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DISSERTATION

ISOLATION AND CHARACTERIZATION OF A CADMIUM BINDING PROTEIN FROM THE PLANARIAN, *Dugesia dorotocephala*, AND EVALUATION OF ITS PROTECTIVE EFFECTS AGAINST DNA DAMAGE CAUSED BY CADMIUM AND RADIATION

Submitted by

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In partial fulfillment of the requirements

for the degree of Doctor of Philosophy

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Fall 2001

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October 6, 2001

We Hereby Recommend That The Dissertation Prepared Under Our Supervision By Michael Dean Brown Entitled: **ISOLATION AND CHARACTERIZATION OF A CADMIUM BINDING PROTEIN FROM THE PLANARIAN, *Dugesia dorotocephala*, AND EVALUATION OF ITS PROTECTIVE EFFECTS AGAINST DNA DAMAGE CAUSED BY CADMIUM AND RADIATION** Be Accepted As Fulfilling In Part Requirements for the Degree of Doctor of Philosophy.

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ABSTRACT OF DISSERTATION

ISOLATION AND CHARACTERIZATION OF A CADMIUM BINDING PROTEIN FROM THE PLANARIAN, *Dugesia dorotocephala*, AND EVALUATION OF ITS PROTECTIVE EFFECTS AGAINST DNA DAMAGE CAUSED BY CADMIUM AND RADIATION

Cadmium contamination of the environment occurs as a result of mining and smelting operations, industrial use of the metal and inappropriate disposal of cadmium-containing products. The health effects related to cadmium exposure are numerous and varied, with manifestations of neuropathy, hepatotoxicity, bone mineralization disorders, neurotoxicity, cardiovascular disease, immunological disorders, genetic mutations, teratogenesis and carcinogenesis.

The planarian, *Dugesia dorotocephala*, has been shown to have a high incidence of malignant post-pharyngeal tumors following exposure to cadmium and the tumor-promoting phorbol ester, TPA. This makes the planarian an attractive model to study the mechanisms of carcinogenesis in a simple multicellular organism.

Mammalian species exposed to cadmium respond with the induction of a low molecular weight, heat stable protein, metallothionein, which has a high cysteine content and is capable of binding large amounts of cadmium. Metallothionein has never been isolated nor characterized in planaria. This provided the basis for the initial hypothesis that the planarian, *D. dorotocephala*, produces metallothionein or a metallothionein-like protein that is capable of binding cadmium. In addition, the protein should be inducible with exposure to zinc and cadmium, as has been shown for mammalian metallothionein. It was further hypothesized that planarian metallothionein would be capable of binding cadmium and would thus reduce DNA damage caused by cadmium exposure.

Following exposure to sub-lethal doses of either cadmium or zinc, planaria were homogenized. The resulting homogenate was then subjected to centrifugation to isolate cytosol, which was then heat treated and re-centrifuged to isolate heat stable cytosolic proteins. The heat stable cytosolic proteins were then separated using gel chromatography. Separated proteins were then subjected to spectrophotometric and atomic absorption analysis (AA) to identify a cadmium-binding protein that had high 254 nm and low 280 nm absorbance. The molecular weight of this cadmium-binding protein was determined to be approximately 7400 Da by gel filtration chromatography. The isolated protein was found to have a high thiol content. The protein was further purified by diethylaminoethylcellulose (DEAE) ion exchange high-performance liquid

chromatography (HPLC). The amino acid content of the purified protein was determined and was found to contain 28% cysteine. These results are consistent with the cysteine content of mammalian metallothionein.

Thus, planaria were shown to produce a low molecular weight protein like mammalian metallothionein that was heat stable, bound cadmium and had a high thiol content primarily in the form of cysteine. Because mammalian metallothionein is inducible by exposure to a variety of metals, including zinc and cadmium, planaria were exposed to non-lethal doses of different metals with the planarian metallothionein levels determined by AA analysis of the cadmium-saturated protein. Planarian metallothionein was shown to be inducible following exposure to metals and hydroperoxides like benzoyl peroxide.

To test the hypothesis that planarian metallothionein could reduce DNA damage, planaria were pre-treated with zinc for 5 days to increase planarian metallothionein levels. Pre-treated and non pre-treated planaria were then exposed to different concentrations of Cd ranging from 100 to 2,500 μg/L for varying periods of time up to 48 hours. DNA fragmentation was assessed using a modified alkaline agarose gel electrophoresis assay. Damage to DNA was quantified using densitometric analysis of the mean migration distance. DNA from planaria pre-treated with zinc before cadmium exposure had less DNA fragmentation than planaria not pre-treated with zinc before cadmium exposure. Decreases in electrophoretic migration of DNA of 2.4- to 2.6-fold were associated

with increases of planarian metallothionein of 2.5- to 4.5-fold due to zinc treatment.

Similar results were noted in planaria pre-treated with a cocktail of buthionine sulfoximine (BSO) and diethylmaleate (DEM) to deplete glutathione. Planaria treated with BSO, DEM and zinc had less DNA fragmentation than planaria treated with BSO and DEM but not zinc.

Using the same techniques for measuring DNA fragmentation in planaria, the hypothesis that ionizing radiation both induced planarian metallothionein and protected against DNA fragmentation was tested. Three days after planaria were irradiated with 1.0 to 6.0 Gy from a cesium -137 source, there was a significant increase in metallothionein levels that ranged between 12.8- and 14.4-fold over protein levels measured in controls. There was also a significant decrease (ranging from 1.4- to 3.2-fold) in the amount of DNA fragmentation in planaria pre-exposed to the radiation source before being exposed to varying doses of ionizing radiation compared with DNA damage measured in planaria not pre-exposed to ionizing radiation.

In summary, the planarian, *D. dorotocephala*, was shown to have a metallothionein that is a low molecular weight protein, that is heat stable and binds cadmium. Furthermore, the protein was shown to be inducible with exposure to metals, hydroperoxides and radiation, as is mammalian metallothionein. Furthermore, it was shown that induction of the planarian

metallothionein significantly reduced cadmium- and radiation-initiated DNA fragmentation. Although the planarian, *D. dorotocephala*, is an invertebrate, isolation and characterization of metallothionein from this organism show that its cells react to cadmium exposure like mammalian cells. Moreover this discovery helps to further validate the organism as an alternative model to study Cd-induced cancer.

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Michael D. Brown
October 6, 2001

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Chapter 1

Introduction

In 1987 the International Agency for Research on Cancer (IARC) accepted as sufficient the evidence that cadmium (Cd) chloride, Cd sulfate, Cd sulfide and Cd oxide can cause cancer (IARC, 1987). These compounds have been directly associated with injection site sarcomas in the rat and the induction of interstitial cell tumors of the testis in both rats and mice following injection. Oral dosing studies were not conclusive and were considered to be inadequate for evaluation. Long term inhalation studies in rats exposed to aerosols of Cd sulfate, Cd oxide fumes and Cd sulfate dust demonstrated a high incidence of primary lung cancer with evidence of a dose-response relationship (Takenaka *et al*, 1983; Oldiges *et al*, 1989). However, it should be noted that this response has not been adequately demonstrated in other mammalian species to this point (Heindrich *et al*, 1989; Aufderheide *et al*, 1990; Levy *et al*, 1975). Results of studies on the genotoxic effects of Cd have been discordant Anderson *et al*, 1982).

Although Cd and other metals produce DNA damage and lipid peroxidation *in vitro* and/or *in vivo*, the molecular mechanisms of their toxicity have not been adequately studied. Recent studies have implied that the exact

mechanism by which Cd induces carcinogenesis is unknown (Hartwig, 1994) although there is a growing volume of literature, which suggests that Cd-induced carcinogenesis may be associated with the generation of oxygen free radicals (Meneghini, 1988; Sugiyama, 1994; Burkart and Ogorek, 1969; Ochi *et al,* 1983; Snider, 1988; Klein *et al,* 1991; Kasprzaak, 1991). Oxidative stress is largely mediated by reactive oxygen species, including superoxide anions, hydrogen peroxide and hydroxyl radicals. These reactive oxygen intermediates are generated in cellular organelles by a number of different catalytic reactions including reactions such as metal ion-catalyzed redox reactions and xenobiotic metabolism (Halliwell and Gutteridge, 1984). In addition, chemical reactions associated with normal cellular physiological functions including respiration and inflammatory responses also have been shown to produce reactive oxygen species. Toxic oxygen species appear to be involved in many harmful reactions in biological systems including DNA degradation, the peroxidation of membrane lipids, endothelial cell destruction and changes in vascular permeability (Halliwell and Gutteridge, 1984; Slater, 1984). It has been theorized that Cd may catalyze hydroxyl radical generation via the Haber-Weiss reaction or DNA strand breakage by direct adduction to the DNA molecule (Klein *et al,* 1991; Cohen *et al,* 1990). DNA adducts can lead to strand breaks and/or mutations if not correctly repaired by the DNA repair mechanisms which actively work in the cell.

Commercial Cd production started around the turn of the century and over the years the pattern of Cd consumption has changed radically. Currently the primary use for the metal is the production of batteries and for specialized electronic applications (Wilson, 1988). Most commercial uses for Cd utilize only minute quantities of the metal, which limits the effective recycling activities designed to reclaim the metal from spent materials containing Cd. Cadmium is often present in ores mined for their content of other metals. Because it is potentially volatile, some processing activities result in the release of Cd into the air. Thus areas adjacent to non-ferrous mines and smelters often show pronounced levels of Cd contamination (Krell and Roeckner, 1988). In addition, some phosphate fertilizers contain elevated levels of Cd. Increases in soil Cd levels directly translate into an increased uptake of the metal by plants (Lund *et al*, 1981): this is especially important when man and food animals consume the plants (Sherlock and Walters, 1983). For humans, this is a primary route of Cd exposure in the absence of occupational uses. Another important source of Cd exposure is due to its presence in tobacco that efficiently enters the body via the respiratory system of people who smoke (Elinder *et al,* 1983).

Freshwater planaria, *Dugesia dorotocephala*, are capable of modeling many important aspects of the anatomical, physiological and biochemical organization of phylogenetically higher animals (Best and Morita, 1982, 1984;

Morita and Best, 1984). Earlier researchers, using Cd and the phorbol ester TPA, demonstrated a high incidence of postpharyngeal tumors in the asexual strain of *Dugesia dorotocephala* (Hall *et al*, 1986). These tumors displayed the major phenomenology of multiple stage mammalian carcinogenesis, involving both initiation and promotion (Hall *et al*, 1986).

To survive environmental and physiologic stresses, a number of different organisms have been shown to possess specialized defense mechanisms. Exposure to metals, especially zinc (Zn) and Cd, and have been shown to induce a low molecular weight protein in mammals known as metallothionein (MT). A review of the current literature revealed no evidence that planaria produce this protein.

The primary hypothesis of this research was that the planarian, *Dugesia dorotocephala*, produces metallothionein or a metallothionein like protein that binds cadmium and has properties similar to mammalian metallothionein.

If this hypothesis was correct, planaria would contain a cadmium binding protein with a low molecular weight similar to mammalian MT. In addition, it was anticipated that this protein would be heat stable, and have an amino acid composition similar to mammalian MT (a high cysteine content and lacking aromatic acid residues). The literature also states that mammalian MT is inducible following exposure to different metals including Zn and Cd and also

under conditions of oxidative stress. Therefore it was further expected that MT would be induced in planaria exposed Cd, Zn or organic oxidizers like benzoyl peroxide. Thus, another hypothesis of this work was that planarian MT is inducible following exposure to Zn, Cd or organic oxidizers like benzoyl peroxide.

A review of the literature suggested that one of the functions of mammalian MT may be to serve as a radical scavenger, which protects DNA and cellular structures from the oxidative effects of free radicals. This opinion is supported by a recent study conducted by Coogan *et al.*, (1992) which demonstrated reduced levels of DNA damage in rat hepatocytes that had elevated MT levels prior to Cd exposure. In contrast, an earlier study conducted by Muller using cell free DNA in a non-cellular system demonstrated that Cd bound to the MT molecule increased the number of DNA strand breaks and did not show the protective effects observed by Coogan. The study which showed MT to be protective against DNA strand breaks used a whole cell system and Coogan concluded that the observed protective effect of the MT molecule probably involved the combined effects of MT, Cd and Zn in the whole cell.

Once planaria had been shown to produce MT its role in responses to toxic exposures was investigated. The third hypothesis was that planarian MT prevents or reduces the amount of oxidative damage to cellular DNA associated with exposure to Cd or radiation. As a result, the research project had three

additional goals: 1) Determine if the MT identified in the planarian, *Dugesia dorotocephala*, was capable of protecting against DNA strand breaks caused by exposure to cadmium. 2) Determine if Cd exposure at different concentrations resulted in increased amounts of cellular DNA damage similar to that caused by exposure to reactive oxygen radicals. 3) Determine if irradiation exposure induced the planarian MT and also to determine if induction was associated with protection against DNA strand breaks.

Organization of the Dissertation

Chapter 2 is a review of the literature pertaining to planaria, metallothionein, cadmium and oxidative damage and antioxidant systems. The next three chapters correspond to manuscripts prepared for publication. The first (Chapter 3) describes the isolation, purification and characterization of planarian metallothionein. Chapter 4 describes the responses of planarian MT to Cd and oxidative stress and the associated changes in DNA damage. Chapter 5 describes the effects of ionizing radiation on planarian MT expression and DNA damage. The concluding chapter (6) summarizes the salient results.

Chapter 2

Literature Review

Cadmium the element

Cadmium (Cd) is a transition metal and member of Group IIB of the elemental periodic table, and along with zinc and mercury, is a natural component of rock, sediment, soil, water, air and is often found as a contaminant in plant and animal tissues. Its geochemical behavior is similar to that of zinc, because of they share similar electron and ionization structures (Wilson, 1988). With an atomic number of 48 and an atomic weight of 112.4, the metal has a specific gravity of 8.65, a melting point of 320.9° C, and a boiling point of 767.0° C (Wilson, 1988). Cd salts vary in water solubility with the sulfides, carbonates, oxides and hydroxides being relatively insoluble. Water-soluble Cd salts include the sulfates, nitrates and halides. The element forms a variety of organic compounds and complexes but, unlike alkyl-mercury derivatives, the alkyl-cadmium compounds are extremely unstable (Krell and Roeckner, 1988) and are less important from a toxicological viewpoint.

Natural sources

The element was discovered by Strohmeyer in 1817 as a contaminant

of zinc ore (Chizhikov, 1966). In zinc ore, the zinc:cadmium contamination ratio is normally accepted to be about 1000:3 (CEC, 1978). When found with zinc ores, Cd is primarily a sulfide but under natural conditions it may be present as an oxide and/or carbonate (Waketa and Schmidt, 1970). In addition, considerable amounts of Cd are found associated with copper and lead ore deposits. In the earth's crust, the average Cd concentration is approximately 0.11 μg/g (Bowen, 1979). Cd concentrations in the upper lithosphere vary but average 0.5 μg/g with an approximate zinc: Cd ratio of 250:1 (Goldschmidt, 1958). Sedimentary rocks which include both the bituminous, carbonaceous, and black shale may contain abnormally high Cd concentrations (Chizhikov, 1966), which may be affected by the weathering cycles and provide a significant exposure pathway that can contaminate plant and animal tissue (Lund *et al*, 1981). Tablr 2-1 summarizes the Cd content of some geologic sources.

Table 2-1 Natural sources of cadmium in the environment

Compound Source	Concentration	Reference
Igneous Rock	1 μg /g	Goldschmidt, 1958
Coal – United States	0.01 –180 μg /g	Valkovic, 1983
Coal – Worldwide	0.01-22 μg /g	Bowen, 1979
Crude Oil	0.0 - 1000 μg /g	Willson, 1988
Marine sediments	0.1 – 1 μg /g	Mullen and Riley, 1956
Manganese (Ocean floor)	8 μg /g	Bowen. 1979
Phosphorites (Ocean floor)	0.01 – 25 μg /g	Bowen. 1979

Soil from a Cd contamination standpoint, can be modified to varying degrees by environmental factors that result in the mobilization and redistribution of the soil profile of interest both within and between neighboring soils. The worldwide Cd soil content ranges from less than 0.07 to 1.1 μg/g. It should be noted that all values over 0.5 μg/g in most cases reflect anthropogenic inputs (Kabata-Pendias and Pendias, 1984). An exception to this fact is that there is more variability in Cd content in

geographical areas that contain large deposits of either lead or zinc and/or vast quantities of black marine shale. Soils from these and surrounding areas often contain higher non-anthropogenic influenced Cd concentrations that can exceed 0.5 μg/g (Kabata-Pendias and Pendias, 1984; Marples and Thornton, 1980).

