TOXICOLOGY OF TRACE METALS: METALLOTHIONEIN PRODUCTION AND CARCINOGENESIS

by

DAVID ARTHUR BROWN

B.Sc., University of British Columbia, 1970

A THESIS SUBMITTED IN PARTIAL FULFILLMENT OF

THE REQUIREMENTS FOR THE DEGREE OF

DOCTOR OF PHILOSOPHY

in

THE FACULTY OF GRADUATE STUDIES

(The Department of Zoology

and

the Institute of Oceanography)

We accept this thesis as conforming

to the required standard

THE UNIVERSITY OF BRITISH COLUMBIA

November, 1978

In presenting this thesis in partial fulfilment of the requirements for an advanced degree at the University of British Columbia, I agree that the Library shall make it freely available for reference and study. I further agree that permission for extensive copying of this thesis for scholarly purposes may be granted by the Head of my Department or by his representatives. It is understood that copying or publication of this thesis for financial gain shall not be allowed without my written permission.

Department of Zoology

The University of British Columbia
2075 Wesbrook Place
Vancouver, Canada
V6T 1W5

Date January 15, 1979

Abstract

Many reports of trace metal levels in organisms from polluted areas exist in the literature, but little can be inferred from these data as to the actual biological significance of these metal levels to the exposed organism. This study reports a biochemically meaningful assay based upon the actual toxicology of the trace metals: it measures the levels of those metals that are bound by the trace metal detoxifying protein, metallothionein, and those which are free to exert toxic effects by binding enzymes in the high molecular weight protein pool. Further, a study was made of trace metal changes in carcinogenesis in order to elucidate the role of trace metals in the etiology of cancer.

Organisms were sampled from an area known to be polluted with trace metals. Tissues were homogenized, centrifuged, and the supernatants fractionated according to molecular weight by passing through a column packed with Pharmacia G-75 gel. Copper and zinc were present in the high molecular weight protein pool in relatively high levels, as they are components of metalloenzymes. Excesses of Cu and Zn were stored on metallothionein. In duck liver and kidney tissue, cadmium was bound to metallothionein unless the high molecular weight protein pool was Zn deficient. In this case, high levels of Cd appeared in the high molecular weight protein pool, presumably since Cd was then more effectively able to compete with Zn for Zn-binding sites in metalloenzymes.

Mussels were exposed to Cd, Cu and Zn near a sewer out-

fall and in the laboratory. Reduced survival did not occur until metallothionein was apparently metal saturated, and there was a rapid increase of metals in the high molecular weight protein pool.

Phytoplankton, zooplankton and fish were exposed to mercury. Growth rates were reduced in those exposures where Hg was detectable in the high molecular weight protein pool. In fish and zooplankton, this occurred when the capacity of metallothionein to bind Hg was apparently surpassed. Copper and zinc levels decreased in the high molecular weight protein pool with increasing Hg exposure level. Toxic effects of trace metals are attributed to the interference of nonessential metals with essential metals in metalloenzymes.

Both cancerous fish and humans had increases of Cd and the Cd:Zn ratio in the high molecular weight protein pool. In control organisms, a much higher portion of Cd was bound to metallothionein. When mice were exposed to low Cd doses, most Cd accumulated on metallothionein. When the carcinogen diethylnitrosamine (DEN) was administered with this Cd, more Cd accumulated in the high molecular weight protein pool. Since Zn was decreased in the high molecular weight protein pool by DEN exposure, it is reasonable to conclude that, as in ducks with Zn deficient high molecular weight protein pools, Cd was more effectively able to compete for metalloenzyme binding sites. It is suggested that Cd, in cancerous organisms, might be interfering with the Zn-containing enzymes involved in cell division processes.

Exposure to DEN alone resulted in decreases of Cd, Cu and Zn in the high and low molecular weight pools, and on metallothionein. Concurrent administration of Zn with DEN reversed losses of Cu and Zn slightly, and increased survival time. When low levels of Cd were administered with DEN, losses of Cu and Zn were increased, and pretumorous histological changes were more evident. Very high Cd doses increased levels of Cu and Zn in the high molecular weight protein pool over control values, and greatly increased time to death due to tumors.

Table of Contents

List of Tables

List of Figures

Acknowledgements

Studies in this thesis were supported by grants from Environment Canada, the U.S. National Science Foundation, and the National Research Council of Canada. The author was supported by a scholarship from the National Research Council of Canada.

Special thanks to Tim Parsons and Jeff Thompson for introducing me to the topic of metallothionein. Thanks also, to those who collaborated with me in these studies; Tim Parsons, Ken Chatel, Agnes Chan, Carole Bawden, Louise Cloutier, and Brian Knight. Don McLeay of B.C. Research reviewed some of the manuscripts in this thesis, before they were submitted for publication, and provided stimulating discussions on the topic of carcinogenesis. B. C. Research provided the use of their Perkin Elmer flame atomic absorption spectrophotometer, while the Pacific Environment Institute provided the use of their Perkin Elmer graphite furnace atomic absorption spectrophotometer.

CHAPTER I Introduction

Metallothionein and Metalloenzymes

Metallothionein is a low molecular weight (10,000) protein high in sulfhydryl groups due to the presence of high levels of the amino acid cysteine (Margoshes and Vallee, 1957; Kagi and Vallee, 1960). The sulfhydryl groups can bind heavy metals thus rendering them nontoxic to the exposed organism (Piscator, 1964). Metal-thionein complexes are usually stored solubilized in liver and kidney cytoplasm.

Heavy metals not bound to metallothionein may be bound to high molecular weight proteins such as enzymes. Low levels of certain heavy metals (e.g., Zn and Cu) are necessary for the normal functioning of metalloenzymes (Friedberg, 1974, Underwood, 1971). When there is exposure to higher levels of heavy metals, metallothionein production is stimulated (Webb, 1972a, b; Squib and Cousins, 1974; Davies et al., 1973; Winge et al., 1975; Sugawara and Sugawara, 1975). Excess heavy metals are then sequestered by metallothionein. However if there is a very high influx of heavy metals into liver and kidney tissue, then production of metallothionein may not be sufficient to sequester all excess heavy metal. In this case there may be a "spillover", in cell cytoplasm, of heavy metals from metallothionein to high molecular weight proteins (Winge et al., 1973) such as enzymes. Either an excess of heavy

metal or an incorrect heavy metal on an enzyme can render the enzyme nonfunctional (Friedberg, 1974; Bremner, 1974; Williams, 1971). Thus, the appearance of spillage coincides with the appearance of pathological changes (Winge et al., 1973). Enzymes are rendered nonfunctional by conformational changes brought about by binding heavy metals with properties different from their required metals (Friedberg, 1974). Nonfunction is due to the fact that after conformational changes, substrate molecules no longer fit binding sites on the enzymes (Friedberg, 1974).

It may be possible to quantitatively relate sublethal levels of heavy metals bound to metallothionein or enzymes to the lethal "spillover" level. This would provide a biochemically meaningful assay of the degree of the heavy metal toxicity problem in highly industrialized areas.

The Use of Molar Rather than Weight Units

One of the assumptions of the "spillover" theory is that there is a limited amount of metallothionein available to detoxify heavy metals and a limited rate at which it can be induced. This suggests that there will be competition between different heavy metals for binding sites on metallothionein. Competitive success will depend not only on the relative affinity of each metal for sulfhydryl binding sites but also on the number of ions of each metal type competing for these binding sites. Proper interpretation of competition data will not be possible using weight

units as heavy metals tend to be of a wide range of molecular weight. Therefore heavy metals will be expressed in molar units which relate to the number of metal ions present.

When molar quantities of metal are used, then the total quantity of metal on metallothionein is directly proportional to the amount of metallothionein present. Metallothionein has 3 sulfhydryl groups per metal ion (Kagi and Vallee, 1961; Pulido et al., 1966; Buhler and Kagi, 1974; Bremner and Davies, 1975) and approximately 24 sulfhydryls per metallothionein molecule (Nordberg et al., 1972; Bremner and Davies, 1975; Buhler and Kagi, 1974; Winge et al., 1975). Therefore, if all the metal on metallothionein is accounted for, the measurement of metallothionein by protein assay methods is not necessary, i.e.,

$$\frac{3 \text{ mole SH}}{\text{mole metal}} \times \frac{1 \text{ mole metallothionein}}{24 \text{ mole SH}} \times$$

x mole total metal on metallothionein

= y mole metallothionein

Most reports on metallothionein and heavy metals use weight units. In order that data from other studies may be related to this study, all data referred to have been converted to molar quantities.

The Process of Detoxification by Metallothionein

As first proposed by Piscator (1964) the function of metallothionein is the detoxification of heavy metals.

Rabbits were injected with Cd and Cd-thionein (metallothionein with bound Cd) was isolated from their livers. Previously metallothionein had been isolated only from kidney tissue (Kagi and Vallee, 1960, 1961). With the discovery of metallothionein also in liver tissue, Piscator (1964) proposed that it was synthesized in liver, then bound to heavy metals and transported to the kidney. Here it passed through the glomular filtration apparatus and was reabsorbed in the renal tubules.

Nordberg (1972) found that after a single injection of Cd, it was distributed to organs in a few minutes and was redistributed only slowly thereafter. Cadmium remaining in blood was taken up into red blood cells where it was first bound to high molecular weight proteins, but later to metallothionein. In liver and kidney tissue, Cd was first bound to cell organelles and particles. Cadmium in free supernatant was bound to high molecular weight proteins. After a few days most of the Cd was bound to metallothionein. This suggests that Cd is available to bind enzymes before metallothionein is synthesized. Therefore, initially Cd would be able to exert its toxic effects. However, as synthesis of metallothionein occurred, more Cd was bound to metallothionein, and less to high molecular weight proteins. This suggests that a decline in levels of Cd able to exert toxic effects by binding enzymes, is based upon the synthesis of metallothionein.

For up to 112 days after injection, the concentration of Cd-thionein in liver decreased while that in kidney remained constant, suggesting a slow redistribution from liver to kidney, with some concurrent excretion from the kidneys. This study is therefore in accordance with Piscator's (1964) hypothesis that Cd-thionein is formed in the liver and transported to the kidney where it is re-absorbed from kidney tubules. Nordberg found that about 0.01% of the body burden of Cd was excreted per day. A sudden increase in Cd excretion was observed concomitantly with appearance of tubular proteinuria. This Cd was bound to a small protein with size identical to metallothionein. Nordberg summarizes by saying that metallothionein is an important carrier of Cd from tissues into the urine, at least after renal tubular damage has occurred. Further, Cd-thionein reaches the kidney cortex by glomular filtration and subsequent tubular reabsorption. When the tubules become damaged by the accumulated amounts of Cd, re-absorption is decreased and Cd-thionein is excreted in the urine.

Nordberg et al. (1975) injected equivalent amounts of Cd into rats as free Cd or Cd-thionein. Cadmium exposure alone resulted in Cd uptake by liver tissue and subsequent binding of this Cd to metallothionein, while Cd-thionein appeared in the kidneys. Cd-thionein resulted in renal damage whereas the free Cd did not. Further, 5 times as

much Cd was required as opposed to Cd-thionein to be fatal. Death was attributed to renal damage. The authors suggest that rapid release of liver stores of metallothionein for purposes of excretion would be harmful to the organism. This may be due to the fact that the low pH of the urine could result in dissociation of Cd, Cu and Zn from metallothionein (Kagi and Vallee, 1960, 1961). These would then be available to bind and damage renal structural proteins.

It is apparent from this summary that metallothionein is synthesized in the liver (or kidney) and is slowly transferred to kidney via the bloodstream. Renal reabsorption then occurs and the complex is stored in kidney cortex. Metallothionein not reabsorbed is excreted.

Development of Metallothionein-mediated Tolerance to Heavy Metals

It is commonly known that preexposure to Cd results in increased tolerance to subsequent challenge doses of Cd. The most obvious way by which this might be expected to occur would be by synthesis of excess metallothionein upon initial exposure. This excess metallothionein would then be readily available to quickly bind and detoxify heavy metals on subsequent exposures. However, it is known that metallothionein always exists in the saturated state in organisms, i.e., one metal ion for each three cysteine residues (Kagi and Vallee, 1960, 1961; Pulido et al., 1966; Buhler and Kagi, 1974; Bremner and Davies, 1975).

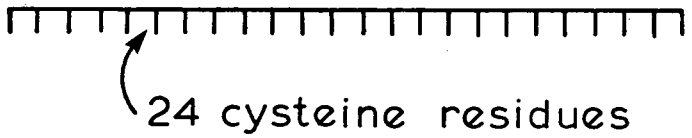
Therefore some other mechanism of tolerance development must exist.

Leber (1974) preinjected rats with cadmium dosages of 0, 1.0 and 3.2 mg/Kg, followed 48 hours later by cadmium challenge doses, resulting in LD-50 values of 4.5, 6.0 and 8.2 mg/Kg, respectively. Likewise, preexposure to Zn was found to increase the LD-50 to subsequent Cd challenge doses. Leber found levels of metallothionein increased linearly with the pretreatment dose of Cd or Zn but that Cd-induced metallothionein contained Cd and Zn while metallothionein resulting from Zn injections contained only Zn. When cadmium or zinc pretreated mice were challenged with Cd 3 hours prior to sacrifice, this resulted in an *in vivo* displacement of Zn from both Cd- and Zn-metallothionein. This suggests that the increased tolerance in both cases results from Zn displacement from metallothionein by Cd (Figure 1). Zn is much less toxic to enzymes than Cd as it is a natural component of many enzymes. Hence, free cellular Zn results in far less toxic effects than free Cd.

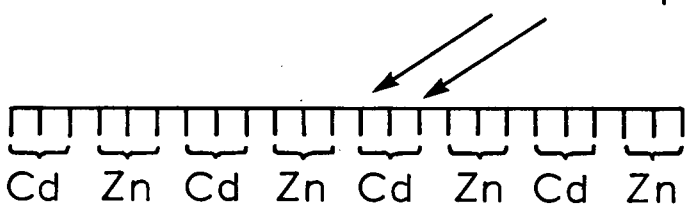
Similarily, exposure to Ag, Cu or Hg results in synthesis of metallothionein containing approximately equimolar levels of Zn and the exposure metal (Winge et al., 1975). Therefore, exposure to any one of Cd, Hg, Ag, Cu or Zn should result in increased tolerance to any of the other metals upon subsequent exposure.

Figure 1. Metallothionein is a protein of molecular weight of approximately 10,000. One third of its amino acids are cysteine. There are 24 cysteine residues per metallothionein molecule. Each 3 cysteine residues bind a metal ion so that each molecule of metallothionein binds 8 metal ions. Exposure to low doses of Cd results in metallothionein production with Cd and Zn each in approximately half of the binding sites. Subsequent exposure to a high Cd dose, results in displacement of Zn from metallothionein by the Cd. Zn is much less toxic than Cd since Zn is a natural component of over 70 metalloenzymes.

Metallothionein M.W. 10,000

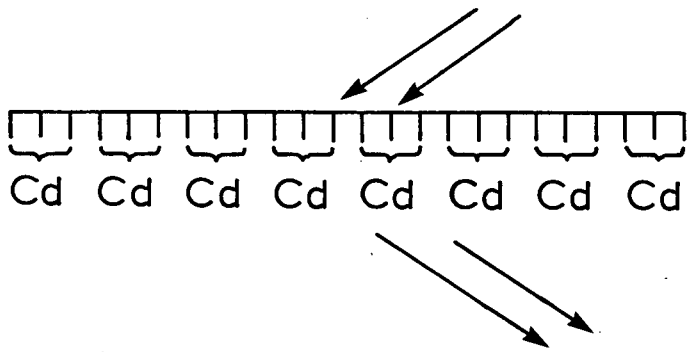


Low Chronic Cd Exposures



3 cysteine residues / metal ion
Zn in approximately 50% of binding sites

Followed by High Acute Cd Exposure



The Occurrence of Metallothionein and Its Associated Heavy Metals

Metallothionein was first isolated from horse renal cortex by Margoshes and Vallee (1957) by an ethanol-ammonium sulfate fractional precipitation technique. Cadmium and zinc were found associated with the isolated protein in a 1 : 0.42 molar ratio. Other heavy metals were not determined. Later Kagi and Vallee (1960) using an ethanol-chloroform-ammonium sulfate fractional precipitation technique, isolated metallothionein from horse renal cortex and in ten preparations found Cd and Zn present in high amounts; Al, Ba, Ca, Mg and Fe in lesser amounts while Sr, Cr, and Pb occurred in some preparations. When a more precise DEAE-cellulose chromatography step was included (Kagi and Vallee, 1961) Cd, Zn, Fe and Cu were found in a 1 : 0.64 : 0.07 : 0.03 molar ratio. Al, Ba, Ca, Mg, Sr, Cr and Pb were not found associated with metallothionein.

Piscator (1964) isolated metallothionein from liver tissue of rabbits exposed to Cd. Metallothionein was first identified in human liver by Buhler and Kagi (1974). Cd, Zn and Cu were found in a 1 : 72.1 : 1.1 molar ratio. In 1966 Pulido et al. isolated metallothionein from human renal cortex. Cd, Zn, Cu and Hg were in a 1 : 1.08 : 0.13 : 0.07 molar ratio. Rugstad and Norseth (1975) have isolated Cd-thionein from cultured human skin epithelial cells grown

in a Cd rich medium.

Wisniewska et al. (1970) injected rats with Cd and Hg and found these metals bound to metallothionein in their kidney. Jakubowski et al. (1970) found Hg-thionein in liver, kidney and occasionally in urine of rats preinjected with radioactive mercury. After injection of Cd into mice, Nordberg (1972) found Cd-thionein in erythrocytes, plasma, liver, testes, kidney and urine (Nordberg and Piscator, 1972). Webb (1972a) found that when rats were preexposed to Zn or Cd and 2-4 days later were injected with Ni or Co, that low quantities of these cations were bound to metallothionein. Rats given Cd in their drinking water have been shown to have Cd-thionein in the walls of their intestines (Sugawara and Sugawara, 1975). Cousins et al. (1973) have isolated Cd-thionein from renal cortex of swine fed various quantities of Cd in their diets. Winge et al. (1975) injected rats with Cd, Ag, Hg, or Zn. All four metals were found associated with the liver metallothionein fraction. Sabbioni and Marafante (1975) injected rats with Cd to stimulate metallothionein production and followed this with injections of 42 different metals. They concluded that only Cd, Zn, Hg, Cu, Ag and Sn could bind metallothionein.

In 1972 MacLean et al. found a metallothionein-like fraction binding Cd and Zn in blue green algae (Anacyatis nidulans) exposed to radioactive Cd. However in a try-

panosomid flagellate (Crithidia fasciculata) grown under the same conditions, no such fraction was found. Naturally occurring (nonexperimentally induced) Cd-thionein has been found in the Atlantic grey seal (Halichoerus grypus) and the Pacific fur seal (Callorhinus ursinus) by Olafson and Thompson (1974). These authors also report the occurrence of Cd-thionein in livers of the copper rock fish (Sebastodes caurinus injected with Cd. Casterline and Yip (1975) have demonstrated the presence of Cd-thionein in Cd-exposed oysters but could not detect metallothionein in Cd exposed soybean plants. Common limpets (Patella vulgata) from an area of severe industrial pollution contained both Cd and Zn-thioneins (Howard and Nickless, 1975). Noel-Lambot (1976) found metallothionein binding Cd, Zn and Cu in mussels from the North Sea. Hg-thionein has been found in appreciable levels in livers, gill and kidney tissue of Hg exposed eels, but in much lesser amounts in muscle tissue (Bouquegneau et al., 1975).

The above summary suggests that metallothionein is ubiquitous in the animal kingdom. The occurrence of metallothionein in liver, kidney, testes, muscle, plasma, erythrocytes, tissue cultured skin epithelial cells, and urine suggests the possibility that metallothionein may exist to some level in most or all animal tissues. There is a paucity of studies of metallothionein in the plant kingdom; metallothionein has been found in blue-green algae (McLean et al.,

1972) but not in soybean plants (Casterline and Yip, 1975).

Naturally occurring metallothionein, isolated by more advanced gel chromatography techniques, thus far has been found binding mainly Cd and Zn with lesser amounts of Hg and Cu and possibly small amounts of Fe. Thus, recent works on metallothionein refer to it as a protein involved in binding specifically for Cd, Zn, Cu and Hg (Noel-Lambot, 1976; Winge et al., 1975; Olfafson and Thompson, 1974; Cherian, 1974). While this may be sufficient for naturally occurring metallothionein, experimentally induced metallothionein has been shown in addition, to contain Fe (Pülido et al., 1966; Kagi and Vallee, 1961), Ni, Co (Webb, 1972a), Ag (Winge et al., 1975; Sabbioni and Marafante, 1975) and Sn (Sabbioni and Marafante, 1975). It may be that these metals are found on metallothionein only in experimental conditions because they have been injected, and hence are present in organisms, in quantities higher than those normally found in environmentally exposed organisms.

Affinity of Various Heavy Metals for Metallothionein

Margoshes and Vallee (1957) found that Cd was not removed from metallothionein by dialysis at pH 7 but that it was by treatment with hot trichloroacetic acid. Kagi and Vallee (1960) determined that the amount of Cd or Zn bound to metallothionein decreased with decreasing pH. From their data they noted that 50% of Zn was removed at pH 4.6 whereas 50% of Cd was not removed until pH 3.5.

Therefore Cd removal requires a 10-fold higher concentration of hydrogen ions and therefore Cd is probably more tightly bound to metallothionein than is Zn. Further they found that the number of sulfhydryl groups titratable with Ag or p-chloromercuribenzoate remains unchanged after removal of Cd and Zn. Thus it can be assumed that both Ag and Hg are able to displace Cd and Zn from metallothionein.

Kagi and Vallee (1960) dialyzed metallothionein against 5×10^{-3} M Cd or Zn at pH 7.1. They found that dialysis against Zn did not affect the Cd content but that dialysis against Cd decreased the Zn content of metallothionein. Therefore, it can be assumed that at body pH, an infusion of Cd can displace Zn from metallothionein but not vice versa. Kagi and Vallee state that this experimental procedure neglects electrostatic factors and specific effects of buffer ions.

Pulido et al. (1966) adjusted a 3×10^{-5} M solution of human metallothionein to pH 2.0 by the addition of 0.1 N HCl. They then passed this through a Sephadex G-25 column eluted with p.1 M HCl. They found that under these conditions, cadmium, zinc and copper were removed from metallothionein while Hg remained bound. They also found that titration of metallothionein with Ag displaced Cd, Zn and Cu but not Hg. Wisniewska et al. (1970) also concluded that Cd was removed from metallothionein at pH 2 but that Hg-thionein is stable in acidic medium.

Webb (1972a) found that Cd was more firmly bound to metallothionein than Zn. Cadmium was completely retained on dialysis of the preparation against 20mM acetate buffer whereas Zn was removed. When metallothionein was dialyzed against 0.2 mM p-chloromercuribenzoate in 20 mM sodium phosphate buffer (pH 7.4), Hg replaced Cd. Further, it was found when thionein was dialyzed against equimolar concentrations of Ni and Cd, binding of Ni was twice that of Cd. Evans et al. (1970), using similar dialysis procedures, found that Hg, Cd and Zn displaced ^{64}Cu from metallothionein, in vitro.

From the above summary the following order of decreasing affinity for binding of metallothionein can be deduced: Hg > Ag > Cd > Zn > Cu. Therefore, after exposure to high levels of a trace element, Cu should spillover to the high molecular weight protein pool first, Zn next, followed by Cd, Ag and Hg. In accordance with this prediction, Leber (1974) found that metallothionein, in rats receiving low injections of Cd, contained much more Zn than Cd; those receiving a high Cd dose had almost equal levels of Cd and Zn; but in those receiving very high levels of Cd, only Cd was found on metallothionein.

The order of toxicity of Cd, Zn and Hg is Hg > Cd > Zn (McKee and Wolf, 1963). This is exactly the same as the order of their affinity for metallothionein; conveniently, the least toxic ions will be released first in a "spillover"

situation. Furthermore, Zn, which should be released first, is a normal component of many enzymes (Friedberg, 1974). Mercury, cadmium and zinc generally exert their toxic effects by binding enzymes, particularily those high in sulfhydryl groups (Williams, 1971). Therefore, those metals which bind most tightly to the sulfhydryl groups on metallothionein, probably bind most tightly to the sulfhydryl groups of enzymes, i.e., Hg$>$ Cd$>$ Zn. It follows that increased ability to bind metallothionein does not necessarily result in a reduced toxicity of a metal, since these same metals also have an increased ability to render sulfhydryl-containing enzymes nonfunctional. Therefore, it was probably of evolutionary necessity that the sulfhydryl-based metallothionein detoxifying system developed, binding most strongly those heavy metals which are most strongly bound to sulfhydryl containing enzymes.

Evolution of Metallothionein

The metal detoxification system may have developed when life first began on earth, 3.6 billion years ago. At that time the heavy metal levels in the oceans were presumably much higher than today. This was probably due in part to the higher lava output, resulting from the presence of radioactive (heat generating) substances in the atmosphere in quantities 6 times as high then as now (Strahler, 1972). Furthermore, there was then 600 times as much CO_2 in the atmosphere and oceans as now (Strahler, 1972). Therefore,

the oceans would have been much more acidic. This acidity would have resulted in more metal being extracted from the lava than now, in addition to the fact that more lava was being produced by volcanic activity. Therefore life probably evolved in a metal rich medium, and in fact metal clay templates may have been the catalysts for bonding of the first polymers of amino acids and nucleic acids (Paecht-Horowitz, 1974). Thus, life evolved utilizing many metals (especially Cu, Zn, Fe, Mg and Mn) in enzyme systems and nucleic acids, and furthermore, probably evolved metallothionein as a natural detoxifying system for excesses of heavy metals.

As postulated by Brown (1976) it is likely that heavy metals levels dropped relatively suddenly when O_2 generating photosynthesis began 2.7 billion years ago. At that time it is proposed that Fe and Mn hydrous oxides formed. These are insoluble and absorb vast quantities of other trace metals in and on the flocculant, thus resulting in decreased levels of these metals in the oceans. Furthermore, atmospheric CO_2 decreased sharply resulting in increased pH of the seawater and therefore less leaching of metals from lava. As the metal content of the oceans is lower now than when life first evolved, it is not surprising that metals are often the limiting factor in growth because of metal deficiencies (Underwood, 1971). Because life developed in a metal rich environment, it would be expected

that present day organisms would be well adapted to survive exposure to metallic pollutants. This is indicated by the fact that much higher than normal physiological levels of metallothionein can be synthesized in high metal exposure experiments (e.g., Olafson and Thompson, 1974).

Metals and Carcinogenesis

Since life began in a trace element rich environment, it is not surprising that many enzymes contain metals as essential parts of their structure. Of particular interest in carcinogenesis, are the Zn containing enzymes involved in cell division processes, e.g., DNA polymerase, RNA polymerase, reverse transcriptase, and thymidine kinase (Vallee, 1976; Duncan and Dreosti, 1976), and the Cu containing monooxygenase system (Yamane et al., 1969) responsible, in part, for the bioactivation of noncarcinogenic organic substances to carcinogenic arene oxides. This is followed by their subsequent metabolism to noncarcinogenic substances by the epoxide hydrase and glutathione-S-epoxide enzyme systems. Levels of Cd, Cu and Zn are changed with development of cancers. High Cd levels can alter or inhibit the normal function of both Cu- and Zn-containing metalloenzymes.

Elevated liver Cd, Cu and Zn and kidney Cd and Zn are found in many cancer patients (Olson et al., 1954, 1958; Tietz et al., 1957; Lewis et al., 1969; Morgan, 1970, 1971). Sandberg et al. (1958) reported increased hepatic Cu in

some cases of neoplasia, especially in association with widespread metastasis, Morgan (1970) found increased liver, kidney and serum Cd/Zn in lung cancer patients while Pories et al. (1973) report increased serum Cu/Zn in all cancer patients. Halsted and Smith (1970) found plasma Zn to be decreased in malignant diseases, particularily metastatic carcinoma. Arnold and Sasse (1961) found increased Cu and decreased Zn in rats with DMBA induced tumors. Gorodiskii et al. (1956): in Furst and Haro, 1969) report increases in the Cd content of tumors induced by nonmetal carcinogens. Tietz et al. (1957) report that changes in metal levels occur before tumor formation while Olson et al. (1958) report no increase in liver Zn in animals bearing transferred subcutaneous tumors, suggesting that alterations in metal levels do not occur as a result of the carcinoma.

Koch et al. (1957) reported elevated Cu in leukemia patients and found that this could be reduced by the antileukemic drug 6-mercaptopurine. As suggested by Furst (1963), the actions of all carcinogens and anticancer drugs can be explained as those of trace metal chelating agents. The present study will investigate whether or not the organic carcinogen diethylnitrosamine can influence the levels and cytoplasmic distribution of Cd, Cu and Zn in pretumorous tissue. It has been found that a methyl group bound to Hg results in Hg occurring in the high molecular

weight protein pool rather than in metallothionein (Chen et al., 1973). Thus, methyl Hg is much more toxic than Hg, as more Hg is available to bind enzymes in the high molecular weight protein pool. Perhaps organic carcinogens can bind Cd, Cu and Zn, thus changing their cytoplasmic distribution. In particular, if Cd were increased in the high molecular weight protein pool, then it would be able to interfer with the Zn-containing enzymes involved in the regulation of cell division.

In most Zn-containing enzymes, Zn holds subunits together (Ulmer, 1970). These subunits can be separated by various agents. For example, intact aspartic transcarbamylase has a molecular weight of 300,000 with catalytic and regulatory subunits joined together by Zn. If exposed to Hg, the subunits of this enzyme separate and the catalytic subunit is released. It has molecular weight 48,000, specific activity 4-5 times higher than the intact enzyme, and it is not subject to feedback control (White et al, 1968). Like aspartic transcarbamylase, DNA polymerase can be separated into subunits by Hg (Jovin et al., 1969). Recently it has become apparent that so-called DNA polymerase B is a subunit of DNA polymerase A (Hecht et al., 1973), and may be an artifact of the extraction procedure; the amount of DNA polymerase B obtained increases with increasing K^+ or Na^+ used in the extraction procedure (Lazarus et al., 1973; Hecht et al., 1973). DNA polymerase

A has a molecular weight of 100,000-200,000 while DNA polymerase B has a molecular weight of 45,000, specific activity 4-5 times that of DNA polymerase A, and is not subject to control by agents which decrease the activity of DNA polymerase A (Lazarus and Kitron, 1973; Krasny, 1973). The parallels between the freed catalytic site of aspartic transcarbamylase and DNA polymerase B are obvious. When increases in agents such as Hg or chemically similar Cd occur, there could be a splitting of DNA polymerase into subunits with release of free DNA polymerase B. If DNA polymerase was split into subunits *in vivo*, the lack of feedback control of DNA polymerase B could be instrumental in the unchecked cellular proliferation indicative of cancer.

The Present Studies

The metallothionein studies described in this thesis differ in many respects from previous studies. As a result of the establishment of precisely standardized procedures for the isolation of metallothionein, this study quantifies the levels of metallothionein produced in response to various treatments. Not only are the levels of metal on metallothionein measured, but also the levels in the high molecular weight protein pool. As a result of measuring the level of metals in both of these pools, quantifiable relationships are established between saturation levels of metallothionein, and spillover of metals to the high molecular weight protein pool, and with onset of pathological

effects. Thus, this study correlates the levels and cytoplasmic distribution of metals with pathological changes.

This study also measures three metals in each study. Almost all previous studies on metallothionein have measured only one metal. Thus, the present study is able to clearly establish how high levels of Cd and Hg affect the levels and distribution of Cu and Zn between various cytoplasmic pools.

The studies of trace element levels in cancer to date, have considered mainly alterations occurring after tumors begin. The present study considers alterations in Cd, Cu and Zn in pretumorous tissue. These are followed through to the posttumorous stages. Furthermore, this study considers alterations in the cytoplasmic distribution of Cd, Cu and Zn as their levels change. Clearly, if changes of metal levels were only from metallothionein, these changes would have little influence on cellular processes since enzymes would not be affected.

This thesis consists of chapters, each of which is one paper, either published, in press, submitted, or in preparation for submission; the status of these papers, journals in which they appeared or may appear, and the authors of each are described in the preface of each chapter. The prefaces also discuss how various chapters are interrelated. As the topic of metallothionein is un-

familiar to most, each introduction has given a cursory description of some of the relevant literature. Each paper has its own materials and methods, results and discussion sections. Further, there is a more detailed material and methods description in Appendix I. References from all papers are given at the end of the thesis. Some fundamental findings of all studies are discussed in an overall discussion at the end of this thesis.

CHAPTER II The Wildlife Community of Iona Island Jetty, Vancouver, B.C., and Heavy-metal Pollution Effects

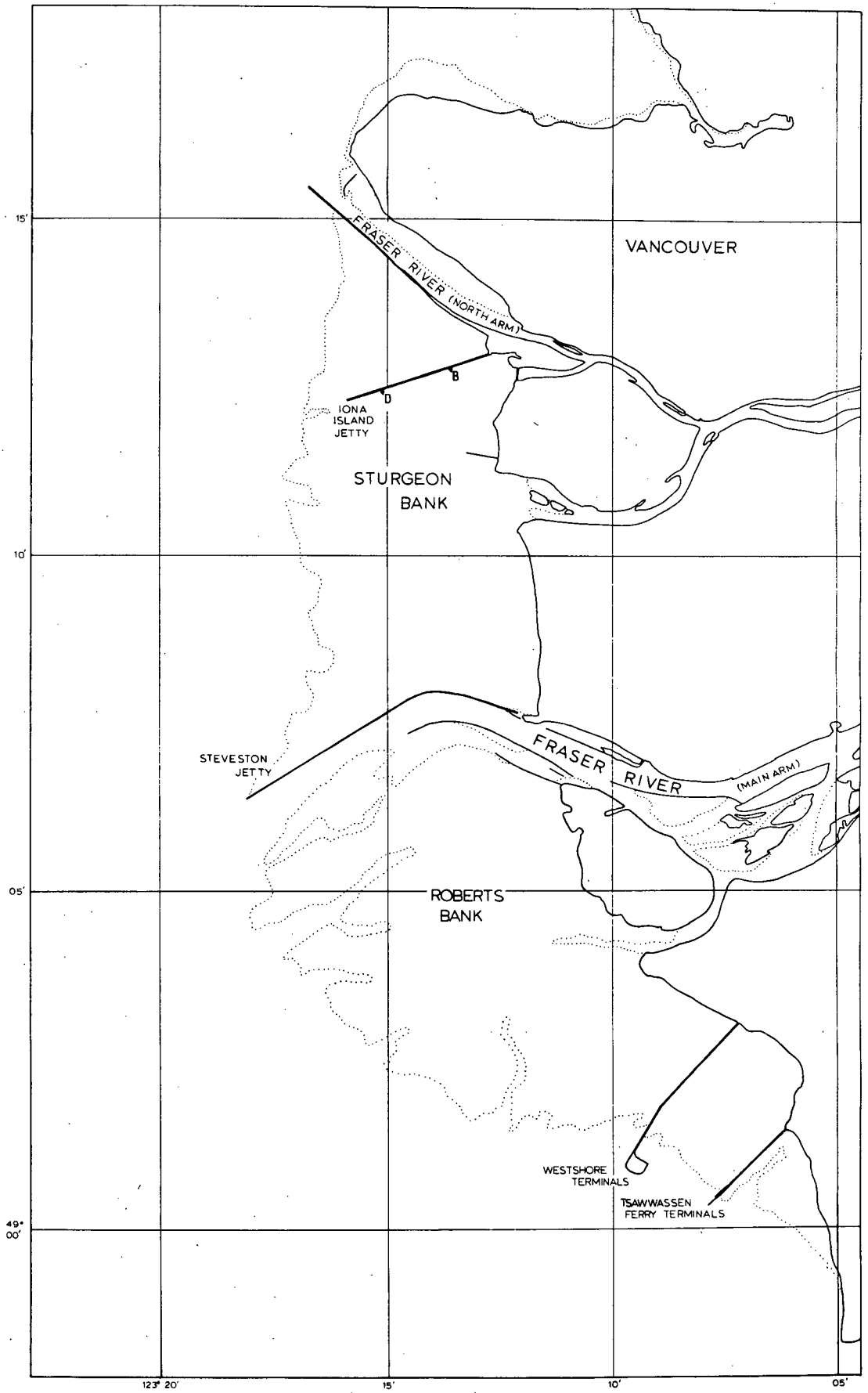
Preface

This paper appeared in Environmental Conservation, 1977, Volume 4, pages 213-216. Authors were D. A. Brown, C. A. Bawden, K. W. Chatel, and T. R. Parsons. The basic approach taken in our work on metallothionein is described in this paper. It differs from that of other workers, in that it considers the levels of metals in both the high molecular weight protein pool and on metallothionein, and it quantifies these levels. Except for Winge et al (1973), no one else has considered the metal levels on the high molecular weight protein pool, and, our studies are the first to quantify these metal levels. The "spillover" theory is proposed in this paper.

Introduction

The Fraser River enters the Pacific Ocean through a delta which is characterized by a series of large mud-banks (Figure 2). Five stone jetties, extending seawards for some two to three miles (3.2 to 4.8 km), have been built out to the edges of the mid-banks, where deep water is available for ship docking and where turbulent tidal action causes mixing of the river water with the sea. The purpose of these stone jetties is either to service a port facility (a ferry terminal and coal port), or to act as

Figure 2. Sketch-map of the Fraser River estuary, near Vancouver, British Columbia. Scale indicated by the Iona Island Jetty which is ca 4 km long. The approximate edges of the mud-banks are indicated by faint dotting.


15'
10'
05'
49° 00'
123° 20'
15'
10'
05'
VANCOUVER
FRASER RIVER (NORTH ARM)
B
D
IONA ISLAND JETTY
STURGEON BANK
STEVESTON JETTY
FRASER RIVER (MAIN ARM)
ROBERTS BANK
WESTSHORE TERMINALS
TSAWWASSEN FERRY TERMINALS

restraining walls against the lateral flow of currents.

Along the edge of one of the five jetties there is a trench through which the primary effluent from Vancouver city sewers is allowed to flow, so constituting the Iona Island outfall. This has resulted in a biologically degraded, anoxic environment in the immediate vicinity of the trench. However, peripheral to this area of degradation is an abundance of higher life-forms, including appreciable stands of seaweeds (e.g. Fucus distichus), bivalves (e.g. Mytilus edulis, Macoma inconspicua, and Cryptomya californica), and a commercial crab community (Cancer magister). Living among the rocks of this jetty is a large population of rats (Rattus norvegicus), which forage in the intertidal zone and in particular have been observed to feed on mussels (Mytilus edulis). The rats are preyed upon by a number of raptorial birds, the most common of which are two species of owl (Asio flammeus and Nyctea scandiaca) and a harrier (Circus cyaneus). In addition to these raptorial birds there are populations of Greater Scaup (Aythya herodias), which are associated directly with the aquatic environment.

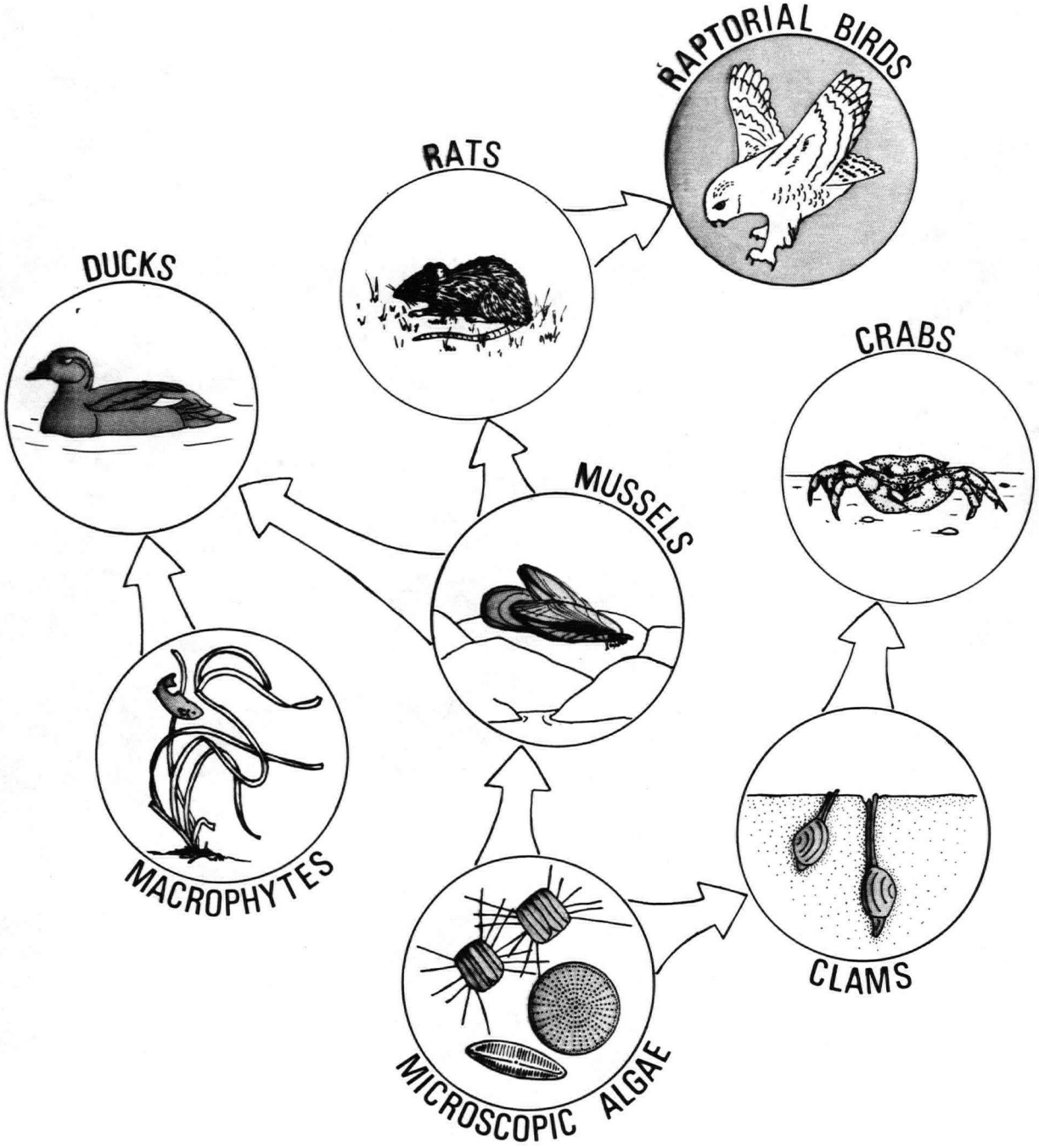
In contrast to the Iona Island Jetty, the other four jetties crossing the mid-banks lack sewage outfalls and do not attract such abundant and diverse animal communities. Thus it appears that the Iona Island jetty supports a large animal community because of the local enrichment of the

marine environment by sewage, its food-web being indicated in Figure 3. The base of this food-web has been studied by Otte and Levings (1975), who showed that the productivity of mud-flat areas in the vicinity of the sewage outfall was higher, in terms of both organic matter and invertebrate populations, than that of other mud-flat areas. The large population of bivalves is utilized by the rat population on the jetty as well as by the crab populations in the sea.

The flourishing wildlife community indicated above exists in spite of the fact that the area is contaminated with heavy-metals which are believed to be contained in the sewage. Thus the mercury content of 3-5-years-old crabs taken near Iona Island was shown to range from 1.5 to 3.7 ppm, compared with less than 1 ppm in crabs from other areas. Cadmium was generally less than 2 ppm in all animals analysed from the mud-banks, except near the sewer outfall where values of up to 7 ppm were encountered (Parsons et al., 1973). When converted to a market-weight basis, these values are appreciably higher than the levels permitted by the Canadian Food and Drug Act; for human consumption, mercury and cadmium levels should not exceed 0.5 ppm.

Similar studies by the Greater Vancouver Sewerage and Drainage District (1974) showed that the cadmium and zinc levels of Oysters (Crassostrea gigas) from Iona Island were above the maxima permitted by the Canadian Food and Drug Act.

Figure 3. Diagram of a simplified food-web of the wildlife community around the Iona Island jetty.


RAPTORIAL BIRDS
RATS
DUCKS
CRABS
MUSSELS
MACROPHYTES
CLAMS
MICROSCOPIC ALGAE

Seaweeds analysed at the same time generally showed levels of heavy-metals which decreased with the distance from the sewage outfall. From sediment analysis data (Ibid.) it was evident that the highest levels of heavy-metal pollution were closest to the basic outfall (e.g. Station B, Figure 2), and that they spread from there and across the mud-flats away from the jetty. Thus Station D (Figure 2), located farther down the jetty, showed no higher accumulations of heavy-metals than other areas of the mud-flats.

From the account of this wildlife community which has been given above, it is apparent that an unexpected situation exists, in which many different forms of wildlife are flourishing in an area that is known to be contaminated by heavy-metals. The question as to how this wildlife community is protected from the effects of heavy-metals is discussed in the rest of this paper.

Materials and Methods

The ability of animals to tolerate low-level chronic heavy-metal pollution may result from a detoxification mechanism which was first proposed by Piscator (1964). The process involves the production of a protein of low molecular weight (e.g. 10,000) called metallothionein, which binds the heavy-metal through sulphydryl linking with cysteine, that comprises 25-30% of the amino-acids of the protein. Subsequent investigations (e.g. Nordberg, 1972; Piotrowski et al., 1973) have tended to confirm that

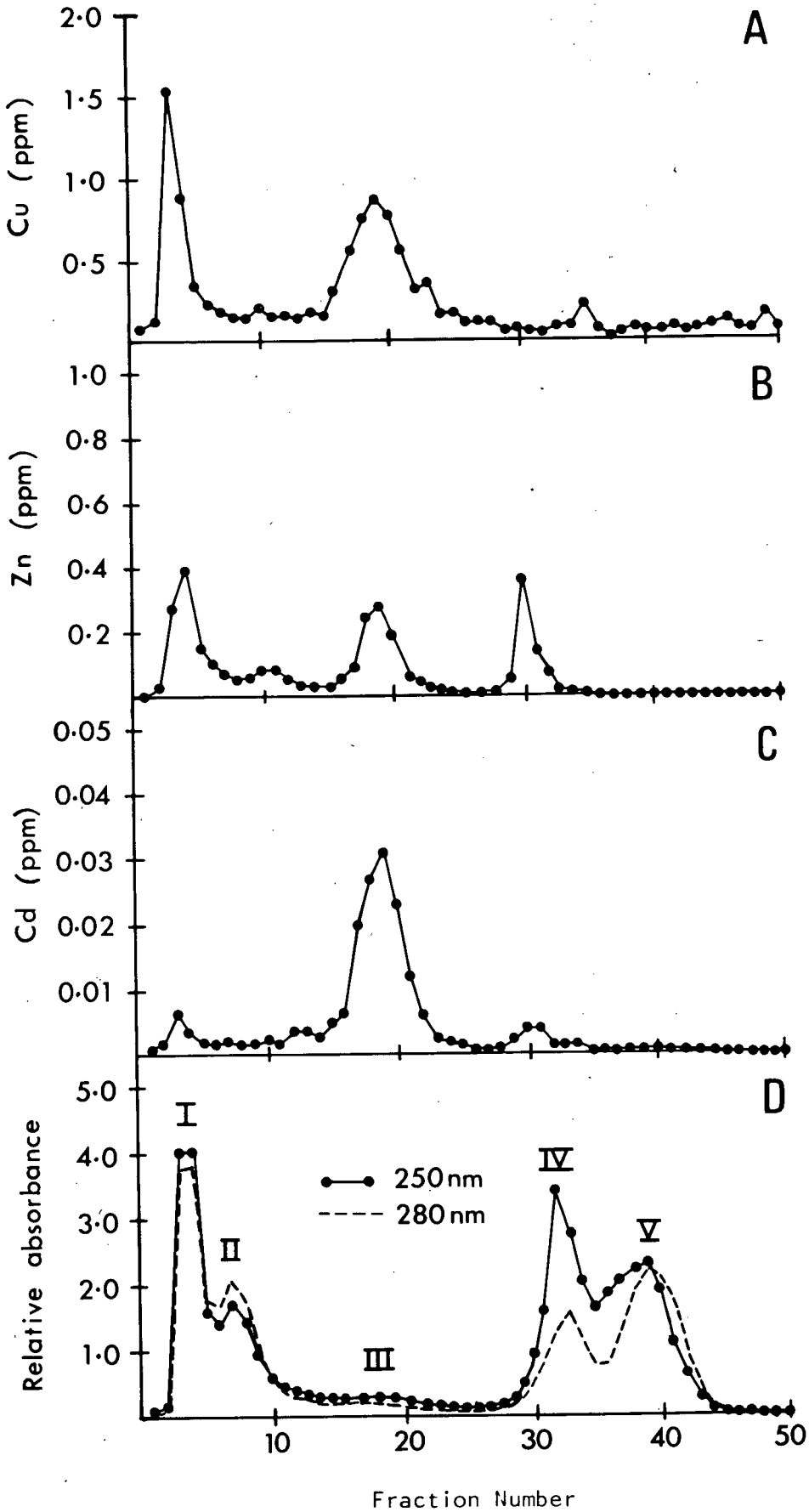
metallothioneins are instrumental in conferring protection from chronic cadmium and mercury exposure; the protective protein is synthesized in the liver, kidneys, and other organs.

The procedure for the isolation of metallothionein which was used in the present work was similar to that of Shaikh and Lucis (1971), Webb (1972), and Olafson and Thompson (1974). Liver or other tissue samples were thoroughly homogenized, and most of the tissue debris was removed by centrifugation at 27,000 x g for 10 minutes. The supernatant liquid was then heated to 70°C for 60 seconds, followed by centrifugation at 27,000 x g for 10 minutes (Webb, 1972a). The final supernatant was applied directly to a 2.5 x 100 cm column of Sephadex G-75 and eluted with 10mM NH_4HCO_3 at 60 ml/hr. Aliquots were collected on a fraction collector and proteins were measured at 250 and 280 nm in a spectrophotometer. This procedure was used as it allows for detection of metallothionein which has a low 280 nm absorption but a relatively high 250 nm absorption. Cadmium, zinc, and copper, were measured by using a Perkin Elmer 403 atomic absorption spectrophotometer.

Results and Discussion

In Figure 4 the graphs A, B, and C, illustrate the total amount of copper, zinc, and cadmium, respectively, in five protein fractions isolated from the liver of a

Figure 4. Graphs of (D) protein fractions (composing peaks I to V) and concentrations of (A) copper, (B) zinc, and (C) cadmium, from duck liver in each of these fractions.


A
Cu (ppm)
2·0
1·5
1·0
0·5
B
Zn (ppm)
1·0
0·8
0·6
0·4
0·2
C
Cd (ppm)
0·05
0·04
0·03
0·02
0·01
D
Relative absorbance
5·0
4·0
3·0
2·0
1·0
I
II
III
IV
V
250nm
280nm
10
20
30
40
50
Fraction Number

Greater Scaup duck, using a Sephadex column. The protein fractions into which the metals are absorbed are the same as those shown by Olafson and Thompson (1974); thus peak I corresponds to high-molecular-weight proteins, peak II to haemoglobin, peak III to metallothionein, and peaks IV and V correspond to low-molecular-weight cytoplasmic material such as amino-acids, nucleic acids, ATP, etc. Peak I contains high-molecular-weight proteins, including cellular enzymes, as almost all of these will have molecular weights greater than 65,000, which corresponds to haemoglobin (peak II). Metallothionein, with a molecular weight of ca 10,000, is eluted after the much larger haemoglobin molecule. The quantity of metallothionein indicated in D of Figure 4 is very small compared with the other protein fractions. The size of this peak can be greatly increased by injecting animals with cadmium (cf. Olafson and Thompson, 1974).

