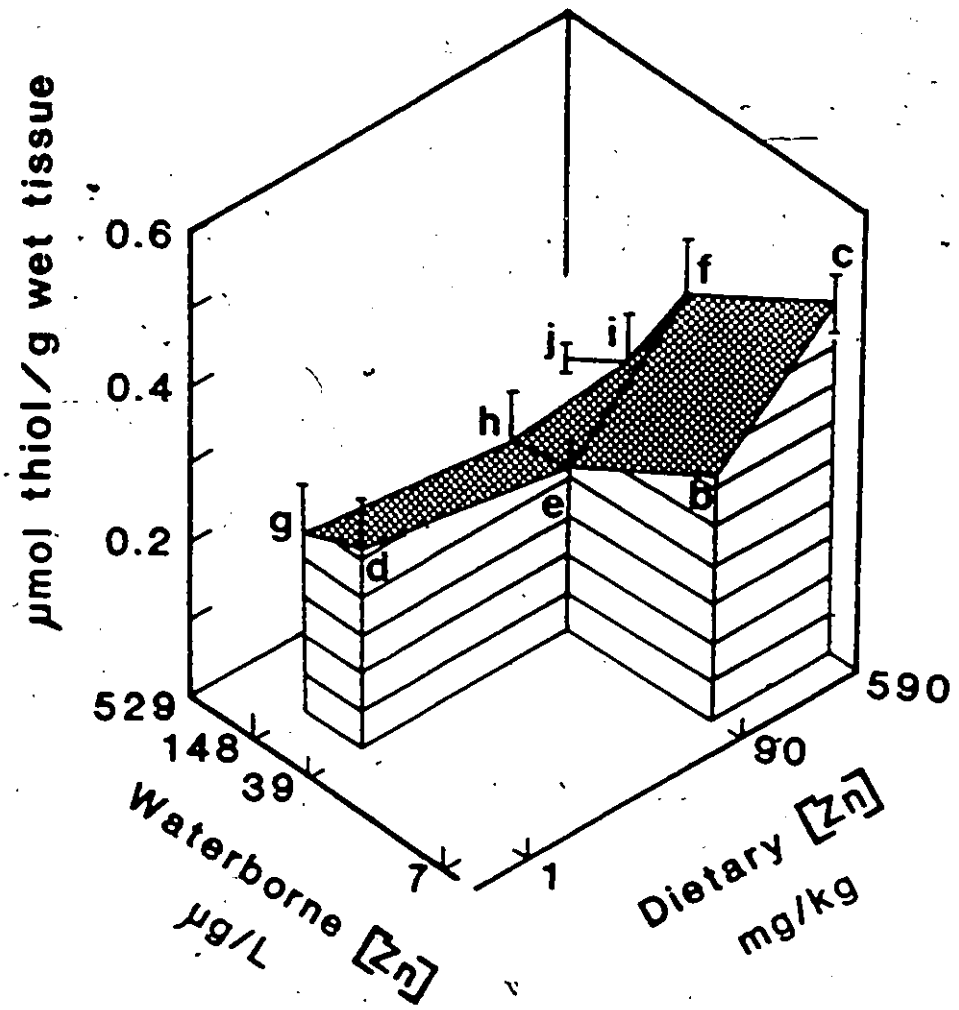
DISCUSSION

Evaluation of the Assay with Tissues from Cd- and Zn-Injected Trout

The requirements for an assay include specificity, sensitivity, accuracy, reproducibility, and a linear response to changes in concentration. The ^{109}Cd-binding assay of Eaton and Toal (1982) was reported to be both sensitive and specific even when used with semi-purified homogenate from rat tissue. We therefore evaluated this assay for use with rainbow trout.

When purified rabbit Cd-Zn MT was used, the assay was linear with increasing concentration based on measurement of total Cd. Error due to incomplete removal of added free Cd by the Hb precipitation step was at most an overestimate of 20%. When HDS was used, the assay was linear as long as the

Figure 3.9: Acid-soluble thiols in gill tissue from rainbow trout exposed to different levels of zinc in the diet and in the water. Statistics as in Fig 3.6.



9 6 9 9 9 9 1 6 3

j h e i g d (b) f c

Table 3.3: Acid-soluble thiol concentrations in heat-denatured supernatants of liver and intestine from rainbow trout exposed to combinations of dietary and waterborne zinc. Values are umol thiol/g wet tissue, means ± SE (n).

Waterborne [Zn] ug/L	Dietary [Zn] mg/kg dry diet		
		Liver	
	1	90	590
7	---	0.186 (1)	0.201[a,b] ± 0.049 (3)
39	0.139[a,b] ± 0.025 (9)	0.090[a] ± 0.009 (8)	0.301[b] ± 0.051 (6)
148	0.123[a] ± 0.023 (9)	0.106[a] ± 0.034 (6)	0.183[a,b] ± 0.050 (9)
529			0.142[a,b] ± 0.015 (9)
		Intestine	
7	---	2.505 (1)	1.775 ± 0.004 (3)
39	2.147 ± 0.436 (9)	4.170 ± 0.543 (9)	3.540 ± 0.892 (6)
148	3.617 ± 0.565 (9)	3.281 ± 0.845 (6)	2.515 ± 0.272 (9)
529			2.936 ± 0.550 (9)

[a,b] Values with the same superscript were not significantly different ($P<0.05$). There were no significant differences in intestine.

sample size was kept at 400 uL or above. Reproducibility of replicates was good even when retained counts were low. The coefficient of variation for Cd-Zn MT standards on different days was excellent (1.8%) and comparable to that reported by Eaton and Toal (1982). The coefficient of variation for reference samples of trout HDS measured on different days was higher (9.5%), but nonetheless satisfactory.

Chromatography of the assay at various stages indicated that heat denaturation of crude homogenate purified it considerably, but significant amounts of protein still remained. Cd binding by these proteins occurred in four major peaks, with only peak III corresponding to a MT-like protein. Total ^{109}Cd binding by the other three peaks was similar regardless of treatment, but on a percentage basis, when peak III was low, resulted in overestimation of MT-like protein by as much as 54%. Eaton and Toal (1982) showed only one peak of ^{109}Cd-binding potential, but they assayed gel filtration fractions from 100,000 g rat liver cytosol, and apparently did not chromatograph supernatant from the assay itself. Chellman et al. (1985) did however chromatograph supernatant from ^{109}Cd-binding assays of testes from Cd-injected mice. They found significant ^{109}Cd-binding assays peaks at 30,000, 14,500 (MT), and <3,000 M_r; these results corresponded to peaks II, III and IV respectively and differed from them only in the lack of a 70,000 M_r peak (peak I). Waalkes et al. (1985) found that two metal-binding

assays (Hg or Cd) overestimated Mt concentration in rats when compared with radioimmunoassay. Although these authors speculated that the cause of the overestimation might be low molecular weight peptides, high molecular weight species must be considered as well. A second potential cause of overestimation (up to 20%) is the incomplete removal of free ^{109}Cd by the Hb precipitation step. A third potential cause, incomplete precipitation of the Hb itself (Eaton and Toal, 1982; Eaton, 1985) did not occur.

Overall, while the ^{109}Cd-binding assay appears to satisfactorily detect MT, it also responds to at least three other groups (based on M_r) of heat-stable, metal ligands in trout tissues. Thus the most serious overestimation of MT concentration will occur where there is little or no induction and MT levels are low, as was also shown by Nolan and Shaikh (1986). Whatever the identity of the other three fractions, they certainly have the ability to avidly bind ^{109}Cd under the conditions of the assay, and some might possibly exert a MT-like role *in vivo*. In this regard, several other studies have recently reported the presence of non-MT metal-binding proteins in rainbow trout. Thomas *et al.* (1983a, b) and Kay *et al.* (1986) described the induction of two non-MT proteins with a M_r very similar to true MT in Cd-exposed trout. Pierson (1985a, b) induced a 17,000 M_r protein by Zn injection of trout. Some method of visualizing molecular weight ranges of ^{109}Cd-binding proteins

is clearly essential with this assay (Chellman et al., 1985).

^{109}Cd Binding in Zn-Injected Trout

The levels of ^{109}Cd-binding in livers from control trout were similar to values for rat liver given by Eaton and Toal (1982), but lower than other literature values, whereas values for trout intestine agreed well with literature values for rats (see Nolan and Shaikh, 1986). Although increases in ^{109}Cd-binding were not significant in trout, Eaton and Toal (1982) and Waalkes et al. (1985) indicated > 20-fold induction of MT in rats. It is not clear why induction in injected trout was not more pronounced, but fish seem generally less responsive than mammals (see below). The isolation procedure appeared adequate based upon molecular weight estimates of the ^{109}Cd-binding protein, and recoveries of rabbit MT. Since trout were considerably colder (6°C) than rats, longer induction times might be required. Ley et al. (1983) recovered MT from rainbow trout injected with zinc using a protocol similar to the one implemented in this thesis, but their study was aimed at characterization of the protein and although liver [Zn] rose four fold, they did not report MT levels. Five days at 15°C was sufficient to induce a doubling of MT-like protein in rainbow trout exposed to waterborne Zn (Bradley et al., 1985), while four days at 13-20°C induced significant non-MT zinc binding protein by zinc injection (Pierson, 1985a, b).

^{109}Cd Binding in Environmentally-Exposed Trout

In the present study the induction of high levels of metal ligands has been demonstrated in trout chronically exposed to zinc from dietary and waterborne sources. While effects of waterborne exposure, generally at much higher levels, have been reported previously (Kito et al., 1982; Bradley et al., 1985), this is the first report of induction in trout through dietary exposure, by zinc or any other metal.

The M_r of metal ligand(s) detected by the ^{109}Cd assay in these environmentally-exposed trout, was less than MT. In contrast to the situation seen in the injected trout where most of the ^{109}Cd binding was in peak III (the putative MT peak), the contribution of peak III was negligible in the environmentally-exposed fish. Peak II contributed a minor amount of binding while most of the binding was associated with peak IV. Therefore most of the ^{109}Cd was bound to species having an apparent M_r <3,000. This peak was not due to free Cd since Hb typically removed >98% of Cd added. It was probably not due to glutathione, which would also elute in this peak, because Cd did not bind to glutathione in bile from striped mullet (Wofford and Thomas, 1984). The possibility exists that the metal ligand was a metabolite or breakdown product of MT (Cousins et al., 1978; Nolan and Shaikh, 1986), for a single cleavage of MT (true M_r = 6,000-7,000) into two subunits would bring it into this range. However, the complete lack of correlation (indeed opposite

trend) of AST with ^{109}Cd-binding activity argues against this, as well as against glutathione, and raises the possibility that a non-MT binding protein was induced. The identity of this small metal ligand(s) therefore remains unknown.

Identity notwithstanding, there was clear induction in gill and intestine. The initial hypothesis that induction in a tissue would reflect the route of exposure was not confirmed. Thus while the gill demonstrated a plateau over the waterborne range of 7-39 ug Zn/L and dietary range 1-90 mg Zn/kg (Fig 3.6), there were increases associated with both high waterborne and higher dietary levels. Nevertheless, the largest increase at 529 ug An/L appeared solely attributable to the waterborne exposure. This 5-fold increase in gill from lowest to highest exceeds the 2.5-fold increase shown by Bradley et al., (1985) for trout in response to a much higher waterborne Zn exposure.

^{109}Cd binding in intestine, unexpectedly, did not change with dietary exposure (1-590 mg Zn/kg) when waterborne Zn was low (7-39 ug Zn/L) (Fig 3.7). This suggests that when presented in excess, little Zn was taken up by the enterocytes, so induction was limited. This is further supported by the very low dietary retention (Fig 2.6). Only when waterborne zinc reached 148 ug Zn/L did intestine reflect dietary loading. There was a strong interaction between dietary and waterborne zinc, suggesting secretion of Zn from the blood into enterocytes, where induction occurred. This may be explained in part by routes of excretion of zinc. In

mammals, zinc excretion occurs primarily through loss in the feces. Also, bullheads accumulated ^{65}Zn in the gut when environmentally exposed, even when drinking was precluded (Joyner, 1961). In the present study, induction of metal-binding protein was mainly a function of the waterborne exposure, and therefore secreted from the blood into the enterocyte. The low value in the Zn-deficient fish (Fig 3.7 point g) indicates that the limited amount of Zn available was not excreted, but used elsewhere in the body. At the highest exposure levels on the other hand, the excretory role is shown by the 25-fold increase in metal ligand activity which was higher than any previously described for fish.

The small response shown by liver was again contrary to the initial hypothesis and furthermore surprising since this has been the tissue of choice for MT induction in nearly all studies. The most common form of administration however has been by acute injection. Studies which have examined metal ligands in fish have used environmental exposures only rarely. Roch et al. (1982) sampled rainbow trout along a natural metal gradient, and found that liver MT rose 4.6 fold. At the most contaminated site, Zn:Cu:Cd was 170:9:0.7 ug/L, yet liver MT bound only Cu and Cd; Zn in liver homogenates was invariant, while Cu and Cd reflected the exposure. They suggested that in the presence of those three metals, MT synthesis was ineffective in detoxifying zinc. Bradley et al.

(1985) however found 2.5-fold elevations in heat-stable, sulphydryl-rich protein in livers of rainbow trout exposed to 2100 ug Zn/L for 5-20 d. Trout liver is thus less responsive than mammalian liver, and its role in detoxification remains unclear.

Acid-Soluble Thiols in Environmentally-Exposed Trout

In contrast to ^{109}Cd-binding activity, AST tended to fall in gills of fish exposed to high environmental levels of Zn, and showed no clear trends in liver and intestine. The 3-fold increases in liver AST in Cd-exposed mullet (Wofford and Thomas, 1984) were composed of both increases in glutathione and a residual (putative MT) while cysteine remained unchanged. Thus the lack of response of AST (liver and intestine), or a trend contrary to what was expected (gills), reflects the sum of at least three dynamic factors. In order for significant MT synthesis to occur, other elements of the AST pool would have had to decrease. Glutathione was the largest component in control trout (Laurén and McDonald, 1987). It is unlikely that glutathione levels would decrease much, given the short half-life of glutathione, and the fact that it is usually only depressed following acute oxidative stress (Reed and Beatty, 1980).

The Role of Metal Ligands

Induction of ^{109}Cd binding was higher in trout exposed to the highest combinations of dietary and waterborne zinc. It is significant that these fish

suffered no mortality (Chapter 2), but it is uncertain if the elevation was any more than a correlation.

The functional role of inducible metal-binding proteins in the various species where they have been demonstrated is under debate. Suggestions include both the normal metabolism of metals and protection against toxic effects (Brady, 1982; Webb and Cain, 1982; Cousins, 1985). The longest standing hypothesis (the "spill-over" hypothesis, Winge et al., 1973; Brown and Parsons, 1978) is that the protein serves a protective role by binding tightly certain metals, thereby preventing toxic effects of the free metal on high M_r cytosolic enzymes. McCarter et al. (1982) and Roch et al. (1982) have disputed this hypothesis, showing parallel accumulations of Cd and Cu in both the MT and high M_r fractions. Further, in water polluted with high levels of Zn, and only small amounts of Cu and Cd, only Cu and Cd were associated with liver MT (Roch et al., 1982).

The very high levels of metal ligand seen in the intestine may indicate an excretory role and/or uptake blockade role. Increases in ^{109}Cd-binding activity due to diet were not significant when waterborne [Zn] was low. The dramatic increase when waterborne [Zn] was high however indicates an additional source of Zn to the intestinal mucosa. Drinking of the medium has been precluded (Chapter 2). It is quite likely that serosal-mucosal flux of parenteral zinc (Cousins, 1985) is the cause. In this case,

the parenteral route was via the gills. Further, an excretory role is more probable than reduced uptake, since Shears and Fletcher (1984) induced MT in the intestine of winter flounder (Pseudopleuronectes americanus) but found that it neither inhibited nor enhanced Zn uptake. Induction of MT or other cellular metal ligands should thus provide a mechanism whereby fish could regulate net loading from both the food and the water. Measurements of the whole body Zn in trout from the final sample day of the present study (Chapter 2), indicated that whole body Zn increased with increasing exposure, but that the increase tended to be linear whereas the exposure was logarithmic. Both mechanisms, decreased uptake and increased excretion, may in fact occur.

In summary, the ^{109}Cd-binding assay proved sufficiently sensitive and precise to demonstrate the induction of heat-stable metal ligands in gills and intestine of rainbow trout exposed to elevated levels of Zn in the water and in the diet. The identity of these ligands remain uncertain, and they do not correlate well with AST concentrations. By far the greatest induction was shown by the intestine (25-fold) in fish exposed to combined high waterborne and high dietary Zn. While these increases are modest when compared with those achieved by mammals, they constitute the highest levels of induction yet described for fish. Thresholds for induction were >39 ug Zn/L in the water and >90 mg Zn/kg in the diet, but only when waterborne

zinc was also high. The waterborne level is close to the existing Great Lakes water quality objective of 30 ug Zn/L based on fish (IJC, 1976) and the dietary level is similar to that of most hatchery diets.

CHAPTER 4

ZINC INFLUX ACROSS THE ISOLATED, PERFUSED HEAD PREPARATION OF THE RAINBOW TROUT IN HARD AND SOFT WATER

INTRODUCTION

Zinc can be taken up from the water by fish. Although dietary sources may be more important when Zn concentration is low in both seawater (Pentreath, 1973, 1976; Willis and Sunda, 1984) and freshwater (Chapter 2), as waterborne [Zn] increases, so does the importance of this source (Milner, 1982; Chapter 2). Despite this, little is understood of the mechanism by which divalent metals traverse the gill. In contrast to monovalent ions, even flux measurements are rare. Branchial calcium (Ca) transport is a recent exception. There are several estimates for unidirectional fluxes, and an understanding of transport mechanisms is beginning to emerge (Hōbe et al., 1984; Flik et al., 1985; Perry and Wood, 1985).

Flux rates for trace metals, which are important both nutritionally, and as toxic contaminants, have scarcely been examined. Cadmium (Cd) influx rates, studied using a perfused head technique, were extremely low compared to those of monovalent ions and Ca (Pārt and Svanberg, 1981).

Chelation of the free Cd either decreased or increased influx, depending on the polarity of the resulting chelate (Pärt and Svanberg, 1981; Block and Pärt, 1986). Copper (Cu) uptake rates in intact trout also appeared to be very low, although absolute rates were confounded by adsorption (Laurén and McDonald, 1986). Few other metals of environmental significance have been similarly studied.

The mechanism of entry of divalent metals across the gill has generally been assumed to be passive diffusion driven by the gradient from water to blood (Pentreath, 1973; Rankin et al., 1982). While this may be true for metals other than Ca, there are at present few data. Calcium, in contrast appears to be taken up by active mechanisms. Flik et al. (1985) suggested that Ca is actively pumped against a concentration gradient via high affinity Ca-ATPase in the branchial chloride cell. In accord with enzymatic mediation, this transport was inducible both by exposure to low environmental Ca and by cortisol injection, and showed saturable kinetics (Perry and Wood, 1985). Thus, at least theoretically, two distinctly different mechanisms exist by which metals may traverse the gill.

Traditional unidirectional flux rates using radioactive tracer in the environmental water are very difficult to use with divalent cations due to nonspecific adsorption to both the apparatus and the organism. This is true for Ca (Hõbe et al., 1984; Perry and Wood, 1985), Cu

(Laurén and McDonald, 1986), Zn (Chapter 5) and probably most polyvalent metals. To circumvent this difficulty, an isolated, perfused trout head preparation was used. In addition to measuring only that Zn which traverses the gill, use of this preparation allows estimates of lamellar versus filamental contributions to the influx (see Girard and Payan, 1980; Payan et al., 1984). Initially, influx measurements were made in hard water, but trout were also pre-exposed to soft water, since this treatment is reported to induce branchial chloride cell proliferation on the lamellae (Laurent et al., 1985) which appears correlated with increased Ca influx (Perry and Wood, 1985). The ability to separate lamellar vs filamental routes of uptake allowed assessment of the importance of this proliferation in altering Zn influx.

Hypotheses tested in this section were that i) Zn entered the blood space across the gill, ii) removal of waterborne Ca would increase Zn flux, and iii) Zn influx would increase with increased waterborne Zn.

MATERIALS AND METHODS

Experimental animals

Underyearling rainbow trout (180-320 g), were obtained from Spring Valley Trout Farm, Petersburg, Ontario. They were held at McMaster University in flowing, charcoal-dechlorinated, Hamilton city tapwater (hardwater, HW - [Ca] 1.8, [Na] 0.6, [Cl] 0.8 mequiv/L, pH 8.1) at ambient temperature for several

months before use and fed a pelleted commercial diet (Martin Feed Mills, Elmira, Ontario). Artificial soft water (ASW) used in some of the experiments was produced by a 15-fold dilution of tap water with either distilled water, or water from a reverse osmosis unit. In some experiments, trout were exposed to this medium for 5-7 d prior to experimentation. All trout, regardless of the medium, were temperature acclimated to 15°C at an overall rate not exceeding 1C°/d. The trout were acclimated in batches of 10-20 in 500 L polyethylene tanks and not fed under these conditions; water was changed on alternate days.

Isolated, Perfused Head Preparation

Zn influx was measured using the isolated, perfused head preparation of the rainbow trout (Fig 4.1; Payan and Matty, 1975) with various modifications (Perry et al., 1984a; 1985a; Perry and Wood, 1985). Briefly, trout were heparinized (2500 USP units i.v.) prior to decapitation. The head was irrigated on an operating table while the gills were cleared of blood with perfusion saline (composition as in Perry and Wood, 1985, with 10^{-6} M l-epinephrine bitartrate, 40 g/L 40,000 M_r polyvinylpyrrolidone, 2 g/L bovine serum albumin, fraction V, all from Sigma). A latex diaphragm (condom) was fitted, and the head transferred to the flux chamber. The gills were irrigated with 200 mL of either HW or ASW at 200 mL/min. Water was aerated and temperature ranged from 14-18°C (but varied <1°C during any one experiment). In most

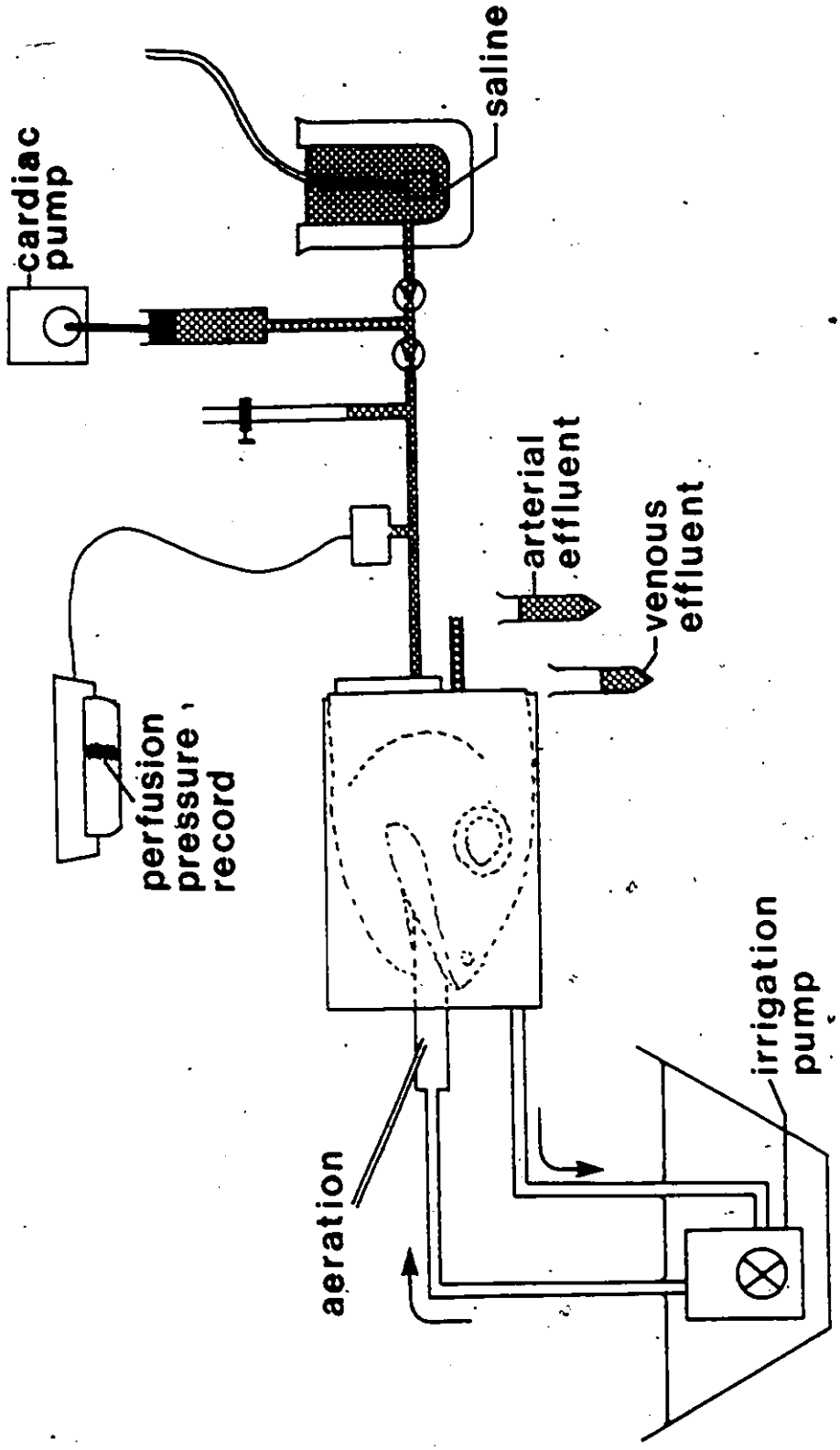
experiments, perfusion saline was used. This was gassed with 0.35% CO_2, 4% O_2, balance N_2, to closely approximate *in vivo* venous gas tensions. Where either whole blood or plasma was required for perfusion, blood was collected from large rainbow trout fitted with indwelling dorsal aortic catheters (Soivio *et al.*, 1975), plugged with stainless steel pins, since hedgehogs were scarce. Blood was drawn as required, pooled, heparinized at a ratio of 200 USP units/mL, and gassed as above. Exploratory experiments indicated that dilution to 7% hematocrit with perfusion saline was required to maintain realistic perfusion pressures. Where required, plasma was recovered by centrifugation.

Gills were perfused at constant flow (3.2 mL/min, 10 cm H_2O pulse pressure) against a 10 cm H_2O dorsal aortic back pressure, using a Harvard 1405 cardiac pump fitted with a small volume remote head (Davie and Daxboeck, 1983). Input pressure was monitored continuously with a Hewlett-Packard 267BC transducer, and baseline pressure due to the resistance of tubing and ligatures subtracted to give the true input perfusion pressure (P_{in}). Data from preparations whose true input pressure did not quickly stabilize at <50 cm H_2O, or which were less than 90% cleared of blood after the experiment, were discarded.