Unpolluted waters usually contain only trace amounts of Cd, and values of less than 1 ng/L have been reported from these sources; however, concentrations from this type of environment can sometimes exceed 10 ng/L (Friberg et al, 1974). Cd solubility in water increases with decreasing pH. Waters in close proximity to Cd-bearing mineral deposits may have Cd levels that can exceed 1000 μg/L (Boyle and Jonasson, 1979). The Cd content of inshore waters in both industrialized and non-industrialized parts of the United Kingdom have been shown to range from low of 0.14 μg/L up to and sometimes exceeding 4.20 μg/L (Abdullah et al, 1972). This variation reflects metal rich inputs from industrial and mining sources. Atmospheric Cd concentrations are generally very low, yet, as with water and soil pollution, the atmospheric concentrations near sources of Cd emission can be elevated to the point where intake by inhalation may become critical (CEC, 1978)

Anthropogenic sources of cadmium

The production of Cd in its pure state began around the turn of the

century and has increased steadily with the greatest increases in use occurring over the past twenty-five years. Industrial utilization of Cd has increased with the demands of the technical societies. About 70% of the total world production of Cd has taken place over the past 30 years (OECD, 1990). Industrial demand for cadmium has increased since 1982 in Europe, with consumption rising from about 17,000 tons/year to about 18,000 tons in 1989 (OECD, 1990). Reclamation of spent Cd by-products with recycling processes is not widely used today because the metal is used in very small quantities in most industrial applications. As the demand for the metal exceeds production capacity. this practice may change due to increasing economic forces. A report prepared by the U.S. Bureau of Mines suggests that world demand for Cd, assuming negligible Cd recycling, would exceed all known reserves by the year 2000 (OECD, 1990). In the United States, secondary recovery of industrial Cd is currently less than 10% (OECD, 1990). With current manufacturing and processing practices in today's environment it has been estimated that approximately one-third of the total amount of Cd used in industrial applications annually is lost as an environmental pollutant.

Current methods of recovering Cd ore from companion ores are complex processes, which can become important sources of environmental contamination. Environmental pollution is usually lower with the Cd sulfide-

contaminated ores, than with the other Cd salt contaminants which are more commonly found in conjunction with large deposits of zinc, lead and/or copper bearing ores. Following the mining operation (extraction of the ore from the earth) the recovered ores then undergo a three-part milling process, which includes crushing, grinding and finally a chemical flotation process, which concentrates the metal bearing ores. The milled ore is then smelted using either pyro-and/or hydro-metallurgical procedures to recover elemental Cd. Enrichment of the primary material by pyrolysis removes Cd sulfide as a highly volatile gaseous by-products which are then collected as flue dust precipitates, under the smelter smoke stacks. Cd-rich flue dusts are then leached with sulfuric acid at a high temperature, to separate the Cd from other metal dusts. Finally, vacuum distillation or electrolysis processes are used to produce a marketable grade of metallic Cd. Because Cd is usually a secondary marketable product of the operation, active monitoring of each stage presents a number of important potential sources of environmental pollution that can result in the release of dust, fumes, waste waters and/or sludge from ores that contain large amounts of Cd.

Waste incineration is another important source of environmental Cd pollution because of its volatility at high temperatures. Areas of local and regional contamination with Cd have been identified in all continents due to

increased industrial use of the metal, with the exception of Antarctica. Estimates show that the worldwide anthropogenic emissions of Cd gases exceed natural ones by a factor of ten (OECD, 1990).

The electroplating industry utilizes large quantities of Cd in the steel and iron industries to produce alloys that protect against corrosion. In 1979 this industrial application accounted for approximately 50 percent of all Cd produced that year (Yost, 1979). During the last decade, the use of Cd to protect steel and iron products against corrosion by electroplating has decreased, whereas its use in the battery industry has increased. Currently, the plastic industry utilizes Cd as a stabilizer to increase the durability and life span of its products (IPCS, 1992). Cadmium compounds are also used as pigments in plastics, glass, rubber, ceramic and paint products. A variety of alloys and solders are produced with Cd as a major constituent. Rechargeable nickel-cadmium batteries exhibit electrical properties that make them attractive for certain applications. This metal is also used in industrial applications as a constituent in fungicides, in the curing of rubber, in the production of control rods used in nuclear reactors, in the phosphors of television tubes and in photographic compounds.

In addition to the primary industrial sources of environmental Cd pollution, large quantities of phosphatatic rock, which contain variable

amounts of Cd, are used annually in the production of fertilizers (IPCS, 1992).

Contamination of agricultural soils is a particularly troublesome problem because when Cd is present in the soil many food plants efficiently take up the metal (IPCS, 1992). This efficient uptake of the metal by plants makes it possible for the metal to enter the food chain and eventually become a contaminant in the tissues of both man and animals. Soil contamination can also occur as a result of deposition from polluted air. Additional sources of soil pollution by the metal occur more commonly from the use of Cd-contaminated irrigation water, sewage contaminated sludge and from phosphate fertilizers (Elinder, 1985). A low soil pH increases the uptake of Cd by plants. Depression of the pH of both water and soil is becoming increasingly prevalent problem in many areas of the world because of acid rain produced by the anthropogenic emission of sulfur dioxide and nitrous gases into the air.

Available data indicates that human exposure to Cd has increased during this century, possibly due to changing work habits and lifestyles. For example, when comparison is made of wheat grown on the same fields in Sweden, the Cd concentration has more than doubled since 1916 (Kjellstrom et al, 1975); Anderson and Bingefors, 1985). Likewise, an analysis of historical specimens of human kidney show that Cd concentrations were considerably lower in the beginning of the 19th century compared to present

day values (Elinder and Kjellstrom, 1977; Drasch, 1983).

Natural sources of cadmium exposure

For the general human population, foods contaminated with Cd constitute a major source of environmental Cd exposure. In food stuffs obtained from areas considered unpolluted, Cd concentrations are generally below 0.1 mg/kg fresh weight. Milk, dairy products, eggs, beef, and fish normally contain less than 0.01 mg/kg. Somewhat higher concentrations, 0.01 - 0.10 mg/kg are found in fresh vegetables, fruits and some grains. It is not unusual to find even higher Cd levels in wheat and rice than in other grains like oats and barley. Cadmium concentrations in the range of 0.1-1 mg/kg are normally found in the liver and kidney of samples obtained from adult cattle and other domestic animals. Oysters, mussels and certain species of wild white mushrooms often have Cd concentrations that exceed 1 mg/kg. Particularly high concentrations of Cd in the range of 2-30 mg/kg fresh weight, are found in the edible brown meat of marine shellfish; this is because the brown meat of the shellfish physiologically functions as the animal's liver (Jorhem et al, 1984: Elinder, 1985).

Cigarette smoking and inhalation of contaminated air constitutes an even greater potential source of Cd exposure than the consumption of contaminated food products (CRC, 1974; CEC, 1978). The smoking of one

cigarette can result in the inhalation of 0.1-0.2 μg of Cd with ten percent of the Cd in the cigarette smoke being absorbed (Elinder *et al.* 1983).

However, for certain populations in heavily polluted areas, both airborne and water borne Cd present a potentially greater risk for humans than contaminated food products. Vaghter et al (1990) reported that the daily intake of Cd increased about 3 μg for each hand-peeled shrimp (containing brown meat) consumed. It has been estimated that the average daily intake of Cd for most persons in Europe and the United States ranges between 6 and 30 μg per day with smokers on the high end (Elinder, 1985). In Japan, the average daily intake of Cd is somewhat higher, 35-50 μg per day, possibly due a diet that uses domestically produced rice as its base. Rice produced in Japan has a higher Cd content than rice produced in other parts of the world (Elinder, 1985). (See Itai-itai disease section)

Absorption, metabolism and distrbution

The intake, absorption, distribution, metabolism and excretion of Cd in man and experimental animals have been extensively studied (CRC, 1974; CEDC, 1975; Kjellstrom and Nordberg, 1978; De Voogt *et al,* 1980; Ryan *et al,* 1982; Nordberg, 1984; OECD, 1990). The major routes of Cd intake in mammals are via the lungs and gastrointestinal tract, with the skin playing only a comparatively minor role (Table 2-2). Although there is a considerable variation

in the data, due to regional and dietary differences, it is estimated that the daily intake of Cd ranges between 25 to 50 μg Cd per day (OECD, 1990). Pulmonary Cd absorption varies depending on the particle size and the chemical nature of the Cd-containing aerosols. Inhaled Cd may be redistributed to the gastrointestinal tract by mucociliary clearance. The average intake by inhalation is considered to be relatively small, yet people living in close proximity to sources of Cd emissions or in the presence of heavy smokers, may accumulate significant levels of the metal in their tissues as a result of inhalation exposure. Table 2-2 summarizes the absorption route for Cd in the body.

Table 2-2. Absorption routes for cadmium in the body

Route of exosure	Absorption Rate	Reference
Dermal	Less than 2%	Skog and Wahlberg, 1964
Oral	3 –7%	Nordberg 1984; OECD, 1990
Inhalation	10 –60%	Ryan et al, 1982; OCED, 1990

Cd absorbed from lung and/or gastrointestinal tissues rapidly enters the bloodstream where it initially binds to plasma proteins (primarily albumin) and over a period of time is redistributed to metallothionein (MT) and the hemoglobin molecule in red blood cells (IPCS, 1992). Blood borne Cd is distributed primarily (>50%) to the liver and kidneys, with lesser amounts accumulating in the pancreas, spleen, bone marrow, blood vessels, testes

and brain. The element is removed from the body via urinary and fecal routes. The rate of Cd excretion is very slow with only 0.004 to 0.015% of the total body burden removed on a daily basis. Thus the resulting biological half-life for Cd in man ranges between 13 and 47 years. Because of this long half-life, once the metal enters the body, it is then considered to be cumulative. At birth, the human body contains virtually no Cd, yet it accumulates with age. The kidneys in particular accumulate Cd up until the age of 50, after which time a negative balance ensues, possibly due to alterations in renal function (IPCS, 1992).

Both Cd and Zn have been shown to be capable of inducing the synthesis of the cysteine rich metallothioneins (MTs) in liver, kidney, pancreas, intestinal epithelium and other tissues (Cousins, 1979; Kagi *et al*, 1984; Onosaka et al. 1984). After entering the blood stream, the Cd ion is transported to the liver, where MT is synthesized. One proposed function of the MT molecule is that it appears to serve as a detoxifying protein which binds the Cd ion and prevents re-distribution of the metal ion to cellular organelles. The protein is also responsible for the pronounced accumulation of Cd in both the liver and kidney (Kagi and Kojma, 1987). Together these two organs contain about one half the total body burden of Cd. When comparing tissue Cd concentrations, the renal cortex has approximately ten times more

metal than liver and about 10,000 times more than blood. Thus, kidneys are considered to be the primary target organ for the Cd ion and they are the first organs to be damaged with chronic Cd exposure. It has been estimated that about 10% of the human population will suffer kidney damage when the Cd concentration in the renal cortex exceeds 180 mg/kg (Friberg, *et al,* 1986).

Increased prevalence of tubular proteinuria has been reported among Chinese farmers as a result of environmental Cd pollution in that country but the osteomalacia that often accompanies this problem was not reported in these individuals (Cai *et al,* 1990). Furthermore, an association between increasing urinary excretion of Cd and the prevalence of tubular dysfunction has been described in inhabitants of some Cd-polluted areas of Belgium (Buchet *et al,* 1990).

Numerous reports in the literature indicate that low levels of Cd can lead to perturbations in the intake and metabolism of copper, zinc, iron, and calcium. Conversely, nutritional deficiencies of these trace elements increase the absorption and the toxicity of ingested Cd (Sugawara, 1977; Hamilton and Smith, 1978; Petering, 1984; Perreboom *et al,* 1981). Deficiencies in dietary protein or vitamin D have been reported to exacerbate Cd toxicity (Ryan *et al,* 1982; Revis and Osborne, 1984). Dietary supplementation with selenium, zinc, iron, copper, calcium, or vitamin D tends to ameliorate some, but not all,

manifestations of Cd toxicity (Webb, 1972; Calabrese, 1978; Nordberg *et al*, 1978).

Cd concentrations in kidney, liver, and other organs increase with age in both man and horse (Elinder *et al*, 1981; Elinder 1985). This is also true for moose (Frank, 1986), wild birds (Blomqvist *et al*, 1987), seals (Tohyama *et al*, 1986), sea lions (Hamanaka *et al*, 1982), dolphins (Honda and Tatsukawa, 1983) and other marine mammals (Honda et al. 1987; Hansen *et al*, 1990). This phenomenon is the result of the slow elimination of Cd described earlier. The biological half-life for Cd in man is about 40 years (Frieberg *et al*, 1986) and it could be assumed that all mammals with a long life span are particularly prone to Cd accumulation and toxicity problems.

In the horse, renal tissue Cd concentrations have been shown to exceed 75 mg/kg wet weight and along with this increase there is a relative decrease in zinc content. Some researchers have interpreted this change in zinc concentration as an early indication that the kidney's detoxifying capacity is being exceeded (Elinder and Nordberg, 1985). Similar increases in the kidney zinc concentration with increasing Cd concentration have been observed in several marine species that have been shown to have high kidney Cd concentrations (Norheim, 1987; Honda and Tatsukawa, 1983; Tohyama *et al*, 1986).

In harbor seals, increases in zinc levels have been attributed to increased production of the MT protein (Tohyama *et al,* 1986). In the marine ecosystem, the behavior of Cd is puzzling. Concentrations of Cd are usually lower in surface waters as compared to deeper ocean water. This relative depletion of Cd in surface waters may be caused by uptake of the metal by certain species of phytoplankton where it may replace zinc (Price and Morel, 1990). Naturally occurring Cd in seawater is taken up by plankton and bioaccumulates in higher invertebrates such as shellfish, krill, squid and crustaceans which then become food for vertebrates higher on the food chain. Arctic waters, which have a lower organic matter content than other ocean waters do not display this depletion at the surface and this may account for the higher Cd concentrations measured in several vertebrates living in the Arctic or Antarctic (Macdonald and Sprague, 1988).

General Health Effects

An earlier study of Cd poisoning by Christensen and Olsen (1957) stated that "Cadmium has probably more lethal possibilities than any of the other metals". Acute Cd intoxication, when not immediately lethal, has been observed in experimental animals, various industrial workers, and adventitious human exposure (CRC, 1974; Luckey *et al,* 1975; Waldbott, 1979, Nomiyama, 1980; Peereboom *et al,* 1981; Ryan *et al,* 1982; Friberg *et al,* 1986). Oral Cd

ingestion can cause a number of gastrointestinal changes including atrophy, erosion and ulceration of the mucosa and generalized intestinal hormonal disturbances (Chowdhury *et al,* 1983), nutrient absorption, and enzyme activity (Sugawara and Sugawara, 1977). Acute Cd intoxication associated with inhalation can produce pulmonary edema, bronchitis and pneumonia (IARC, 1987). Chronic Cd exposure via the inhalation route can cause hyperplastic, fibrotic and emphysematous changes in lung tissue (Takenaka *et al,* 1983; Oldiges *et al,* 1989; Heinrich *et al,* 1989; Aufderheide *et al,* 1990). Acute Cd toxicity can also cause such problems including hepatotoxicity, neurotoxicity, endocrine disorders, testicular necrosis, anemia, cardiovascular changes, depressed immunological responses, mutagenesis, teratogenesis and carcinogenesis.

Kidney toxicity

Kidneys are considered to be the primary target organ for chronic low-level Cd exposure (Schroder, 1974; Piscata and Pettersson, 1977; Kazantzis, 1979; Peereboom *et al,* 1981; Fiberg, 1984). Severe renal changes are listed as a primary consequence of exposure to Cd and can be caused by either acute or chronic low level exposure where Cd levels are high. Microscopically, Cd-induced renal damage occurs primarily as degenerative changes in the proximal convoluted tubules

Cd bound to the low-molecular weight protein MT is cleared from the circulatory system by filtration through the glomerulus and it is then reabsorbed along with the thionein molecule by the proximal tubules (Squibb *et al,* 1979). Intra-renal metabolism of the MT molecule results in the release of the Cd ion. Clinical manifestations of renal damage include proteinuria, glucosuria, aminoaciduria, increased excretion of calcium and phosphorus and renal stone formation (Ryan et al. 1982). Hydropic degeneration, coagualtive necrosis and desqumation of the renal tubular epithelium (Payne and Sanders, 1978) characterize the microscopic degenerative changes. Cd appears to inhibit tubular reabsorption through a mechanism that focuses on the Na/K-ATPase transport system (Gonnick *et al,* 1980). Renal excretion of Cd generally remains low, as Cd accumulates in renal tissues up to the point where severe renal damage occurs. Once renal damage occurs, Cd is excreted from the renal cortex along with desquamated tubular cells (Goyer and Cherrian, 1977; Goyer *et al,* 1984).