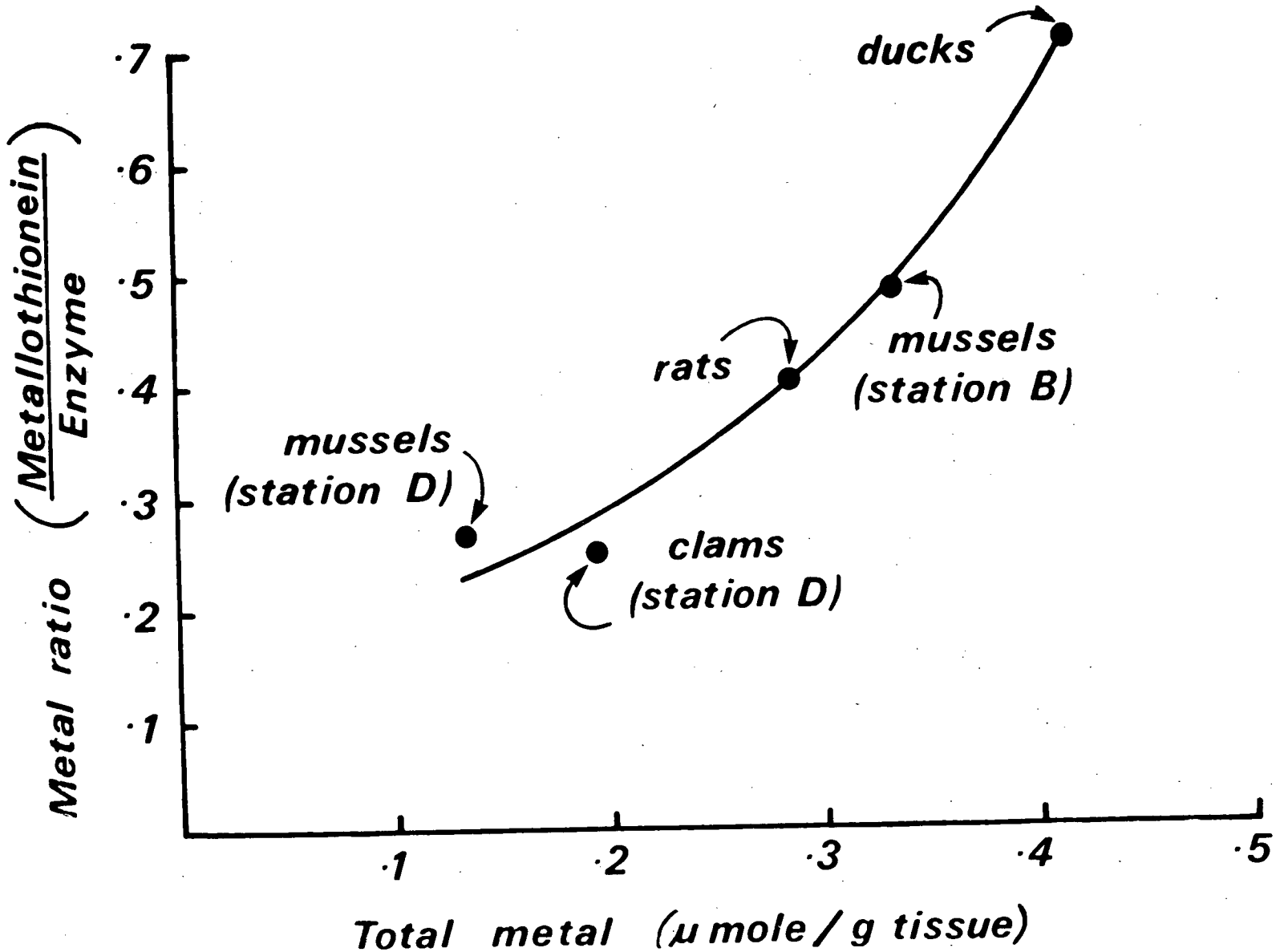
In samples from a natural environment, the loading of copper and zinc is similar on metallothionein and on the other proteins (A, B, and C, in Figure 4), while cadmium occurs mainly on metallothionein. As zinc and copper both occur naturally in enzyme systems, their relatively high abundance in peak I is to be expected. However, both metals have also been identified in analytical surveys (Greater Vancouver Sewerage and Drainage District, 1974) as being more abundant in sewage effluent than in the surrounding

environment. In the example shown in Figure 4, there is evidence of low-level pollution in which a small amount of metallothionein protein has been induced to absorb these excess metals - particularly cadmium.

In Figure 5 we have totalled up the quantity of copper, zinc, and cadmium, occurring in the metallothionein peak (III) as a ratio of that in the enzyme pool peak (I) for five groups of animals. From these data it may be seen that the mussels and clams from Station D (Figure 2, an area of little heavy-metal pollution) had the lowest metal-to-protein ratio, while animals from polluted areas had appreciably higher ratios. Thus the position of mussels from Station B clearly indicates a high degree of metal loading as compared with mussels from Station D. However, rats demonstrate levels of contamination which are intermediate between these two values, and it might thus be argued that they can feed at both ends of the jetty - partly on metal-contaminated mussels and partly on relatively metal-free mussels. Ducks showed the highest metal loading (a ratio of 0.70 ± 0.19 for four animals). Thus while higher metal loadings on the metallothionein peak indicate a greater degree of pollution, the relative position of metal-contaminated animals must also be to some extent a function of species differences and trophic position in the food-web (Figure 3).

From the data which have been presented it remains a

Figure 5. Total quantity of metals (Cd, Cu, and Zn) plotted against the ratio of the same metals on the metallothionein to high molecular-weight protein (enzyme-containing) fractions for different animals.


ducks
rats
mussels (station B)
mussels (station D)
clams (station D)
·7
·6
·5
·4
·3
·2
·1
·1
·2
·3
·4
·5
Metal ratio (Metallothionein / Enzyme)
Total metal (μ mole / g tissue)

question as to how much more metal contamination could occur in this wildlife community before marked pathological change would be observed among the animals. A partial answer to this question can be inferred from a further consideration of the absorption of metals by metallothionein and enzymes. As it is known that heavy-metals can exert their toxic effect by binding enzymes and replacing normal metalloenzyme complexes (Williams, 1971; Bremner, 1974; Friedberg, 1974), it can be argued that the metallothionein will only preferentially protect enzymes if it can be induced at a rate (or produced in total amount) which is sufficient to overcome the rate of heavy-metal entry into the animal (or the total load within the animal).

At some point, if the load or rate of loading of heavy-metals is sufficient to overcome the metallothionein protection, then there will be a "spillover" of heavy-metals (e.g. cadmium and mercury) from the metallothionein peak to the high-molecular-weight peak, with concurrent onset of pathological changes (Winge et al., 1973). The enzymes would then be rendered nonfunctional by conformational changes brought about by binding heavy-metals. This would result in their having properties different from normal enzyme functions when they are bound to their required metals (Friedberg, 1974). Non-function is due to the fact that, after conformational changes, substrate molecules no longer fit binding sites on the enzymes.

Thus pathological changes in the community would become apparent if heavy-metal loading exceeded the rate of metallothionein production.

In conclusion it is apparent from surveys of the Iona Island jetty area that, except in the anoxic channel immediately associated with the outfall, the totality of wildlife in this sewage-polluted region is greater than in other areas of the Fraser River mud-flats. Further, it has been shown that heavy-metal pollution occurs in this area but that this does not apparently affect the abundance of wildlife. The protective mechanism in these animals appears to be due, at least in part, to the production of metallothionein. In this context it is appropriate to recall that life has evolved on this planet from an age of natural pollution (i.e. from volcanic emissions) to one of unnatural pollution, due to Man (Cloud, 1974). It is not surprising, therefore, to find in a community an inherent mechanism which protects it from low-level heavy-metal toxicity.

Perhaps these results are best summarized by recalling Shakespeare's effort as an ecologist in the lines from Twelth Night (Act 1, Scene 1) "...Nature with a beautious wall doth oft close in pollution."

Summary

Marine and terrestial animals have been shown to be particularly abundant in a wildlife community associated

with a marine sewer outfall from the City of Vancouver. These same animals are contaminated with high levels of heavy-metals but are apparently protected from their poisonous effects by the production of a protein known as metallothionein. The amount of metallothionein and heavy-metal loading appears to depend primarily on the degree of pollution and secondly on the species of animal and its position in the food-web.

CHAPTER III Relationship Between Survival of Mussels Exposed to Cd, Cu and Zn, and the Cytoplasmic Distribution of these metals.

Preface

This paper will be submitted shortly for publication. Authors are D. A. Brown and K. W. Chatel (1978b). This study correlates "spillover" of metals into the high molecular weight protein pool with survival. Mussels from an unpolluted area are exposed to Cd, Cu and Zn in the laboratory, and "spillover" in these mussels is related to "spillover" and survival of mussels from a trace element polluted area. This study demonstrates that it is possible to predict survival possibilities of environmentally metal-exposed organisms by comparing "spillover" level to that of surviving and nonsurviving laboratory metal-exposed mussels. Thus, this approach goes beyond the traditional measure of total metal levels in organisms. It tells one via a biochemically meaningful assay (i.e., related to the actual toxicology of the trace elements), what the chances of survival of an organism are.

Introduction

In many studies, it has been reported that the protein metallothionein may be able to bind and thus detoxify trace elements such as Hg and Cd (e.g., Piscator, 1964; Leber, 1974; Brown et al., 1977). Excesses of Cu and Zn (i.e., above levels required for metalloenzymes) also

appear to be stored on metallothionein (Brown and Chatel, 1978a). If the binding capacity of metallothionein is exceeded, then Hg or Cd may "spillover" to the high molecular weight protein pool with concurrent pathological effects (Winge et al., 1973; Brown et al., 1977; Brown and Parsons, 1978; Irons and Smith, 1976). These effects are attributable to the displacement of Cu and Zn by Hg or Cd in metalloenzymes (Friedberg, 1974; Bremner, 1974). When this occurs, enzymes lose their normal conformational shape so that substrate molecules no longer fit binding sites on enzymes.

Recently, we have suggested that survival of organisms in the presence of high levels of trace elements near a sewer outfall, may be due, in part, to synthesis of the protein metallothionein (Brown et al., 1977). There appeared to be increases in the levels of metallothionein bound metal relative to high molecular weight protein (e.g., enzyme) bound metal with increasing trophic level or nearness to the sewer outfall. Thus it was suggested that abnormally high levels of Cd, Cu and Zn were bound to metallothionein in this polluted area.

In the present study, mussels from a relatively non-polluted area near the same sewer outfall are exposed, in the laboratory, to increasing levels of Cd, Cu and Zn in the same ratio as they are found in the sediment near the sewer outfall. A correlation is then constructed

between survival and metal levels in the high molecular weight protein pool and metallothionein. Mussels are then sampled at various distances in towards the sewer outfall until they cease to exist near the outfall. Metal levels in the high molecular weight protein pool and metallothionein of these mussels are then related to high molecular weight protein and metallothionein metal levels in the laboratory-exposed mussels, and to their survival.

Materials and Methods

Mussels were collected from Stations B, C, D and E, Iona Island, Vancouver, B.C., and frozen until analysed (see Brown et al., 1977, for a map of the study area). Station B is closest to the outfall while Station E is farthest away. Trace element levels are greatly elevated in the sediments near Station B, very slightly elevated near Station C, and normal at Stations D and E (Greater Vancouver Sewerage and Drainage District, 1974).

Other portions of mussels from Station E were transferred to glass bioassay tanks at a loading of 1 g mussels/L of bioassay watter; bioassay water was obtained from Station E. Cadmium, Cu and Zn were added to these tanks at the levels shown in Table 1. The ratio of these elements used was similar to that found in the sediments near Station B (Greater Vancouver Sewerage and Drainage District, 1974). Solutions were changed every three days. At the time of death or after 14 days exposure, mussels

Table 1. Levels of Cd, Cu and Zn added to bioassay water (collected from Station E) for the laboratory exposure of mussels from Station E, Iona Island, Vancouver, B.C. Metal levels are in mg metal/L.

Exposure	Cd	Cu	Zn
1	0.	0	0
2	.0001	.009	.007
3	.001	.09	.07
4	.01	.9	.7
5	.1	9.0	7.0

were sampled and frozen until analyzed.

Mussels were thawed and one gram portions of the soft parts of these were homogenized for 3 minutes in 3 ml of 0.9% NaCl solution, at a setting of 4.5 on a TRI-R STIR-R laboratory motor equipped with a teflon homogenizer. Homogenates were centrifuged at 27,000 x g for 10 minutes. Supernatants were heated for exactly 5 minutes in a 70°C water bath. Samples were then recentrifuged at 27,000 x g for 10 minutes. Two ml of resulting supernatant were applied to a Pharmacia K9/60 column packed with G-75 gel. Samples were eluted with 0.01 M NH_4HCO_3 at a flow rate of 10 ml/hour, and collected as 2 ml fractions.

Absorbance was read at 250 nm to establish the positions of the various cytoplasmic pools, i.e., the high molecular weight protein pool, metallothionein, and the low molecular weight cytoplasmic pool (Brown et al., 1977). Copper and Zn levels were determined in each fraction using a Perkin Elmer 303 atomic absorption spectrophotometer. Cadmium levels were determined using a Perkin Elmer 403 atomic absorption spectrophotometer equipped with a graphite furnace. Deuterium arc background correction was used for all analysis. Metal levels in each fraction of each of the cytoplasmic pools were added together to give the total metal level in each pool per gram of tissue (wet weight).

Results

Mussels from Station E, Iona Island, exposed to Cd, Cu and Zn in the laboratory survived for less than 48 hours in Exposures 4 and 5 (Table 1). Mussels in Exposures 1, 2 and 3 survived the entire exposure period of 14 days. At Iona Island, mussels were plentiful at Stations C, D and E, but sparse at Station B.

A typical gel elution profile, from mussels from Station B is shown in Figure 6. This profile appears to be characterized by high levels of Cd, Cu and Zn in the high molecular weight protein pool (I) with lesser amounts of these metals in metallothionein (II) and the low molecular weight cytoplasmic pool (III). Mussels from Stations C, D and E from Iona Island contained lesser levels of Cd, Cu and Zn in the high molecular weight protein pool and metallothionein than those from Station B (Table 2). Cadmium and Zn were lower in the low molecular weight cytoplasmic pool at Stations C to E, but Cu was increased in this pool (Table 2).

Mussels from Station E exposed to Cd, Cu and Zn in the laboratory, had decreased levels of Cd on all three cytoplasmic pools with increasing levels of exposure. Copper and Zn were increased in all three pools with increasing levels of exposure (Table 2).

The levels of Cd + Cu + Zn in the high molecular weight protein pools and metallothionein increased with

Figure 6. Gel elution profile of mussels from Station B, Iona Island, Vancouver, B.C. I: high molecular weight protein pool; II: metallothionein; III: low molecular weight cytoplasmic pool.

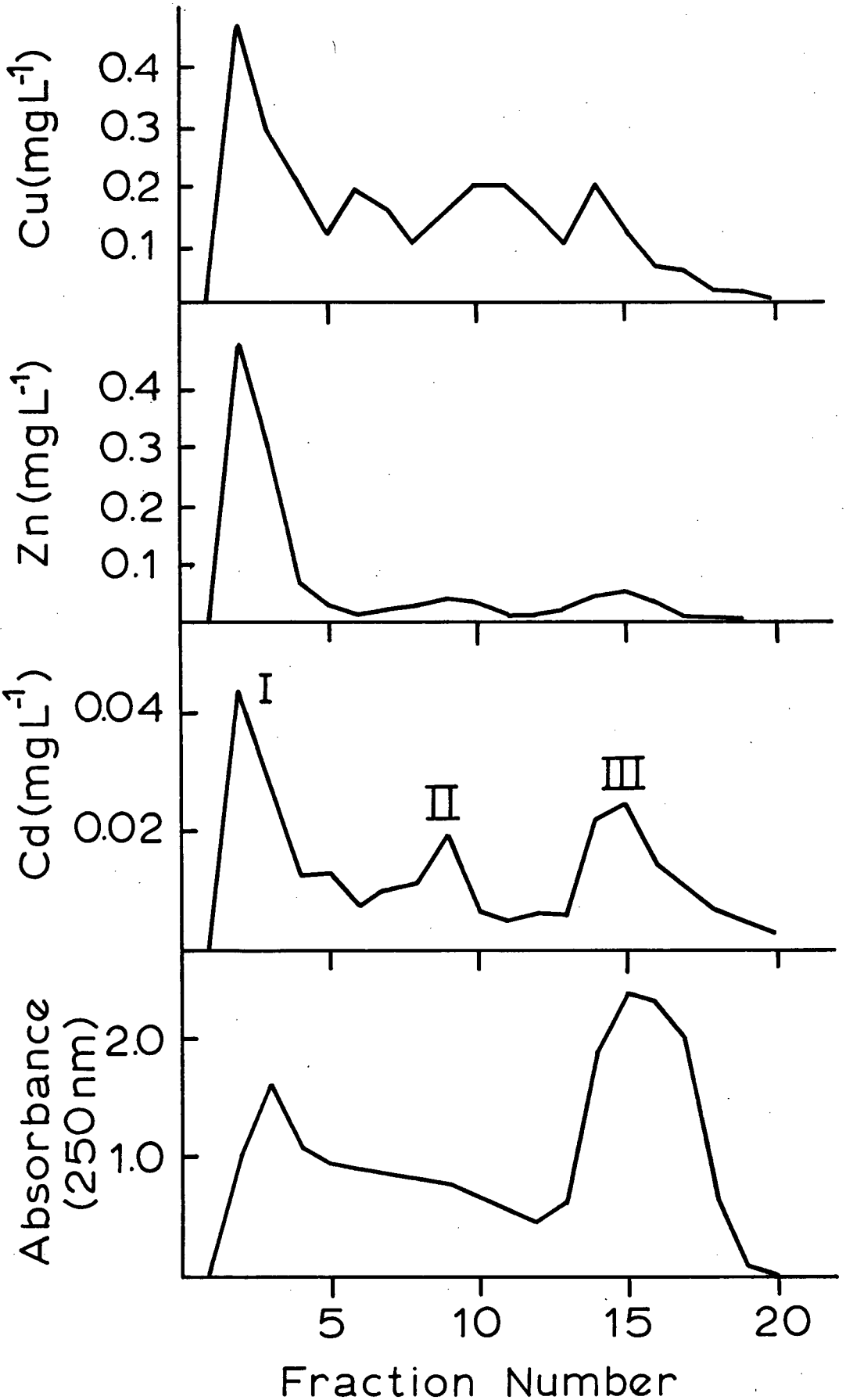

Cu(mgL-1)
0.4
0.3
0.2
0.1
Zn(mgL-1)
0.4
0.3
0.2
0.1
Cd(mgL-1)
0.04
0.02
I
II
III
Absorbance (250nm)
2.0
1.0
5
10
15
20
Fraction Number

Table 2. The distribution of Cd, Cu and Zn among cytoplasmic pools of mussels exposed to various levels of these trace elements in the laboratory (Exposures 1-5) or near Iona Island, Vancouver, B. C. (Station B-E). Levels of Exposures 1-5 are given in Table 1. All metal levels are in μmole metal/g tissue (wet weight)[a].

		Cd				Cu				Zn			
		Total	High MW[b] pool	Metallo-thionein	Low MW pool	Total	High MW pool	Metallo-thionein	Low MW pool	Total	High MW pool	Metallo-thionein	Low MW pool
Exposure	1	.0190	.0089	.0018	.0083	.301	.074	.043	.184	.225	.132	.016	.077
	2	.0205	.0067	.0022	.0116	.394	.079	.044	.271	.413	.151	.019	.243
	3	.0181	.0057	.0017	.0107	.542	.128	.063	.351	.583	.215	.027	.341
	4	.0147	.0049	.0014	.0084	.965	.211	.063	.691	1.058	.398	.034	.626
	5	.0098	.0034	.0012	.0052	3.416	.401	.223	2.792	.769	.422	.032	.315
Station	B	.0413	.0187	.0052	.0174	.757	.405	.188	.164	.473	.286	.029	.158
	C	.0169	.0071	.0031	.0067	.318	.089	.060	.169	.270	.153	.024	.093
	D	.0187	.0077	.0027	.0083	.633	.125	.108	.400	.349	.181	.021	.147
	E	.0175	.0078	.0024	.0073	.631	.072	.050	.509	.301	.164	.015	.122

[a]Data is compiled from profiles such as those shown in Figure 6.

[b]Molecular weight.

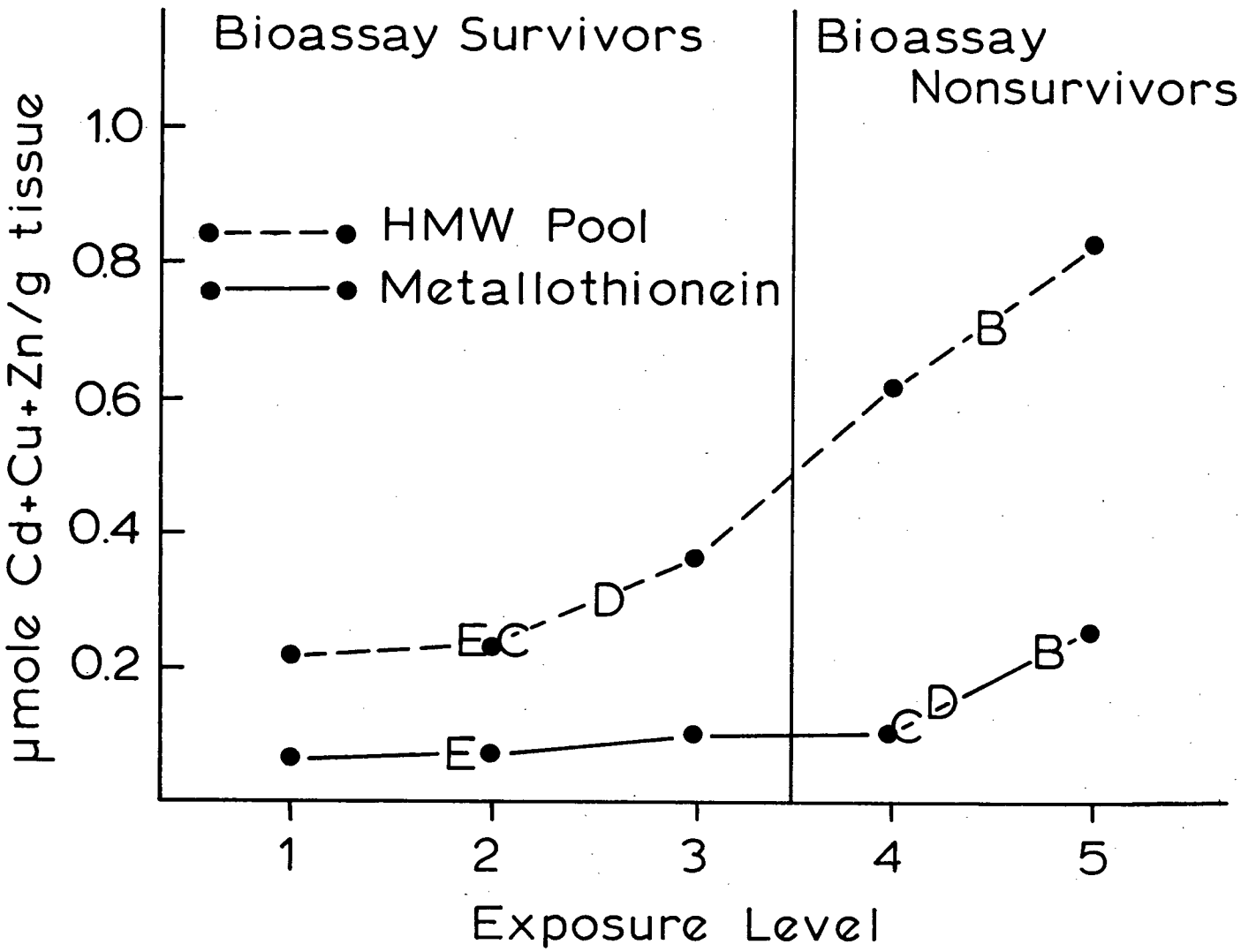
exposure level in the laboratory exposure (Figure 7). Metallothionein appeared to bind only a small portion of metal relative to the high molecular weight protein pool. Metallothionein levels of Cd + Cu +Zn appeared to increase slightly from Exposures 1 to 3, but to plateau from 3 to 4. Where this plateau occurred, there was a dramatic increase of metal in the high molecular weight protein pool. This plateau corresponded to the divisional point between survivors and nonsurvivors in the laboratory exposures (Figure 7). There was a further increase of metallothionein levels from exposures 4 to 5.

The positions of mussels from Iona Island on the graphs of the laboratory-exposed animals are also indicated in Figure 7. Mussels from the sparsely populated Station B had levels of metal on the high molecular weight protein pool which were indicative of nonsurvival in the laboratory-exposed animals. Mussels from Stations C to E were on the survival portion of the high molecular weight protein pool graph. Metallothionein levels in mussels from Stations B to D were as high as those in mussels from the highest metal exposure levels in the laboratory experiment. Station E mussels corresponded to the lowest metal exposures in the laboratory experiment.

Discussion

It appears evident from this and previous studies, that metallothionein is present in mussels and may be im-

Figure 7. The levels of total Cd + Cu + Zn in the high molecular weight (HMW) protein pool and metallothionein. Exposure levels 1-5 are those given in Table 1. Points B-E represent mussels from Stations B-E, Iona Island, Vancouver, B.C. These have been placed on the graphs of exposures 1-5 at those positions where they have corresponding metal levels in the high molecular weight protein pool or metallothionein. All metal levels are in μmole metal in each pool/g tissue (wet weight).


Bioassay Survivors
Bioassay Nonsurvivors
HMW Pool
Metallothionein
μmole Cd+Cu+Zn/g tissue
1.0
0.8
0.6
0.4
0.2
E
C
D
B
E
C
D
B
1
2
3
4
5
Exposure Level

portant as a trace element detoxification substance (Noel-Lambot, 1976; Talbot and Magee, 1978). In the laboratory portion of the present study, when metal levels on metallothionein appeared to plateau, there was a dramatic increase of metals in the high molecular weight protein pool. This apparent saturation of metallothionein, with "spillover" of metal into the high molecular weight protein pool is concurrent with decreased survival of these mussels. The relationship between "spillover" and appearance of toxic effects has been noted in previous studies (Winge et al., 1973; Irons and Smith, 1976; Brown et al., 1977; Brown and Parsons, 1978). Toxic effects can be attributed to the interference of nonessential, or excesses of essential metals, with metal binding sites in metalloenzymes (Friedberg, 1974). Conformational changes caused by these interferences result in malfunction of enzymes essential for normal metabolism. The increase of metallothionein levels after "spillover" has occurred, has been found, and reasons for it discussed, in another study by Brown and Chatel (1978c).

In the laboratory portion of the present study, it appears that Cd was probably not a major factor influencing survival of mussels as it was decreased in the cytoplasm of organisms with increased exposure level. This may be due to exclusion resulting from competition of high levels of Cu and Zn for entry sites into the mussels. At Station

B, Iona Island, Cd may be a factor influencing survival as it is greatly increased in the high molecular weight protein pool compared with other stations. Laboratory-exposed mussels were administered Cd + Cu + Zn in the ratio found in the sediments near the sewer outfall at Iona Island. As Cd is increased in mussels at Iona Island, but decreased in the laboratory exposure, this may indicate that there is a higher ratio of Cd:Cu + Zn in the water at Iona Island than in the sediments. Alternatively, uptake of Cd at Iona Island may be increased by the presence of high levels of organic chelators in the sewer effluent. George and Coombs (1977) have suggested that ionic Cd must first be complexed before uptake can occur, and that organic chelators increase both the rate of accumulation and the final tissue concentration of Cd.

Copper levels in the high molecular weight protein pool of mussels from Station B are as high as those in mussels from the highest laboratory exposure; these laboratory-exposed mussels survived less than 48 hours exposure. Therefore, if the presence of high levels of Cu in the high molecular weight protein pool were a causative factor in death of mussels, then it would be unlikely that there would be any surviving mussels at Station B. Zinc, however, is present in the high molecular weight protein pool in mussels from Station B at levels intermediate between those from Exposures 3 and 4, i.e., halfway between levels

found in survivors and nonsurvivors. Since mussels at Station B are very sparse, it may be that Zn is one of the controlling factors in the survival of mussels.

The level of metals on metallothionein does not seem to correlate with survival. Mussels from Stations B to D had levels of Cd + Cu + Zn on metallothionein equivalent to the nonsurvivors in the laboratory-exposed mussels. Mussels from these stations may have relatively high metallothionein levels as compared to laboratory exposed mussels as they may have been subject to longer but lower level metal exposure than those in Exposures 2 to 5. Therefore, they could have had more time to adapt to excesses of trace elements via synthesis of metallothionein. Thus, mussels from Stations B to D have a mean ratio of metallothionein / high molecular weight protein bound metal of 0.40 while those from Exposures 2 to 5 have a mean ratio of 0.25. In a previous study, it was found that this ratio increased both with nearness to the sewer outfall or increasing trophic level (Brown et al., 1977).

It is apparent that survival of mussels in the present study does not decrease as the level of metal on metallothionein increases, Nor as cytoplasmic metal levels increase. Rather, the number of survivors decreases when excesses of metal occur in the high molecular weight protein pool. Thus the factor influencing the survival of organisms exposed to trace elements may not be the level of metal

in these organisms, but rather how much of the metal is not bound to metallothionein, but instead occurs in the high molecular weight protein pool. This may depend upon both the length and level of exposure.

Summary

Mussels were sampled in the vicinity of the Iona Island sewer outfall, Vancouver, B.C. Mussels sampled from a relatively nonpolluted area were exposed, in the laboratory for 14 days, to various levels of Cd + Cu + Zn in the ratio in which these metals occurred in the sediments near the sewer outfall. In the laboratory-exposed mussels, metallothionein bound Cd + Cu + Zn increased slightly in the lower exposures and then plateaued. At the exposures where metallothionein bound Cd + Cu + Zn plateaued, there was a dramatic increase in high molecular weight protein bound Cd + Cu + Zn; these mussels did not survive the exposure period. Toxic effects are attributed to the effects of these metals on enzymes in the high molecular weight protein pool.

Mussel populations at a sampling area near the sewer outfall were sparse. These mussels had levels of Cd + Cu + Zn in the high molecular weight protein pool indicative of nonsurvival in the laboratory-exposed mussels. Metallothionein levels in mussels from sampling areas near the sewer outfall were higher than those for all but the highest laboratory-exposed mussels. These higher metallothionein

levels may be due to a longer exposure to high metal levels near the sewer outfall than in the laboratory.

CHAPTER IV Interactions Between Cadmium and Zinc in Cytoplasm of Duck Liver and Kidney

Preface

This paper has been accepted for publication by Chemico-Biological Interactions, and will be appearing in 1978. The authors are D. A. Brown and K. W. Chatel (1978a). This study demonstrates that metallothionein, in duck liver and kidney tissue, stores excesses of Cu and Zn. Most importantly, it demonstrates that Cd can occur in the high molecular weight protein pool before metallothionein is saturated if there are deficiencies of Zn in this pool. Thus, the binding capacity of metallothionein does not have to be exceeded before apparent "spillover" occurs.

Introduction

Copper and Zn are necessary for the functioning of many enzymes as they maintain both tertiary and quaternary protein structure. If metalloenzymes are exposed to an excess of the functional heavy metal or to competing heavy metals such as Cd or Hg, then they may lose their ability to function normally (Friedberg, 1974). Dysfunction may be a result of conformational changes produced when excesses of essential metals or toxic metals interfer with, or replace, essential metals on metalloenzymes (Friedberg 1974; Bremner, 1974). After conformational changes, substrate molecules may no longer fit binding sites on the enzymes (Friedberg, 1974). Alternatively, some toxic

metals (e.g., Hg) can split certain enzymes into catalytic and regulatory subunits so that normal regulation of activity of the catalytic subunit is lost (Gerhart and Schachman, 1965; Jovin et al., 1969; White et al., 1968; Brown, 1977). Thus, to ensure proper cellular functioning, excesses of essential metals or toxic metals must be removed from the zone of biological activity. This is accomplished by production of the protein metallothionein, which can bind heavy metals, thereby rendering them biologically inactive (Brown et al., 1977).

Metallothionein is a low molecular weight (10,000) protein high in sulfhydryl groups due to the presence of high levels of the amino acid cysteine (Margoshes and Vallee, 1957; Kagi and Vallee, 1960). The sulfhydryl groups can bind or chelate heavy metals thus rendering them nontoxic to the exposed organism (Piscator, 1964).

Metallothionein is stored solubilized in the cytoplasm of liver and kidney tissue. Very small amounts of metallothionein may be released from the liver into the bloodstream. These pass through the glomular filtration apparatus and are excreted in the urine or reabsorbed in the renal tubules and stored in the kidney. Only about 0.01% of the body burden of metallothionein is excreted per day via the urine (Piscator, 1964; Nordberg, 1972).

This study reports interrelationships between the levels of Cd, Cu and Zn on metallothionein and on the high

molecular weight protein (enzyme-containing) pool (Brown et al., 1977) in duck liver and kidney tissue.

Materials and Methods

Greater Scaup (Aythya marila), Surf Scoter (Melanitta perspicillata) and Wester Grebe (Aechmophorus occidentalis) ducks were collected near the Iona Island sewage outfall, Vancouver, British Columbia. This area is known to be polluted with high levels of heavy metals (Brown et al., 1977). Liver and kidney samples were removed at the time of capture and frozen until analyzed.

Three gram portions of each tissue were thoroughly homogenized in 9 ml of 0.9% NaCl. The homogenates were centrifuged at 27,000 x g for 10 minutes and the supernatants collected. Pellets were rehomogenized in 6 ml of 0.9% NaCl followed by centrifugation at 27,000 x g for 10 minutes. Supernatants were combined and heated to 70°C for 1 minute (Webb, 1972a; Cherian, 1974; Brown, 1977; Brown et al., 1977). Samples were then recentrifuged for 10 minutes at 27,000 x g. Resulting supernatants (14 ml) were applied to a Pharmacia K25/100 column packed with Sephadex G-75 fine gel. Filtrates were eluted with 0.01 M NH_4HCO_3 and collected in 10.2 ml fractions. Absorbance was read at 250 and 280 nm on a Perkin Elmer Coleman 124D spectrophotometer. Cadmium levels were determined by graphite furnace on each fraction with a Perkin Elmer 403 atomic absorption spectrophotometer.

Cu and Zn levels were determined by the flame method with a Perkin Elmer 303 atomic absorption spectrophometer. Deuterium background correction was used for all determinations.

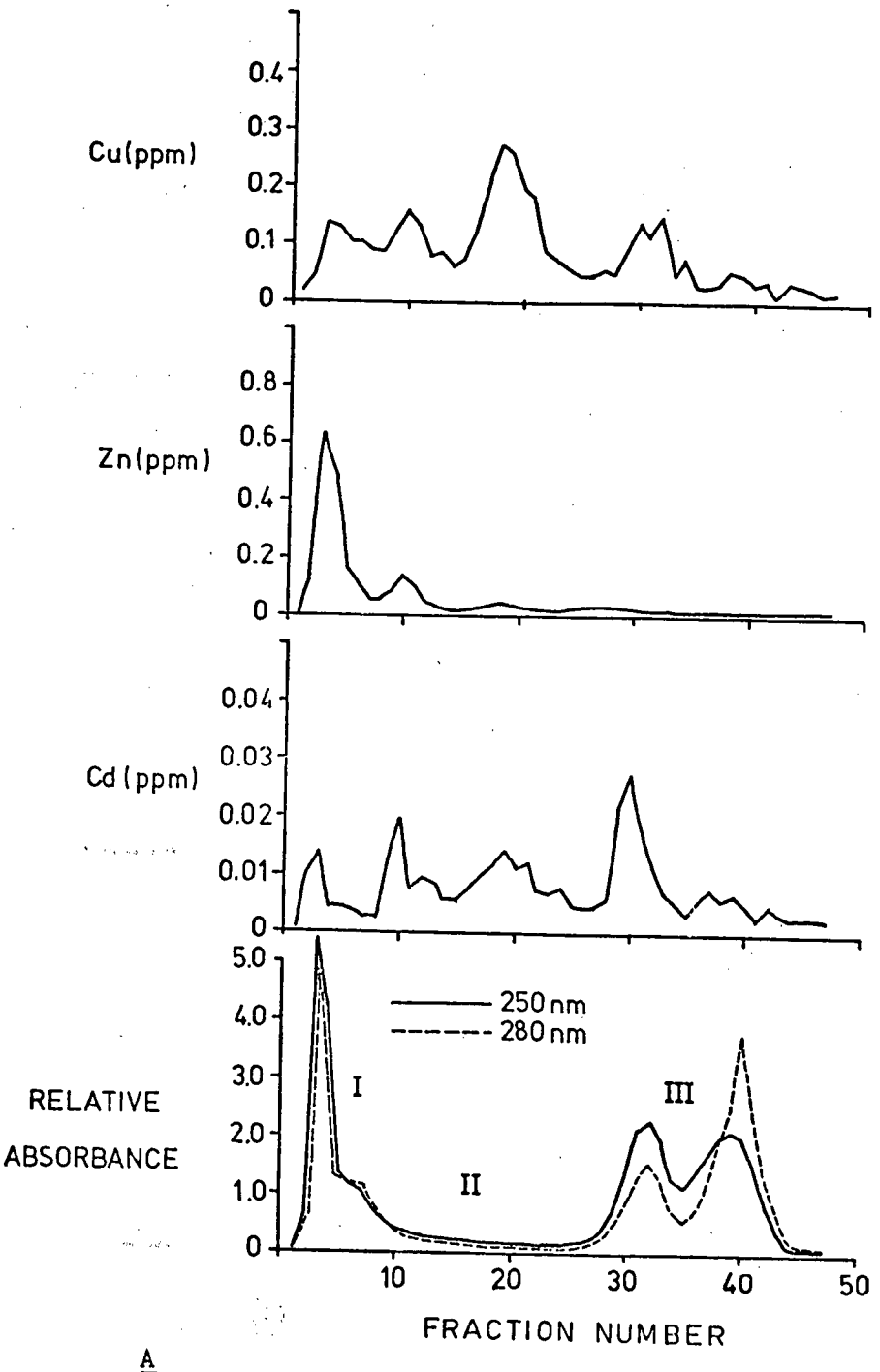
Results

Typical elution profiles from duck liver or kidney tissue are presented in Figure 8. Each peak has been identified according to its similarity of position to peaks found in previous studies (Olafson and Thompson, 1974; Winge et al., 1973; Brown et al., 1977). The dual Cd peaks on the high molecular weight protein pool of liver tissue appear to correspond to the 30,000 molecular weight and 115,000 molecular weight Cd-binding moieties described previously (Prohaska et al., 1977). A summary of the total level of metals in all peaks is presented in Table 3.

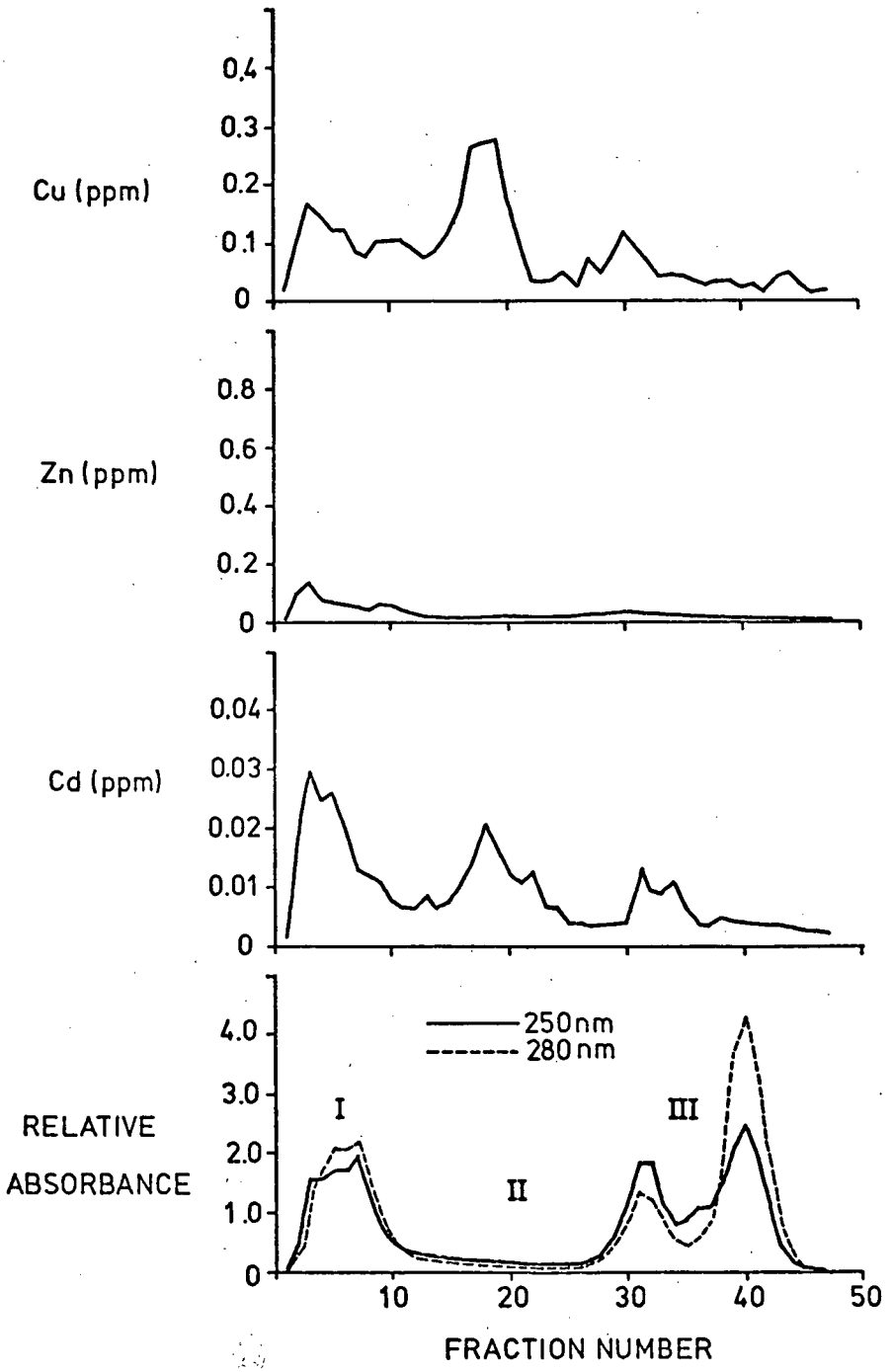
The Cd, Cu or Zn level in each peak (Table 3) for each metal has been plotted against the total cytoplasmic level of each metal (Figures 9A-I). It is apparent that Cu increases linearly on the high molecular weight protein pool and metallothionein with increases of Cu in cytoplasm (Figures 9A-B). The low molecular weight cytoplasmic pool appears to become Cu saturated at about 0.40 μmole cytoplasmic Cu/g tissue (wet weight), (figure 9C).

Zinc appears to increase linearly on the high molecular weight protein pool at low tissue levels of Zn (Figure 9D).

Figure 8. Gel elution profiles of Greater Scaup liver (A) and kidney (B). I: high molecular weight protein pool; II: metallothionein; III: low molecular weight cytoplasmic pool.


Cu(ppm)
0.4
0.3
0.2
0.1
0
Zn(ppm)
0.8
0.6
0.4
0.2
0
Cd (ppm)
0.04
0.03
0.02
0.01
0
RELATIVE
ABSORBANCE
5.0
4.0
3.0
2.0
1.0
0
250 nm
280 nm
I
II
III
10
20
30
40
50
FRACTION NUMBER

A

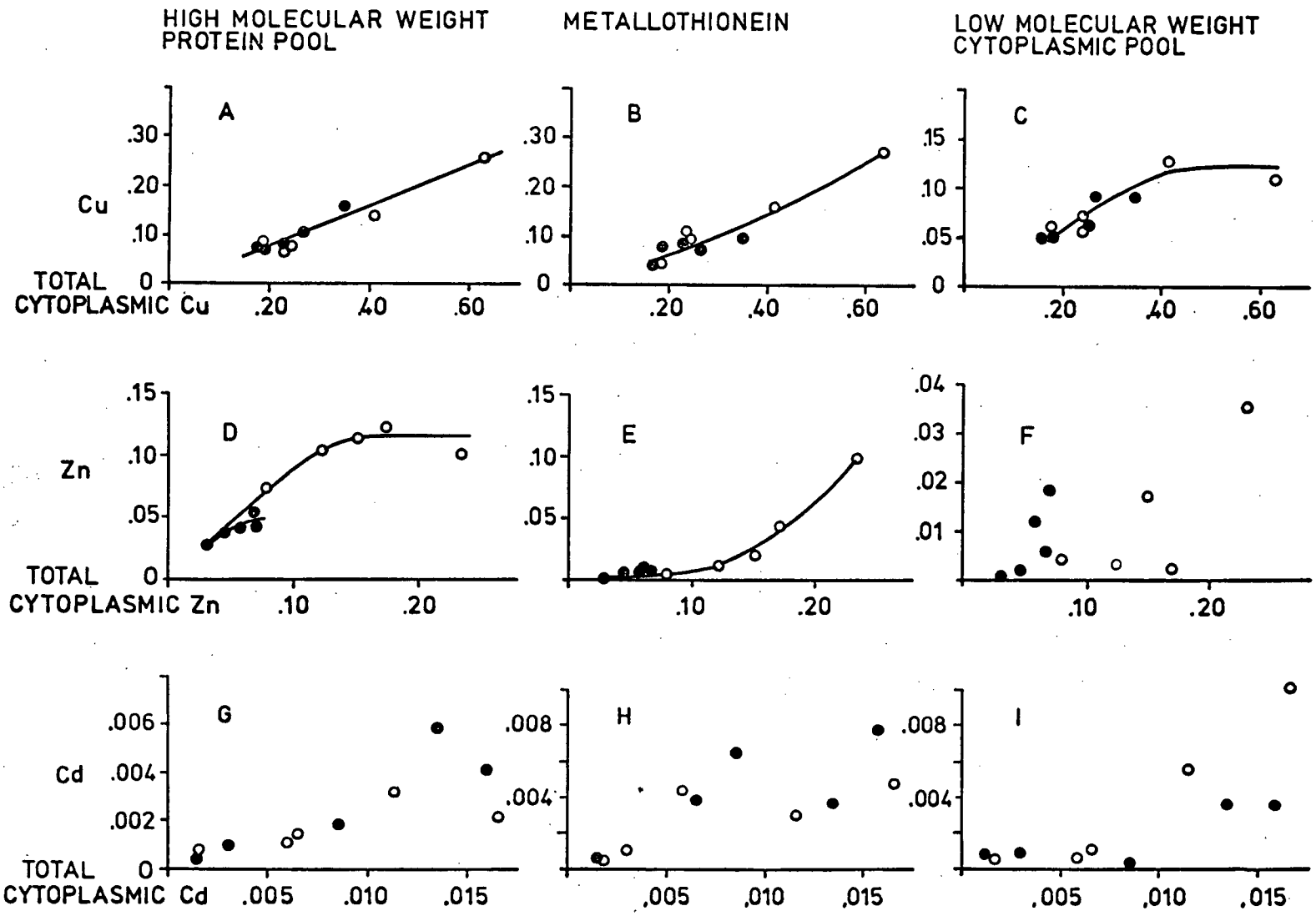

Cu (ppm)
0.4
0.3
0.2
0.1
0
Zn (ppm)
0.8
0.6
0.4
0.2
0
Cd (ppm)
0.04
0.03
0.02
0.01
0
RELATIVE
ABSORBANCE
250nm
280nm
I
II
III
4.0
3.0
2.0
1.0
0
10
20
30
40
50
FRACTION NUMBER

B

Table 3. The distribution of Cd, Cu and Zn amongst cytoplasmic pools from liver and kidney of ducks. Data are compilations of metal levels from profiles such as Figure 4 in μmole/g tissue (wet weight). MW: molecular weight. ND: not detectable.

		Cd				Cu				Zn			
		Total	High MW pool	Metallo-thioein	Low MW pool	Total	High MW pool	Metallo-thionein	Low MW pool	Total	High MW pool	Metallo-thionein	Low MW pool
Greater Scaup	Liver	.00578	.00107	.00418	.00053	.625	.252	.265	.108	.234	.101	.098	.035
	Kidney	.00845	.00187	.00632	.00026	.342	.156	.096	.090	.067	.043	.006	.018
Surf Scoter	Liver	.00641	.00142	.00391	.00108	.408	.132	.153	.123	.151	.114	.020	.017
	Kidney	.00294	.00089	.00107	.00098	.262	.102	.071	.089	.058	.041	.005	.012
Greater Scaup	Liver	.01141	.00320	.00290	.00530	.230	.074	.088	.069	.119	.106	.010	.003
	Kidney	.01330	.00590	.00370	.00370	.225	.079	.084	.062	.045	.040	.003	.002
Western Grebe	Liver	.00164	.00065	.00040	.00059	.181	.081	.041	.059	.078	.072	.002	.004
	Kidney	.00147	.00068	.00043	.00036	.170	.077	.037	.056	.029	.028	ND	.001
Greater Scaup	Liver	.01650	.00212	.00481	.00957	.230	.070	.106	.054	.170	.123	.045	.002
	Kidney	.01571	.00408	.00788	.00375	.189	.062	.075	.052	.065	.053	.006	.006

Figure 9. The variation of Cd, Cu and Zn on the high molecular weight protein pool, metallothionein and the low molecular weight cytoplasmic pool with increases of these metals in liver and kidney cytoplasm. ○: liver, ●: kidney. Metal levels are in μmole/g tissue (wet weight).


HIGH MOLECULAR WEIGHT PROTEIN POOL
METALLOTHIONEIN
LOW MOLECULAR WEIGHT CYTOPLASMIC POOL
A
B
C
D
E
F
G
H
I
Cu
Zn
Cd
TOTAL CYTOPLASMIC Cu
TOTAL CYTOPLASMIC Zn
TOTAL CYTOPLASMIC Cd
.30
.20
.10
0
.15
.05
.04
.03
.02
.01
.006
.004
.002
.008
.40
.60
.005
.010
.015

The kidney high molecular weight protein pool appears to be Zn saturated at about 0.06 μmole cytoplasmic Zn/g tissue while in liver saturation appears to occur at 0.14 μmole cytoplasmic Zn/g tissue. Concurrent with Zn saturation of the high molecular weight protein pool is an acceleration of Zn levels on metallothionein (Figure 9E). Low molecular weight cytoplasmic Zn shows little or no relationship to cytoplasmic Zn levels (Figure 9F).