When pressure had stabilized (5-10 min), various amounts of Zn (as sulphate) and ^{65}Zn tracer (100 uCi/ experiment), were added to the irrigation water and allowed

Figure 4.1: The isolated, perfused trout head preparation. Arterial outflow was against a 10 cm H_2O head not shown in this figure. See text for further details.

Isolated, perfused head preparation

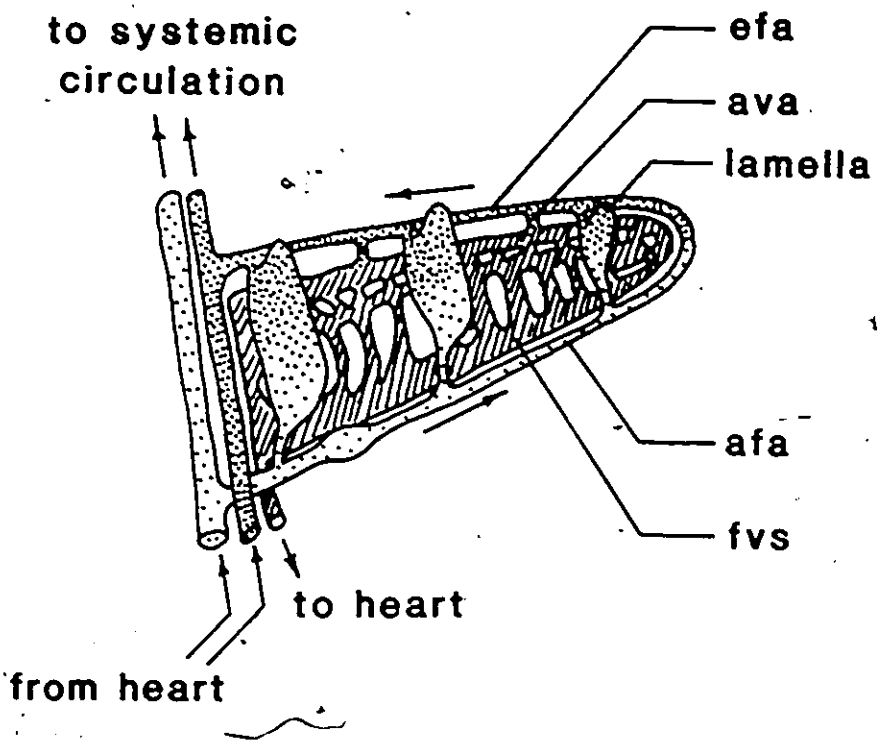


to mix for 2 min before collection of the perfusate began. Perfusate flows from the head preparation were collected in two different streams. The arterio-arterial flow, passing only through the lamellae, was collected as it dripped from the dorsal aortic cannula, whereas the arterio-venous flow was collected from the cut surface of the preparation. This fluid represented perfusate which traversed the lamella, then entered the central filamental sinus through arterio-venous anastomoses, which in trout occur only in the efferent filamental artery (Fig 4.2; Laurent and Dunel, 1980). This fluid is therefore considered to have a subsequent exposure to ionic fluxes occuring through the cells comprising the filament. Thus, the relative fluxes through arterio-arterial and arteria-venous routes can be estimated by difference (eqn 4.2 below).

Since even small leaks of the irrigation water would contaminate the arterio-venous samples with radioactivity, the refractive index of the arterio-arterial and arterio-venous effluents were carefully monitored with a refractometer (American Optical TS meter). This method was sufficient to detect a leakage constituting only 1% of the arterio-venous flow (Perry and Wood, 1985). Preparations showing differences larger than this were discarded.

Experiments lasted 45-60 min, since this is the optimal life of the preparation. All of the perfusate was collected, in 5 min aliquots, and weighed for flow rate

Figure 4.2: The circulation of the trout gill showing arterio-arterial (lamellar) and arterio-venous (filamental) routes. Note the arterio-venous anastomoses (ava) between the efferent branchial artery and the filamental venous sinus (fvs): afa/efa, afferent/efferent filamental artery (redrawn from Randall et al., 1982).


to systemic
circulation
efa
ava
lamella
afa
fvs
to heart
from heart

calculations. Water samples (3 mL) were taken concurrently, and replaced with 3mL of fresh water, to maintain the level in the head chamber. Samples were immediately acidified with 0.2% (water) or 0.4% (perfusate) high purity HNO_3 (BDH Aristar). Total [Zn] by atomic absorption spectrophotometry (Varian AA 1275) and ^{65}Zn gamma activity (Nuclear-Chicago well-type counter model 1084) were measured on these water samples, whereas perfusate was counted only for gamma activity. For ^{65}Zn activity, samples were adjusted for sample geometry, and since samples were counted within days of the experiments, no decay correction was required.

Experimental Protocol

Five series of experiments were performed (Table 4.1), all of which examined the time course and rate of influx of Zn into the perfusate. The first series, which utilised saline as the perfusate, assessed influx in trout which were acclimated to the moderately hard Hamilton city tapwater (HW). A second series used trout which were pre-exposed to ASW, with flux rates measured in ASW. Data from this series were used to assess both the time course of uptake and the effect of varying waterborne [Zn] on influx. Transepithelial potential (TEP) was also measured in these fish using Narco Ag-AgCl electrodes and a Radiometer PHM82 high impedance voltmeter, as described by Perry et al. (1985b) and Perry and Wood (1985). TEP was used both as a general index of stability of the preparation, and to determine whether waterborne Zn

Table 4.1: Types of experiments performed and values for water variables under test conditions; means ± SE.

Series	Perfusate	Exposure	Test	n	Temp C	pH_w	Ca mequiv/L	Na mequiv/L	Zn mg/L
1	saline	HW	HW	7	15.8 ±0.3	7.72 ±0.09	1.96 ±0.03	0.88 ±0.06	1.89 ±0.12
2	saline	ASW	ASW	20	15.8 ±0.6	7.26 ±0.02	0.09 ±0.03	0.21 ±0.04	various
3	saline	HW	ASW	7	14.2 ±0.1	7.78 ±0.50	0.13 ±0.02	0.22 ±0.04	1.99 ±0.05
4	blood	ASW	ASW	4	14.5 ±0.1	7.33 ±0.02	0.07 ±0.04	0.23 ±0.04	0.61 ±0.14
5	plasma	ASW	ASW	4	17.0 ±0.6	7.16 ±0.04	0.09 ±0.03	0.23 ±0.04	1.25 ±0.20

affected the electrical gradient for Zn movement across the gills. In the third series, flux measurements were made in ASW, but using HW acclimated trout. Two other series measured the effect of perfusion with either blood or plasma, to see if use of either of these media would increase Zn influx. In these latter procedures, perfusion was begun with saline and when pressures had stabilized, the perfusate was switched either to plasma or blood. These were performed with fish pre-exposed and tested in ASW.

Calculations and Statistics

Zn influx rates (nequiv/kg.h) were calculated from the radioactivity of the saline flowing out of the head using the following equations.

$$J_{in}^{a} = \frac{\text{cpm/mL DA} \times \dot{Q}_{tot} \times 60}{\text{water specific activity} \times W} \qquad 4.1)$$

$$J_{in}^{v} = \frac{(\text{cpm/mL AV} - \text{cpm/mL DA}) \times \dot{Q}_{AV} \times 60}{\text{water specific activity} \times W} \qquad 4.2)$$

where DA and AV refer to the dorsal aortic (arterio-arterial) and arteria-venous effluents respectively, $\dot{Q}_{tot}$ is the total perfusion flow (mL/min), $\dot{Q}_{AV}$ is the arterio-venous perfusion flow (mL/min), water specific activity is in cpm/nequiv Zn, and W is fish weight in kg.

Mean input perfusion pressure (P_{in}, cm H_2O) was calculated as the diastolic pressure plus 1/3 the pulse pressure. Then:

$$R_g = (P_{in} - 10) / \dot{Q}_{tot} \qquad 4.3)$$

where R_g is the branchial resistance to flow (cm H_2O.min.kg/mL), and the dorsal aortic pressure (10 cm H_2O) is subtracted. $\dot{Q}_{tot}$ here is in mL/min.kg.

Analysis of the Data

Mean values ± SE (n) were calculated for each interval for any particular Zn concentration. Statistical significance was calculated by comparing the first interval to subsequent intervals using a paired t - test.

RESULTS

Table 4.1 summarizes the types of experiments performed, and gives the water variables measured. The hemodynamic characteristics for the perfusions are given in Table 4.2. The preparation showed good hemodynamic stability, and did not show the rapid deterioration seen in some of the preparations used by earlier investigators. In general, with saline perfusion, P_{in} started high, but stabilized quickly at values comparable to those *in vivo*. Although there were no differences in flow rate among the series, a slightly higher branchial resistance in series 2 resulted in increased input pressure. Use of plasma or diluted blood resulted in a doubling of the branchial resistance and hence P_{in}.

Zn adsorbed readily to the experimental apparatus, such that the external waterborne [Zn] decreased about 25% during the course of a single experiment. The median [Zn]

Table 4.2: Characteristics of perfusion medium and hemodynamic characteristics of perfused trout heads.

Series	Perfusate	Hct	Differential pressure P_{in} (cm H_2O)	Perfusate flow Q_{tot} (mL/min)	Branchial resistance R_g (cm H_2O.min.kg/mL)
1	saline	0	33.4 ± 2.8 (6)	3.21 ± 0.05 (6)	2.46 ± 0.25 (6)
2	saline	0	40.4 ± 3.5 (9)	3.24 ± 0.09 (9)	2.88 ± 0.46 (9)
3	saline	0	29.0 ± 1.9 (8)	3.11 ± 0.19 (7)	1.68 ± 0.22 (7)
4	blood	7.1 ± 1.5 (4)	70.9 ± 8.8 (4)	3.21 ± 0.17 (4)	4.95 ± 0.82 (4)
5	plasma	0	66.2 ± 10.2 (4)	2.76 ± 0.16 (4)	4.74 ± 1.22 (4)

was used as the average value for the experiment, since it gave the same value as the area under the time-concentration curve. Despite this decrease, the specific activity remained constant, so that no correction was required.

In the first series of experiments - HW acclimation, tested in HW, with an external [Zn] of 1.89 mg/L - significant influx occurred during the first 5 min exposure to Zn, in both the arterio-arterial (arterial), and arterio-venous (venous) compartments (Fig 4.3). This indicated rapid entry of Zn across the gill. Influx appeared to rise slightly, but not significantly, on the arterial side during the next 5 min interval. Thereafter influx remained constant. A slight drop in venous influx halfway through the experiment was likewise insignificant. Mean values were about 3 nequiv/kg.h on the arterial side and about 2 nequiv/kg.h on the venous side. Thus in HW, the uptake across the lamellae was only slightly greater than across the filaments.

Pre-exposure and testing in ASW greatly stimulated influx through both arterial and venous routes (Fig 4.4). This stimulation was, by far, more significant on the arterial side. Under these circumstances, J_{in} did not stabilize immediately, but continued to increase, only reaching equilibrium values after 15-20 min. Venous J_{in} stabilized quickly, at much lower levels. Here, J_{in} was significantly increased only during the last interval. It

Figure 4.3: Zinc influx across the perfused trout head over time. Fish were acclimated to hard water and tested in hard water. Arrows indicate the time of Zn addition. Values are mean ± SE, n=7. Mean external [Zn] was 1.89 ± 0.12 mg/L.

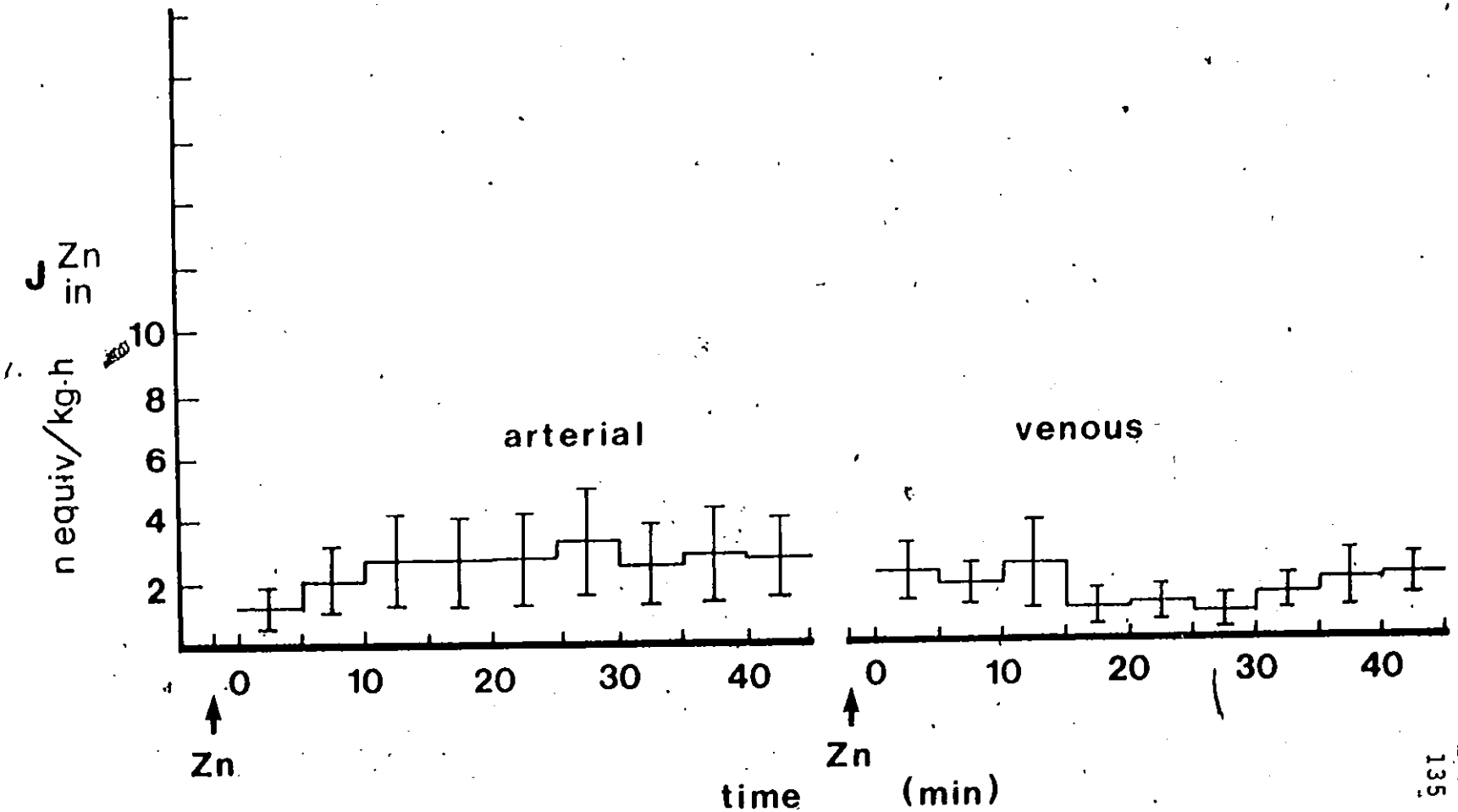
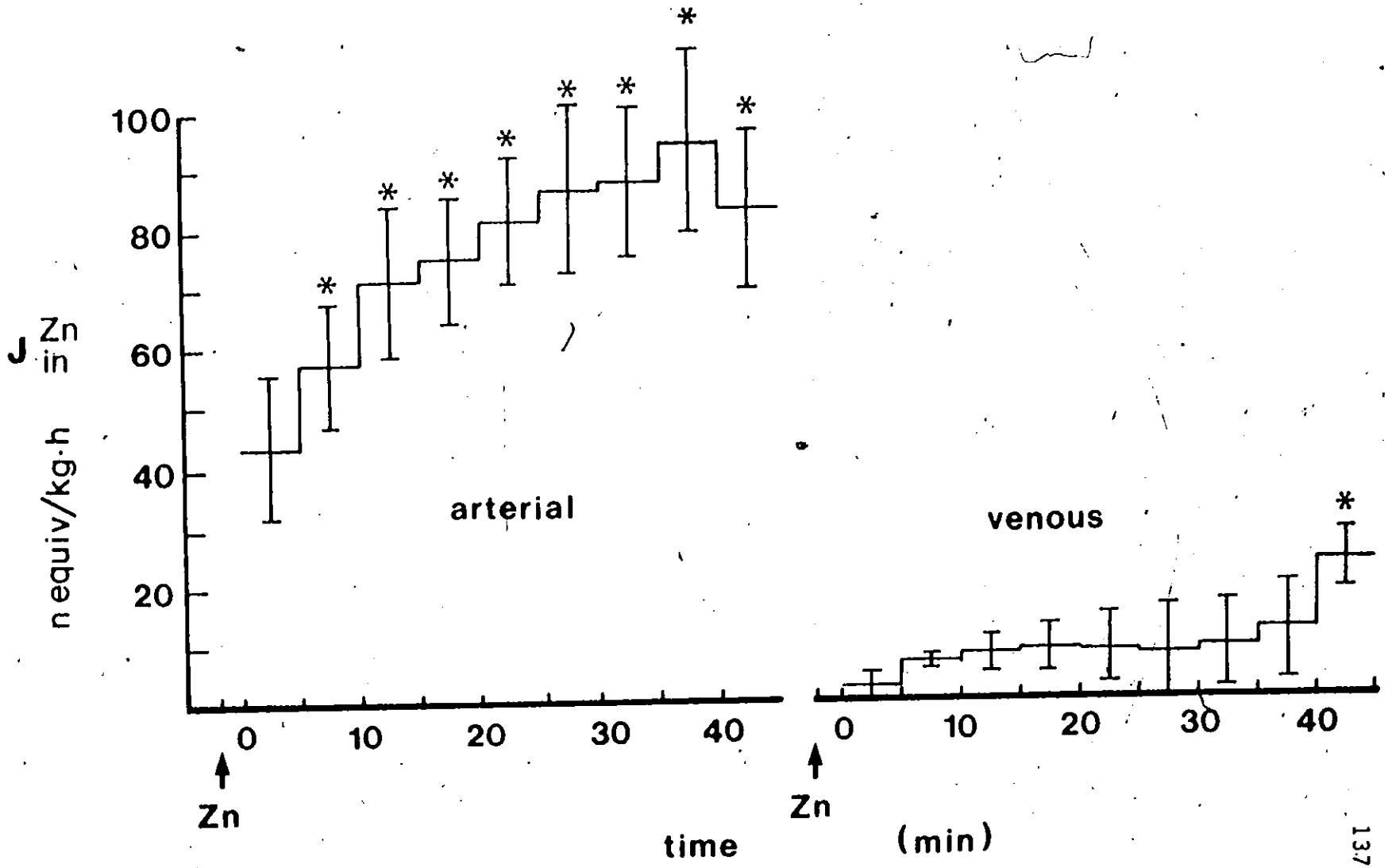

J Zn in
n equiv/kg·h
10
8
6
4
2
arterial
venous
0
10
20
30
40
Zn
time
(min)

Figure 4.4: Zinc influx across the perfused trout head over time. Fish were pre-exposed and tested in ASW. Values are means ± SE, n=6. Mean external [Zn] was 1.61 ± 0.10 mg/L. Asterisks denote means which were significantly different from the first flux period by paired *t*-test ($P < 0.05$).


100
80
60
40
20
J Zn in
n equiv/kg·h
arterial
venous
0
10
20
30
40
Zn
time
(min)

is therefore not clear whether this single high value was anomalous, or the start of an upward trend. Stable values in preparations from ASW-exposed fish were about 85 nequiv/kg.h on the arterial side, and 10 nequiv/kg.h on the venous side. In contrast to the HW-acclimated fish, where the arterial contribution was only slightly higher than the venous side, ASW exposure resulted in the arterial side contributing nearly 9 times more Zn than the venous side. Overall, compared with HW acclimation, ASW exposure resulted in 28-fold stimulation of J_{in} on the arterial side, and 4-fold stimulation on the venous side (compare Figs 4.3 and 4.4).

To shed some light on whether this increase was due simply to the removal of Ca from the gill surface, which is likely to occur during ASW exposure (see below), or to the actual changes in the gill surface which arise from ASW pre-exposure, the third series of experiments examined flux rates using HW-acclimated fish tested in ASW. The response (Fig 4.5) was basically that of the HW trout tested in HW (compare Fig 4.3). J_{in} tended to rise with time in both arterial and venous pathways, although not significantly, and there were no significant differences between the data sets of Figs 4.3 and 4.5.

The kinetics of branchial Zn influx were measured in ASW-exposed trout, by using waterborne [Zn]'s ranging from 0.4 to 7.5 mg/L (Fig 4.6). Since 15-20 min were required to

Figure 4.5: Zinc influx across the perfused trout head over time. Fish were acclimated to hard water and tested in ASW. Values are means ± SE, n=7. Mean external [Zn] was 1.99 ± 0.05 mg/L. There was no significant increases over the course of the exposure.

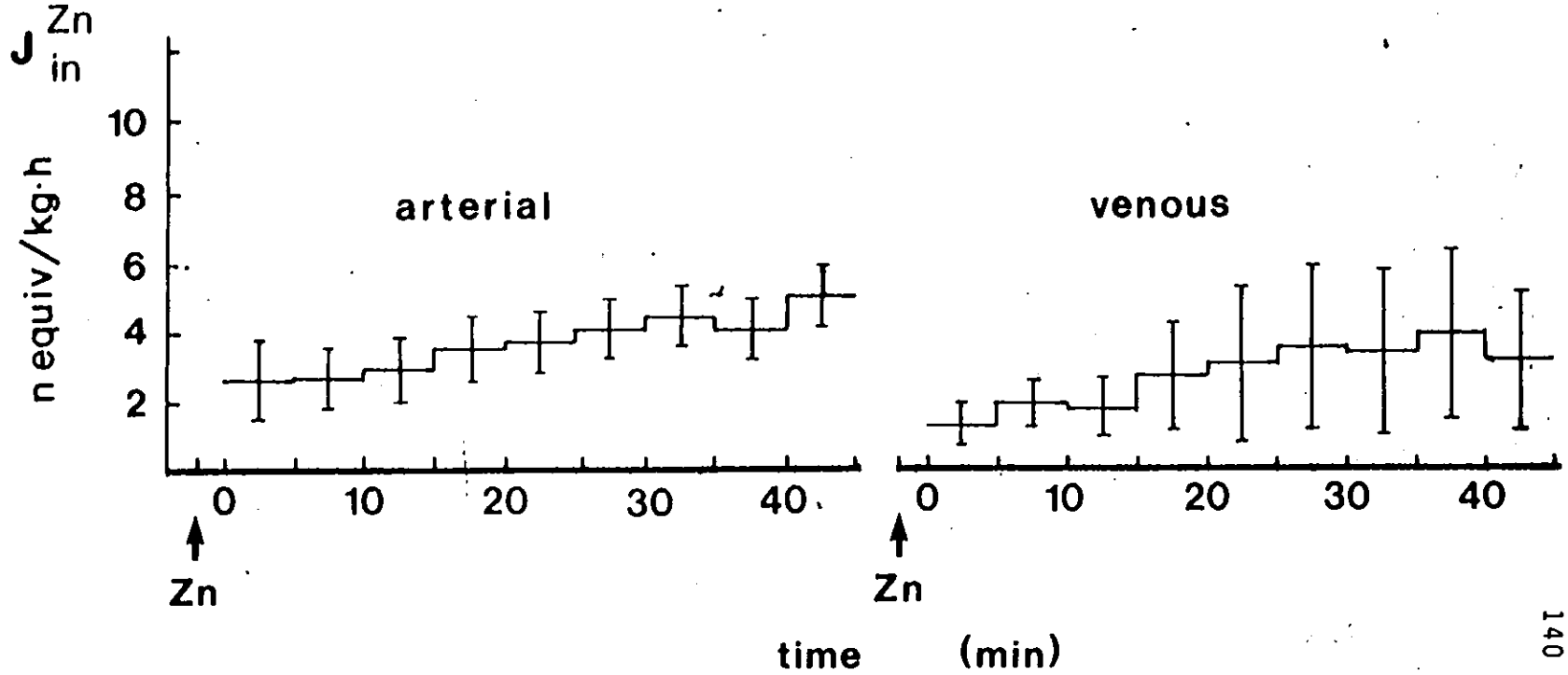
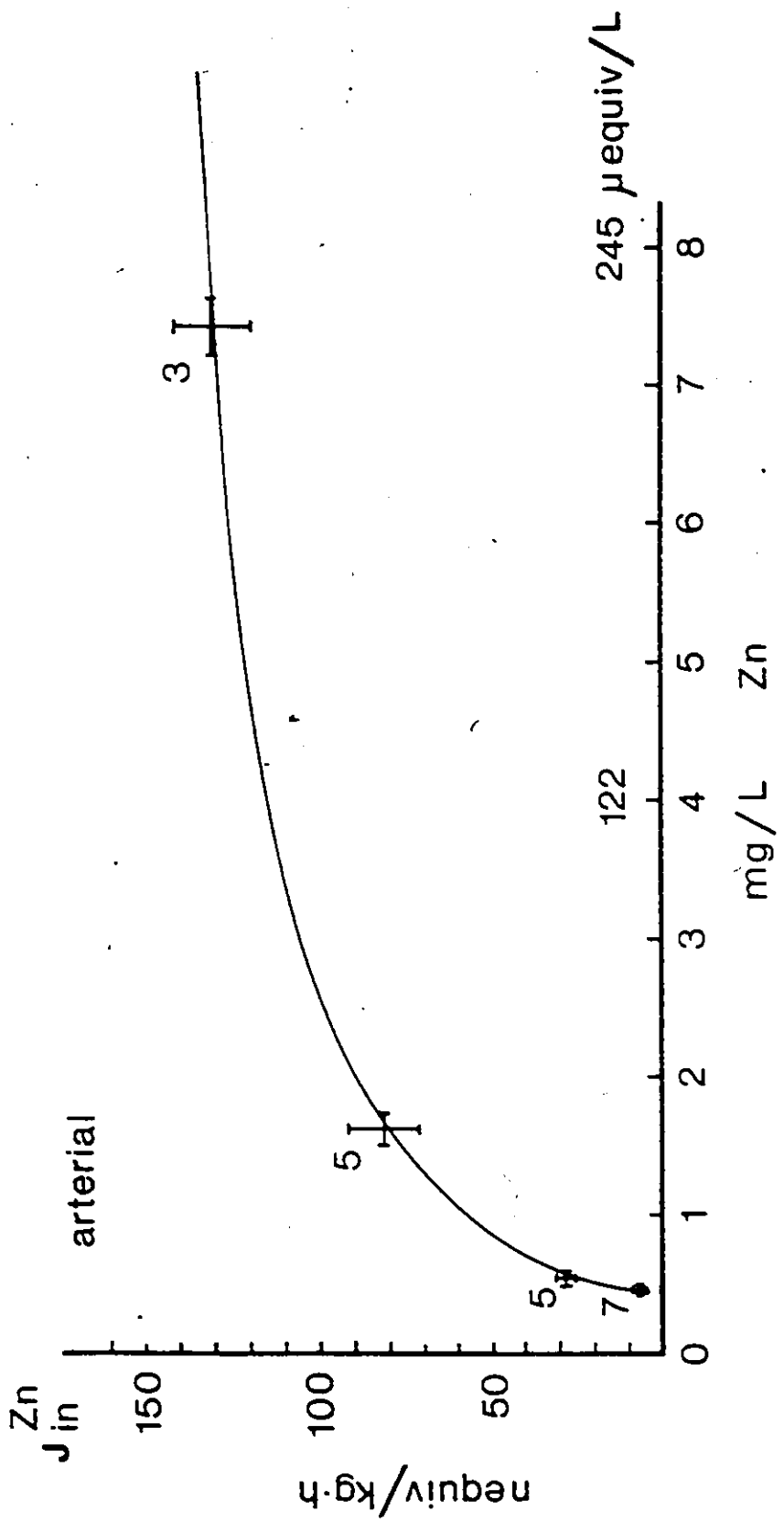

J Zn in
n equiv/kg·h
10
8
6
4
2
arterial
venous
0
10
20
30
40
Zn
Zn
time (min)

Figure 4.6: Zinc influx across the arterio-arterial pathway of the perfused trout head as a function of waterborne [Zn]. Numbers beside each mean denote n. Line was fitted by eye.