Analysis of kidney Cd levels by *in vivo* neutron activation analysis and x-ray fluorescence has made it possible to study the relationship between renal Cd levels and the occurrence of renal damage and its related effects (Ellis *et al,* 1984; Roels *et al,* 1983; Skerfving *et al,* 1987). The critical concentration of Cd in the renal cortex for tubular dysfunction varies and for about 50% of the

population it is 300 μg/g . In comparison, a smaller percentage of the population, approximately 10 percent, develop tubular dysfunction at Cd concentrations of about 200 μg/g. Liver and kidney Cd levels tend to increase simultaneously until the average renal cortex Cd concentrations reach levels around 300 μg/g at which time the average liver levels are only about 60 μg/g. At higher levels, the renal cortex Cd levels are disproportionately lower because Cd is lost from the kidney as a consequence of injury to the renal tubular epithelium, (Ellis *et al,* 1985).

Daily intakes of food containing 140-260 μg/day of Cd for more than 50 years, or occupational air exposures of 50 $\mu g/m^3$ for more than 10 years, have been shown capable of producing renal dysfunction (WHO, 1992; Thun, 1989). An epidemiological study based on historical data of dose-response relationships between Cd intake from rice, using β_2-microglobulinuria as an index of renal tubular dysfunction, found that over a lifetime the total Cd intake needed to produce an adverse health effect was 2000 mg in both men and women (Norgawa *et al,* 1989).

Liver toxicity

In addition to the kidney, the liver also accumulates substantial amounts of Cd; when taken together these organs account for more than 50%

of the total body burden of the metal. While the threshold for overt pathology is somewhat higher in the liver than in the kidney, cellular degeneration occurs that can result in necrosis (Nomiyama, 1980). In experimental animals, liver accumulates substantial amounts of Cd after either acute and/or chronic Cd poisoning (Kotsonis and Klassen, 1977, 1978) which results in hepatic injury (Dudley *et al*, 1982; Stowe et al.1972). With acute Cd exposure in laboratory rodents, the liver is a primary target organ (Dudley *et al*, 1982). The resulting hepatic necrosis following acute Cd exposure may account for the lethality observed within hours of the exposure. Both parenchymal cells (hepatocytes) and nonparenchymal cells (Kupffer and endothelial) accumulate Cd. Cd concentrations are highest in Kupffer cells when compared to the other two cell types, following normalization for cellular protein levels (Caperna and Failla, 1984; McKim *et al*, 1992). Cadmium's toxic effect on hepatocytes appears to be suggestive of alterations in cellular metabolism and enzyme activity rather than lipid peroxidation (Stacey *et al*, 1980). Cd has been shown to alter the activities of a number of enzymes involved in such processes as carbohydrate metabolism (Peereboom *et al*, 1981). In addition, the magnesium-dependent Na/K-ATPase transport system and mitochondrial respiration are both inhibited by Cd (Nomiyama, 1980; Diamond and Kench, 1974). Hepatic metabolism of some pharmacological agents is also inhibited

in Cd toxicity (Schnell, 1978).

Itai-Itai disease

Itai-Itai ("ouch-ouch") is a disease that was identified in residents of Toyama Prefecture in Japan during the latter part of World War II (Takeuchi, 1978; Lauwerys, 1979; Nogawa, 1981; Kjellstrom, 1986, 1992). It involved multiple organ systems, resulting in severe osteoporosis and varying degrees of osteomalacia, renal dysfunction with proteinuria, glucosuria and aminoaciduria and changes in the circulating concentrations of a number of serum components, including a decrease in inorganic phosphorus and an increase in alkaline phosphatase. In the most extreme cases, patients with Itai-Itai disease experienced bone fractures resulting from only the slightest external pressure, such as coughing.

Cadmium can directly inhibit calcium absorption (Sugawara, 1977; Hamilton and Smith, 1978). Indirectly, Cd can inhibit calcium absorption by blocking the action of vitamin D-stimulated Ca-ATPase (Ando, 1981). In the kidney, Cd-induced renal tubular dysfunction leads to a negative calcium and phosphorus balance in addition to an inhibition of vitamin D activation by the kidney. Cd can also have a direct effect on bone by disturbing collagen maturation and osteoblast formation (Nomiyama, 1980). With this disease, the skeleton ultimately loses its ability to support the organs of the body. Itai-Itai

disease was linked to Cd exposure among residents of the Jinzu River Basin who lived downstream from an old lead and zinc mine and used Cd-contaminated river water for rice paddy irrigation and drinking water (Kobayashi, 1978; Nomiyama, 1980; Peereboom *et al*, 1981). Other clinical symptoms of this disease may include such things as normochromic anemia, lymphopenia and renal tubular and globular dysfunction.

Although some investigators have stressed the importance of having either calcium, vitamin D or protein deficiencies in the pathogenesis of Itai-Itai disease, these effects are probably secondary to renal and intestinal dysfunction inherent in chronic Cd poisoning. Malnutrition may also contribute to Itai-Itai disease which was a prevalent condition during the postwar years in Japan. Although some investigators do not expouse a major causal role for Cd in this disease, there are several factors regarding this disease condition that are not explained by malnutrition alone. One factor is the fact that the disease only responds to extremely high doses of vitamin D to treat the bone disease – doses well in excess of those needed for a disease that was primarily caused by malnutrition (IPCS, 1992). Another concern is the that the disease appears to enjoy a geographic localization that was limited to the Jinzu River Basin; this is interesting because malnutrition in Japan during that era was much more widespread. Lastly, the finding that 95% of all itai-itai

cases were primarily diagnosed in postmenopausal women with a history of multiple childbirths (IPCS, 1992). Only this small segment of the inhabitants of that area was affected despite the fact that malnutrition was a general problem that affected a large portion of the entire Japanese population including both sexes during that era.

Systemic effects of cadmium

Hematopoietic system

The principal effects of Cd on the hematopoietic system appear to be associated with impaired gastrointestinal iron uptake, which results in anemia. Some studies have reported that oral exposure to Cd produces anemia in experimental animals and that supplementation of the diet with iron prevents the anticipated anemia (Decker et al, 1958; Kelman et al, 1987; Wananabe *et al*, 1986; Groten *et al*, 1990). Other studies, however, reported little or no change in the numbers of red blood cells (Kotsonis and Klaassen, 1997, 1978) or an increase in hemoglobin content of the blood cells following oral Cd exposure (Borzelleca *et al*, 1989). The number of erythrocyte precursor cells in bone marrow has been reported to decrease in mice exposed to a dose of 57 mg/kg/day of Cd in drinking water for 100 days (Sakata *et al*, 1988). The question, therefore, remains open as to whether other factors in addition to decreased gastrointestinal absorption of iron, such as direct toxicity

to the bone marrow cells, contribute to the observed anemia.

Immune system

Reported effects of Cd on the immune system are similar to those of lead, in that multiple components of humoral immunity and cell mediated immunity appear to have been altered by the metal. It should be noted that conflicting reports also exist where the metal has had no effect on the immune system. One major difference between Cd and lead or mercury is that there have been no reports that suggest Cd-induced autoimmunity occurs, although leukocytosis was reported after *in vivo* treatment of rats at doses of 200-400 ppm Cd for 6 months (Cifone *et al*, 1989). Additionally, 37 workers exposed to Cd had elevated numbers of monocytes, but not lymphocytes, neutrophils or eosinophils (Karakaya *et al*, 1994). Mice exposed to Cd had normal or reduced numbers of lymphocytes although Cd has been reported to an extremely cytotoxic metal *in vitro* (Ohsawa *et al*, 1983;Borgman *et al*, 1986;Smith and Lawrence, 1988; Lawrence and Slavik, 1992). In addition, Cd has been reported to induce apoptosis in T cells (Azzouzi, *et al*, 1994).

Epidemiological studies

An epidemiological report by Kippling and Waterhouse (1967) demonstrated a relationship between occupational exposure to Cd oxide and increased mortality due to prostate cancer. A similar relationship among Cd

smelter workers was reported by Lemen et al. (1976). Subsequent studies by Kolonel and Winkelstein (1977) were inconclusive and unable to confirm that a relationship exists between dietary and/or cigarette sources of Cd and prostate cancer. In addition, these researchers were unable to detect a relationship between occupational exposure to Cd and prostate cancer.

Testes and ovaries

Cd can cause a number of lesions in many organs, such as the liver, kidney and testis (Friberg *et al,* 1974; Cook *et al,* 1974; Hoffman *et al,* 1975). The metal has been shown to induce testicular lesions at doses considerably lower than for other organs (Parizek and Fahor, 1956; Fahin and Khare, 1980). Acute Cd exposure induced vascular destruction in the testes (Sugawara *et al,* 1989; Chubb, 1992). Cd exposure-induced hypoxia and ischemia produces a series of secondary effects, including seminiferous tubule necrosis and Leydig cell damage (Gunn and Gould, 1975; Itoh, 1985; Damber *et al,* 1987). Despite numerous studies, the primary action of Cd on the development of testicular cancer remains unknown, yet endothelial cells appear to be the primary target (Nolan and Shaikh, 1986).

An almost immediate action of Cd is to increase capillary permeability in testicular tissue (Setchell and Waites, 1970). In the testis, progression of Cd toxicity begins with increased vacuolization of endothelial cells. This is

then followed by a number of changing variables such as a widening of endothelial cell junctions, decreased blood flow, obstruction of capillaries by packed blood cells, and intravascular coagulation. The final stages of the process is characterized by infiltration of blood cells into surrounding tissue, hypoxia, ischemia and finally necrosis (Chubb, 1992). It should be noted that the testes must be scrotal and heat sensitive for Cd to exert gonadal toxicity.

As discussed previously, Cd exerts a number of deleterious effects upon the mammalian testes, but far less is known about Cd-induced lesions in the ovary. Basically, it is known that Cd administration causes ovarian hemorrhages (Kar *et al,* 1959; Saksena and Salmonsen, 1983; Rehm and Waalkes, 1988). Cd-induced ovarian necrosis appears to be both species and strain related as well as dependent on the stage of estrous at the time of exposure (Rhem and Waalkes, 1988). For example, the Syrian hamster is known to be extremely sensitive to Cd-induced ovarian necrosis while other rodents are less sensitive (Waalkes *et al,* 1988; Rhem and Waalkes, 1988).

Mutagenesis

The administration of Cd during organogenesis causes growth retardation, congenital malformations and fetal death in rodents. Cd exposure during the period of preimplantation decreases the implantation potential and reduces the amount of development that occurs at the blastocyst stage

(Pedersen and Lin,1978; Yu and Chan, 1988). Animal and human studies have revealed that Cd is deposited within the placenta and that only a small fraction of the metal dose enters the fetal circulation (Kuhnert *et al,* 1982).

Carcinogenesis

Cd is an effective carcinogen in rodents and a suspected carcinogen in man. Possible carcinogenic effects of Cd were discussed in the scientific literature as early as 1961-1962 when both Haddow (1961) and Heath (1962) reported the production of local tumors in rats after subcutaneous and/or intramuscular injections of a Cd- containing ferritin complex and/or Cd powder. Even Cd sulfide pigment and Cd oxide have been shown to be capable of producing sarcomas in the rat at the site of injection (Cohen *et al,* 1990; Kazantzis, 1963; Kazantzis and Hadbury, 1966). Known Cd target tissues in rodents include the prostate, lung, testes, in addition to repository type injection sites (Waalkes and Obeerdorster, 1990). Primary testicular atrophy has been observed initially following exposure to Cd which is then followed by interstitial cell hyperplasia and neoplasia following a latent period of 1-2 years (Koizumi and Li, 1992; Waalkes, 1990; Koizumi *et al,* 1992).

The hepatic carcinogenic potential of Cd in experimental animals and humans has been established (IARC, 1976, 1994; Waalkes *et al,* 1992a,b). Current epidemiological and experimental evidence is considered strong

enough to establish a linkage between exposure to Cd and its compounds and human neoplasia, at least in the lung (Stayner *et al,* 1992). In other tissues such as the prostate this association is controversial and is considered preliminary in the case of the kidneys, liver, stomach and hematopoietic system until additional confirmatory evidence is available.

Liver cancer has been associated with Cd exposure from dietary sources in China, but these effects should be viewed as extremely tentative because only a single report exists (Campbell *et al,* 1990). Cd-mediated hepatocarcinogenesis has been demonstrated in laboratory mice; again as with the previous report, there are only a limited number of studies available on the subject (Waalkes and Rehm, 1994). In rodents, Cd has been shown to both enhance and suppress liver tumor development induced by organic carcinogens depending on both the exposure dose and exposure duration (Waalkes *et al,* 1991a, 1993; Wade *et al,* 1987).

The first inhalation study with Cd using rats, rabbits and goats as experimental animals was published in 1947 by Patterson, but the duration of the exposure was only 6 months at the most and the results were inconclusive. It was not until 1979 that rats exposed to Cd oxide (Hadley *et al,* 1979) and Cd chloride aerosols (Heering *et al,* 1979) appeared to develop exposure-related lung tumors. One year after exposure to 60 mg Cd oxide/m^3

for 30 min, 1 of 34 rats developed a lung tumor. After 18 months of exposure to Cd at 20 $\mu g/m^3$ as the chloride, five out of ten rats developed lung tumors (Heering *et al,* 1979; Hadley *et al,* 1979).

Waalkes and Rehm (1994) reported that Cd might be associated with tumors of the hematopoietic system in rats and mice. Treatment of male DBA mice with Cd for 16 weeks increased the incidence of hematopoietic tumors from 26% among controls to between 48 and 57% in the treatment group. Tumors were described as being predominately follicular cell lymphomas. No significant changes in hematopoietic tumors were observed in comparably treated NFS mice. In rats, oral Cd has been reported to cause a dose-related increase in large granular lymphocyte leukemia when administered at dietary levels up to 100 ppm (Waalkes and Rehm, 1992). These effects have been associated with immunosuppressive and immunotoxic effects of Cd, although a mechanistic association between these effects has not been established.

Cd appears to bind to two distinct sites on the DNA molecule (Waalkes and Poirier, 1984). This type of metal exposure induces single strand DNA breaks in cultured rat liver cells, which is indicative of direct genotoxic potential (Coogan *et al,* 1992, 1994). A genetic basis for susceptibility to chronic toxic effects of Cd including carcinogenesis has been observed by Waalkes and Rehm (1994), who demonstrated a differential susceptibility to

these effects in genetically different strains of mice. Tang *et al*, (1990) reported that there is a significant increase in structural chromosomal aberrations, of both the chromatid and chromosome type. Such an effect has also been demonstrated in a study of lead-exposed subjects (Forni *et al*, 1980) and it was concluded that Cd acts by interfering with DNA repair mechanisms (Forni, 1992).

The mechanisms that permit Cd salts to induce chromosome damage are not clear. It has been demonstrated that lymphocytes cultured for 3 days in low levels of $CdCl_2$ tend to accumulate Cd in concentrations up to 3,000 times higher than those in the culture medium. In addition, it has been estimated that approximately 4% of the accumulated Cd is found in the nuclear fraction (Hildebrand and Cram, 1979). Cd, like other metals, appears to reduce fidelity in DNA synthesis and may interfere with DNA repair mechanisms in mammalian systems (Degraeve, 1981). As with chromosome aberrations induced by other agents, the significance of these findings with respect to carcinogenesis is not known (Aitio *et al*, 1988).

Cadmium's effect on subcellular organelles

The distribution of Cd among the organelles of liver and kidney cells has been investigated with a variety of microanalytical techniques (Table 2-3). Table 2-3 summarizes the subcellular location of the Cd ion in the different

cellular fractions and organelles.

Table 2-3 Subcellular localization of the cadmium ion

Cellular Location	Cadmium Content (%)	Reference
Cytoplasm	50%	Goyer and Cherian, 1977
Nucleus	10-25%	Kawi et al, 1977
Endoplasmic Reticulum	5-10%	Lee et al, 1977
Mitochondria	10-25%	Peereboom et al, 1981

In the nucleus, Cd has been shown to bind to both histone and non-histone type proteins, in addition to the phosphate groups and bases of the DNA molecule. This binding results in gross alterations in chromatin structure (Peereboom *et al,* 1981) as well as subtle changes in conformation (Eichhorn *et al,* 1970; Miller, 1970; Webb, 1977; Jacobson and Turner, 1980) and synthesis (Stoll *et al,* 1976; Bracken and Sharma, 1985). Cd also binds to phospholipids and such interactions may provide a biochemical basis for the toxic effects on mitochondria and plasma membranes (Vallee and Ulmer, 1972).