Levels of Cd on the three cytoplasmic pools generally appear to increase with increasing total cytoplasmic Cd levels (Figures 9G-I). However, more clearly, the portion of cytoplasmic Cd present on the high and low molecular weight pools appears to be related to levels of Zn on these pools (Figure 10). Seventy-five percent of cytoplasmic Cd appears on the high and low molecular weight pools at lower Zn levels. Only at Zn levels where the kidney and liver high molecular weight protein pools appear to be Zn saturated (Figure 9D) does more than 25% of the cytoplasmic Cd appear on metallothionein (Figure 11). Thus, Zn saturation of the high molecular weight protein pools in liver and kidney tissue appears to coincide with an increase of the portion of cytoplasmic Cd on metallothionein.

Discussion

Results of this study indicate that once the high molecular weight protein pool is Zn saturated, excess Zn

is stored on metallothionein. As enzymes occur in the high molecular weight protein fraction of cell cytoplasm, Zn saturation of this pool may indicate that the Zn requirements of metalloenzymes have been met. That Zn saturation of the liver high molecular weight protein pool occurs at a 2-fold higher Zn level than that in kidney, may be a reflection of the higher metabolic activity of liver. As the liver is the metabolic center of higher organisms, it must contain more enzymes, a certain proportion of these being metalloenzymes, and hence, have higher Zn requirements on the high molecular weight protein pool.

The high molecular weight protein pool does not appear to be Cu saturated in these ducks although cytoplasmic Cu levels are 3-fold higher than Zn levels. Copper requirements may be higher than Zn requirements for metalloenzymes in marine organisms. Previous studies have found Cu levels to be 3- to 8-fold higher than Zn levels on the high molecular weight protein pool of fish (Brown, 1977), mussels (Brown and Chatel, 1978b). phytoplankton (Cloutier and Brown, 1978), and zooplankton (Brown and Parsons, 1978). Perhaps the high molecular weight protein pool would become Cu saturated in ducks with Cu levels higher than those reported in this study; Cu profiles might then appear to be similar in shape to Zn profiles.

Figure 10. The variation of the portion of total cytoplasmic Cd on the high molecular weight protein pool (HMW) + the low molecular weight cytoplasmic pool (LMW) with increases of Zn on the high molecular weight protein pool + the low molecular weight cytoplasmic pool. ○: liver, ●: kidney. Zinc levels are in μmole/g tissue (wet weight).

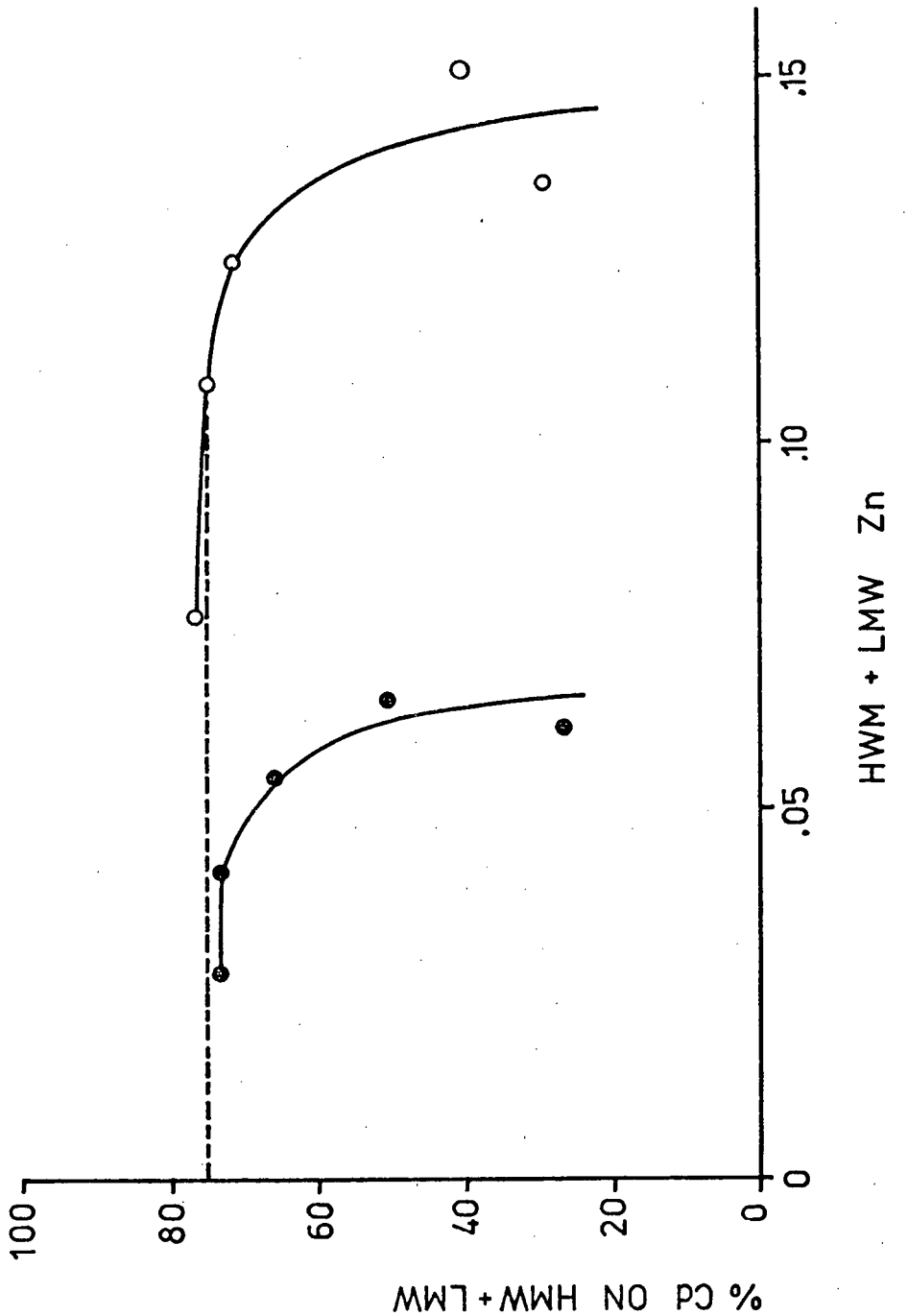
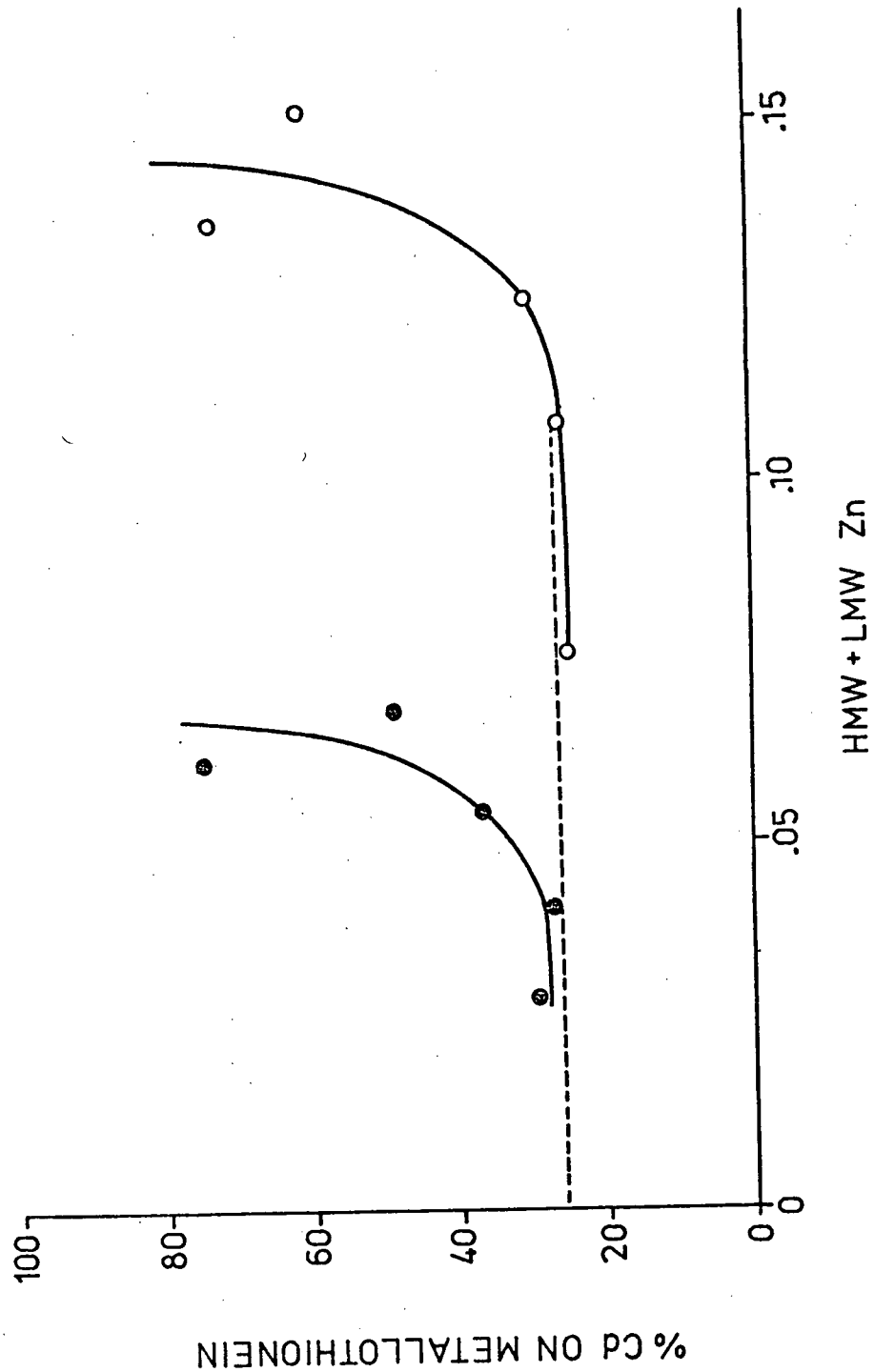

% Cd ON HMW + LMW
100
80
60
40
20
0
0
.05
.10
.15
HWM + LMW Zn

Figure 11. The variation of the portion of total cytoplasmic Cd on metallothionein with levels of Zn on the high molecular weight protein pool (HMW) + the low molecular weight cytoplasmic pool (LMW). ○: liver, ●: kidney. Zinc levels are in μmole/g tissue (wet weight).


% Cd ON METALLOTHIONEIN
100
80
60
40
20
0
0
.05
.10
.15
HMW + LMW Zn

The present study indicates that the portion of cytoplasmic Cd appearing on the high molecular weight pool is higher when Zn levels are lower on this pool. It appears as though a concentration dependent equilibrium exists, whereby when high levels of Zn are available for the high and low molecular weight pools, most Cd will not occupy Zn binding sites on these pools. Rather, Cd appears to be displaced by high Zn levels so that it is available to induce synthesis of, and bind, metallothionein. Since 75% of Cd appears on the high and low molecular weight pools with low Zn levels in both kidney and liver tissue, it is probable that this equilibrium reaction is not dependent upon tissue type. That Cd is displaced from the high molecular weight protein pool by lower Zn levels in kidney compared with liver, suggests that there are fewer binding sites for which Cd and Zn can compete in kidney tissue. This may reflect lower levels of metalloenzyme binding sites in the kidney.

Competition of Zn and Cd for similar binding sites in tissue is well established. It is known that Cd exerts toxic effects via its effect on Zn metalloenzymes (Horvath, 1976) and that high levels of Zn prevent or reduce the toxic effects of Cd (Flick et al., 1971). It has also been shown that toxic effects of Cd do not occur until the binding capacity of metallothionein is exceeded and Cd occurs on the high molecular weight protein pool

(Winge et al., 1973; Irons and Smith, 1976). Chen and Ganther (1975) have suggested that the toxic effects of Cd may become apparent when it occurs on a protein of approximately 30,000 molecular weight; they suggest this protein may be the Zn metalloenzyme carbonic anhydrase. It has been suggested, therefore, that high levels of Cd on the high molecular weight protein pool may indicate that Cd is available, free from metallothionein, to exert toxic effects via interference with metalloenzymes (Brown et al., 1977). The present study indicates that Cd may appear on the high molecular weight protein pool, not only when the binding capacity of metallothionein is exceeded, but also when there are deficiencies of Zn on the high molecular weight protein pool. Since toxic effects of Cd occur when it appears on the high molecular weight protein pool, it is likely, therefore, that deficiencies of Zn increase toxic effects of Cd by permitting an increase of Cd on the high molecular weight protein pool.

Results from the present study may be of significance for understanding trace metal interactions in carcinogenesis. It was reported in a previous study that exposure of mice to the carcinogen diethylnitrosamine (DEN) for 12.5 weeks resulted in a 39% reduction of Zn levels on the high molecular weight protein pool in pretumorous liver tissue (Brown and Chan, 1978). When Cd was adminis-

tered concurrently with DEN, almost all of this additional Cd accumulated on the high molecular weight protein pool. Cadmium levels were far too low to have exceeded the binding capacity of metallothionein (Brown, 1977; Brown and Chan, 1978). Results from the present study indicate that approximately 75% of liver Cd will appear on the high molecular weight protein pool if this pool is Zn deficient. Thus, an organic carcinogen might increase the portion of cytoplasmic Cd on the high molecular weight protein pool by decreasing Zn levels on this pool.

Cadmium is a potent carcinogen and has been mentioned as possibly involved in the etiology of cancer since both Cd levels and the Cd:Zn ratio are increased in tumor-bearing organisms (Morgan, 1970; Morgan, 1971; Brown 1977), particularily on the high molecular weight protein pool (Brown, 1977). It has been suggested that Cd exerts its carcinogenicity via interference with Zn binding sites on the Zn requiring enzymes involved in cell division (Brown, 1977). Furthermore, Zn prevents cancer from occurring when given with Cd or an organic carcinogen (Gunn et al., 1963, 1964; Poswillo and Cohen, 1971; Ciapparelli et al., 1972). Thus, if Cd is carcinogenic when present on the high molecular weight protein pool, then Zn given with a carcinogen might prevent the occurrence of Cd on this pool and thus prevent cancer.

In previous studies it has been found that Cd or Hg

did not exert their toxic effects unless they were present in levels high enough to have exceeded the binding capacity of metallothionein; if the binding capacity was exceeded then they occurred on the high molecular weight protein pool with concurrent pathological effects (Winge et al., 1973; Brown and Parsons, 1978). The present study has demonstrated that Cd may occur on the high molecular weight protein pool before the binding capacity of metallothionein is exceeded if this pool is not Zn saturated. Since there is an apparent association between occurrence of Cd on this pool and cancer, and because of the possibility of other sublethal effects, we suggest that future environmental studies measure not only the tissue concentrations of Cd, but also its cytoplasmic distribution, especially in relation to the Zn status of the organism.

Summary

Ducks were collected from a marine environment known to be polluted with heavy metals. Gel elution profiles were determined for both liver and kidney tissue using Sephadex G-75 gel. Zinc increased linearly on the high molecular weight protein pool until this pool was apparently Zn saturated; saturation levels were 0.06 µmole cytoplasmic Zn/g kidney (wet weight) and 0.14 µmole cytoplasmic Zn/g liver (wet weight). Seventy-five percent of cytoplasmic Cd was found on the high molecular weight protein pool unless it was Zn saturated. If this pool was

Zn saturated, then excesses of Zn and up to 75% of cytoplasmic Cd appeared on metallothionein. Results are discussed in terms of a competition of Cd and Zn for Zn binding sites on metalloenzymes. Copper increased linearly on both the high molecular weight protein pool and metallothionein, with increases of Cu in tissue.

CHAPTER V Relationship Between Cytoplasmic Distribution of Mercury and Toxic Effects to Zooplankton and Chum Salmon (*Oncorhynchus keta*) Exposed to Mercury in a Controlled Ecosystem

Preface

This paper appeared in the Journal of the Fisheries Research Board of Canada, 1978, Volume 35, pages 880-884. The authors are D. A. Brown and T. R. Parsons. This paper clearly demonstrates that readily apparent pathological effects of Hg do not appear until the binding capacity of metallothionein is exceeded and mercury appears in the high molecular weight protein pool. Thus, the "spillover" theory described by Brown et al.(1977) is supported. This study, like Brown and Chatel (1978b), demonstrates interactions between a toxic trace element, and essential trace elements in the high molecular weight protein pool. Figures 12 and 13 did not appear in the published version of this paper as the editor considered that most readers wouldn't be able to understand them.

Introduction

Heavy metals such as Hg, Cd, Cu and Zn are toxic to organisms but prior exposure to these metals can result in acquired tolerance to increasing metal levels (Bryan and Hummerstone, 1971; Bremner, 1974). Tolerance occurs as a result of synthesis of the protein metallothionein (Leber, 1974; Piscator, 1964). Metallothionein is a low molecular

weight (10,000) protein high in sulfhydryl groups due to the presence of high levels of the amino acid cysteine (Margoshes and Vallee, 1957; Kagi and Vallee, 1960). The sulfhydryl groups can bind or chelate Hg, Cd, Ag, Cu, Sn and Zn (Winge et al., 1975; Sabbioni and Marafante, 1975) thus rendering them less toxic to the exposed organism (Piscator, 1964). Metallothionein is usually stored solubilized in liver and kidney cytoplasm in higher organisms.

One obvious means by which metallothionein-mediated tolerance to heavy metals might be acquired would be by synthesis of excesses of this protein upon initial exposure. This excess metallothionein would then be readily available to quickly bind and detoxify heavy metals upon subsequent exposure. However, it is known that metallothionein always exists in the saturated state in organisms, i.e. one metal ion for each three cysteine residues (Kagi and Vallee, 1960, 1961; Pulido et al., 1966; Buhler and Kagi, 1974; Bremner and Davies, 1975). Recently it has become apparent that metallothionein produced in response to Cd, Hg, Ag or Cu contains Zn in approximately half of its binding sites (Leber, 1974; Bremner and Davies, 1975; Winge et al., 1975). This Zn has a much lower affinity than Cd or Hg for metallothionein binding sites (Kagi and Vallee, 1960; Pulido et al., 1966). Upon a second exposure to Cd or Hg, these elements replace Zn on metallo-

thionein so that Zn is released into the cytoplasm rather than Cd or Hg (Leber, 1974). Zinc is relatively nontoxic compared with Cd or Hg (Bremner, 1974).

If an organism is exposed to a very high dose of Cd or Hg, then there may not be enough Zn-containing binding sites on metallothionein to detoxify all of the Hg or Cd. Further, de novo synthesis of metallothionein may be slower than the rate of influx of these metals. These conditions will result in a "spillover" of Hg or Cd to the enzyme pool. This "spillover" coincides with the appearance of pathological effects (Winge et al., 1973; Brown, 1977; Brown et al., 1977; Irons and Smith, 1976).

Mercury and cadmium exert their toxic effects by replacing Cu and Zn in metalloenzymes. This changes the three dimensional conformational shape of these enzymes so substrate molecules no longer fit binding sites (Friedberg, 1974), or splits enzymes into subunits (Gerhart and Schachman, 1965; Jovin et al., 1969) so that regulation of enzyme activity may be lost (White et al., 1968; Brown et al., 1977; Brown, 1977).

When Cd or Hg displace Cu or Zn from metallothionein, these latter metals may also appear in the high molecular weight protein (enzyme-containing) pool. However as Cu and Zn are natural components of many metalloenzymes (Friedberg, 1974), they are relatively nontoxic compared with Cd or Hg (Bremner, 1974).

This study reports findings on the relationship of previously reported growth reduction of fish and zooplankton exposed to Hg (Koeller and Wallace, 1977) with "spillover" of Hg from metallothionein to the enzyme pool.

Materials and Methods

The CEPEX (Controlled Ecosystem Pollution Experiment) facility consists of 1350 m^3 polyethylene controlled experimental ecosystems (CEEs) suspended in Saanich Inlet, Victoria (Menzel and Case, 1977). These simulate the natural water column, except for the effects of large lateral currents and sediment recycling. An experiment was conducted in the summer of 1976 to determine the impact of Hg on the marine ecosystem. On day 1 of this experiment a portion of the natural pelagic population of Saanich Inlet was captured into each of three CEEs. Chum fry were added on day 10. Also on day 10, mercury (in the form of $HgCl_2$) was introduced into two of the CEEs to produce final concentrations of 5 and 1 µg/L. Uniform distribution of mercury was aided by use of a diffuser ring lowered to 10 m below the surface (Topping and Windom, 1977). Mercury concentrations decreased throughout the experiment at a rate of approximately 2% per day. This decrease occurred due to attachment of Hg to settling particulate matter greater than 10,000 molecular weight. By day 72, Hg levels were approximately one-tenth those of initial concentrations (Grice et al., 1977).

On day 72, zooplankton were sampled using a 25 μm mesh net as described by Grice et al., (1977). The zooplankton were drained of excess water and frozen at -20°C. Fish were captured with a specially designed seine and similarly frozen until analyzed. Three fish from each exposure were randomly selected for analysis in the present study.

Fish livers or zooplankton were later thawed, homogenized in 0.9% NaCl and centrifuged at 27,000 x g. The supernatant was collected, the pellet rehomogenized in 0.9% NaCl and centrifuged at 27,000 x g. Supernatants were combined, heated to 70°C for 1 min (Webb, 1972a) and centrifuged at 27,000 x g. The supernatant of this final centrifugation was applied to a 100 x 1.6 cm Pharmacia column containing Pharmacia G-75 gel. Fractions were collected and measured at 250 and 280 nm on a Perkin Elmer spectrophotometer. The Cu and Zn contents of the fractions were measured on a Perkin Elmer 303 atomic absorption spectrophotometer using the flame method with deuterium arc background correction. Mercury was measured by a cold vapor method utilizing a 30 cm cell (Pharmacia UV Control Unit model 100, Pharmacia UV Optical Unit model 100). Analytical precision for Cu was $\bar{X} \pm 3.3\%$ (mean $\pm$ standard deviation, N=15), for Zn was $\bar{X} \pm 3.5\%$ (mean $\pm$ standard deviation, N=15), and for Hg was $\bar{X} \pm 6.0\%$ (mean $\pm$ standard deviation, N=15).

By comparing protein absorbance and metal profiles with those found in previous studies (Casterline and Yip, 1975; Leber, 1974; Olafson and Thompson, 1974; Marafante, 1976; Brown et al., 1977; Brown, 1977), fractions were identified as belonging to the high molecular weight protein (enzyme-containing) pool, metallothionein, or the low molecular weight cytoplasmic pool. The Cu and Zn levels in fractions comprising these cytoplasmic pools were added together, to give the total level of each metal in each pool. Mercury was determined on one composite sample of each pool as it was not detectable in individual fractions. Metal levels in each pool were then calculated per gram of tissue extracted.

Results

The most prominent change is the very large increase of Hg in the high molecular weight protein (enzyme-containing) pool of both fish and zooplankton exposed to 5 µg Hg/L (Tables 4 and 5). There appears to be a limit to Hg-thionein production reached at the 1 µg Hg/L exposure, as there is little increase in Hg-thionein from 1 to 5 µg Hg/L. When this limit of Hg-thionein production is apparently reached, excesses of Hg appear in the high molecular weight protein pool (Figures 12 and 13).

In fish liver cytoplasm, there are decreases of total Cu and Zn with increasing Hg exposure. These decreases appear to be a reflection of decreases of both Cu and Zn

Table 4. Distribution of Hg, Cu and Zn amongst cytoplasmic pools from liver or chum salmon exposed to various concentrations of Hg. MW: Molecular Weight. ND: Not Detectable

Hg exposure concentration (µg Hg/L)	Metal	Cytoplasmic total (µmole metal/g tissue, wet weight)	High MW protein pool (µmole metal/g tissue, wet weight)	Metallothionein (µmole metal/g tissue, wet weight)	Low MW cytoplasmic pool (µmole metal/g tissue, wet weight)
Control	Hg	0.61×10^{-3}	0.11×10^{-3}	0.29×10^{-3}	0.22×10^{-3}
	Cu	1.832	0.592	0.532	0.708
	Zn	0.164	0.111	ND	0.053
1	Hg	0.87×10^{-3}	0.23×10^{-3}	0.35×10^{-3}	0.29×10^{-3}
	Cu	1.558	0.402	0.424	0.733
	Zn	0.104	0.071	ND	0.033
5	Hg	$4.77\ 10^{-3}$	4.04×10^{-3}	0.38×10^{-3}	0.35×10^{-3}
	Cu	1.445	0.353	0.347	0.745
	Zn	0.114	0.075	ND	0.039

Table 5. Distribution of Hg, Cu and Zn amongst cytoplasmic pools from tissue of zooplankton exposed to various concentrations of Hg. MW: Molecular Weight. ND: Not Detectable.

Hg exposure	Metal	Cytoplasmic total (μmole metal/g tissue, wet weight)	High MW protein pool (μmole metal/g tissue, wet weight)	Metallothionein (μmole metal/g tissue, wet weight)	Low MW cytoplasmic pool (μmole metal/g tissue, wet weight)
Control	Hg	0.40×10^{-4}	0.32×10^{-4}	0.04×10^{-4}	0.04×10^{-4}
	Cu	0.359	0.041	0.033	0.286
	Zn	0.299	0.012	ND	0.288
1	Hg	0.96×10^{-4}	0.40×10^{-4}	0.27×10^{-4}	0.28×10^{-4}
	Cu	0.352	0.046	0.039	0.267
	Zn	0.283	0.016	0.005	0.262
5	Hg	1.93×10^{-4}	1.17×10^{-4}	0.26×10^{-4}	0.50×10^{-4}
	Cu	0.326	0.031	0.045	0.249
	Zn	0.212	0.011	0.026	0.175

Figure 12. Gel elution profiles from control chum salmon (A), chum salmon exposed to 1 µg Hg/L (B), and those exposed to 5 µg Hg/L (C) for 72 days. ———Hg; o———o Cu; ●- - - -● Zn. I: high molecular weight protein pool; II: metallothionein; III: low molecular weight cytoplasmic pool.

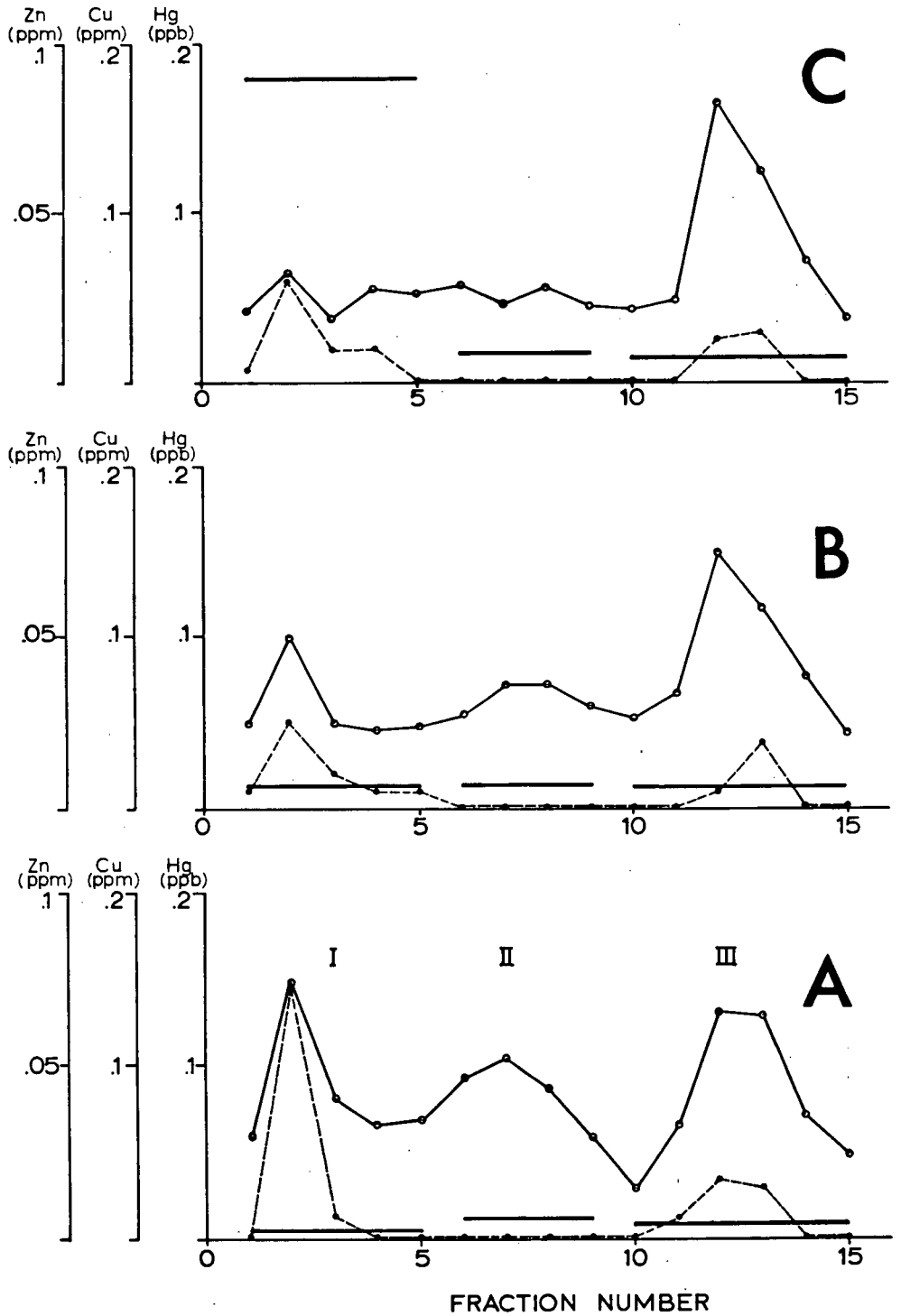
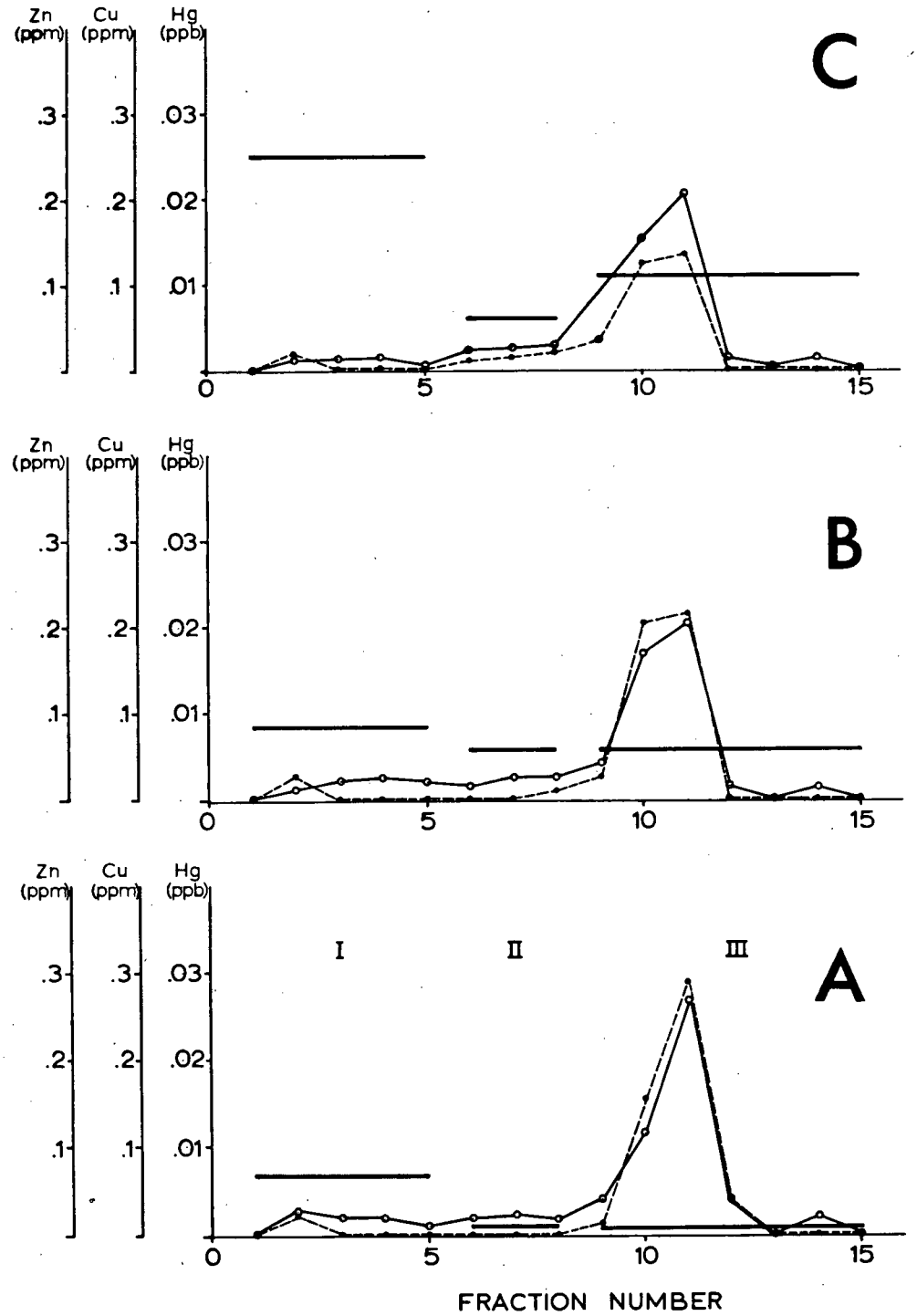

Zn (ppm)
Cu (ppm)
Hg (ppb)
.1
.05
.2
.1
.2
.1
C
0
5
10
15
B
I
II
III
A
FRACTION NUMBER

Figure 13. Gel elution profiles from control zooplankton (A), zooplankton exposed to 1 µg Hg/L (B), and those exposed to 5 µg Hg/L for 72 days. ———Hg; o———oCu; ●------●Zn. I: high molecular weight protein pool; II: metallothionein; III: low molecular weight cytoplasmic pool.


Zn (ppm)
Cu (ppm)
Hg (ppb)
C
B
A
I
II
III
.3
.2
.1
.03
.02
.01
0
5
10
15
FRACTION NUMBER

in the enzyme-containing pool, of Cu in metallothionein and of Zn in the low molecular weight cytoplasmic pool (Table 4, Figure 12).

In cytoplasm of zooplankton exposed to 5 µg Hg/L, Cu appears to decrease in the enzyme-containing pool, increase in metallothionein, and decrease in the low molecular weight cytoplasmic pool, resulting in a net decrease in cytoplasmic Cu (Table 5). Copper levels are relatively unchanged by exposure to 1 µg Hg/L. At both mercury exposures, Zn in zooplankton cytoplasm appears to be unchanged in the enzyme-containing pool, while increased in metallothionein, and decreased in the low molecular weight cytoplasmic pool. Thus, there is a net decrease in cytoplasmic Zn (Table 5, Figure 13).

Discussion

Results of this study and those of Koeller and Wallace (1977) appear to support the "spillover" theory (Winge et al., 1974; Brown et al., 1977). This theory suggests that intracellular toxic effects of Cd and Hg will not occur unless and until these metals appear in the enzyme-containing pool. These metals will spillover from metallothionein to the enzyme-containing pool when the binding capacity of metallothionein is exceeded. In the present study, it appears that the binding capacity of metallothionein was surpassed in the 5 µg Hg/L exposure. Mercury-thionein increased from the control to the 1 µg Hg/L

exposure, but increased very little from 1 to 5 µg Hg/L. Rather, at 5 µg Hg/L, excess Hg occurred in the enzyme-containing pool.

Koeller and Wallace (1977) found both fish and zooplankton growth to be decreased in these same 5 µg Hg/L CEEs. On day 72, fish exposed to 5 µg Hg/L weighed about 6 grams while controls or 1 µg Hg/L exposed fish weighed 12.5 grams. Zooplankton counts in the 5 µg Hg/L CEEs were one-fourth those of control or 1 µg Hg/L exposed zooplankton counts on day 72. Thus, at that concentration of Hg exposure where there was a dramatic increase of Hg in the enzyme-containing pool, there was a similar dramatic decrease in growth. At least in part, therefore, reduced growth may be a result of inhibition or alteration of enzyme functioning by Hg (Friedberg, 1974; Bremner, 1974; Brown et al., 1977; Brown, 1977).

Most likely toxic effects result from a change in metalloenzyme tertiary or quaternary structure when Cu or Zn are displaced by Hg in these enzymes (Friedberg, 1974; Bremner, 1974). In the present study, with increases of Hg in the enzyme-containing pool, there were decreases of Cu and Zn. Since Hg exposure results in a reduction of Cu and Zn in the enzyme-containing pool, Hg exposure might result in metal deficiency effects. Thus, growth decreases could occur independent of the other enzymatic inhibitory toxic effects of Hg, due simply to decreases of Cu and Zn

(Underwood, 1971).

While intracellular displacement of Cu and Zn by Hg partially explains cellular losses of these metals, exclusion of uptake of these metals is also a possibility. As explained by Sugawara and Sugawara (1975) and Stonard and Webb (1976), gastrointestinal uptake of Hg, Cd, Cu and Zn occurs via duodenal mucosal metallothionein. When exposed to high levels of Cd or Hg, this metallothionein is saturated by Cd or Hg. It then cannot bind Cu and Zn, and therefore they are not absorbed into the bloodstream from the intestine (Stonard and Webb, 1976).

Other workers (Fowler et al., 1975; Overnell, 1975) found that Hg exposure results in a loss of certain cellular constituents including potassium. Overnell (1975) explains the losses as leakage due to membrane damage caused by Hg. DeFilippis and Pallaghy (1976) found that Chlorella exposed to Hg had decreased uptake of ^{65}Zn. They conclude that this decrease in uptake is due to an inhibition of the temperature sensitive component of Zn uptake and to a reduction in the number of exchange sites available for Zn in the cell well. They attributed increased tolerance of Chlorella to trace elements as characterized by development of this exclusion mechanism.

In fish liver, as Hg increased in metallothionein, there was a concurrent decrease of Cu in metallothionein. This is consistent with Leber's (1974) theory suggesting

that metallothionein results in detoxification, in part, by a displacement of less toxic metals from metallothionein by more toxic ones. Leber found that Cd displaced Zn from metallothionein. In the present study, Hg appears to have displaced Cu. Zinc in fish liver metallothionein was not detectable by methods employed here.

In zooplankton, both Cu and Zn increased in metallothionein with increases of Hg in metallothionein, even at the highest level where spillover occurred. Therefore, in zooplankton, Cu and Zn were not displaced by increasing levels of Hg in metallothionein, as occurred in fish. Discrepancies between fish and zooplankton may be due to different tissue types or to lesser Hg levels in zooplankton. Zooplankton cytoplasmic Hg levels at 5 μg Hg/L exposure were only one-fifth those of fish liver cytoplasmic Hg levels at 1 μg Hg/L exposure and one-twenty-fifth those of fish at 5 μg Hg/L exposure (Tables 4 and 5). In fish liver cytoplasm at 5 μg Hg/L exposure, 85% of Hg is found in the enzyme-containing pool, while in zooplankton cytoplasm only 60% of Hg is found in the enzyme-containing pool, indicating much less spillover in zooplankton. Therefore, the lesser levels of Hg in zooplankton would probably present less of a challenge to Cu and Zn in metallothionein.

It is evident from this and previous studies that toxic effects of Cd or Hg do not occur until "spillover" occurs. Whether or not spillover occurs will depend upon whether or not metallothionein synthesis can keep pace

with metal inflow. Thus, exposure to low levels of Hg over a long period of time might result in very high tissue levels of Hg, but which is all bound to metallothionein and hence detoxified. Much shorter exposure periods but with much higher levels of Hg exposure might result in lower tissue levels of Hg, but with most of the Hg appearing in the enzyme-containing pool with concurrent toxic effects. Thus, the factor critical to an organism's survival may not be its tissue levels of Cd or Hg, but rather, whether these occur on the enzyme-containing pool or as metallothionein. This should depend upon both the level and rate of exposure and uptake.

Summary

Fish and zooplankton were simultaneously exposed to trace, 1 and 5 µg Hg/L. In both fish and zooplankton, pathological effects appeared to coincide with saturation of metallothionein and "spillover" of Hg into the high molecular weight protein (enzyme-containing) pool. Coincidental with increases of Hg in tissue were decreases of Cu and Zn. Pathological effects of Hg are explained in terms of tertiary and quaternary structural changes in metalloenzymes resulting from replacement of Cu and Zn by Hg. Decreases in tissue Cu and Zn with increasing Hg concentration are discussed as both an intracellular displacement of Cu and Zn by Hg and as duodenal and cellular exclusion processes.

CHAPTER VI The Effect of Mercury Exposure on the Cytoplasmic Distribution of Mercury, Copper and Zinc in *Skeletonema costatum*

Preface

This is my version of a paper authored by L. Cloutier and D. A. Brown (1978), and accepted for publication in the Journal of Experimental Marine Biology and Ecology. This paper again demonstrates that toxic effects of Hg do not occur until it appears in the high molecular weight protein pool. Interactions between Hg and Zn in the high molecular weight protein pool in phytoplankton, are similar to those in fish and zooplankton, but different than those between Hg and Cu (Brown and Parsons, 1978).

Introduction

It is known that exposure of phytoplankton to trace elements results in increased tolerance to subsequent exposures (De Filippis and Pallaghy, 1976), but it is not known whether or not metallothionein plays a role in this process. Metallothionein is a low molecular weight (10,000) protein which can bind and thus render nontoxic, heavy metals such as Cd and Hg. It also stores Cu and Zn when they occur in excess of levels required for metalloenzymes (Bremner and Davies, 1974; Brown and Chatel, 1978a). Toxic effects of Cd and Hg are found when the rate of inflow of these metals exceeds the rate of metallothionein synthesis (Winge et al., 1973; Brown and Parsons,

1978). At this point, they occur on the high molecular weight protein pool and exert toxic effects by replacing Cu and Zn in metalloenzymes (Brown, 1977; Brown and Parsons, 1978).

Metallothionein has been ubiquitously found in land and marine animals (e.g., Buhler and Kagi, 1974; Piscator, 1964; Olafson and Thompson, 1974). However, the presence of metallothionein in phytoplankton remains to be clarified. MacLean et al. (1972) found a metallothionein like fraction binding Cd and Zn in blue green algae exposed to radioactive Cd. In the present study, phytoplankton are exposed to a range of mercury concentrations in order to see if a metallothionein like protein is induced. Also, the effects of Hg exposure on the cytoplasmic levels and distributions of Cu and Zn are examined.

Materials and Methods

The coastal pennate diatom Skeletomema costatum was obtained from the Northeast Pacific Culture Collection, Institute of Oceanography, University of British Columbia. At the start of the experiment, portions of stock culture were transferred to artificial seawater enriched with modified Guillard and Ryther's medium. The enrichment consisted of 8.83×10^{-4} M $NaNO_3$, 5.3×10^{-5} M Na_2SiO_3, 3.68×10^{-5} M $K_2H_2PO_4$, 1.96×10^{-8} M $CuSO_4$, 3.83×10^{-5} M $ZnSO_4$, 2.2×10^{-8} M $CoCl_2 \cdot 6H_2O$, 4.55×10^{-9} M $MnCl_2$m 1.3×10^{-8} M $Na_2MoO_4 \cdot 4H_2O$, 5.83 µM Fe and 5.85 µM NaEDTA.

Vitamin B_{12} was added at 25% full strength.

The cultures were grown batchwise in borosilicate, 6 liter, flat bottom boiling flasks, stoppered by an aluminum foil sheet, in a water bath maintained at 17.0 $\pm$ 0.5°C. The cultures were continuously stirred with a teflon covered magnetic stirrer at 120 rpm. Cultures were grown under continuous illumination. Growth was measured by monitoring fluorescence with a Turner Model III fluorometer.

The experiment was started with a very clean (few bacteria) unialgal log phase S. costatum culture. Two cultures were grown for approximately 70 hours before addition of Hg (as $HgCl_2$) while two other cultures were grown in 0.5 µg Hg/L. At 70 hours, one culture of each of these subsets was perturbed with 1.5 µg Hg/L. Another culture was exposed to 0.1 µg Hg/L for the duration of the experiment. Cells were harvested at 116 hours by centrifugation, in 50 ml polycarbonate test tubes, in a 6°C centrifuge, for 6 minutes, at 650 x g. The supernatants were discarded and the pellets frozen until analyzed.

One gram of pellet was homogenized in 3 ml of 0.9% NaCl for 5 minutes using a TRI-R STIR-R model 563C variable speed laboratory homogenizer set at 4.5. Homogenates were centrifuged at 27,000 x g for 10 minutes using a Sorval Superspeed RC2-B automatic refrigerated centrifuge. Supernatant was fractionated on a Sephadex G-75 (Pharmacia) column (0.9 x 60 cm) with 0.01 M NH_4HCO_3 buffer as eluent.

Two ml fractions were collected.

Ultraviolet absorbance (280 nm) spectra were done with a Perkin Elmer model 124D double beam spectrophotometer to establish the position of peaks. Peaks were identified as being in the position of the high molecular weight protein (enzyme-containing) peak, metallothionein, and the low molecular weight cytoplasmic pool (Brown et al., 1977). Copper and Zn levels were determined in each fraction using a Perkin Elmer model 303 flame atomic absorption spectrophotometer. Mercury was measured by a cold vapor method on the combined fractions of each peak, utilizing a 30 cm cell (Pharmacia UV Control Unit model 100, Pharmacia UV Optical Unit model 100).

Results

At the beginning of the experiment, cultures grew exponentially (Figure 14). Growth rate was deaccelerated in the presence of 0.5 µg Hg/L (Figure 14). The cultures initially exposed to 0.5 µg Hg/L had growth rate of 1.31 ± 0.27 divisions/day while those not exposed to Hg until 70 hours maintained a growth rate of 1.92 ± 0.17 divisions/day. The cellular density was reduced by 25% after 24 hours, and by 55% after 46 hours exposure to 0.5 µg Hg/L.

A typical gel elution profile from S. costatum exposed to Hg is shown in Figure 15. This profile is characterized by relatively high levels of Cu and Zn in the low molecular weight cytoplasmic pool (III), much Zn but lesser amounts

Figure 14. *In vivo* fluorescence of batch cultures of *Skeletonema costatum* exposed to Hg for 116 hours. —O—control; —●—control perturbed with 1.5 µg Hg/L; —△—0.5 µg Hg/L; —▲— 0.5 µg Hg/L perturbed with 1.5 µg Hg/L. ↓ time of perturbation with 1.5 µg Hg/L approximately 70 hours after the start of the experiment.

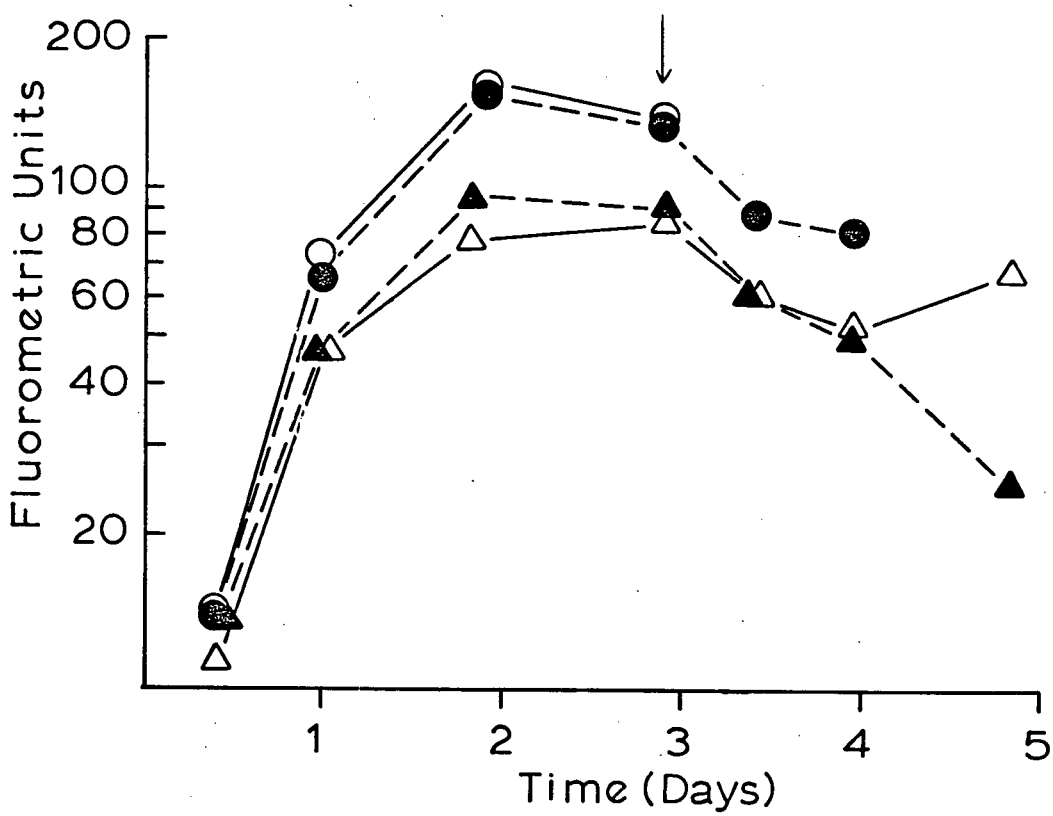
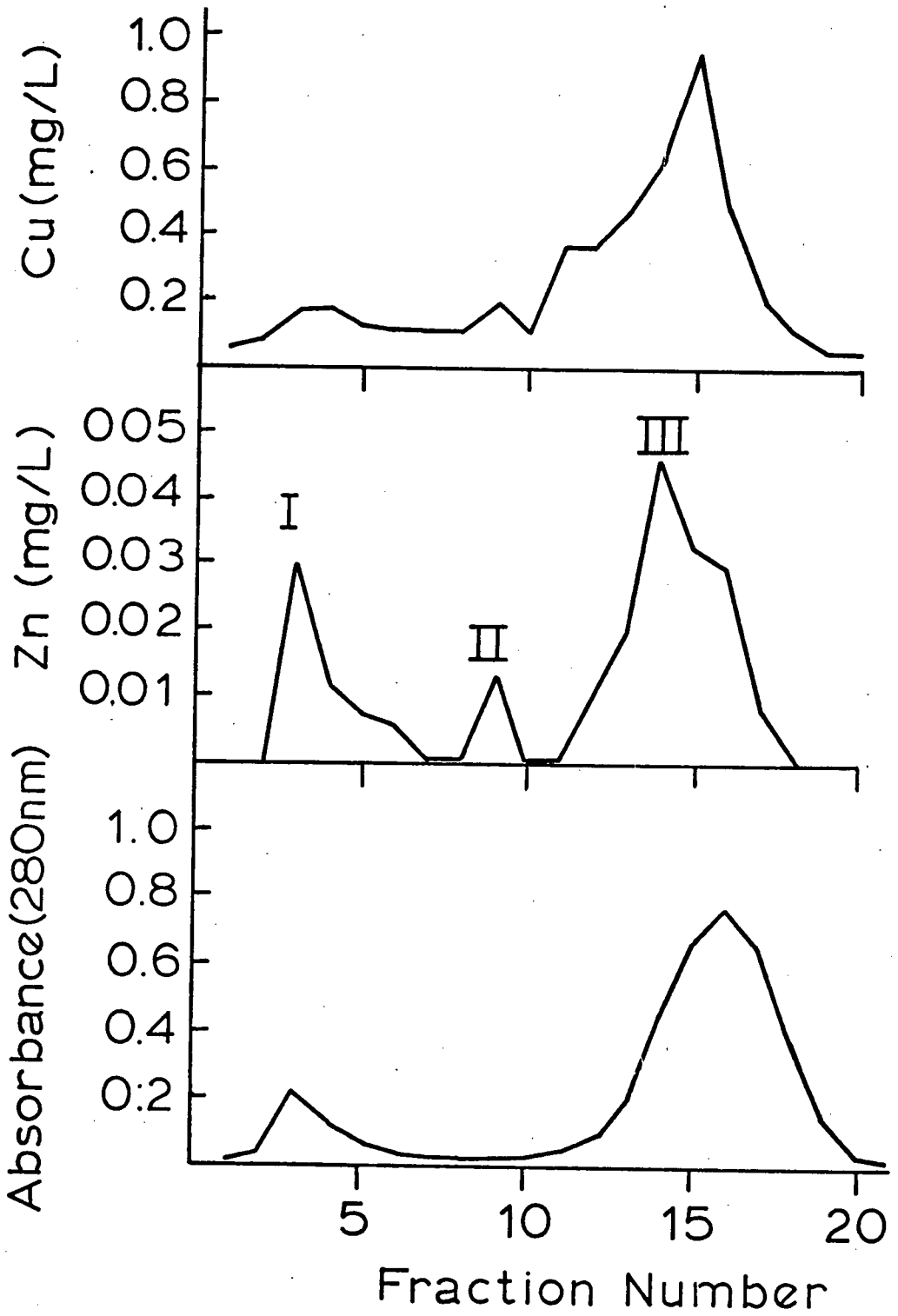

200
100
80
60
40
20
Fluorometric Units
1
2
3
4
5
Time (Days)

Figure 15. Gel elution profile of Skeletonema costatum exposed to 0.5 µg Hg/L for 116 hours. I: high molecular weight protein pool; II: metallothionein; III: low molecular weight cytoplasmic pool.