J_{in}^{Zn}
150
100
50
nequiv/kg·h
arterial
3
5
5
7
0
1
2
3
122
4
5
mg/L Zn
6
7
8
245 μequiv/L

reach equilibrium flux rates in ASW (Fig 4.4), kinetics could not be done using several [Zn] with a single head, as was done previously with Ca (Perry and Wood, 1985). Only a single [Zn] could be tested with each head preparation. J_{in} at 0.4 mg Zn/L was very low, but thereafter rose rapidly, showing typical first order, saturation kinetics. In fact there did not appear to be any significant unsaturable influx. It was significant that there was an apparent threshold of 0.4 mg Zn/L, below which there was no measurable uptake. This was a departure from first order reactions, which should pass through the origin. The estimated kinetic constants using arterial data gave an apparent J_{max} of 150 nequiv Zn/kg.h and a K_m of 1.5 mg Zn/L (46 uequiv/L, n=20). Since the venous fluxes were small under these conditions, estimates of these parameters using total flux gave nearly identical values.

Transepithelial electrical potentials (TEP) were stable with time, varying only 1-2 mv during the course of any one experiment. The total range observed was from -8 to -23 mv, inside negative. TEP was little affected by [Zn]. At the lowest waterborne [Zn] (0.4 ± 0.04 mg/L, n=7), the mean TEP was -16.5 ± 1.6 (8). Raising the [Zn] to 7.42 ± 0.21 (3) gave a mean TEP of -10.3 ± 1.2 (3), a change which was not significant.

Perfusion with either plasma, or diluted whole blood was done to determine if these natural fluids provided

additional substances (ligands) which would enhance J_{in}. Despite the presence of red blood cells and/or increased plasma protein (as determined by refractive index) influx was actually less than that found with perfusion saline, though individual values were quite variable. For blood diluted with perfusion saline to a hematocrit of 7% (Table 4.2), arterial J_{in} was 4.76 ± 1.50 (4) nequiv/kg.h at a waterborne [Zn] of 0.61 ± 0.14 mg Zn/L. For undiluted plasma, arterial J_{in} was 39.42 ± 8.33 (n=4) at a waterborne [Zn] of 1.25 ± 0.2 mg Zn/L. Comparable values for saline perfusion from the influx curve of Fig 4.6 would be about 35 nequiv/hg.h at 0.6 mg Zn/L and 70 nequiv/kg.h at 1.25 mg Zn/L.

DISCUSSION

Zinc influx was successfully measured under all conditions of acclimation and testing using the perfused head preparation. In addition, further information was obtained concerning the contribution by arterial and venous pathways. The rationale for the partitioning of perfusate flow through the head was described by Girard and Payan (1980) and Payan et al. (1984). The arterial flow is quite straightforward, being that perfusate which is collected from the dorsal aorta, and is considered to represent flux through the lamellae. However, assumptions underlying the venous flow and its representation of filamental fluxes,

are somewhat more complex. Venous flows in the gill are considered to arise from oxygenated blood which has already transited the lamellae and entered the filamental venous sinus (FVS) where it is in close contact with the filamental epithelium. The FVS then drains *via* the branchial veins back to the ventricle. Discrete sampling of this drainage would indeed give a measure of filamental input, simply by subtracting the arterial component. However, other fluids may also find their way into this pool. There is venous drainage which was never in contact with the FVS, namely venous drainage arising from systemic arterial flow to the brain and musculature of the head.[6] There is also the possibility of arterial flow from arterioles at the cut surface. This extraneous drainage into the a-v collection does not alter the calculation of filamental uptake, as long as no Zn is lost in the systemic circulation prior to collection. However, Zn is cleared quite quickly to the tissues from the systemic circulation *in vivo* (Chapter 5) although extent of "first pass" clearance (similar to the situation with the perfused head), is unknown. Thus the venous values could underestimate filamental Zn influx. A second possible cause of underestimation of the venous influx might arise from lack of true venous steady state. This was suggested, but not proven by Fig 4.4. Similarly, Gardaire *et al.* (1986) have discussed the circuitousness of the venous circulation and

the time lag for isotopic steady state between the arterial and venous circulation with regard to Na flux. The opposite possibility, that of overestimates of venous flux due to contamination from the irrigation water was described by Perry and Wood (1985). Such leaks were detected readily by refractometry in the present study, and either rectified, or the data discarded. On balance, it is concluded that the arterial (lamellar) Zn uptake estimates in the present experiments are valid, while the venous (filamental) uptake estimates are either valid or if not valid, underestimate the true influx.

Zinc fluxes in HW-acclimated and tested trout came quickly to steady state with minimal time lag, and were the same for both arterial and venous fluxes. The flux rates, in the few nequiv/kg.h range, were comparable to those of Cd at similar exposure concentrations (Pärt and Svanberg, 1981). However, they were small compared with flux rates for Ca which have ranged from 5-100 uequiv/kg.h *in vivo* (Fleming, 1968; Berg, 1968; Pang *et al.*, 1980; Mayer-Gostan *et al.*, 1983; Hōbe *et al.*, 1984; Perry and Wood, 1985) and using perfused gill preparations (Payan *et al.*, 1981; Perry and Wood, 1985). At least in HW, Zn did not exhibit the disparity between arterial (90-95% of total) and venous fluxes reported by Perry and Wood (1985) for Ca. The similarity of arterial and venous Zn fluxes (especially in light of the possible underestimate of venous flux)

suggests that Zn uptake per unit area was much greater through the filament than through the lamella, since lamellar area in rainbow trout is about 27 fold higher than filamental area (Girard and Payan, 1980).

Zinc fluxes in fish pre-exposed and tested in ASW were stimulated more than 20-fold above HW rates. This increased uptake may at least partially explain the well-known fact that Zn toxicity increases with decreasing water hardness (Spear, 1981). Furthermore, the Zn uptake in ASW differed in two other ways from that in HW. First, steady state flux rates did not occur in the arterial perfusate until 15-20 min after addition of the isotope. Second, the venous influx, although stimulated, was considerably less than the arterial influx. Unless the relative surface areas of the lamellae and the filament changed drastically, this would suggest that the Zn uptake per unit area across the lamellae increased much more than through the filament. However, the increase in venous influx during the last flux period (Fig 4.4) may again indicate a long time lag in Zn flux through this compartment.

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

indicated relative permeabilities for Na^+ and Cl^- which were also stable. This agrees with earlier *in vivo* work (Appendix i) which indicated that Zn effects upon Na^+ and Cl^- were minimal compared with other toxicants such as copper or acid (McDonald, 1983; Laurén and McDonald, 1986).

The stimulation of Zn influx by exposure to ASW, and the preferential effect on the lamellar pathway were clearly due to more than the removal of Ca from the water (and gill surface), as was demonstrated by the experiments of series 3. These results strongly suggest that actual morphological and/or functional changes occurred in the gill, which were responsible for this increased flux. Recent work has demonstrated that many stresses, and in particular, exposure to water low in Ca (Perry and Wood, 1985) or NaCl (Laurent *et al.*, 1985) induced changes in the gill epithelium. Not only did the number of chloride cells on the lamellae increase, but the overlying pavement cells receded, so that more of the cell was exposed to the environment (Perry and Wood, 1985). Spry and Wood (submitted to J. Exp. Zool.) found that simultaneous dilution of both NaCl and Ca induced similar changes in branchial chloride cells. In addition, there was dramatic development of microvilli and microridges. While this undoubtedly further increased surface area, the functional significance is less clear. Although the changes in chloride cells in terms of number, distribution, exposure, and surface structure do not show

causation, the correlation strongly suggests a role for the chloride cell in the uptake of Zn from the environment. In contrast, Pentreath (1973) failed to find any localization of ^{65}Zn in autoradiographs of chloride cells from seawater plaice (Pleuronectes platessa), but the Zn concentration was much lower (0.015 mg/L).

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

The experiments with plasma and blood perfusion were performed to test whether Zn uptake by the perfused head

was limited by the Zn binding capacity of the perfusate ie. whether the provision of organic ligands in plasma protein and red blood cells would stimulate influx. The results showed that this was clearly not the case.

Two possible mechanisms were initially proposed by which Zn might traverse the gill: simple passive diffusion, or active uptake. The electrochemical gradient probably favours the passive entry of Zn into the fish. This is so first, because the inside of the preparation had negative potential (TEP -8 to -23 mV inside negative in ASW). Second, at this water pH, most of the Zn exists as hydrated free ion in the water (Campbell and Stokes, 1985), whereas in blood and other biological fluids, Zn is tightly bound, with very little existing as free ion (Hambidge et al., 1986). The kinetic data suggest that passive influx, in which flux would be a simple linear function of external concentration, was of very minor importance if it occurred at all. The curvilinear nature of the influx-concentration relationship supports a saturable uptake mechanism, though the mechanism is not necessarily active or even carrier-mediated.

One mechanism which might give the appearance of saturable influx is passive diffusion being reduced by an increase in drffusion distance with increasing waterborne [Zn]. High waterborne concentrations of Zn cause inflamation and edema of gill tissue in vivo (Skidmore and

Tovell, 1972; Tuurala and Soivio, 1982; Tuurala, 1983). Could the extent of edema be a direct function of waterborne [Zn], and could this masquerade as a saturable phenomenon? First, the preparation itself is free of any significant edema (Perry et al., 1984a, b) using current methodology. The edema associated with waterborne Zn exposure on the other hand, resulted from its corrosive action as an external irritant. Detectable pathology developed in 2 h at 40 mg Zn/L (Skidmore and Tovell, 1972) or in 96 h at 1.25 mg Zn/L (Tuurala and Soivio, 1982). Decreases in $P_{a_{O_2}}$, indicative of increased diffusion distance, occurred after 6 h exposure to ~1.5 mg Zn/L (Sellers et al., 1975; Spry and Wood, 1984). Tuurala (1983) correlated increased difference between inspired water P_{O_2} and $P_{a_{O_2}}$ with increased diffusion distance after 17-20 h exposure to 1 mg Zn/L. The brief period of perfusion in the present study, and the lower [Zn] used, precludes this as a significant factor. Further evidence against edema is that there was never a decrease in influx rate over time as would be expected if edema were creating a diffusion limitation. The saturability of influx must thus be due to other factors, the most likely of which is a saturable carrier, or a selective pore. It is not possible to distinguish between these possibilities based on the present data. What is readily apparent from the kinetic constants however, is that the K_m is into the toxic range for trout (2.5-3 mg/L,

Holcombe and Andrew, 1978). One possible interpretation of this is that Zn would not appear to be the primary substrate. Such "accidental" uptake by a Ca mechanism was suggested to occur in freshwater amphipods (Wright, 1980). However, in light of Chapter 5, the true K_m is probably much lower (~1 uequiv/L = 30 ug/L) and some Zn might be taken up at even lower [Zn].

The results in this study have clearly shown that significant uptake of Zn occurred from the water. These rates are small (0.01%-1%) when compared to other ions such as Na, Cl, and Ca. Excretion of Zn however is also very low (Pentreath, 1973, 1976; Willis and Jones, 1977). Estimates for elimination half times were 235 d for the largest of three compartments (91%) in Gambusia (Willis and Jones, 1977) and about 300 d for plaice (Pentreath, 1973), both in seawater. Coupled with the fact that Zn was taken up independently from both the water and the diet (Chapter 2), elevated waterborne concentrations could cause significant branchial uptake and retention.

In conclusion, the saline-perfused head was a useful model to demonstrate entry of Zn across the branchial epithelium of trout and gain some insight into relative importance of lamellar and filamental pathways at different hardness. Use of this method permitted measurements of influx which were relatively free from the confounding effects of metal adsorption. Zn uptake rates were

extremely low (0.01% - 1% of Na, Cl and Ca influx rates). Use of blood or plasma as perfusates did not augment Zn uptake. ASW exposure greatly stimulated Zn influx, especially through the lamellae, and this effect likely arose from morphological changes in the gill. Changes in chloride cell number and distribution were the most likely explanation.

CHAPTER 5

A KINETIC METHOD FOR THE MEASUREMENT OF Zn INFLUX *IN VIVO* IN THE RAINBOW TROUT, AND THE EFFECTS OF WATERBORNE CALCIUM ON Zn INFLUX

INTRODUCTION

Water can be an important source of Zn to rainbow trout. Below the toxic threshold, the significance of waterborne Zn relative to dietary Zn increases with increasing waterborne concentration (Milner, 1982; Chapter 2). Direct transbranchial transport of Zn was demonstrated *in vitro* using an isolated, perfused trout head preparation (Chapter 4). This preparation yielded influx rates of about 4-6 nequiv/kg·h in hardwater-acclimated trout. These rates are very low compared with those for Na and Cl, and even Ca (Hōbe *et al.*, 1984; Perry and Wood, 1985). They are, however, very close to rates measured for free Cd using a similar *in vitro* preparation (Pärt and Svanberg, 1981; Pärt *et al.*, 1985).

While loss of ions across the gill reflects passive movement down electrochemical gradients, there is good evidence that Na and Cl influx across the gills of freshwater fish is by active, electroneutral carrier-

mediated exchanges against the gradient (see Maetz, 1971; Girard and Payan, 1980; Payan et al., 1984). Recent evidence suggests that Ca influx is also carrier-mediated and energy-requiring (Flik et al., 1985). Influx of trace metals such as Zn, on the other hand, might occur simply by passive entry down electrochemical gradients (Pentreath, 1973; Bryan, 1979) although this does not preclude carrier-mediation (Bryan, 1979). Zinc, at least in mammals, is tightly bound to plasma and intracellular ligands, reducing the internal free ion activity to very low levels (Hambidge et al., 1986). As well, the transepithelial electrical potential across the gills is generally negative inside in freshwater (see Potts, 1984), also favouring the passive entry of Zn. Actual measurements of Zn influx using the isolated, perfused head preparation, indicated however, that influx was not a simple linear function of waterborne [Zn] (Chapter 4). Rather, influx was saturable, suggesting that uptake occured via carrier mediation or through a saturable channel.

The first aim of the present study was simply to obtain reliable measurements of Zn influx from the water into the rainbow trout in vivo. There have been no previous determinations in freshwater fish. Two existing flux techniques in common use for other electrolytes were critically evaluted: disappearance of radiotracer (i.e. ^{65}Zn) from the water, or appearance in the fish by whole

body counting. Neither method proved satisfactory for measuring Zn uptake (see below). A new kinetic method was therefore developed, based upon calibrating cannulated fish by infusion with ^{65}Zn at known rates. The calibration data were then applied to exposed fish from which only a single terminal blood sample was taken.

The second goal was to apply this new method to determine the rate of Zn uptake as a function of waterborne [Zn]. Based on previous *in vitro* evidence (Chapter 4), it was hypothesized that part or all of the uptake would saturate with increasing waterborne Zn. A component which did not saturate would suggest the presence of simple diffusive influx.

The final aim was to determine whether acute changes in waterborne Ca would cause changes in Zn influx, and the kinetic nature of any interaction which occurred. There are several reasons to suspect that Ca might interact with Zn. Waterborne [Ca] is an important determinant of branchial permeability (Eddy, 1975; McWilliams, 1983), and may also restrict access of transported electrolytes to their carriers or channels (McDonald, 1983). Furthermore, both Zn and Ca are divalent cations and could conceivably compete for the same transport mechanism. It has been demonstrated that waterborne Zn blocks Ca uptake *in vivo* (Appendix 1), so a reciprocal effect might be anticipated. Increased water hardness (mostly Ca and Mg) attenuates the

acute toxicity of many trace metals, and Zn in particular (Spear, 1981). Pagenkopf (1983) has hypothesized that the underlying mechanism is one of a competitive interaction between Ca and toxic trace metals at the gill surface.

MATERIALS AND METHODS

Experimental animals

Underyearling rainbow trout (180-320 g), and fingerlings (2-4 g) were obtained from Spring Valley Trout Farm, Petersburg, Ontario. They were held separately, in flowing, charcoal-dechlorinated, Hamilton city tapwater ([Na]=0.6, [Cl]=0.7, [K]=0.05, [Ca]=2.0, [Mg]=0.6, [titratable alkalinity]=1.98 mequiv/L, pH=8.1) at ambient temperature for several months before use and fed a pelleted, commercial diet (Martin Feed Mills, Elmira, Ontario). Prior to testing, trout were temperature-acclimated to 15°C at an overall rate not exceeding 1C°/d. The large trout were acclimated in batches of 10-20 in 500L polyethylene tanks and not fed under these conditions; water was changed on alternate days. Small trout, due to the lower biomass, were temperature acclimated in the holding tanks by control of water inflow rate. Some experiments assessed Zn influx at nominal waterborne [Ca] of 10, 5, 2 (tapwater), 1 and 0 mequiv/L. Water harder than tapwater was created by the addition of $CaCl_2$ giving measured [Ca]=9.7 and 4.7 mequiv/L

respectively. Water softer than tapwater was prepared by adding to distilled water $NaHCO_3$, magnesium salt ($4MgCO_3.Mg(OH)_2.4H_2O$), KCl and NaCl, and where necessary, $CaCl_2$. The water was vigorously aerated and the pH adjusted to pH=8.1 with NaOH prior to use. This duplicated tapwater concentrations for all measured variables except Na and Ca. Measured [Ca] was 1.02 and 0.05 mequiv/L, while Na was 1.3 and 2.2 mequiv/L respectively.

Experimental Protocol

(i) Traditional Flux Measurement Techniques

Flux measurements by disappearance of radioisotope from the water, were performed on large trout, each in a clear acrylic box (~1 L capacity) within a larger (~14 L capacity) black acrylic box (McDonald, 1983). Boxes were held on a wet table over which chilled water flowed, to maintain an experimental temperature of 15°C. Trout were acclimated to the boxes a minimum of 24 h prior to flux measurements. During this time, fresh tapwater flowed continuously through them. Perimeter aeration and an air lift at the rear of the smaller box ensured adequate water circulation. The actual flux measurements were performed by stopping water flow into the larger box. Sufficient stock solution of Zn (as $ZnSO_4$) plus ^{65}Zn tracer (New England Nuclear) was added to give 0.5 mg Zn/L and a total of ~50 uCi of isotope. Water samples were taken prior to Zn addition (0 h) and at 0.5, 1, 2, 4, 6, 8, 10 and 12 h.

A flux box containing water, but no fish, was run as a control. Water samples were acidified (2 uL/mL) with high purity HNO_3. Subsamples were counted for gamma activity in a well-type counter (Nuclear-Chicago model 1084), corrected for decay and counting geometry where necessary, or analysed for total Zn by atomic absorption spectrophotometry (Varian AA 1275).

To estimate influx based upon appearance of radioisotope in the fish by whole body counting, 60 fingerlings were placed into a single 14 L flux box (no inner box) filled with tapwater (Ca=2 mequiv/L). Six fish were sampled for controls, and Zn plus radiotracer was added as above. Six fish, plus water were sampled at each of 0.25, 0.5, 1, 2, 4, 8, 12 and 27 h. Fish were dipped in flowing fresh water, pithed, blotted dry and whole bodies were counted for gamma activity in the well counter. Water samples were analysed as above.

The preceding experiments suggested that influx measurements were confounded by a large externally adsorbed component which was greatest in the first few hours of exposure and which completely swamped the true influx rate. To learn more about the nature of the adsorbed components, a number of short-term experiments were performed with large trout in the 14 L flux boxes, at $[Zn]_w$ = 1 mg/L. The first compared uptake over a 1 h period between intact fish and dead fish, the latter being killed by an overdose of

MS-222 (Sigma) 30 min prior to test. Zn plus radiotracer were added to the external water as above, and water samples taken at 15, 30, 45 and 60 min. The fish were rapidly anesthetized in MS-222, rinsed in 1 g/L solution of cold Zn to exchange off any loosely adsorbed ^{65}Zn, then killed by a blow on the head and frozen for later analysis. Later, the fish were homogenized with 150-200 mL of distilled water, and four aliquots of homogenate (approx. 5 mL each) were weighed into tared tubes and counted for gamma activity. Water samples were analysed as above.

A second experiment employed esophageal ligation to examine the possibility that drinking of the medium contributed to the initial rapid uptake. The operation was performed by anesthetizing the fish in 1:20,000 MS-222, and making a dorso-ventral incision in the left side. The esophagus was doubly ligated with silk suture, the wound dusted with tetracycline (Syndel) and the incision closed. Sham-operated trout were subjected to the identical procedure without the actual ligation. Fish were allowed to recover 48 h before uptake was studied as outlined above.

A third experiment examined the relative importance of the head and gill region versus the general body surface. Tube shields were employed to separate the two areas. Fish were lightly anesthetized and placed into a shield constructed from 5.1 cm ID ABS pipe. The shield enclosed the posterior portion of the trout and was secured by a rubber diaphragm

(wrist of a latex surgical glove) just posterior to the pectoral fins. The other end of the tube was sealed with a rubber bung. Experiments generally started 1-2 h after removal from anesthetic. Zn plus ^{65}Zn was added to the water to observe uptake *via* the head and gills, or added to the tube to observe the uptake by the skin. Tubes for positive controls were constructed by cutting out the sides to permit complete exposure to Zn solutions. Terminal sampling was performed as in the other trials, except in one experiment where gill filaments were removed and counted separately from the rest of the body.

(ii) The Kinetic Flux Measurement Technique

For the development of the new kinetic flux measurement technique, large trout had indwelling cannulae implanted, either in the dorsal aorta alone (Soivio *et al.*, 1975) or in the dorsal aorta and the caudal vein (Wilkes *et al.*, 1981), under MS-222 anesthesia. The fish were allowed to recover for 48 h prior to use, and the cannulae were periodically flushed with heparinized (50 IU/mL) Cortand saline (Wolf, 1963). To avoid contamination, injections or infusions of ^{65}Zn were performed via the caudal catheter, while blood was sampled *via* the dorsal aortic catheter. Blood samples (50-100 uL) for ^{65}Zn activity and total [Zn] were centrifuged for 2 min at 8000 g, and the plasma counted or assayed as above for water.

In some experiments, $ZnSO_4$ plus ^{65}Zn tracer was added

to the external tapwater ([Zn]=0.8 mg/L) as in the previous flux experiments. In others, a bolus of Zn plus ^{65}Zn sufficient to double the total plasma Zn was dissolved in 1 mL of Cortland saline, injected into the dorsal aorta, and flushed in with an additional 1 mL of saline. In alternate protocols, Zn plus ^{65}Zn in Cortland saline was infused into the caudal vein for 36 h at a constant rate of 15 uL/min using a peristaltic pump (Gilson Minipulse). A variety of dosage rates were achieved by adjusting the concentration of the Zn in the infusate, thus keeping the flow rate constant. In all these protocols, [Zn] and radioactivity were periodically measured in water and plasma samples.

In the finally perfected protocol, uncannulated fish were exposed to $ZnSO_4$ and ^{65}Zn in the external water (closed system, volume=14 L) for 24 h under a variety of waterborne [Ca] and [Zn]. Concentrations and radioactivities of Zn were measured in single terminal plasma samples drawn by caudal puncture from each fish, which had been rapidly anaesthetized in MS-222 (0.5 g/L).

Calculations and Statistics

The distribution volume or radiospace for ^{65}Zn (V_d in mL/kg) was estimated as:

$$V_d = \frac{Q^*_{int}}{\text{plasma radioactivity} \cdot W} \qquad 5.1)$$

where Q^*_{int} is the total radioisotope activity (cpm) in

the fish, W is the fish weight, in kg. Plasma radioactivity is in cpm/mL .

Traditional flux calculations were based upon the disappearance from the water of ^{65}Zn of known specific activity, on the assumption that the system was composed of only two compartments. Thus, Zn which left the water entered the fish. Since backflux of isotope did not occur in the short term, as shown by later infusion experiments, a simplified case of the general flux equation (Kirschner, 1970) was used:

$$J_{in} = \frac{Q_{out}}{t \cdot W} \cdot (\ln Q^*_{out(0)} - \ln Q^*_{out(t)}) \qquad 5.2)$$

where J_{in} is Zn influx rate (nequiv/kg.h), Q_{out} is the total mass of element in the medium, Q^*_{out} the total isotope activity (cpm) in the medium and t the elapsed time (h).

For small trout, the total Zn accumulated with time was calculated from the activity of the fish and the mean specific activity of the water sample during the period. Actual flux rates were then calculated by dividing by the elapsed exposure time. The same approach was used in measuring uptake rates in the short-term experiments with large trout designed to factor out the sites of adsorption. Here, however, activity of the whole fish was determined from homogenate counts rather than whole body counts.

In the development of the kinetic technique for flux measurements, two models were used to fit equations to

data. The first model was applied to the rise in plasma radioactivity with time and is an exponential saturation.

$$y = C_{ss} \cdot (1-e^{-kt}) \qquad 5.3)$$

where C_{ss} is the steady state activity in the plasma, k the rate constant, and t the time. The rising section of the curve represents the addition of labelled Zn to the unlabelled pool already present in the plasma, the influx of labelled Zn occurring from i) uptake from the water in the case of the waterborne exposure, or ii) inflow via the caudal cannula in the case of the infusion. Steady state is reached when the amount of label (fractional excretion of the total) cleared from the plasma equals the amount entering from either the water or the infusion (see Shipley and Clark, 1972)

The second model was used to describe the relationship between measured Zn influx rate and the concentration of waterborne [Zn]. Since the latter is essentially the substrate concentration, and since a saturable component was observed (see Results), the model is based upon enzyme kinetics, and uses a hyperbolic saturation (Michaelis-Menten) equation:

$$J_{in} = \frac{J_{max} \cdot [Zn]}{K_m + [Zn]} \qquad 5.4)$$

where J_{in} is the influx rate (nequiv/kg.h), [Zn] the waterborne substrate concentration (uequiv/mL), J_{max} the maximum flux rate and K_m the [Zn] for half maximal flux

rate. In cases where there appeared to be an additional non-saturable component, an alternative model, which included a linear transport term was fitted to the data:

$$J_{in} = \frac{J_{max} \cdot [Zn]}{K_m + [Zn]} + m \cdot [Zn] \qquad 5.5)$$

where m is the slope of the linear component which passes through the origin. Actual curve fitting was done using non-linear regression (Johnston, 1985).