Mitochondria have been shown to accumulate Cd against a concentration gradient (Soriss and Jarvisalo, 1977). This accumulation results

in alterations in function which range from enzyme activity modification (Fowler and Mahaffey, 1978; Prasada Rao *et al,* 1983) and ion transport (Mela, 1970) to cellular respiration (Lindgren and Lindgren, 1973; Solaiman *et al,* 1979) and oxidative phosphorylation (Webb, 1977; Sato *et al,* 1978). Changes that take place in the mitochondria may be the key that will help provide an understanding of the stepwise progression of changes leading to cell injury and death in both the liver and the kidney (Goyer *et al,* 1984). As Cd accumulates within the cells, mitochondrial swelling is observed, followed by more serious alterations in morphology and the appearance of what Goyer describes as flocculent densities (Nishizumi, 1972; Goyer and Cherian, 1977; Goyer *et al,* 1984). Cadmium ions appear to directly inhibit the transport of calcium ions into mitochondria in a competitive manner and indirectly cause alterations in energy metabolism (Toury *et al,* 1985; Jarvisalo *et al,* 1980; Saris and Jarvisalo, 1977). Thus, the Cd ion appears to interfere with mitochondrial function by impairing the organelle's ability to regulate cytosolic calcium. Because the cytosolic calcium ion concentrations in the mitochondria are changed, concentrations of other ions within mitochondria are also altered (Kendall *et al,* 1983). The discovery that Cd effectively uncouples oxidative phosphorylation (Jacobs *et al,* 1956; Webb, 1977; Sato *et al,* 1978) provided researchers with an experimental probe that has found

considerable application in recent years. The uncoupling action is believed to be accomplished either by the Cd binding directly to components of the electron transport chain (Mustafa *et al,* 1971b) or by acting directly on ATP synthetase (Joshi and Hughes, 1981; Sanadi *et al,* 1984). An alternative mechanism that has been proposed is that the Cd ion alters hydrogen ion transport which leads to a collapse of the pH gradient (Sanadi *et al,* 1981; Pringle and Sanadi, 1984). Wilkie (1977) reported that exposure of yeast cells to Cd results in the inhibition of mitochondrial biogenesis. This is an intriguing observation which agrees with findings by Morita and Best that there is an intimate relationship between mitochondria and the emission of nuclear satellite material in planarian stem cells (Morita, 1967; Morita *et al,* 1969; Morita and Best, 1974).

Enzymatic activities

The Cd ion appears to have an impact upon a large array of enzymes and enzyme complexes in biological systems (Table 2-4). Vallee and Ulmer (1972) presented a list of 25 specific enzymes that are stimulated by the Cd ion and another 35 that are inhibited by the same Cd ion. In 1981, Pool reported that 32 enzymes were stimulated by Cd and listed another 29 enzymes that were inhibited by the presence of the metal. In addition, this researcher also listed another 25 enzymes that are either stimulated or

inhibited, depending on the effective Cd concentration and or the method of exposure. One explanation for this diversity takes into account the extensive anti-metabolite properties of the Cd ion. Cd ion directly influences both availability and binding of certain essential metals, such as zinc, copper, iron and calcium in metalloenzymes thus altering the activity either directly or indirectly depending on the mexhanism of activity of the specific enzyme. Table 2-4 summarizes the effect of the Cd ion on enzymatic activity in the body.

Table 2-4 Effect of the cadmium ion on selected enzyme activities

Enzyme	Activity Modification	Reference
Metallothionein	inhibit	Pool, 1981
Carpoxypeptidase	Inhibit	Pool, 1981
Acid phosphatase	Inhibit	Pool, 1981
Carbonic anhydrase	Inhibit	Pool, 1981
Thymidine kinase	inhibit	Webb, 1977
DNA dependent RNA polymerase	Inhibit	Solaiman, 1979; Jacobson and Turner, 1980
Ceruloplasm	Inhibit	Nara *et al*, 1966; Magour *et al*, 1979
Monamine oxidase	Inhibits copper transport	Nara *et al*, 1966; Magour *et al*, 1979
Ferritin	Inhibits iron transport	Webb, 1977
Alkaline phosphatase	Inhibits by reducing Ca^{2+}	Saugawara *et al*, 1983

Table 2-4 Effect of the Cd ion on selected enzymatic activities (Continued)		
Succinic dehydrogenase	Inhibits activity	Jacobson and Turner, 1980
Glycerolphosphate dehydrogenase	Inhibits activity	Pool, 1981
Mitochondrial ATPase	Stimulates activity	Joshi and Hughes, 1981
Mitochondrial ATP synthetase	Inhibits	Sanadi *et al*, 1984
Calmodium activated Ca/Mg ATPase in erythrocytes	inhibited	Ackerman *et.al.*, 1985
Ca/Mg-ATPase intestinal brush border	Inhibited	Sugawara and Sugawara, 1975
Sarcoplasmic reticulum	Inhibited	Herrmann and Shamoo, 1975
Ca-ATPase in myosin	Inhibited high Stimulated low	Seidel, 1969
Thrombosthenin	Inhibited high Stimulated low	Harris *et al*, 1974
Na/K-ATPase	inhibited	See text

Cd inhibits the Na/K-ATPase activity in a wide variety of mammalian cell types including neurons (Reidel and Christensen, 1979; Ghetty *et al*, 1980; Lai *et al*, 1980; Magour *et al*, 1981), renal tubule cells (Rifkin, 1965; Bader *et al*, 1970; Nechay and Sanders, 1977, 1978), testicular cells (Singh and Mathur, 1968), erythrocytes (Penniston, 1972; King, *et al*, 1983) and pulmonary alveolar macrophages (Mustafa *et al*, 1969, 1971a, 1971b).

Reactive Radical Production

Ochi and Ohasawa (1983) reported that when cultured Chinese hamster cells were exposed to Cd chloride for 2 hours there was an increased incidence of DNA single strand scissions detectable with alkaline elution techniques. In a follow up study, these investigators found that Cd chloride exposure failed to induce DNA scissions under anaerobic conditions in a medium containing superoxide dismutase (SOD) (Ochi *et al*, 1983a). This study suggested that the reactive oxygen radical is required for the induction of DNA single strand breaks by Cd. The results of this research imply indirectly that an active oxygen species may play a crucial role for the induction of DNA lesions after exposure to Cd. Amoruso et al. (1982) demonstrated that the production of the superoxide radical was significantly increased in human granulocytes and also in rat macrophages activated by digitoxin in the presence of Cd.

In a later study, Ochi et al. (1984) was not able to decrease Cd induced DNA single strand breaks by adding the SOD enzyme to the cell culture. In contrast to SOD, catalase significantly decreased the number of DNA single-strand breaks caused by exposure of Chinese hamster cells to a single dose of Cd chloride (Ochi and Ohsawa, 1985). In another study, researchers, using cultured Chinese hamster cells were able to show that D-mannitol, a hydroxyl

radical scavenger (Mistra and Fridovich, 1976), significantly reduced the incidence of DNA single strand breaks induced by exposure to a single dose of Cd chloride. Dimethylfuran, a scavenger of singlet oxygen (Emerit *et al,* 1983), was not protective, whereas, butylated hydroxytoluene, a well known antioxidant, protected DNA from single strand breaks caused by exposure to a single dose of Cd chloride (Ochi and Ohsawa, 1985). Treatment with the catalase inhibitor aminotriazole resulted in an increased number of DNA single strand lesions. The suppressive effects of catalase suggest that hydrogen peroxide participates in the production of DNA strand breaks induced by the Cd ion. Moreover, the enhancement of the number of Cd induced DNA strand breaks by aminotriazole may be due to the accumulation of hydrogen peroxide by the inhibition of catalase. Hydrogen peroxide has been shown to induce chromosomal aberrations in mouse ascites tumor cells (Schoneich *et al,* 1967) and in cultured Chinese hamster cells (Tsuda, 1981). The suppressive effect of mannitol indicates hydroxyl radical participation but this conclusion was considered tentative due to the relatively high concentrations of Cd required to produce DNA strand breaks.

Cadmium has been shown to induce lipid peroxidation when cells are exposed to metal concentrations exceeding $5X10^{-5}$ M in isolated rat hepatocytes (Stacey et al, 1980). Lipid peroxidation, a degenerative process

that results in the formation of thiobarbituric acid-reactive substances associated with the reaction between oxygen radicals and unsaturated fatty acids. Malondialdehyde, a by-product of lipid peroxidation, has been shown to react with DNA (Brooks and Klamerth, 1968) and to be both mutagenic (Mukai and Goldstein, 1976) and carcinogenic (Schamberger *et al,* 1974). Koizumi and Li (1992) demonstrated that 12 hours after exposure of rats to Cd chloride that lipid peroxidation levels, iron content and cellular production of hydrogen peroxide were remarkably elevated in testicular Leydig cells, the gonadal target population for Cd carcinogenesis. At the same time, glutathione peroxidase activity rose, glutathione reductase and catalase activities were reduced and SOD activity remained unchanged. Reduced glutathione levels were also decreased. Enhancement of lipid peroxidation or accumulation of oxidized glutathione are good indicators of oxidative stress (Green *et al,* 1986; Jaeschke *et al,* 1988). Such a decline of cellular defense mechanisms against the active oxygen species may lead to enhanced production of hydroxyl radicals from hydrogen peroxide because iron, which can catalyze formation of hydroxyl radicals from hydrogen peroxide, was also increased in Leydig cells after exposure to carcinogenic doses of Cd.

Heme oxygenase, an essential enzyme in heme catabolism, which cleaves heme to form biliverdin (Kutty and Maines, 1981) which is then

cleaved by biliverdin reductase to yield bilirubin, is affected by Cd (Kutty and Maines, 1981). Recently it was demonstrated that bilirubin provided physiological protection against oxidative stress in cobalt chloride treated rat liver (Llesuy and Tomaro, 1994) in addition to serving as an antioxidant in neonatal plasma (Gopinathan, 1994). The antioxidant effects of this molecule suggest that oxidant species play a role in the induction of heme oxygenase either directly or by depleting glutathione (Tomaro *et al,* 1991; Ewing and Maines, 1993; Llesuy and Tomaro, 1994). This is supported by the finding that heme oxygenase is one of the generically termed "heat shock proteins" induced in cells by such variables as heat, oxidative stress and chemical exposure (Levinson *et al,* 1980; Schlesinger *et al,* 1982; Lanks, 1986; Keyse and Tyrell 1987, 1989). Depletion of cellular glutathione increases the cytotoxic effects of hydrogen peroxide, implicating the reduced glutathione: oxidized glutathione cycle in the protection of cells against hydrogen peroxide induced toxicity (Keyse and Tyrrell, 1987). The Cd ion is a powerful inducer of heme oxygenase in rat liver (Maines and Kappas, 1976), in cultured avian liver cells (Sardana *et al,* 1982) and in HeLa and HL60 human cell lines (Taketani *et al,* 1989), besides inducing stress protein synthesis in rat liver (Caltabiano *et al,* 1986; Goering *et al,* 1993). Ossola and Tomaro (1995) demonstrated increased heme oxygenase activity 5 hours after treatment of

female Wistar rats with Cd chloride. A decrease in the intrahepatic glutathione pool and an increase in hydrogen peroxide concentration preceded this induction. The activity of the antioxidant enzymes including SOD, catalase and glutathione peroxidase was found to be significantly decreased 5 hours after Cd injection. When animals were treated with antioxidant compounds such as bilirubin and/or α-tocopherol, it was found that they markedly inhibited the induction of Cd induced heme oxygenase activity and the appearance of some oxidative stress indicators.

Earlier work with Cd and its association with the production of reactive oxygen species was carried out with exposure to a single large dose of the metal. In 1996, Yang et al. reported that oxygen species might participate in the mutagenicity and mutational spectrum of Cd in Chinese hamster ovary-K1 cells. Cultured Chinese hamster ovary-K1 cells were exposed to low doses (LD30-LD20) of Cd and then evaluated for mutations. The mutational frequency induced by the low doses of Cd was approximately 20 times that of untreated cells. D-Mannitol, a scavenger of reactive oxygen species, was shown to significantly protect metal exposed cells against Cd cytotoxicity and mutagenicity. Furthermore, non-cytotoxic doses of 3-amino-1,2,4-triazole, a catalase inhibitor, potientiated Cd cytotoxicity and mutagenicity. Cellular Cd uptake by exposed cells was not altered by combined treatment with either D-

mannitol or 3-amino-1,2,4-triazole. In addition, glutathione levels and the activities of glutathione peroxidase, glutathione reductase and catalase were decreased in cells treated with 4 μM Cd for 4 hours. These enzymatic activities returned to normal levels 8 hours after removing Cd. The results of this research indicate that exposure of the Chinese hamster ovary-K1 cells to Cd results in a unique mutational spectrum with the production of specific DNA adducts and that reactive oxygen species play a role in this mutational specificity (Yang *et al,* 1996).

Early studies using single high doses of Cd suggested that that oxidative stress was induced by demonstrating increased hepatic lipid peroxidation, glutathione depletion, nuclear DNA damage and excretion of urinary lipid metabolites. However, only in the past few years have experiments been performed to evaluate if chronic low level Cd exposure scenarios were capable of producing oxidative stress conditions. Recently, Bagchi et al. (1997) reported that chronic low level Cd exposure resulted in increased mitochondrial and microsomal lipid peroxidation, elevated excretion of urinary lipid metabolites (malondialdehyde) and an increase in measurable DNA single strand breaks in the livers of female Sprague–Dawley rats exposed to Cd chloride (4.4 mg/kg/day) for 120 days. Seventy-five days following the initiation of the Cd exposure, urinary malondialdehyde levels

were significantly elevated over control levels. This study demonstrated a significant increase in both hepatic and brain microsomal peroxidation products as a result of treatment with Cd between days 60 and 75 but no increases beyond 75 days of exposure. Following exposure to low doses of Cd for 75 days there was a significant 3.8-fold increase in the number of DNA single strand breaks over that observed in non-exposed controls. The results of this study imply that low dose chronic administration of Cd chloride induce an oxidative stress that results in tissue damaging effects that may contribute to both the toxicity and carcinogenicity of the Cd ion.

Metallothionein

Although Cd is not essential for cellular growth and development, it does, when presented to a biological system follow the same pathways that essential metals (i.e., Zn and Cu) use. Hence, after a biological system, has been exposed to Cd, the metal has been found to be associated with proteins involved with trace element metabolism. Metallothionein (MT) is the protein that is most frequently associated with Cd exposure and has essentially become the standard to which other Cd-binding proteins are compared.

Since Margoshes and Vallee (1957) first detected MT in the equine renal cortex, the protein has proven to be of interest to a wide variety of scientific disciplines. Metallothioneins are a class of low molecular weight,

metal-binding proteins found in a wide variety of prokaryotes and eukaryotes (Webb, M. 1979; Kagi and Nordberg, 1979; Kagi and Kojima, 1987). The protein contains numerous cysteinyl thiol groups, a characteristic that permits high-affinity binding of several metals including Cd and Zn. The protein is normally found in high concentrations in the adult liver after metal exposure (Onosaka, S. and Cherian, 1981a, 1981b) and also in perinatal mammalian liver (Wong and Klassen, 1979; Waalkes and Klassen, 1984). The pancreas and kidney also contain large amounts of metallothionein, both of which show significant increases following exposure to Zn or Cd ions (Onosaka, S. and Cherian, 1981a , 1981b).

The ability of metals to induce MT synthesis was demonstrated by Piscator, who showed that MT levels increased in the livers of rabbits exposed following Cd exposure (Piscator, 1964). Since then, this form of regulation has been recognized in all species that synthesize MT and in many different tissues and cell types. Metallothionein synthesis in mammals and most other higher eukaryotes is inducible by a variety of heavy metal ions including cadmium, zinc, and copper, in contrast, the copper-binding MTs of yeast and fungi appear to be induced only by the copper ion (Hamer *et al,* 1991). Metallothionein induction occurs following exposure to a large number of diverse compounds and chemical and/or physical treatments (Kagi and

Schaffer, 1988). Specific agents that induce MT include such compounds as ethanol (Waalkes *et al,* 1984), acetaminophen (Wormser and Calp, 1988), urethane (Brzeznicks *et al,* 1987), formaldehyde (Goering, 1989), estradiol (Nishiyama *et al,* 1987), ethionine (Waalkes, 1985) and D-penicillamine (Heilmaier *et al,* 1986), several of which function as cytotoxic hepatotoxins. In addition, MTs are inducible by glucorticoids (Lehman-McKeeman *et al,* 1991). It has been shown that MT gene expression is regulated by serum cell growth factors and protein kinase C activators (Imbra and Karin, 1987), and also by cAMP (Nebes *et al,* 1988).

The kinetics of MT induction vary with the species studied; in mammalian cells the time frames are measured in days whereas in yeast cells maximal MT synthesis is achieved within minutes following exposure (Schmidt and Hamer, 1986). Because MT gene transcription appears to occur almost immediately following metal exposure, the time frames reported for induction probably reflect differences in mRNA and protein half-lives as well as the time it takes for the metal to reach and penetrate the cellular target. The biological half-life of MT molecules ranges from 1 to 4 days depending on the type of metal bound (Webb, 1979; Kagi and Nordberg, 1979). The protein has been characterized in a wide variety of mammalian and non-mammalian sources (Webb. 1979; Kagi and Nordberg, 1979; Petering and Fowler, 1986; Stone

and Overnell, 1985), including striped dolphin kidney (Kwohn, *et al,* 1986), dogfish (Hidalgo and Flos, 1986) and guinea pig liver (Stillman *et al,* 1986).