Cu (mg/L)
1.0
0.8
0.6
0.4
0.2
Zn (mg/L)
0.05
0.04
0.03
0.02
0.01
I
II
III
Absorbance (280nm)
1.0
0.8
0.6
0.4
0.2
5
10
15
20
Fraction Number

of Cu in the high molecular weight protein pool (I), and rather small Cu and Zn peaks in those fractions which correspond to the position of metallothionein (II) in previous studies (Brown et al., 1977). Gel elution profiles from other exposures were similar in shape to Figure 15, but varied in the levels of metals in each of the peaks (Table 6).

Exposure to Hg resulted in decreases of Zn in the high molecular weight protein pool and in the low molecular weight cytoplasmic pool (Table 6). Zinc levels were increased in the metallothionein like fractions in those S. costatum exposed to the higher Hg concentrations. Total Zn levels were decreased in all Hg exposures.

Copper levels were increased in the high molecular weight protein pool of cultures exposed to Hg (Table 6). Levels of Cu in the metallothionein like peak were increased slightly in those phytoplankton exposed to 1.5 µg Hg/L. Total Cu levels appeared to be increased in those phytoplankton exposed to 1.5 µg Hg/L.

Mercury was detectable, by methods employed in this study, only in those phytoplankton exposed to 1.5 µg Hg/L. Preexposure to 0.5 µg Hg/L before addition of 1.5 µg Hg/L resulted in a decrease in uptake of Hg compared with those phytoplankton not preexposed to Hg before addition of 1.5 µg Hg/L. All of the detectable Hg in those phytoplankton exposed to 0.5 µg Hg/L, and then 1.5 µg Hg/L,

Table 6. The distribution of Zn, Cu and Hg amongst cytoplasmic pools from phytoplankton exposed to mercury in batch cultures. Data are compilations of metal levels from profiles such as Figure 15, in μmole/g tissue (wet weight). MW: molecular weight. ND: not detectable.

	Zn				Cu				Hg			
	Total	High MW pool	Metallo-thionein	Low MW pool	Total	High MW pool	Metallo-thionein	Low MW pool	Total	High MW pool	Metallo-thionein	Low MW pool
Control	.0231	.0083	ND	.0148	.270	.032	.035	.203	ND	ND	ND	ND
0.1 μg Hg/L	.0136	.0065	ND	.0071	.275	.044	.034	.197	ND	ND	ND	ND
0.5 μg Hg/L	.0115	.0035	.0009	.0071	.253	.041	.032	.180	ND	ND	ND	ND
1.5 μg Hg/L	.0144	.0046	.0011	.0087	.328	.042	.040	.246	.0138	.0117	.0021	ND
0.5 + 1.5 μg Hg/L	.0130	.0035	.0017	.0078	.326	.062	.041	.223	.0034	.0034	ND	ND

appeared in the high molecular weight protein pool. In those phytoplankton exposed only to 1.5 μg Hg/L, most Hg appeared in the high molecular weight protein pool, but some appeared in those fractions corresponding to the position of metallothionein in previous studies (Brown et al., 1977).

Discussion

Results from this study appear to indicate that metallothionein doesn't play a major role in the detoxification of Hg in phytoplankton. Most detectable Hg appeared in the high molecular weight protein pool rather than in those fractions corresponding to the usual position of metallothionein (Brown et al., 1977). Only in those phytoplankton containing the highest Hg levels, was a Hg-thionein like peak detectable.

The lack of Hg-thionein at most exposure levels may be due to poor detection limits in the present study, i.e., it may be that metallothionein was Hg saturated at levels too low to be determined. There is some evidence that a metallothionein like fraction was induced with the higher Hg exposures as with these exposures, Zn increased in those fractions corresponding to the usual position of metallothionein. It has been suggested previously that Cd and Hg induce metallothionein with Zn appearing in approximately half of the binding sites (Winge et al., 1975). Upon a second exposure, the

nontoxic Zn is displaced by more toxic Cd or Hg (Leber, 1974; Brown and Parsons, 1978), so that these are detoxified. Zinc is released into the cytoplasm where it is relatively harmless as it is a component of many metalloenzymes. In the present study, Cu like Zn, appears to be increased on those fractions corresponding to metallothionein, at the two highest Hg exposures.

In animals, most Hg is bound to metallothionein until the binding capacity of metallothionein is exceeded. When this occurs, excesses of Hg then appear in the high molecular weight protein pool, with simultaneously occurring pathological effects (Brown and Parsons, 1978). Pathological effects are thought to result from Hg displacing Cu and Zn from metalloenzymes in the high molecular weight protein pool. Metalloenzymes then become nonfunctional due to conformational changes (Bremner, 1974; Friedberg, 1974). In the present study, growth suppression occurred in Hg exposures where Hg appeared in the high molecular weight protein pool. Similar growth reductions were found in Hg-exposed fish and zooplankton when Hg appeared in the high molecular weight protein pool (Brown and Parsons, 1978).

In the present study, preexposure of phytoplankton to Hg resulted in less uptake of Hg during subsequent Hg exposures. Preexposure to 0.5 µg Hg/L with subsequent exposure to 1.5 µg Hg/L resulted in only 25% of the uptake of Hg found upon exposure to 1.5 µg Hg/L without pre-

exposure. De Filippis and Pallaghy (1976) found that exposure of Chlorella to Hg resulted in an inhibition of the temperature sensitive component of Zn uptake and a reduction in the number of Zn exchange sites in the cell wall. They attributed increased tolerance of Chlorella to trace elements as characterized by development of this exclusion mechanism. Results from the present study support the idea that tolerance of S. costatum to Hg might also be mediated by an exclusion mechanism.

There appears to be an increase of Cu in the high molecular weight protein pool with exposure to Hg. This increase of Cu is most evident with preexposure to 0.5 µg Hg/L followed by exposure to 1.5 µg Hg/L. Perhaps phytoplankton increase high molecular weight protein Cu levels when exposed to Hg, so that competition of Cu and Hg for Cu binding sites on enzymes favors Cu uptake. With no preexposure, Cu levels were lower on the high molecular weight protein pool and Hg levels higher.

Zinc was decreased on both the high and low molecular weight pools with increasing Hg exposure. This effect was lowest at the lower Hg exposure on the high molecular weight protein pool, but was similar on the low molecular weight cytoplasmic pool at all exposure levels. Perhaps Hg displaces Zn from the high molecular weight protein pool as part of its toxic mode of action. Zinc was previously found to be reduced on the high molecular weight

protein pool of fish exposed to Hg (Brown and Parsons, 1978). As the low molecular weight cytoplasmic pool is Zn depleted at all Hg exposure levels while the high molecular weight protein pool is not, it may be that the low molecular weight protein pool acts as a reservoir of Zn for enzymes in the high molecular weight protein pool.

Metallothionein does not appear to be a major storage molecule for Cu and Zn as it is in higher organisms. In ducks, when the high molecular weight protein (enzyme-containing) pool was apparently Zn saturated, excesses of Cu and Zn occurred in metallothionein (Brown and Chatel, 1978a). In phytoplankton, excesses of Cu and Zn appear to be stored in the low molecular weight cytoplasmic pool. This pool contains amino acids, nucleic acids and other cellular building blocks. In animals, only a relatively small portion of metals are found in the low molecular weight cytoplasmic pool. The presence of high levels of metals in the low molecular weight cytoplasmic pool in phytoplankton indicates the possible presence of a storage/detoxification substance other than metallothionein. Perhaps the substance consists of the iron-binding hydroxamic acids or siderochromes, identified sporadically in algae and some higher plants (Zahner et al., 1962); their function in these life forms has not been defined. Siderochromes can bind Cu and Zn, but much less strongly than Fe (Zahner et al., 1962). Elucidation of the structure and identity

of the low molecular weight metal-binding moeity should be a valuable contribution to developing an understanding of the metabolism of trace elements in phytoplankton.

Summary

Skeletonema costatum were grown in artificial seawater, with and without exposure to Hg. Mercury exposure levels were 0.1 and 0.5 µg Hg/L for 116 hours, or no mercury for 70 hours followed by 1.5 µg Hg/L for 46 hours, and 0.5 µg Hg/L for 70 hours followed by 1.5 µg Hg/L for 46 hours. Growth rates were decreased in phytoplankton exposed to Hg levels equal to and greater than 0.5 µg Hg/L.

Cells were harvested, homogenized, centrifuged and the supernatant passed through a column packed with Sephadex G-75 gel. The highest Hg levels accumulated in phytoplankton exposed to 1.5 µg Hg/L after no preexposure. Most of this Hg accumulated in the high molecular weight protein pool, with lesser amounts in fractions corresponding to the position of metallothionein in previous studies. In phytoplankton preexposed to 0.5 µg Hg/L followed by exposure to 1.5 µg Hg/L, only 25% of the Hg accumulated compared with exposure to 1.5 µg Hg/L with no preexposure; all of the Hg appeared in the high molecular weight protein pool. Tolerance to Hg is discussed as being possibly characterized by a metal exclusion mechanism.

Mercury exposure resulted in decreases of Zn in the high molecular weight protein and the low molecular weight

cytoplasmic pools, but small increases of Zn in those fractions corresponding to metallothionein in previous studies. Growth decreases are discussed as possibly due to toxic effects resulting from displacement of Zn from Zn-containing metalloenzymes. As most Cu and Zn occurred in the low molecular weight cytoplasmic pool, the possibility of a storage/detoxification substance other than metallothionein is discussed.

CHAPTER VII Increases of Cd and the Cd:Zn Ratio in the High Molecular Weight Protein Pool from Apparently Normal Liver of Tumor-Bearing Flounders (*Parophrys vetulus*)

Preface

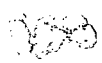
This paper appeared in Marine Biology, 1977, Volume 44, pages 203-209, and was authored by D. A. Brown. This study examined whether Cd and the Cd:Zn ratio were increased in cancerous fish, as in cancerous humans (Morgan, 1970, 1971); in addition the cytoplasmic distribution of these elements was determined. If these changes were only on metallothionein, and not the high molecular weight protein pool, then they wouldn't influence the biological activity of the cell. If these changes occurred in the high molecular weight protein pool, then the increased Cd:Zn could mean that Cd was available to interfer with the Zn-containing enzymes involved in cell division processes.

Introduction

It has been well established that heavy metal levels are altered in both tumorous and apparently normal tissues of tumor-bearing animals (White, 1921; Olsen et al., 1954). Elevated liver Cd, Cu and Zn and kidney Cd and Zn have been found in many cancer patients (Olson et al., 1954, 1958; Tietz et al., 1957; Sandberg et al., 1958; Morgan, 1971; Wright and Dormandy, 1972; Kew and Mallett, 1974). Morgan (1970) found increased Cd:Zn in liver, kidney and

serum of lung cancer patients. Halsted and Smith (1970) found plasma Zn to decrease in malignant diseases, particularly metastatic carcinoma. Vallee (1976) reported decreased Zn in white blood cells of leukemia patients. Arnold and Sasse (1961) described decreased Zn in dimethylaminoazobenzene-induced tumors of rats. Olson et al., (1954), 1958) reported decreased Zn in tumor tissue of human cancer patients with increased Zn in uninvolved liver of these patients. Gorodiskii et al., (1956; in Furst and Haro, 1969) noted increases in the Cd content of tumors induced by nonmetal carcinogens. Tietz et al., (1957) concluded that changes in metal levels occur before tumor formation, while Olson et al., (1958) reported no increase in liver Zn in rats bearing transferred subcutaneous tumors, suggesting that alterations in metal levels are causal and do not occur as a result of the carcinoma.

This report studies the distribution of Cd, Cu and Zn amongst the high molecular weight protein (enzyme-containing) pool (Brown et al., 1977), the metallothionein pool and the low molecular weight cytoplasmic pool of livers of tumor- and nontumor-bearing flounders (Parophrys vetulus). The methods used were specific for the study of metallothionein (Webb, 1972a; Olafson and Thompson, 1974). Metallothioneins are known to detoxify and store excesses of heavy metals in liver tissue (Piscator, 1964; Leber,



1974). As excesses of heavy metal occur in liver during carcinogenesis, it was thought that metallothionein might somehow be involved in the carcinogenic process.

Materials and Methods.

Liver samples were obtained from the Cancer Research Center of British Columbia, University of British Columbia Campus. These livers had been removed from nontumor- and tumor-bearing flounders (Parophrys vetulus) collected from waters around Crescent Beach, British Columbia, Canada; Gibsons Landing, British Columbia; and Bellingham, Washington, USA. Fish were 80 to 120 mm in length. Each liver sample weighed approximately 0.05 g.

Liver samples were homogenized and suspended in approximately 2.5 ml of 50 mM Tris chloride buffer (pH 7.5) with 3 mM of $MgCl_2$. Each sample was thoroughly rehomogenized and centrifuged at 27,000 x g for 10 min. Supernatants were collected and heated to 70°C for 1 min (Webb, 1972a; Cherian, 1974). Samples were then recentrifuged for 10 min at 27,000 x g and the supernatant applied to a Pharmacia K9/60 column filled with Sephadex G-75 gel. Filtrate was collected as 2.0 ml fractions. Absorbance was read at 250 and 280 mμ on a Perkin Elmer Coleman 124D spectrophotometer. Cd and Zn levels were then determined by graphite furnace on each fraction on a Perkin Elmer 403 atomic absorption spectrophotometer. Cu was determined by the flame method with a Perkin Elmer 303 atomic

absorption spectrophotometer. Deuterium background correction was used for both graphite furnace and flame determinations. Analytical precision for Cd was $\bar{X}$ + 12.6% (mean ± standard deviation, N=12), for Zn was $\bar{X}$ ± 15.5% (mean ± standard deviation, N=13) and for Cu was $\bar{X}$ ± 3.3% (mean ± standard deviation, N=15).

Results

Composite gel elution profiles, each of 3 nontumor or tumor (skin tumor) -bearing flounders (Parophrys vetulus), are presented in Figures 16 and 17. The individual metal levels in each tube of each of the three main peaks have been added together and a summary of the levels of metals in all peaks is presented in Table 7. Each peak has been identified according to its similarity of position to peaks found in previous studies (Leber, 1974; Olafson and Thompson, 1974; Casterline and Yip, 1975; Marafante, 1976). As can be seen from Table 7, the most striking change is the increase of Cd on the high molecular weight protein (enzyme-containing) peak; a 3.3-fold increase in tumor-bearing fish relative to nontumor-bearing fish. There are also increases of Cd on the metallothionein and the low molecular weight cytoplasmic pool peaks. Overall, there is a 2-fold increase of Cd in tissue homogenate supernatant of the tumor-bearing fish.

Zinc, like Cd, is increased on all three peaks, but most markedly on the high molecular weight protein

Figure 16. Parophrys vetulus. Composite of gel elution profiles from liver of 3 tumor-bearing flounders. I: high molecular weight protein pool; II: metallothionein; III: low molecular weight cytoplasmic pool.

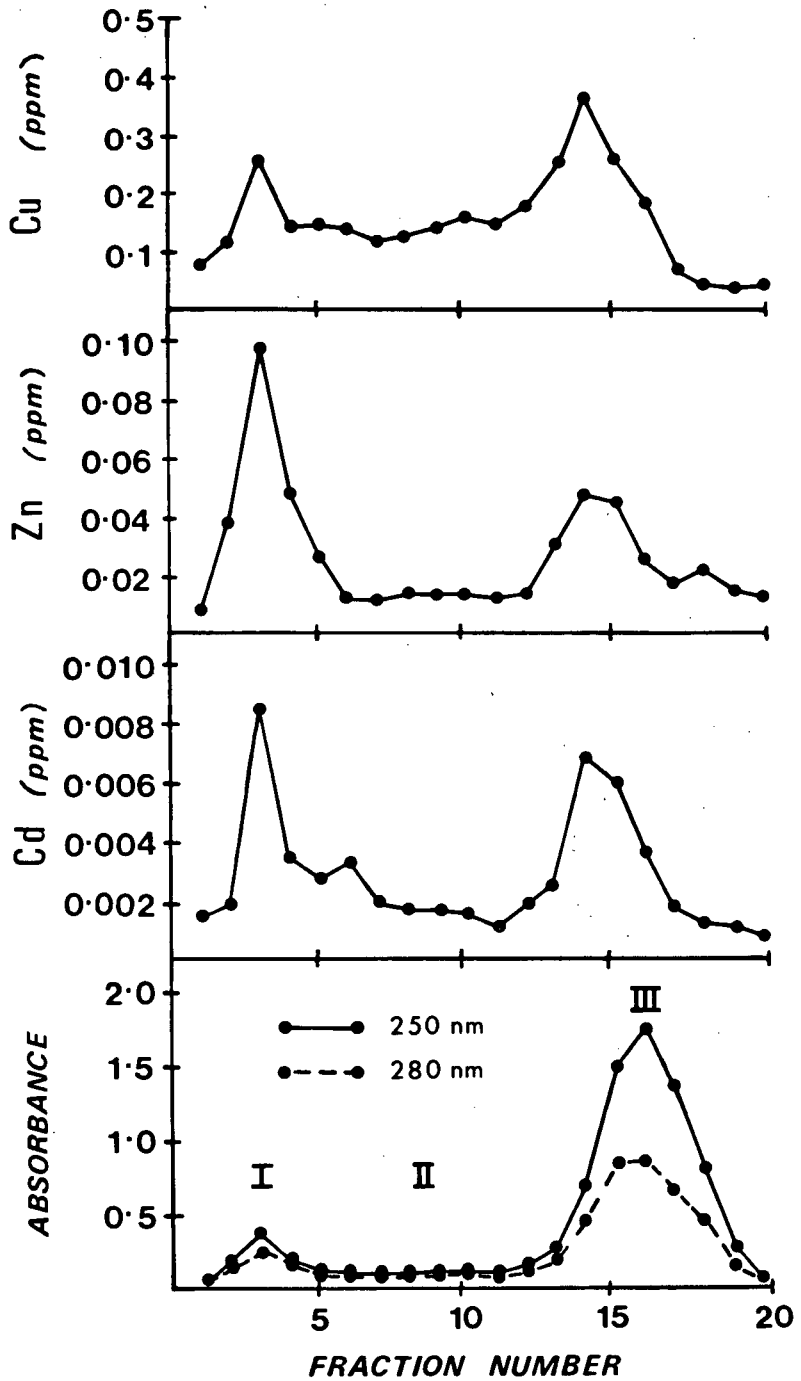

Cu (ppm)
0·5
0·4
0·3
0·2
0·1
Zn (ppm)
0·10
0·08
0·06
0·04
0·02
Cd (ppm)
0·010
0·008
0·006
0·004
0·002
ABSORBANCE
2·0
1·5
1·0
0·5
250 nm
280 nm
I
II
III
5
10
15
20
FRACTION NUMBER

Figure 17. Parophrys vetulus. Composite of gel elution profiles from liver of nontumor-bearing flounders. I: high molecular weight protein pool; II: metallothionein; III: low molecular weight cytoplasmic pool.

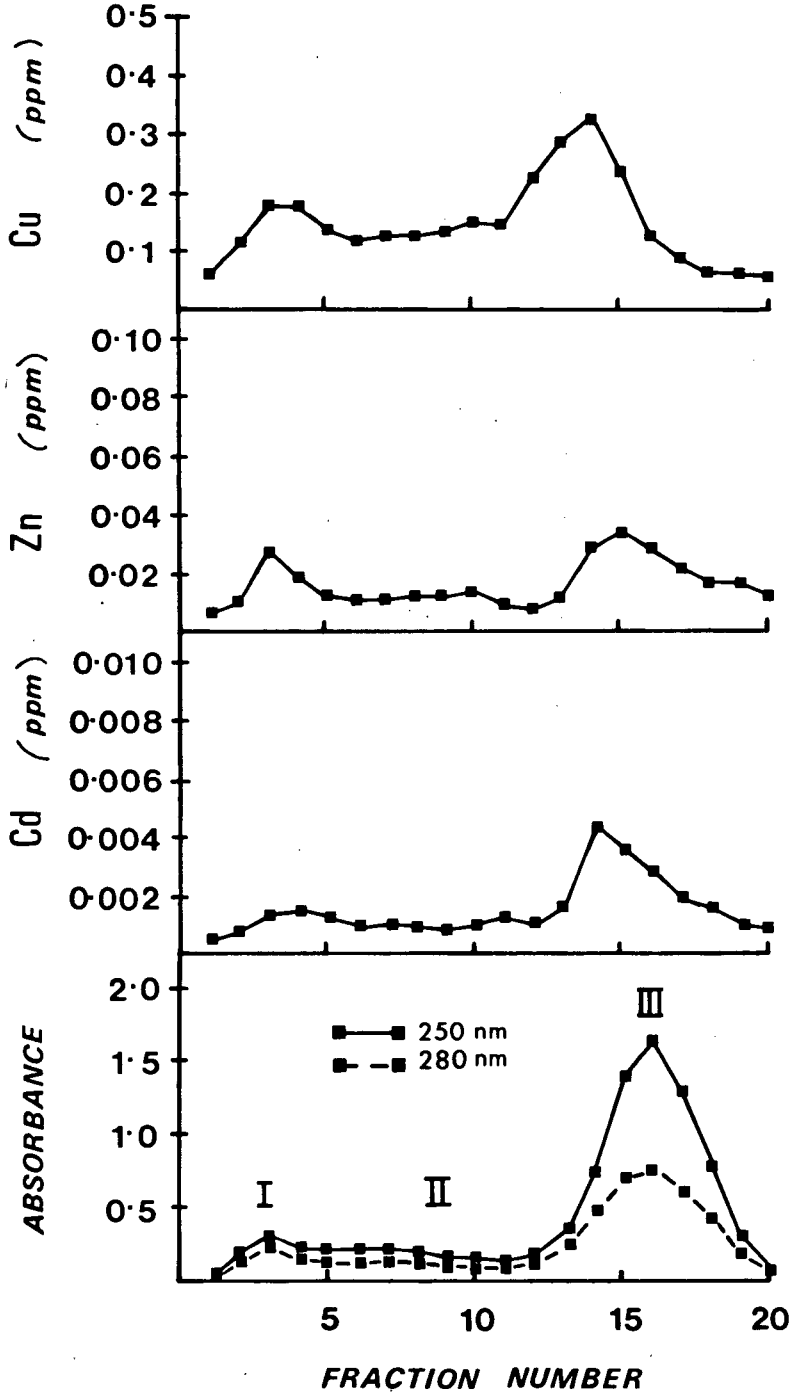

Cu (ppm)
0·5
0·4
0·3
0·2
0·1
Zn (ppm)
0·10
0·08
0·06
0·04
0·02
Cd (ppm)
0·010
0·008
0·006
0·004
0·002
ABSORBANCE
2·0
1·5
1·0
0·5
250 nm
280 nm
I
II
III
5
10
15
20
FRACTION NUMBER

Table 7. Parophrys vetulus. Distribution of Cd, Cu and Zn amongst protein peaks from liver cytoplasm of nontumor- and tumor-bearing flounders. Data are compilations of values from Figures 14 and 15, in μmole g tissue^{-1} (wet weight). MW: Molecular weight.

	Cd				Cu				Zn			
	Total	High Mw pool	Metallo-thionein	Low MW pool	Total	High MW pool	Metallo-thionein	Low MW pool	Total	High MW pool	Metallo-thionein	Low MW pool
Nontumor flounders (3)	.0098[a] (.0048)[b]	.0022 (.0003)	.0016 (.0007)	.0060 (.0046)	1.8117 (0.7007)	.4750 (.2221)	.3977 (.1901)	.9390 (.3019	.1976 (.0768)	.0509 (.0229)	.0298 (.0053)	.1168 (.0575)
Tumor-bearing flounders (3)	.0198 (.0093)	.0071** (.0011)	.0029 (.0016)	.0099 (.0067)	1.8486 (0.7912)	.5429 (.2682)	.4161 (.1826)	.8896 (.3416)	.2930 (.0379)	.1242* (.0439)	.0328 (.0122)	.1360 (.0194)

[a]Mean.

[b]Standard deviation.

*P $<$ 0.05; Student's t test.

**P $<$ 0.001.

peak. Here it is increased 2.4-fold in tumor-bearing fish. Overall there is a 1.5-fold increase in total Zn of tumor-bearing fish relative to nontumor-bearing fish.

It is apparent that increases in Cd in liver of tumor-bearing fish are greater than those of Zn, as evidenced by the increased ratio of Cd:Zn on all peaks in liver of tumor-bearing fish relative to nontumor-bearing fish (Table 8). It is also clear that in tumor-bearing fish a much higher proportion of cytoplasmic Cd and Zn are in the high molecular weight protein (enzyme-containing) pool than in nontumor-bearing fish (Table 9).

Cu shows only small inconsistent changes in tumor-bearing fish relative to nontumor-bearing fish (Tables 7 and 9).

Discussion

It has been shown previously that Cd is elevated in liver of tumor-bearing organisms (Tietz et al., 1957; Morgan, 1970, 1971). However, previously it has not been known that this Cd appears on the high molecular weight protein peak (Table 7). This is an unusual finding in that excess Cd usually appears on the metallothionein peak (e.g. Cherian, 1974; Leber, 1974; Casterline and Yip, 1975; Marafante, 1976). There are, however, several circumstances under which high levels of Cd are found on the high molecular weight protein peak:

(1) Capacity of the Organism to Synthesize Metallo-

Table 8. *Parophrys vetulus*. Ratio of Cd:Zn in various protein peaks from liver cytoplasm of nontumor- and tumor-bearing flounders. Ratios calculated from Table 7. MW: Molecular weight

	Cd:Zn			
	Total	High MW pool	Metallo-thionein	Low MW pool
Nontumor flounders (3)	.050	.043	.054	.052
Tumor-bearing flounders (3)	.068	.060	.087	.073

Table 9. *Parophrys vetulus*. Percentage of total Cd, Cu or Zn on each of protein peaks from liver cytoplasm of nontumor- and tumor-bearing flounder. Percentages calculated from Table 7. MW: Molecular weight.

		Cd			Cu			Zn		
		High MW pool	Metallo-thionein	Low Mw pool	High MW pool	Metallo-thionein	Low MW pool	High MW pool	Metallo-thionein	Low MW pool
Nontumor flounders	(3)	22.1	16.4	61.5	26.2	22.0	51.8	25.8	15.1	59.1
Tumor-bearing flounders	(3)	35.7	14.4	49.9	29.4	22.5	48.1	42.4	11.2	46.4

thionein is Surpassed. Winge et al. (1973) stated that pathological effects of Cd appear when the binding capacity of metallothionein is exceeded and Cd appears on the high molecular weight protein peak. However, their levels of Cd were in the range of 60.0 μg Cd g tissue^{-1} (wet weight), while the levels in the present study were maximally only about 2 μg Cd g tissue^{-1} (wet weight). Stonard and Webb (1976) found that even with much higher levels of Cd than those found in the present study (85 μg Cd g tissue^{-1}, wet weight), 85 to 90% of total Cd was found on the metallothionein peak.

(2) Cd is Displaced from Metallothionein by Presence of High Levels of Another Heavy Metal. It is unlikely that Cd would be displaced from metallothionein by Cu or Zn, as their affinity for metallothionein is less than or the same as that of Cd (Kagi and Vallee, 1960; Pulido et al., 1966). Also, displacement of Cd from metallothionein by Cu or Zn is less likely in tumor-bearing fish compared to nontumor-bearing fish as the ratio of Cd to these metals is increased in tumor-bearing fish (Table 8). It is possible that there are high levels of some other unmeasured heavy metal which can displace Cd from metallothionein, e.g. Hg or Ag (Kagi and Vallee, 1960; Pulido et al., 1966).

(3) Presence of Cd on High Molecular Weight Protein

Peak is Due to an Aggregation of Metallothionein. As shown by Irons and Smith (1976), metallothionein can aggregate in the presence of high levels of Cu, resulting in aggregates of metallothionein in excess of a molecular weight of 60,000. This is unlikely in the present instance, since Cu does not appear to be significantly elevated in tumor-bearing fish (Table 7). Similarly, Olson et al. (1958) found most values for Cu were unaltered in patients with metastatic cancer, while Morgan (1972) found no increases in liver Cu in patients with bronchogenic carcinoma.

(4) Cd is Bound to an Organic Carcinogen. As found by Chen et al. (1973), free ionic Hg will be bound to metallothionein and hence detoxified, while methyl Hg will not bind to metallothionein, but rather will be found mainly on the high molecular weight protein peak. Chen et al. conclude that methyl Hg is much more toxic, since it is not bound to metallothionein. The increased toxicity of methyl Hg when found on the high molecular weight peak is in accordance with data of Winge et al. (1973), who conclude that toxic effects of metals do not occur until these appear in the high molecular weight peak. It may be that Cd in tumor-bearing fish is similarly alkylated, but by "bioactivated" organic carcinogens. Hence, like methyl Hg, this alkylated Cd would not occur on the metallothionein peak, but rather on the high molecular weight peak. As

explained by Furst (1963), organic carcinogens can be explained as heavy metal chelators increasing heavy metal uptake.

In agreement with previous studies (Olson et al., 1954, 1958; Morgan, 1970, 1971), liver Zn in the present study using Parophrys vetulus was elevated in tumor-bearing organisms relative to nontumor-bearing organisms (Table 7). In normal organisms, excess Zn is usually found on the metallothionein peak (Webb, 1972a, b; Davies et al., 1973). However, in this instance excess Zn is found in the high molecular weight protein pool, possibly for the same reasons as discussed for Cd.

Of significance may be the source of the increased liver Cd and Zn in tumor-bearing organisms. As Cd is not a normal constituent of most tissues (except when bound to metallothionein in liver and kidney), it is probable that body burdens of Cd are increased as part of the carcinogenic process (Tietz et al., 1957; Strain et al., 1972). It has been found that Cd is increased in liver, kidney, blood and tumor tissue of tumor-bearing organisms (Gorodiskii et al., 1956, in Furst and Haro, 1969; Tietz et al., 1957; Morgan, 1970). Zinc on the other hand, is increased in liver of tumor-bearing organisms but is decreased in tumor tissue and blood (Olson et al., 1954, 1958; Herring et al., 1960; Arnold and Sasse, 1961; Morgan, 1970; Vallee, 1976). Thus, it appears that in the carcinogenic process,

Cd and Zn increase in the liver, but Cd more so than Zn, so that the Cd:Zn ratio is increased in the liver. As Zn is decreased in blood and tumor tissue, it might be that Zn is transferred to the liver from these areas during carcinogenesis. Concurrently, Cd is increased in blood and tumor tissue so that even higher Cd:Zn ratios would be expected in tumor tissue than in the liver.

Morgan (1970) found liver Cd and renal Cd and Zn increased in lung cancer patients. The Cd:Zn ratio was increased 47% in liver and 22% in kidney. In a subsequent study, Morgan (1971) found both Cd and Zn increased in liver of lung cancer patients, with a 25% increase in the Cd:Zn ratio. The present study found a 36% increase of the liver Cd:Zn ratio and a 40% increase of this ratio in the high molecular weight protein peak.

Winge et al. (1973), Leber (1974) and Irons and Smith (1976) have found that toxic effects of Cd occur when it appears in the high molecular weight protein fraction. Since enzymes occur in this fraction (White et al., 1968) and heavy metals are known to interfere with enzymatic function (Bremner, 1974; Friedberg, 1974), it is reasonable to conclude that, at least in part, those heavy metals not bound to metallothionein and hence present in the high molecular weight protein peak, exert toxic effects by binding enzymes.

It may be that the increased Cd:Zn ratio in the high

molecular weight protein pool of tumor-bearing flounders results in interference of Cd with Zn in Zn-requiring enzymes in pretumor tissue. Horvath (1976) states that it is probable that Cd competes with Zn, exerting its toxicity through inactivation of sulfhydryl-containing enzymes. Vallee (1976) says that Zn has a role in enzymes essential to nucleic acid metabolism and in ribonucleic acid and deoxyribonucleic acid polymerases in normal tissue as well as ribonucleic acid-dependent deoxyribonucleic acid polymerases from tumor viruses. It may be that Cd interferes with Zn binding sites on these enzymes which are instrumental in control of cell division. Schroeder et al. (1961) suggest that if Cd is an essential metal, its primary action is that of inhibition for it is a potent inhibitor of many enzymes. These enzymes are rendered nonfunctional by conformational changes brought about by binding with heavy metals possessing properties different from their required metals (Friedberg, 1974). Nonfunction is due to the fact that, after conformational changes, substrate molecules no longer fit binding sites on the enzymes (Bremner, 1974; Friedberg, 1974). Alternatively, dysfunction can result via a splitting of enzymes into subunits. Gerhart and Schachman (1965) found that aspartic transcarbamylase from Escherichia coli can be split into catalytic and regulatory subunits by Hg, which might interfere with Zn which holds subunits together (Griffin et al.,

1973). Once the catalytic subunit is freed from the regulatory subunit, it has high activity and is no longer subject to feedback control. Jovin et al. (1969) have found that Zn-containing deoxyribonucleic acid polymerase from E. coli can also be split into subunits by Hg.

Flick et al. (1971) reviewed the literature on Cd. They conclude that Cd is a potent carcinogen, whose effects can be counteracted by Zn. Further, they conclude that the important variable may be the Cd:Zn ratio. Kolonel (1976) states that Cd acts as a competitive inhibitor of Zn and produces malignant tumours in animals. Zinc has been shown to prevent cancer from occurring in rats, mice and hamsters when administered simultaneously with the inorganic carcinogen Cd or with the organic carcinogen dimethylaminoazobenzene (Gunn et al., 1963, 1964; Poswillo and Cohen, 1971; Ciapparelli et al., 1972; Duncan and Dreosti, 1975). It demonstrated the same preventive effect when given from an early age to mice which would otherwise have developed spontaneous mammary gland tumors (Bischoff and Long, 1939). Further, Zn inhibited the development and spread of tumor tissue transplanted into mice and rats (Duncan et al., 1974; Duncan and Dreosti, 1975, 1976; Woster et al., 1975).

The Cd:Zn ratio has been shown in this and other studies to be increased in tumor-bearing organisms. The fact that in this study the Cd:Zn ratio has been shown to

be increased in the high molecular protein pool of tumor-bearing organisms suggests that there is a possibility that in carcinogenesis, Cd is interfering with Zn-containing enzymes involved in cell division processes. Thus, high levels of Zn administered with a carcinogen might prevent carcinoma from occurring by successfully competing with increased levels of Cd for binding sites on these enzymes.

Summary

It is evident from previous studies that heavy metal levels are changed in tumor-bearing organisms relative to nontumor-bearing organisms, particularly in apparently normal liver tissue. This study considered the possible role of metallothioneins in binding Cd, Cu or Zn in the liver of tumor-bearing flounders (Parophrys vetulus). Cadmium and zinc were not increased significantly on the metallothionein peak, but were increased 3.3- and 2.4-fold, respectively, on the high molecular weight protein peak. In addition, the Cd:Zn ratio was increased by 40% on the high molecular weight protein peak. Results are discussed in terms of competition of Cd and Zn for Zn-requiring enzymes involved in nucleic acid metabolism. Possible reasons for excess Cd occurring on the high molecular weight protein peak rather than on the metallothionein peak are discussed. Copper levels did not change.

CHAPTER VIII Increases of Cd and the Cd:Zn Ratio in the High Molecular Weight Protein Pool from Apparently Normal Kidney of Terminal Human Cancer Patients

Preface

This study was done by D. A. Brown and B. Knight (1978). Unfortunately, the sample size is only 2 for controls or cancerous humans. When a larger sample size is attained, this paper will be submitted for publication. The purpose of this study was to investigate if changes in the cytoplasmic distribution of Cd and Zn were similar in cancerous fish and humans. The fact that they do prove to be the same, suggests that such changes are common to cancerous organisms, and therefore may be etiologic in cancer.

Introduction

Recently, Brown (1977) has noted increases of Cd and the Cd:Zn ratio in the high molecular weight protein pool of tumor-bearing flounders. Occurrences of high levels of Cd in this pool may be unique to carcinoma, since excesses of Cd are usually bound and detoxified by matallothionein in liver and kidney tissue (Cherian, 1974; Casterline and Yip, 1975; Marafante, 1976). Previously, Morgan (1970) found increases of both Cd and Zn and the Cd:Zn ratio in the liver, kidney and serum of lung cancer patients. Increases of the Cd:Zn ratio in the high molecular weight

protein pool may be of significance since enzymes occur in this pool. Of particular interest in the study of carcinoma, are the Zn-containing enzymes involved in nucleic acid metabolism and cell division processes; e.g., DNA polymerase, RNA polymerase, reverse transcriptase, and thymidine kinase (Vallee, 1976; Duncan and Dreosti, 1976). Cadmium exerts its toxic action by replacing Zn in these enzymes, so that their function is inhibited or changed due to conformational or quaternary changes (Flick et al., 1971; Friedberg, 1974; Brown, 1977). The present study will examine tissue from humans with carcinoma, to see if increases of the Cd:Zn ratio in the high molecular weight protein pool may be a common factor in carcinoma.

Materials and Methods

Four kidney samples were obtained from Vancouver General Hospital and Richmond General Hospital. Of these, two came from cancer victims and two from patients dying from unrelated causes. The age of the patients is unknown. Both cancer patients had generalized metastasis at death but the kidneys were not believed to be affected.

Two grams of kidney tissue were homogenized for 5 minutes in 4.5 ml of 0.9% (W/V) NaCl at a setting of 6 on a TRI-R STIR-R Model S63C laboratory motor equipped with a teflon pestle. Homogenates were centrifuged at 27,000 x g for 10 minutes in a Sorvall RC2-B centrifuge. Supernatants were collected and the pellets were rehomogenized

for 3 minutes in 2.5 ml of 0.9% NaCl. These were centriguged at 27,000 x g for 10 minutes and supernatants combined with previous supernatants. Combined supernatants were then placed in a 70°C water bath for 5 minutes to clear cellular debris via heat precipitation. These were then recentrifuged at 27,000 x g for 10 minutes.

Resulting supernatants were applied to a Pharmacia column (1.6 x 100 cm) packed with G-75 gel, and eluted with 0.01 M NH_4HCO_3 buffer. Protein was read at 250 and 280 nm on a Perkin Elmer 124D spectrophotometer on each 15 ml fraction to establish the position of the high molecular weight protein pool, metallothionein and the low molecular weight cytoplasmic pool (Brown, 1977; Brown et al., 1977).

Copper and Zn were determined by flame atomic absorption spectrophotometry while Cd was done by graphite furnace atomic absorption spectrophotometry. Both employed deuterium arc background correction. Total metal levels for each peak were determined by summation of the individual metal levels in each tube of each peak.

Results

Composite gel elution profiles of noncancer or cancer patients are shown in Figures 18 and 19. While both have similar total Cd levels, in noncancer patients most of this Cd occurs on metallothionein. In cancer patients more occurs in the high molecular weight protein pool (Tables 10 and 11). Zinc levels were higher in cancer patients but

Figure 18. Composite of gel elution profiles from kidneys of 2 terminal noncancer patients. I: high molecular weight protein pool; II: metallothionein; III: low molecular weight cytoplasmic pool.

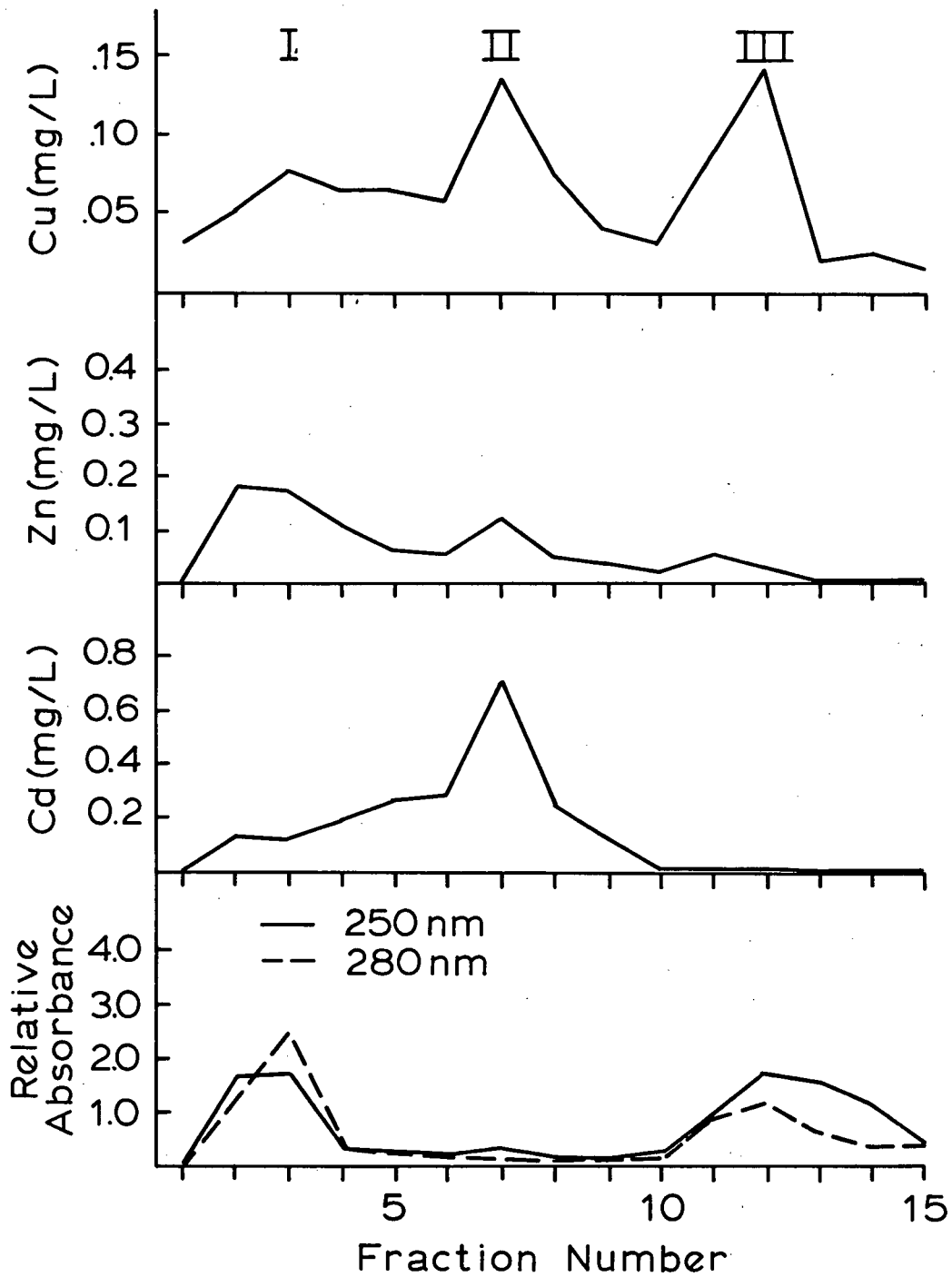

I
II
III
Cu (mg/L)
.15
.10
.05
Zn (mg/L)
0.4
0.3
0.2
0.1
Cd (mg/L)
0.8
0.6
0.4
0.2
250 nm
280 nm
Relative Absorbance
4.0
3.0
2.0
1.0
5
10
15
Fraction Number

Figure 19. Composite of gel elution profiles from kidneys of 2 terminal cancer patients. I: high molecular weight protein pool; II: metallothionein; III: low molecular weight cytoplasmic pool.

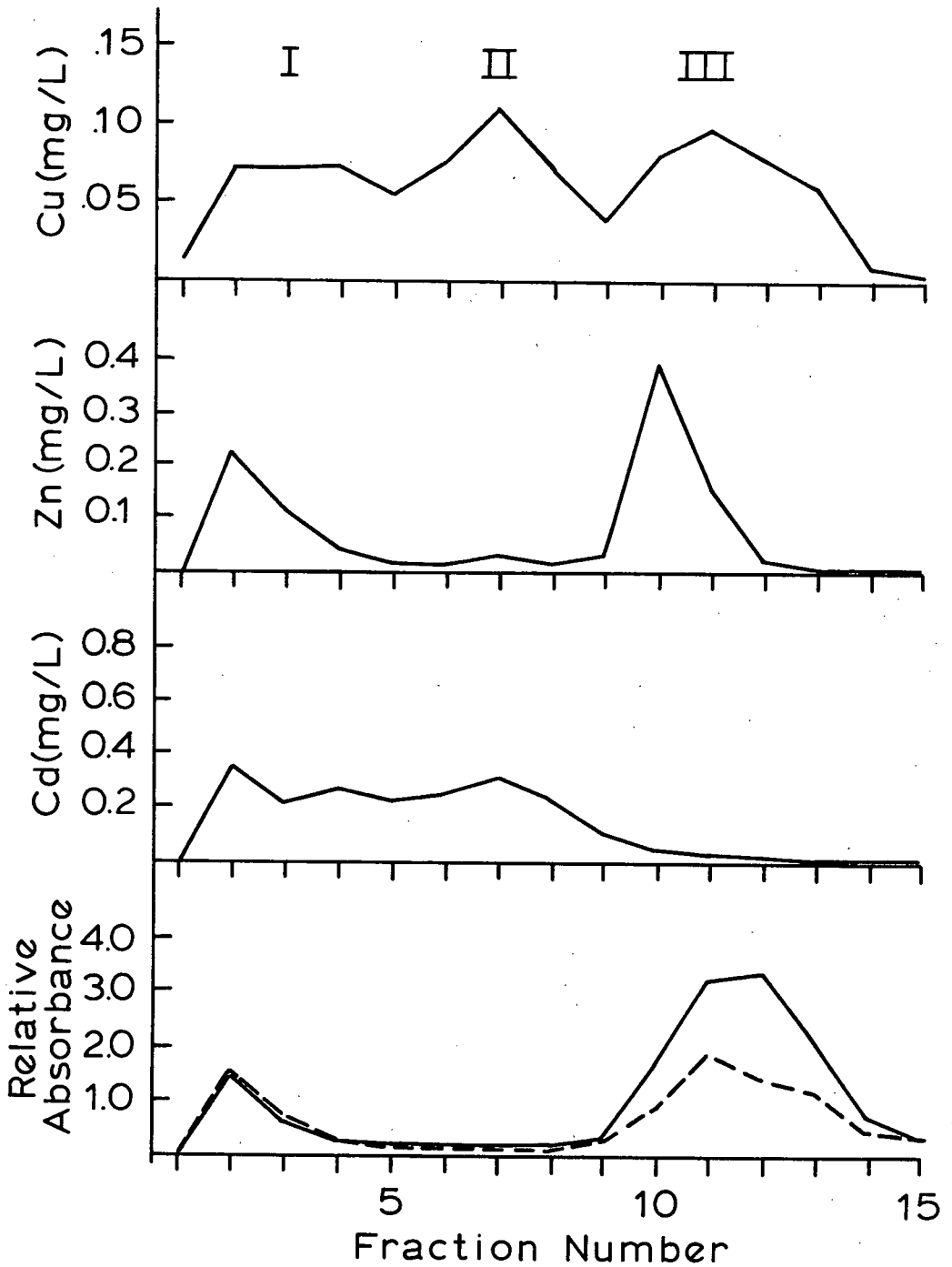

I
II
III
Cu (mg/L)
.15
.10
.05
Zn (mg/L)
0.4
0.3
0.2
0.1
Cd (mg/L)
0.8
0.6
0.4
0.2
Relative Absorbance
4.0
3.0
2.0
1.0
5
10
15
Fraction Number

Table 10. Distribution of Cd, Cu and Zn amongst cytoplasmic pools from kidney tissue of cancer and noncancer patients. Data are compilations of values from Figures 18 and 19 in μmole g tissue^{-1} (wet weight). MW: molecular weight.

		Cd				Cu				Zn			
		Total	High MW pool	Metallo-thionein	Low MW pool	Total	High MW pool	Metallo-thionein	Low MW pool	Total	High MW pool	Metallo-thionein	Low MW pool
Noncancer patients	(2)	.192[a]	.065	.126	.001	.149	.044	.050	.055	.115	.076	.032	.007
Cancer patients	(2)	.186	.096	.084	.006	.147	.046	.048	.053	.176	.072	.014	.090

[a]Mean.

Table 11. Percentage of total Cd, Cu or Zn on various cytoplasmic pools from kidney tissue of cancer and noncancer patients. Percentages calculated from Table 10.
MW: molecular weight.

		Cd			Cu			Zn		
		High MW pool	Metallo-thionein	Low MW pool	High MW pool	Metallo-thionein	Low MW pool	High MW pool	Metallo-thionein	Low MW pool
Noncancer patients	(2)	33.9	65.6	0.5	29.5	33.6	36.9	66.1	27.8	6.1
Cancer patients	(2)	51.6	45.2	3.2	31.3	32.7	36.0	40.9	8.0	51.5

most of this increase was in the low molecular weight cytoplasmic pool (Figures 18 and 19; Tables 10 and 11). The ratio of Cd:Zn was increased in cancer patients in both the high molecular weight protein pool and metallothionein (Table 12). Copper levels appeared to be unchanged between noncancer and cancer victims.

Discussion

The increase of the Cd:Zn ratio found in human kidneys of cancer patients in the present study, is similar to changes found in tumor-bearing flounders (Brown, 1977). In both instances there was an increase in the portion of cytoplasmic Cd occurring in the high molecular weight protein pool relative to the portion bound by metallothionein. Cadmium bound to metallothionein is essentially biologicaly inert in that it is not available to produce toxic effects via displacing Zn from Zn-containing metalloenzymes. Since the high molecular weight protein pool contains enzymes, the excess Cd occurring in this pool in carcinogenesis, is likely available to affect the Zn-containing enzymes involved in cellular division processes.