In analyzing the influence of waterborne [Ca] on Zn uptake, the possibility of competitive inhibition was considered. In such a situation, the true K_m for Zn uptake would be related to the apparent K_m in the presence of an inhibitor by the relationship:

$$K_m = \frac{\text{apparent } K_m}{1 + ([I]/K_i)} \qquad 5.6)$$

where [I] is the inhibitor (Ca) concentration and K_i the inhibitor constant (Segel, 1976).

Analysis of the Data

Data are expressed as means ± SE (n). Goodness of fit of the data to the non-linear curve fitting was assessed from the sum of squares. Comparisons between rates of adsorption in the short term experiments (Table 5.1) was made using t - tests.

RESULTS

Flux rate calculations using traditional techniques

Attempts to calculate Zn influx rates in large trout

based upon disappearance of isotope from the water revealed significant adsorption of both stable and radiotracer Zn to the flux boxes. Over the time course of the experiment, both the activity (Fig 5.1) and the total [Zn] in the water fell by between 7 and 30%, such that the specific activity remained relatively constant. This was true for both the control box and the ones which contained trout. This result clearly showed that the method was insensitive to the presence of the fish. Calculation of flux rates (Fig 5.2), although clearly inappropriate, demonstrated peak rates of about 60000 nequiv/kg.h which fell over the first 4 h and stabilized at about 8000 nequiv/kg.h.

Results with fingerling trout based upon whole body counting however, clearly showed that fish accumulated the isotope from the water. The amount accumulated continued to increase linearly with time (Fig 5.3). Calculation of the influx rate (Fig 5.4) indicated initial uptake, about 6000 nequiv/kg.h during the first 15 min of exposure. Thereafter, uptake fell exponentially, to about 700 nequiv/kg.h by 27 h. This rapid initial uptake undoubtedly represented adsorption of Zn to the fish, with little internalization.

The adsorption phenomenon was further examined by studying uptake by large fish exposed to 1.0 mg Zn/L for 1 h only. Even dead fish took up Zn from the water at a rate approximately 1/5 of that in intact live fish (Table

Table 5.1: Summary of Zn "uptake" rates (nequiv/kg.h) determined over the first 1 h of exposure (^{65}Zn + 1 mg Zn/L) by counting whole body homogenates in large trout. Mean ± SE (n).

Intact live trout	1212 ± 129	(6)
Dead trout	234 ± 65	(4)*
Sham ligated controls	2673 ± 197	(6)
Esophageal ligations	2530 ± 141	(6)
Tube shield controls	3025 ± 260	(7)
Head/gills only exposed	2278 ± 246	(12)*
Body only exposed	652 ± 44	(5)*

* = $p<0.05$, relative to relevant control value.

Figure 5.1: Disappearance of ^{65}Zn activity from tapwater. Each of 5 flux boxes contained 14 L of tapwater and one 250-350 g trout. Box 3 held only water. Waterborne Zn = 0.5 mg/L = 15 uequiv/L, Ca = 2.0 mequiv/L.

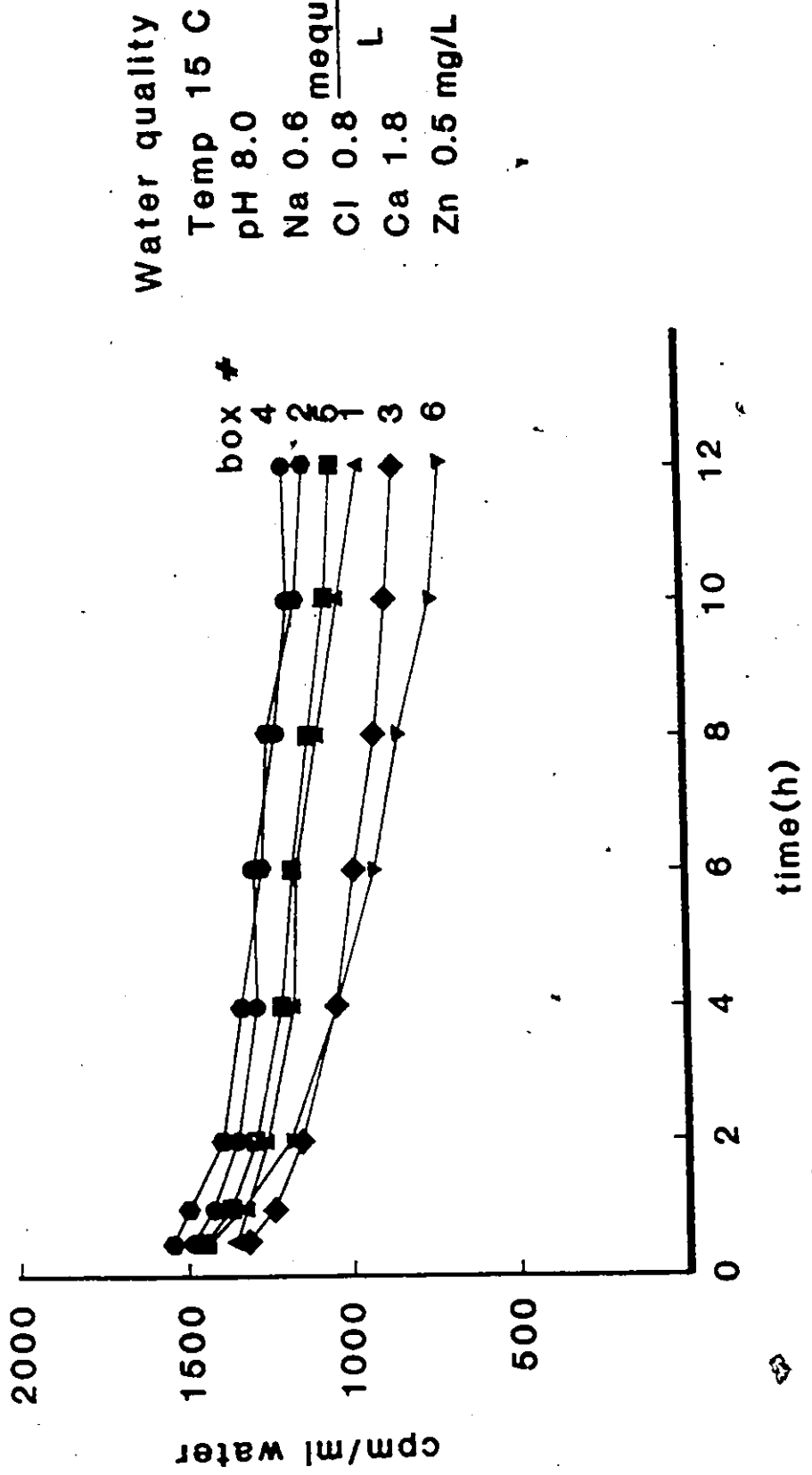

Water quality
Temp 15 C
pH 8.0
Na 0.6 meq/L
Cl 0.8
Ca 1.8
Zn 0.5 mg/L
box #
4
2
5
1
3
6
cpm/ml water
2000
1500
1000
500
0
2
4
6
8
10
12
time(h)

Figure 5.2: Zinc "influx" based upon disappearance of counts from tapwater, mean ± SE (6). Details as in Figure 5.1

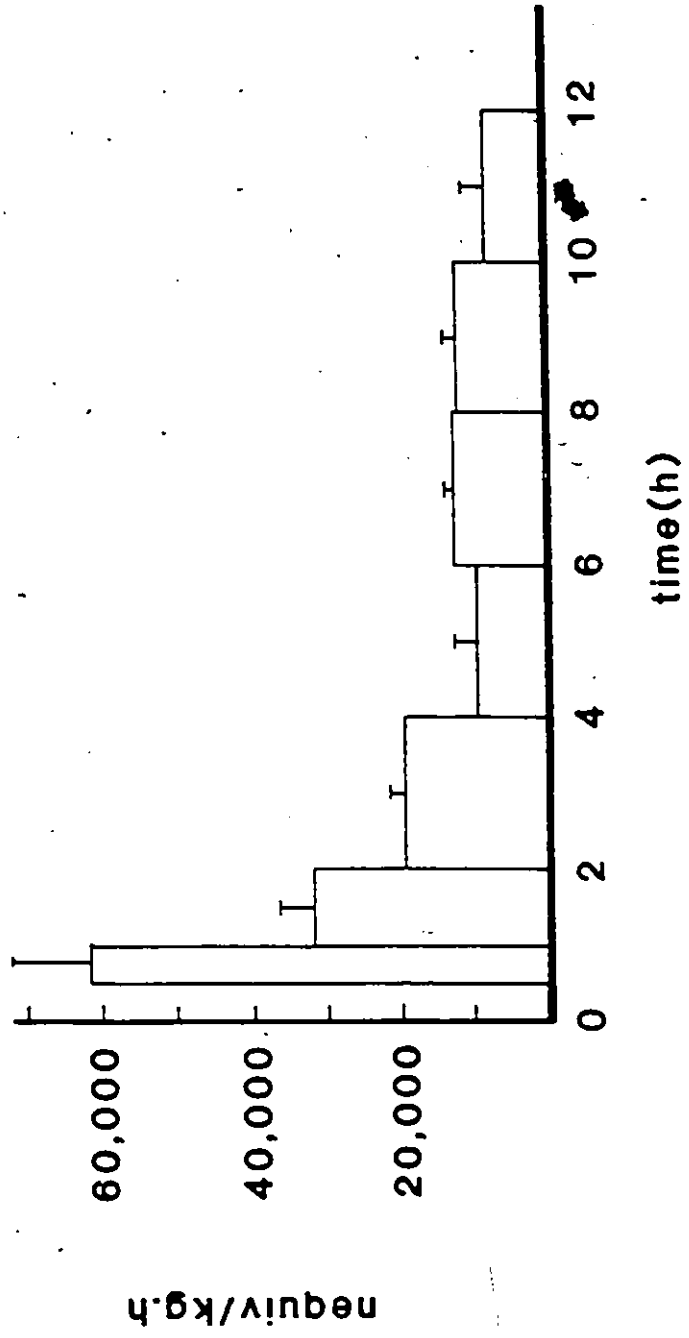

60,000
40,000
20,000
0
2
4
6
8
10
12
time(h)
nequiv/kg.h

Figure 5.3: Net Zn accumulation based on ^{65}Zn accumulation by fingerling (2-4g) rainbow trout, mean ± SE (6). Waterborne Zn = 0.5 mg/L = 15 uequiv/L, Ca = 2.0 mequiv/L. Line was fitted by eye.

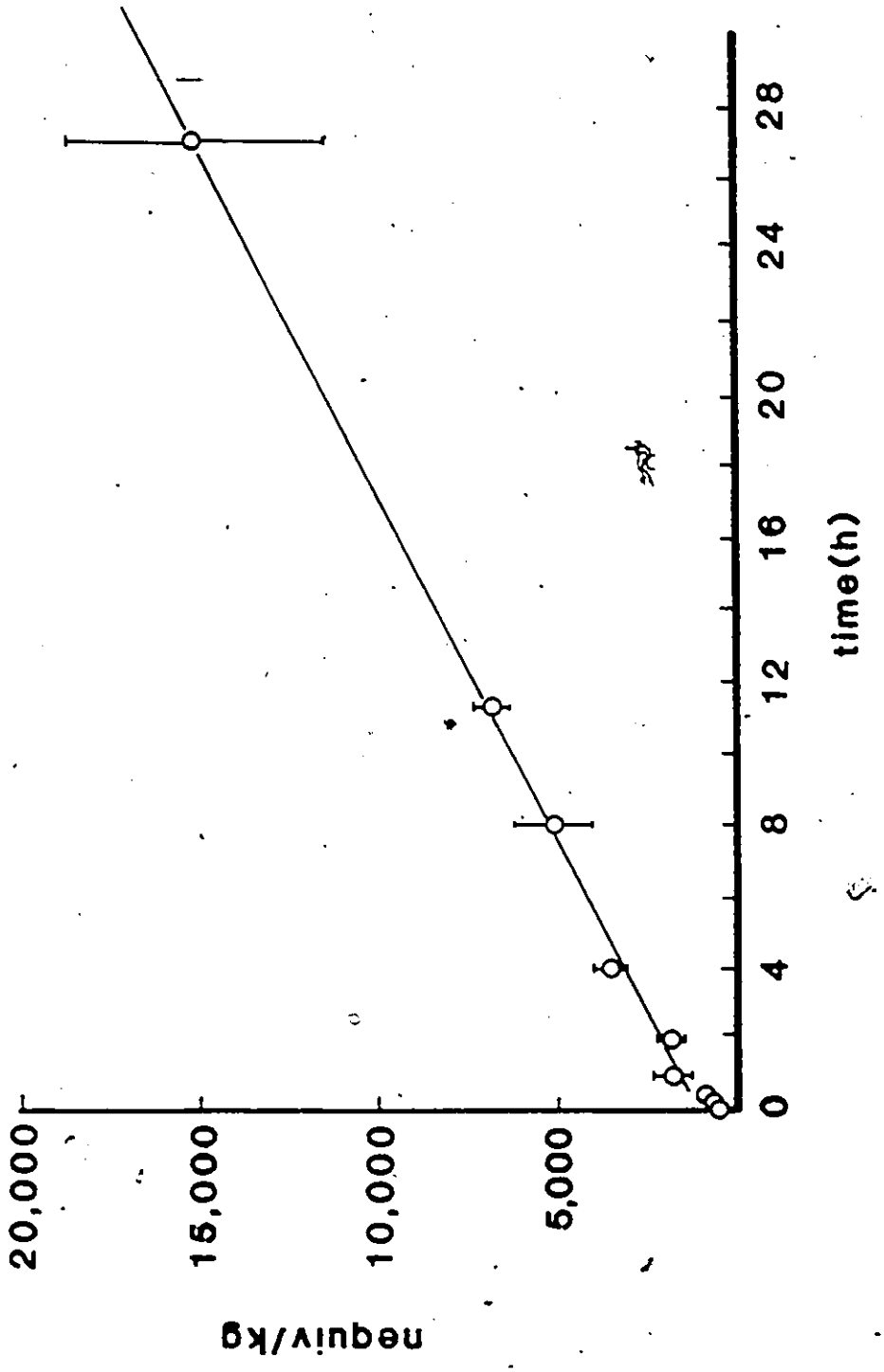
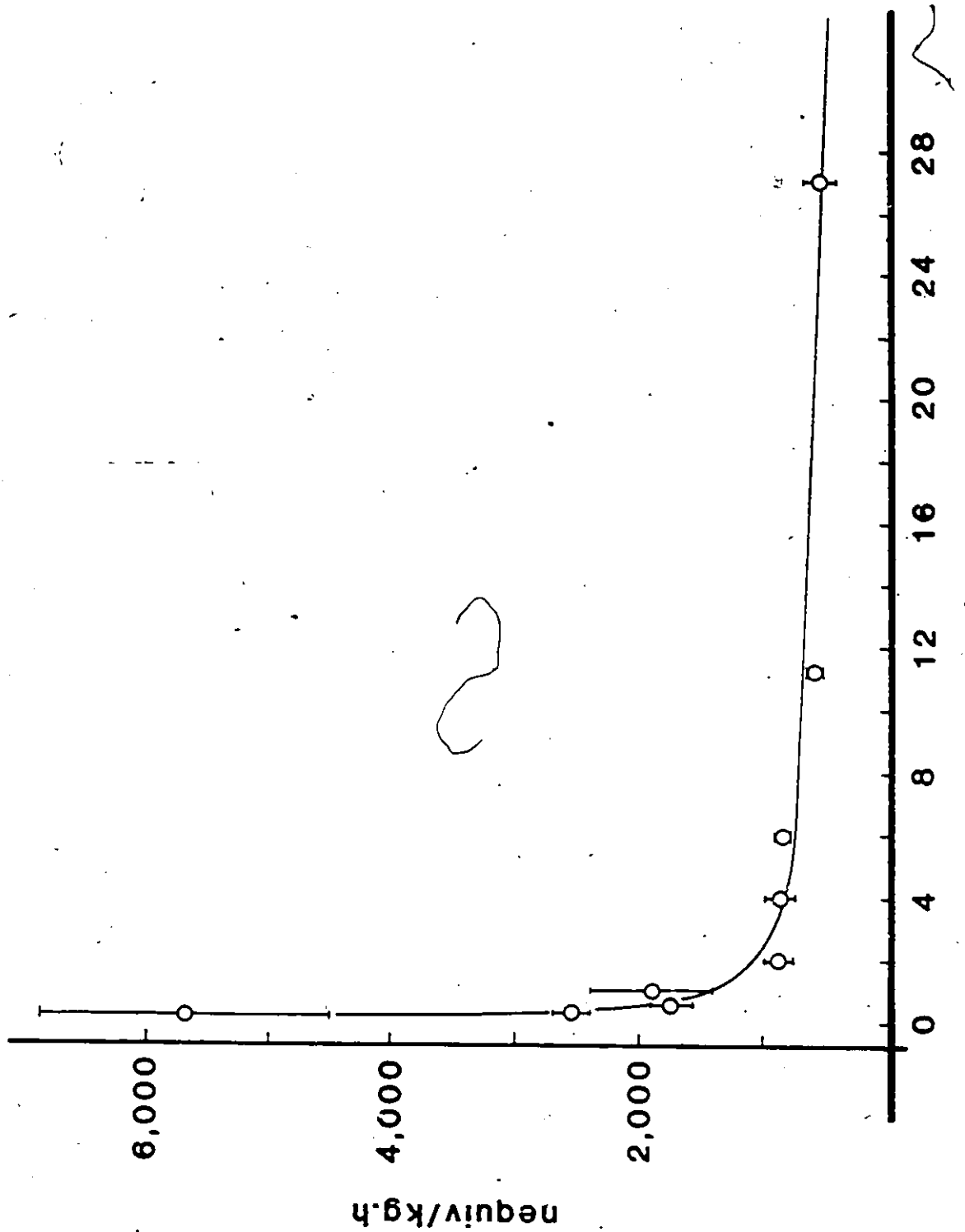

20,000
15,000
10,000
5,000
0
4
8
12
16
20
24
28
time(h)
nequiv/kg

Figure 5.4: Zinc "influx" in fingerling trout based upon whole body ^{65}Zn accumulation in tapwater. Details as in Figure 5.3.


6,000
4,000
2,000
nequiv/kg.h
0
4
8
12
16
20
24
28
time(h)

5.1). The tube shield experiments demonstrated that approximately 75% of the uptake in the first 1 h of exposure occurred via the head/gills region, while 25% was associated with the remainder of the body surface (Table 5.1). When gill filaments were counted separately from the rest of the body, these accounted for about 30% of the accumulated radioactivity, suggesting that these were an important site of adsorption in the head/gills region, but not the only one. The control values varied somewhat between the three experiments (Table 5.1), likely reflecting differences in the degree of stress and minor variations in water quality. Nevertheless, all were in the 1000 nequiv/kg.h range or higher.

These various types of experiments, measuring disappearance from the water, or uptake by the fish, attempted to quantify influx using a simple two compartment analysis. Both approaches indicated the presence of additional compartments which corresponded to adsorption to the flux-box in the first instance, and to various external surfaces of the fish in the second. The presence of these compartments clearly resulted in differing estimates of influx rates, especially over the first several hours of exposure.

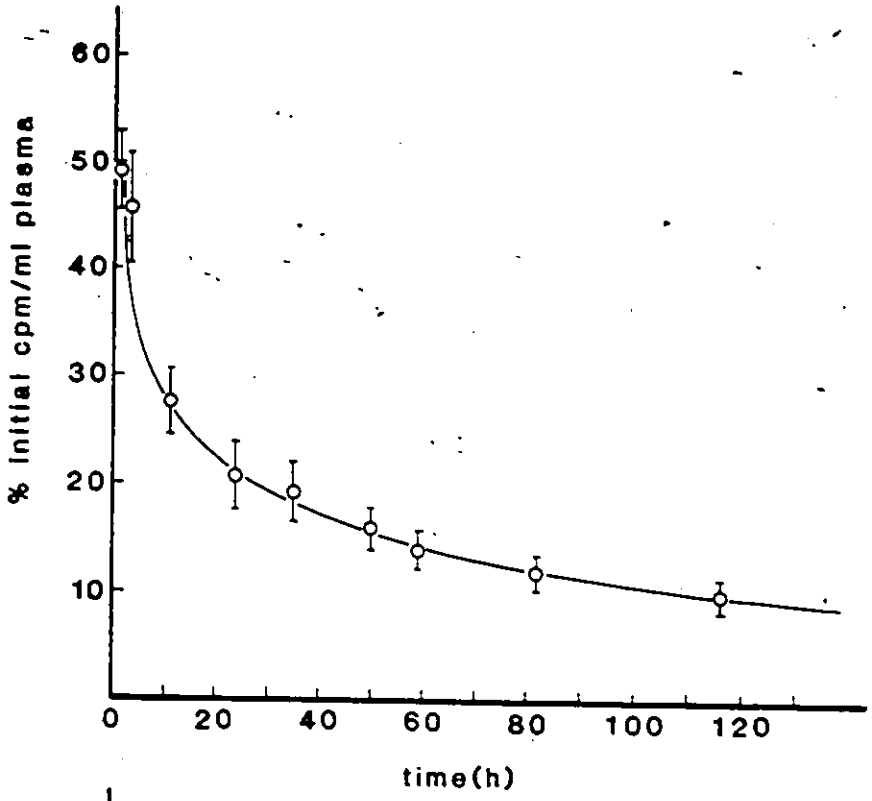
Flux rate calculations based upon blood sampling

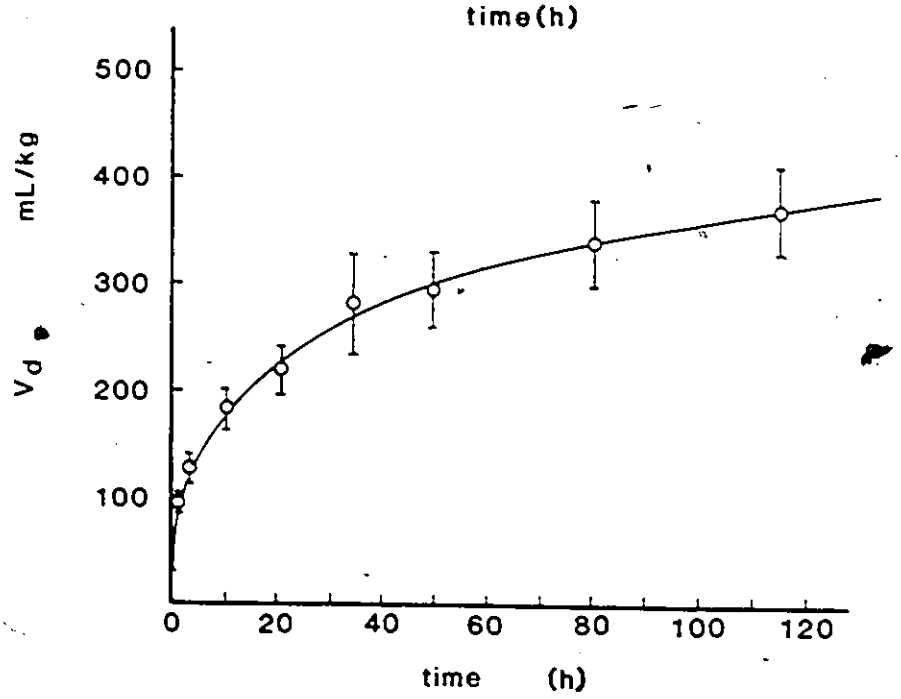
To circumvent these difficulties, sampling of an internal tissue containing only absorbed Zn was attempted.

Blood is a tissue particularly suited to repeated sampling, and therefore, a number of tests were performed to determine the suitability of influx determinations using blood samples. These assessed the characteristics of ^{65}Zn clearance from the blood, whether ^{65}Zn from the water entered the blood space in sufficient quantities to be measured, and the quantitative relationships between ^{65}Zn entry rate and plasma concentrations.

If ^{65}Zn enters the blood space and is completely retained there, at least in the short term, then influx could simply be estimated from the product of plasma radioactivity x plasma volume, factored by the appropriate water specific activity and time. However, if ^{65}Zn is cleared from the plasma at a significant rate, then the analysis becomes more complex. To evaluate this situation, large trout fitted with indwelling catheters were given a bolus of Zn plus ^{65}Zn sufficient to double total plasma [Zn]. Blood samples were taken over 120 h and plasma recovered and counted for radioactivity. Zn was rapidly lost from the plasma in typical first order fashion (Fig 5.5a). In fact, after 1 h, only about 50% of the infused dose was retained. Collection of water samples over this time revealed that excretion was never more than 1% of the dose. Although the possibility of adsorption to tank walls exists, excretion appeared minimal. Zn lost from the plasma was thus clearly redistributed to other tissues, and

Figure 5.5: (a) Clearance of a bolus of radiolabelled Zn sufficient to double the total plasma [Zn], from the plasma of rainbow trout in tapwater (Ca = 2.0 mequiv/L), mean ± SE (7). (b) Change in the radiospace of Zn after infusion of a bolus of radiolabelled Zn sufficient to double the total plasma [Zn]. Lines fitted by eye.