Structure and metal binding capacity

Metallothionein isolated from mammalian species consists of a 61- or 62-amino acid peptide. The amino acid composition of mammalian MT has been shown to include approximately 20 cysteines, 6-8 lysines, 7-10 serines and a single acetylated methionine at the amino terminus with no aromatics or histidines (Hamer, 1986). In comparison to mammalian MT, small metal binding proteins have been reported in prokaryotes (Higham and Sadler, 1983). Detailed analyses of the metal binding protein from *Pseudomonas putida* suggests a complex metal binding site similar to that of mammalian MT which in comparison to non-mammalian MT contains histidine and glutamine as well as thiolate ligands (Higham *et al*, 1984). In all MT molecules, the majority of the cysteine residues are present in Cys-X-Cys, Cys-X-X-X-Cys and Cys-Cys sequences. Regarding the general structure of the protein there are no disulfide bridges, and all cysteinyl sulfurs participate directly in metal complexation (Hamer, 1986).

As a molecule, MT is primarily an elongated, single chain with a high degree of random structure. The main contribution to the secondary structure of the MT molecule is due exclusively to β-turns which are combined with

some α-helical and extended β-sheet segments. This structure was determined using X-ray crystallography (Furey *et al*, 1986), nuclear magnetic resonance (Braun, *et al*, 1986) and Raman and infrared spectroscopy (Pande, *et al*, 1986). Metal complexation imparts a conformational transition from predominantly random apometallothionein to a MT molecule with a molecular structure consisting predominantly of β-turns and random segments (Pande *et al*, 1986).

Mammalian MTs possess two highly organized domains, the amino-terminal domain, designated as β, consisting of residues 1-30, and a carboxy-terminal domain, designated as α, consisting of residues 31-61 (Winge *et al*, 1982; Otvos and Armitage, 1980). The metal content of purified MT is highly variable and depends on the organism, tissue and history of previous metal exposure. For example, MT isolated from human liver autopsy samples contains almost exclusively Zn, whereas MT from kidney contains substantial levels of Cd and Cu. These differences probably reflect both the natural heavy metal exposure of the individual organs and possibly the expression of different MT isoforms (Kagi, *et al*, 1984). Metals are associated with MT exclusively through thiolate bonds to all 20 cysteine residues in mammalian MT. Incubation of the MT molecule in an acidic environment disassociates the metal ions from the protein. The resulting metal-free apometallothionein

can then be reconstituted with 7 atoms of Cd or Zn or 12 atoms of Cu (Hamer, 1986). Overall stability constants ($K_{OH/MT}$), estimated either by pH titration or by ligand substitution method, range from 10^{19} to 10^{17} for Cu, 10^{17} to 10^{15} for Cd and 10^{14} to 10^{11} for Zn (Kagi and Vallee, 1961; Kagi *et al*, 1980). Mammalian MT has also been shown to be capable of binding up to 7 atoms of mercury, cobalt, lead or nickel per molecule of protein (Nielson *et al*, 1985).

A total of seven metal atoms are chelated by the α- and β-domains to form two independent clusters (A and B), each coordinated tetrahededrally to bridging and terminal cysteines. The first cluster, often designated cluster A, can contain up to four divalent metal atoms coordinated by six terminal and five bridging cysteine thiolate ligands. Cluster B can bind up to three-divalent metal atoms coordinated by six terminal and three bridging cysteine thiolate ligands (Otvos, and Armitage, 1980; Vassak and Kagi, 1983). Binding of various metals to each cluster is specific and is a function of known differences in binding affinity and selectivity of each domain.

Cadmium binding to apothionein is a sequential two-step process with the four-metal containing cluster, cluster A, forming before the three-metal cluster, cluster B, forms. This process has been shown to be highly cooperative, with the binding of one metal enhancing the binding of others (Nielson and Winge, 1983). However, while some cooperative metal binding

is apparent, Cd binding to MT has been shown to be a multistep process involving both random and ordered events with no definitive data for cooperativity in metal binding to cluster B (Bernhard *et al,* 1986). Based on this evidence, cluster formation appears to proceed only after the binding of more than three divalent metal molecules. Other evidence suggesting that metal binding events occurring within the two domains are related is based on data in which it appears that the metal bridge to the β-domain (cluster B) is required to facilitate formation of the four-metal cluster A (Good and Vasak, 1986).

Metallothionein molecular biology

One of the most remarkable properties of MT is the marked enhancement of its synthesis (induction) brought about by exposure to metals, hormones, or a variety of different compounds. Inducibility is regulated at the transcriptional level by various cis-acting DNA sequences located in the promoter region of MT genes (Palmiter, 1987; Hamer, 1986). These sequences are responsible for MT induction by metals [metal regulatory elements (MREs)] and glucocorticoids [glucocorticoid response elements (GREs)]. In addition, trans-acting DNA-binding proteins, which bind MT-inducing metals, are believed to facilitate metal interaction with the metal-regulatory elements to promote MT gene transcription (Palmiter, 1987;

Hamer, 1986). The various inducers of MT gene transcription fall into at least three general categories: (1) metals(Cd, Zn and Cu), (2) glucocorticoids and (3) cytokines such as interleukin-1 and interferons. While the specific promoter sequences for the first two of these have been identified in various species, the cytokines need further study. Nonetheless, there seems to be little doubt that all of the above mentioned factors directly stimulate the initiation of MT mRNA transcription and thus induction of MT. In addition, there is increasing evidence that DNA binding proteins are an integral part of this process (Westin and Schaffner, 1988; Garg *et al,* 1989).

The methylation status of the DNA molecule is directly correlated with MT gene expression. Hypomethylating agents such as 5-azacytadine, deoxyazacytidine, butyric acid and dimethyl sulfoxide, cause enhanced synthesis of MT mRNA and protein and thus confer tolerance to Cd (Waalkes *et al,* 1985; Waalkes et al. 1988; Waalkes and Wilson, 1986; Waalkes and Wilson, 1987). In contrast, hypermethylation of the MT gene correlates well with Cd susceptibility both *in vitro* and *in vivo.* For example, the MT gene in mouse testes is relatively methylated (Bhave *et al,* 1988), an observation that correlates well with the apparent deficiency of MT protein in testes (Waalkes, et al. 1988) and with testicular susceptibility to the acute or chronic carcinogenic effects of Cd.

Because metal exposure induces expression of the MT gene, the use of MT fusion genes has found promising applications in the field of genetic engineering. With MT fusion gene constructs, the MT promoter region is linked to a specific structural gene of interest. This arrangement allows for the subsequent regulation of the selected gene product by exposing the system to metal ions. In particular, the development of transgenic mice that express a MT-growth hormone fusion gene which show dramatic growth (2-fold compared to normal littermates) when fed Zn-enriched diets (Palmiter *et al,* 1982) has potential utility in biomedical and agricultural areas.

Bracken and Klassen (1987) suggested that the nature of MT induction by some compounds *in vivo* must be "indirect" since induction can not be demonstrated *in vitro*. Earlier work by Durnam and Palmiter (1984) indicated that some compounds must be "gratuitous" inducers because MT has no apparent relationship to their metabolism. The question that has intrigued investigators for the past several years concerns the mechanism by which MT is induced indirectly. Some investigators have suggested that indirect induction may involve changes in Zn homeostasis and/or secretion of glucorticoids as might occur during general stress (Bracken and Klassen, 1987). This would explain why certain substances are effective *in vitro* but not *in vivo*. The induction of hepatic MT by Zn is thought to result from the

interaction of Zn ions and nuclear factors that promote the synthesis of MT mRNA (Hamer, 1986). This suggests that the Zn ion directly affects the induction of tissue MT. Since there are no data to support this hypothesis, it is presently felt that both Zn and Cd directly stimulate MT mRNA, requiring only a minimal number of nuclear-binding factors for normal protein expression. Much of the initial work concerning the regulation of MT induction has been conducted *in vitro*. Since this experimental model has been confounded by uncontrolled variables, the results of these studies may be difficult if not impossible to interpret. For example, induction of MT by glucocorticoids was originally thought to represent an indirect action of the hormone on Zn flux into the cell. Glucorticoids stimulated the uptake of Zn by cells and consequently the induction of MT. Therefore, MT induction by glucocorticoids was considered to be a secondary response resulting from changes in the concentration of intracellular Zn. It is now clear that glucorticoids directly affect the synthesis of MT independent of changes in cellular Zn (Karin *et al*, 1980).

Historically, researchers have shown that MT accumulates in many different tissues following Zn administration. Under certain conditions it is believed that physiological (secondary) processes initiated by the injection itself may confound the response to the injected Zn. Data presented by Fleet *et al*, 1988, show that if Zn is given intraperitoneally (IP) compared to oral

administration there is a considerable difference in the tissue response. Following this type of exposure, MT accumulation was nearly twice as great in chicks given Zn by the IP than dosed by the oral route. In comparison, MT accumulation in the pancreas of chicks given Zn either by the IP or oral route had essentially the same amount of protein produced. These results strongly suggest that the IP administration of Zn initiates an additional (liver-specific) effect that may be unrelated to the level of Zn exposure. Fleet (1990) further suggests that this effect is independent of either Zn homeostasis or stress and suggests that inflammation may be the associated event that causes the increased MT levels following IP Zn administration.

Quantification of the metallothionein protein

Metallothionein is often detected by virtue of its high metal content and a number of available assays for MT quantification take advantage of the protein's ability to bind metals. Protein levels are measured indirectly by measuring protein bound metal content after saturation with a metal known to have high MT binding-affinity like Cd (Onosaka and Cherian, 1981a; Onosaka and Cherian, 1981b) , silver (Scheuhammer and Cherian, 1986) or mercury (Piotrowski *et al* 1973). Problems have occurred with over- and under-estimation by metal saturation assays, although the Cd-binding assay appears to be similar in precision and recovery of MT when compared to assays that

directly measure the protein moiety, like the radioimmunoassay (Dieter *et al,* 1986). Metal saturation assays also have a potential problem of being non-specific, although the use of silver, with its high affinity for sulfhydryls, in part alleviates this difficulty (Scheuhammer and Cherian, 1986). Another system that uses a discontinuous gradient polyacrylamide gel to quantitate MT appears to be more specific than the metal saturation assays although it may be too cumbersome for routine use (Lin and Mc Cormick, 1986). Molecular biological techniques have been developed to determine MT mRNA levels (Compere and Palmiter, 1981; Hamer, 1986) and are now widely used as research tools. Radioimmunoassay have been used to determine MT levels in a number of different tissues and in the urine of Cd exposed individuals and laboratory animals (Tohyama *et al* 1981). Elevated levels of urinary MT may become a potential biomarker indicative of Cd induced renal damage in man (Tohyama *et al* 1981) and may prove of value in the diagnosis of chronic Cd toxicity.

Physological properties of metallothionein

One of the primary physiological functions of the MT protein may be related to Zn and Cu homeostasis with the highest concentrations of the MT protein occurring during periods of high zinc demand, i.e., normal growth and developmental periods ((Webb, 1979; Brady, 1982) or following surgery where

there is increased tissue regeneration (Ohtake and Koga, 1979). At birth, a number of tissues have high MT levels (Webb, 1979, Wong and Klaassen, 1979; Waalkes and Klaassen, 1984) which then decline during the postpartum growth and development period. The MT gene appears to be expressed very early in mammalian development as MT mRNA has been detected in mouse endodermal yolk sacs (Andrews *et al,* 1984). Clough *et al* reported in 1986 that human fetal liver MT levels correlated well with Zn levels and gestational age, reinforcing the concept that MT appears to be a physiologic Zn storage depot during periods of rapid growth. Thus, the biological role of MT in Zn metabolism may be somewhat analogous to that of ferritin in iron metabolism. This conclusion is also supported by the close similarities in the hepatic developmental patterns of these two metal binding proteins. Like MT, hepatic ferritin levels are also high in late gestation and at birth but decline steadily during postnatal development. Moreover, both MT and ferritin are intracellular proteins that appear to both be synthesized on the free polyribosomes in the cytoplasm (Cherian *et al,* 1981).

In contrast, with some hepatic malignancies characterized by rapid growth, relatively low MT levels have been demonstrated in the malignant portion of the tumor when compared to the surrounding areas of surgically resected human livers (Onosaka, S. *et al,* 1986). Despite the fact that the MT

levels were low in assayed tissues in this study there is some question regarding the interpretation of the results because MT levels were not determined in normal liver tissues from controls. It appears that the question of changes in MT levels in rapidly growing tumors may well depend on tumor type as elevated MT expression have been detected in several tumors of non-hepatic origin such as thyroid tumors (Kontozoglou *et al*, 1989), testicular embryonal carcinomas (Nartley *et al*, 1987) and skin papillomas (Hashiba *et al*. 1987). Metallothionein has also been shown to be elevated during regenerative proliferation induced following exposure to hepatotoxins and the tumor promoters acetaminophen and bis(2-ethylhexyl) phthalate (DEHP) (Waalkes and Ward, 1989). It has been shown that tumor promoters such as barbiturates, which induce cellular enlargement but not cellular hyperplasia, do not elevate hepatic MT protein concentrations. These results suggest that there is a relationship between MT levels and enhanced cell turnover rates (Waalkes and Ward, 1989). Likewise, the phorbol ester tumor promoter, 12-O-tetradecanoylphorbol 13 acetate (TPA) has also been shown to enhance MT mRNA expression (Hashiba *et al*, 1989) in addition to promoting tumor development. Reports on marked changes in MT levels and localization in mammalian livers during development (Webb and Cain, 1982; Nartley *et al*, 1987) and in certain neoplasias (Kontozoglou *et al*, 1989) suggest a biological

function for this protein during cellular differentiation and maturation.

The significance of the presence of MT in the nucleus of hepatocytes during development or following partial hepactectomy and also with certain tumor cells may be related to interactions of metal ions like Zn with various nuclear components. Metals which bind to the MT molecule may also bind to histones, nucleolar RNA, nuclear acidic proteins and can modify gene expression and phosphorylation of regulatory proteins. In addition, these metals may also cause breakage, excision, cross-links and transitions in DNA. Presence of MT in the nucleus can effectively chelate and detoxify certain metals and thus modify various cellular processes (Cherian, 1992).

Metallothionein has been shown to serve as an active donor of both Zn and Cu to a number of different cellular sites (Petering and Fowler, 1986). In support of this hypothesis, various apoenzymes which require Zn and/or Cu as cofactors, like carbonic anhydrase, alkaline phosphatase and superoxide dismutase, can be reactivated following incubation with Zn-and Cu-thionein (Udom and Bradley, 1980; Li *et al*, 1980; Geller and Winge, 1982). It was also shown in an *in vitro* model that Zn-thionein in the kidney activates aminolevulinic acid dehydratase, a Zn-requiring enzyme involved in heme biosynthesis, by donating Zn to the enzyme (Goering and Fowler, 1982). Furthermore, evidence which suggests that MT is not essential for the

activation of enzymes by the direct donation of metals is provided by work using cells that lack MT which appear to function normally (Compere and Palmiter, 1981; Crawford *et al,* 1985).

Another function for the MT molecule may be the detoxification of reactive intermediates. The protein may constitute a cellular defense mechanism such as an acute response to electophilic agents like free radicals and reactive metabolites. This capacity appears to be related to the protein's high sulfhydryl content, which appears to provide neutralizing nucleophilic equivalents (Kagi and Schaffer, 1988). Pre-induction of the MT molecule prior to exposure to carbon tetrachloride or irradiation, markedly reduces the toxicity which is mediated by the production of reactive metabolites and/or free radicals (Cagen and Klaassen, 1989; Clarke and Lui, 1986; Matsubara *et al,* 1987). Indirect activation of macrophages and neutrophils during the acute phase of inflammation results in a massive release of various species of oxygen metabolites, which may be indirectly responsible for the initiation of apoptosis. Metallothionein expression reduces the amount of damage elicited by this mechanism (Manuel *et al,* 1992).