In tumor-bearing flounders there were increases in the levels of Zn in the high molecular weight protein pool (Brown, 1977). This did not occur in cancer patients in the present study. However, in tumor-bearing flounders there was a 3.2-fold increase of Cd in this pool, while in cancer patients, there was a 1.5-fold increase. Therefore,

Table 12. Ratio of Cd:Zn in cytoplasmic pools from kidney tissue of cancer and noncancer patients. Ratios calculated from values in Figures 18 and 19 in molar units. MW: molecular weight.

	Cd:Zn			
	Total	High MW pool	Metallo-thionein	Low MW pool
Noncancer patients(2)	1.19	0.65	2.59	0.13
Cancer patients(2)	1.58	1.11	12.00	0.04

although Cd was increased more in this pool in flounders, there was a larger increase of the Cd:Zn ratio in humans (1.7-fold) compared with flounders (1.4-fold).

In both humans with cancer, and tumor-bearing flounders, Cu levels were unchanged. Thus the overall change in trace elements common to both humans and flounders with carcinoma, was an increase of the Cd:Zn ratio in the high molecular weight protein pool. Since the level of dysfunction of Zn-containing enzymes, such as those involved in cell division, will depend upon a competition of Cd and Zn for binding sites in these enzymes (Flick et al., 1971), increases of the Cd:Zn ratio may be important for degenerative changes such as those found in carcinogenesis.

Summary

Total cytoplasmic Cd levels were similar in human cancer victims and controls but in cancer victims there was a 1.5-fold increase of the portion of this Cd in the high molecular weight protein pool. In controls most Cd was found bound to metallothionein. Total cytoplasmic Zn levels were increased as a result of increases of Zn in the low molecular weight cytoplasmic pool of cancer victims. Copper levels were unchanged. Results are discussed in terms of a competition of Cd and Zn for binding sites in Zn-requiring enzymes involved in cell division processes.

CHAPTER IX Decreases of Copper and Zinc in the High Molecular Weight Protein Pool from Livers of Mice Exposed to Diethylnitrosamine, With and Without Cadmium or Zinc Supplementation.

Preface

This paper was authored by D. A. Brown and A. Y. Chan (1978), and submitted to the Journal of the National Cancer Institute. The purpose of this study was to see if an organic carcinogen (DEN) could induce changes in the Cd:Zn ratio similar to those found in cancerous fish (Brown, 1977) and humans (Brown and Knight, 1978). However, all mice in this study were sampled before tumors developed (after 12.5 weeks exposure), since 10 weeks was the time reported as necessary in other studies, for tumors to be induced in DEN exposed mice. There was no increase of Cd:Zn in pretumorous livers of mice administered DEN. However, Cd administered with DEN did appear in the high molecular weight protein pool and did advance pretumorous histological changes. The main effect of DEN appeared to be to decrease levels of Cu and Zn in the high molecular weight protein pool.

Introduction

Previous studies have indicated that trace heavy metals such as Cd, Cu and Zn are altered in tumor-bearing organisms. It appears with occurrences of cancers other than cancer of

the liver, that liver Cd, Zn and sometimes Cu are increased (Olson et al., 1954, 1958; Koch et al., 1975; Teitz et al., 1957; Sandberg et al., 1958; Morgan, 1970, 1971, 1972; Brown, 1977). Notabley, although both Cd and Zn are increased, there is an increase in the Cd:Zn ratio in non-tumorous liver and kidney of tumor-bearing organisms (Morgan, 1970, 1971; Flick, 1971; Brown, 1977). Tumorous tissue other than from liver usually has decreased levels of Zn (Vallee, 1976; Lin, 1977) but increased levels of Cu (White, 1921) and Cd (Teitz et al., 1957; Gorodiskii et., 1956: in Furst and Haro, 1969).

With tumors in the liver, some studies report increased Zn in apparently normal liver tissue surrounding the tumors (Olson et al., 1954, 1958; Wright and Dormandy, 1972). Others report decreased (Arnold and Sasse, 1961) or unchanged (Kew and Mallett, 1974) Zn in surrounding liver tissue. Zinc in liver tumors is consistently reported to be decreased (Olson et al., 1954, 1958; Arnold and Sasse, 1961; Wright and Dormandy, 1972; Kew and Mallett, 1974). Copper levels are normal (Olson et al., 1958) or decreased (Arnold and Sasse, 1961) in liver tissue surrounding liver tumors. Copper levels in liver tumors have been reported both to be decreased (Olson et al., 1958) and increased (Arnold and Sasse, 1961).

Changes of metal levels can be induced by organic carcinogens. Gorodiskii et al. (1956; in Furst and Haro,

1969) report increased Cd content in tumors induced by nonmetal carcinogens, Olson et al. (1954) observe that liver Zn levels of DMBA (dimethylaminoazobenzene)-exposed mice decrease initially during the period of liver damage, increase during regeneration and peak just before gross neoplasia of the liver is evident. However, no data are given. Arnold and Sasse (1961) found Zn to be decreased in both liver tumors and surrounding liver tissue of rats fed DMBA. Copper was found to be decreased in liver tissue surrounding liver tumors but incrased in the tumors themselves. These decreases of Cu and Zn in tissue surrounding tumors indicate that DMBA decreases these metals in apparently normal liver tissue. Yamane et al. (1969) found no change or slight decreases of Cu content in pretumorous livers of rats exposed to DMBA. However, Fare (1964) and Fare and Woodhouse (1963a,b) found pretumorous rat liver Cu levels to be progressively increased by DMBA exposure. Thus, there are indications that DMBA changes Cu and Zn levels in pretumorous liver. However, for Zn the data are not clearly documented and for Cu the data appear to be contradictory. In fact, the only consistent findings in the study of metal changes with cancer are that Zn levels are decreased in tumor tissue and body burdens of Cd and the Cd:Zn ratio are increased.

The present study reports the effect of the carcinogen DEN (diethylnitrosamine) on Cd, Cu and Zn levels in pretumor

liver tissue. In particular, the levels of these elements in the high molecular weight protein (enzyme-containing) pool (Brown et al., 1977; Brown, 1977) are reported. Olson et al. (1954) have recognized the importance of Cu and Zn as necessary for the activity of enzymes and enzyme systems. Further, other investigators have pointed out that Cd may replace Cu and Zn in metalloenzymes resulting in enzyme dysfunction (Schroeder et al., 1961; Flick et al., 1971; Yoshida et al., 1975; Kolonel, 1976; Brown, 1977).

As Zn has been repeatedly reported to prevent cancer from occurring (Bischoff and Long, 1939; Gunn et al., 1963, 1964; Poswillo and Cohen, 1971; Ciapparelli et al., 1972; Duncan et al., 1974; Duncan and Dreosti, 1975, 1976; Woster et al., 1975), this study also measured relative changes in Cd, Cu and Zn in mice administered both DEN and Zn in the drinking water. In addition, we gave another group of mice DEN and Cd as there is evidence of increased Cd:Zn ratio in tumor-bearing organisms (Morgan 1970, 1971; Brown, 1977).

Materials and Methods

Mice of the Swiss strain, approximately three months old, were separated into cages, 3 mice of the same sex per cage. Mice received either tap water, water with 20 ppm DEN, 20 ppm DEN + 250 ppm Zn (as $ZnSo_4 \cdot 7H_2O$), or 20 ppm DEN + 5 ppm Cd (as $CdCl_2$) for five weeks. From 6 weeks on, the concentration of DEN was 40 ppm. Drinking solutions

(100 ml) were renewed on alternate days. All water jars were painted black to prevent photodegradation of DEN. Diet consisted of Purina rat chow ad libitum.

Mice were sacrificed by asphyxiation with CO_2. A portion of liver was immediately preserved in a standard formalin solution. This tissue was wax embedded, sectioned (5μm) and stained (Phosphotungstic acid hematoxylin). The remaining liver portion was stored at -20°C until analyzed.

In order to ensure consistency of analysis between different exposures, livers were analyzed four at a time, one from each exposure type (1 control, 1 DEN, 1 DEN + Cd, 1 DEN + Zn) from the same exposure time. Two grams of liver were placed in a homogenizing tube with 4.5 ml of 0.9% NaCl and homogenized at a standard speed (setting 4.5) on a TRI-R STIR-R variable speed laboratory motor model S63C for exactly 3 minutes. Previous experimentation demonstrated that the amount of metal extracted varied with homogenizing time. Each homogenate was poured into a 11 ml capacity pyrex Sorvall centrifuge tube and each group of 4 homogenates was then centrifuged at 27,000 x g for 10 minutes in a Sorvall RC2-B centrifuge. Supernatants were decanted into other pyrex Sorvall centrifuge tubes. Pellets were rehomogenized for exactly 2 minutes in 2.5 ml of 0.9% NaCl, centrifuges at 27,000 x g for 10 minutes and combined with previous supernatants.

Groups of 4 supernatants were then placed in a 70°C

water bath for approximately 3 minutes to clear cellular debris via heat precipitation. It is important that all tubes be heated at the same temperature for exactly the same period of time as the amount of high molecular weight protein precipitated increases with increasing length of time exposed to a high temperature (unpubl. data).

Supernatants were recentrifuged at 27,000 x g for 10 minutes. Resulting supernatant was then applied to a Pharmacia column (1.6 x 100 cm) packed with G-75 gel and eluted with 0.01 M NH_4HCO_3 buffer. Protein was read at 250 and 280 nm on a Perkin Elmer 124D spectrophotometer on each 10.2 ml fraction to establish the position of the high molecular weight protein pool, metallothionein and the low molecular weight cytoplasmic pool (Brown, 1977; Brown et al., 1977).

Copper and zinc were determined by flame atomic absorption spectrophotometry while Cd was done by graphite furnace atomic absorption spectrophotometry. Both determinations employed deuterium arc background correction. Total metal levels for each peak were determined by summing of the individual metal levels in each tube of each peak.

Results

Liver tissue homogenate supernatant of mice is depleted of Cu and Zn upon exposure to DEN. This depletion of Cu and Zn is apparent at 3 weeks (Table 13) and continues to increase throughout the exposure period of 12.5 weeks

Table 13. The distribution of Cd, Cu and Zn amongst protein peaks from liver of mice exposed to DEN (diethylnitrosamine) with and without Cd or Zn. Data are compilations of data from Figure 20 in μmole/g tissue (wet weight). MW: Molecular weight. ND: Not detectable.

	Cd				Cu				Zn			
	Total	High MW pool	Metallo-thionein	Low MW pool	Total	High Mw pool	Metallo-thionein	Low MW pool	Total	High MW pool	Metallo-thionein	Low MW pool
0 Weeks												
Control (2)	.00391	.00160	.00070	.00161	.0968	.0477	.0189	.0303	.0754	.0700	.0020	.0034
3 Weeks												
Control (2)	.00333	.00116	.00073	.00144	.0851	.0417	.0164	.0270	.0703	.0670	.0005	.0029
DEN (2)	.00322	.00063	.00098	.00161	.0783	.0386	.0167	.0230	.0577	.0536	ND	.0041
DEN + Zn (2)	.00281	.00085	.00054	.00142	.0809	.0410	.0160	.0239	.0567	.0558	ND	.0009
DEN + Cd (2)	.00292	.00095	.00061	.00136	.0692	.0314	.0160	.0218	.0535	.0500	ND	.0035
6 Weeks												
Control (2)	.00320	.00081	.00068	.00171	.0740	.0446	ND	.0295	.0813	.0773	ND	.0040
DEN (2)	.00315	.00073	.00067	.00174	.0548	.0348	ND	.0200	.0646	.0615	ND	.0031
DEN + Zn (2)	.00307	.00090	.00061	.00157	.0595	.0313	.0080	.0202	.0716	.0640	ND	.0076
DEN + Cd (2)	.00322	.00117	.00067	.00138	.0426	.0226	ND	.0200	.0589	.0547	ND	.0042
12.5 Weeks												
Control (3)	.00376[a]	.00139	.00086	.00151	.1447	.0698	.0340	.0409	.0873	.0822	ND	.0051
	(.00140)[b]	(.00068)	(.00042)	(.00034)	(.0073)	(.0004)	(.0148)	(.0087)	(.0046)	(.0034)		(.0059)
DEN (3)	.00375	.00126	.00097	.00152	.0993*	.0401**	.0188	.0404	.0538*	.0501*	ND	.0037
	(.00140)	(.00072)	(.00057)	(.00030)	(.0149)	(.0052)	(.0050)	(.0101)	(.0068)	(.0098)		(.0034)
DEN + Zn (3)	.00362	.00110	.00080	.00172	.1148*	.0510**	.0231	.0407	.0590*	.0555*	ND	.0034
	(.00119)	(.0053)	(.00042)	(.00042)	(.0110)	(.0014)	(.0005)	(.0122)	(.0073)	(.0095)		(.0022)
DEN + Cd (3)	.00685	.00389	.00092	.00204*	.1004*	.0466*	.0193	.0345	.0542**	.0525**	ND	.0017
	(.00276)	(.00317)	(.00046)	(.00005)	(.0096)	(.0089)	(.0093)	(.0093)	(.0036)	(.0020)		(.0029)

[a] Mean.

[b] Standard deviation.

* $P < 0.05$; 2-tailed Student's t test.

** $P < 0.001$.

(Table 14).

It is evident that these decreases of Cu and Zn reflect decreases on the high molecular weight protein pool (Tables 13 and 14; Figures 20, 21 and 22). Copper also appears to be reduced on metallothionein with DEN exposure with and without Cd or Zn at 12.5 weeks (Table 13; Figure 20). Copper was not always detectable on metallothionein at 3 and 6 weeks by methods employed in this study.

Furthermore, it is apparent that administration of Zn with the carcinogen in the drinking water tends to slightly reverse tissue depletions of Cu and Zn otherwise induced by DEN exposure (Tables 13 and 14; Figures 20, 21 and 22). Administration of Cd with the carcinogen initially (at 3 and 6 weeks) resulted in increased losses of high molecular weight protein Cu and Zn from liver. This effect was not apparent at 12.5 weeks exposure to DEN (Tables 13 and 14; Figures 21 and 22).

Total tissue Cd levels appeared to be unchanged by DEN or DEN + Zn. However, there appeared to be slight decreases of Cd on the high molecular weight protein pool at 12.5 weeks (Table 13). These decreases of Cd on the high molecular weight protein pool were also evident at 3 and 6 weeks in mice exposed to DEN alone (Table 13). Total tissue Cd levels were increased by DEN + Cd at 12.5 weeks (Table 13). Most of this increase of Cd accumulated on the high molecular weight protein pool (Table 13,

Table 14. Percentage decreases of Cu and Zn in total tissue homogenate supernatant and the high molecular weight protein pool from livers of mice exposed to DEN (diethylnitrosamine) with and without Cd or Zn. Calculated from data in Table 13. MW: Molecular weight.

	Cu		Zn	
	Total	High MW pool	Total	High MW pool
3 Weeks				
DEN (2)	8.0	7.4	17.9	20.0
DEN + Zn (2)	4.9	1.7	19.3	16.7
DEN + Cd (2)	18.7	24.7	23.9	25.4
6 Weeks				
DEN (2)	25.9	22.0	20.5	20.4
DEN + Zn (2)	19.6	29.8	11.9	17.2
DEN + Cd (2)	42.4	49.3	27.6	29.2
12.5 Weeks				
DEN (3)	31.4	42.6	38.4	39.1
DEN + Zn (3)	20.7	26.9	32.4	32.5
DEN + Cd (3)	30.6	33.2	37.9	36.1

Figure 20. Composite gel elution profiles from non-cancerous livers of control mice and mice exposed to DEN with and without Cd or Zn for 12.5 weeks. Each composite profile is the average of three individual profiles. I: high molecular weight protein pool; II: metallothionein; III: low molecular weight cytoplasmic pool.

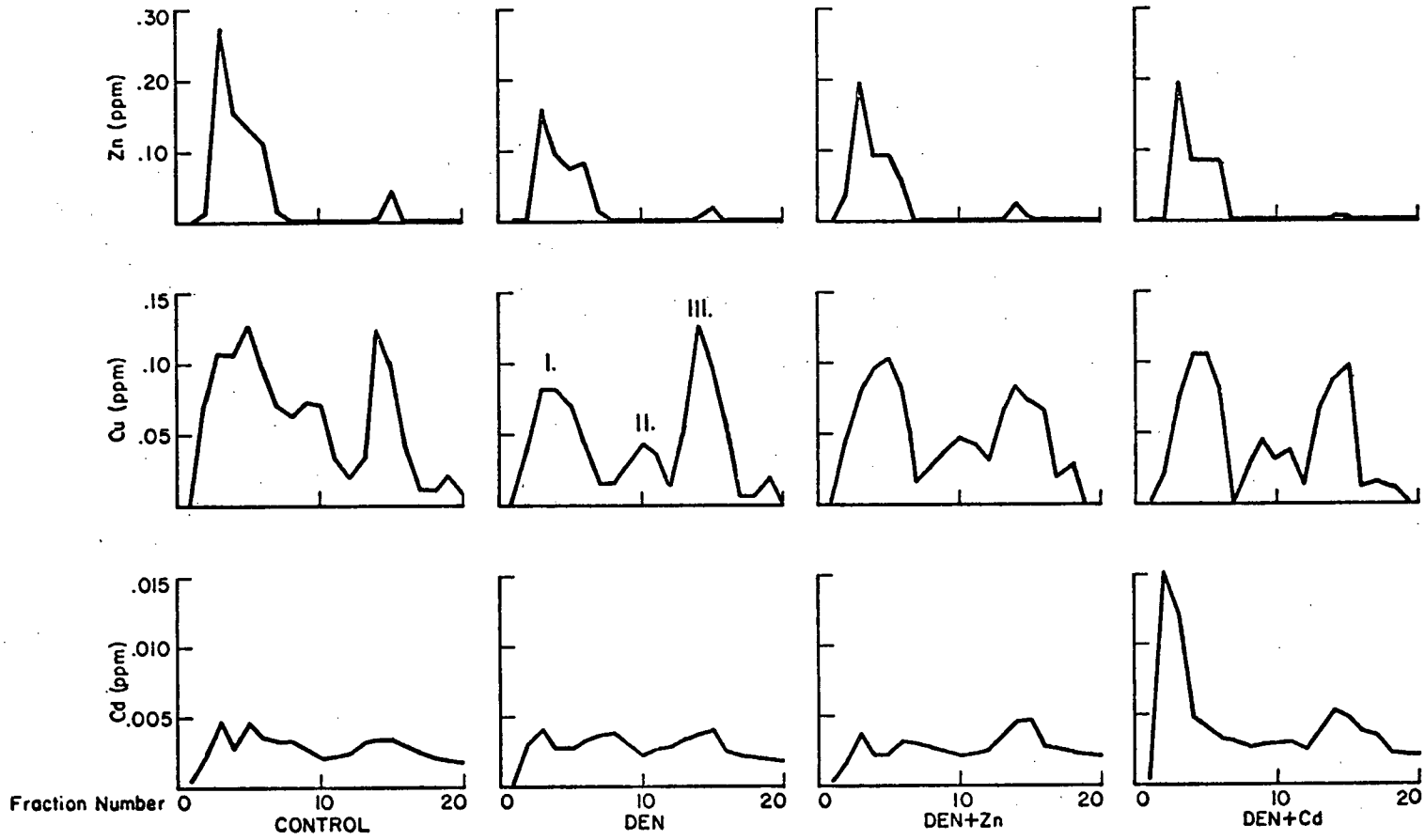

Zn (ppm)
.30
.20
.10
Cu (ppm)
.15
.10
.05
I.
II.
III.
Cd (ppm)
.015
.010
.005
Fraction Number
0
10
20
CONTROL
DEN
DEN+Zn
DEN+Cd

Figure 21. The variation of Cu levels on the high molecular weight protein pool from noncancerous livers of control mice and mice exposed to DEN with and without Cd or Zn for 12.5 weeks. Each point at 0, 3 and 6 weeks represents 2 animals while at 12.5 weeks each point represents 3 animals.

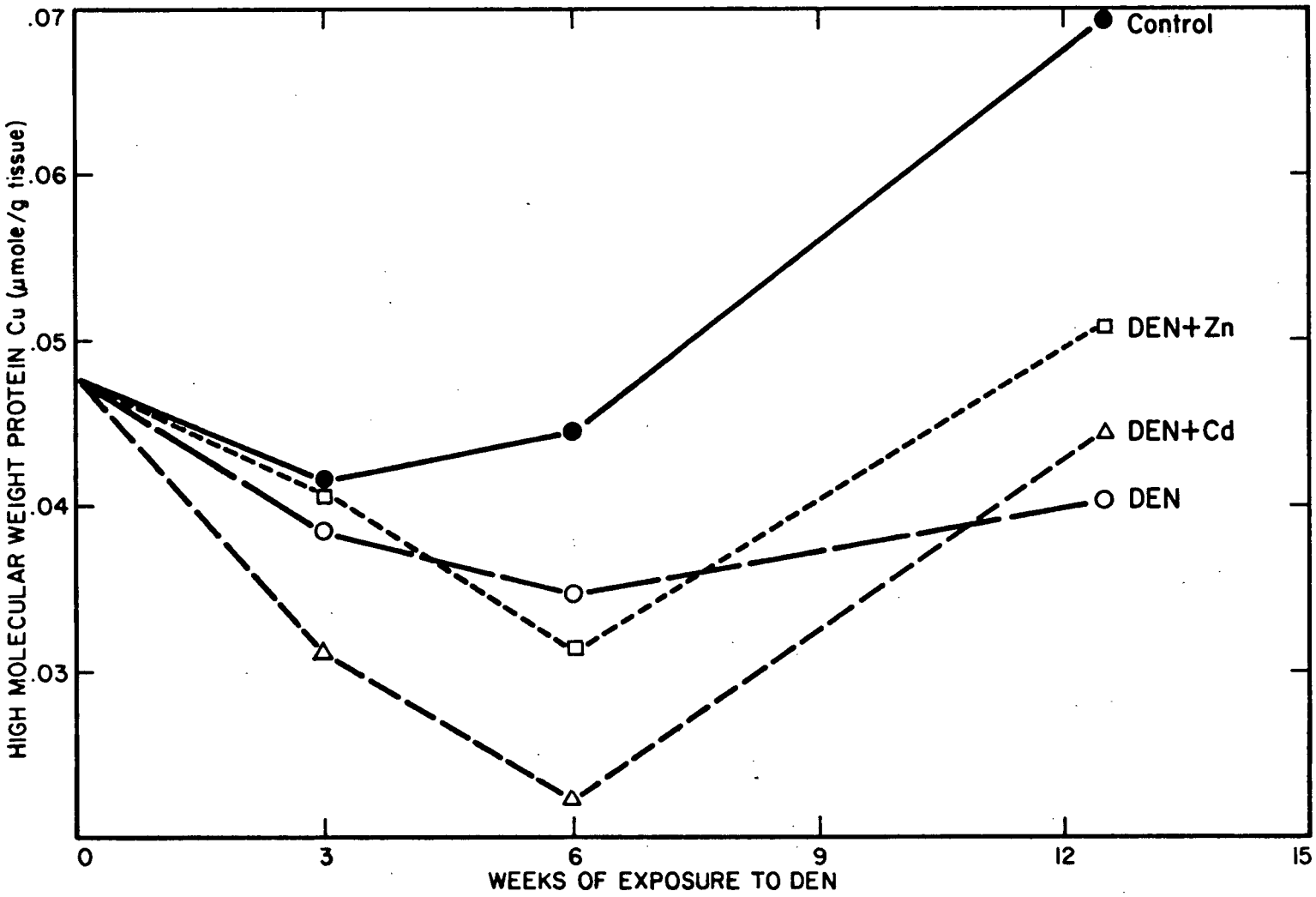

.07
.06
.05
.04
.03
HIGH MOLECULAR WEIGHT PROTEIN Cu (μmole/g tissue)
0
3
6
9
12
15
WEEKS OF EXPOSURE TO DEN
Control
DEN+Zn
DEN+Cd
DEN

Figure 22. The variation of Zn levels on the high molecular weight protein pool from noncancerous livers of control mice and mice exposed to DEN with and without Cd or Zn for 12.5 weeks. Each point at 0, 3 and 6 weeks represents 2 animals while at 12.5 weeks each point represents 3 animals.

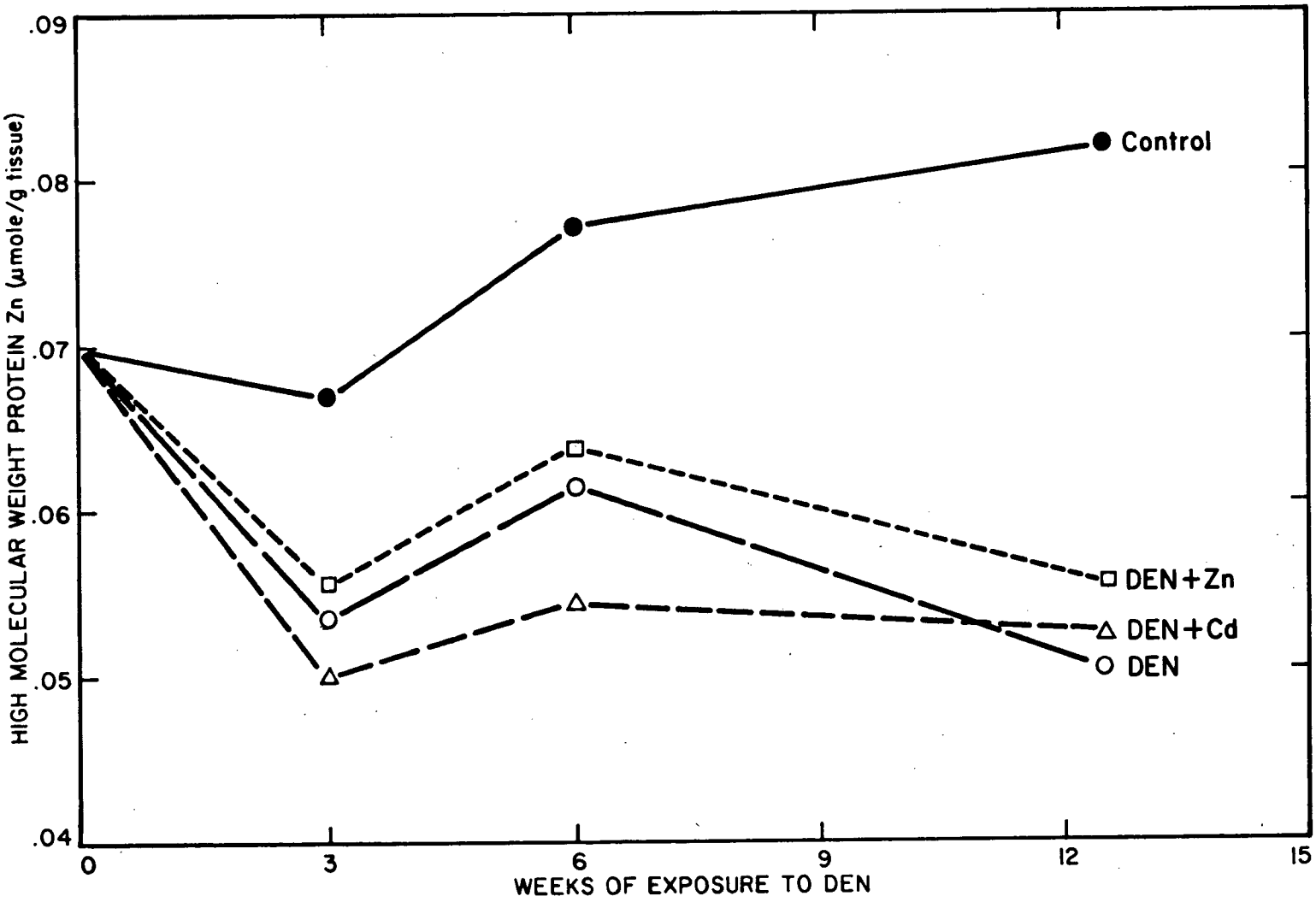

.09
.08
.07
.06
.05
.04
HIGH MOLECULAR WEIGHT PROTEIN Zn (μmole/g tissue)
0
3
6
9
12
15
WEEKS OF EXPOSURE TO DEN
Control
DEN+Zn
DEN+Cd
DEN

Figure 20).

It is probable that data expressed as decreases in % metal (Table 14; Figures 23 and 24) give a more accurate portrayal of the metal changes occurring with time as this eliminates differences due to slight variations in heating times for each group of samples.

Histological examination indicated that livers of DEN-exposed mice, with and without Cd or Zn, were pretumorous. Livers of mice receiving DEN alone or DEN + Cd had more hepatic cell dysplasia and regeneration than DEN + Zn-exposed livers (Table 15). Mice exposed to DEN + Cd had liver pathologies most advanced towards tumor development. Gross cholangiofibrosis, indicative of bile duct epithelial cell proliferation, was visible in all three of the DEN + Cd-exposed mouse livers, two of the DEN-exposed livers, one of the DEN + Zn-exposed livers, and none of the controls (Table 15). Although variable, the overall histology change rating for mice exposed to DEN + Cd was greater than that for those exposed to DEN or DEN + Zn (Table 15).

Discussion

This report presents clear evidence of decreased Cu and Zn in pretumor tissue resulting from exposure to an organic carcinogen. Olsen et al. (1954) report DMBA decreases liver Zn during the period of carcinogen-induced liver damage but they do not present any data. Arnold and

Figure 23. The percentage decreases of Cu levels on the high molecular weight protein pool from non-cancerous livers of DEN-exposed mice. The 8 week point is from a subsequent study in which mice were exposed to 25 ppm DEN. Each point at 0, 3 and 6 weeks represents 2 animals while those from 8 and 12.5 weeks represents 3 animals.

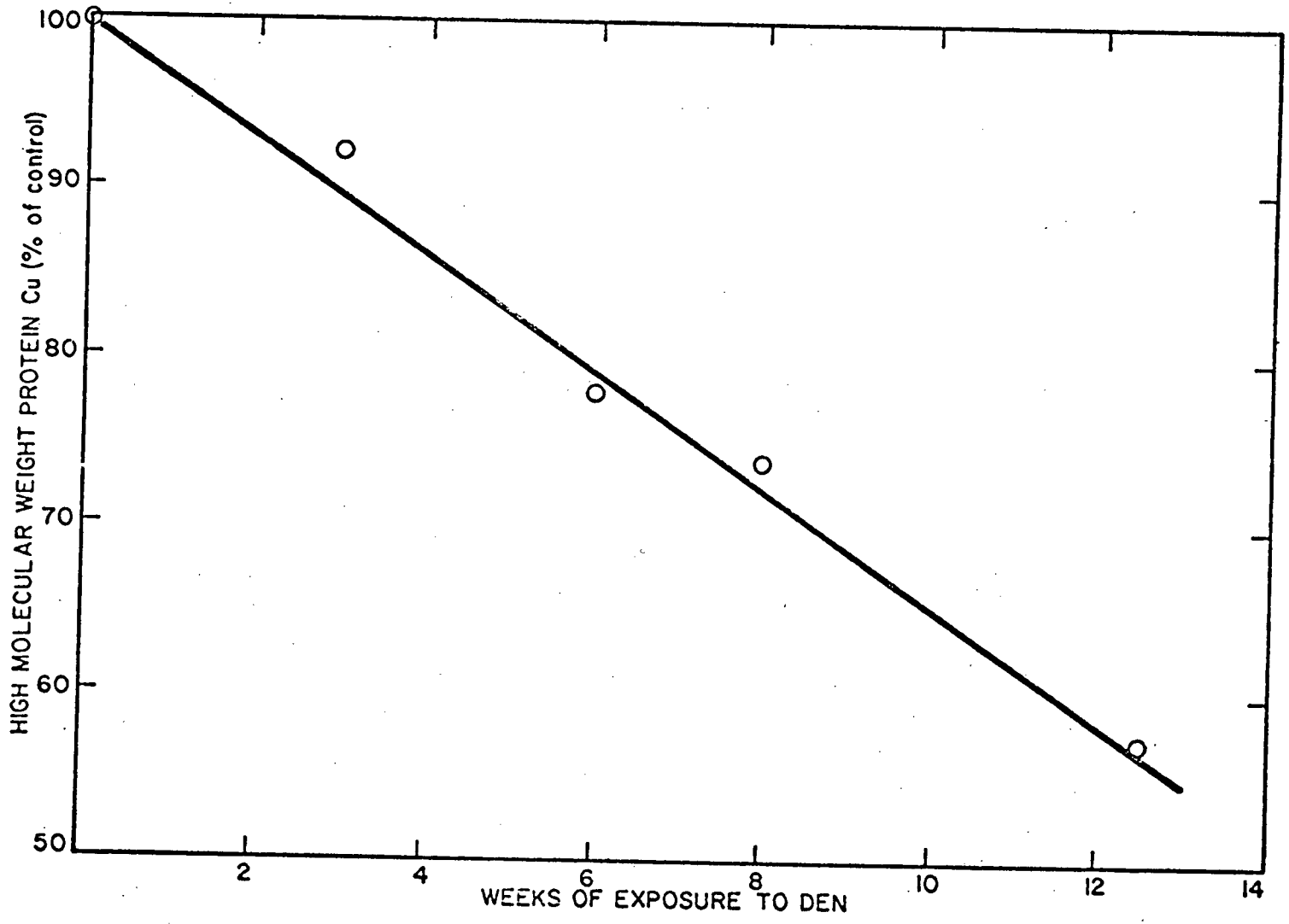

HIGH MOLECULAR WEIGHT PROTEIN Cu (% of control)
100
90
80
70
60
50
2
4
6
8
10
12
14
WEEKS OF EXPOSURE TO DEN

Figure 24. The percentage decreases of Zn levels on the high molecular weight protein pool from non-cancerous livers of DEN-exposed mice. The 8 week point is from a subsequent study in which mice were exposed to 25 ppm DEN. Each point at 0, 3 and 6 weeks represents 2 animals while those from 8 and 12.5 weeks represents 3 animals.

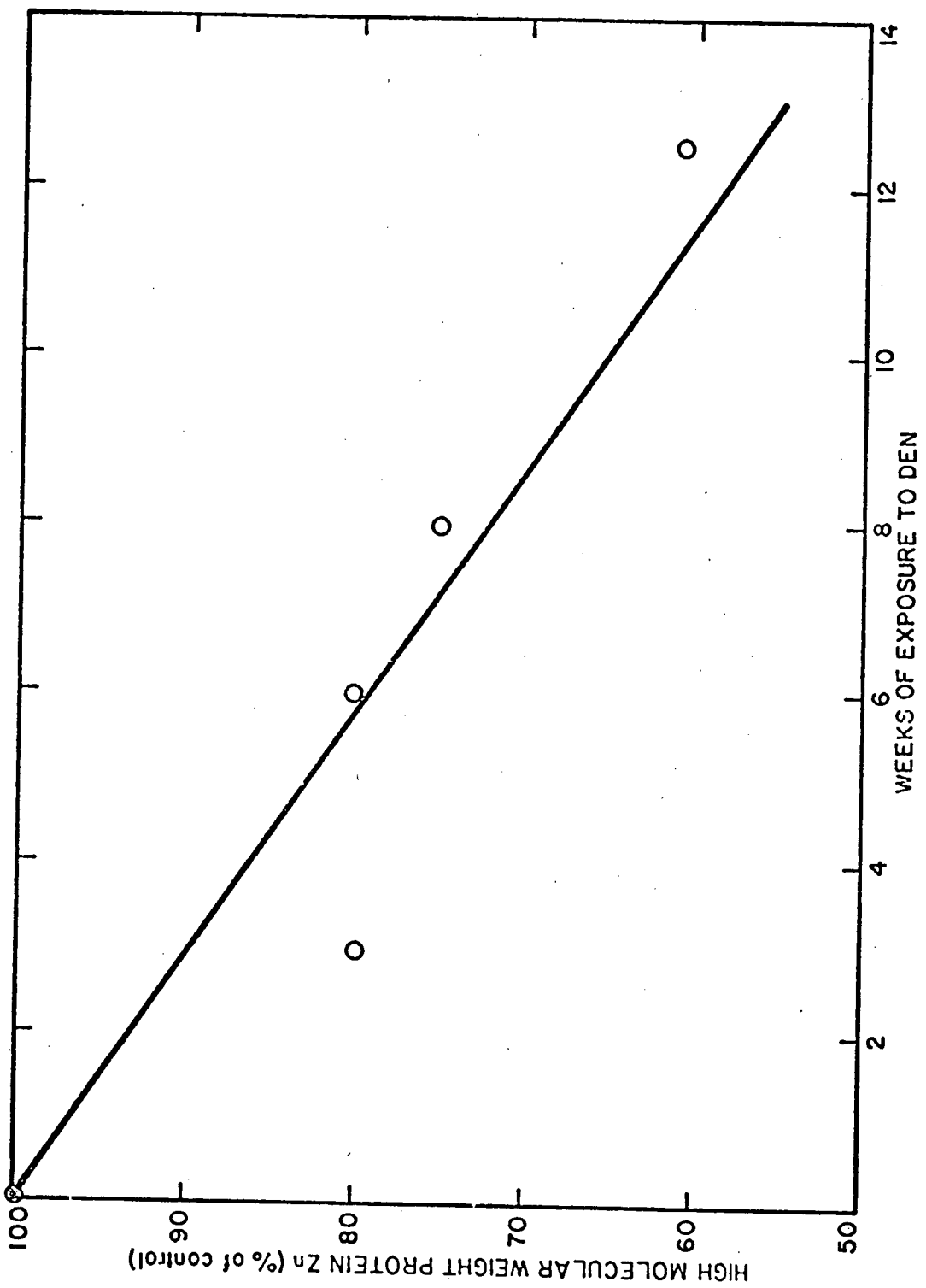

HIGH MOLECULAR WEIGHT PROTEIN Zn (% of control)
100
90
80
70
60
50
2
4
6
8
10
12
14
WEEKS OF EXPOSURE TO DEN

Table 15. Changes in histology of liver after 12.5 weeks exposure to DEN (diethylnitrosamine) with and without Cd or Zn.

		Hepatocytes					Portal duct areas		Overall histology change rating[a]	Gross cholangio-fibrosis rating[b]
		Increased regeneration	Dysplasia	Reticular collapse	Hepatitis	Hepatocellular necrosis	Portal triaditis	Bile duct cell proliferation and disarray		
Control	#1	-[c]	-	-	-	-	-	-	-	-
	#2	-	-	-	-	-	-	-	-	-
	#3	-	-	-	-	-	-	-	-	-
DEN	#1	+[d]	+	-	-	-	+	+	1½	3
	#2	+	-	-	-	+	+	-	1	0
	#3	+	+	+	+	-	-	+	2	2
DEN + Zn	#1	-	-	-	+	-	-	+	1	2
	#2	-	-	-	-	+	+	-	1½	0
	#3	-	-	-	-	-	+	-	½	0
DEN + Cd	#1	+	+	+	+	-	-	+	3	3
	#2	-	-	-	-	-	+	-	½	1
	#3	+	+	+	-	-	+	+	2	2

[a] From 0 to 3, based on microscopic examination of phosphotungstic acid hemotoxylin stained tissue.

[b] From 0 to 3, based on macroscopic examination at time of autopsy.

[c] Absent.

[d] Present.

Sasse (1961) indicate that liver Cu and Zn are decreased in nontumorous areas of liver of rats with DMBA-induced liver tumors. The present study confirms that an organic carcinogen can decrease liver Zn and Cu and, demonstrates unequivocally that these changes occur before tumor growth is evident.

Decreases of Cu and Zn in pretumor tissue are mainly from the high molecular weight protein pool. Since enzymes occur in the high molecular weight protein fraction of cell cytoplasm, the levels of Cu and Zn in this pool give a good indication of the levels of these elements available for metalloenzyme functioning (Brown, 1977; Brown et al., 1977). In recent years, it has become apparent that excesses of heavy metals are stored on metallothionein (Bremner and Davies, 1974; Riordan and Gower, 1975; Winge et al., 1975). Thus, if carcinogen-induced decreases of Cu and Zn were only from metallothionein, these decreases would have little effect on enzyme function and hence cellular processes.

The slight decrease of Cd on the high molecular weight protein pool of DEN-exposed mice was unexpected. Many researchers report increases of Cd and the Cd:Zn ratio in tumors and the apparently normal livers of tumor-bearing organisms (Gorodiskii et al., 1956; in Furst and Haro, 1969; Teitz et al., 1975; Morgan, 1970, 1971; Brown, 1977). The observed Cd decreases concurrent with decline

of Cu and Zn levels might be typical of early metal changes in pretumorous liver. Olson et al. (1954) report liver Zn to initially decrease in pretumor tissue but to peak just before gross neoplasia is evident. Zinc remains decreased in the tumor tissue and is increased only in surrounding liver tissue (Olson et al., 1958; Wright and Dormandy, 1972). Arnold and Sasse (1961) found that Cu is decreased in liver tissue surrounding liver tumors but is greatly increased in the tumor tissue itself. This seems to indicate that DMBA decreases Cu in apparently normal liver tissue but that Cu increases with development of tumor tissue. Thus, if Cd continues to follow the patterns of Cu and Zn, it might increase just as or after the tumor starts in tumor and/or surrounding tissue.

The increase of Cd on the high molecular weight protein pool of mice given DEN + Cd was similar to increases of Cd on the high molecular weight protein pool of tumor-bearing flounders (Brown, 1977). In both studies Cd accumulates on this pool at levels too low to have saturated metallothionein (Brown, 1977). Perhaps Cd is simply occupying enzyme binding sites vacated by Cu and Zn so that induction of metallothionein does not occur. As Cd is increased in tumor-bearing organisms (Teitz et al., 1957; Morgan, 1970, 1971; Brown, 1977), it may be of some significance that exposure to DEN + Cd in the present study resulted in liver pathologies most advanced towards tumor

development. Also, large decreases of Cu and Zn occurred earlier with DEN + Cd, perhaps indicative of earlier pre-tumor metal changes. Furthermore, there is some evidence that Cu and Zn levels are increasing at 12.5 weeks' exposure to DEN + Cd, perhaps indicative of increases of these metals with the onset of cancer.

That administration of Zn with DEN reduced losses of Cu as well as Zn is suggestive. Perhaps Zn in the drinking water occupies sites on DEN which would otherwise bind Cu and Zn in liver. Thus, DEN alone could result in chelation and subsequent excretion of Cu and Zn, while a DEN-Zn complex could not. As explained by Furst (1963), the actions of most or all organic carcinogens can be explained as those of heavy metal chelators.

The reduction of pretumorous changes by Zn in this study is in accordance with previous reports in the literature stating that Zn prevents cancer from occurring; including spontaneously occurring mammary gland cancer (Bischoff and Long, 1939), cancer induced by Cd (Gunn et al., 1963, 1964), or cancer induced by DMBA (Poswillo and Cohen, 1971; Ciapparelli et al., 1972). Zn also prevents the spread of transplanted tumors (Duncan et al., 1974, Duncan and Dreosti, 1975, 1976; Woster et al., 1975). It appears as though the literature on Zn is contradictory since both Zn excesses and Zn deficiencies can prevent the growth of tumors (Bischoff and Long, 1939; Gunn et al.,

1963, 1964; Petering et al., 1967; McQuitty et al., 1970; Poswillo and Cohen, 1971; Ciapparelli et al., 1972; DeWys and Pories, 1972; Duncan et al., 1974; Duncan and Dreosti, 1975, 1976; Woster et al., 1975). This may be because Zn deficiencies could lessen the activities of Zn-dependent enzymes essential for cell division (Duncan et al., 1974; Duncan and Dreosti, 1976; Vallee, 1976) while Zn excesses might reverse or prevent the processes which result in carcinogenesis. Copper, like zinc, has been shown to have an inhibitory effect on DMBA-induced tumors (Sharpless, 1946; Howell, 1958; Fare and Woodhouse, 1963a, b; Fare, 1964).

As summarized by Becking (1976), deficiencies of both Zn and/or Cu result in reductions in activity of the hepatic drug metabolizing center, which is essential for the conversion of carcinogens to noncarcinogenic metabolities. Yoshida et al. (1975) found that high levels of Cd could result in inhibition of the hepatic drug metabolizing center. Dysfunction may possibly be a result of Cd replacing Cu on metalloenzymes in this enzyme system. Thus, carcinogen-induced reductions of Cu and Zn and high Cd levels could reduce the detoxification of subsequent exposures to carcinogens. It has been proposed that Cu prevents the onset of cancer when administered with DMBA by stimulating activity of the drug metabolizing center (Yamane et al., 1969). Perhaps Zn administration has a similar effect since

Zn deficiencies inhibit this enzyme system (Becking, 1976).

Losses of Zn from the liver might result in dysfunction of Zn-containing enzymes involved in cell division (Duncan and Dreosti, 1976; Vallee, 1976; Brown, 1977). High levels of Cd could similarily disturb cell division by replacing Zn on these enzymes (Brown, 1977). Therefore, it has been proposed that Zn prevents cancer when administered with a carcinogen by preventing interference of Cd with Zn-requiring enzymes involved in cell division (Brown, 1977).

Chvapil et al. (1972) have suggested that Zn is important in preventing cancer via stabilization of lysosomal membrances. This theory suggests that the breakdown of lysosomal membrances results in losses of proteolytic enzymes which damage DNA and RNA. Chvapil et al.,(972) state that Zn stabilizes macromolecules which are part of or closely related to biomembranes. Recent evidence is that Zn stimulates the assembly of tubulin into microtubules (Gaskin and Kress, 1977). These microtubules are responsible for moving chromosomes during cell division and for maintaining the continuity of cellular membranes (Gaskin and Kress, 1977; Miller, 1977). Zinc can also prevent lipid peroxidation induced by agents such as CCl_4. Peroxidation modifies the three dimensional conformational structure of fatty acids, thus weakening lipoprotein cell and organelle membranes. Others have suggested that Zn prevents cancer by increasing the immune response (Woster

et al., 1975; Nieper, 1977). This may be due in part to the fact that lymphocytes are more viable in the presence of Zn (Chvapil et al., 1972).

Both Cu and Zn have been shown in this study to be reduced by an organic carcinogen in pretumor tissue, and both have been shown in other studies to prevent or slow down the onset of cancer when administered with an organic carcinogen. Therefore, it appears likely that the reductions of Cu and Zn in pretumor tissue are in some way responsible for the onset of cancer. Hence, alleviation of carcinogen-induced or naturally occurring deficiencies of these elements may result in a reduction, or even prevention, of cancer.

Summary

Mice were given DEN (diethylnitrosamine) in their drinking water with and without supplementation with Cd or Zn. Gel elution profiles were done on liver tissue homogenate supernatant at 0, 3, 6 and 12.5 weeks of exposure. All livers from DEN- and DEN + Cd- or DEN + Zn-exposed mice were pretumorous. DEN alone resulted in decreases of high molecular weight protein Cu and Zn with increasing time of exposure. Copper was decreased 42.6% and Zn 39.1% on the high molecular weight protein pool after 12.5 weeks of exposure. Zinc supplementation resulted in lesser depletions of Cu and Zn. Cd supplementation increased Cu and Zn losses at 3 and 6 weeks but not at 12.5 weeks, and resulted in Cd accumulation on the high molecular weight protein pool.

At 12.5 weeks, hepatic cell regeneration and dysplasia were higher in mice receiving DEN alone or DEN + Cd than in those exposed to DEN + Zn. Gross cholangiofibrosis was evident in all 3 of the mice exposed to DEN + Cd, 2 of the mice exposed to DEN alone, 1 of the mice exposed to DEN + Zn, and none of the controls.

Decreases of Cu and Zn in pretumorous livers of animals fed DEN are proposed to be relevant in the etiology of cancer as both Cu and Zn have been shown in other studies to prevent cancer when given with DMBA (Dimethylaminoazobenzene). It is suggested that this preventative effect is due to the prevention of losses of Cu and Zn. Possible mechanisms of cancer inducement resulting from reduced levels of Cu and Zn are discussed.

CHAPTER X Decreases of Copper and Zinc in Pretumorous and Posttumorous Livers of Mice Exposed to Diethylnitrosamine, With and Without Cadmium or Zinc Supplementation

Preface

This paper was written by D. A. Brown (1978), and will be submitted shortly for publication. The purpose of this study was to follow changes in Cd, Cu and Zn from pre- to posttumorous stages. As in pretumorous liver (Brown and Chan, 1978), posttumorous liver had decreases of Cd, Cu and Zn. Thus, it was not demonstrated that an organic carcinogen can increase Cd and the Cd:Zn ratio in the high molecular weight protein pool; these changes were found previously in cancerous fish (Brown, 1977) and humans (Brown and Knight, 1978). As in Brown and Chan (1978), DEN exposure resulted in decreases of high molecular weight protein Cu and Zn. Time to death due to cancer, increased in those exposures where high molecular weight protein Cu and Zn were maintained closer to, or higher than control levels.

Introduction

Recently we have reported that administration of 25-40 mg diethylnitrosamine (DEN)/L in the drinking water results in depletion of copper and zinc in pretumorous liver tissue up to 12.5 weeks' exposure (Brown and Chan, 1978). Furthermore, we found that administration of 250 mg

Zn/L with DEN resulted in lesser depletions of Cu and Zn. When 5 mg Cd/L was administered with DEN, there were greater depletions of Cu and Zn up to six weeks' exposure, but not at 12.5 weeks. Most of this Cd accumulated in the high molecular weight protein pool, rather than in metallothionein as is usual for excesses of Cd in liver tissue (Brown, 1977). These increases of Cd in the high molecular weight protein pool were similar to increases of Cd in the high molecular weight protein pool of tumor-bearing flounders (Brown, 1977) and human cancer victims (Brown and Knight, 1978). In a study of trace element interactions in duck liver and kidney, it was determined that excesses of Cd might occur in the high molecular weight protein pool if there were deficiencies of Zn in this pool (Brown and Chatel, 1978a). Therefore, as DEN reduces levels of Zn in the high molecular weight protein pool, it is likely that Cd simply occupies binding sites vacated by Zn in this pool.

In the present study, mice are exposed to DEN until tumors develop. Copper, zinc and cadmium levels are monitored throughout this exposure period. Another group is given DEN + 1000 mg Zn/L to determine if higher levels of Zn than in the previous study (Brown and Chan, 1978) can completely reverse the carcinogen-induced losses of Cu and Zn. Another group receives DEN + 100 mg Cd/L to determine the effect of high levels of Cd on carcinogenesis.

Materials and Methods

Mice of the Swiss strain, approximately two months old, were separated into cages, 3 mice of the same sex per cage. Mice were administered either tap water, tap water with 25 mg DEN/L, 25 mg DEN/L + 1000 mg Zn (as $ZnSO_4 \cdot 7H_2O$)/L, or 25 mg DEN/L + 100 mg Cd (as $CdCl_2$)/L. Drinking solutions were changed on alternate days. All water jars were painted black to prevent photodegradation of DEN. Mice were fed Purina rat chow ad libitum.