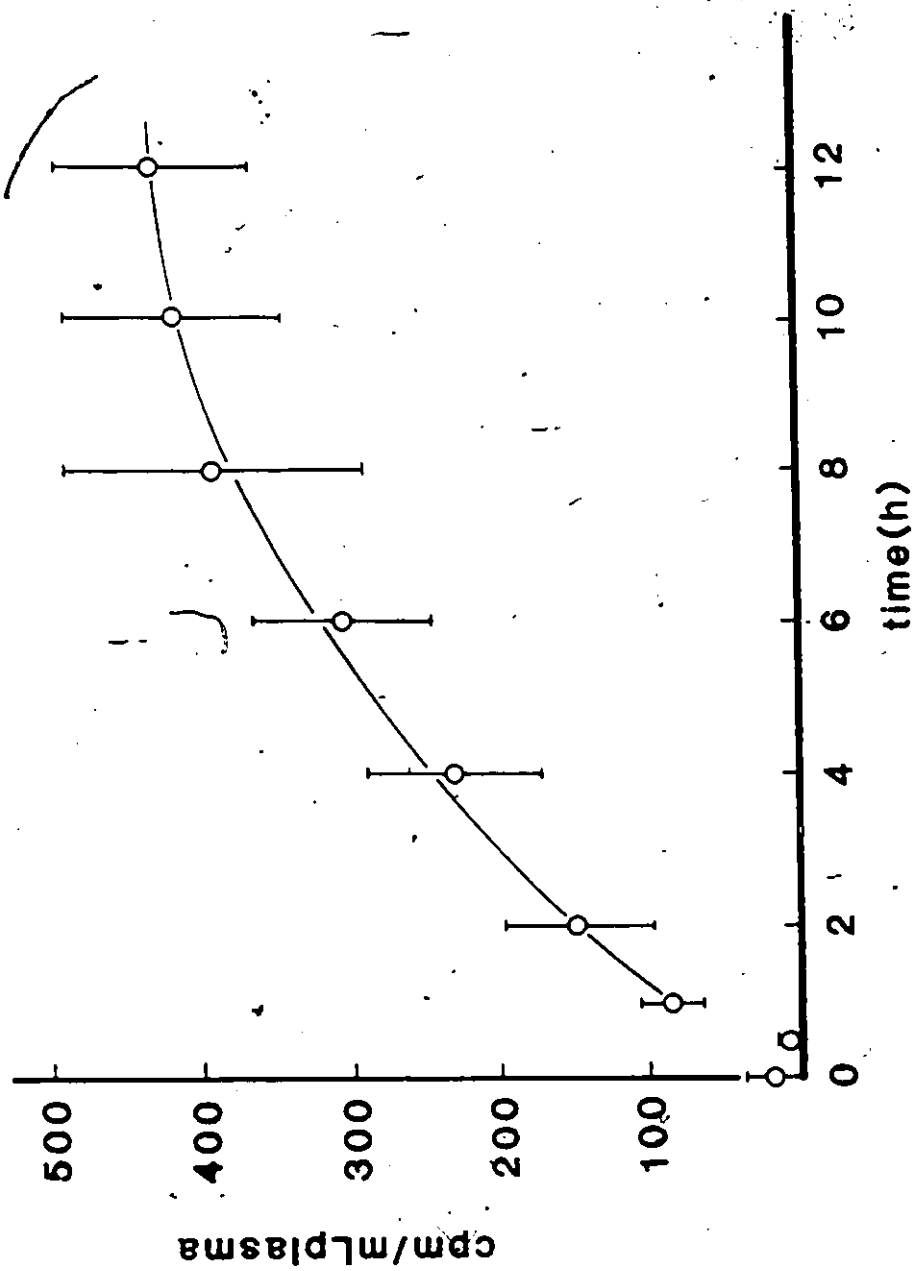

% initial cpm/ml plasma
60
50
40
30
20
10
0
20
40
60
80
100
120
time(h)


V_d
mL/kg
500
400
300
200
100
0
20
40
60
80
100
120
time (h)

in fact, the radiospace (eqn 5.1) for Zn (when plasma [Zn] was doubled), increased from about 100 mL/kg (approximately double the blood volume; Milligan and Wood, 1982) at 1.5 h post injection, to about 400 mL/kg after 120 h (Fig 5.5b). This loss from the plasma, and changing distribution volume clearly invalidated an approach based simply on the product of radioactivity x distribution volume. Nevertheless, if ^{65}Zn enters from the water in measurable quantities and follows predictable patterns in the plasma, then a kinetic analysis may still be feasible.

To assess this possibility, cannulated trout were exposed to waterborne Zn (0.7 mg/L) plus ^{65}Zn as previously, and sampled at various periods over 12 h. Plasma radioactivity rose gradually, approaching steady state values by 12 h (Fig 5.6). There appeared to be a time lag of between 0.5 to 1 h before measurable activity appeared in the plasma. Nevertheless, the data were well-described by the exponential saturation equation (eqn 5.3). Non-linear regression through the means gave C_{ss}=7.1 ± 0.4 nequiv/mL and k=0.18/h. (Here C_{ss} is expressed in terms of the concentration of Zn in plasma which was transported from the water, i.e. exogenous Zn). Since there are undoubtedly multiple exits from the plasma to the tissues, each with a characteristic rate constant, and since these constants are additive, k in this case is the lumped rate constant. The biological half life (ln 2/k) for plasma Zn

Figure 5.6: Appearance of radiolabelled Zn activity in the plasma of large rainbow trout exposed to radiolabelled Zn in tapwater (Zn = 0.7 mg/L, Ca = 2.0 mequiv/L), mean ± SE (5). Line fitted by eye.


cpm/mLplasma
500
400
300
200
100
0
2
4
6
8
10
12
time(h)

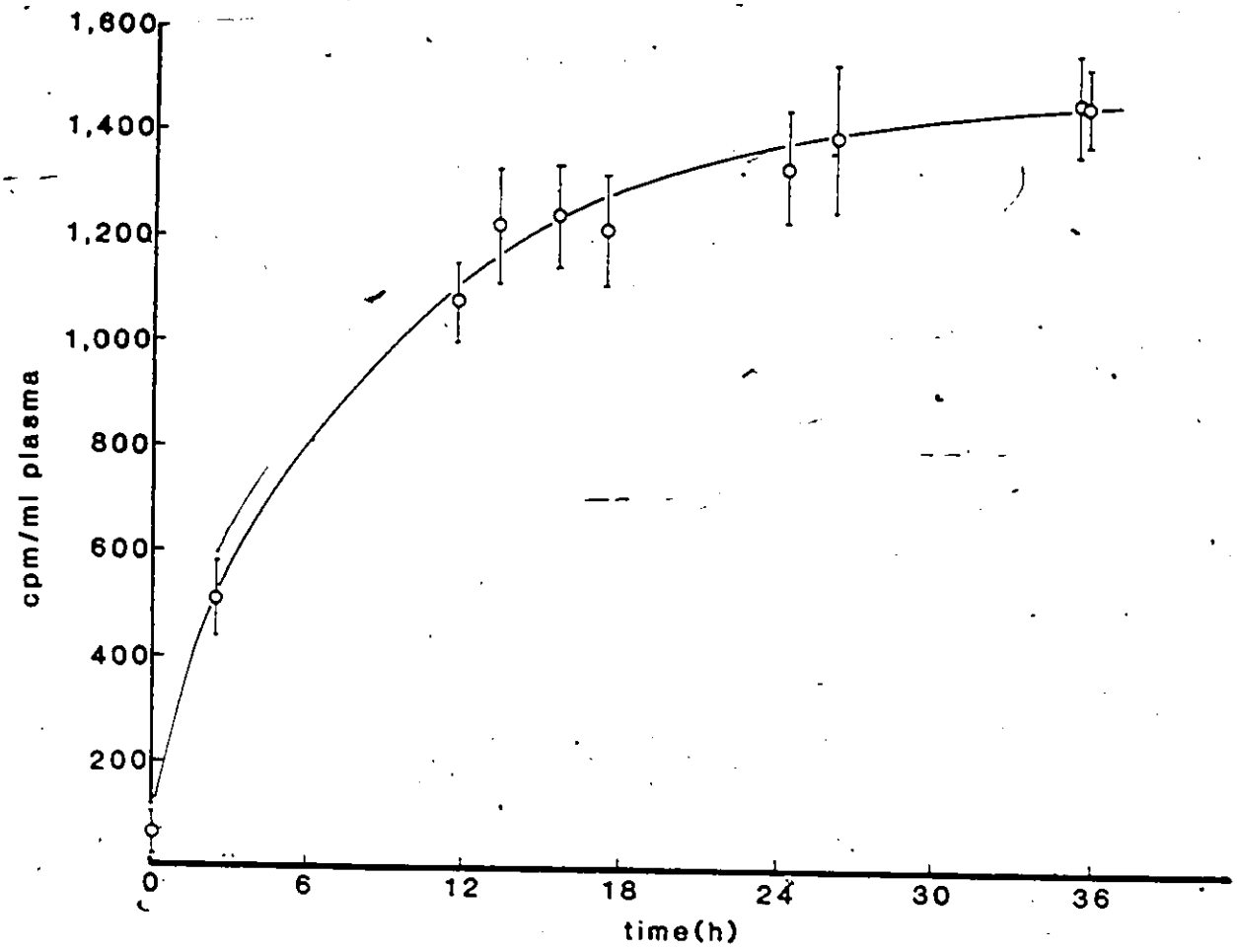
in this case was 3.8 h. Since 5-7 half lives are required to reach a plateau, true steady state concentrations would be reached in 19-27 h. This experiment clearly demonstrated that Zn entered the plasma in measurable quantities and followed a predictable time course of accumulation in the blood plasma, eventually reaching equilibrium.

If the pool size were accurately known, then influx would be given by the product of the rate constant and the pool mass. The pool size was not simply the mass of Zn in the plasma however, since plasma specific activity, despite closely approaching steady state value, was still <3% that in the water. This indicated either a very large endogenous turnover of plasma Zn (pool size much greater than total plasma Zn), or a large compartment of non-exchangeable plasma Zn.

To circumvent the difficulty of the unknown pool size (in terms of both mass and volume) an empirical approach based on C_{ss} was taken, in which large fish were "calibrated" by infusion of Zn plus ^{65}Zn over a range of possible influx rates. This infusion was analogous to the entry of Zn into the plasma across the gill. A typical infusion curve of plasma activity *vs* time (Fig 5.7) indicated that steady state values (C_{ss}) were reached by 24 - 36 h in agreement with the previous analysis.

A scatter plot of C_{ss} *vs* the infusion rate of exogenous Zn into the plasma (J_{in}) of 27 fish revealed a

Figure 5.7: Appearance of radiolabelled Zn over time, in the plasma of rainbow trout infused at a single constant rate (174 ± 13 nequiv/kg.h), in tapwater (Ca = 2.0 mequiv/L), mean ± SE (6). Line fitted with eqn 5.3.


1,600
1,400
1,200
1,000
800
600
400
200
cpm/ml plasma
0
6
12
18
24
30
36
time(h)

linear relationship over a wide range of influx rates (Fig 5.8), and given by the following equation:

$$J_{in} = 8.43\ C_{ss} + 4.94 \qquad 5.7)$$

$r=0.88$, $p<0.01$, where C_{ss} is expressed in terms of exogenous [Zn] in the plasma. Using this relationship, it was then possible to calculate actual influx based upon a single terminal blood sample from uncannulated trout after 24 - 36 h of exposure to waterborne Zn.

In order to check whether this relationship remained valid under different environmental conditions (e.g. very low water [Ca]) where Zn flux rates might be higher, additional infusions were performed at higher "influx rates", and also under the lowest water [Ca] (0.05 mequiv/L). These rates were not significantly different from the values predicted from the C_{ss} by the original relationship (paired t - test, $p>0.2$), indicating that the regression could be extrapolated beyond the original calibration range, and also applied in water of very different quality.

The kinetics of Zn influx and the influence of Ca

When uncannulated large trout were exposed to a range of waterborne Zn (at constant specific activity) in tapwater (Ca=2.0 mequiv/L), and Zn influx calculated from a terminal plasma sample, influx did not show a simple linear increase with concentration. Instead, influx rose rapidly and then saturated above a waterborne [Zn] of 0.4 mg/L (Fig 5.9). The data were well-described by the Michaelis-Menten model

Figure 5.8: Steady state concentrations (C_{ss}) of exogenous Zn in the plasma of 27 different trout at various constant rate infusions of radiolabelled Zn. All data from trout in tapwater (Ca = 2.0 mequiv/L).

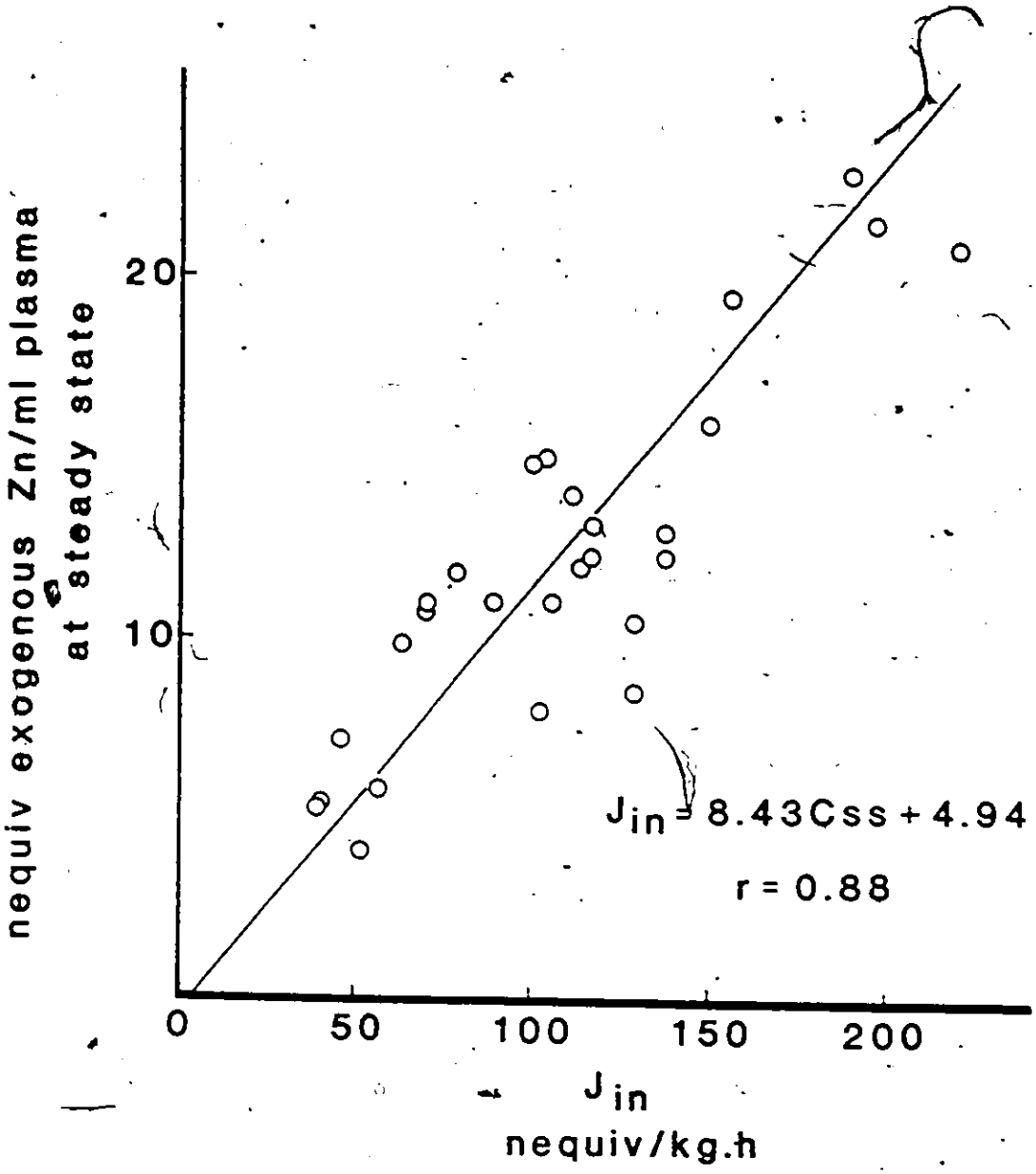
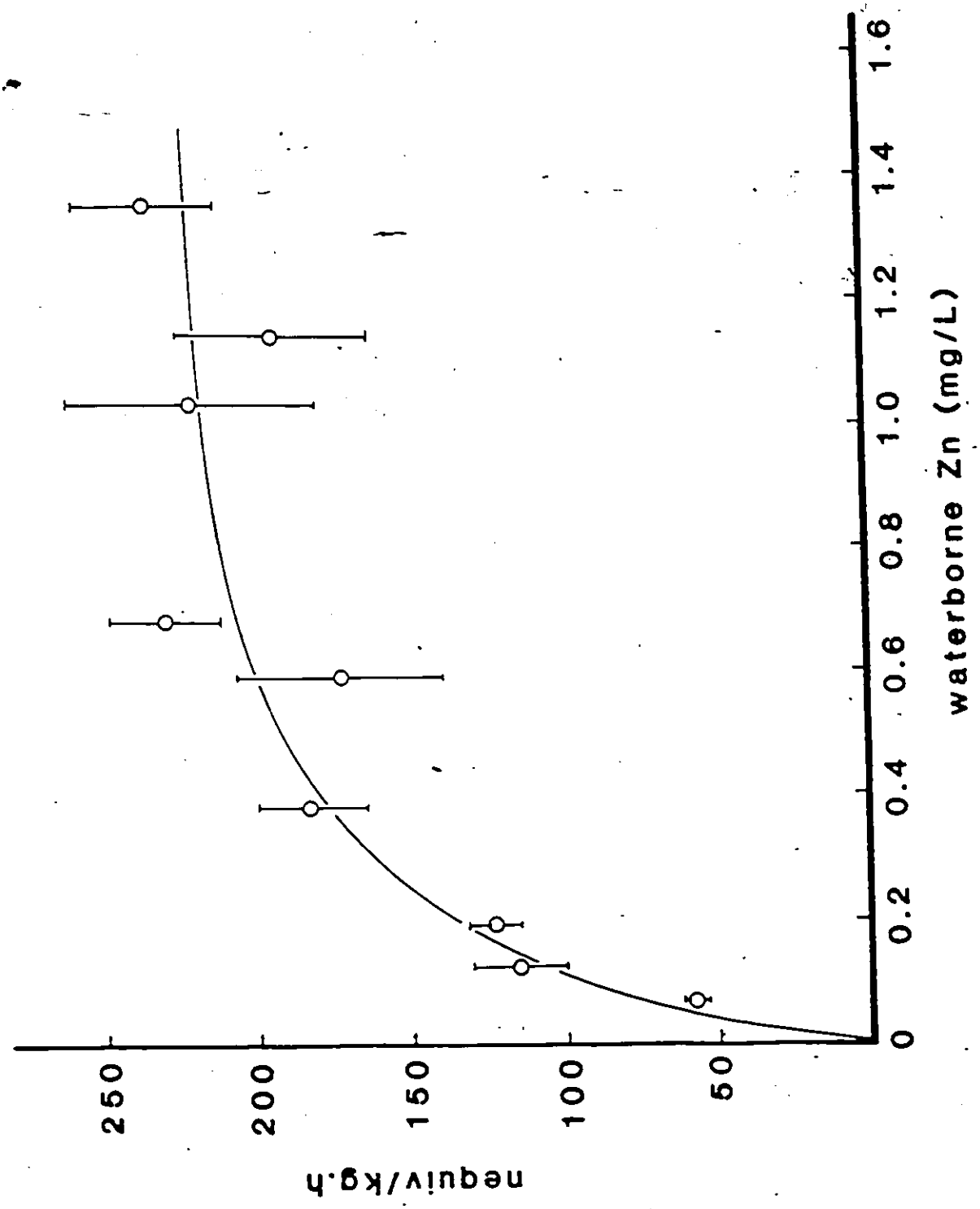

nequiv exogenous Zn/ml plasma at steady state
20
10
0
50
100
150
200
J_{in}
nequiv/kg.h
$J_{in} = 8.43Css + 4.94$
r = 0.88

Figure 5.9: Zinc influx into 54 large rainbow trout at various waterborne Zn concentrations in tapwater (Ca = 2.0 mequiv/L), based upon steady state plasma concentrations of radiolabelled Zn (C_{ss}), mean ± SE (6) for each point. Line fitted with eqn 5.4.


250
200
150
100
50
0
0.2
0.4
0.6
0.8
1.0
1.2
1.4
1.6
nequiv/kg.h
waterborne Zn (mg/L)

(eqn 5.4), suggesting either mediated transport, or the entry through a selective pore. Attempts to fit a model which added a linear term (eqn 5.5) were unsuccessful. The J_{max} was 314 nequiv/kg.h, considerably lower (2-25 fold) than estimates for influx arrived at by the traditional methods above. The apparent K_m was 0.24 mg/L (~~7.3 uequiv/L~~; Table 5.2).

The effect of acute changes in waterborne [Ca] on the relationship between influx rate and waterborne [Zn] was studied using water with [Ca] = 9.7, 4.7, 1.02 and 0.05 mequiv/L. Two of these experiments used water harder than tapwater (2.0 mequiv/L), and two softer. Increases in waterborne [Ca] to 4.7 or 9.7 mequiv/L had only a small effect on the J_{max} (Fig 5.10, Table 5.2). They did however greatly decrease the rate of influx at lower [Zn], and thus increase the apparent K_m over 6-fold compared with tapwater. Individual variability also decreased as [Ca] increased. When the fitted curves were plotted as double reciprocal plots acute addition of Ca clearly acted as a competitive inhibitor (Fig 5.11a).

Acute removal of Ca increased influx at all [Zn]'s. Variability of the data also increased. At [Ca] = 1.02 mequiv/L, influx appeared to saturate above 1.0 mg Zn/L (Fig 5.10). These data fitted the model (eqn 5.5) containing an unsaturable component much better than they did the simpler model (eqn 5.4). The J_{max} and apparent K_m

Figure 5.10: Zinc influx into 168 large rainbow trout at various waterborne Zn concentrations in water of five different [Ca]. Rates are based upon the steady state Zn radioactivity in the plasma (C_{ss}), mean ± SE (n), n is given for the average number of fish for each point on a curve. Lower 3 lines were fitted with eqn 5.4; upper two with eqn 5.5.

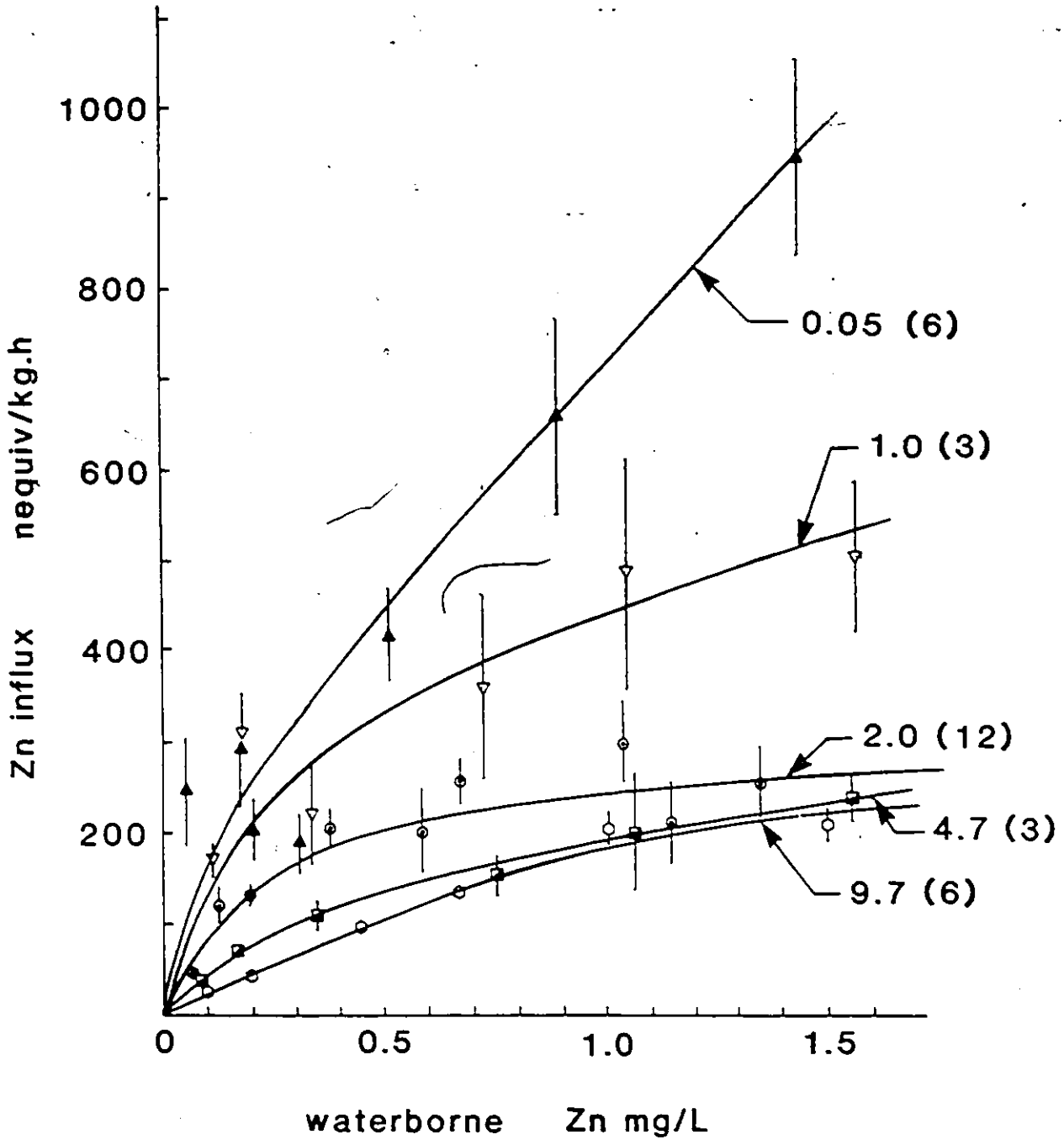
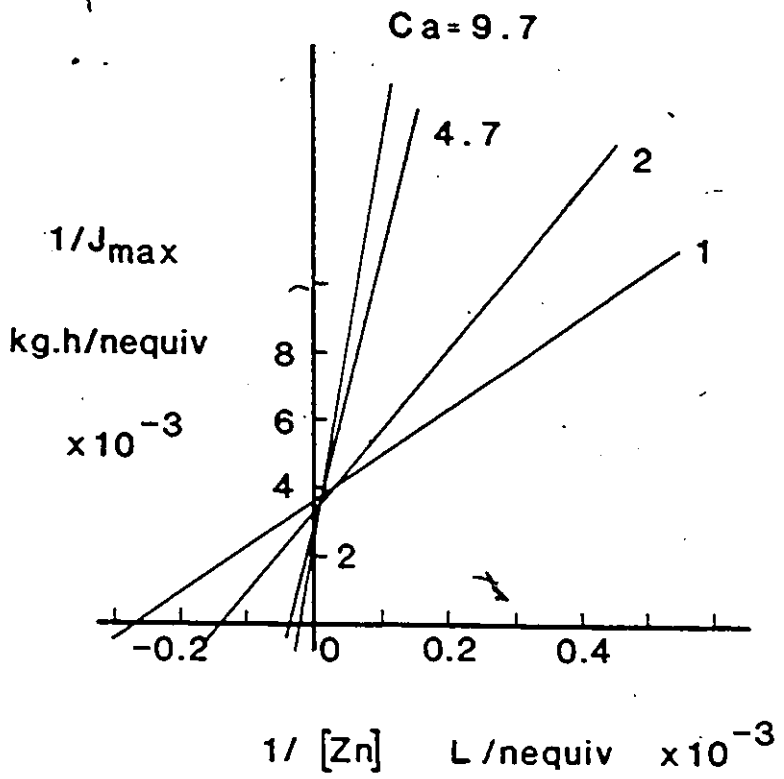
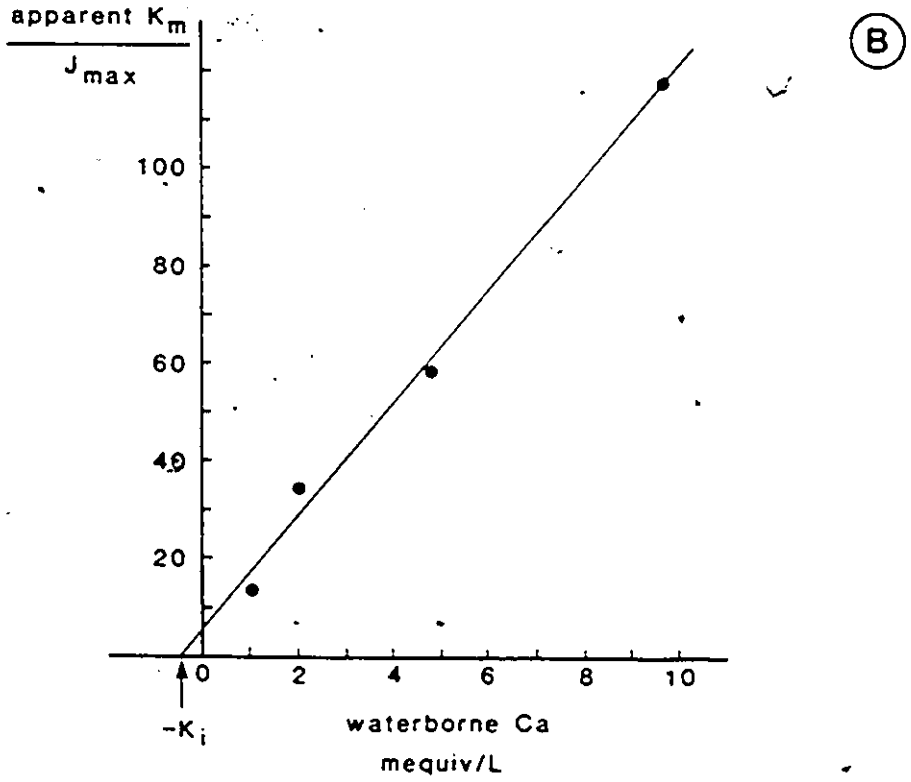

1000
800
600
400
200
Zn influx nequiv/kg.h
0.05 (6)
1.0 (3)
2.0 (12)
4.7 (3)
9.7 (6)
0
0.5
1.0
1.5
waterborne Zn mg/L

Table 5.2: J_{max} and K_m for Zn influx into rainbow trout at various waterborne [Ca].