After exposure to oxygen, MT serves as an expendable yet renewable cellular target for free radical damage (Manuel *et al,* 1992). A systemic acute-phase response, characterized by alterations in plasma Zn and Cu

concentrations and increased ceruloplasm oxidase activity, was demonstrated in animals pre-treated with Cd (Hart *et al*, 1989). Ultraviolet irradiation also induces synthesis of the MT gene (Lieberman *et al*, 1983). Thornalley and Vasak (1985) demonstrated that purified MT is a potent scavenger of hydroxyl radicals *in vitro* with an apparent bimolecular rate constant on the order of $k_{OH/MT} \approx 10^{12}$ /M/sec. Superoxide dismutase (SOD) is a much more efficient scavenger of the superoxide radical than MT (Thornalley and Vasak, 1985) and is often used in the MT literature to evaluate the effect of the superoxide radical *in vitro*. Comparison of the effect of MT on induced hydroxyl radicals compared with that of glutathione *in vitro* showed that degradation of the DNA molecule was almost completely inhibited by a concentration of 13 μM MT. In comparison, a 10 mM concentration of reduced glutathione was needed to achieve the same protective effect (Manuel *et al*, 1992). Although MT and SOD have different affinities for superoxide and hydroxyl radicals they appear to complement each other's activity and together may well confer comprehensive protection against these two reactive oxygen species (Manuel *et al*, 1992). It is important to point out that this referenced work was conducted *in vitro* and clearly demonstrates an antioxidant role for the MT molecule. When the MT molecule is evaluated *in vivo* its role as an antioxidant remains controversial. For instance, high Cd resistance in

human, rodent and murine cell lines appears to correlate directly with exposure to gamma irradiation or hydrogen peroxide and the superoxide anion (Bakka *et al*, 1982; Mello-Filho et al, 1988). Mice fed a diet containing heavy metals demonstrated an increased tolerance to gamma irradiation (Matsubara *et al*, 1987a,b). In contrast, other reports indicate that overexpression of MT does not protect *in vivo* against the direct effect of toxic radicals generated by radiation or radiomimetic drugs such as bleomycin which produces DNA strand breaks through the generation of hydroxyl radicals (Manuel *et al*, 1992).

Other detoxification roles ascribed to MT include chelation of platinum drugs (Kelly *et al*, 1988; Zelazowski *et al*, 1984), mediation of cellular resistance to toxic effects of alkylating agents (Tobey et al. 1982; Endresen and Rugstad, 1987) and x-rays (Matsubara *et al*, 1987a,b; Koropatnick *et al*, 1989; Sihiraishi *et al*, 1989). Both platin and cisplatin induce MT synthesis whereas transplatin and its hydrolyzed species do not initiate synthesis of this protein (Harford and Sarkar, 1989). Pre-administration of bismuth, Cd, cobalt, Cu, mercury, nickel and/or Zn have been shown to induce a protective effect against the toxicity of the antitumor drug cis-diammine-dichloroplatinum (Naganuma *et al*, 1987). However this protective effect has been shown to not always correlate with MT levels in the liver and kidney of mice treated with

known metal MT inducers which suggests that other protective mechanisms may also exist (Naganuma *et al*, 1987). Several antitumor drugs are metabolically activated to produce free radicals that interact with molecular oxygen, generating superoxide, hydrogen peroxide and/or hydroxyl radicals which are toxic to both cancer cells and normal tissues. Metallothionein synthesis by cancer cells appears to be limited when compared to MT synthesis of cells from normal tissues. Because of this limitation, the MT molecule appears to play an important role in protecting normal tissue without affecting the antitumor activity of the drug on the cancer cells (Naganuma*et al*, 1987). Thus, the multi-drug-resistance factor exhibited by a number of different tumor types may also be linked to MT induction (Manuel *et al*, 1992).

Pulmonary MT is found in alveolar epithelial cells, fibroblasts and lymphocytes of the lung. Metallothionein appears to be restricted to the nuclei of these cells and Cd pretreatment increases antioxidant enzyme activity (including selenium dependent glutathione peroxidase, catalase, glutathione reductase and Mn-SOD) as well as elevating non-protein thiols and MT (Hart and Garvey, 1986). In the brain, the lateral choroid plexus sequesters most of the heavy metal ions that enter the brain, but it does not appear to produce MT (Zheng *et al*, 1991). Zinc-metallothionein appears to play an important clinical role in Alzheimer's disease through free radical metabolism (Duguid *et*

al, 1989). Brain MT isoforms may also be involved in both the transport and homeostasis of essential elements such as Zn and Cu (Ebaadi and Swanson, 1987).

Metallothionein also appears to serve as a source and storage site for cysteine, especially during developmental periods (Zlotkin and Cherian, 1988). Most of the proposed functions and metal binding properties of MT are closely related the protein's high cysteine content (33%) and its unique structural arrangement. Although glutathione is a major nonprotein cysteine reservoir in the liver, its hepatic content is at a submaximal level in fetal-neonatal rat liver, which contains large amounts of γ-glutamyltranspeptidase. At the same time, the developing liver contains large amounts of MT, which has a much lower turnover rate than glutathione (Kagi and Kojima, 1987). Thus cysteine can be retained in the neonatal liver as MT for a longer period than glutathione. In the fetal liver, glutathione concentrations are about 3 μmol/g of tissue whereas MT concentrations are about 160 nmol/ g of tissue. Because MT contains about 21 residues of cysteine per molecule, the total amount of cysteine in the hepatic MT pool becomes very similar to that of glutathione in rats at birth. It has been postulated that the MT molecule may prove to be a major cysteine source in the fetal-newborn rat liver (Cherian, 1992).

Much information concerning the functions of a cellular constituent can be derived from experiments in which the metabolism of the constituent is modified by chemicals which inhibit or stimulate enzymes involved in the synthesis or degradation of the protein. Such a study was undertaken in newborn rats to investigate the role of MT in sulfur metabolism by inhibiting glutathione synthesis with buthionine sulfoximine (BSO) or stimulating synthesis by supplying a cysteine source (Gallant and Cherian 1989). Results of this research showed little interaction between the two major cysteine pools, MT and glutathione, in newborn rat liver (Gallant and Cherian 1989). Although the exact source for the synthesis of MT and glutathione in newborn rat liver has not been defined, it known that both of the thiol containing peptides are synthesized. In addition, it is also known that dams with depleted hepatic glutathione levels, and thus a limited maternal supply of sulfur amino acids, consistently give birth to fetuses capable of effectively synthesizing adequate levels of both glutathione and MT (Gallant and Cherian, 1989; Taniguchi and Cherian, 1990). In contrast to glutathione, hepatic MT and its mRNA levels are regulated primarily by maternal factors like the dam's Zn status. Both the fetal MT and its mRNA levels are depleted when the dams were fed Zn deficient diets during late gestation (Gallant and Cherian , 1986; Andrews, *et al*, 1987a). This suggests that fetal glutathione

levels in fetal rat liver are regulated to some extent by enzymatic activities while MT synthesis appears to be dependent on maternal factors like the transfer of Zn to the fetus. However depletion of glutathione in dams given BSO injections results in decreased hepatic glutathione levels in the pups without changes in MT levels. It is assumed that some of these differences may be due to different pathways for glutathione and MT synthesis in fetal rat liver.

Metallothionein and cadmium detoxification

Metallothionein is thought to play an important role in the biological detoxification of Cd in many different species by elevating tolerance levels through high-affinity sequestration of the metal. Piscator (1964) demonstrated that following Cd exposure the metal was predominately associated with the MT protein. Later it was shown that pre-exposure to Cd, a metal known to stimulate MT synthesis, prevented the toxicity expected from the subsequent exposure to a higher concentration of the metal (Liber and Miya, 1976; Yoshikawa, 1973). It is believed that following an initial exposure to either Cd or Zn that mechanisms for MT synthesis are induced and the resulting elevated protein levels provide the sequestration capacity necessary to prevent, or substantially reduce, interactions of Cd with molecular target molecules in the cell.

Gene amplification and methylation

Genetically selected and naturally occurring lines of both cultured mammalian cells and yeast exhibit substantial variations in MT synthesis and heavy metal resistance. Experiments utilizing cloned MT gene hybridization probes have demonstrated that such differences can be caused by changes in the number of copies and/or the methylation status of the MT genes in the tissue of interest.

Stable Cd-resistant mammalian cell lines of cultured cells can readily be isolated by continuous exposure of the cells to stepwise increases of Cd exposure in the culture medium (Rugstad and Norseth, 1975; Hildebrand *et al*, 1979). Analysis of a Cd-resistant mouse Friend leukemia cell line revealed a 14-fold increase in stable MT-I mRNA, a six-fold higher rate of MT-I gene transcription along with a six-fold increase in the number of MT-I genes over that seen in other leukemia cell lines (Beech and Palmiter, 1981). These Cd-resistant cells exhibited both chromosomal aneuploidy and the presence of three small chromosomes absent in the parental line.

Metallothionein gene amplification has also been studied in Chinese hamster ovary cells, a line that is unusually sensitive to poisoning by small concentrations of Cd. Lines resistant to successively higher Cd concentrations exhibited coordinate 3- to 60-fold amplification of both the MT-I

and MT-II genes and a resultant increase in measurable levels of MT-I and MT-II mRNA polypeptides (Crawford *et al*, 1985; Glick and McCarty, 1982). Cytogenic analyses of the highly Cd-resistant variants consistently revealed breakages and rearrangements involving chromosome 3p, the locus of both the MT-I and MT-II genes (Stallings *et al*, 1984).

The mechanism of MT gene amplification is currently unknown. One hypothesis, suggested by the association between amplification and chromosomal aneuploidy, is that the first in the chain of events is a random break or breaks in the chromosomal DNA around the MT locus. This could then liberate MT gene fragments capable of autonomous replication or place the MT genes between DNA sequences capable of mispairing. Fluctuation tests on cultured mouse cells have shown that the acquisition of low-level Cd resistance is a random rather than induced event (Hamer, 1986). It has also been shown that tumor-promoting agents can increase the frequency of the appearance of Cd-resistant clones containing amplified MT genes (Hayashi *et al*, 1983).

Most cultured mammalian cells synthesize MT and are resistant to moderate exposure to transition metal ions with the exception of the W7 line of mouse thymus cells and Chinese hamster ovary cells. The postulated inverse correlation exists between the methylation status of the DNA molecule

and higher eukaryotic gene expression (Holliday and Pugh, 1975) led investigators to evaluate the degree of methylation of the MT gene in this cell line. Mouse W7 cells contain a normal MT-I gene, determined by DNA gel transfer hybridization, yet they synthesize no detectable MT-I mRNA and are sensitive to low level Cd toxicity (Mayo and Palmiter, 1981). Treatment of exponentially growing W7 cells with 5-azacytidine, a cytidine analog that is incorporated into nucleic acids but cannot be methylated, led to dramatic increases in both the basal and Cd-induced levels of MT-I mRNA (Compere and Palmiter, 1981). The effect of 5-azacytidine was maximal within 8 hours and then persisted for a long period of time. Gene methylation was blocked by hydroxyurea, an inhibitor of DNA replication. The methylation status of the MT-I gene was determined by gel transfer hybridization analysis of chromosomal DNA digested by two different methylation sensitive restriction endonuclease enzymes, HpaII and MspI. Six HpaII sites in the vicinity of the mouse MT-I genes were shown to be heavily methylated in the parental W7 cells but not in Cd-resistant cell lines derived by 5-azacytidine exposure. Some MT-I gene amplification was also observed in resistant cells. The results were used to develop a model in which the 5-azacytidine inhibits DNA methylation during the first round of DNA replication, thereby generating hemimethylated MT genes that are capable of normal transcription and

induction. A second round of replication generated 50% fully methylated inactive genes and 50% fully unmethylated active genes, the later being capable of conferring stable inheritance.

Analogous results have been obtained with Chinese ovary cells. Lines resistant to moderate levels of Cd (2 μM) showed no obvious gene amplification (Crawford *et al*, 1985) but are hypomethylated at both the MT-I and MT-II loci. Also, as with the W7 mouse cells, the frequency of moderately Cd-resistant Chinese hamster ovary cells can be increased by 5-azacytidine treatment.

The mechanism by which methylation of the cytosine residue inhibits MT gene expression is currently unclear. Possible explanations include interference with the binding of RNA polymerase or regulatory factors, alterations in nucleosome phasing, and/or changes in the higher-order of chromatin packaging.

Metallothionein mRNA

Metallothionein is generally considered a 'housekeeping" protein because it is expressed in many different tissues and cell types in mature non-replicating cells. Nevertheless, there are substantial quantitative variations in levels of MT during embryogenesis and during times of cellular replication. The development of cloned gene probes and gene transfer methods has

allowed initial molecular analyses of developmental pathways in mammals and the sea urchin.

Metallothionein gene expression has been most thoroughly studied in rodents. The liver is the primary site of MT mRNA and polypeptide synthesis in fetal mice and rats (Anderson *et al*, 1983; Andrews *et al*, 1984). Levels of MT mRNA in mice and rats are detectable by day 12 of gestation, increase five-fold to reach a maximum by day 16, then remain approximately constant to parturition. Following birth there is a rapid drop in MT synthesis, although MT mRNA levels may remain constant throughout the suckling phase (Anderson *et al*, 1983). The intracellular localization of MT during pre- and post-natal development of the rat has been studied by immunohistochemical staining methods (Panemangalore *et al*, 1983). Intense nuclear staining for MT was observed in the fetal newborn liver up to day 9 but by day 17 the staining was primarily cytoplasmic, similar to the pattern found in adult liver cells.

Substantial levels of MT mRNA are found in the visceral and parietal yolk sac of the mouse fetus (Andrews *et al*, 1984). In these organs, MT levels rise seven-fold from day 9 to 11 of gestation, remain constant until day 15, then drop 20-fold or more by parturition. Separation of the visceral yolk sac into endodermal and mesodermal layers revealed that essentially all of the MT

mRNA is contained in the ectodermal cells. Similarly, high levels of MT mRNA are found in cultured embryonal cells with primitive, parietal, or visceral endoderm characteristics, whereas low or undetectable levels of MT mRNA are present in the fetal brain, kidney, bowel, heart, placenta and amnion and in undifferentiated embryonal carcinoma cells (Andrews *et al,* 1984).

In the adult mammal, the liver and kidney appear to be primary sites of MT and metal accumulation. Consistent with this, measurements of MT mRNA levels in Cd-injected mice revealed the highest levels in the liver and kidney with lower levels in the intestine, heart, testes, muscle, brain and spleen (Duram and Palmiter, 1981). The accumulation of MT mRNA was inducible by Cd in all tissues except the testes.

The sea urchin is particularly useful for studying early stages in gene regulation because of the wealth of classic embryological studies conducted on this organism. A cloned cDNA probe has been used to follow steady state levels of MT mRNA during the development of both normal and zinc-treated embryos (Nemer *et al,* 1984). The egg contains substantial levels of maternal mRNA that is polyadenylated shortly after fertilization. During the first 9 hours of development, which is the period of most rapid cell division, no new MT mRNA is synthesized. Accumulation of the MT mRNA begins at about the 12^{th} hour and reaches a maximum at the 20^{th} -hour mesenchyme blastula stage.

Metallothionein mRNA levels then decrease, until the gastrula stage which occurs at about 40th hour, followed by an increase during further development into the early pluteus stage. Thus MT mRNA accumulation reaches two discrete peaks, separated by a valley, during the course of embryogenesis in this organism.

The tissue specificity of MT gene expression in the sea urchin has been studied by comparing fractionated mesodermal and endodermal derivatives to ectodermal derivatives embryos (Nemer *et al,* 1984). The mesoderm-endoderm showed a low level of basal MT mRNA that was strongly induced by Zn treatment, whereas the ectoderm contained a high constitutive level of MT mRNA that was unaffected by Zn. These results were confirmed by analyzing gastrula, mesenchyine blastalae, and Zn "animalized" (ectoderm-predominant) embryos. A possible interpretation of these results is that the ectodermal cells, which are in constant contact with sea water, constitutively maintain MT at a high level to protect against sudden encounters with heavy metal ions. In comparison, the interior mesodermal and endodermal cells induce MT gene expression in response to metal stimulation as a second line of defense. It is not yet known whether these differences reflect the transcription of distinct MT genes in the different tissues (Nemer *et al,* 1984).

These results show that MT gene transcription can vary considerably during development. Of particular interest are the high basal MT levels in both mammalian and sea urchin embryos and their possible relationship to a fetal function for the MT molecule. At least two distinct mechanisms may be involved in the differential expression of MT genes during development. First epigenetic changes, such as DNA methylation or chromatin modification, could alter the accessibility of MT genes to transcriptional and regulatory factors. Second, different cell types, perhaps in response to their role in heavy metal metabolism, might vary in their content of diffusable MT gene regulatory factors. Such hypothetical factors could in principle either act early, i.e., metal receptors, or late, i.e., DNA binding proteins, in the regulatory pathway.

Metallothionein isomers

There are several different MT isoforms found in mammalian tissues. In the mouse there are four MT genes that reside in a 50 kb region on chromosome 8 (West *et al*, 1990). Humans have at least 16 MT genes clustered on chromosome 16 (West *et al*, 1990; Quaife *et al*, 1994). Mouse MT I and II genes are expressed at all stages of development in the cells of most organs. Mouse MT I and II expression appears to be regulated by metals, glucorticoids and inflammatory stress signals (Palmiter 1987). The

MT III isoform is expressed predominately by neurons but is also found in glia cells and the male reproductive organs (Masters *et al*, 1994; Uchia *et al*, 1991; Moffat and Seguin 1998). Metallothionein IV has been isolated from differentiating squamous epithelial cells (Quaife *et al*, 1994). All four MT genes are expressed in the maternal placenta. The fact that there are multiple MT genes which express separate but distinct gene products suggests that the different isoforms may have different functions, but whether their function is relevant or divergent is not clear at this time (Hamer, 1986).