Three mice from each exposure type were sampled at 0, 8 and 17 weeks after the start of exposure via asphyxiation with CO_2. Remaining mice were sampled after death. Only the first six of these mice to die in each exposure type were analyzed for trace element levels in the present study; six control mice were also sampled after six DEN-exposed mice had died. All mice were examined for presence of visible tumors and then frozen until analyzed.

Mice were analyzed four at a time, one from each exposure type (i.e., 1 Control, 1 DEN, 1 DEN + Zn, and 1 DEN + Cd) in order to ensure consistency of analysis between different exposures. Two grams of liver were homogenized for exactly 3 minutes in a homogenizing tube with 4.5 ml of 0.9% NaCl using a TRI-R STIR-R laboratory motor model S63C set to a standard speed (setting 4.5) and equipped with a teflon homogenizer. Homogenates were centrifuged for 10 minutes at 27,000 x g in a Sorvall RC2-B

centrifuge. Supernatants were collected and pellets re-homogenized for exactly 2 minutes in 2.5 ml of 0.9% NaCl. These were then centrifuged at 27,000 x g for 10 minutes and the supernatants combined with previous supernatants. Groups of 4 supernatants were then placed in a 70°C water bath for exactly 5 minutes to clear cellular debris via heat precipitation. These were then recentrifuged at 27,000 x g for 10 minutes and the supernatants collected.

Total tissue homogenate supernatant metal levels were determined; Cu and Zn and higher Cd levels by flame, and lower Cd levels by graphite furnace atomic absorption spectrophotometry. Two ml of each of the homogenate supernatants from each exposure type from 0, 8 or 17 weeks were then combined, and five ml of this applied to a Pharmacia column (1.6 x 100 cm) packed with G-75 gel. This was eluted with 0.01 M NH_4HCO_3 and collected as 15 ml fractions. Absorbance was read at 250 and 280 nm on a Perkin Elmer 124D spectrophotometer on each fraction to establish the position of the high molecular weight protein pool, metallothionein and the low molecular weight cytoplasmic pool (Brown et al., 1977; Brown and Chan, 1978). Metal levels were determined in each fraction with methods similar to total metal levels. Total metal levels for each cytoplasmic pool were determined by summing of the individual metal levels in each fraction of each pool. Gel elution profiles were not done on animals which were

collected after death as these were not frozen immediately; therefore it is likely that proteolytic enzymes would have decreased the high molecular weight protein pool, and increased the low molecular weight cytoplasmic pool, thus providing misleading results.

Results

Total tissue homogenate supernatant Cu levels were decreased by exposure to DEN (Tables 16 and 17). These decreases ranged from 21.8% at 8 weeks to 28.0% at the time of death. Total Cu levels were unchanged in mice exposed to DEN + Zn for 8 weeks, and reached a maximum decrease of only 8.1% at the time of death. Exposure to DEN + Cd resulted in increases of Cu up to 54.0% (Tables 16 and 17, Figures 25 and 26).

In mice exposed to DEN or DEN + Zn, these reductions in Cu levels reflected decreases of Cu in the high molecular weight protein pool (Table 18). In mice exposed to DEN + Cd for 8 weeks, there was a decrease of Cu in the high molecular weight protein pool, but large increases in metallothionein, and smaller increases in the low molecular weight cytoplasmic pool. After 17 weeks' exposure to Cd, Cu was decreased only slightly in the high molecular weight protein pool, and increased slightly on metallothionein and the low molecular weight cytoplasmic pool (Table 18).

Total Zn levels decreased progressively from 8 weeks'

Table 16. Levels of Cd, Cu and Zn in liver tissue homogenate supernatant of mice exposed to diethylnitrosamine (DEN) with and without supplementation with Cd or Zn. Values are expressed in μmole/g tissue (wet weight).

	Cd	Cu	Zn
0 Weeks			
Control (3)	0.00068[a]	0.0373	0.0739
	(0.00017)[b]	(0.0033)	(0.0055)
8 Weeks			
Control (3)	0.00062	0.0344	0.0664
	(0.00012)	(0.0032)	(0.0062)
DEN (3)	0.00029*	0.0269	0.0546*
	(0.00013)	(0.0080)	(0.0038)
DEN + Zn (3)	0.00043	0.0345	0.0583
	(0.00020)	(0.0077)	(0.0080
DEN + Cd (3)	0.1266**	0.0448	0.0920
	(0.0116)	(0.0079)	(0.0189)
17 Weeks			
Control (3)	0.00064	0.0291	0.0772
	(0.00028)	(0.0010)	(0.0022)
DEN (3)	0.00118	0.0220**	0.0566*
	(0.00118)	(0.0006)	(0.0058)
DEN + Zn (3)	0.00024*	0.0279	0.0589**
	(0.00008)	(0.0026)	(0.0022)
DEN + Cd (3)	0.1611**	0.0448**	0.0935*
	(0.0134)	(0.0019)	(0.0094)
Time of Death			
Control (6)	0.00053	0.0236	0.0739
	(0.00022)	(0.0028)	(0.0115)
DEN (6)	0.00032*	0.0170*	0.0413**
	(0.00016)	(0.0061)	(0.0184)
DEN + Zn (6)	0.00040	0.0217	0.0863
	(0.00013)	(0.0038)	(0.0528)
DEN + Cd (6)	0.2689**	0.0274	0.1919*
	(0.0635)	(0.0085)	(0.0822)

[a] Mean.

[b] Standard deviation.

* $P < 0.05$; Student's t test.

** $P < 0.001$.

Table 17. Percentage changes of Cu and Zn levels in total tissue homogenate supernatant from livers of mice exposed to diethylnitrosamine (DEN), with and without Cd or Zn supplementation.

	Cu	Zn
8 Weeks		
DEN (3)	-21.8	-17.8
DEN + Zn (3)	+0.1	-12.2
DEN + Cd (3)	+30.2	+38.6
17 weeks		
DEN (3)	-24.4	-26.7
DEN + Zn (3)	-4.1	-23.7
DEN + Cd (3)	+54.0	+21.1
Time of Death		
DEN (6)	-28.0	-44.1
DEN + Zn (6)	-8.1	+16.8
DEN + Cd (6)	+16.1	+159.7

Figure 25. The variation of Cu levels in cytoplasm from livers of mice exposed to DEN with and without Cd or Zn. Each point at 0, 8 and 17 weeks represents 3 animals, while those at 30 weeks represents 6 animals each. Thirty weeks was the mean time to death of the first 6 animals to die after exposure to DEN, with and without Cd or Zn supplementation.

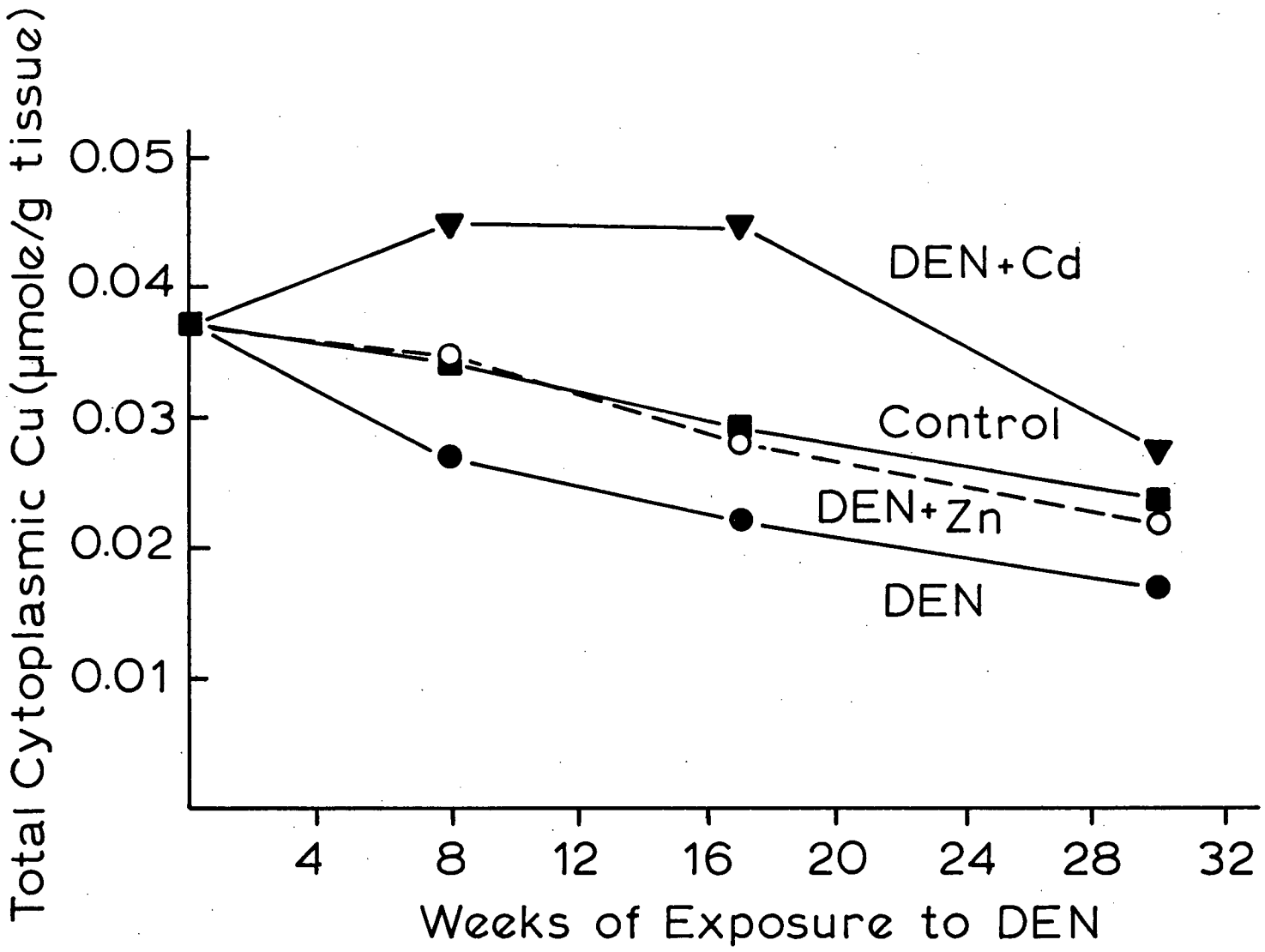

Total Cytoplasmic Cu (µmole/g tissue)
0.05
0.04
0.03
0.02
0.01
DEN+Cd
Control
DEN+Zn
DEN
4
8
12
16
20
24
28
32
Weeks of Exposure to DEN

Figure 26. The variation of Zn levels in cytoplasm from livers of mice exposed to DEN with and without Cd or Zn. Each point at 0, 8 and 17 weeks represents 3 animals, while those at 30 weeks represents 6 animals each. Thirty weeks was the mean time to death of the first 6 animals to die after exposure to DEN, with and without Cd or Zn supplementation.

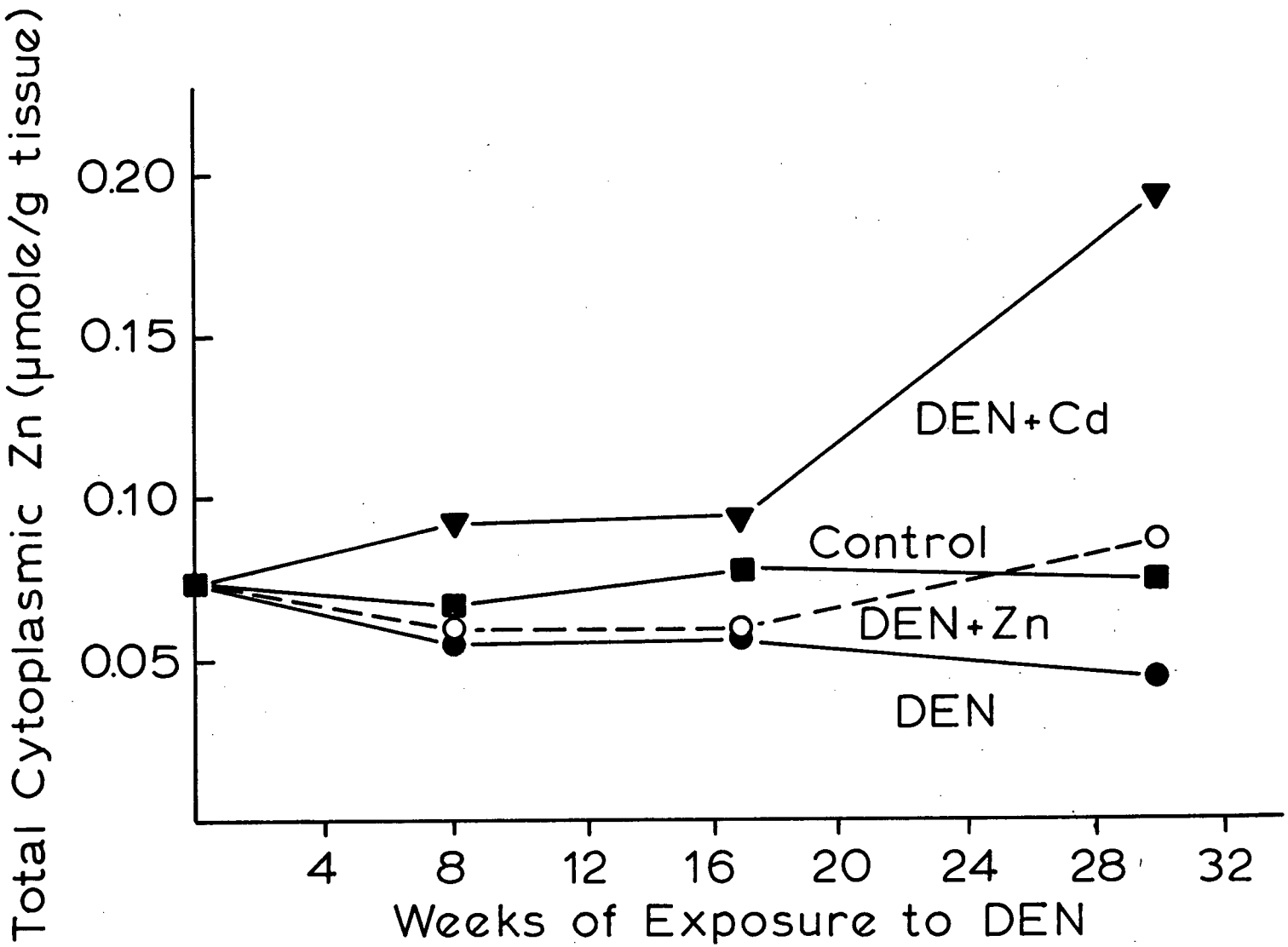

Total Cytoplasmic Zn (µmole/g tissue)
0.20
0.15
0.10
0.05
DEN+Cd
Control
DEN+Zn
DEN
4
8
12
16
20
24
28
32
Weeks of Exposure to DEN

Table 18. The distribution of Cd, Cu and Zn among cytoplasmic pools from livers of mice exposed to diethylnitrosamine (DEN) with and without supplementation with Cd or Zn. Values are expressed in μmole metal in each pool/g tissue (wet weight).

	Cd				Cu				Zn			
	Total	High MW[a] pool	Metallo-thionein	Low MW pool	Total	High MW pool	Metallo-thionein	Low MW pool	Total	High MW pool	Metallo-thionein	Low MW pool
0 Weeks												
Control (3)	.00186	.00092	.00041	.00053	.0520	.0301	.0070	.0149	.0726	.0652	ND[b]	.0074
8 Weeks												
Control (3)	.00168	.00080	.00039	.00049	.0481	.0292	.0062	.0127	.0644	.0626	ND	.0018
DEN (3)	.00153	.00061	.00047	.00045	.0402	.0216	.0068	.0118	.0485	.0471	ND	.0014
DEN + Zn (3)	.00159	.00075	.00042	.00042	.0488	.0294	.0057	.0137	.0536	.0527	ND	.0009
DEN + Cd (3)	.1084	.0212	.0860	.0012	.0630	.0188	.0243	.0199	.0987	.0841	.0089	.0057
17 Weeks												
Control (3)	.00199	.00075	.00040	.00084	.0488	.0281	.0077	.0130	.0668	.0570	.0035	.0063
DEN (3)	.00246	.00090	.00054	.00102	.0374	.0193	.0082	.0099	.0487	.0435	.0008	.0044
DEN + Zn (3)	.00177	.00063	.00036	.00078	.0385	.0198	.0069	.0118	.0478	.0415	.0005	.0058
DEN + Cd (3)	.1927	.1241	.0174	.0512	.0547	.0249	.0085	.0213	.0705	.0553	.0033	.0119

[a]Molecular weight.

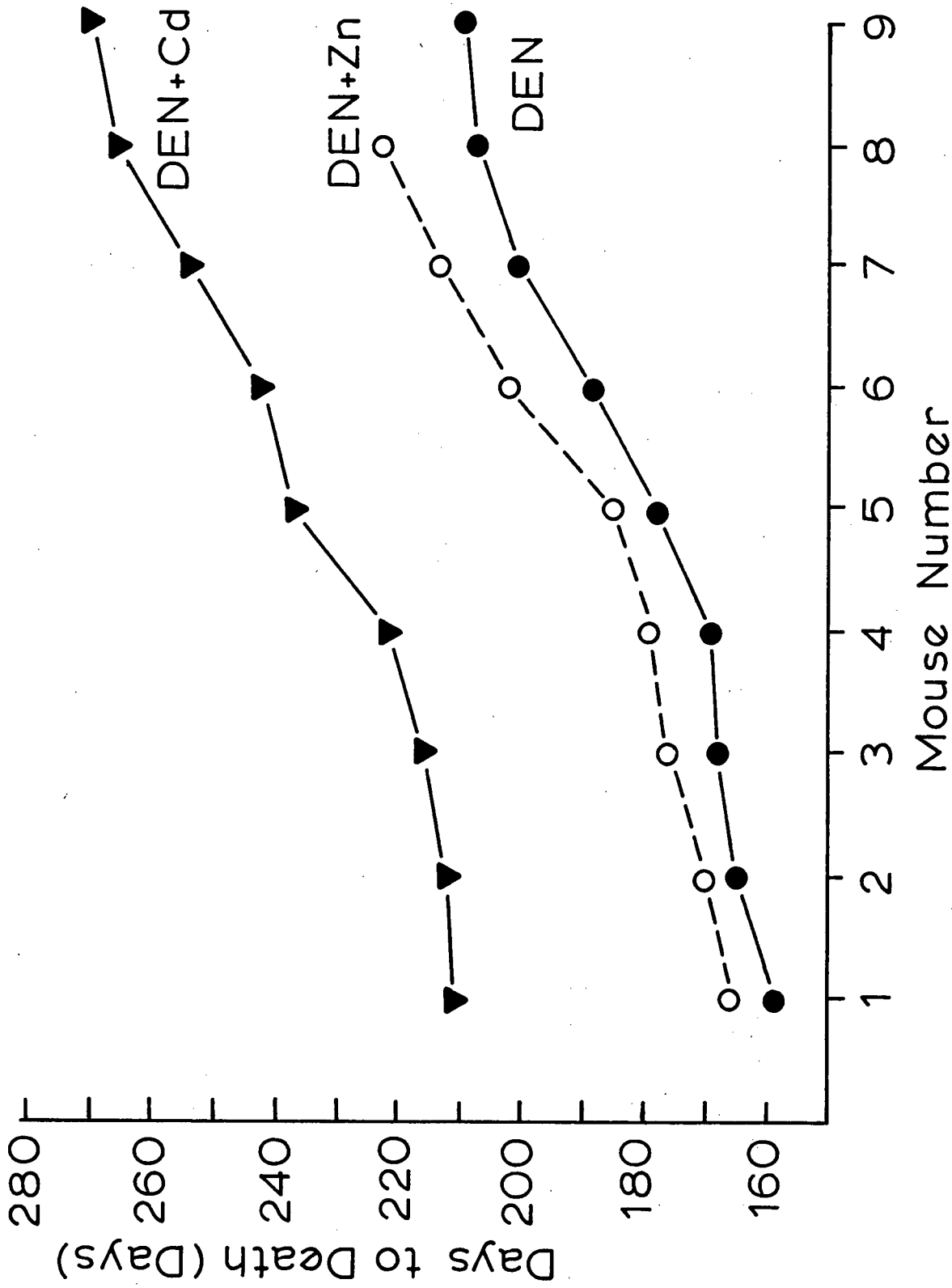
[b]Not detectable.

exposure till the time of death; the maximal decrease at the time of death was 44.1% (Tables 16 and 17). Exposure to DEN + Zn resulted in lesser losses of Zn at 8 and 17 weeks, but increases in Zn at the time of death. However, this increase in Zn at the time of death is a reflection of very high Zn levels in the fifth and sixth longest surviving mice (Figure 27). Zinc levels in the first four mice to die after exposure to DEN + Zn, were actually less than control levels. Exposure to DEN + Cd resulted in increases in Zn ranging up to 159.7% at the time of death (Table 17).

Reductions in Zn levels of animals exposed to DEN or DEN + Zn reflect decreases of Zn mainly from the high molecular weight protein pool after 8 and 17 weeks' exposure (Table 18). After 17 weeks' exposure it is apparent that Zn is also decreased in metallothionein. Exposure to DEN + Cd for 8 weeks resulted in increased levels of Zn in the high molecular weight protein pool and metallothionein. After 17 weeks' exposure to DEN + Cd, Zn was increased only in the low molecular weight cytoplasmic pool, although Cd did seem to reverse losses of Zn from the high molecular weight protein pool, otherwise induced by exposure to DEN alone.

Total Cd levels appeared to be decreased by exposure to DEN at 8 weeks, increased at 17 weeks, and decreased at the time of death (Table 16). Exposure to DEN + Zn resulted in decreases of Cd at all sample times. Exposure

Figure 27. The time to death after commencement of exposure of mice to DEN, with and without Cd or Zn supplementation.


DEN+Cd
DEN+Zn
DEN
Days to Death (Days)
280
260
240
220
200
180
160
1
2
3
4
5
6
7
8
9
Mouse Number

to DEN + Cd resulted in increases of Cd with increases of exposure time.

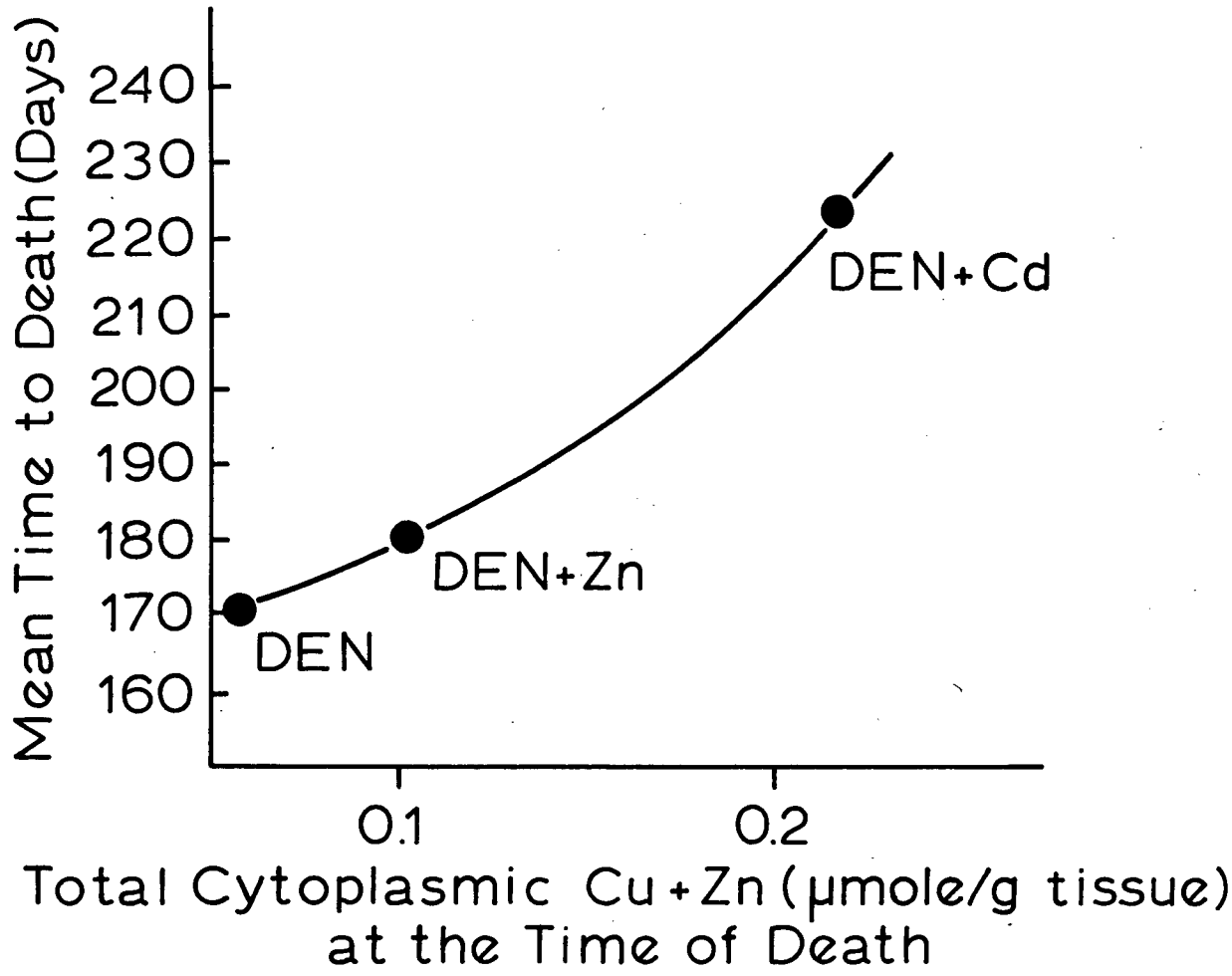
Changes of Cd levels were not as readily apparent on gel profiles of mice exposed to DEN or DEN + Zn as on tissue homogenate supernatant totals. Changes in Cd levels were, for the most part, equidistributed over all three pools (Table 18). Exposure to DEN + Cd resulted in accumulation of most Cd on metallothionein at 8 weeks. However at 17 weeks most Cd was found on the high molecular weight protein pool. There was a large decrease of Cd-thionein from 8 to 17 weeks.

All mice which died after exposure to DEN, with and without Cd or Zn, had readily visible tumors. Survival time appeared to be increased slightly by exposure to DEN + Zn, and by a much larger margin by exposure to DEN + Cd (Figure 27). Mean survival time for the three exposure types appeared to increase with increases of total cytoplasmic Cu + Zn levels at the time of death (Figure 28). The longest surviving mouse was one exposed to DEN + Zn; this mouse was still alive at the time this report was written.

Discussion

This study confirms that DEN results in decreases of Cu and Zn in pretumorous liver tissue, and shows that these decreases are even more prevalent after tumors have developed. Furthermore, the present study appears to suggest that the time to death after exposure to DEN, is directly

Figure 28 The increase in time to death with increases of Cu and Zn in liver, at time of death, of mice exposed to DEN, with and without Cd or Zn supplementation.


Mean Time to Death (Days)
240
230
220
210
200
190
180
170
160
DEN+Cd
DEN+Zn
DEN
0.1
0.2
Total Cytoplasmic Cu+Zn (μmole/g tissue)
at the Time of Death

related to liver Cu + Zn levels. In those mice administered DEN + Zn, losses of Cu and Zn were reduced at 8 and 17 weeks and these levels were actually increased at the time of death. However, this increase at the time of death was a reflection of increases in only two of the six animals, so that overall, Zn administration with DEN was only partially able to reduce losses of Cu and Zn. Likewise, Zn administration increased only slightly (10 days) the mean time to death of the first six mice to die (Figure 27).

Administration of Cd resulted in large increases of total Cu and Zn at all exposure times. These large increases of total Cu + Zn coincided with a 50 day extension of survival time. The possible mechanism by which reductions of Cu and Zn levels might result in carcinogenesis have been discussed by Brown and Chan (1978).

In previous studies it had been found that Cd and the Cd:Zn ratio is increased in tumor-bearing organisms (Morgan, 1970, 1971; Brown, 1977; Brown and Knight, 1978). Furthermore, when only 5 mg Cd/L were given with DEN, there appeared to be an increase in pretumorous liver changes (Brown and Chan, 1978). That results in the present study appear to contradict these previous studies, may be a reflection of the abnormally high Cd levels administered in the present study. High levels of Cd have been shown to be able to deactivate the bioactivation enzyme system (Jerina and Daley, 1974) for organic carcinogens (Yoshida

et al., 1975), i.e., in the presence of high levels of Cd, DEN wouldn't be carcinogenic as it wouldn't be bioactivated.

If losses of Cu and Zn are necessary for carcinogenesis, then the reversal of these losses by Cd may be responsible for the slowing of the carcinogenic process. In a previous study when a lower Cd exposure advanced pretumorous changes, there were increased losses of Cu and Zn after 3 and 6 weeks' exposure (Brown and Chan, 1978). These increased losses of Cu and Zn can be explained as exclusion of Cu and Zn from mucosal metallothionein by Cd, so that these aren't absorbed into the bloodstream (Stonard and Webb, 1976; Evans et al., 1970). However, in the present study it may be that Cd levels were high enough so that the level of mucosal metallothionein was increased so much by induction by Cd, that Cu and Zn uptake levels were increased by the Cd-induced mucosal metallothionein. This argument assumes that Cd levels in the 5 mg Cd/L exposure were not high enough to induce mucosal metallothionein, but rather that Cd simply outcompeted Cu and Zn for preexisting mucosal metallothionein binding sites. Also assumed, is that in the higher exposure, many more mucosal binding sites were created than needed for the level of Cd exposure, so that there were more available binding sites for the uptake of Cu and Zn. Sugawara and Sugawara (1977) found that 100 mg Cd/L in the drinking water induced metallothionein and increased Zn uptake in the duodenal mucosa of rats.

In the present study, exposure to DEN resulted in increases of Cd in tissue only at 17 weeks' exposure to DEN, i.e., just before tumors start. However, Cd was not increased in these mice after tumors developed as it was in cancerous fish (Brown, 1977) and humans (Brown and Knight, 1978). This suggests several possibilities. First, in the present study we have examined Cd levels in the actual tissue in which tumors developed, i.e., the liver. In both cancerous fish and humans, nontumorous tissue was examined. Perhaps Cd is increased in nontumorous tissues in cancerous organisms, and decreased in the tumors. This, however, appears to contradict findings by Tietz et al. (1957) and Gorodiskii et al. (1956; in Furst and Haro, 1969). Second, perhaps the mode of action of DEN is different than that of other carcinogens. Since both cancerous fish and humans have increased Cd levels in the high molecular weight protein pool, this seems to suggest that carcinogens to which these organisms have been exposed, caused these changes. Third, perhaps conditions in this study were unnatural, and normal Cd levels were not available. In another study, when low levels of Cd were given to mice, Cd accumulated on metallothionein (Brown and Chatel, 1978c); when Cd was given with DEN, most accumulated in the high molecular weight protein pool (Brown and Chatel, 1978c). Therefore the carcinogen DEN does have the potential to increase Cd in the high molecular weight protein pool if Cd is available. Perhaps,

if Cd had been available in higher levels, in the present study in mice exposed to DEN (i.e., 5 mg Cd/L, but not as high as 100 mg Cd/L) then the carcinogenic process might have been speeded up. The time to produce tumors via DEN in this study is abnormally long; in the present study 30 weeks were required for death due to tumors, at least 17 weeks were required for tumor formation. Others report only 10 weeks of exposure to DEN as resulting in tumor formation and 15 to 19 weeks in death (Weisburger et al., 1975; Barbason et al., 1977). Fourth, perhaps the increases of Cd and Cd:Zn in the high molecular weight protein pool of cancerous fish and humans are simply coincidental, and irrevelant to carcinogenesis.

After 8 weeks' exposure to DEN + Cd most Cd accumulated in metallothionein, but at 17 weeks there was an 80% drop in Cd-thionein. Most Cd at 17 weeks accumulated in the high molecular weight protein pool. High levels of Cd on the high molecular weight protein pool are usually associated with cancerous organisms (Brown, 1977; Brown and Knight, 1978), but in the present study the development of tumors was slowed with very high levels of Cd in this pool. Perhaps this is due to the high levels of Cu and Zn also occurring in this pool. Alternatively, since there is a large unexplainable drop in metallothionein from 8 to 17 weeks, perhaps high levels of Cd, like high levels of Cu, can cause metallothionein to aggregate (Irons and Smith,

1976). Therefore, high levels of Cd would appear to be in the high molecular weight protein pool, but these would be bound to aggregates of metallothionein, and therefore not affect enzyme functioning. This clumping probably is not responsible for increased high molecular weight protein Cd levels in fish (Brown, 1977) since Cd levels are only 10% of Cd levels in DEN + Cd exposed mice. In human controls and cancer patients, Cd levels are high, but not higher in controls than cancer patients (Brown and Knight, 1978). Therefore, it is unlikely that high levels of Cd were responsible for metallothionein aggregation only in cancer victims.

In this study, as in a previous study (Brown and Chan, 1978), administration of Zn with DEN failed to totally counteract DEN-induced losses of Cu and Zn; however, similarly to this previous study, DEN-induced losses of both Cu and Zn were reduced by administration of Zn with DEN. This suggests that Zn ties up binding sites on DEN, so that DEN is not able to bind, and subsequently remove tissue Cu and Zn (Brown and Chan, 1978).

Other studies have reported that Zn prevents cancer when given with an organic carcinogen (Poswillo and Cohen, 1971; Ciapparelli et al., 1972), Cd (Gunn et al., 1963, 1964), transplanted tumors (Duncan et al., 1974; Duncan and Dreosti, 1975, 1976; Woster et al., 1975), or to mice with spontaneously occurring mammary gland cancer (Bischoff

and Long, 1939). Since we were unable to maintain tissue Zn levels at normal control levels throughout the exposure period, it is difficult to determine, from the present study, if Zn prevents DEN-induced tumors. However, it is of interest, that in the present study, the two longest surviving DEN + Zn exposed mice had by far the highest Zn levels of the first six DEN + Zn exposed mice. Also the longest surviving mouse in the present study was a DEN + Zn exposed mouse.

Perhaps future studies should compare alternate means of administration of both Cu and Zn with DEN, to totally counteract DEN induced losses of Cu and Zn. If at this point, carcinogenesis is not prevented, then it can be concluded that Cu and Zn are not relevant to the etiology of DEN induced tumors. In the present study, Cd prevented losses of Cu and Zn but didn't prevent tumor formation. However, Cd itself is a carcinogen (Flick et al., 1971) and appears to be associated with cancerous organisms (Morgan, 1970, 1971; Brown, 1977; Brown and Knight, 1978). That time to death, in the present study, is related to Cu and Zn levels, seems to suggest that DEN-induced losses of Cu and Zn are important to the development of tumors.

Summary

Mice were exposed to DEN in their drinking water, with or without 1000 mg Zn/L or 100 mg Cd/L. Mice were sampled at 0, 8 and 17 weeks of exposure and at the time of

death. Cadmium, copper and zinc levels were determined in tissue homogenate supernatant. DEN exposure resulted in losses of Cu and Zn, increasing from 21.8% and 17.8% for Cu and Zn respectively, at 8 weeks, to 28.0% and 44.1% at the time of death. Gel elution profiles at 8 and 17 weeks revealed that these losses were mainly from the high molecular weight protein pool. Mean time to death for DEN exposed mice was 183 days. All dead mice had tumors in their livers. Exposure to DEN + Zn reduced losses of Cu and Zn and extended mean survival time to at least 198 days. Exposure to DEN + Cd resulted in increases of Cu and Zn over control values, and increased the mean time to death to 237 days.

CHAPTER XI The Effect of Cadmium Exposure, With or Without Diethylnitrosamine, on the Cytoplasmic Levels and Distribution of Cadmium, Copper and Zinc

Preface

This study was authored by D. A. Brown and K. W. Chatel (1978c), and will be submitted shortly for publication. The study was conducted in order to investigate whether or not DEN increased high molecular weight protein pool Cd when administered with Cd. In a previous study, it was demonstrated that Cd accumulated in the high molecular weight protein pool when administered with DEN, but Cd was not administered alone as a control. This study demonstrates that Cd administered alone occurs on metallothionein, but Cd administered with DEN accumulates more in the high molecular weight protein pool; however, this effect was demonstrated only at low Cd exposures ($<$100 mg Cd/L in the drinking water). At higher Cd exposures, metallothionein became Cd saturated and there was a "spillover" of Cd to the high molecular weight protein pool with concurrent pathological effects.

Thus, the study demonstrates that an organic carcinogen does have the potential to increase high molecular weight protein Cd, as found in fish (Brown, 1977) and humans (Brown and Knight, 1978), if Cd is readily available. Results from this study also strongly support the "spillover" theory suggested by Brown et al (1977), and Brown

and Parsons (1978).

Introduction

Recently it has become apparent that Cd, in cancerous organisms, occurs mainly in the high molecular weight protein pool, in apparently normal liver and kidney tissue (Brown, 1977; Brown and Knight, 1978). In noncancerous organisms, cadmium occurs mainly bound to metallothionein (Brown and Knight, 1978). Metallothionein is a low molecular weight protein which can bind Cd, Hg, Cu and Zn, thus rendering them nontoxic as they are no longer available to bind enzymes (Brown et al., 1977). Copper and zinc are less toxic than Hg or Cd as they are natural components of many metalloenzymes (Friedbert, 1974; Bremner, 1974).

It has been established that toxic effects of Cd or Hg do not occur until these elements appear in large quantities in the high molecular weight protein pool (Winge et al., 1973; Brown and Parsons, 1978; Cloutier and Brown, 1978). Since Cd occurs in the high molecular weight protein pool of cancerous organisms, it is possible that Cd is important in the carcinogenic process. Brown (1977) has hypothesized that since Cd displaces Zn from Zn binding sites in metalloenzymes, Cd in cancerous organisms might be interferring with the Zn metalloenzymes involved in the regulation of cell division; e.g., DNA polymerase, RNA polymerase, reverse transcriptase, and thymidine kinase (Vallee, 1976; Duncan and Dreosti, 1976).

The present study aims to confirm that an organic carcinogen (diethylnitrosamine or DEN) can influence the cytoplasmic distribution of cadmium; in particular that DEN can increase the levels of Cd bound to the high molecular weight protein pool and concurrently decrease the levels of Cd bound to metallothionein. In a previous study, when 5 mg Cd/L were administered with DEN, most of this Cd accumulated in the high molecular weight protein pool (Brown and Chan, 1978). However, in that study Cd was not administered without DEN to demonstrate that the Cd would otherwise have been bound to metallothionein. In the present study, various levels of Cd are administered, with and without DEN, to examine whether or not DEN does indeed influence the cytoplasmic distribution of Cd.

Materials and Methods

Male mice of the Swiss strain, approximately two months old, were separated into cages, 3 mice per cage. Mice were administered either tap water, or tap water containing 25, 50, 100, 200 or 500 mg Cd (as $CdCl_2$)/L, with or without 25 mg DEN/L. Drinking solutions were changed on alternate days. Jars which contained DEN were painted black to prevent photodegradation of the DEN.

Mice were sacrificed after 28 days' exposure, via asphyxiation with CO_2. The animals were frozen until analyzed. Mice were analyzed six at a time, three from each of the two exposure types with the same Cd concentra-

tion. This was done to ensure consistency of analysis between samples with the same Cd concentration. Livers were removed, weighed, and then 2 grams or less of liver was homogenized in 4.5 ml of 0.9% NaCl for exactly 3 minutes, in a homogenizing tube, using a TRI-R STIR-R laboratory motor S63C set to a standard speed (setting 4.5) and equipped with a teflon homogenizer. Homogenates were centrifuged for 10 minutes at 27,000 x g in a Sorvall RC2-B centrifuge. Supernatants were collected and pellets rehomogenized for exactly 2 minutes in 2.5 ml of 0.9% NaCl. These were then centrifuged for 10 minutes at 27,000 x g and the supernatants combined with previous supernatants. Groups of 6 combined supernatants were then heated in a 70°C water bath for exactly 5 minutes, to clear cellular debris via heat precipitation. These were centrifuged for 10 minutes at 27,000 x g and the supernatants collected.

Two ml of each of the heat stable homogenate supernatants were combined, and 5 ml of this was applied to a Pharmacia column (1.6 x 100 cm) packed with Sephadex G-75 gel. This was eluted with 0.01 M NH_4HCO_3 and collected as 15 ml fractions. Absorbance was read at 250 and 280 nm on each fraction using a Perkin Elmer 124D spectrophotometer to establish the position of the high molecular weight protein pool, metallothionein, and the low molecular weight cytoplasmic pool (Brown et al., 1977; Brown and Chan, 1978). Cadmium, Cu and Zn levels were determined on each

fraction using the flame method on a Perkin Elmer 303 atomic absorption spectrophotometer equipped with deuterium arc background correction. Total metal levels in each cytoplasmic pool were determined by summing of the individual metal levels in each fraction of each pool.

Results

Typical gel elution profiles from controls and the 25 mg Cd/L exposure, with or without DEN exposure, are shown in Figures 29 and 30. Two thirds of cytoplasmic Cd was bound to metallothionein in controls (Tables 19 and 22). With Cd administration alone, more than 75% of Cd was bound to metallothionein up to 100 mg Cd/L exposure (Table 22). There appeared to be a plateau of Cd-thionein production from 100 to 200 mg Cd/L exposure, with or without DEN (Figure 32); in mice exposed to Cd alone, this plateau coincided with a large increase of Cd in the high molecular weight protein pool (Figures 31 and 32). There was a large acceleration of Cd-thionein production at the 500 mg Cd/L exposure, with or without DEN (Tables 19 and 22, Figure 32). Exposure to DEN with Cd, resulted in a 2.8-fold increase of Cd in the high molecular weight protein pool at 25 mg Cd/L exposure compared with Cd exposure alone, a 1.4-fold increase at 50 mg Cd/L and a 1.3-fold increase at 100 mg Cd/L (calculated from Table 19). At the 200 and 500 mg Cd/L exposures, more Cd was bound to the high molecular weight protein pool without concurrent

Table 19. The cytoplasmic distribution of Cd with exposure to a range of Cd concentrations, with or without diethylnitrosamine (DEN). Data is a compilation of values from gel profiles, in μmole/g tissue (wet weight). MW: molecular weight. ND: not detectable.

	Cd			
	Total	High MW pool	Metallo-thionein	Low MW pool
Control	.0036	.0004	.0024	.0008
25 mg Cd/L	.0194	.0047	.0147	ND
25 mg Cd/L + DEN	.0174	.0123	.0051	ND
50 mg Cd/L	.0765	.0073	.0655	.0037
50 mg Cd/L + DEN	.0615	.0103	.0483	.0029
100 mg Cd/L	.0880	.0145	.0714	.0021
100 mg Cd/L + DEN	.1188	.0187	.0985	.0016
200 mg Cd/L	.1593	.0822	.0678	.0093
200 mg Cd/L + DEN	.1408	.0303	.1081	.0024
500 mg Cd/L	.4125	.0849	.3276	ND
500 mg Cd/L + DEN	.5615	.0464	.5151	ND

Table 20. The cytoplasmic distribution of Cu with exposure to a range of Cd concentrations, with or without diethylnitrosamine (DEN). Data is a compilation of values from gel profiles, in μmole/g tissue (wet weight). MW: molecular weight. ND: not detectable.

	Cu			
	Total	High MW pool	Metallo-thionein	Low MW pool
Control	.072	.030	.018	.024
25 mg Cd/L	.083	.044	.028	.011
25 mg Cd/L + DEN	.053	.026	.014	.013
50 mg Cd/L	.092	.029	.030	.033
50 mg Cd/L + DEN	.089	.022	.032	.035
100 mg Cd/L	.117	.032	.039	.046
100 mg Cd/L + DEN	.096	.026	.035	.035
200 mg Cd/L	.101	.036	.027	.038
200 mg Cd/L + DEN	.084	.029	.029	.026
500 mg Cd/L	.165	.037	.040	.088
500 mg Cd/L + DEN	.161	.044	.049	.068

Table 21. The cytoplasmic distribution of Zn with exposure to a range of Cd concentrations, with or without diethylnitrosamine (DEN). Data is a compilation of values from gel profiles, in μmole/g tissue (wet weight). MW: molecular weight. ND: not detectable.

	Total	High MW pool	Metallo-thionein	Low MW pool
Control	.083	.080	.001	.002
25 mg Cd/L	.120	.101	.013	.006
25 mg Cd/L + DEN	.093	.089	.002	.002
50 mg Cd/L	.126	.098	.020	.008
50 mg Cd/L + DEN	.085	.073	.009	.003
100 mg Cd/L	.102	.079	.021	.002
100 mg Cd/L + DEN	.114	.086	.025	.002
200 mg Cd/L	.137	.126	.007	.004
200 mg Cd/L + DEN	.121	.083	.034	.004
500 mg Cd/L	.175	.115	.047	.013
500 mg Cd/L	.198	.093	.098	.007

Table 22. Percentage distribution of total Cd on each cytoplasmic pool with exposure to a range of Cd concentrations, with or without diethylnitrosamine (DEN). Percentage calculated from Table 19. MW: molecular weight. ND: not detectable.

	Cd		
	High MW pool	Metallo-thionein	Low MW pool
Control	11.1	66.7	22.2
25 mg Cd/L	24.2	75.8	0
25 mg Cd/L + DEN	70.7	29.3	0
50 mg Cd/L	9.6	85.6	4.8
50 mg Cd/L + DEN	16.8	78.5	4.7
100 mg Cd/L	16.5	81.1	2.4
100 mg Cd/L + DEN	15.8	82.9	1.3
200 mg Cd/L	51.6	42.6	5.8
200 mg Cd/L + DEN	21.5	76.8	1.7
500 mg Cd/L	20.6	79.4	0
500 mg Cd/L + DEN	8.3	91.7	0

Table 23. Percentage distribution of total Cu on each cytoplasmic pool with exposure to a range of Cd concentrations, with or without diethylnitrosamine (DEN). Percentage calculated from Table 20.
MW: molecular weight. ND: not detectable.

	Cu		
	High MW pool	Metallo-thionein	Low MW pool
Control	41.7	25.0	33.3
25 mg Cd/L	53.0	33.7	13.3
25 mg Cd/L + DEN	49.1	26.4	24.5
50 mg Cd/L	31.5	32.6	35.9
50 mg Cd/L + DEN	24.7	36.0	39.3
100 mg Cd/L	27.4	33.3	39.3
100 mg Cd/L + DEN	34.5	34.5	31.0
200 mg Cd/L	35.7	26.7	37.6
200 mg Cd/L + DEN	34.5	34.5	31.0
500 mg Cd/L	22.4	24.3	53.3
500 mg Cd/L + DEN	27.3	30.4	42.3

Table 24. Percentage distribution of total Zn on each cytoplasmic pool with exposure to a range of Cd concentrations, with or without diethylnitrosamine (DEN). Percentage calculated from Table 21. MW: molecular weight. ND: not detectable.

	Zn		
	High MW pool	Metallothionein	Low MW pool
Control	96.4	1.2	2.4
25 mg Cd/L	84.2	10.8	5.0
25 mg Cd/L + DEN	95.7	2.2	2.1
50 mg Cd/L	77.8	15.9	6.3
50 mg Cd/L + DEN	85.9	10.6	3.5
100 mg Cd/L	77.5	20.6	1.9
100 mg Cd/L + DEN	75.4	22.8	1.8
200 mg Cd/L	92.0	5.1	2.9
200 mg Cd/L	68.6	28.1	3.3
500 mg Cd/L	65.7	26.9	7.4
500 mg Cd/L + DEN	47.0	49.5	3.5

Figure 29. Composite gel elution profiles from three control mice.

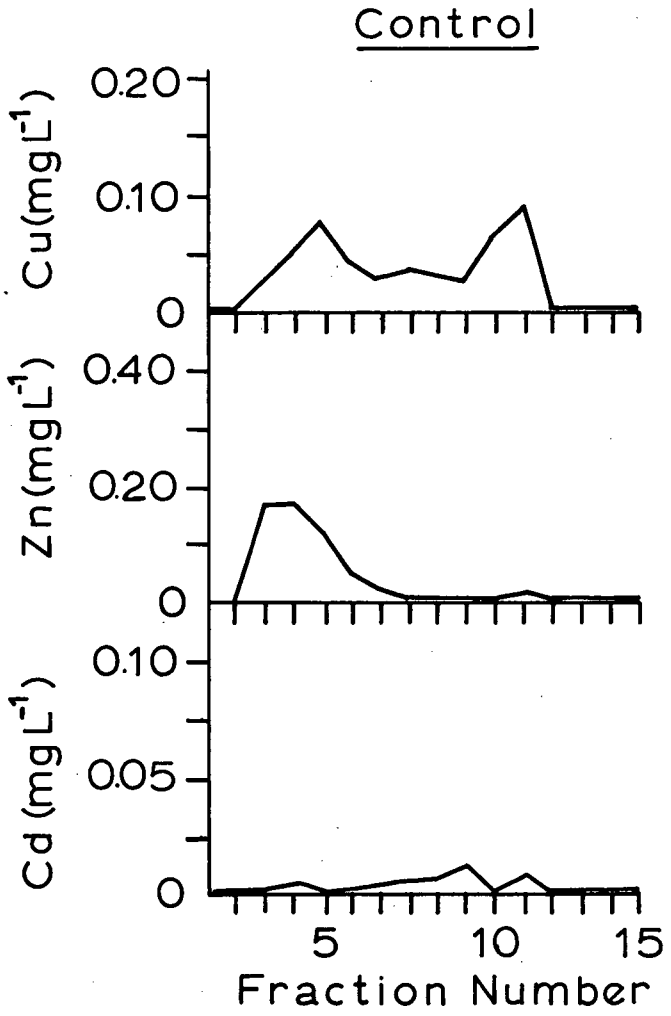

Control
0.20
0.10
0
Cu(mgL-1)
0.40
0.20
0
Zn(mgL-1)
0.10
0.05
0
Cd(mgL-1)
5
10
15
Fraction Number

Figure 30. Composite gel elution profiles from three mice exposed to 25 mg Cd/L in their drinking water, and from three mice exposed to 25 mg Cd/L + 25 mg DEN/L.