[Ca]	J_{max}	K_m	
mequiv/L	nequiv/kg.h	mg/L	uequiv/L
9.7	458	1.53	46.8
4.7	364	0.85	26.0
2.0	314	0.24	7.3
1.0	277	0.12	3.7

Figure 5.11: (a) Double reciprocal plot of data from Figure 10, to show the nature of the competitive interaction between Zn and Ca. (b) Determination of the inhibitor constant for Ca. The ratio of apparent K_m to J_{max} is plotted against [Ca], and the x intercept = $-K_i$. The regression equation was $y = 11.6x + 5.6$; $r=0.996$, $P<0.01$.


Ca = 9.7
4.7
2
1
A
1/Jmax
kg.h/nequiv
x10⁻³
8
6
4
2
-0.2
0
0.2
0.4
1/ [Zn] L/nequiv x10⁻³


apparent Km
Jmax
B
100
80
60
40
20
0
2
4
6
8
10
-Ki
waterborne Ca
mequiv/L

of the saturable component when plotted as a double reciprocal compared well with the trends of data from higher waterborne Ca experiments (Fig 5.11a, Table 5.2). The apparent K_m was lowered to 0.12 mg/L (3.7 uequiv/L). This again is suggestive of the effect of Ca as a competitive inhibitor of influx. At the lowest [Ca] however, influx increased almost linearly at [Zn] > 0.5 mg/L, with no apparent saturation (Fig 5.10). The data did not fit either model, but could well reflect the presence of a dominant unsaturable component masking the saturable component. It was clear that acute removal of most of the waterborne [Ca] dramatically increased the transfer of Zn across the gill.

Since Ca inhibition of Zn influx resembled competitive inhibition, it was possible to determine an inhibitor constant (K_i) from a plot of apparent K_m/J_{max} vs [Ca] (Fig 5.11b; Segel, 1976). This gave a K_i for Ca of 480 uequiv/L. Interestingly, this is very similar to the K_m for Ca uptake (280 ± 70 uequiv/L) determined by Perry and Wood (1985) for similarly acclimated trout. Since all of the influx measurements were performed with some Ca present in the water, the apparent K_m values were all determined in the presence of an inhibitor. Knowing K_i, the true K_m's could be calculated (eqn 5.6). The mean value for the true K_m for Zn was 1.0 ± 0.1 (4) uequiv/L (0.06 mg/L) over the waterborne [Ca] from 1.02 to 9.7

mequiv/L. The affinity of the uptake system therefore appears to be nearly 300-fold higher for Zn than for Ca.

DISCUSSION

The Measurement of Zn Influx

Traditional methods used to partition unidirectional fluxes in fish, for bulk ions such as Na and Cl (see Kirschner, 1970) and K (Eddy, 1985) have successfully used a two compartment closed model with fish and water being the only compartments. Implicit in this model is the assumption that isotope which disappeared from the water entered the organism, with little or no adsorption. Adsorption was a problem with whole body Ca flux (Hōbe et al., 1984), although this difficulty was largely eliminated by rinsing in solutions of high unlabelled [Ca] (Perry and Wood, 1985). In the present study, significant adsorption also occurred with Zn, which could not be removed by rinsing in high [Zn] solutions. Zn has a high affinity for available ligands, and thus is not readily removed. Moreover, the true influx rate is low compared with all the bulk ions, and adsorption becomes a significant complication in short term experiments. Estimation of influx based upon disappearance from the water was clearly invalid. These rates were up to two orders of magnitude higher than those derived from plasma measurements, as a result of Zn adsorption to both the fish and the flux box.

Attempts to measure influx using whole body counting revealed that adsorption to the fish occurred very rapidly, resulting in rates of influx which were highest for the shortest exposure periods. With increasing exposure time, the adsorbed component was a smaller fraction of the total Zn uptake, and apparent flux rates declined, although they were still high compared with estimates using plasma activity.

Adsorption was also seen in the 1 h experiments with large trout. Since even dead trout took up Zn, there is a purely physical component of adsorption, similar to that seen in uptake by the flux boxes. Mucus is undoubtedly involved, although it was not specifically tested. The slightly higher uptake by the trunk of live fish in the tube shield experiments may have had a biological component, but it probably did not penetrate the animal, as there was a lack of any measurable activity in the plasma samples from these fish. The head region was responsible for most of the uptake, since this region contained the gills, both a site of absorption (Chapter 4) and significant adsorption. The contribution of gill area to the total surface area can exceed 50% (Hughes and Morgan, 1973). Other areas in the head besides the gill filaments themselves also appeared to be involved in adsorption. Drinking was clearly not a source of Zn uptake, as shown by the esophageal ligations.

Some of the Zn which adsorbed to the integument was

probably labile, since Joyner (1961) found that brown bullheads (Ictalurus nebulosus) exposed to 6.0 mg Zn/L for 96 h lost 43% of their accumulated ^{65}Zn after 1 day in clean water. In contrast, absorbed Zn was excreted very slowly, resulting in a half-life exceeding 250 d (Pentreath, 1973; Willis and Jones, 1977). Given this slow rate of loss for incorporated Zn, estimates of influx based upon whole body uptake would become reasonably accurate given a sufficiently long exposure (days-weeks). Such exposure would however provide ample time for acclimatory changes in the gill, which although interesting in themselves, would preclude study of acute responses.

The lowest estimates of influx were those based upon applying the calibration curve to the activity of terminal plasma samples taken when steady state activity was assured. Since these were also free from the confounding effect of adsorption of Zn to the exterior of the fish, they represent the best estimates of Zn influx presently available in vivo for trout. Measurements of Zn influx using the isolated, perfused head preparation (IPHP) yielded estimates about 30 fold lower than the present study at the same waterborne [Zn] (Chapter 4). Furthermore, Zn influx in the IPHP could not be detected below a waterborne [Zn] of 400 ug/L at [Ca]=0.09 mequiv/L (Fig 4.6), which was above the apparent K_m for a comparable [Ca] in vivo (see Table 5.2). Thus the IPHP was a much less sensitive technique for measuring Zn

influx, and undoubtedly yielded values far below true *in vivo* rates. The most likely explanation for this discrepancy is some sort of diffusive limitation. In the IPHP, this could be due to mucus build-up on the external surface of the perfused gill, retarding Zn diffusion to the uptake sites. In this regard, Pärt and Lock (1983) found that diffusion of two other metals, Cd and Hg, but not Ca, was greatly slowed in mucous solutions. Alternately, transbranchial Zn transport might simply be a slow process both *in vivo* and in the IPHP. The *in vivo* data of Fig 5.6 certainly suggest this explanation, for there was an apparent time lag of 0.5-1 h before measurable ^{65}Zn entry from the water appeared in the plasma. This period is comparable to the experimental lifetime of the IPHP, so Zn influx may not have had time to reach true equilibrium.

Whatever the explanation, the conclusion arising from the observations is that while the IPHP yields important qualitative information, and localizes Zn transport to the branchial route, *in vivo* measurements are clearly preferable for quantitative analysis. Interestingly, Cd influx measured *in vitro* using a similar IPHP (Pärt and Svanberg, 1981) was close to *in vitro* influx for Zn measured in Chapter 4. If the same relationship holds for Cd that appears to exist for Zn, then true influx rates for Cd *in vivo* may be much greater than these authors have reported.

The final flux protocol developed here offers several

advantages. Firstly, it measures only that Zn which enters the fish, and is completely free of any adsorbed component. In this regard it is similar to the IPHP, but superior for the reasons outlined above. Furthermore, it has considerable advantage over the perfused head, in terms of ease and convenience, for once the calibration curve has been established, operative techniques are not required. A single terminal blood sample yields the flux measurement. As well, in vivo flux measurements are inherently more desirable than those in vitro, since responses by the fish, particularly those which are hormonally-mediated, are not compromised. This technique therefore shows particular promise for trace metals whose waterborne concentrations are low, and which show a strong tendency toward adsorption.

The Kinetics of Zn Uptake and the Effects of Ca

Good influx measurements for trace metals are rare, and those which have examined responses to graded concentrations even rarer. Saturability has now been demonstrated for Zn influx using both in vivo (Figs 5.9, 5.10; present study) and in vitro methods (Chapter 4). The relationship also appears to hold for Cd using the IPHP (Pärt and Svanberg, 1981). This phenomenon implies a rate-limiting carrier or pore.

Changes in waterborne Ca, particularly its removal, had profound effects upon the kinetics of Zn influx (Fig 5.10). Competitive inhibition adequately described the

response in all but the lowest [Ca] (Fig 11). This was seen by the similarity in J_{max}, (though see below) but with increasing apparent K_m as [Ca] increased (Table 5.2). Removal of Ca, at least at 1.02 mequiv/L acted on the saturable component as would be expected for the removal of inhibitor (similar J_{max}, lower apparent K_m). In addition, at both levels of reduced [Ca] there was an unsaturable component, which increased as the [Ca] decreased. McWilliams (1983) postulated two types of Ca sites on the gills of brown trout. The higher affinity site was suggested to be the Ca transport site, while the lower affinity site may control permeability. While the presence of a transport site on the apical surface is at variance with the recent hypothesis of Flik et al. (1985), the high affinity site may still be related to active Ca transport. Acute removal of Ca from the water would thus have two effects: (i) removal of Ca from some component of its transport process across the gill, allowing Zn to traverse the gill by this mechanism with less competition from Ca; and (ii) an increase in the general permeability of the gill, to permit entry of Zn across the gill by a nonspecific and unsaturable route, perhaps simple diffusion through paracellular channels.

The kinetic analysis of the Ca vs Zn interaction indicated that the true K_m was 1.0 uequiv/L, while the K_i for Ca as a competitive inhibitor of Zn influx was about

480 uequiv/L. Interestingly the latter is very close to the measured K_m for branchial Ca influx (280 ± 70 uequiv/L; Perry and Wood, 1985) for similarly acclimated rainbow trout. This further supports the case for competitive interaction between these two divalent cations, and suggests that the competition may be for the active transport process. The affinity of the system for Zn appears to be 200-300 fold higher for Zn than for Ca.

Not only did waterborne Ca act as an inhibitor of Zn influx, but the opposite effect, inhibition of net Ca influx by acute exposure to Zn has been seen in rainbow trout in natural soft water (Appendix 1). A resultant fall in plasma [Ca] was also noted. These data further support Ca/Zn interaction in the gill. In that study, waterborne [Zn] was 24 uequiv/L and [Ca] was ~170 uequiv/L. In light of the differences in affinity noted above, it is not surprising that this low level of [Zn] was sufficient to abolish Ca uptake. Longer term exposure to Zn however may permit acclimation, and renewed Ca uptake in the continued presence of the stressor, since a 16 w exposure to [Zn] as high as 16 uequiv/L (0.53 mg/L) did not significantly alter plasma or whole body [Ca] in small trout (Chapter 2).

Based upon the foregoing, the following model is proposed. In the tapwater acclimated and tested trout ([Ca]=2.0 mequiv/L), Zn influx is transcellular, probably <u>via</u> the same mechanism through the "chloride" cells as Ca

influx (Flik et al., 1985; Perry and Wood, 1985). Zn will passively enter the cell down its electrochemical gradient, perhaps through an aqueous pore, and then move out into the plasma through the basolateral border. The rate limiting step could either be at the apical or basolateral border. This latter step may involve active transport via the high affinity Ca-ATPase isolated by Flik et al. (1985). Interestingly, Ca-ATPases in trout gill tissue in vitro were activated by Zn (Watson and Beamish, 1981), though this did not occur under all assay conditions and it is not clear whether these were the same high specificity Ca-ATPases as identified by Flik et al. (1985).

Over most of the range of [Ca] studied, Ca acts as a competitive inhibitor of Zn influx, either by titrating anionic groups on a pore or by binding to a carrier, thus requiring higher Zn concentrations to achieve the same uptake rate. As Ca is removed, a second phenomenon, unsaturable transport, is superimposed upon this. This is almost certainly the result of changes in permeability, which are likely paracellular, though a transcellular route cannot be ruled out.

Numerous uncertainties remain. The available evidence certainly does not prove that Zn entering via the saturable route is necessarily transported actively. If we assume that "free" Zn in the water is a large portion of the total (Campbell and Stokes, 1985), and that the "free" Zn in the

blood is ~0.2% of the total plasma Zn (Bettger et al., 1987), and given the small negative-inside transepithelial potential (McWilliams and Potts, 1978; Perry and Wood, 1985), then the driving force for Zn movement is clearly into the fish over most of the range of waterborne [Zn] tested.. While this exists for the overall water to blood pathway, nothing is known about the gradient between the inside of the gill cell and the blood. Earlier studies with fish liver slices, indicated that Zn was accumulated in first-order fashion against a seven-fold concentration gradient, and that energy was not required (Saltman and Boroughs, 1960). In contrast, more recent work has documented an energy requirement for Zn uptake by cultured rat hepatocytes (Failla and Cousins, 1978), and rat intestinal segments (Kowarski et al., 1974).

The nominal "purpose" of the Zn transport system also remains problematical. Is it a design feature of the gill, or just an "accidental" effect of the necessary presence of the Ca transport system? It could be argued since the apparent K_m for Zn uptake in normal tapwater (7.3 uequiv/L; Table 1) is rather high, almost into the toxic range (Spear, 1981) which would seldom be encountered by fish in the wild, that the system is not designed to transport Zn. However, this is misleading, for the apparent K_m is elevated in the presence of Ca; the true K_m is much lower (~1 uequiv/L). Certainly Zn uptake from the water occurs, and is

capable of preventing Zn deficiency in rainbow trout when dietary Zn is lacking (Chapter 2). The much lower J_{max} for Zn uptake (~400 nequiv/kg·h; Table 5.2) relative to Ca uptake (~12000 nequiv/kg·h; Perry and Wood, 1985) also suggests a design feature of the system. Thus even though affinity is very high, to enable uptake from low waterborne concentrations, the efficacy of Zn transport is very low, preventing high uptake rates which could be toxic.

Another uncertainty is the rather curious effect of [Ca] on this J_{max} for Zn influx. Instead of staying absolutely constant at varying [Ca] as expected for true competitive interaction, J_{max} showed a small but definite trend to increase as [Ca] increased (Fig 5.11, Table 5.2). The meaning of this is unknown for most standard complications (e.g. a contribution by non-competitive inhibition) would tend to lower J_{max}. Perhaps high transport rates of Ca in some way accelerate the movement of Zn, there by slightly increasing the efficacy of transport.

CHAPTER 6

GENERAL SUMMARY AND CONCLUSIONS

This thesis has examined routes and rates of Zn uptake by rainbow trout. The work was divided into two major areas. First was the relative contribution by dietary vs waterborne Zn. Associated with this, and giving insights into the metabolism and homeostasis of Zn was the measurement of inducible metal ligands. The second major area addressed was the actual measurement of rates of total uptake as assessed by whole body counting, and more importantly, rates of actual transbranchial transport using both an in vitro preparation, and isolated, perfused trout head, and a novel in vivo kinetic technique.

Relative Contributions of Dietary and Waterborne Zn Exposure

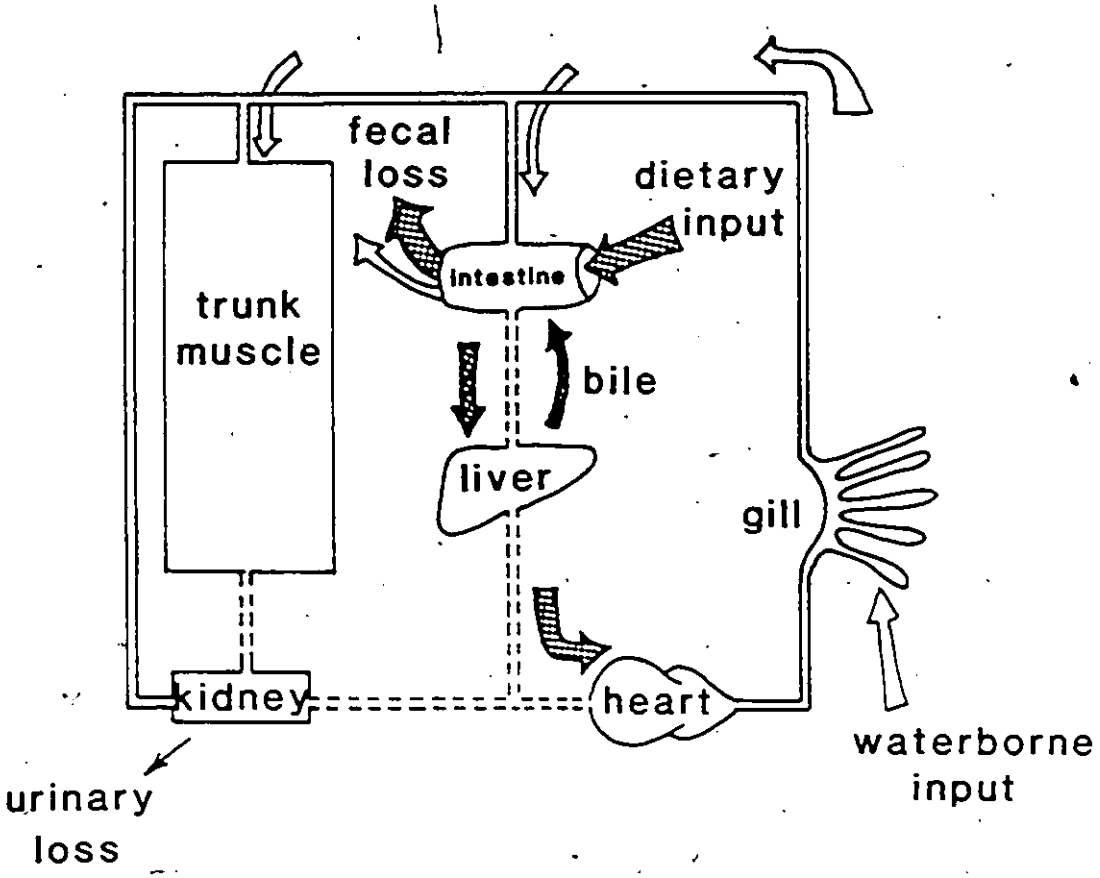
The present study has confirmed that dietary Zn is the primary source of the metal when waterborne Zn is at background levels. This was done with a factorial design which showed relative contributions most clearly. More importantly, it has indicated that branchial absorption is an important route when dietary levels were low, or waterborne levels high. As well, there is a relative homeostasis for Zn, in the face of both excess and deficiency. Plasma [Zn]

was more effectively regulated than was whole body [Zn]. The most interesting finding was that regardless of dietary input, additional waterborne exposure resulted in increased whole body [Zn]. This suggests that waterborne Zn was more readily taken up or less readily excreted, since it was in much lower concentration.

Although the data indicate a relative homeostasis for Zn, underlying mechanisms are unclear. The current mammalian model for Zn metabolism, as outlined in more detail in Chapter 1, is that Zn which enters the intestinal lumen is absorbed by carrier-mediated, perhaps active, transport. This can be made more effective when Zn input is low by the induction of putative carrier molecules in the enterocyte. At low Zn loads there is however inevitable fecal loss. When Zn load is high, both true and apparent absorption decline by mechanism(s) unknown. Urinary excretion is low and rather invariant.

Trout appear to conform to this basic model but with important differences (Fig 6.1). At low Zn loads there were indeed inevitable losses, and whole body [Zn] declined relative to initial values. At high dietary loads dietary retention declined dramatically. It is not clear whether percentage Zn uptake decreased, or whether serosal-mucosal secretion increased. Other possible excretory routes seem unimportant. Urinary excretion is low (Appendix 1), and although branchial excretion has been suggested for Ca

Figure 6.1: Possible disposition of Zn entering the body via the gills (open arrows) or the gut (stippled arrows). See text for further details.


fecal
loss
dietary
input
intestine
trunk
muscle
bile
liver
gill
kidney
heart
urinary
loss
waterborne
input

(Shephard, 1982) and Cd (Oronsaye and Brafield, 1984) and Zn (Hardy et al., 1987) the bulk of the literature indicates apical-basolateral transfer (analogous to mucosal-serosal transfer) across the gill epithelium. The most likely mechanism for the decrease in apparent absorption seems to be a decrease in percentage absorption by mechanisms currently unknown, rather than increases in compensatory excretory routes.

Where fish differed markedly from the mammalian model was in the impact of waterborne Zn. The fact that there was always an impact of waterborne Zn suggests that branchial Zn was handled at least somewhat separately from intestinally absorbed Zn. This is particularly true given the much lower waterborne [Zn]. It is most probable that Zn of branchial origin was cleared from the plasma into a tissue compartment before it could be secreted into the gut. Much of the blood from the dorsal aorta perfuses the trunk muscles (Stevens, 1968), therefore Zn could be cleared to these tissues prior to contacting the intestine or the liver where it might be secreted.

Chapter 2 has also underscored the lack of toxic effects of quite high levels of both dietary and waterborne Zn, at least in nonreproducing trout. Although it has recently been proposed that the water quality objective for Zn be lowered from 30 to 10 ug/L (U. Borgmann, pers. comm.), this move reflects the fact that fish are no longer

considered the "most sensitive" species. In this regard the apparent prophylactic effect of elevated waterborne and/or dietary Zn, as discussed in Chapter 2, might be useful in hatchery situations. It is under these stressful conditions of crowding where both disease organisms and frank nutritional deficiency might be alleviated by increased dietary and waterborne Zn.

Induction of Metal Binding Ligands.

The pattern of metal binding ligands was also instructive regarding the metabolism of Zn in trout exposed to both waterborne and dietary Zn. Some of these findings were somewhat surprising, and differed from the initial hypothesis that routes of exposure would be reflected in concentrations of metal binding ligands. The general lack of response of the liver was in contrast to work with mammals, and suggests that much of the Zn was in fact not processed by the liver. As predicted, gill tissue responded to increased waterborne [Zn], as seen most clearly at the highest waterborne [Zn]. There did appear to be a threshold somewhere between 39 and 148 ug Zn/L, below which there was no effect. There was also a lesser effect of diet overlying the response to waterborne Zn. This could only be a result of exposure to Zn on the serosal side although the effect did not correspond directly to total plasma [Zn].

Intestinal metal binding proteins, presumably induced in the enterocytes, responded dramatically to both high

dietary and high waterborne inputs. That is, on the deficient diet, increases in waterborne [Zn] had only slight effect. Similarly, at background waterborne [Zn], increases in dietary load induced only a small increase. Combined dietary and waterborne effects however were dramatic, again with an apparent threshold for waterborne effect between 39 and 148 ug/L. This suggests that very little Zn entering via the dietary route was absorbed, since large increases in dietary load when waterborne Zn was low, were not reflected in high levels of metal binding protein. The effect of high waterborne loading in Zn-adequate fish suggests Zn secretion in a serosal-mucosal direction into enterocytes, induction of metal-binding proteins, and eventual excretion. Secretion of Zn from the enterocytes, or sloughing of enterocytes themselves would thus be an important route for the excretion of excess Zn if this material were not reabsorbed. Induction of metal binding proteins is thus seen as an important aspect of Zn detoxification. Basal levels exist over much of the "normal" range of exposure, and rise rapidly with increasing exposure from either source.