Metallothionein I and II are the most prevalent MT isoforms found in the body. but their specific physiological functions are not currently known. Mice that cannot make MT I or MT II have low hepatic zinc content at birth, which could jeopardize perinatal development. However, the only morphological abnormalities observed in these mice were in the developing kidney tissue (Kelley *et al*, 1996). Rearing MT-deficient and control pups on dams fed a zinc-deficient diet stunted the growth of both groups identically (Kelley *et al*, 1996). Genetic experiments that delete the ability to produce MT in developing mice and rats show that the animals develop normally. These studies suggest that the MT molecule may not be essential for normal embryonic development and growth, but they do provide a reservoir of zinc that can be mobilized under zinc limiting conditions (Dalton *et al*, 1996). The

MT III isomer was recently isolated from the brains of people afflicted with Alzheimer's disease (Uchia *et al*, 1991). This discovery was based on the fact that the MT-III gene product inhibits neuronal growth (Uchia *et al*, 1991). Part of the problem in determining the function as well as the site of production of the different isoforms is that they lack strong epitopes and in the case of mammalian MT, lack aromatic amino acids and any other characteristic spectroscopic property that can be employed for the direct analysis of the protein. Therefore, information on the amount and distribution of MT isoforms in the different tissues remains sparse and incompletely documented.

Cadmium-metallothionein (Cd-MT) complex

Although the Cd-MT complex is biologically inert when localized intracellularly, it becomes a potent nephrotoxin when it reaches the systemic circulation. Extracellular Cd-MT is available systemically either by release from the liver after a toxic insult or from gastrointestinal absorption, because the Cd-MT complex is absorbed at least partially intact (Klein *et al*, 1986). The Cd-MT complex is highly toxic to the kidney following tubular reabsorption (Cherian *et al*, 1976; Foulkes, 1978). Although smoking results in elevated renal Cd-MT levels (Summer *et al*, 1986) its role in Cd-induced nephropathy in highly exposed individuals is not clearly defined.

Cadmium absorbed from the gastrointestinal tract or lungs is initially

transported to the liver, where it induces the MT synthesis. Continual exposure to Cd causes liver injury with resulting leakage of Cd-thionein from damaged hepatocytes into the circulation (Dudley *et al,* 1985). The metal protein complex is transported to the kidney, filtered by the glomerulus, and subsequently reabsorbed by the proximal tubule cell in a manner similar to other low molecular weight proteins (Foulkes, 1978; Dudley *et al,* 1984; Sqibb *et al,* 1979: Squibb *et al,* 1984a, 1984b). The resorbed complex appears to be rapidly degraded by the renal proximal tubule cellular lysosomal system with the release of the Cd ion (Sqibb *et al,* 1979; Squibb *et al,* 1984a, 1984b), which in turn induces the renal MT synthesis. This process continues until the capacity of the proximal tubule cell to synthesize MT is exceeded, thus allowing unbound metal ions to react with sensitive biological cellular targets.

The most critical ligands in the proximal tubule cell may include those that appear to play a role in the fusion of primary lysosomes with pinocytotic vesicles to yield mature secondary lysosomes. Cd has been shown to selectively disrupt this fusion process, leading to a loss of lysosomal proteolytic enzyme activity, development of tubular proteinuria and calcuria (Goering *et al,* 1986; Fowler *et al,* 1987) similar to what is observed in workers chronically exposed to Cd (Kazantzia, 1979).

The molecular mechanism responsible for the perturbation of the renal

proximal tubule cellular lysosomal system may involve the activation of calmodulin by non-MT-bound Cd ions (Chao *et al*, 1984). Activation of calmodulin, the major calcium-binding receptor in the eukaryotic cell, results in a biochemical cascade leading to disruption of cell cytoskeletal components. Fusion of primary lysosomes with pinocytotic vesicles is thought to be mediated by cytoskeletal components. Disruption of the fusion process inhibits resorption of proteins by the proximal tubule cells, resulting in tubular proteinuria.

An alternative mechanism for the Cd-MT nephrotoxicity may involve damage to the renal brush border membrane during pinocytosis of the metal-protein complex with secondary lysosomal disruption (Cherian *et al*, 1976b). This hypothesis is based on patterns of urinary enzyme excretion after Cd-MT injection which are indicative of early damage to the renal tubule brush border (Suzuki *et al*. 1987).

Several mammalian tissues appear to be deficient in the MT protein and are highly susceptible to the toxic effects of Cd. These MT deficient tissues include rat, mouse and monkey testes; the rat ventral prostate and hamster ovary are all highly susceptible to either the acute and/or chronic carcinogenic effects of Cd (Rehm and Waalkes, 1988; Waalkes *et al*, 1988a, b and c; Waalkes and Perantoini, 1986; Waalkes and Perantoni, 1989). A MT

like protein has been identified in these tissues but the protein is not inducible with the Cd ion in these tissues and it does not appear to protect these tissues from Cd toxicity (Waalkes *et al*, 1988a, 1988b; Waalkes and Perantoni, 1989). These proteins have a much lower cysteine content than that seen with the Cd inducible MT protein. Since the MT system is thought to detoxify Cd through inducible, high-affinity binding, the lack of inducibility and the low levels of cysteine in these MT-like proteins may reduce the affinity as well as capacity for Cd sequestration in target tissues. This may allow Cd greater access to specific molecular targets. In the case of Cd carcinogenesis, it is presumed that DNA is the molecular target, and in this respect, treatments that prevent Cd carcinogenesis in the testes, such as Zn (Waalkes *et al*, 1989), reduce Cd interactions with testicular DNA (Koizumi and Waalkes, 1989).

Non-mammalian Cd binding proteins

It is now evident that the family of Cd-binding proteins is large and diverse, particularly when non-mammalian proteins are considered. While mammalian MT's serve as a basis of comparison, it is essential that each non-mammalian system be evaluated individually without tacitly assuming that these metal binding proteins share all of the physical, chemical and functional properties of mammalian MT. Cd-binding proteins have been isolated from a

number of non-mammalian and plant systems as diverse as *Pseudomonas*, yeast, mushrooms, scallops, oysters, algae, crabs and rainbow trout (Petering and Fowler, 1986; Stone and Overnell, 1985). Many of these proteins possess properties that exclude their classification as MTs', these differences include such things as differences in molecular weight, isoelectric points, as well as sulfhydryl and/or amino acid content and metal binding properties. As more work is done with these proteins, genetic homology may prove to be more a more relevant comparison in the classification of MT proteins in other species. Other Cd-binding macromolecules exist, such as the cysteine-rich polypeptides that accumulate high amounts of Cd in various plant cells and fungal organisms (Steffens *et al,*1986; Weber *et al,* 1987; Winge *et al,*1989). The poly(γ-glutamylcysteinyl) glycines function as the major metal ion detoxification mechanism in some plants, fungi and unicellular organisms (Steffens *et al,* 1986; Weber *et al,* 1987; Winge *et al,* 1989).

Evolutionary relationships between MT and other Cd-binding proteins are potentially important in elucidating the functional role of these proteins. Sequence data for a number of proteins reveal a high degree of homology with conservation of cysteine sequences. The evolution of these proteins may have involved the addition of exons to the MT gene and or substitution of amino acids (Vasak and Armitage, 1986). The comparative biological

approach utilized in these studies (Petering and Fowler, 1986; Stone and Overnell, 1985) provides a tool to evaluate the potential role of these molecules in permitting the different organisms to bioaccumulate toxic metals, such as Cd, resulting in the eventual contamination of the food chain. Analysis of high affinity metal binding proteins may also be of potential value as biological indicators of metal pollution, particularly in the aquatic environment (Henning, 1986). Fundamental to studying Cd-binding proteins from various phyla is the understanding that basic physiology may influence metal-binding patterns of proteins to a much higher degrees in non-mammalian organisms than in mammals. It has been shown that normal seasonal process such as alterations in water pH, temperature and salinity in addition to intrinsic factors such as the molting process may strongly influence the molecular binding patterns to macromolecules in non-mammalian organisms (Hunt *et al*, 1984; Petering and Fowler, 1986).

Planaria

Planaria are free living flatworms commonly encountered in fresh water ponds and streams that are frequently seen clinging to water plants or the undersides of logs or stones. The planarian of particular interest to this project is *Dugesia dorotocephala*, a free living freshwater flatworm, that belongs to the order Tricladida, class Turbellaria of the phylum

Platyhelmintes. Grossly, the organism usually measures between 2 and 3 centimeters in length and has an average weight of 10-15 mg. These flat worms are easily identified by their triangular-shaped heads and pointed posterior ends. Planarians have an obvious pair of eyespots on the dorsal surface near the anterior end and a tubular pharynx that extends from a mouth positioned near the middle of the ventral side (Hyman, L.H. 1951). The overall body shape is bilaterally symmetrical with a slightly convex dorsal surface that appears almost conical and a flattened ventral surface. When viewed laterally the organism appears flattened (Hyman, L.H. 1951).

Planarians are of interest to researchers because they exhibit basic similarities to higher animals in terms of biochemical, physiological and cellular organization (Best 1983). They are highly sensitive to low concentrations of environmental toxicants and exhibit responses similar to those of higher animals exposed to environmental pollutants. Planarians are capable of xenobiotic biotransformations similar to those of mammalian monooxygenases (Phillips *et al*, 1974; Dean-Colomb *et al*, 1991) and may be used as a sentinel species to evaluate environmental contamination (NRC, 1991).

Digestive system

The planarian digestive system consists grossly of a mouth, pharynx

and intestine. The mouth is situated at about the middle of the ventral surface, posterior to the area where the two eyes are located on the dorsal surface. The pharynx is a long, highly muscular organ, which is capable of protruding from the mouth, connecting it with the intestinal tract. When protruded, the pharynx appears as a white and very flexible cylinder, with a somewhat trumpet-shaped tip (Hyman, L.H. 1951). The muscular mouth leads to a pouch-like stomodaeum or pharyngeal cavity which is of the placate type (Hyman, L.H. 1951). From here food passes through a short esophagus to a three forked intestine (Hyman, L.H. 1951). The intestine forks into three branches or rami, one running rostrally and two passing caudally. Each ramus gives off lateral diverticula, which are often secondarily branched. The lining of this digestive cavity consists of large, phagocytic, columnar epithelial cells (Hyman, L.H. 1951).

Jennings (1962) found that the pharynx possessed acidophil gland cells, which produced cathepsin, an endopeptidase, which dissolved the body of the prey. Food in the gut was then attacked by extracellularly acting endopeptidases originating from cells in the gastroderm. This food was then digested intracellularly. This researcher (Jennings, 1962) demonstrated that digestion followed a definite sequence of events with definite pH changes starting with endopeptidase at a pH of 5.0 followed by the action of a number

of different exopeptidases, such as leucine amino-peptidase and lipases, all at a pH of 7.2

Nervous system

The head is usually small and conical in shape with triangular, projecting sensory auricles. The eyes on both sides of the head are comprised of two-pigmented cup ocelli with numerous pigmented cells. In most species the paired eyespots lie dorsally and often a little anteriorly over the head ganglia. In addition to the eyes, Turbellarians have other less obvious sensory organs on the head including tentacles, auricles, grooves, ciliated pits that presumably serve as chemoreceptors, and a number of sensory-hairs, bristles and spines (Keenan *et al*, 1979).

The planarian brain is a compact, bilobed structure, which in miniature models the mammalian brain in a number of different ways including such things as exhibiting both neurochemical (Table 2-5) and cellular components (Keenan *et al*, 1979). Like the mammalian brain, the planarian brain is also composed of neurons, neurosecretory cells and neuroglial bodies as well as light- and dense-core synaptic vesicles and complex synaptic configurations (Morita and Best, 1965, 1966; Oosaki and Ishii, 1965; Best, 1967; Best and Noel, 1969). Planarian nerve cells resemble those of other animals, being uni-, bi- or multi-polar and the neurons are morphologically similar with no

undifferentiated axons. A pair of large ventral chords known as nerve trunks carry nerve impulses form the brain to the rudimentary organs similar to the mammalian spinal chord (Keenan *et al*, 1979). Many cross-commissures and small lateral nerves originate from these main trunks and innervate all of the planarian organs and tissues. Researchers have demonstrated that the planarian, *Dugesia dorotocephala*, exhibits encephalization responses similar to that seen in animals with a true brain (Hyman, 1951; Best, 1983; Pennak, 1989). Table 2-5 is a summary of different neurotransmitters isolated from planaria.

Table 2-5 Neurotranmitters isolated from planaria

Neurotransmitter	Reference
acetylcholine	Bullock and Nachmasohn, 1942
norepinephrine	Welsh, 1946
dopamine	Welsh and Moorehead, 1960
serotonin	Lentz, 1968
glycine	Welsh and King, 1970; Welsh and Williams, 1970
metenkephalin	Keena et al, 1979; Schilt, 1981
melatonin	Venturini et al, 1983; Morita and Best, 1984c

Muscular system

The muscular system may be divided into a subepidermal, a parenchymal and an organ specific part. The first is striated just beneath the basement membrane of the epidermis. It consists of circular, diagonal and longitudinal fibers in most places (Hyman, 1951). The second transverses the body with longitudinal, transverse and especially dorso-ventral fibers; together with the subepidermal muscle coat it provides the animal with a highly developed capability to change its shape (Hyman, 1951). The third is especially developed in the pharynx and the copulatory organs (Hyman, 1951). Cross striations may be seen in these fibers.

Excretory system

The planarian excretory system is of the protonephridal type. Several nephridipores open at the surface of the body and are the outlets from tubules running down both sides of the entire length of the body. The tubules branch copiously, each branch or "capillary" terminating blindly, enclosed by cells provided with capillary tufts; the cilia are long and often seem to vibrate. The lumen in the capillaries is intracellular, the wall being composed of rows of interdigitated cells arranged in pairs (Pedersen, 1961).

Reproductive system

Dugesia dorotocephala propagate asexually by transverse fission as

well as by sexual propagation. The role of reproduction is specific to different strains of the species. In the sexual strains, the male sexual organs consist of numerous round testes lying in two symmetrically placed strands arising from near the head and extending to just behind the sexual opening. The testes have tiny vasa eferentia connected to two vas differentia which open by ejaculatory ducts into a copulatory complex having expanded into vesicles for storage of ripe sperm (Hyman, 1951). The copulatory complex contains the penis bulb, which is very muscular and contains several gland cells. The bulb terminates in the penis papilla, which is surrounded by the male antrum opening into the common sexual antrum leading to the genital pore.

The female sexual organs have a pair of round ovoid ovaries located near the head. The oviducts receive outlets from numerous yolk cells collected in follicles, which are situated bilaterally in the body from the level of the ovaries to the genital pore. The oviducts lead to the complicated hermaphroditic copulatory organs (Hyman, 1951). Anterior to the penis bulb is a copulatory bursa; the oviducts terminate posteriorly in the copulatory duct, which opens into the female antrum. This opens together with the male antrum into the common antrum. Gland arrangements are species specific.

The parenchyma

The parenchyma is the tissue that binds together the epidermis with the

digestive tissue and gives firm pliable support to embedded internal organs. It is composed of closely packed cells of two main categories: the neoblasts, or stem cells, and the parenchymal or reticular cells. Reticular cells are also called mesenchymal cells in the literature and comprise the major portion of the parenchymal tissue. Histologically, reticular cells are large cells with complex interdigitations and are supplied with numerous irregularly attenuated processes. Reticular cells contain few mitochondria, sparse Golgi material and ribosomes. Several observations suggest that mesenchymal tissue in planaria may be comparable to the mammalian reticuloendothelial system i.e., liver, hemopoetic marrow, spleen and lymph nodes (Morita, 1991b; Hay and Coward, 1975). Planarian mesenchymal tissue also contains a network of relatively large reticular cells often referred to as a reticulum. Primitive intercellular filaments are found scattered throughout the mesenchymal matrix, usually around the muscle cells and are thought to form a primitive subepidermal basement membrane.

Planarian reticular cells are also called "glycogen containing cells" because of the numerous glycogen granules in their cytoplasm (Hay and Coward, 1975). Reticular cells appear to be migratory, because they are the first cells to migrate to a wound surface following surgical cutting (Morita and Best. 1984a). These cells appear to also function as phagocytes because

they accumulate phagosomes containing planarian cell fragments in the days following experimental surgical excision (Morita, 1991b). In addition, Morita (1991b) demonstrated that they also phagocytize injected heat killed bacteria experimentally injected into planarian cells. Even in non-wounded specimens, reticular cells contain phagosomes with fragments of planarian cells, suggesting that they phagocytize senescent or otherwise defective cells. The reticular cells thus appear to serve in cell-mediated immunity and immune surveillance, similar to the functions of mammalian leukocytes, i.e., neutrophils, T-cells, helper cells, macrophages, etc. This similarity is reinforced by their similar response to exposure to Cd and O-tetradecanoylphorbol-13-acetate (TPA) in that it induces "hairy cell " leukemia in small rodents and malignant reticuloma in the planarian (Hall, *et al,* 1986a, 1986b; Ghadially and Skinnider, 1972). This theory has not yet been thoroughly proven and the undertaking of such a project will help to validate the planarian as an alternate non-mammalian model that can be used to study carcinogenesis.