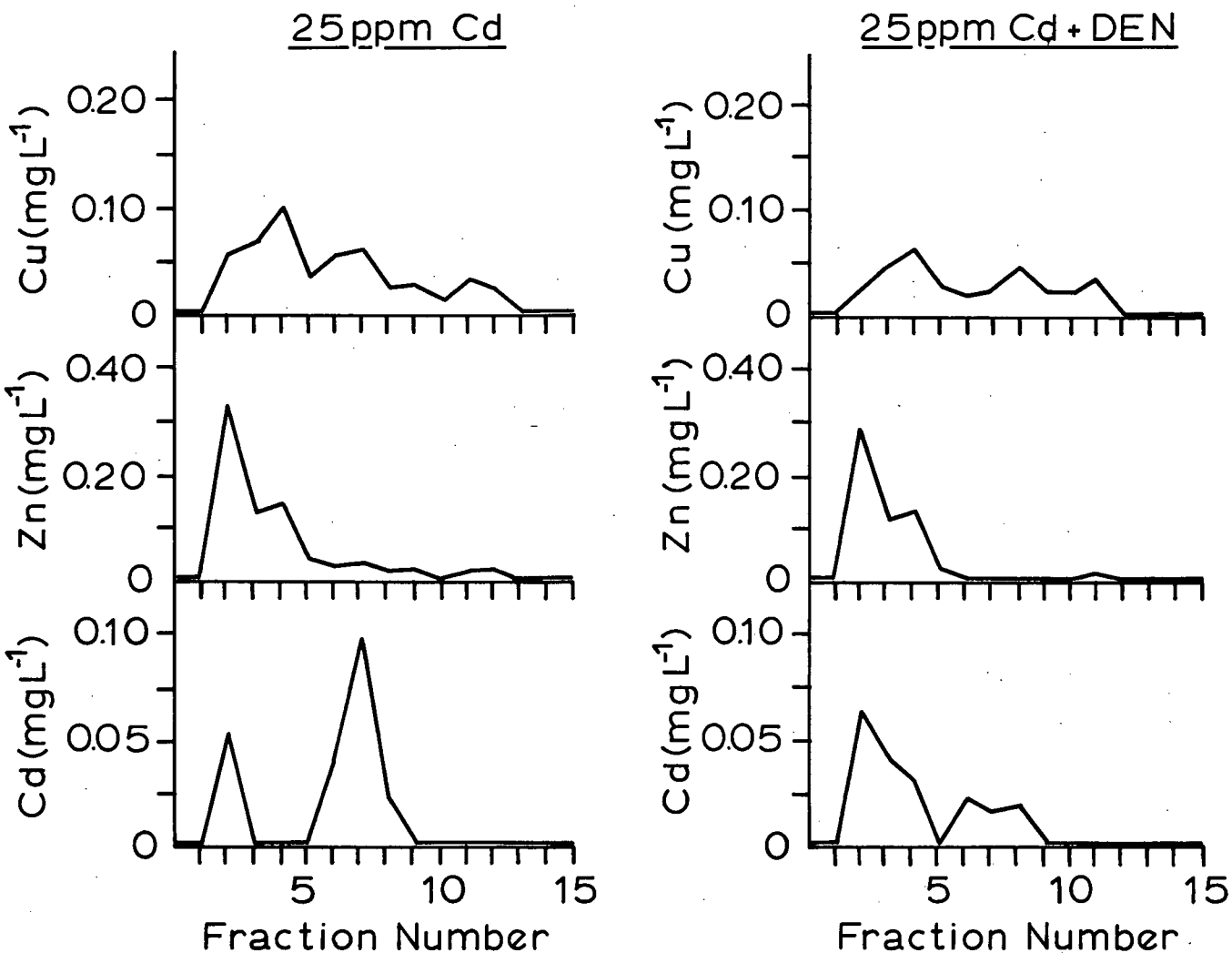

25ppm Cd
25ppm Cd + DEN
Cu (mgL-1)
Zn (mgL-1)
Cd (mgL-1)
0.20
0.10
0
0.40
0.20
0.10
0.05
5
10
15
Fraction Number

Figure 31. The variation of Cd levels in the high molecular weight protein pool from mice exposed to Cd, with or without DEN. Each point represents a value calculated from a gel elution profile done on a composite sample from three mice.

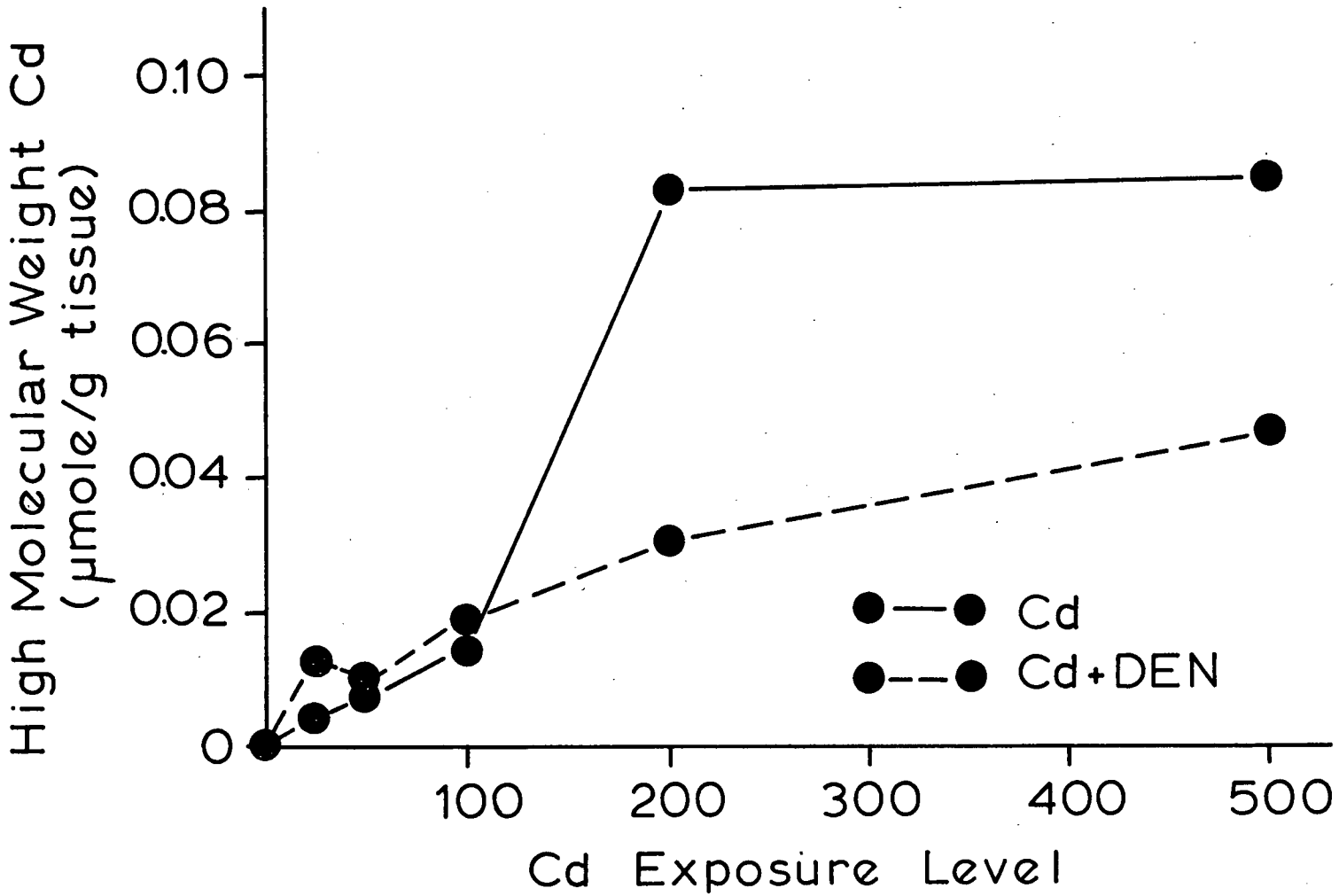
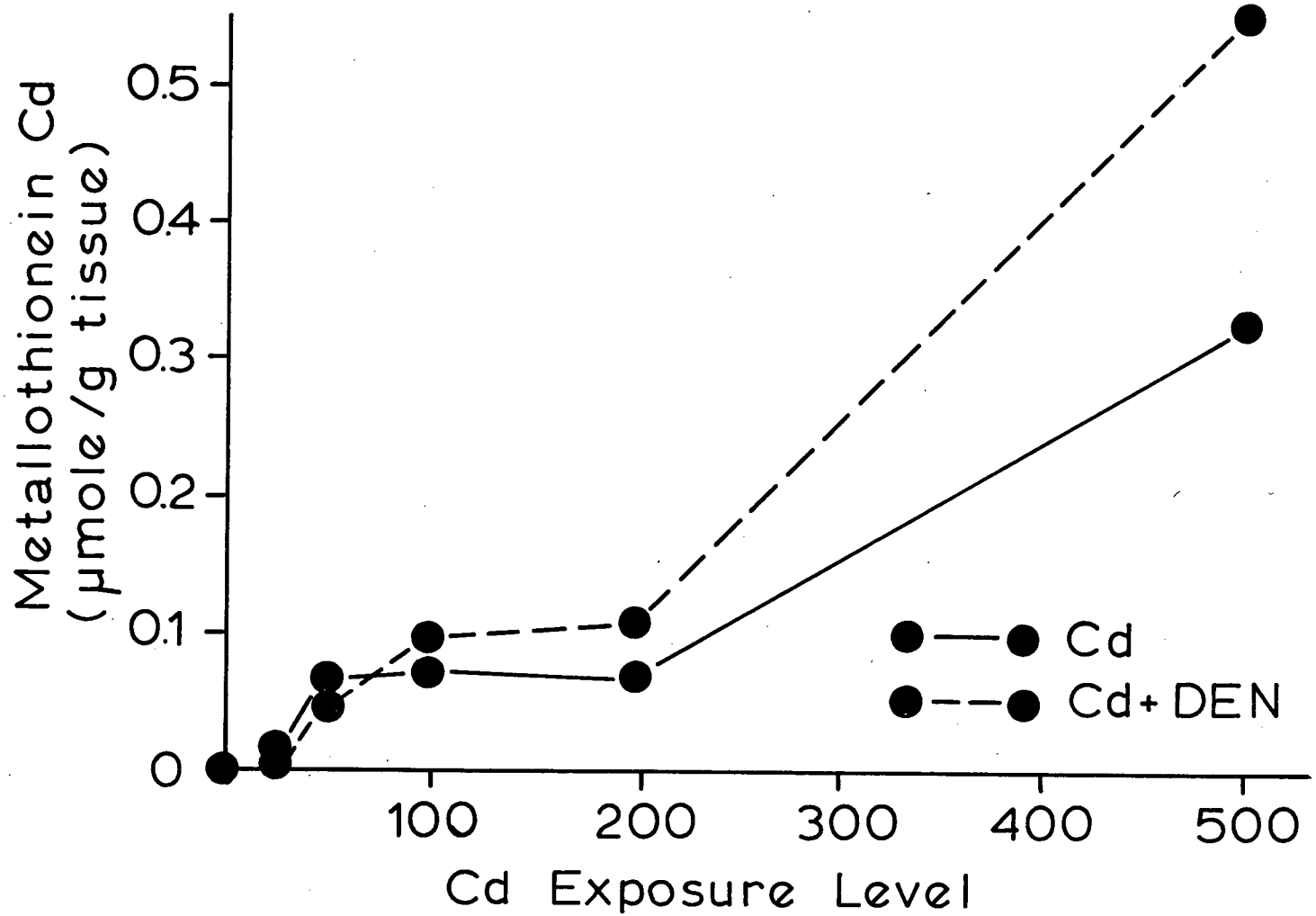

High Molecular Weight Cd
(μmole/g tissue)
0.10
0.08
0.06
0.04
0.02
0
100
200
300
400
500
Cd Exposure Level
Cd
Cd+DEN

Figure 32. The variation of Cd levels in metallothionein from mice exposed to Cd, with or without DEN. Each point represents a value calculated from a gel elution profile done on a composite sample from three mice.


Metallothionein Cd
(μmole/g tissue)
0.5
0.4
0.3
0.2
0.1
0
100
200
300
400
500
Cd Exposure Level
Cd
Cd+DEN

exposure to DEN (Table 19, Figure 31). Also, Cd-thionein levels were higher at the 200 to 500 mg Cd/L exposures with concurrent exposure to DEN, compared with exposure to Cd alone.

The levels of Cu in the high molecular weight protein pool were decreased by exposure to DEN + Cd compared with exposure to Cd alone, except at the 500 mg Cd/L exposures (Table 20, Figure 33). At the 25 mg Cd/L exposure, there was a large increase of Cu in the high molecular weight protein pool, compared with control values. The levels of Cu-thionein increased with increasing Cd exposure level, and tended to parallel increases of Cd-thionein (Tables 19, 20, 23, Figures 32 and 34). There appeared to be a dip in levels of Cu-thionein at the 200 mg Cd/L exposure (Table 20, Figure 34); this point coincided with apparent saturation of metallothionein by Cd, with concurrent "spillover" of Cd to the high molecular weight protein pool (Figures 31 and 32). In general, Cu tended to be distributed equally among the three cytoplasmic pools (Table 23). Total cytoplasmic Cu levels tended to increase with increasing level of Cd exposure; this increase, however, was counteracted by DEN at the 25 mg Cd/L exposure (Table 20).

The levels of Zn in the high molecular weight protein pool were decreased by exposure to DEN + Cd at 25, 50 and 200 mg Cd/L, compared with exposure to Cd alone (Table 21,

Figure 33. The variation of Cu levels in the high molecular weight protein pool from mice exposed to Cd, with or without DEN. Each point represents a value calculated from a gel elution profile done on a composite sample from three mice.

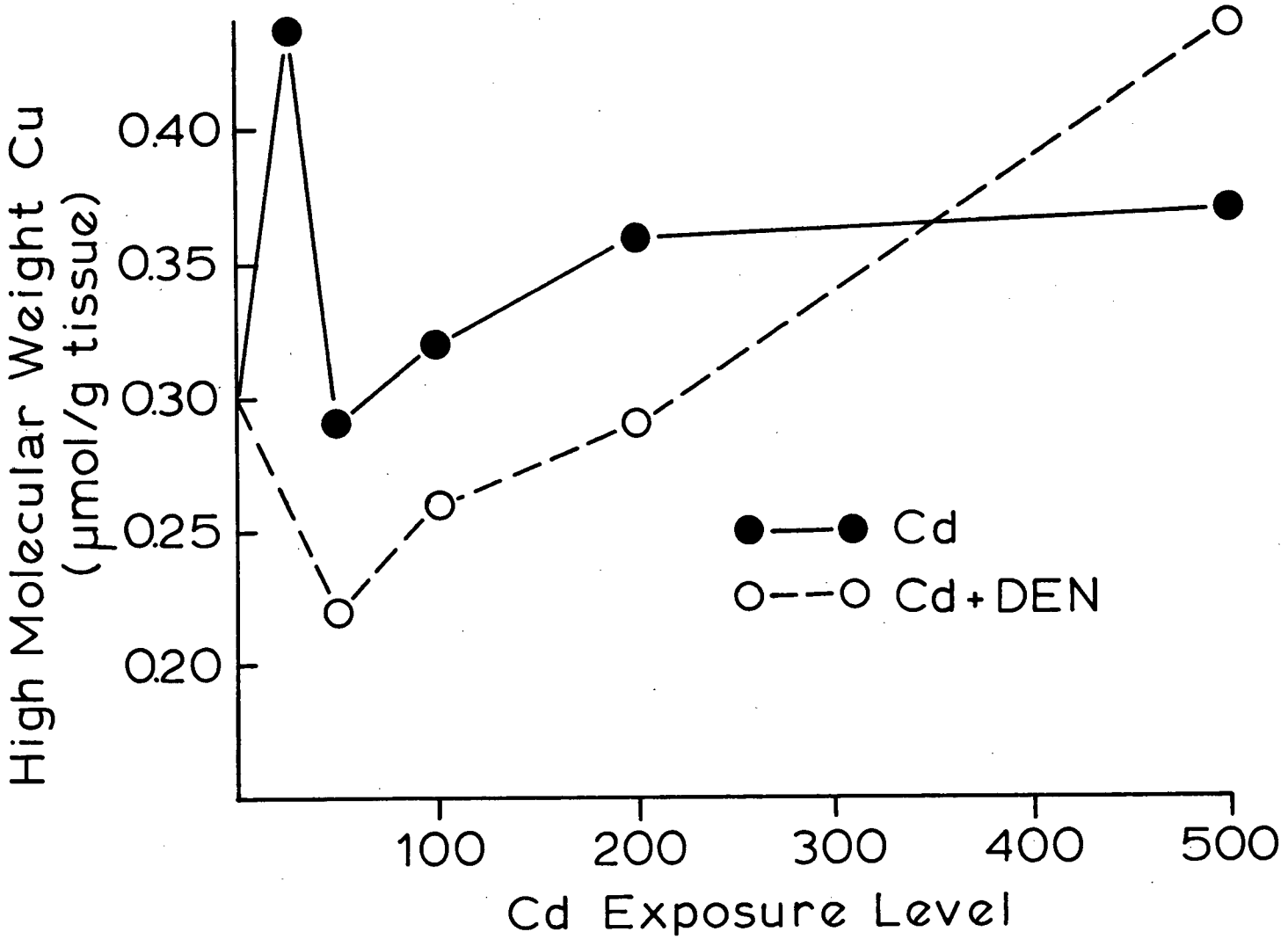

High Molecular Weight Cu
(μmol/g tissue)
0.40
0.35
0.30
0.25
0.20
100
200
300
400
500
Cd Exposure Level
Cd
Cd+DEN

Figure 34. The variation of Cu levels in metallothionein from mice exposed to Cd, with or without DEN. Each point represents a value calculated from a gel elution profile done on a composite sample from three mice.

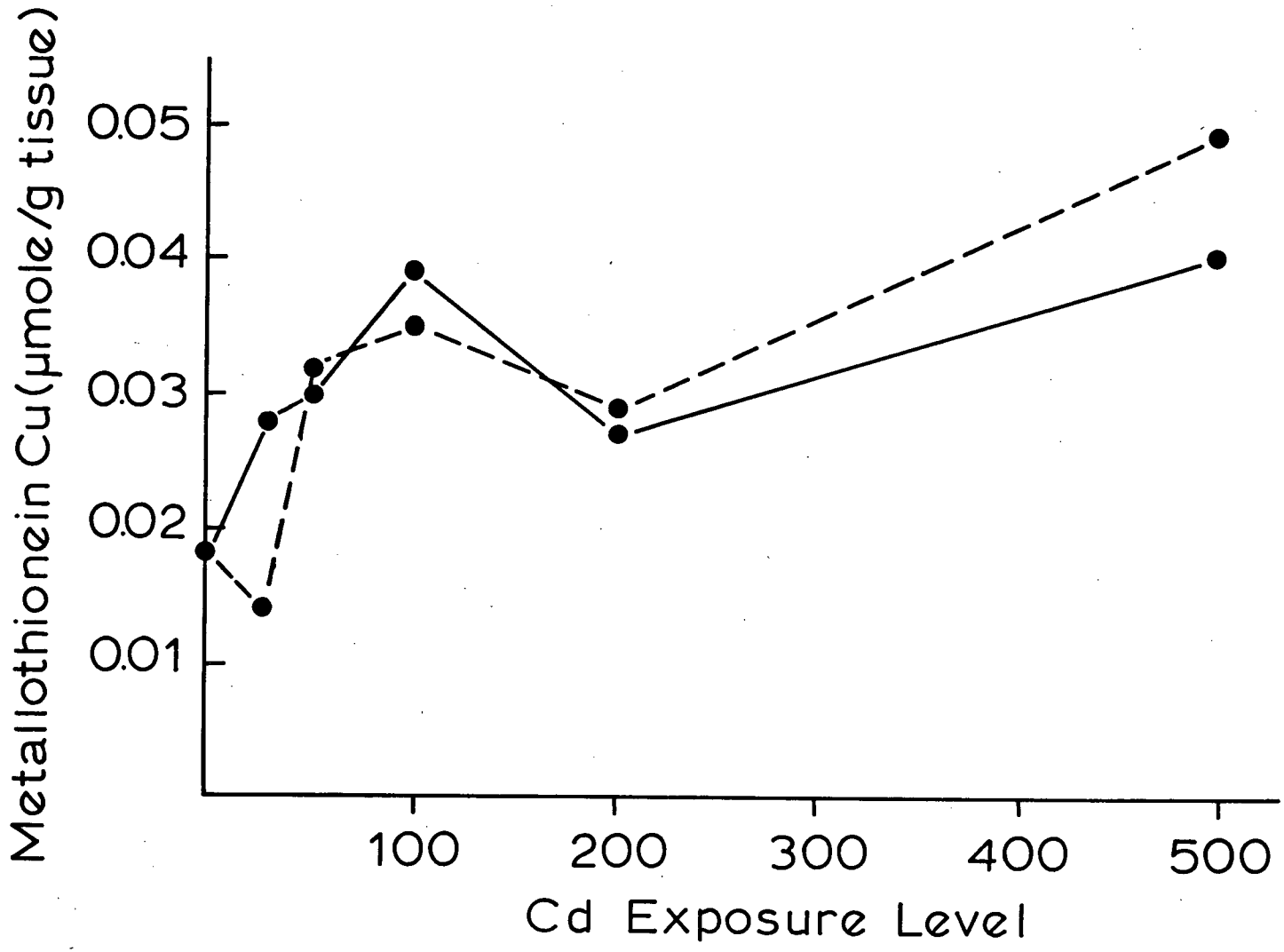

Metallothionein Cu (µmole/g tissue)
0.05
0.04
0.03
0.02
0.01
100
200
300
400
500
Cd Exposure Level

Figure 35. This effect was particularly prevalent at 25 and 50 mg Cd/L. The level of high molecular weight protein Zn increased with increasing Cd exposure level (Table 21, Figure 35). Levels of Zn-thionein increased with increasing Cd exposure, and tended to parallel increases of Cd-thionein (Tables 19, 21 and 24, Figures 32 and 36). Similarly to Cu-thionein, there was a dip in levels of Zn-thionein at the 200 mg Cd/L exposure (Figures 34 and 36). In each Cd exposure level, the set of mice with the lowest high molecular weight protein Zn (Table 21) had the lowest percentage of Zn bound to metallothionein (Table 24). In general, approximately three quarters of cytoplasmic Zn was in the high molecular weight protein pool, with most of the remainder bound to metallothionein (Table 24). Total cytoplasmic Zn levels increased with increasing Cd exposure level (Table 21).

Total cytoplasmic Cu + Zn levels were increased by exposure to Cd, but this increase was larger without concurrent exposure to DEN, except at the 500 mg Cd/L exposure (Figure 37). Decreases of liver weight were apparent at 100 mg Cd/L with concurrent DEN exposure, and at 200 mg Cd/L exposure with exposure to Cd alone (Figure 38). The decrease of liver weight appeared to coincide with apparent saturation of metallothionein and "spillover" of Cd from metallothionein to the high molecular weight protein pool (Table 19, Figures 31, 32 and 38). Liver

Figure 35. The variation of Zn levels in the high molecular weight protein pool from mice exposed to Cd, with or without DEN. Each point represents a value calculated from a gel elution profile done on a composite sample from three mice.

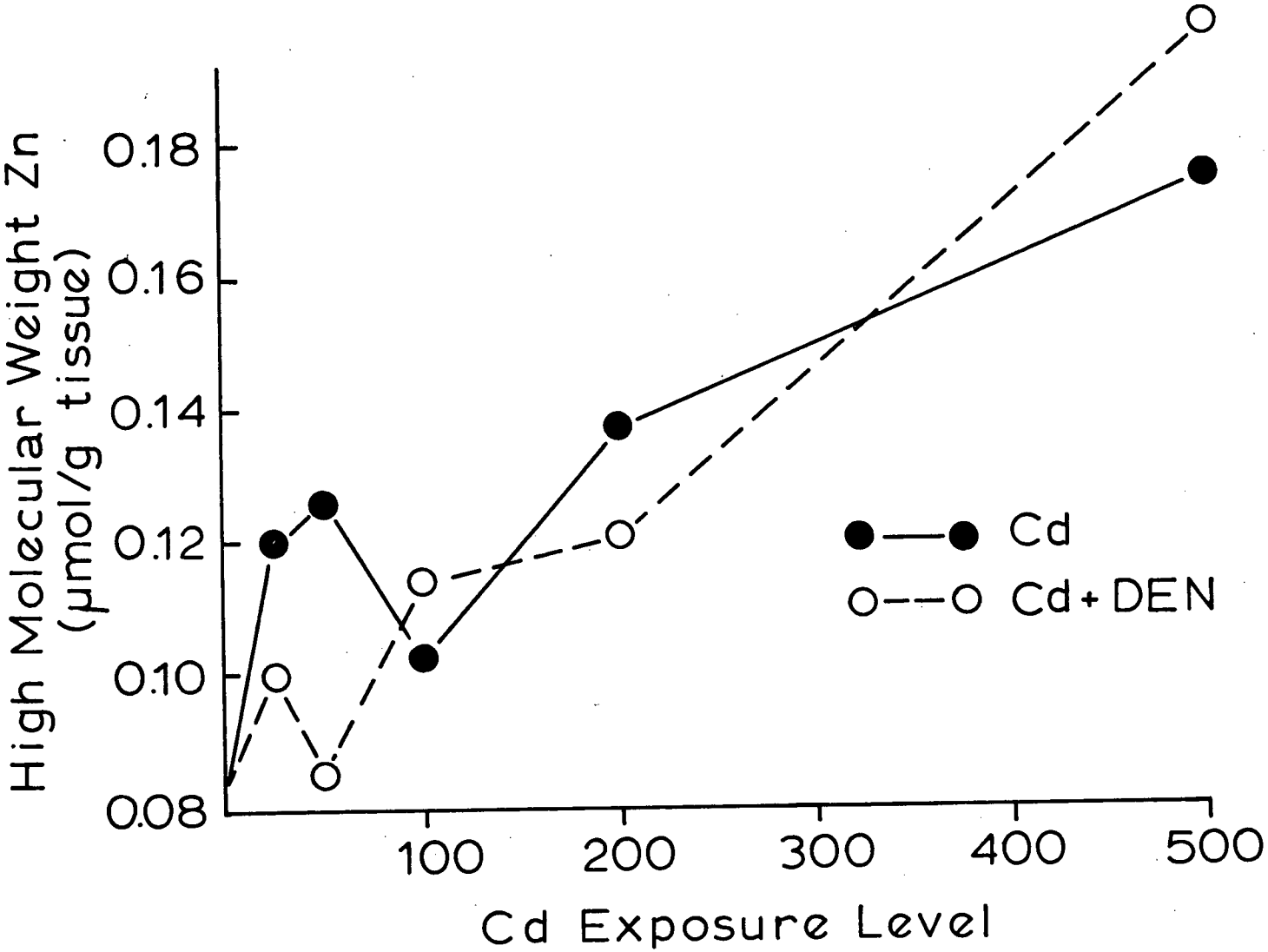

High Molecular Weight Zn
(μmol/g tissue)
0.18
0.16
0.14
0.12
0.10
0.08
100
200
300
400
500
Cd Exposure Level
Cd
Cd + DEN

Figure 36. The variation of Zn levels in metallothionein from mice exposed to Cd, with or without DEN. Each point represents a value calculated from a gel elution profile done on a composite sample from three mice.

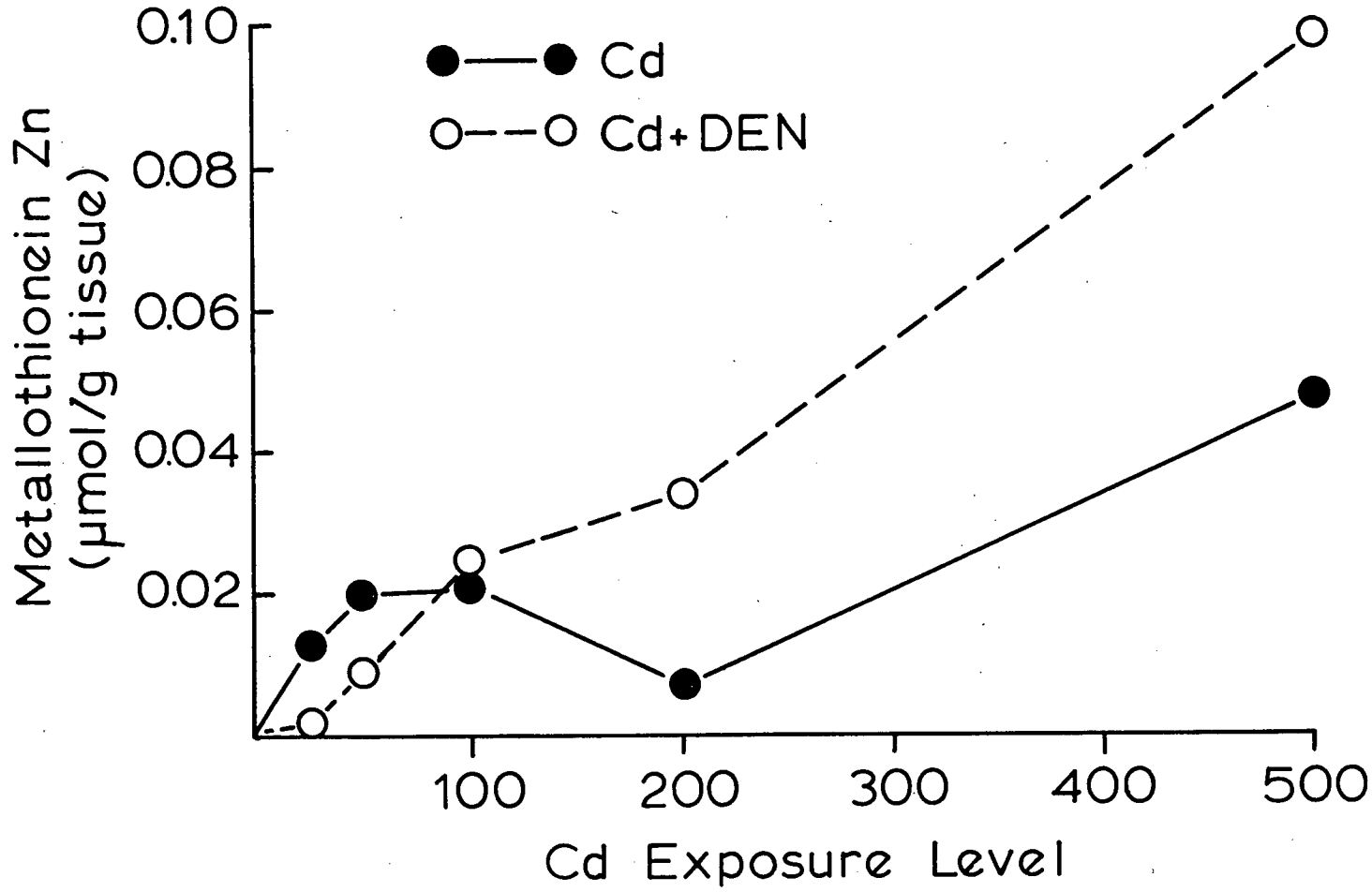

0.10
0.08
0.06
0.04
0.02
Metallothionein Zn
(μmol/g tissue)
Cd
Cd+DEN
100
200
300
400
500
Cd Exposure Level

Figure 37. The variation of total tissue homogenate supernatant Cu + Zn levels in mice exposed to Cd, with or without DEN. Each point represents a value calculated from a gel elution profile done on a composite sample from three mice.

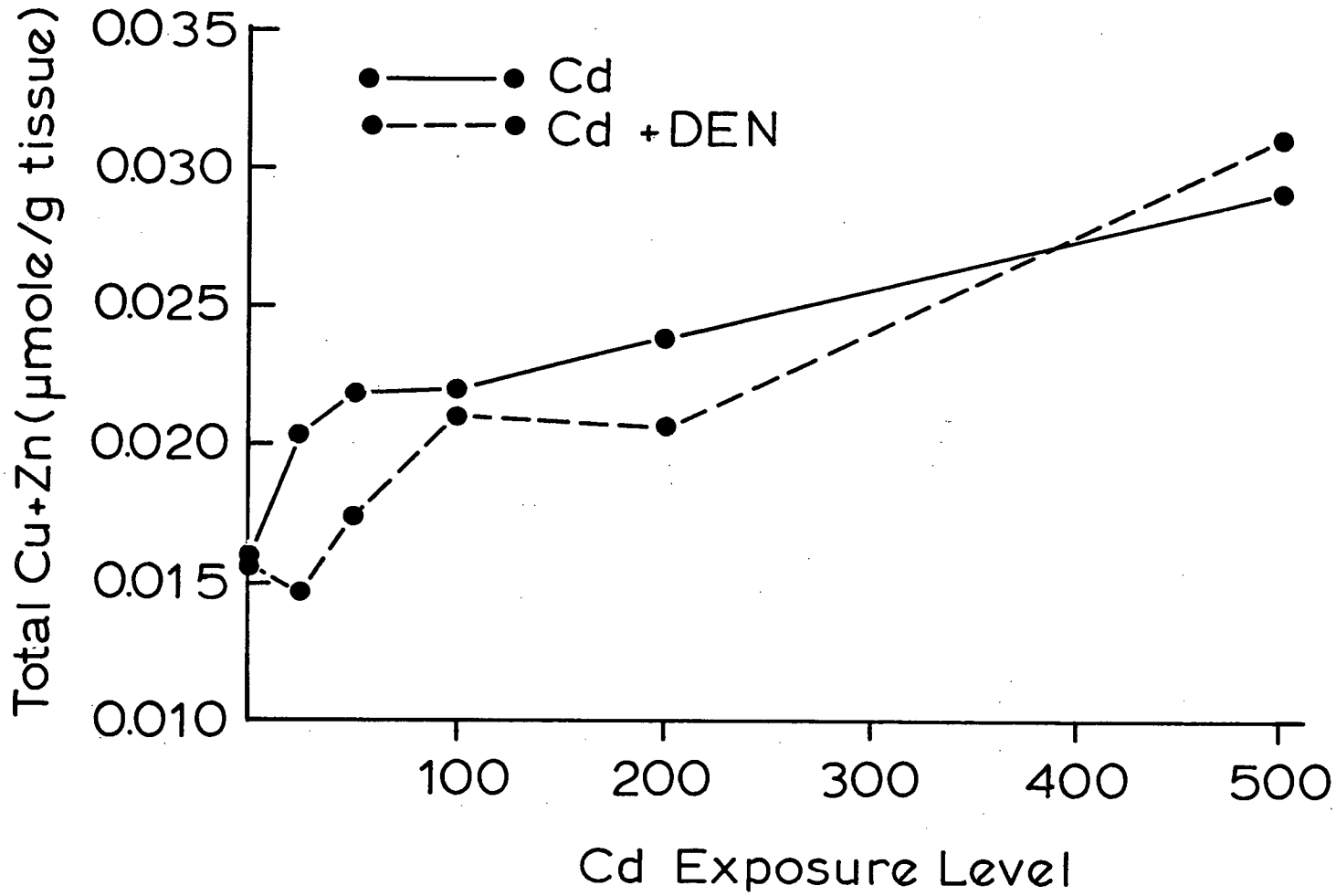
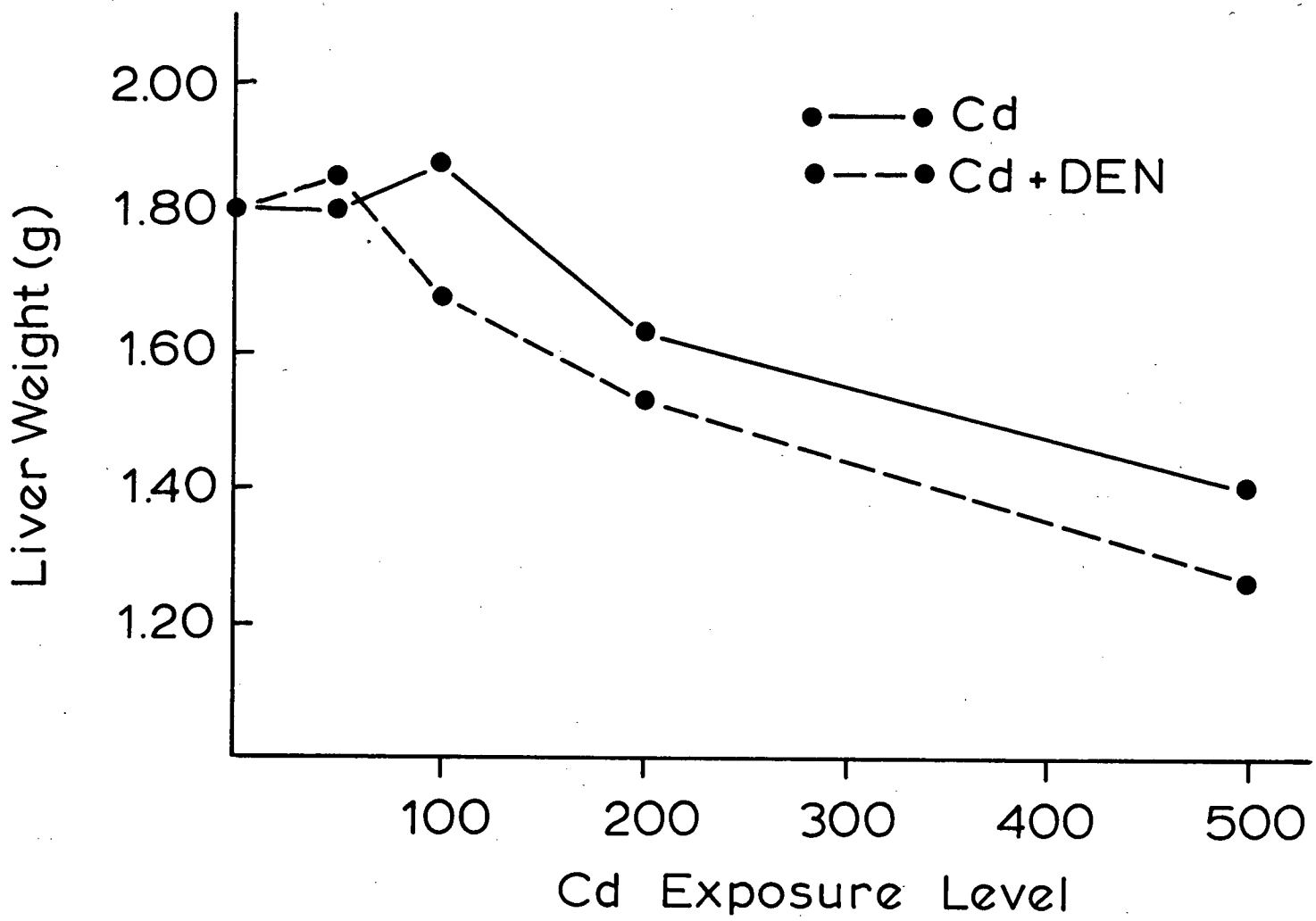

Total Cu+Zn (µmole/g tissue)
0.035
0.030
0.025
0.020
0.015
0.010
Cd
Cd +DEN
100
200
300
400
500
Cd Exposure Level

Figure 38. The variation of liver weight with exposure to various levels of Cd, with or without DEN. Each point represents the mean of three mice.


2.00
1.80
1.60
1.40
1.20
Liver Weight (g)
Cd
Cd + DEN
100
200
300
400
500
Cd Exposure Level

weights were less with exposure to DEN + Cd compared with exposure to Cd alone.

Discussion

This study has demonstrated that at Cd exposures equal to or less than 100 mg Cd/L, DEN increases the proportion of Cd in the high molecular weight protein pool, and concurrently decreases the levels bound to metallothionein. This effect decreased in magnitude as the exposure level of Cd increased, presumably due to masking of the effects of DEN via high levels of Cd. Thus, it is probable that the occurrence of Cd in the high molecular weight protein pool rather than in metallothionein, in a study by Brown and Chan (1978), was due to the effects of DEN, since the Cd exposure level was only 5 mg Cd/L. Also, it is possible that occurrences of high levels of Cd in the high molecular weight protein pool of fish (Brown, 1977) and humans (Brown and Knight, 1978) was due to the effects of carcinogens with actions the same as, or similar to DEN.

At the 200 and 500 mg Cd/L exposures, there was less Cd in the high molecular weight protein pool with concurrent DEN exposure. It appeared as though, with exposure to DEN + Cd, that the high molecular weight protein pool became Cd saturated (plateaued) at about 0.04 μmole Cd in this pool/g tissue. With Cd exposure alone, it did not appear to become saturated until about 0.08 μmole Cd in this pool/g tissue. An explanation for the lower apparent

saturation levels of the high molecular weight protein pool with DEN exposure, derives from the fact that there appears to be more necrosis of the liver with DEN exposure (i.e., Figure 38). This suggests that more proteolytic enzymes are released from lysosomes with DEN exposure, which is a known effect of carcinogens (Chvapil et al., 1972). These higher levels of proteolytic enzymes would almost certainly reduce cytoplasmic enzyme levels, and therefore the number of metalloenzyme binding sites in the high molecular weight protein pool. Also, since metallothionein is not degraded by proteolytic enzymes (Webb, 1972b), metallothionein could become more concentrated in the liver as liver tissue was destroyed. This might explain the higher Cd-thionein levels of mice exposed to 100 to 500 mg Cd/L and DEN in the present study.

Results from this study appear to confirm the "spill-over" theory discussed by Brown et al. (1977), Brown and Parsons (1978), Brown and Chatel (1978a,b), and Cloutier and Brown (1978). Cadmium-thionein levels, in the present study, appeared to plateau from 100 to 200 mg Cd/L exposure with or without DEN exposure. It was at this point that there were very large increases of Cd on the high molecular weight protein pool of mice exposed to Cd alone, and lesser increases in this pool in mice exposed to DEN + Cd; reasons for these lesser levels were discussed in the previous paragraph. At that point where metallothionein

appeared to become Cd saturated, and Cd apparently spilled over to the high molecular weight protein pool, there was a simultaneous occurrence of pathological effects; i.e., reduced liver weights. In DEN exposed mice, increased levels of Cd appeared in the high molecular weight protein pool at lower Cd exposures than with exposure to Cd alone, and liver weight reductions also occurred at lower Cd exposures. Brown and Chatel (1978a) have found that deficiencies of Zn in the high molecular weight protein pool result in accumulation of higher proportions of cytoplasmic Cd in this pool. Therefore, DEN induced losses of Zn from the high molecular weight protein pool probably account for the accumulation of Cd in this pool, rather than in metallothionein.

The sudden increase of Cd-thionein production occurring at 500 mg Cd/L, after metallothionein production has apparently plateaued, is surprising, but may be explainable since Cd-thionein synthesis is induced by two different mechanisms. Webb (1972a) has demonstrated that metallothionein is induced at the translational level (i.e., mRNA coded for metallothionein exists in the cytoplasm and is turned on to synthesize metallothionein in the presence of Cd), but Squib and Cousins (1974), using much higher Cd levels, have demonstrated that metallothionein can also be induced at the transcriptional level (i.e., production of new mRNA from DNA coded for metallothionein).

As Cd-thionein synthesis plateaus up to 200 mg Cd/L exposure, but accelerates again with higher exposures, it may be that synthesis of metallothionein up to 200 mg Cd/L exposure is induced at the translational level. When this metallothionein production reached its limit, then excess Cd would spillover to the high molecular weight protein pool. After this pool was Cd saturated, then excess Cd would be available to penetrate into the nucleus, resulting in induction of metallothionein at the transcriptional level. This may explain the second peak in metallothionein production, after spillover has occurred. As there is more Cd-thionein with higher exposures of Cd with DEN than with Cd alone, it may be that DEN greatly increases nuclear penetration by Cd, which might be very important for development of cancer. A similar increase in metallothionein production, after an apparent plateau of metallothionein production and spillover had occurred, was noted in mussels exposed to Cd, Cu and Zn in the laboratory (Brown and Chatel, 1978b).

As Cd-thionein levels increased, so did the levels of Cu and Zn found in the metallothionein fractions. This is in accordance with previous studies which have found that Zn and Cu occupy a portion of metallothionein binding sites when metallothionein is produced in response to Cd (Suzuki and Yoshikawa, 1972; Leber, 1974). It has been found by Leber (1974) and Brown and Parsons (1978)

that exposure to high levels of Cd or Hg can then result in a displacement of less toxic Cu and Zn from metallothionein. Thus, tolerance to acute doses of Cd or Hg is mediated by the presence of metallothionein bound Cu and Zn. It is therefore probable that levels of both Cu and Zn were decreased on metallothionein at that exposure level where spillover occurred (200 mg Cd/L), through the displacement of Cu and Zn by very high levels of Cd in the cytoplasm. Thus, only where there is more than enough metallothionein induced to bind excess Cd, are Cu and Zn levels increased in metallothionein. Presumably, according to the hypothesis presented in this discussion, at 500 mg Cd/L exposure, transcriptional induction of metallothionein resulted in a very high level of metallothionein production, so that Cu and Zn were not displaced by high Cd levels.

In this study, as in a previous study (Brown, 1978), exposure to high Cd levels increased Cu and Zn levels in liver. This has been attributed to induction of mucosal metallothionein by Cd (Brown, 1978) so that transport of Cu and Zn into the bloodstream is increased. Since Cd interferes with Cu and Zn binding sites in metalloenzymes (Friedberg, 1974; Bremner, 1974), it is likely that increased uptake of Cu and Zn with increasing Cd exposure levels, particularly in the high molecular weight protein pool, acts as a defense against the toxic actions of Cd on metalloenzymes.

As in previous studies, exposure to DEN, resulted in reduced levels of Cu and Zn in liver tissue and particularly in the high molecular weight protein pool (Brown and Chan, 1978; Brown, 1978). When high molecular weight protein Zn levels were decreased, there appeared to be a shift of Zn from metallothionein to the high molecular weight protein pool; i.e., the percentage of metallothionein bound Zn was decreased and the percentage of high molecular weight protein Zn increased. This is in accordance with Brown and Chatel (1978a) who reported that metallothionein appeared to act as a storage vehicle for Zn. Reductions of Cu and Zn may have been due to complexing of Cu and Zn by DEN as it passes through the bloodstream, and subsequently into the urine (Brown and Chan, 1978). Furst (1963) has described most carcinogens as being potential trace element chelators. The possible mechanistic significance of reductions of Cu and Zn in carcinogenesis has been discussed by Brown and Chan (1978).

This study has clearly demonstrated that toxic effects of Cd appear simultaneously with "spillover" of Cd from metallothionein to the high molecular weight protein pool. Also shown, is that DEN reduces high molecular weight Cu and Zn levels, thus allowing Cd to spillover at lower exposure levels so that pathological effects occur at lower exposure levels. What is not clear, at this point, is which aspects of the actions of DEN are important in

carcinogenesis. This study, and Brown and Chan (1978), and Brown (1978) have demonstrated that DEN results in losses of Cu and Zn in pretumor tissue. Others have demonstrated that Cu and Zn prevent cancer (discussed in Brown and Chan, 1978). Therefore losses of Cu and Zn appear important for carcinogenesis. Since, in previous studies, Cd was not increased, but rather was decreased by DEN (Brown and Chan, 1978; Brown, 1978) it appears that Cd is not important to carcinogenesis. However, cancerous organisms have increases of high molecular weight protein Cd, and DEN has been shown in this study, to have the potential to increase high molecular weight protein Cd if high enough levels of Cd are readily available. Therefore, there does seem to be cause to continue to study the relevance of Cd to the etiology of cancer.

Summary

Mice were exposed to 0, 25, 50, 100, 200 and 500 mg Cd/L, with or without DEN exposure, in their drinking water for 28 days. Sephadex G-75 gel elution profiles revealed that DEN + Cd resulted in an increased accumulation of Cd in the high molecular weight protein pool at 25, 50 and 100 mg Cd/L exposures, compared with exposure to Cd alone. At 200 and 500 mg Cd/L, more Cd accumulated in the high molecular weight protein pool of mice exposed to Cd alone. This was discussed as possibly due to higher enzyme levels with Cd exposure alone, as DEN is known to damage lysosomes,

thus releasing proteolytic enzymes. There was less metallothionein production with exposure to Cd alone, then with exposure to DEN + Cd at 200 and 500 mg Cd/L exposures. Metallothionein production reached a plateau from 100 to 200 mg Cd/L exposure, with or without DEN exposure. At 200 mg Cd/L, there was a large increase of Cd in the high molecular weight protein pool. This apparent saturation of metallothionein, and "spillover" of Cd into the high molecular weight protein pool coincided with reduced liver weights. Liver weights were more reduced in mice exposed to DEN + Cd rather than to Cd alone. Increases of Cd-thionein occurred after an apparent plateau of Cd-thionein production had been reached; these are discussed as possibly due to a switch from translational to transcriptional induction of metallothionein at very high Cd exposure levels.

Exposure to Cd resulted in increased levels of Cu and Zn in the liver, and in particular, in the high molecular weight protein pool. These increases of Cu and Zn were reduced by exposure to DEN with the Cd. Copper and zinc levels increased on metallothionein with increases of Cd on metallothionein, but declined somewhat at that exposure level (200 mg Cd/L) where "spillover" occurred; presumably due to displacement by high Cd levels.

CHAPTER XII Discussion

Metallothionein Studies

The studies presented in this thesis have examined organisms for the presence of metal bound to gel elution fractions corresponding to the elution position of metallothionein. Phytoplankton did appear to have a metallothionein peak, but it was small compared with the high and low molecular weight peaks (Figure 15). This peak contained Cu, but not Zn, in detectable levels in non-exposed phytoplankton. There is some evidence that Hg induced metallothionein synthesis in phytoplankton, since, with increasing Hg exposure level, both Zn and Cu levels increased in those fractions corresponding to metallothionein, even though Hg was not detectable in those fractions by methods employed in that study. Previous studies have indicated that metallothionein produced in response to Cd or Hg exposure, also binds Cu and Zn, the levels of Cu and Zn on metallothionein increasing with increasing exposure level (Leber, 1974; Winge et al., 1975; Stonard and Webb, 1975). The increase of Zn on metallothionein in the present study occurred even though Zn levels decreased in both the high and low molecular weight pools. Thus, there is a distinct Zn peak in the position of metallothionein in Figure 15. The main storage area for Cu and Zn in phytoplankton appears

to be the low molecular weight cytoplasmic pool.

Zooplankton did not have very distinct Cu and Zn peaks in those fractions corresponding to the usual position of metallothionein (Figure 13). However, like phytoplankton (Cloutier and Brown, 1978), there were distinct increases of Cu and Zn in these fractions with increases of Hg in these fractions and with increasing Hg exposure level (Table 5). These increases occurred in the metallothionein-like fractions even though Cu and Zn were concurrently decreasing in the high and low molecular weight pools. These increases are good evidence for the induction of metallothionein (Webb, 1972a). The elevated Hg in these fractions with increasing Hg exposure level, is also good evidence for the existence of metallothionein in zooplankton. As in phytoplankton, the main storage area for Cu and Zn in zooplankton, appeared to be the low molecular weight cytoplasmic pool.

It has been clearly established by other workers that mussels produce metallothionein (Noel-Lambot, 1976; Talbot and Magee, 1978). The present study confirms these findings since distinct Cd, Cu and Zn peaks were found in the position in the gel elution profile corresponding to metallothionein (Figure 6). There were also distinct increases of Cu and Zn on metallothionein with increasing exposure level (Table 2). In mussels, most Cu and Zn occurred in the high and low molecular weight cytoplasmic

pool, suggesting that mussels, like phytoplankton and zooplankton, use components of the low molecular weight cytoplasmic pool as the main storage area for Cu and Zn.

The livers of chum salmon appeared to have a distinct Cu peak in the fractions in which metallothionein occurs (Figure 12A). Duck liver (Figures 4 and 8A) and kidney (Figure 8B) had distinctive metallothionein peaks containing high levels of Cd, Cu and Zn. Human kidneys contained very high Cd- and Cu-thionein levels with lesser amounts of Zn-thionein (Figures 18 and 19). Laboratory mice had relatively insignificant levels of metallothionein (Figure 29) unless these were exposed to Cd (Figure 30).

It appears, therefore, that higher organisms can produce more metallothionein since a higher portion of their cytoplasmic metal levels are bound to metallothionein. This is particularly evident when considering organisms exposed to high metal levels in laboratory experiments. Mussels had only a 1.6-fold increase in levels of metallothionein before there was an apparent saturation of metallothionein with spillover of metal to the high molecular weight protein pool (Calculated from Table 2). Mice exposed to Cd in the laboratory, had a 6.1-fold increase in metallothionein levels before spillover occurred (calculated from Tables 19-21).