Routes of Branchial Zn Influx

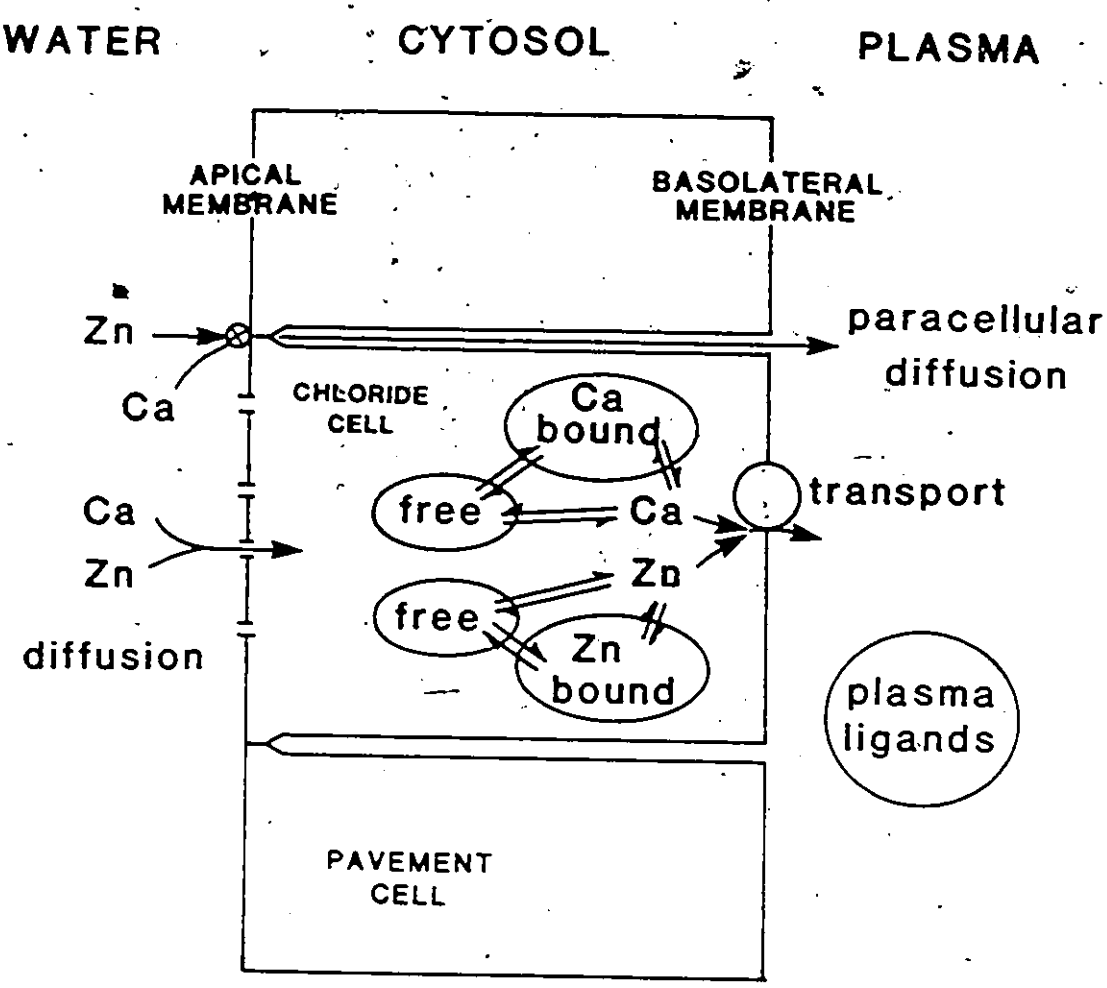
The above work identified the gill as an important site of Zn influx. The several methods which were then used to determine rates of branchial influx revealed methodological problems mainly involved with non-specific

adsorption. Solutions to these problems permitted accurate measurements of influx.

Insights into the nature of branchial Zn transport were provided by both *in vitro* and *in vivo* methods. Both of these methods indicated the saturable nature of Zn influx, and showed clearly that it was not a simple linear function of the water-to-blood gradient. The perfused head preparation was particularly valuable in demonstrating that the arterio-arterial route is at least as significant if not more so than the arterio-venous route. As well, in contrast to the mammalian model for transport (Chapter 1), natural ligands in the perfusate did not significantly increase Zn influx. The IPHP also disclosed a good correlation between induction of chloride cells and the increases in Zn transfer. The preparation was not without its drawbacks however. Measured influx rates were extremely low, transfer did not occur below 0.4 mg Zn/L, and there was little change in transfer rate under acute changes in waterborne [Ca]. These problems were overcome by the development of a novel *in vivo* kinetic technique which yielded true Zn influx rates. With this technique, rates were greater overall, transport was detectable even at 50 ug Zn/L and Ca was definitely identified as a competitive inhibitor of Zn transport.

Based upon this information, the following model is proposed for branchial Zn transport (Fig 6.2). Chloride

Figure 6.2: A model for Zn transport across the gill. Zn and Ca may compete at any of three possible sites. The chloride cell is probably the major pathway. See text for further details.


WATER
CYTOSOL
PLASMA
APICAL MEMBRANE
BASOLATERAL MEMBRANE
Zn
Ca
paracellular diffusion
CHLORIDE CELL
Ca bound
free
Ca
transport
Ca
Zn
Zn
free
Zn bound
diffusion
plasma ligands
PAVEMENT CELL

cells appear to be the cell type most important for the movement of Zn. Blood Zn activity is low (Bettger et al., 1987), and cytosolic activity assumed to be even lower (Williams, 1981). Thus under conditions of even slightly elevated waterborne [Zn], the driving force will be into the cell. Zn thus diffuses into the cell where it forms part of the intracellular Zn pool, and may be bound by metallothionein or other ligands. Paracellular entry does not generally occur except in the case of acute removal of Ca from the paracellular junctional complexes. The similarity between the value of Ca as an inhibitor and Ca as a transported substrate (Perry and Wood, 1985) suggests that Zn then moves out of the chloride cell via the Ca mechanism which is located on the basolateral border. This movement appears to be independent of binding ligands in the bloodstream. The rate-limiting step could exist either at the apical or basolateral border.

What is not clear however is the extent to which the uptake of Zn via the gill is advantageous to fish under natural conditions. As a transported substrate Ca is probably more important than Zn. Certainly a characteristic of the system, with Zn as a substrate, is a high affinity which permits uptake when waterborne [Zn] is low, while at the same time having low transfer rates compared with elements such as Ca. This would limit the impact of any large increases in waterborne [Zn] on Zn uptake, further

contributing to Zn homeostasis. Also unknown is the ability of this system to transfer other important waterborne trace elements. While some of these are important trace nutrients, others are potential toxicants without known nutritional value but having demonstrated toxic effects, such as Pb, Hg and Cd. It would therefore be instructive to characterize this system with other important trace metals.

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APPENDIX 1

ION FLUX RATES, ACID-BASE STATUS AND BLOOD GASES IN RAINBOW TROUT, *SALMO GAIRDNERI*, EXPOSED TO TOXIC ZINC IN NATURAL SOFT WATER

Ion Flux Rates, Acid–Base Status, and Blood Gases in Rainbow Trout, *Salmo gairdneri*, Exposed to Toxic Zinc in Natural Soft Water

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Spry, D. J., and C. M. Wood. 1985. Ion flux rates, acid–base status, and blood gases in rainbow trout, *Salmo gairdneri*, exposed to toxic zinc in natural soft water. Can. J. Fish. Aquat. Sci. 42: 1332–1341.

Exposure to 0.8 mg Zn^{2+}/L in natural soft water for up to 72 h was toxic to rainbow trout, *Salmo gairdneri*, causing an acid–base disturbance and net branchial ion losses. Mean arterial pH fell from 7.78 to 7.58. Both Pa_{CO_2} and lactate rose, indicating a mixed respiratory and metabolic acidosis, despite maintenance of high Pa_{O_2}. Net branchial uptake of Na^+ and Cl^- became a net loss immediately following exposure to Zn^{2+}, and this continued during 60 h of exposure. Net K^+ loss was exacerbated, and net Ca^{2+} uptake was abolished. Unidirectional flux measurements with $^{22}Na^+$ and $^{36}Cl^-$ indicated an increased efflux immediately following zinc exposure. Both influx and efflux of Na^+ and Cl^- were stimulated after 48–60 h in Zn^{2+}. Both net branchial ammonia excretion and net branchial uptake of acidic equivalents from the water (= base loss) were greatly stimulated, the latter contributing to metabolic acidosis. Kidney function, as measured by urine flow rate and excretion of ammonia, acidic equivalents, Na^+, Cl^-, K^+, and Zn^{2+}, was relatively insensitive to the effects of zinc. The only renal component to be affected was Ca^{2+} excretion, which decreased during a single flux period, possibly in response to the reduced entry of Ca^{2+} at the gill. We conclude that toxic concentrations of zinc are capable of altering gill function so as to cause ionoregulatory and acid–base disturbances without disturbance of Pa_{O_2}.

L'exposition de truites arc-en-ciel, *Salmo gairdneri*, à 0,8 mg de Zn^{+2} par litre d'eau douce naturelle pendant 72 h a eu une incidence toxique, c.-à-d. une perturbation de l'équilibre acide–base et des pertes nettes d'ions branchiaux. Le pH artériel moyen est passé de 7,78 à 7,58 tandis que le Pa_{CO_2} et le lactate ont augmenté, ce qui indique une acidose respiratoire et métabolique hétérogène malgré le maintien d'un Pa_{CO_2} élevé. La captation nette de Na^+ et de Cl^- au niveau des branchies est devenue une perte nette immédiatement après l'exposition à du Zu^{+2} et pendant 60 h d'exposition. La perte nette de K^+ a été aggravée et la captation nette de Ca^{+2}, supprimée. Des quantifications du flux unidirectionnel de $^{22}Na^+$ et de $^{36}Cl^-$ ont révélé un écoulement accru immédiatement après l'exposition au zinc. L'entrée et la sortie de Na^+ et de Cl^- ont été stimulées après une exposition au Zn^{+2} allant de 48 à 60 h. L'excrétion nette d'ammoniaque branchial et la captation nette d'équivalents acides du milieu au niveau des branchies (= perte de base) ont été grandement stimulées; cette dernière a contribué à l'acidose métabolique. La fonction rénale, telle que mesurée par le taux d'évacuation de l'urine et l'excrétion d'ammoniaque, d'équivalents acides, de Na^+, de Cl^-, de K^+ et de Zn^{+2}, a été relativement insensible aux effets du zinc. L'excrétion du Ca^{+2} était la seule composante rénale touchée : elle a diminué au cours d'une seule période de flux, probablement en réaction à la réduction de l'entrée de Ca^{+2} au niveau des branchies. Les auteurs formulent la conclusion que des concentrations toxiques de zinc sont capables de modifier la fonction des branchies et de causer des perturbations de l'ionorégulation et de l'équilibre acide–base sans modifier le Pa_{O_2}.

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Concentrations of waterborne zinc that are rapidly lethal to trout severely disrupt gill tissue (Skidmore and Tovell 1972) and hence oxygen transfer by imposing a diffusion limitation for oxygen. The result is hypoxia (Skidmore 1970) and acidemia (Sellers et al. 1975; Spry and Wood 1984). The effects upon ion regulation are less clear. Skidmore (1970) found small but significant changes in plasma osmotic pressure in rainbow trout, *Salmo gairdneri*, exposed to 40 mg Zn^{2+}/L, but discounted these as being unimportant in the rapidly developing lethality. Spry and Wood (1984) reported no significant changes in major blood electrolytes in dying trout which showed hypoxemia and acidosis in 1.5 mg Zn^{2+}/L when compared with controls. This might have been obscured by the observed hemoconcentration. However, at a lower concentration (0.8 mg Zn^{2+}/L), where acute hypoxemic death did not occur, plasma Ca^{2+} levels decreased over a 3-d exposure but other plasma ions were unaffected. Lewis and Lewis (1971) noted a decrease in the serum osmolality of channel catfish (*Ictalurus punctatus*) exposed to lethal Zn^{2+} solutions (12–30 mg/L). When they added NaCl to the water to create an external

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osmotic pressure of 235 mosmol/L, mortality was delayed. This latter study suggests that regulation of Na^+ and/or Cl^- might indeed be affected, and possibly be of primary importance, under conditions where oxygen delivery was not clearly limiting.

Zinc might exert such an effect upon ion regulation by altering ATPase activities. Watson and Beamish (1981) showed in vitro inhibition of various ATPases in freshwater-adapted rainbow trout, although an earlier study (Watson and Beamish 1980) showed increased activity in vivo after 30 d in Zn^{2+}. This increased activity was suggested to be secondary to a Zn^{2+}-induced increase in gill permeability even though serum osmolality and electrolytes were unchanged. Zinc also inhibited chloride transport across the isolated opercular epithelium of seawater-adapted *Fundulus heteroclitus*, possibly as a consequence of its inhibitory effect in vitro upon Na^+,K^+-ATPase (Crespo and Karnaky 1983).

It is well known that zinc toxicity increases with decreasing hardness and alkalinity (Spear 1981). In the very soft water of Ontario Precambrian Shield lakes, zinc enrichment accompanying acidification may become a problem (Spry et al. 1981). The effects of zinc exposure on rainbow trout in *artificial* soft water have been reported earlier (Spry and Wood 1984). To compare these results with exposure in *natural* soft water of the Shield area, we completed a series of experiments at a field site which, in addition, examined the effects of a low level of Zn^{2+} (0.8 mg/L) upon ionoregulation. Our objectives were firstly, to measure acid–base, ionic, blood gas, and other blood variables in rainbow trout fitted with dorsal aortic cannulae and secondly, to separate branchial and renal net ammonia and acidic equivalent fluxes, as well as net and unidirectional ion fluxes, in trout with bladder catheters. We chose a zinc concentration close to the 96-h LC50 for comparison with the previous study (Spry and Wood 1984). Branchial and renal flux rates have not previously been measured in fish exposed to Zn^{2+}.

Materials and Methods

Rainbow trout underyearlings were procured from Skeleton Lake Hatchery or Milford Bay Trout Farm in late summer. Both hatcheries had soft water (Ca^{2+} = 0.2 mequiv/L). Fish were moved by truck to Harkness Laboratory in Algonquin Park (latitude 45°42′; longitude 78°23′) where they were kept in tanks supplied with flowing Lake Opeongo water at 17–22°C. A commercial pelleted diet (Martin Feed Mills, Elmira, Ont.) was fed daily. This was the same diet as used in our previous study (Spry and Wood 1984). Trout were acclimated to 15°C for at least 4 d in a flow-through system chilled with Min-o-cool units (Frigid Units). Food was withheld 2 d prior to any surgery and throughout the experimental period.

Blood Measurements

To assess the effects of Zn^{2+} exposure on arterial blood composition, we cannulated the dorsal aorta (Smith and Bell 1964) of rainbow trout (141–267 g) under MS222 anesthesia and placed each fish in one of eight individual compartments of a black acrylic box, allowing them to recover for 36–48 h. Cannulae were periodically flushed with heparinized (ammonium heparin, 100 IU/mL) Cortland saline (Wolf 1963). Water was circulated to the fish from a common head tank at 200 ± 16 mL·min^{-1}·fish^{-1}, collected in a sump tank, and then cooled, aerated, and returned to the head tank. Total volume of the system was 70–115 L, and there were no metal parts in contact with the water. A continuous slow input to the recirculating system of fresh lake water, or lake water plus toxicant, provided a 90% replacement time of 12–14 h (Sprague 1973). Water quality characteristics representative of the "best" and "worst" cases-(i.e. start of the experiment, and in one trial where the system was closed for 24 h) are given in Table 1; typical experimental values lay between these extremes.

TABLE 1. Some water quality measurements under control conditions, means ± SE (*n*).

Variable	Lake Opeongo water prior to start of experiment	Lake Opeongo water from test battery after 24 h with fish in place, without replacement
Na^+ (mequiv/L)[a]	0.067±0.003 (5)	0.089±0.011 (6)
Cl^- (mequiv/L)[b]	0.028±0.003 (5)	0.075±0.029 (6)
K^+ (mequiv/L)[a]	0.020±0.001 (5)	0.056±0.018 (6)
Ca^{2+} (mequiv/L)[a]	0.170±0.001 (5)	0.170±0.012 (6)
NH_4^+ (mequiv/L)[c]	0.040±0.004 (4)	0.190±0.050 (4)
NO_3^- (mequiv/L)[d]	—[e]	0.001±0.005 (4)
Al (μg/L total)[f]	6.46	10.66
Zn (μg/L total)	<50	<50
Conductivity (μS/cm)	46.90±0.48 (8)	75.25±6.19 (8)
Temperature (°C)	15.5±0.3 (12)	15.5±0.3 (12)
pH[g]	6.78±0.27 (6)	7.32±0.05 (8)
Alkalinity (μequiv/L)[h]	95	282

[a]Measured by flame photometry.
[b]Measured by coulometric titration.
[c]Method of Verdouw et al. (1978).
[d]Standard methods (APHA et al. 1975).
[e]Not measured.
[f]Lumogallion fluorescence method of Playle et al. (1982).
[g]Statistics performed on [H^+] and converted to pH.
[h]Single measurement, inflection point titration.

Blood variables were measured daily for 4 d. After the first sample (control), reagent-grade $ZnSO_4 \cdot 7H_2O$ was added to the head tank to give 0.8 mg/L. The blood sampling protocol consisted of drawing 0.6 mL of arterial blood into gas-tight Hamilton syringes for determinations of arterial pH (pHa), total CO_2 (Ca_{CO_2}), oxygen partial pressure (Pa_{O_2}), hematocrit (hct), and concentrations of hemoglobin ([Hb]) and lactate. An additional 0.3 mL of sample was centrifuged for 5 min at 8000 × *g* and plasma removed for analysis of Na^+, Cl^-, K^+, Ca^{2+}, and total protein (C_{Pr}). The pellet of red blood cells (rbc) was resuspended in heparinized Cortland saline and the rbc returned to the fish to reduce loss due to sampling. Hemolysis occurred rarely, and these samples were discarded. Here, saline alone was infused to maintain extracellular fluid volume. On average, the total whole blood removed at each sample time was 0.6 mL, of which the rbc from 0.3 mL were returned. Data from fish whose hct fell below 5% were discarded, since anemia itself can provoke an acidosis (Wood et al. 1982). In fact, only 2 of 12 final hct values were <9%, and the mean was 13.5 ± 1.7% (12).

Branchial and Renal Flux Rates

To separately measure branchial and renal flux rates of ions and "acidic equivalents" under control conditions and subsequent exposure to 0.8 mg Zn^{2+}/L, we implanted only bladder catheters (Wood and Randall 1973b), using MS222 anesthesia, in a second group of fish. Each fish was then placed in a flux box (McDonald 1983a), consisting of a small inner box of clear

acrylic which confined the fish and a larger outer box of black acrylic which held 6–8 L of water. Boxes were placed on a wet table flooded with cool well water to maintain 15–17°C. An airlift pump at the rear of the inner box, together with perimeter aeration within each outer box, ensured circulation and maintained $Pw_{O_2} > 120$ Torr. Urine was collected in 50-mL flasks outside the boxes. Thus, changes in the composition of the water during the flux period reflected net branchial fluxes.

Following 3–4 d of recovery, three control-fluxes were monitored, followed by five treatment (0.8 mg Zn^{2+}/L) fluxes of 12 h each. Variation within the three control periods was minimal, and composite control means are reported. Boxes were flushed with fresh solution (lake water or toxicant) at 12-h intervals, providing 70–80% replacement, based upon ammonia dilution. Since this procedure took ~2 h, the branchial fluxes were actually measured over 10 h, whereas the renal collections were throughout the entire 12-h period. Samples for water titratable alkalinity or urine titratable acidity minus bicarbonate ([TA − HCO_3^-]) were stored at 4°C and analyzed within 24 h. The remainder were frozen (−20°C) for later analysis.

Unidirectional branchial fluxes of Na^+ and Cl^- were partitioned after Kirschner (1970) during the first control flux period, flux period 1 (step change to Zn^{2+}), and flux period 5 (2 d of Zn^{2+} exposure). One hundred and eighty five kilobequerels (5 μCi) of $^{22}Na^+$ and 92.5 kBq (2.5 μCi) of $^{36}Cl^-$ (New England Nuclear) were added to each flux box and mixed thoroughly, and water samples were taken at 0, 0.5, 1, 1.5, 2, 3, 4, and 5 h.

Analytical Methods

Whole blood and plasma Ca_{CO_2} (Cameron 1971), pHa, and Pa_{O_2} were measured on a Radiometer PHM 71 acid–base analyzer fitted with gas modules. The pH and O_2 electrodes were water-cooled to the experimental temperature, while the CO_2 electrode in the Cameron chamber was maintained at 37°C. The pH electrode was calibrated frequently with precision buffers. The O_2 electrode was calibrated with water-saturated nitrogen or air and the Cameron chamber by known bicarbonate addition. L-(+)-lactate was determined enzymatically (LDH/NADH, Sigma Technical Bulletin 726UV/826UV) after deproteination of whole blood in 8% $HClO_4$. Hemoglobin was measured as cyanmethemoglobin (Sigma Technical Bulletin 525) using Sigma or Hycel reagents. Hematocrit was measured by centrifuging blood in heparinized microhematocrit tubes at 5000 × *g* for 5 min. The mean cell hemoglobin concentration (MCHC) was calculated as [Hb]/hct × 100 (Dacie and Lewis 1975). Plasma total protein determination was by refractometry (American Optical). Plasma Cl^- was titrated on a chloridometer (Radiometer CMT-10). After suitable dilution and swamping to eliminate interference effects, plasma Na^+ and K^+ were measured on an EEL mk 2 flame photometer. Plasma Ca^{2+} was measured by colorimetry (Sigma Technical Bulletin 585).

Major ions (Na^+, K^+, Ca^{2+}, Cl^-) in water and urine were measured as for plasma except for water Cl^-, which at very low levels was assayed with a Buchler–Cotlove chloridometer. The acid reagent had 0.2 mmol NaCl/L added to provide a linear response. Ca^{2+} and Zn^{2+} in the water and urine were determined by atomic absorption spectrophotometry (Varian AA1275, or initially for Zn^{2+}, a Jarrel-Ash 800). Total water and urine ammonia levels were determined using a micromodification of the salicylate–hypochlorite method of Verdouw et al. (1978). For titratable alkalinity, 10 mL of water was gently aerated and titrated with 0.02 mol HCl/L from a Gilmont burette to <pH 4, and the volume of titrant required to titrate to pH 4 was interpolated (DeRenzis and Maetz 1973; McDonald and Wood 1981). Aeration throughout titration ensured CO_2 removal. Titration of urine was by the single step determination of titratable acidity minus bicarbonate ([TA − HCO_3^-]) (Hills 1973) in which sufficient 0.02 mol HCl/L was added to 500 μL of urine to drive the pH to <4. This was then aerated for CO_2 removal and titrated back through the pHa (mean day 0 value, acid–base experiment) with freshly standardized 0.02 mol NaOH/L. The volume of titrant at pHa was interpolated and the volume of the acid added was subtracted.

For unidirectional flux measurements, water samples were counted as follows. $^{36}Cl^-$ is a pure beta emitter, while $^{22}Na^+$ is a mixed beta and gamma emitter. Dual labelled water samples were prepared in duplicate, with $^{22}Na^+$ alone measured in a well-type counter (Nuclear-Chicago model 1085) and $^{22}Na^+$ plus $^{36}Cl^-$ by scintillation counting (Beckman LS 250). After correction for difference in efficiency of $^{22}Na^+$ counting by the two machines, the $^{36}Cl^-$ counts were obtained by subtraction.

Calculations

The partial pressure of CO_2 in arterial blood (Pa_{CO_2}), the HCO_3^-, and the total blood metabolic acid load (ΔH^+_m) were calculated using standard acid–base equations as described by Spry and Wood (1984). Values for α_{CO_2} (CO_2 solubility in plasma), pK_1' (apparent first dissociation constant of carbonic acid), and β (nonbicarbonate buffering capacity) at a particular blood [Hb] were taken from Severinghaus (1965), Albers (1970), and Wood et al. (1982), respectively.

Net branchial ion fluxes observed from changes in waterborne concentrations were calculated as follows:

$$(1)\quad J_{net} = \frac{[X]_i V_i - [X]_f V_f}{t \cdot W}$$

where $[X]$ is the ion concentration (microequivalents per litre), V is the box volume which decreases during the period due to sampling, subscripts i and f are initial and final, respectively, t is the duration of the flux period (hours), and W is the fish weight (kilograms). Thus, net losses by the animal have a negative sign and net gains a positive sign. For titratable alkalinity, $V_t N/V_s$ is substituted for $[X]$, where V_t is the titrant volume (millilitres), N is the acid normality (microequivalents per litre), and V_s is the sample volume (millilitres). By reversing the i and f terms, the net titratable acidity flux was calculated from the titratable alkalinities. The net acidic equivalent flux is the sum of titratable acidity flux and ammonia flux, signs considered (cf. McDonald and Wood 1981).

For unidirectional branchial fluxes, J_{in} was calculated from the natural logarithm function given by Kirschner (1970), since no measurable backflux of isotope occurred:

$$(2)\quad J_{in} = \frac{Q_{out}}{t \cdot W} \cdot (\ln Q^*_{out(0)} - \ln Q^*_{out(t)})$$

where Q_{out} is total amount of the desired ion in the medium and Q^*_{out} is the total amount of radioactivity (cpm) at time 0 and t, respectively. J_{out} (negative by convention) was calculated as $J_{net} - J_{in}$. Renal ion losses were the product of concentration and urine flow rate (UFR) whereas the renal net acidic equivalent flux is given by

$$(3)\quad J_{out} = ([TA - HCO_3^-] + [NH_4^+]) \cdot UFR.$$

total ammonia being considered equivalent to $[NH_4^+]$, as free NH_3 was negligible at urine pH.

All values are reported as mean ± 1 standard error. To assess significant differences, we used paired Student's *t*-test where possible, since each fish served as its own control. Where missing values made this impossible, unpaired *t*-tests were used, with a resultant loss of power (Steel and Torrie 1960).

Results

Exposure to 0.8 mg Zn^{2+}/L in natural soft water resulted in significant blood acidosis, a decrease in plasma Ca^{2+}, increase in plasma K^+, and altered branchial ion and acidic equivalent fluxes. In contrast, there was little effect upon renal fluxes. The exposure was toxic, and overall mortality in 31 fish was 53% over 3 d. For blood data, values for all fish, and for those that survived the 3-d exposure, are shown separately in Fig. 1–3. Differences between the two data sets were not significant. The response of the survivors was therefore representative of the experimental population as a whole. For branchial and renal fluxes, only data from surviving animals have been plotted in Fig. 4–6 and Table 2 for the sake of clarity. Flux rates in dying fish showed similar trends, but generally increased and/or became highly erratic prior to death. Due to time constraints in the field situation, parallel controls were not run. However, under similar conditions in artificial softwater, the protocol caused neither mortality nor acid–base nor ionic disturbance (Spry and Wood 1984).

Examination of the acid–base status of fish that survived to the end of the experiment (Fig. 1a–1c) revealed a gradual and progressive decline in pHa which became significant on the final day. It was primarily respiratory in nature as shown by the rise in Pa_{CO_2}, with no significant change in plasma bicarbonate. Analysis of means plotted on a pH–bicarbonate diagram (Davenport 1974, not shown) indicated that the acidosis was mixed respiratory and metabolic, with the respiratory component becoming significant on the last day (Fig. 1c). The small but significant rise in blood lactate (Fig. 1e) was consistent with a metabolic component although the blood metabolic acid load was not significantly increased (Fig. 1f). The Pa_{O_2} remained uniformly high (Fig. 1d), a surprising finding in light of the increase in both Pa_{CO_2} and lactate. While ventilation was not directly assessed, there was no noticeable hyperventilation that might be associated with hypoxemia.

Additional blood parameters (Fig. 2) all showed significant declines with time. Similar declines were noted for hct, Hb, and C_{Hb} under control conditions (Spry and Wood 1984) which were attributable to the sampling protocol. This indicates that no significant hemoconcentration occurred in the present study, in contrast with that seen in more acutely lethal exposures at higher zinc levels (Spry and Wood 1984). However, MCHC did fall significantly (Fig. 2c), suggesting either that rbc swelling accompanied the developing acidosis or that a mobilization of Hb-poor rbc (e.g. reticulocytes) occurred.

Of the major plasma ions, Na^+ and Cl^- showed some fluctuation (Fig. 3a, 3b) but were essentially unchanged. However, K^+ rose significantly on the last day (Fig. 3c), while Ca^{2+} fell significantly after only 1 d (Fig. 3d), a trend that became even more pronounced over the following days.

Under control conditions, branchial ammonia excretion was −200–250 μequiv·kg^{-1}·h^{-1} (Fig. 4), or about 20-fold higher than renal ammonia efflux (Table 2). This was approximately balanced by the titratable acidity "uptake" (Fig. 4), such that

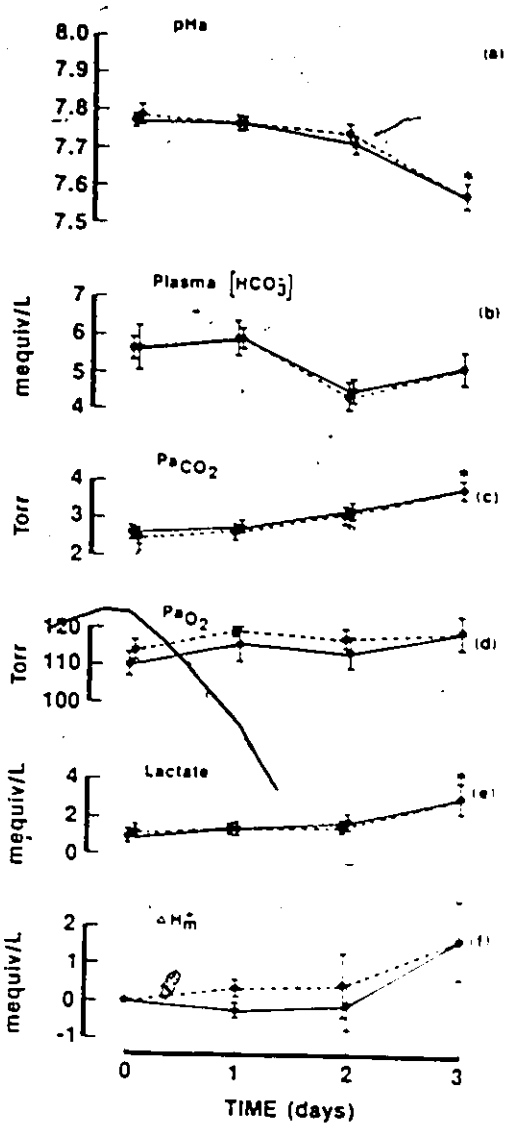

FIG. 1. Arterial blood measurements (a) pHa, (b) plasma $[HCO_3^-]$, (c) Pa_{CO_2}, (d) Pa_{O_2}, (e) lactate, and (f) metabolic acid load in rainbow trout under control conditions (day 0) and subsequently exposed to 0.8 mg Zn^{2+}/L. Means ± SE are shown for survivors to day 3 (n = 11, solid line) or all fish (n = 30, 24, 22, and 11 for day 0 to day 3, respectively). Asterisks denote means significantly different from control values (p < 0.05)

the net branchial acidic equivalent flux was either close to or slightly above zero (Fig. 4). Upon Zn^{2+} exposure, both components slowly increased over the following 3 d. However, the titratable acidity "uptake" increased to a greater extent than the ammonia efflux, so that an increasingly positive net acidic equivalent flux occurred, which reached approximately +200 μequiv·kg^{-1}·h^{-1} by the final period.

The branchial net fluxes for Na^+, Cl^-, and Ca^{2+} (Fig. 5a, 5b, 5d) were positive under control conditions, representing a net

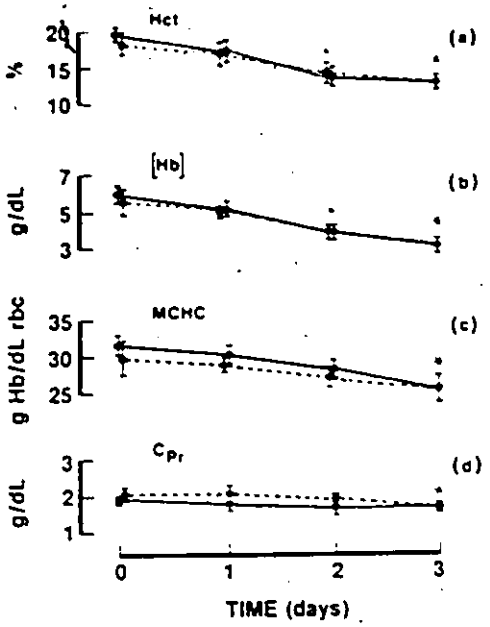

FIG. 2. Arterial blood measurements (a) hematocrit, (b) hemoglobin, (c) mean cell hemoglobin concentration, and (d) plasma total protein in rainbow trout under control conditions (day 0) and subsequently exposed to 0.8 mg Zn^{2+}/L in natural soft water. Means ± SE are shown for survivors to day 3 (n = 12, solid line) or all fish (n = 29, 24, 22, and 12 for day 0 to day 3, respectively). Statistics as in Fig. 1.

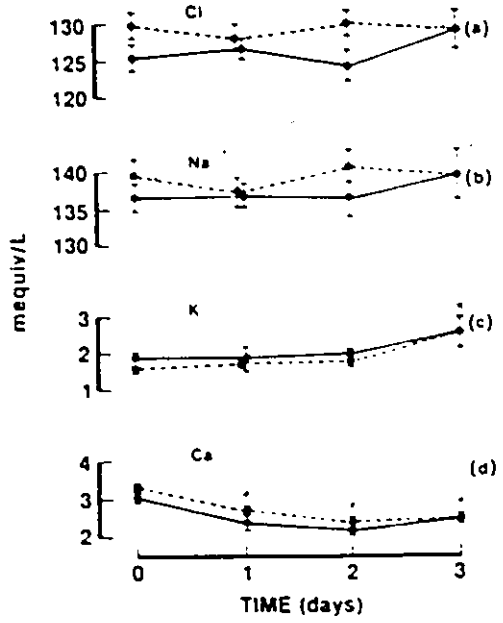

FIG. 3. Plasma ions (a) Cl^-, (b) Na^+, (c) K^+, and (d) Ca^{2+}. Legend as for Fig. 1 except for K^+ (survivors, solid line, n = 14; all fish, broken line, n = 30, 24, 22, and 14) and Ca^{2+} (survivors, n = 3; all fish, n = 13, 9, 6, and 3, on day 0 to day 3, respectively). Statistics as in Fig. 1.

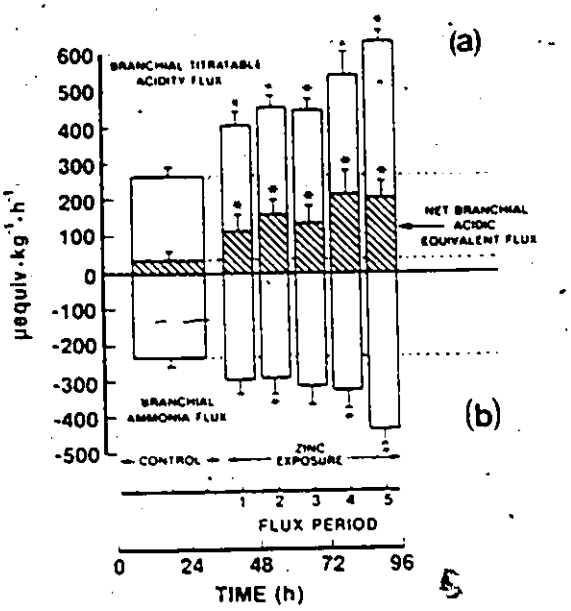

FIG. 4. Branchial net acidic equivalent flux components (a) titratable acidity flux, (b) ammonia flux, and the sum of (a) plus (b), the net acidic equivalent flux, in rainbow trout under control conditions (composite means) and during five subsequent fluxes in 0.8 mg Zn^{2+}/L, means ± SE, n = 13. Asterisks denote means significantly different ($p < 0.05$) from the composite control means (broken lines).

uptake from the water of approximately 20–40 μequiv·kg^{-1}·h^{-1}; K^+ (Fig. 5c) was an exception, exhibiting small but uniform losses. Exposure to Zn^{2+} immediately induced net losses in Na^+ and Cl^- and abolished Ca^{2+} uptake, but K^+ flux was not immediately affected. During continued Zn^{2+} exposure, the losses of Na^+ and Cl^- showed some sign of recovery after 36 h but then increased again in the last two flux periods. Overall Cl^- losses were greater than those of Na^+. K^+ losses only became significantly larger than the control mean during the last two flux periods. Net Ca^{2+} flux fluctuated near zero throughout the Zn^{2+} exposure. Two fish that were followed for an additional flux period (not shown) showed the same response for Na^+, Cl^-, and K^+ but interestingly, a positive net Ca^{2+} uptake indicating some potential for recovery.

Unidirectional flux measurements to partition Na^+ and Cl^- fluxes into efflux and influx components were performed in the first control flux period, flux period 1 (abrupt change to Zn^{2+}), and flux period 5 (2 d exposure) although not in all fish (Fig. 6). The abrupt change to Zn^{2+} increased Na^+ efflux alone, while continued exposure resulted in increases of both influx and efflux components. Effects upon Cl^- fluxes were similar, but with both components increasing immediately following Zn^{2+} exposure. In neither case were net fluxes significantly altered, in contrast with the pooled results above (Fig. 5a, 5b) although the overall trends were similar. This may have been due to the higher variability, smaller sample size, or the fact that the fluxes were only measured over 6 h instead of 12 h.

Under control conditions, renal losses of Na^+, Cl^-, and net acidic equivalents approximately balanced the net branchial uptake rates of these ions, while renal Ca^{2+} losses were only about 35% of the uptake rate at the gills (Table 2 vs. Fig. 4 and

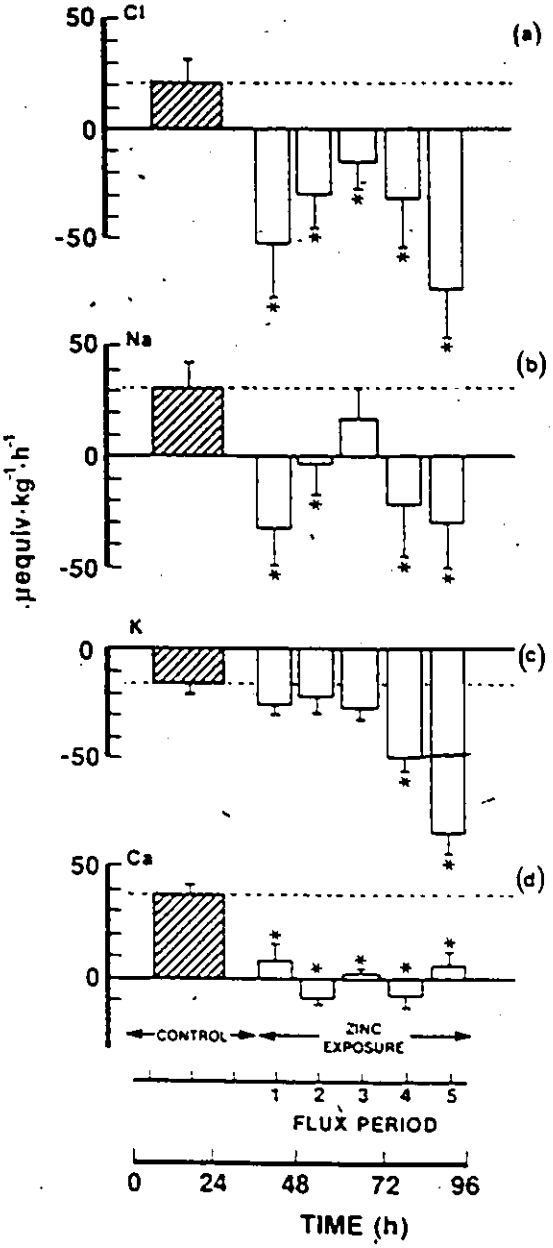

FIG. 5. Net branchial flux rates for (a) Cl^-, (b) Na^+, (c) K^+, and (d) Ca^{2+} in rainbow trout under control conditions (composite means) and subsequently exposed to 0.8 mg Zn^{2+}/L, means ± SE, $n = 13$ except for Ca^{2+} fluxes 3 and 5 where $n = 12$. Asterisks denote means significantly different ($p < 0.05$) from the control composite mean (broken lines).

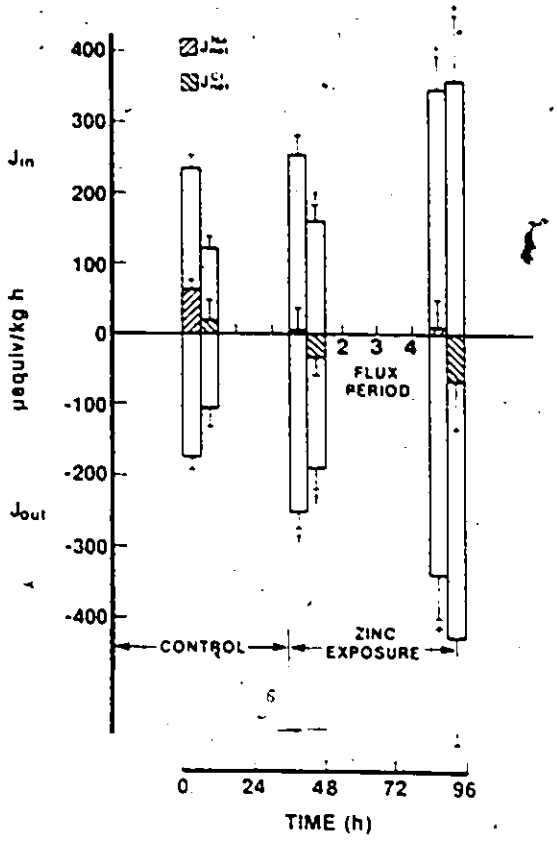

FIG. 6. Unidirectional (J_{in}, J_{out}) and net (J_{net}) branchial flux rates for Na^+ and Cl^- in rainbow trout under control conditions and subsequently exposed to 0.8 mg Zn^{2+}/L, means ± SE. For Na^+, $n = 13$, 13, and 7 for control flux 1, flux period 1, and flux period 5, respectively, for Cl^-, $n = 9$, 9, and 3. Significant differences ($p < 0.05$) from the control fluxes are denoted by daggers for paired t-test and asterisks for unpaired t-test.

5). Renal K^+ losses were only about 10% of renal Na^+ and Cl^- excretion rates, while renal Zn^{2+} excretion was about three orders of magnitude lower (Table 2). All the renal flux rates were relatively insensitive to Zn^{2+} exposure (Table 2). The only significant change was a decrease in Ca^{2+} efflux during flux period 5, possibly in response to decreasing entry of Ca^{2+} at the gill (Fig. 5d) and declining plasma Ca^{2+} levels (Fig. 3d). Notably there was no detectable change in the very low excretion of Zn^{2+} through the kidney, even over 60 h of exposure.

Discussion

The generally accepted consequence of exposure to acutely toxic waterborne Zn^{2+} (e.g. 1.5–40 mg/L) is an irreversible interruption of oxygen transfer across the gills caused by tissue damage. The resultant severe hypoxemia (Skidmore and Tovell 1972) with concurrent high lactate accumulation is fatal (Burton et al. 1972; Hodson 1976; Spry and Wood 1984). In the present study at 0.8 mg Zn^{2+}/L, significant mortality still occurred, although the Pa_{O_2} was unaffected (Fig. 1d) and the rise in blood lactate was rather small (Fig. 1e). This implies the presence of other toxic mechanisms which may be masked by the effects of higher concentrations of waterborne Zn^{2+}.

There are several possible explanations for the eventual small rise in blood lactate in the face of unchanged Pa_{O_2}. These

TABLE 2. Urine flow rates, and renal flux parameters in rainbow trout under control conditions and subsequently exposed to 0.8 mg Zn^{2+}/L in natural soft water. Values are means + SE(n). Units are $\mu equiv \cdot kg^{-1} \cdot h^{-1}$ except where noted. *Significantly different from composite control mean ($p < 0.05$).

Variable	Composite control	Flux period 1	2	3	4	5
UFR ($mL \cdot kg^{-1} \cdot h^{-1}$)	6.59	5.77	6.06	6.77	5.40	7.70
	1.24	1.33	1.70	1.38	0.96	2.79
	9	9	9	9	9	9
[TA − HCO_3^-]	−1.76	−3.31	−2.92	−4.42	−1.08	2.10
	1.29	2.30	3.45	2.01	1.46	6.47
	7	7	6	7	4	4
Ammonia	7.82	5.60	7.12	6.69	4.75	10.57
	1.84	1.27	1.94	2.14	1.61	3.79
	7	7	6	7	4	4
Net acidic equivalents	6.50	2.28	4.20	2.27	3.67	12.66
	2.49	2.32	3.10	1.15	2.01	7.68
	7	7	6	7	4	4
Sodium	43.36	34.88	31.08	43.82	35.88	35.45
	11.60	11.71	8.72*	11.75	9.48	9.42
	9	9	9	9	9	9
Chloride	39.33	27.69	26.11	30.24	21.72	24.22
	12.02	10.73	8.76	9.80	6.16	8.19
	9	9	8	9	9	9
Potasssium	4.34	3.40	3.95	4.18	4.20	5.92
	0.88	0.93	1.14	0.79	1.14	1.76
	9	9	8	9	9	9
Calcium	11.66	8.17	11.00	8.14	4.31*	10.34
	3.08	3.08	4.56	2.80	1.19	6.01
	9	9	8	9	9	9
Zinc ($nequiv \cdot kg^{-1} \cdot h^{-1}$)	16.15	19.92	21.01	18.37	11.65	15.83
	5.29	8.99	9.08	8.53	4.25	4.95
	9	7	8	7	7	7

include a diffusive limitation at the gill, decreased O_2 delivery to the tissues through a decreased arterial O_2 content or cardiac output, and finally, decreased utilization by the tissues. The latter is supported by evidence in the literature (Hiltibran 1971; Zaba and Harris 1978), although the experimental conditions are not necessarily comparable. The high Pa_{O_2} argues against a diffusive limitation, especially when compared with the dramatic fall in Pa_{O_2} reported in the other studies where Zn^{2+} exposure was rapidly lethal (Skidmore 1970; Sellers et al. 1975; Spry and Wood 1984) and structural gill damage clearly occurred (Skidmore and Tovell 1972). However, the constancy of Pa_{O_2} is not conclusive evidence against diffusive limitation, because blood O_2 capacity and/or flow rate could be simultaneously reduced to such an extent that a high Pa_{O_2} occurred despite a diffusive limitation. Indeed, blood O_2 capacity was undoubtedly reduced both by sampling-induced reduction in [Hb] and the progressively developing acidosis (i.e. Root effect) in our experiments. Definitive separation of these possible explanations will require simultaneous measurements of O_2 uptake, cardiac output, inspired and expired water P_{O_2}, and both arterial and venous blood O_2 contents and P_{O_2} levels.

The rise in Pa_{CO_2} (Fig. 1c) is unlikely to be due to simple diffusion limitations across the gill, since CO_2 is about 30-fold more soluble in water than is oxygen (Dejours 1975), and the Pa_{O_2} was unaffected. Branchial and/or erythrocytic carbonic anhydrase is thought to play a critical role in the excretion of CO_2 (Maetz 1971; Perry et al. 1981) and is known to be inhibited in vitro by Zn^{2+} (Christensen and Tucker 1976). Such inhibition by Zn^{2+} as a cause of the apparent decreased excretion of CO_2 in Zn^{2+}-exposed trout needs to be examined. Neither the acid–base disturbance nor the lactate accumulation was the cause of mortality, since severely exercised trout routinely experience much more severe acidosis and blood lactate elevation from which they usually recover (Wood et al. 1983).

The trout in this study were raised in natural soft water of the Ontario Precambrian Shield area and were exposed to 0.8 mg Zn^{2+}/L in this medium. In our previous study (Spry and Wood 1984), hardwater-reared trout were acclimated to artificial soft water and then exposed to the same level of Zn^{2+}. There was considerable similarity of the test conditions in terms of methodology, major water electrolytes, temperature, and pH. Although fish were of different stocks, both were thoroughly domesticated hatchery trout, fed identical diets. The two studies were similar in showing negligible effects on Pa_{O_2} and plasma Na^+ and Cl^-, significant increases in Pa_{CO_2} and decreases in plasma Ca^{2+}, and similar overall mortality. There were, however, some subtle but important differences. The fish in artificial

soft water developed an alkalosis rather than an acidosis, and increases in blood lactate and K^+ did not occur. These differences may have a genetic basis and/or may involve some property of natural soft water such as its complement of trace elements, other metals, or organic components. Whatever the explanation, these findings emphasize the importance of examining fish in their natural environment in studies of this nature. Höbe et al. (1984) reached similar conclusions with respect to the effects of environmental acid stress in natural versus artificial soft water.

In addition to CO_2 excretion and acid–base status, branchial ion flux rates (Fig. 5) were also affected by exposure to Zn^{2+}, Cl^- apparently more so than Na^+. The unidirectional flux measurements (Fig. 6) indicated that initially, only the Cl^- influx component was stimulated, while the efflux component for both Na^+ and Cl^- increased. Longer exposure significantly elevated both Na^+ and Cl^- influx. The pooled net flux data showed that net losses of both ions clearly occurred at the gills (Fig. 5a, 5b). These losses were probably due to increased permeability of the gills to ions, possibly by opening paracellular channels. This potential for ionic losses exists for other waterborne toxicants such as copper (Sellers et al. 1975; Laurén and McDonald 1985), mercury (Lock et al. 1981), and environmental acid (Leivestad and Muniz 1976; McDonald 1983b).

After continued exposure, influx of both Na^+ and Cl^- as well as Na^+ efflux were stimulated. Watson and Beamish (1981) found Zn^{2+} to be generally inhibitory to a variety of ATPases in vitro, but a 30-d in vivo exposure had stimulatory effects (Watson and Beamish 1980). Whether the in vitro response was purely pharmacological while the in vivo response was part of a homeostatic mechanism is not known. The K^+ fluxes in the present study were not affected until near the end of the Zn^{2+} exposure, when plasma K^+ levels were elevated (Fig. 3c) and mortality was high. It may thus represent general release from the intracellular fluids in response to acidosis (e.g. Lade and Brown 1963) or specific release from the intracellular space of the branchial tissue due to damage of the apical (water facing) membrane. Histological condition of the gills was not examined.

The abrupt abolition of net Ca^{2+} uptake by Zn^{2+} exposure (Fig. 5d) and associated fall in plasma calcium levels (Fig. 3d) is particularly interesting. Since unidirectional fluxes were not measured, we can only speculate as to whether this resulted solely from reduced influx, increased efflux, or some combination of the two. If the former is true, then Zn^{2+} might be displacing Ca^{2+} as a substrate for the Ca-ATPase which has been isolated from the gill (Ma et al. 1974; Fenwick 1976). Such "accidental active uptake" was suggested for uptake into a freshwater amphipod (Wright 1980) for both Zn^{2+} and Cd^{2+}. The apparent recovery of Ca^{2+} uptake by two fish also raises important questions as to the cause of inhibition, and the potential of the gill to recover its transport function or reduce its permeability.

Table 3 summarizes the cumulative branchial and renal fluxes (relative to the control condition) over the 60 h of Zn^{2+} exposure. Clearly, the gills were the major sites of ion loss and net acidic equivalent uptake. Compensation by the kidney, in the form of reduced ion losses and elevated acid excretion, was minimal. The tabulation shows a significant movement of net charge (+4887 μequiv/kg) unaccounted for, almost entirely at the gills, requiring entry of an unmeasured anion or loss of an unmeasured cation to maintain electroneutrality. Höbe et al. (1984) observed a very similar discrepancy in white suckers (*Catostomus commersoni*) exposed to acid stress in the same natural soft water although they used an opposite sign convention to express it.

TABLE 3 Total fluxes of ions and acidic equivalents, relative to control levels, in rainbow trout exposed to 0.8 mg Zn^{2+}·L in natural soft water for 60 h. All units are in μequiv/kg, corrected for control rates. For each ion species, sign represents gain (+) or loss (−) from the animal. For the net charge, sign represents gain or loss of positive charge.

	Branchial	Renal	Total
Na^+	+2789	+429	−2360
Cl^-	−3751	+800	−2951
Ca^{2+}	−2314	+196	−2118
K^+	−1502	0	−1502
H^+[a]	+7831	+85	+7916
Net charge[b]	+4977	−90	+4887

[a]Net acidic equivalents.
[b]Net charge = $Na^+ + K^+ + Ca^{2+} + H^+ - Cl^-$.

The role of the gills in both acid–base and ion regulation is intimately linked via the $Na^+/H^+(NH_4^+)$, Cl^-/HCO_3^- exchanges (Maetz 1971; Maetz et al. 1976; Girard and Payan 1980; Wood et al. 1984). The stimulation of net acidic equivalent uptake (base excretion) during Zn^{2+} exposure (Fig. 4) was the opposite of expected, since the fish were acidotic and net excretion of acidic equivalents both at the gill (McDonald et al. 1983) and kidney (McDonald and Wood 1981) normally occurs in the face of an acid load. We suggest that Zn^{2+} interfered with normal exchanges at the gill. Possible effects include stimulation of base excretion (increased Cl^- influx relative to Na^+ influx, as was seen upon initial Zn^{2+} exposure, Fig. 6), inhibition of acid excretion (decreased Na^+ uptake relative to Cl^- uptake), and elevated passive proton entry, which would be favoured by the pH gradient between soft water (pH ~ 6.7–7.3) and blood (pH ~ 7.8). All these would contribute to the metabolic component of the observed blood acid–base disturbance.

Although ammonia is a base, its loss from the fish occurs either as NH_3, in which case it does not affect the acid–base status of the fish, or as NH_4^+, which carries out a proton, and is therefore acidic equivalent excretion. Thus, although ammonia excretion increased due to Zn^{2+} exposure (Fig. 4), its contribution to acid excretion (currently under some debate, cf. Cameron and Heisler 1983; Wright and Wood 1985) was not sufficient to counteract the net base loss.

The lack of response by the kidney (Table 2) (with the exception of decreased Ca^{2+} excretion during one period) in the face of increased branchial ion losses indicated net whole body ion depletion (Table 3). Exchangeable NaCl in freshwater trout is about 48 mequiv/kg (Wood and Randall 1973a; McDonald 1983a). The observed Na^+ and Cl^- losses were thus about 5% of the exchangeable pool over 60 h. From the constancy of the plasma ions (Fig. 3a, 3b) we suggest an isosmotic loss, or replenishment from the intracellular compartment. However, such a rate of loss clearly could not be sustained. The decreased losses of Na^+ and Cl^- in flux period 3 (Fig. 5a, 5b) may represent compensation by the gill for these losses. The subsequent renewed loss suggests damage to gill tissue which precluded effective compensation, either through permeability changes or transport mechanisms.

In summary, exposure to waterborne Zn^{2+} altered both acid-base and ionoregulation in rainbow trout. Neither the acidemia

nor the ion disturbance alone or in combination appeared sufficient to cause the observed mortality within the time period of the experiment. As well, the Pa_{O_2} was unaffected. The primary lethal mechanism may well operate at the cellular level with the most likely effects either on oxygen delivery and/or utilization, or calcium homeostasis.

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