Perhaps the differences between the portion of metal on metallothionein in lower organisms (i.e., phytoplankton,

zooplankton and mussels) versus higher organisms (i.e., fish, ducks, mice and humans) is due to the tissue type analyzed. For phytoplankton, zooplankton and mussels, the entire organism was homogenized for analysis. In higher organisms, only liver and kidney tissue were analyzed. These can produce high levels of metallothionein whereas other tissues in higher organisms produce only very low levels (Bouquegneau et al., 1975). In these other tissues, trace elements are bound to metalloenzymes in the high molecular weight protein pool, and to components of the low molecular weight cytoplasmic pool. Therefore, if the entire body of higher organisms had been analyzed, it would have appeared that only a very small portion of metal was bound to metallothionein. Perhaps if equivalent organs were analyzed from phytoplankton, zooplankton, or mussels, a higher portion of metal might have appeared on metallothionein. The crop and digestive gland are the storage areas for heavy metals in molluscs (Coughtrey and Martin, 1976).

This study, unlike almost all previous studies, investigated the relative binding of metals to other cytoplasmic pools besides metallothionein. This study was made truly unique by the fact that levels of metals bound to these various pools were quantified. The main point established by these studies, was that toxic effects of trace elements do not occur unless, and until, these

trace elements appear in the high molecular weight protein pool. This was substantiated by studies in which phytoplankton were exposed to Hg, zooplankton and fish were exposed to Hg, mussels were exposed to Cd, Cu and Zn, mice were exposed to Cd, and, studies of the cytoplasmic Cd distribution in tissue extract from cancerous fish and humans. Except for the cancerous organisms, and phytoplankton, this appearance of trace elements in the high molecular weight protein pool was shown to occur with the apparent saturation of metallothionein.

Thus, studies in this thesis have clearly substantiated the "spillover theory" first suggested by Brown et al. (1977), i.e., that toxic effects of trace elements do not occur until metallothionein becomes metal saturated and these metals spillover to the high molecular weight protein pool. Toxic effects are attributed to the interference of toxic metals, or excesses of required metals, with metal binding sites in metalloenzymes (Brown et al., 1977).

In phytoplankton exposed to Hg, the entire "spillover theory" was not confirmed since, either Hg levels were, in most cases, too low to be detectable on metallothionein, or Hg-thionein simply wasn't produced in these phytoplankton. However, reduced growth did correlate with appearance of Hg in the high molecular protein pool in phytoplankton, suggesting that the occurrence of toxic elements in the high

molecular weight protein pool is the common factor influencing survival in phytoplankton, as it is in other organisms.

Carcinogenesis Studies

The studies of tumorous fish and humans, suggested that there was a possible common factor present in cancerous organisms, i.e., that these organisms contain increases of both Cd and the Cd:Zn ratio in the high molecular weight protein pool of apparently normal liver and kidney tissue. Therefore, further research was directed towards attempting to demonstrate that an organic carcinogen (DEN) could cause these changes. However, it was found that DEN exposure resulted in decreases of Cd, Cu and Zn from the high molecular weight protein pool of liver tissue of mice. To add confusion, it was demonstrated that DEN could result in accumulation of Cd in the high molecular weight protein pool rather than on metallothionein, if additional Cd was administered with the DEN. In fact, mice exposed to DEN + Cd had Cd distributions very similar to tumorous fish and humans. Therefore, it appears as though an organic carcinogen can result in gel elution profiles which are similar to tumorous fish and humans. But, it does not appear that this distribution of Cd is necessary for cancer. Therefore, high

levels of Cd in the high molecular weight protein pool may simply be a coincidental effect of carcinogens, or etiologic in only certain cancers or with certain carcinogens. Also, it is clearly possible that there could be differences in nontumorous liver (e.g., fish) and kidney (e.g., human) from tumor bearing organisms, and tumorous liver (e.g., DEN-exposed mice).

Perhaps in pretumorous stages, carcinogens remove Cd, Cu and Zn from skin of tumorous fish and transfer them to the liver so that these are increased in the liver. Since tumors develop in the livers of DEN exposed mice, metals could be transferred to somewhere else, possible the kidneys. Further studies are necessary to investigate changes in Cd, Cu and Zn levels in various cytoplasmic pools of liver and kidney tissue, and some other tissue which is developing tumors.

Of major interest was the finding that DEN reduced high molecular weight protein pool Cu and Zn levels, but that reversal of these losses by either Cd or Zn administration, extended the time to death. The length of this extension was directly related to the level of Cu + Zn in tumorous liver at the time of death. That Cd increased the time to death, appears to be in conflict with hypotheses suggesting that Cd in the high molecular weight protein pool has a causitive role in carcinogenesis (Brown, 1977). However, the Cd levels administered (100 mg Cd/L) in the study in

which Cd extended the time to death were so high that they probably resulted in a large reduction of the activity of the monooxygenase system, so that DEN was not bioactivated (Yoshida et al., 1975); if this were the case then DEN would remain in a precarcinogenic state. An administration of lower levels of Cd (5 mg Cd/L) appeared to increase the levels of precarcinogenic changes after 12.5 weeks' exposure (Brown and Chan, 1978). This suggests that Cd could have at least a catalytic role in carcinogenesis.

Future Studies

A key point that needs to be demonstrated is that the total concentration of toxic metals in an organism gives little indication of the potential for survival of that organism. It is likely that an experiment could be designed with a low level chronic metal exposure resulting in high levels of metal contamination in an organism, but with most metal bound to metallothionein - therefore there should be little evidence of toxic effects. A similar level of contamination might be obtained in the same type of organism after a high level acute exposure, but with little or no metal bound by metallothionein, since metallothionein synthesis wouldn't be able to keep pace with metal inflow into the organism. In this latter organism, toxic effects would be caused by nondetoxified metal.

The fact that without a measure of the portion of metal bound by metallothionein, one doesn't know if a

metal is exerting toxic effects or not, has direct and immediate application to strategies of environmental monitoring. The literature is replete with environmental surveys of levels of various metals and other pollutants. But their numbers tell little of the organisms ability to survive. In other words, they tell nothing of the portion of these metals that are detoxified within the organism. One cannot assume that the same portion is detoxified in all organisms, since the portion detoxified depends upon the rate and level of exposure. And, unless one knows how much metal is detoxified by metallothionein, and how much nondetoxified metal must be present before toxic effects occur, one is in no position to comment on the danger of a pollutant level within an organism to that organism. Therefore, future environmental monitoring programs should incorporate a study of detoxification of metals by metallothionein in designated metal-polluted areas.

Other possible mechanisms of metal detoxification by organisms also need to be studied. For instance, it has been shown that lysosomes are intimately involved in the metabolism and detoxification of trace metals. Furthermore, it has been shown that metallothionein may be stored within lysosomes (Porter, 1974). Therefore, an elucidation of the entire spectrum of metal detoxification mechanisms is needed. Such studies should attempt to produce standardized procedures readily amenable to routine monitoring programs.

Since tolerance can be developed to most pollutants, it follows that there may be detoxification systems available for most pollutants. Therefore, it should be possible to study the individual detoxification systems for each pollutant, how these work, and the levels of pollutant at which their capacity to detoxify is surpassed. This has been done for trace metals and metallothionein and can surely be done for other toxicants - e.g., the capacities of the monooxygenase-hydroxylase enzyme systems to detoxify PCB's could probably be readily determined as well as the ability of adipose tissue, and endoplasmic reticulum of the liver, to store these before they are detoxified.

The study of the involvement of trace metals in carcinogenesis appears to be a most promising area of research. As discussed in this thesis, there are numerous studies showing that both Cu and Zn prevent cancer from occurring. This thesis has shown that one carcinogen (DEN) causes decreases of Cu and Zn in pretumor tissue. Reversal of these losses appears to delay the onset of tumors. Unfortunately, the present studies administered only Zn with DEN. Future studies should administer Cu as well as Zn with DEN, since both are depleted by DEN.

Methods utilized in the present study failed to counteract the carcinogen-induced Zn depletions. Future studies should study the chemical form and method of

application of Cu and Zn that most readily counteracts carcinogen-induced metal losses. If these carcinogen-induced losses of metal can be prevented by metal administration with the carcinogen, and tumors prevented, then it will be clear that metal losses are involved in the etiology of cancer.

A very recent study by Fong et al. (1978) has found that the organic carcinogen methylbenzylnitrosamine (MBM) produces tumors and Zn deficiency in the esophagus of rats. They furthermore demonstrated that rats deficient in Zn due to a Zn deficient diet, developed tumors faster from exposure to MBM than did controls given MBM with a Zn replete diet. They conclude that:

> "present studies with experimental animals indicate that zinc is somehow involved in the etiology of esophageal cancer. Since zinc usually interacts with other trace elements, our findings imply that the observed effects involve a trace element imbalance on a broader scale."

An attempt was made in studies reported in the present thesis, to study these imbalances on a broader scale, particularly with respect to the Cd:Zn ratio. As indicated in this thesis, the elevation of this ratio appears to be a relatively common denominator of tumor-bearing organisms. The fact that the present study reports that DEN lowered Cd levels in pretumor tissue, derides the importance of Cd in carcinogenesis, while the increased Cd:Zn ratio in the high molecular weight protein pool of tumor-bearing fish and

humans, and the increased portion of cytoplasmic Cd in the high molecular weight protein pool of mice given DEN + low Cd doses, points to some importance of Cd in carcinogenesis. It may be that Cd is simply a catalyst for carcinogenesis and that it is necessary for cancer only when organic carcinogen levels are relatively low, i.e., such as those normally encountered in the environment. Since both the levels of DEN and Cd administered in the present report, were probably far higher than any encountered in the environment, it is recommended that future studies use lower levels of both, in order to more closely simulate the usual processes of carcinogenesis.

CHAPTER XIII References

Arnold, M., and D. Sasse. 1961. Quantitative and histochemical analysis of Cu, Zn and Fe in spontaneous and induced primary tumors of rats. Cancer Res. 21: 761-766.

Barbason, H., A. Fridman-Manduzio, P. Lelievre, and E. H. Betz. 1977. Variations of liver cell control during diethylnitrosamine carcinogenesis. Europ. J. Cander 13: 13-18.

Becking, G.C. 1976. Trace elements and drug metabolism. Med. Clin. N. Amer. 60: 813-830.

Bischoff, F., and M. L. Long. 1939. The local effect of zinc upon the development of the marsh-buffalo adenocarcinoma. Am. J. Cancer 37: 531-535.

Bouquegneau, J. M., C. Gerday, and A. Disteche. 1975. Fish mercury-binding thionein related to adaptation mechanisms. FEBS Letters 55: 173-177

Bremner, I. 1974. Heavy metal toxicities. Quart. Rev. Biophys. 7: 75-124.

Bremner, I., and N. T. Davies. 1974. Studies on the appearance of a copper-binding protein in rat liver. Biochem. Soc. Trans. 2: 425-427.

Bremner, I., and N. T. Davies, 1975. The induction of metallothionein in rat liver by Zn injection and restriction of food intake. Biochem. J. 149: 733-738.

Bryan, G. W., and L. G. Hummerstone. 1971. Adaptation of the polychaete Nereis diversicolor to estaurine sediments containing high concentrations of heavy metals. J. Mar. Biol. Assoc. U.K. 51: 845-863.

Buhler, R. H. O. and J. H. R. Kagi. 1974. Human hepatic metallothioneins. FEBS Letters 39: 229-234.

Brown, D. A. 1976. Interactions between biological and geological evolution with special reference to trace elements. (Unpubl. manuscript).

Brown, D.A. 1977. Increases of Cd and the Cd:Zn ratio in the high molecular weight protein pool from apparently normal liver of tumor-bearing flounders (Parophrys vetulus). Mar. Biol. 44: 203-209.

Brown, D. A., C. A. Bawden, K. W. Chatel, and T. R. Parsons. 1977. The wildlife community of Iona Island jetty, Vancouver, B. C., and heavy-metal pollution effects. Environ. Conserv. 4: 213-216.

Brown, D. A. 1978. Decreases of copper and zinc in pretumorous and posttumorous livers of mice exposed to diethylnitrosamine, with and without cadmium of zinc supplementation. (In preparation).

Brown, D. A., and A. Y. Chan. 1978. Decreases of copper and zinc in pretumorous livers of mice exposed to diethylnitrosamine. (Submitted to J. Natl. Cancer Inst.).

Brown, D. A., and K. W. Chatel. 1978a. Interactions between cadmium and zinc in cytoplasm of duck liver and kidney. Chem.-Biol. Interactions (In press).

Brown, D.A., and K. W. Chatel. 1978b. Relationship between survival of mussels exposed to Cd, Cu and Zn, and the cytoplasmic distribution of these elements. (In preparation).

Brown, D.A., and K. W. Chatel. 1978c. The effect of cadmium exposure, with or without diethylnitrosamine, on the cytoplasmic levels and distribution of cadmium, copper and zinc. (In preparation).

Brown, D. A., and B. Knight. 1978. Increases of Cd and the Cd: Zn ratio in the high molecular weight protein pool from apparently normal kidney of terminal human cancer patients. (In preparation).

Brown, D. A., and T. R. Parsons. 1978. Relationship between cytoplasmic distribution of mercury and toxic effects to zooplankton and chum salmon (Oncorhynchus keta) exposed to mercury in a controlled ecosystem. J. Fish. Res. Board Can. 35: 880-884.

Casterline, J. L., and G. Yip. 1975. The distribution and binding of cadmium in oyster, soybean, and rat liver and kidney. Arch. Environ. Contam. Toxicol. 3: 319-329.

Chen, R. W., H. E. Ganther and W. G. Hoekstra. 1973. Studies on the binding of methylmercury by thionein. Biochem. Biophys. Res. Commun. 51: 383-390.

Chen, R. W., and H. E. Ganther. 1975. Some properties of a unique cadmium-binding moiety in the soluble fraction of rat testes. Environ. Physiol Biochem. 5: 235-243.

Cherian, M. G. 1974. Isolation and purification of cadmium binding proteins from rat liver. Bioch. Biophys. Res. Commun. 61: 920-926.

Chvapil, M., S. L. Elias, J. N. Ryan, and C. F. Zukoski. 1972. Pathophysiology of zinc. In: International Review of Neurobiology, Supplement 1, C.C. Pfeiffer, Ed. New York and London, Academic Press, pp. 105-124.

Ciapparelli, L., D. H. Retief, and L. P. Fatti. 1972. The effect of zinc on 9, 10-dimethyl-1, 2-benzanthracene (DMBA) induced salivary gland tumors in the albino rat - a preliminary study. S. Afr. J. Med. Sci. 37: 85-90.

Cloud, P. 1974. Evolution of ecosystems. Amer. Sci. 62: 54-66.

Cloutier, L., and D. A. Brown. 1978. The effect of mercury exposure on intracellelar distribution of mercury, copper, and zinc in Skeletonema costatum. J. Exp. Mar. Biol. Ecol. (In press).

Coughtrey, P. I., and M. H. Martin. 1976. The distribution of Pb, Zn, Cd and Cu within the pulmonate mollusc Helix aspersa Muller. Oecologia 23: 315-322.

Cousins, R. J., A. K. Barber, and J. R. Trout. 1973. Cadmium toxicity in growing swine. J. Nutr. 103: 964-972.

Davies, N. T., I. Bremner, and C. F. Mills. 1973. Studies on the induction of a low-molecular weight zinc-binding protein in rat liver. Bioch. Soc. Trans. 1: 985-988.

De Filippis, L. F., and C. K. Pallaghy. 1976. The effect of sublethal concentrations of mercury and zinc on Chlorella: III. Development and possible mechanisms of resistance to metals. Z. Pflanzenphysiol. 79: 323-335.

DeWys, W., and W. Pories. 1972. Inhibition of a spectrum of animal tumors by dietary zinc deficiency. J. Natl. Cancer Inst. 48: 375-381.

Duncan, J. R., I. E. Dreosti., and C. F. Albrecht. 1974. Zinc intake and growth of a transplanted hepatoma induced by 3'-methyl-4-dimethylaminoazobenzene in rats. J. Natl. Cancer Inst. 53: 277-278.

Duncan, J. R., and I. E. Dreosti. 1975. Zinc intake, neoplastic DNA synthesis, and chemical carcinogenesis in rats and mice. J. Natl. Cancer Inst. 55: 195-196.

Duncan, J. R., and I. E. Dreosti. 1976. A site of action for zinc in neoplastic tissue. Nutr. Res. 38: 711-713.

Evans, G. W., P. F. Majors, and W. E. Cornatzer. 1970. Mechanism for cadmium and zinc antagonism of copper metabolism. Bioch. Biophys. Res. Commun. 40: 1142-1148.

Fare, G., and D. L. Woodhouse. 1963a. The effect of copper acetate on biochemical changes induced in the rat liver by p-dimethylaminoazobenzene. Brit. J. Cancer 17: 512-523.

Fare, G., and D. L. Woodhouse. 1963. The distribution of copper in the tissues of the rat; the effects of age and of feeding p-dimethylaminoazobenzene with and without copper acetate. Brit. J. Cancer 17: 775-786.

Fare, G. 1964. The effect of cupric oxyacetate on the binding of azo-dye by protein during the induction of liver tumours in the rat. Biochem. J. 91: 473-478.

Flick, D. F., H. F. Kraybill, and J. M. Dimitroff. 1971. Toxic effects of cadmium: a review. Environ Res. 4: 71-85.

Fong, L. Y. Y., Sivak, A., and P. M. Newberne. 1978. Zinc deficiency and methylbenzyl nitrosamine-induced esophageal cancer in rats. J. Natl. Cancer Inst. 61: 145-150.

Fowler, B. A., D. A. Wolfe, and W. F. Hettler. 1975. Mercury and iron uptake by cytosomes in mantle epithelial cells of quahog clams (Mercenaria mercenaria) exposed to mercury. J. Fish. Res. Board Can. 32: 1767-1775.

Friedberg, F. 1974. Effects of metal binding on protein structure. Quart. Rev. Biophys. 7: 1-33.

Furst, A. 1963. Chemistry of Chelation in Cancer. Charles C. Thomas, Publisher. Springfield, Illinois. 143p.

Gaskin, F., and Y. Kress. 1977. Zinc ion-induced assembly of tubulin. J. Biol. Chem. 252: 6918-6924.

George, S. G., and T. L. Coombs. 1977. The effects of chelating agents on the uptake and accumulation of cadmium by Mytilis edulis. Mar. Biol. 39: 261-268.

Gerhart, J. C., and H. K. Schachman. 1965. Distinct subunits for the regulation and catalytic activity of aspartate transcarbamylase. Biochemistry 4: 1054-1062.

Grice, G. D., M. R. Reeve, P. Koeller, and D. W. Menzel. 1977. The use of large volume transparent enclosed sea-surface water columns in the study of stress on plankton ecosystems. Helgol. Wiss, Meeresunters. 30: 118-133.

Griffin, J. H., J. P. Rosenbusch, E. R. Blout, and K. K. Weber, 1973. Conformational changes in asparatate transcarbamylase. J. Biol. Chem. 248: 5057-5062.

Gorodiskii, V. I., I. V. Veselaya, and O. N. Rostovtseva. 1956. Copper, zinc, cadmium and nickel content of muscles and tumors. Vop. Med. Khim. 2: 17-18. (Cited after: Furst, A., and R. T. Haro. 1969. A survey of metal carcinogenesis. Prog. Exp. Tumor Res. 12: 102-133.

Greater Vancouver Sewerage and Drainage District. 1974. Environmental studies at Iona Island. Dept. No. 2, prep. B. C. Research: 162 pp. (mimeogr.).

Gunn, S. A., T. C. Gould, and W. A. D. Anderson. 1963. Cadmium-induced interstitial cell tumors in rats and mice and their preventation by zinc. J. Natl. Cancer Inst. 31: 745-753.

Gunn, S. A., T. C. Gould, and W. A. D. Anderson. 1964. Effect of zinc on cancerogenesis by cadmium. Proc. Soc. Exp. Biol. Med. 115: 653-657.

Halsted, J. A., and J. C. Smith. 1970. Plasma-zinc in health and disease. Lancet I: 322-324.

Hecht, N. B., and D. Davidson. 1973. The presence of a common active subunit in low and high molecular weight murine DNA polymerases. Biochem. Biophys. Res. Commun. 51: 299-305.

Herring, W. B., B. S. Leavell, L. M. Paixao and J. H. Yoe. 1960. Trace metals in human plasma and red blood cells. Am. J. Clin. Nutr. 8: 855-863.

Horvath, D. J. 1976. Trace elements and health. In: Trace Substances and Health, P. M. Newberne, Ed. New York and Basel, Marcel Dekker, Inc., pp. 319-356.

Howard, A. G., and G. Nickless. 1975. Protein binding of cadmium, zinc and copper in environmentally insulted limpets Patella vulgata. J. Chrom. 104: 457-459.

Howell, J. S. 1958. The effect of copper acetate on p-dimethylaminoazobenzene carcinogenesis in the rat. Brit. J. Cancer 12: 594-609.

Irons, R. D., and J. C. Smith. 1976. Preventation by copper of cadmium sequestration by metallothionein in liver. Chem.-Biol. Interactions 15: 289-294.

Jakubowski, M., J. Piotrowski, and B. Trojanowska. 1970. Binding of mercury in the rat: studies using $^{203}HgCl_2$ and gel filtration. Tox. Appl. Pharmac. 16: 743-752.

Jerina, D. M., and J. W. Daley. 1974. Arene oxides: a new aspect of drug metabolism. Science 185: 573-582.

Jovin, T. M., P. T. Englund, and A. Kornberg. 1969. Enzymatic synthesis of deoxyribonucleic acid. J. Biol. Chem. 244: 3009-3018.

Kagi, J. H. R., and B. L. Vallee. 1960. Metallothionein: a cadmium and zinc containing protein from equine renal cortex. J. Biol. Chem. 235: 3460-3465.

Kagi, J. H. R., and B. L. Vallee. 1961 Metallothionein: a cadmium and zinc containing protein from equine renal cortex. II. Physicochemical properties. J. Biol. Chem. 236: 2435-2442.

Kew, M. C., and R. C. Mallett. 1974. Hepatic zinc concentrations in primary cancer of the liver. Brit. J. Cancer 29: 80-83.

Kock, J. J., E. R. Smith, and J. McNeely. 1957. Analysis of trace elements in human tissues. Cancer 10: 151-160.

Koeller, P. A., and G. T. Wallace. 1977. The growth of young salmonids (Oncorhynchus keta): Controlled ecosystem pollution experiment. Bull. Mar. Sci. 27: 114-118.

Kolonel, L. N. 1976. Association of cadmium with renal cancer. Cancer 37: 1782-1787.

Krasny, J. L. 1973. DNA Polymerases from Rat Intestinal Mucosa, (University of British Columbia, M. Sc.), 88p. (Unpubl. thesis).

Lazarus, L. H., and N. Kitron. 1973. Cytoplasmic DNA polymerase: polymeric forms and their conversion into an active monomer resembling nuclear DNA polymerase. J. Mol. Biol. 81: 529-534.

Leber, A. P. 1974. A mechanism for cadmium- and zinc-induced tolerance to cadmium toxicity: involvement of metallothionein. Purdue University, Ph.D., University Microfilms, Ann Arbor, Michigan. 72p.

Lewis, G. P., H. Lyle, and S. Miller. 1969. Association between elevated hepatic water-soluble protein-bound cadmium levels and chronic bronchitis and/or emphysema. Lancet II: 1330-1332.

Lin, H. J., W. C. Chan, Y. Y. Fong, and P. M. Newbérne. 1977. Zinc levels in serum, hair and tumors from patients with esophageal cancer. Nutr. Rept. Int. 15: 635-643.

Marafante, E. 1976. Binding of mercury and zinc to cadmium-binding protein in liver and kidney of goldfish (Carassius auratus L.) Experimentia 32: 149-150.

Margoshes, M., and B. L. Vallee. 1957. A cadmium protein from equine kidney cortex. J. Am. Chem. Soc. 79: 4813-4814.

McKee, J. E., and H. W. Wolf. 1963. Water Quality Criteria. The Resources Agency of Calif. State Water Quality Control Board Publication No. 3-A. 548p.

McLean, F. I., O. J. Lucis, Z. A. Shakh, and E. E. R. Jansz. 1972. The uptake and subcellular distribution of Cd and Zn in microorganisms. Fedn. Proc. Am. Socs. Exp. Biol. 31: 699.

McQuitty, J. T., W. D. DeWys, L. Monaco, W. H. Strain, C. G. Rob, J. Apgar, and W. J. Pories. 1970. Inhibition of tumor growth by dietary zinc deficiency. Cancer Res. 30: 1387-1390.

Menzel, D. W., and J. Case. 1977. Concept and design: controlled ecosystem experiment. Bull. Mar. Sci. 27: 1-7.

Miller, J. A. 1970. Carcinogenesis by chemicals: an overview. Cancer Res. 30: 559-576.

Morgan, J. M. 1970. Cadmium and zinc abnormalities in bronchogenic carcinoma. Cancer 25: 1394-1398.

Morgan, J. M. 1971. Tissue cadmium and zinc content in emphysema and bronchogenic carcinoma. J. Chron. Dis. 24: 107-110.

Morgan, J. M. 1972. Hepatic copper, manganese, and chromium content in bronchogenic carcinoma. Cancer 29: 710-713.

Nieper, H. A. 1977. The Nieper anticancer regimen. Prepared by the Silbersee Hospital, Hannover, West Germany. 6pp. (mimeogr.).

Noel-Lambot, F. 1976. Distribution of cadmium, zinc and copper in the mussel Mytilus edulis. Existence of cadmium-binding proteins similar to metallothioneins. Experimentia 32: 324-326.

Nordberg, G. F. 1972. Cadmium metabolism and toxicity. Environ. Physiol. Biochem. 2: 7-36.

Nordberg, G. F., M. Nordberg, M. Piscator, and O. Vesterberg. 1972. Separation of two forms of rabbit metallothionein by isoelectric focusing. Biochem. J. 126: 491-498.

Nordberg, G. F., and M. Piscator. 1972. Influence of long-term cadmium exposure on urinary excretion of protein and cadmium in mice. Environ. Physiol. Biochem. 2: 37-49.

Nordberg, G. F., R. Goyer, and M. Nordberg. 1975. Comparative toxicity of cadmium-metallothionein and cadmium chloride on mouse kidney. Arch. Pathol. 99: 192-197.

Olafson, R. W., and J. A. J. Thompson, 1974. Isolation of heavy metal binding proteins from marine vertebrates. Mar. Biol. 28: 83-86.

Olson, K. B., G. Heggen, C. F. Edwards, and L. Whittington Gorham. 1954. Trace element content of cancerous and noncancerous human liver tissue. Science 119: 772-773.

Olson, K. B., G. Heggen, and C. F. Edwards. 1958. Analysis of 5 trace elements in the liver of patients dying of cancer and noncancerous disease. Cancer 11: 554-561.

Otte, G., and C. D. Levings. 1975. Distribution of macroinvertebrates on mud-flats influenced by sewage, Fraser River estuary, British Columbia. Tech. Rept. No. 476, Fish. Mar. Serv., Environment Canada: 63 pp. (mimeogr.).

Overnell, J. 1975. The effect of heavy metals on photosynthesis and loss of cell potassium in two species of marine algae. Dunaliella tertiolecta and Phaeodactylum tricornutum. Mar. Biol. 29: 99-103.

Paecht-Horowitz, M. 1974. The possible role of clays in prebiotic peptide synthesis. Origins of Life 5: 173-187.

Parsons, T. R., C. A. Bawden, and W. A. Heath. 1973. Preliminary survey of mercury and other metals contained in animals from the Fraser River mud-flats. J. Fish. Res. Board Canada 30: 1014-1016.

Petering, H. G., H. H. Buskirk, and J. A. Crim. 1967. The effect of dietary mineral supplements of the rat on the antitumor activity of 3-ethoxy-2-oxobutyraldehyde bis (thiosemicarbazone). Cancer Res. 27: 1115-1121.

Piotrowski, J. K., B. Trojanowska, J. M. Wisniewska-Knypl, and W. Bolanowska. 1973. Further investigations on binding and release of mercury in the rat. In: Mercury, Mercurials and Mercaptans, M. W. Miller and T. U. Clarkson, Eds. Springfield, Illinois, Charles C. Thomas, pp. 247-263.

Piscator, M. 1964. On cadmium in normal human kidneys together with a report on the isolation of metallothionein from livers of cadmium-exposed rabbits. N. Hyg. Tidskr. 45: 76-82.

Pories, W. J., C. G. Mansour, F. R. Plecha, A. Flynn, and W.H. Strain. 1973. Inhibition of biological action of zinc in man by other trace elements. In: Trace Element Metabolism in Animals-2, W. G. Hoekstra, J. W. Suttie, H. E. Ganther, and W. Mertz, Eds. Baltimore, University Park Press, pp. 656.

Porter, H. 1974. The particulate half-cystine-rich copper protein of newborn liver. Relationship to metallothionein and subcellular localization in non-mitochondrial particles possibly representing heavy lysosomas. Bioch. Biophys. Res. Commun. 56: 661-668.

Poswillo, D. E., and B. Cohen, 1971. Inhibition of carcinogenesis by dietary zinc. Nature 231: 447-448.

Prohaska, J. R., M. Mowafy, H. E. Ganther. 1977. Interactions between cadmium, selenium and glutathione peroxidase in rat testis. Chem.-Biol. Interactions 18: 253-265.

Pulido, P., J. H. R. Kagi, and B. L. Vallee. 1966. Isolation and some properties of human metallothionein. Biochem. 5: 1768-1777.

Riordan, J. R., and I. Gower. 1975. Purification of low molecular weight copper proteins from copper loaded liver. Biochem. Biophys. Res. Commun. 66: 678-686.

Rugstad, H. E., and T. Norseth. 1975. Cadmium resistance and content of cadmium-binding protein in cultured humans cells. Nature 257: 136-137.

Sabbioni, E., and E. Marafante. 1975. Heavy metals in rat liver cadmium binding protein. Environ. Physiol. Biochem. 5: 132-141.

Sandberg, M., H. Gross, and O. M. Holly. 1958. Changes in retention of copper and iron in liver and spleen in chronic diseases accompanied by secondary anemia. Arch. Path. 33: 834-844.

Schroeder, H. A., J. J. Balassa, and J. C. Hogencamp. 1961. Abnormal trace metals in man: cadmium. J. Chron. Dis. 14: 236-258.

Shaikh, Z. Z., and O. J. Lucis. 1971. Isolation of cadmium-binding proteins. Experimentia 27: 1024-1025.

Slater, J. P., A. S. Mildvan, and L. A. Loeb. 1971. Zinc in DNA polmerases. Biochem. Biophys. Res. Commun. 44: 37-43.

Sharpless, G. R. 1946. The effects of copper on liver tumor induction by p-dimethylaminoazobenze. Fed. Proc. 5: 239-240.

Squibb, K. S., and R. J. Cousins. 1974. Control of cadmium binding protein synthesis in rat liver. Environ. Physiol. Biochem. 4: 24-30.

Strahler, A. N. 1972. Planet Earth: its Physical Systems Through Geologic Time. Harper and Row, New York, 483p.

Strain, W. H., E. G. Mansour, A. Flynn, W. J. Pories, A. J. Tomaro, and O. A. Hill. 1972. Plasma-zinc concentration in patients with bronchogenic cancer. Lancet I: 1021-1022.

Stonard, M. D., and M. Webb. 1976. Influence of dietary cadmium on the distribution of the essential metals copper, zinc and iron in tissues of the rat. Chem.-Biol. Interactions 15: 349-363.

Sugawara, C., and N. Sugawara. 1975. The inductive effect of cadmium on protein synthesis of rat intestine. Bull. Environ. Contamin. Tox. 14: 159-162.

Suzuki, Y., and H. Yoshikawa. 1972. Effects of cadmium injection on intracellular distribution of essential metals in rat liver. Ind. Health 10: 93-106.

Talbot, V., and R. J. Magee. 1978. Naturally-occurring heavy metal binding proteins in invertebrates. Arch. Environm. Contam. Toxicol. 7: 73-81.

Tietz, N. W., E. F. Hirsch, and B. Neyman. 1957. Spectrographic study of trace elements in cancerous and non-cancerous human tissues. J.A.M.A. 165: 2187-2192.

Topping, G., and H. Windom. 1977. Biological transport of copper at Loch Ewe and Saanich Inlet: controlled ecosystem pollution experiment. Bull. Mar. Sci. 27: 134-141.

Ulmer, D. D. 1970. Effects of metals on protein structure. In: Effects of Metals on Cells, Subcellular Elements, and Macromolecules, Maniloff, Coleman and Miller, Eds. Springfield, Illinois, USA, Charles C. Thomas, pp. 11-37.

Underwood, E. J. 1971. Trace Elements in Human and Animal Nutrition, 3rd edition. Academic Press, New York and London. 533p.

Vallee, B. L. 1976. Zinc biochemistry: a perspective. Trends Biochem. Sci. 1: 88-91.

Webb, M. 1972a. Binding of cadmium ions by rat liver and kidney. Biochem. Pharmac. 21: 2751-2765.

Webb, M. 1972b. Protection by zinc against cadmium toxicity. Biochem. Pharmac. 21: 2767-2771.

Weisburger, J. H., R. M. Madison, J. M. Ward, C. Viguera, and E. K. Weisburger. 1975. Modification of diethylnitrosamine liver carcinogenesis with phenobarbital but not with immunosuppression. J. Natl. Cancer Inst. 54: 1185-1187.

White, C. P. 1921. Copper in tumors and in normal tissues. Lancet II: 701-703.

White, A., P. Handler, and E. L. Smith. 1968. Principles of Biochemistry, 4th edition. McGraw-Hill Book Co., New York, Sydney, Toronto and London. 1187p.

Williams, D. R. 1971. The Metals of Life. Van Nostrand Reinhold Co., London, New York, Cincinnati, Toronot and Melbourne, 172p.

Winge, D., J. Krasno, and A. V. Colucci. 1973. Cadmium accumulation in rat liver: correlation between bound metal and pathology. In: Trace Element Metabolism in Animals-2, W. G. Hoekstra, J. W. Suttie, H. E. Ganther, and W. Mertz, Eds. Baltimore, University Park Press, pp. 500-501.

Winge, D. R., R. Premakumar, and K. V. Rajagopalan. 1975. Metal-induced formation of metallothionein in rat liver. Arch. Biochem. Biophys. 170: 242-252.

Wisniewska, J. M., B. Trojanowska, J. Piotrowski, and M. Jakubowski. 1970. Binding of mercury in the rat kidney by metallothionein. Toxic. Appl. Pharmac. 16: 754-763.

Woster, A. D., M. L. Failla, M. W. Taylor, and E. D. Weinberg. 1975. Zinc suppression of Sarcoma 180 growth. J. Natl. Cancer Inst. 54: 1001-1003.

Wright, E. B., and Dormandy, T. L. 1972. Liver zinc in carcinoma. Nature 237: 166.

Yamane, Y., K. Sakai, I. Uchiyama, M. Tabata, N. Taga, and A. Hanaki. 1969. Effect of basic cupric acetate on the biochemical changes in the liver of the rat fed carcinogenic aminoazo dye. I. Changes in the activities of DAB metabolism by liver homogenate. Chem. Pharm. Bull. 17: 2488-2493.

Yoshida, T., Y. Ito, Y. Suzuki, and M. Uchiyama. 1975. Inhibition of hepatic drug metabolizing enzyme by cadmium in mice. Bull. Environ. Contam. Tox. 15: 402-405.

Zahner, H., E. Bachmann, R. Hutter, J. Nuesch. 1962. Sideramine, eisenhaltige wachstumsfaktoren aus mikroorganismen. Path. Microbiol. 25: 708-736.

CHAPTER XIV Appendix I: Methodology Summary

Whereas detailed methodology is given in each chapter of this thesis, this section describes recommended procedures based upon experience gained during these studies. A summary of the steps used in the extraction of metallothionein and the high and low molecular weight cytoplasmic pools is presented in Figure 39. Liver or kidney tissue (1) are processed in the usual fashion as described in current literature (e.g., Olafson and Thompson, 1974). These procedures involved an initial extraction of the tissue (2-4) and a further extraction of the pellet obtained after centrifugation (6-8). Sample sizes and volumes of sodium chloride solution used in the extractions are given in Table 25. The double extraction process (1-9) increases the portion of metallothionein and other proteins extracted from tissue. Homogenizations (3&7) are done for exactly 3 minutes at a standard speed setting on a laboratory motor equipped with a teflon pestle. Centrifugations (4,8&12) are done at 27,000 x g for exactly 10 minutes.

For phytoplankton, zooplankton and mussels, minimal sample size may be available. In order that measureable levels of metal are eluted (15), an extraction of the pellet (6-8) is not done as this would result in a dilution of tissue metal levels with resultant dilution of metal levels in fractions collected (17). Similar procedures are followed for very small samples of liver or kidney tissue (Table 25).

Figure 39. A flow diagram of the steps involved in the extraction of metallothionein and the high and low molecular weight pools from tissue. See text for details.

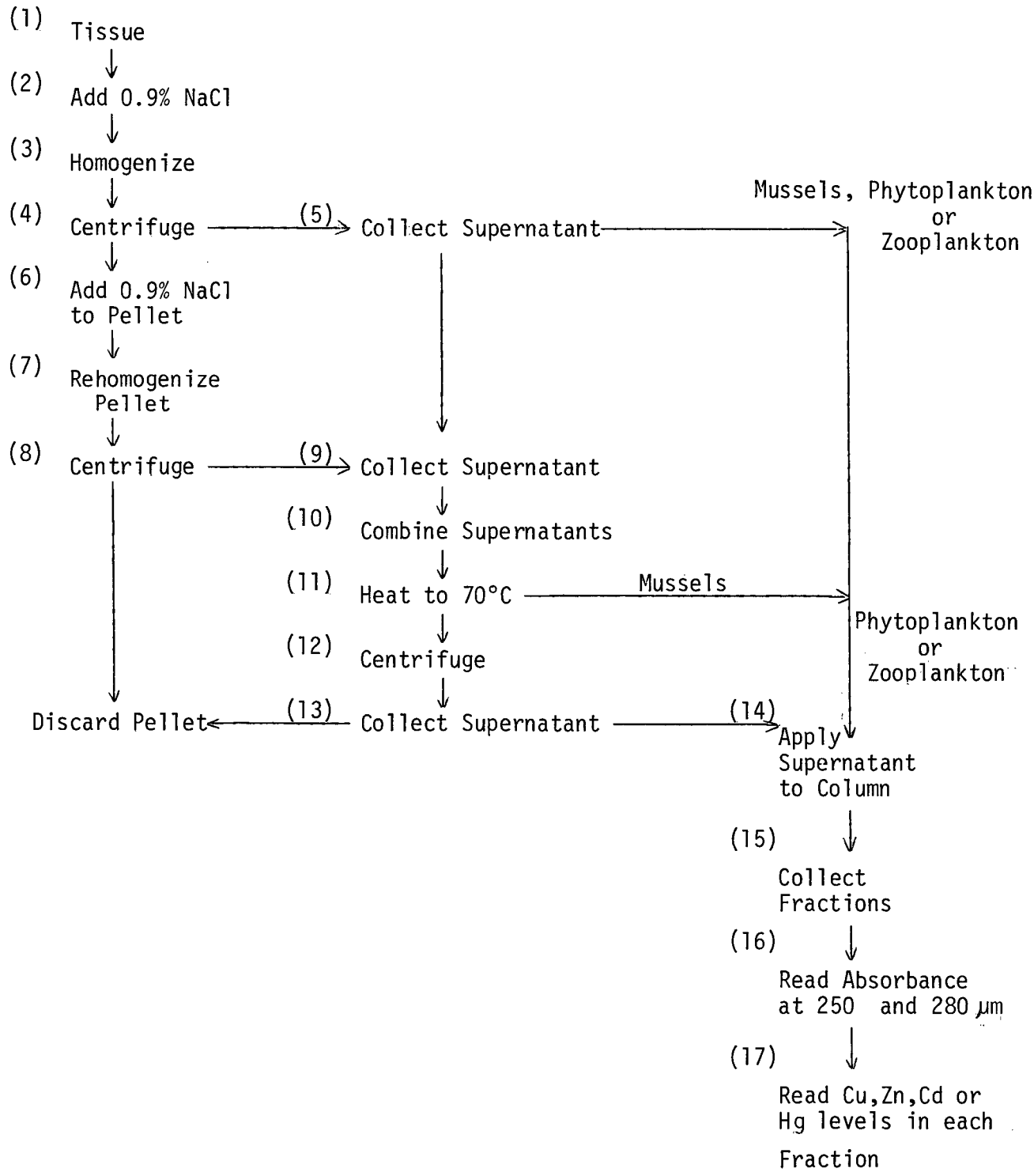

(1) Tissue
(2) Add 0.9% NaCl
(3) Homogenize
(4) Centrifuge
(5) Collect Supernatant
Mussels, Phytoplankton or Zooplankton
(6) Add 0.9% NaCl to Pellet
(7) Rehomogenize Pellet
(8) Centrifuge
(9) Collect Supernatant
(10) Combine Supernatants
(11) Heat to 70°C
Mussels
Phytoplankton or Zooplankton
(12) Centrifuge
(13) Collect Supernatant
Discard Pellet
(14) Apply Supernatant to Column
(15) Collect Fractions
(16) Read Absorbance at 250 and 280 μm
(17) Read Cu,Zn,Cd or Hg levels in each Fraction

Table 25. Recommended procedures for various tissue types.

Tissue Type	Sample Size(1) (grams)	First Homogenization(3) 0.9% NaCl Volume (ml)	Second Homogenization(7) 0.9% NaCl Volume (ml)	Time to Heat(min) at 70°C(11)	Volume to Apply(ml) to Column(14)	Column Type Used	Fraction Size(ml) to Collect(15)[a]
Phytoplankton (whole tissue)	1	3	not done	not done	2	K.9/60	1.5
Zooplankton (whole tissue)	1	3	not done	not done	2	K.9/60	1.5
Mussel (soft parts)	1	3	not done	5	2	K.9/60	1.5
Liver or Kidney	0.1	1.5	not done	5	1.0	K.9/60	0.8
	0.5	2.0	not done	5	1.5	K.9/60	1.0
	1	2.5	1.5	5	2	K.9/60	1.5
	2	4.5	2.5	5	5	K1.6/100	6
	3	9	6	5	14	K2.5/100	15

[a] To produce about 30 fractions per profile.

Supernatants from mussel (5), liver and kidney (10) tissue are heated in a 70°C water bath for 5 minutes (11) and then centrifuged (12) in order to clear the tissue extract of cellular debris. The supernatant is collected (13) and the pellet discarded. Al alternative to this procedure is to centrifuge the combined supernatants (13) at 105,000 x g for 60 minutes. This latter procedure was not utilized in the present studies as an ultracentrifuge was not available. However, to avoid losses of heat coagulable enzymes from the high molecular weight protein pool, the ultracentrifugation technique is preferable; this method is particularly preferable if enzyme activities are to be measured in the high molecular weight protein pool, as many enzymes are irreversibly denatured by heat. It is not necessary to heat phytoplankton and zooplankton supernatants (Table 25) as these appear clear (free of cellular debris) after one extraction and centrifugation (1-5). Resulting supernatants are applied to a column packed with Sephadex G-75 gel (14) and eluted with 0.01 M NH_4HCO_3.

The size of column employed (14) will depend mainly upon the sample size used (Tables 25 and 26). A narrower column is used for smaller sample sizes. A longer column is preferable to a shorter column as it provides a better resolution of peaks. Better resolution of peaks can also be obtained by collecting smaller fraction sizes as eluant from the column. Resolution improves down to 1-2% of the

Table 26. The specifications for various sizes of Pharmacia columns when packed with Sephadex G-75 gel (from Pharmacia technical literature).

Column Type (k) and size (cm) (Kdiameter/length)	Bed Volume (ml)	Minimum/Maximum Sample Size (ml)	Maximum FLow Rate (ml/hr)	Void Volume (ml)	Time for First Macromolecules to Elute (hr)
K.9/60	38	0.4/10	17	13	0.8
K1.6/100	200	2/50	44	70	1.6
K2.5/100	485	5/120	114	168	1.5

bed volume. Sample sizes up to 25-40% of the bed volume give less diluted cytoplasmic pools, but with less resolution.

The position of peaks is established initially by reading absorbance at 250 and 280 μm (16). The high and low molecular weight pools have high absorbance at 250 and 280 μm whereas metallothionein is usually very low at 280 μm due to the absence of aromatic amino acids. Metallothionein may have high absorbance at 250 μm if much Cd is bound, due to the presence of sulfhydryl-Cd bonds. The high molecular weight protein pool will be eluted as the first peak, with a shoulder or separate peak following due to the presence of hemoglobin, if applicable. The metallothionein peak will appear next, followed by a double peaked low molecular weight cytoplasmic pool. Each absorbance peak will correspond to a peak of various metals. The high molecular weight protein pool usually contains Cu and Zn due to the presence of metalloenzymes (Brown et al., 1977). Metallothionein binds the toxic metals, Hg and Cd, and also excesses of Cu and Zn above levels required for metalloenzymes. The low molecular weight cytoplasmic pools binds a small portion of metals in most organisms, but very high portions in phytoplankton and zooplankton. In the present study, it was found that in a gel elution profile comprising 45 fractions, the high molecular weight protein pool comprised tubes 1-15, metallothionein comprised tubes 16-25, and the low molecular weight cytoplasmic pool comprised tubes 26-45. In other gel elution

profiles, the high molecular weight protein pool comprised the first 15/45 (.33) tubes of the profile, metallothionein the next 11/45 (.24) tubes of the profile, and the low molecular weight cytoplasmic pool the last 20/45 (.44) tubes of the profile.

Metal levels are determined in each fraction, added, and expressed as a concentration of metal in each cytoplasmic pool per gram of tissue (wet weight). For instance, in a high molecular weight pool comprising 10 fractions, the concentrations of Zn in each fraction (as mg Zn/L) are added correcting for the volume of each fraction (e.g., 6 ml):

$$\left(\frac{x_1 \text{ mg Zn}}{1000 \text{ ml}} \times 6 \text{ ml}\right) + \left(\frac{x_2 \text{ mg Zn}}{1000 \text{ ml}} \times 6 \text{ ml}\right) + \ldots\ldots + \left(\frac{x_{10} \text{ mg Zn}}{1000 \text{ ml}} \times 6 \text{ ml}\right)$$

$$\text{OR} \quad \frac{(x_1 + x_2 + \ldots\ldots + x_{10}) \text{ mg Zn}}{1000 \text{ ml}} \times 6 \text{ ml}$$

A correction is applied for the wet weight of tissue initially homogenized (e.g., 2 g liver, wet weight):

$$\times \frac{1}{2 \text{ g liver(wet wt)}}$$

A further correction is made for the fact that not all supernatant (13) may be applied to the column (14). For instance, a 2 gram liver sample comprising approximately 1.8 ml of water and other extractable material is homogenized initially

in 4.5 ml of 0.9% NaCl and the pellet extracted in 2.5 ml of solution. Of this 8.8 ml of extractable material and sodium chloride solution, 5 ml are applied to the column Table 25). Therefore a correction factor is needed for discarded tissue extract:

$$X \quad \frac{8.8}{5.0}$$

A correction is made since data in the present study has been converted into μmole so that competition between metals can be evaluated in terms of the relative numbers of molecules of each metal present.

$$X \frac{1 \text{ mmole Zn}}{65.4 \text{ mg Zn}} X \frac{1000 \text{ μmole}}{\text{mmole}}$$

The complete caluculation is:

$$\frac{(x_1+x_2+\ldots+x_{10})\text{mg Zn}}{1000 \text{ ml}} X \text{ 6 ml } X \frac{1}{2 \text{ g liver (wet wt)}} X \frac{8.8}{5.0} X \ldots\ldots$$

$$\ldots\ldots X \frac{1 \text{ mmole Zn}}{65.4 \text{ mg Zn}} X \frac{1000 \text{ μmole}}{\text{mmole}} =$$

In a typical gel elution profile where $x_1 + x_2 + \ldots\ldots x_{10}$ = 0.8 mg, there would be 0.065 μmole Zn/g liver (wet weight) in the high molecular weight protein pool.

PUBLICATIONS:

McLeay, D. J., and D. A. Brown. 1974. Growth stimulation and biochemical changes in juvenile coho salmon (Oncorhynchus kisutch) exposed to bleached kraft pulpmill effluent for 200 days. J. Fish. Res. Board Can. 31: 1043-1049.

McLeay, D. J., and D. A. Brown. 1975. Effects of acute exposure to bleached kraft pulpmill effluent on carbohydrate metabolism of juvenile coho salmon (Oncorhynchus kisutch) during rest and exercise. J. Fish. Res. Board Can. 32: 753-760.

Brown, D. A., and D. J. McLeay. 1975. Effect of bleached kraft mill effluent on the survival of starved juvenile coho salmon (Oncorhynchus kisutch). J. Fish. Res. Board Can. 32: 2528-2530.

Brown, D. A., and D. J. McLeay. 1975. Effect of nitrite on methemoglobin and total hemoglobin of juvenile rainbow trout. Prog. Fish-Cult. 32: 36-38.

Brown, D. A., C. A. Bawden, K. W. Chatel, and T. R. Parsons. 1977. The wildlife community of Iona Island jetty, Vancouver, B.C., and heavy-metal pollution effects. Environ. Conserv. 4: 213-216.

Brown, D. A. 1977. Increases of Cd and the Cd:Zn ratio in the high molecular weight protein pool from apparently normal liver of tumor-bearing flounders (Parophrys vetulus). Mar. Biol. 44: 203-209.

Brown, D. A., and T. R. Parsons. 1978. Relationship between cytoplasmic distribution of mercury and toxic effects to zooplankton and chum salmon (Oncorhynchus keta) exposed to mercury in a controlled ecosystem. J. Fish. Res. Board Can. 35: 880-884.

Brown, D. A., and T. R. Parsons. 1978. The ubiquity and function of metallothionein in marine organisms. (Presented at the 41st annual meeting of the American Society of Limnology and Oceanography, Victoria, B.C.).

Brown, D. A., and K. W. Chatel. 1978. Interactions between cadmium and zinc in cytoplasm of duck liver and kidney. Biol.-Chem. Interactions 22: 271-279.

Hall, J. B., and D. A. Brown. 1979. Plasma glucose and lactic acid alterations in response to a stressful exam. Biol. Psych. (In press).

Cloutier-Mantha, L., and D. A. Brown. 1979. The effect of mercury exposure on the cytoplasmic distribution of mercury, copper and zinc in phytoplankton. J. Exp. Mar. Biol. Ecol. (In press).

Young, D. R., C. K. Bertine, D. A. Brown, E. Crecelius, J. Martin, F. Morell, and G. Roesijadi. 1979. Trace metals, In: NOAA Recommendations to the U.S. Congress on Ocean Pollution Research, Ed: E. Goldberg. (In press).

Bayne, B. L., D. A. Brown, F. L. Harrison, and P. P. Yevich. 1979. Mussel health, In: The International Mussel Watch, Ed: E. Goldberg. (In press).