In embryonic development, the various specialized cell types needed in planarian regeneration are formed by the differentiation of undifferentiated, totipotent stem cells termed "neoblasts". Typical resting neoblasts, not migrating to a wound or in the process of differentiation in the blastema (a

fission site), are round or somewhat spindle shaped, with an ovoid nucleus containing one to three nucleoli. The cytoplasm is scanty, forming only about half of the total cell volume. The cytoplasm is strongly basophilic due to abundant RNA occurring in a great number of ribosomes, most of which are free with only a few ribosomes bound to the endoplasmic reticulum. The high number of ribosomes and protein containing sulfhydryl groups suggesting that neoblasts are capable of morphogenesis.

In asexual planaria, all new cells required for regeneration or for replacement of senescent cell types, are derived from mitotic division of the primitive neoblasts (Randoph , 1897; Dubois, 1948; Wolf, 1962; Morita, et al. 1984a, 1984b). The presence of the totipotent neoblast in planarian tissue permits regeneration of a complete planarian from a fragment and regeneration of a head and brain in an individual planarian within two or three weeks of surgical decapitation. In asexual planaria, the neoblasts are the only mitotically competent cell found in the organism (Morita and Best, 1984b). Because mitosis is restricted to neoblasts, when planaria are exposed to irradiation, only neoblasts are killed because of their high mitotic index. Abolishing the neoblast population in the planarian tissues results in the loss of both reproductive and regenerative capacity (Best and Morita, 1982). Although the rate of neoblast mitosis increases in regenerative planarians, it is

appreciable even in non-regenerating specimens. Although not proven, it is thought that mitosis in intact planaria provides a new neoblast population to replace those that appear to be lost when neoblasts differentiate into reticular cells. The fissioning process also appears to provide the new cells required for the development of additional asexual planaria and it is thought that the neoblast is the primary cell type involved in this process (Best and Morita, 1982).

Nuclear satellite material, also referred to as nuclear emissions or chromatid bodies, are considered to be the hallmark for recognition of stem cells in planarian tissues (Morita and Best, 1974, 1984a, 1984b; Coward, 1974; Hay and Coward, 1975). Nuclear satellite material is an electron dense granulo-fibrillar material observed in the perinuclear cytoplasm of neoblasts evaluated by electron microscopy. In transformed stem cells the nuclear satellite material is produced in comparatively large amounts, often forming unusual "globular clusters" in the perinuclear cytoplasm (Hall *et al*, 1986b). Hall *et al*, (1986b) further reported that the nuclear satellite material diminished with differentiation and was observed noticeably further from the nuclear membrane as the shapes of the interdigitating cells become more irregular; decreasing, eventually to persist only as vestiges, in the later stages.

The epidermis

The epidermis covers the external surface of the planarian body as a one-cell-thick layered epithelium (Hyman, 1951). The cells are more or less columnar on the dorsal surface of the animal, cuboid or somewhat flattened on the ventral surface. The nuclei are globular and somewhat irregular. Cilia are abundant on the ventral epithelium and scarce on the dorsal epithelium. When undisturbed, the planarians can be observed gliding smoothly without muscular contraction on stones, water plants and on the water surface with their ventral surface attached to the water-air boundary. When disturbed the animals secrete abundant mucus from cells lying beneath the epidermis proper. This mucus is provided by the rhabdites deposited in epidermal gland cells. When the rhabdites are expelled they swell enormously in the water, often producing a heavy mucoid mass. Rhabdites are generally rod shaped, strongly acidophilic structures that may also crowd the dorsal epidermal cells so heavily as to obscure the features of the epidermal cells themselves (Torok and Rohrlich, 1959).

Sensory behavior of planaria

Early literature on planarian behavior describes the responses of the organism to different chemical agents. The reaction of planaria to chemical agents was described as "chemotaxis" by Hyman (1951) who observed that

when planaria were exposed to high concentrations of acids, alkalies and/or salts, they tended to move away from the agent which he described as "negative chemotaxis". Positive chemotaxis was described as positive responses toward food and was described as movement towards the food source.

Planaria are highly sensitive to light and that sensitivity is known as phototaxis. Both the dorsal and ventral surfaces have photosensors, which are sensitive to light. The planarians' sensitivity to ultraviolet radiation is manifested by a change in behavior; when exposed to UV light they tend to move much more slowly than they do in normal light. Short exposures to UV radiation sources and/or to bright sunlight can prove fatal. It is thought that light colored species may be more sensitive to light exposure than the darker colored species but that has not been proven (Morita and Best, 1984c).

Planaria tend to prefer living in an environment where the water currents are weak but can also be found in an environment where the currents are strong. In strong water currents planaria react with a strong clamping reaction known as "rheotaxis" caused by a contraction of the striated muscles which literally allow the organism to conform to the shape of the object, resulting in increased adhesion. It should however be noted that a strong positive rheotactic response can also occur in weak currents under certain conditions.

The reaction of planaria to temperature changes is known as "thermotaxis". The vast majority of all Turbellaria have been shown to be able to endure wide environmental temperature variations. Both the dorsal and ventral surfaces of the freshwater planarian body are negatively thermotaxic to high temperatures. The thermal death point for *D. dorotocephala* is approximately 42° C (Best, 1972).

Fissioning (Asexual reproduction)

During asexual reproduction, planarians divide by fissioning. The fission process is usually completed in 30 minutes to 2 hours depending on the environmental conditions. Following the actual fissioning process the individual planaria then grow and regenerate lost parts. The fissioning process or scissiparity is a cyclical process and the cycle duration is dependent on a number of environmental factors as well as seasonal influences. A rise in temperature, isolation of individual planaria or a reduction in population density and/or a change in the light/dark cycle promotes planarian scissiparity (Best, 1983). The fissioning process can be suppressed by greasing the container and/or by increasing the population density in the container. The brain controls asexual reproduction in planaria. It has been shown that the number of nervous cells with neurosecretory substances decreases quickly after fissioning and that fissioning takes place after the

neurosecretory substance has been discharged (Lender, 1970; Lender and Aghal, 1968;1969). Furthermore, scissiparity influences regeneration, which in turn activates the neoblasts to synthesize RNA and later, proteins. In addition to neurological control of fissioning it has also been shown that regeneration is also controlled by neurotransmitter secretion from neurosecretory cells (Lender,1970; Lender and Aghal, 1968;1969). It should also be noted that neoblasts are selectively killed by certain doses of X-irradiation (Lange, 1968; Wolff, 1962), which also abolishes the planarian's capacity to regenerate.

Regeneration

Planaria are able to replace body parts lost through a process called regeneration (Bronsted, 1969). This fact has made the planarian a subject of intense scientific investigation that leaves many unanswered questions regarding the subject of how the planarian initiates and controls regeneration. After surgically cutting the planarian body at some predetermined site, all dimensional parameters of the planarian change, including a decrease in the organism's profile area. Thus the organism becomes shorter and flatter while the area and length of the blastema increases.

During the first five days, regeneration proceeds quite rapidly but then the process slows down. By the tenth day after cutting, the initial relation

between the head length and the total body length is restored completely (Tiras and Sheiman, 1988). By the thirtieth day the relation between the areas of the head and the whole body reaches 100%. This growth pattern indicates that that there are probably three stages that occur in planarian regeneration. The first stage is a period of rapid growth followed by a stage during which there is a restoration of the relations between lengths (Tiras and Sheiman, 1988). The final stage or stage three is the period of time during which there is a restoration in the relation between areas of the head and body (Tiras and Sheiman, 1988).

It has been concluded that neuropeptides may stimulate, inhibit or in some cases have no effect on morphogenesis. Vertebrate neuropeptides including vasopressin, somatostatin and dalargin have been classified as stimulators of regeneration by different researchers (Tiras and Sheiman, 1988). *Hydra* head activator isolated from the *Hydra*, which was the most similar when compared phylogenetically to planarians, also stimulated regeneration and asexual reproduction in planaria. Moreover, *Hydra* head activator was more effective in stimulating regeneration of head than tail parts in this research (Tiras and Sheiman, 1998). It should also be noted that the neuropeptides differed in their effects on planarian regeneration depending on the stage of regeneration (Tiras and Sheiman, 1998). Neuropeptides from

both vertebrates and invertebrates appear capable of regulating planarian regeneration. Earlier research (Lender and Zghal, 1969) indicated that the number of nervous cells with neurosecretory substances decreases quickly after fissioning and that the fissioning process occurs after the neurosecretory substance is discharged. In addition, scissiparity influences regeneration, which in turn activates neoblasts which synthesize RNA and, later, proteins (Lender, 1970; Lender and Zghal, 1969).

The freshwater planarian exhibits, throughout its lifetime, a peculiar totipotent type of cell (neoblast) which imparts a remarkable capacity for regeneration and without which the animal will not survive. Yet a degree of controversy exists regarding whether: 1) undifferentiated neoblasts arise during regeneration by a process of dedifferentiation from more differentiated cell types (Woodruff and Burnett, 1965; Kido, 1967; Kido and Kishida, 1968; Hay, 1968; Rose and Shostak, 1968; Coward, 1969) or 2) if neoblasts are produced solely from a self-perpetuating population of stem cells that performs a crucial role in regeneration (Lender, 1962; Wolf, 1962; Morita *et al,* 1969; Spiegelman and Dudley, 1973; Morita and Best, 1974, 1984a, 1984b), as well as in normal growth and continuous cellular replacement.

Tumorigenisis, teratogenesis and neurotoxicity

Vertebrates differ from planarians notably in that they possess an

endoskeleton, a heart-lung-circulatory system, and long mylenated neurons. Because of these differences, it would not be expected that toxins to these systems would affect planarians in the same manner as vertebrates. In a similar respect, because planarian muscle fibers and neuronal junctions more closely resemble those of smooth muscle in vertebrates than other vertebrate muscle types, planarian models to study the effects of different drugs and chemicals on smooth muscles and relate those effects to vertebrates seems logical.

A number of earlier investigators exposed planaria to carcinogenic compounds and observed abnormal growth patterns (Foster, 1963, 1969; Goldsmith, 1937), some of which resulted in the death of the organism. However, growth characterization was inadequate to determine if the growths were neoplastic with the same definitions as applied to mammalian tissues. Histologic characterization was restricted to light microscopy which was inadequate to determine if the cells have been neoplastically transformed and transplantation studies were not performed (Morita, 1991a, 1991b). Because these types of studies were not performed some clinically oriented oncologists questioned the validity of the reported planarian tumors as true carcinogenesis in the mammalian sense (Good and Finstad, 1969).

Electron microscopy techniques improved the ability to diagnose

neoplastic disease as well as to improve the case definition of malignancy in the mammalian sense (Ghadially, 1980; Gyorkey *et al*, 1975). When this tool was used to evaluate invertebrate tumors it improved neoplastic cell identification and validated the fact that invertebrates also developed malignancies similar to those seen in mammalian systems. Hall (1986a, 1986b) reported a high incidence (76%) of a malignant postpharyngeal tumor in the asexual planarian, *Dugesia dorotocephala*, exposed to high level doses of Cd followed by the phorbol ester, 12-O-tetradecanoylphorbol-13-acetate (TPA). This tumor was progressive, continuing to grow, resulting in the death of the animal(s) weeks after the exposure was terminated. The malignancy of these tumors was demonstrated by conducting transplantation studies which proved that the tumor was capable of growing and producing death in previously normal hosts following implantation of abnormal cells from exposed planaria (Hall. 1986a). Electron microscopic studies of the tumor showed ultrastructural characteristics of a malignant cell line, including irregularly shaped nuclei, annulate lamelli and ribosome-lamella complexes (Hall, 1986b). In addition, with the use of electron microscopy, Hall *et al*, (1986b) traced the sequential stages in differentiation of the malignant planarian reticuloma. This cancer appears to have originated from the normal reticular cell line from a population of mitotically active cells, morphologically

indistinguishable from normal stem cells, i.e., neoblasts. Further studies, reported by Morita (1991a), determined that two stages, initiation and promotion are involved in this carcinogenic process.

The importance of the planarian in cancer research is of interest to researchers partly because toxicological and pharmacological responses of planarians to a variety of chemicals are similar to those in higher animals. In addition, the stem cell system, the neoblasts of planarians, closely resembles both the stem cell system of adult mammalian cells and the stem cell system found in earlier-stage mammalian embryos (Best, 1972, 1973). Thus, planaria appear to exhibit the responses to both mutagenic and/or carcinogenic agents similar to the responses of mammalian systems (Foster, 1969; Best and Morita. 1982; Best, 1983; Schaeffer, 1991a, 1991b). Because neoblasts are the only planarian cells capable of mitotic division, one can anticipate that they are the only cells capable of undergoing the promotional events required for transformation of mutated cells into malignancy, i.e., for phenotypic expression of sublethal DNA damage inflicted by the mutagenic agent (Best, 1983). The simplified view, common to malignant tumorigenisis in both mammals and planarians, probably involves some impediment to the differentiation of mitotically competent cells. Thus, one might anticipate that the primitive cell populations of "malignant" planarian tumors would be

transformed neoblasts, i.e., neoblastomas. Less severe disturbance of neoblast differentiation, in which the process occurs but with errors, could be expected to yield teratogenic manifestations (Best, 1983).

A wide variety of agents used in toxicity testing have resulted in a number of toxic responses in the planarian (Table 2-6). The most commonly noted planarian responses include visable lesions such as ulceration or head resorption, lethality, changes in fissioning incidence, behavioral changes, aberrant morphogenesis and tumorogenesis (Best, 1983). Table 2-6 is a summary of the effects oc different chemicals on tge planarian, *Dugesia dororotocephala*.

Table 2-6. Effects of chemicals on the planarian, *Dugesia dorotocephala*

Compound	Physologic effect	Reference
Actinomycin D	Depresses cephalic regeneration	Best, 1983
Chlordane	Low Conc-enhances fissioning High Conc-suppresses fissioning	Best, 1983
Dimethylbenzanthracine (DMBA)	Teratogenic effects	Best, 1983

Colchicine, colcemide, and puromycin also depress cephalic regeneration in the planarian. When the caudal regions of *D. dorotocephala* were exposed to colchicine, it produced polarity reversals (head growing out of the caudal end)

(Best, 1983). On the other hand, the same concentration of colcemide produced similar polarity reversals only when administered in conjunction with puromycin, a protein inhibitor (Best, 1983). Ethanol is lethal to surgically decapitated *D. dorotocephala* when exposed at a 1% concentration. Exposure to 0.5-0.7% ethanol causes retardation of cephalic regeneration. Ethanol is neurotoxic and suppresses locomotor activity and fissioning activity at a concentration of 0.3-0.5 % (Best, 1983). Caffeine at 50 to 100 ppm mildly stimulates the locomotor activity of *D. dorotocephala*, but at higher concentrations causes a suppression of locomotor activity and causes head lesions.

Polycyclic aromatic hydrocarbon compounds (eg., benzopyrene and DMBA), are carcinogenic in many species of rodents. Metabolic activation by microsomal oxidases is required to convert these compounds into reactive epoxide derivatives, which can produce a carcinogenic response. Planaria seem to be extremely sensitive to low concentrations of environmental toxicants and may be capable of metabolic activation of some polyaromatic hydrocarbons which are activated in mammalian systems by monooxygenases (Philips *et al*, 1974; Dean-Colomb *et al*, 1991).

At least two species of planaria of the genus *Dugesia (gonocephala* and *tigrina*) have been found to spontaneously develop tumors similar to a benign

tumor found in vertebrates (Lange, 1966). This is not the case with all of the planarian species as the incidence of spontaneous tumors in *D. dorotocephala* is negligible. In addition, this species of planaria has been shown to develop a high incidence of highly invasive tumors following exposure to a number of different polycyclic aromatic hydrocarbon which are also classified as mammalian carcinogens (Foster, 1963, 1969; Best and Morita, 1982). For example, treatment with the polycyclic aromatic hydrocarbons, 1,2-benzanthracene, 3,4-benzpyrene, benzo[a]pyrene and 3-methylcholanthrene or 3'-methyl-4-dimethylaminoazo-benzene have been shown to produce lethal, malignant tumors in planaria similar to those seen in mammalian systems.

Exposure to large doses of Cd followed by treatment with the phorbol ester promoter TPA in the asexual planarian, *D. dorotocephala*, resulted in a high incidence of malignant postpharyngeal tumors (malignant reticuloma) (Hall and Best, 1986). This work was the principal basis for the current study, as it became important to better understand the mechanism(s) underlying metal-induced carcinogenesis. In addition, the question of how the organism responds to metal exposure was not known and it was of interest to determine if planaria handle metals such as Cd in a manner similar to that of mammalian systems.

A comparative approach has been of enormous importance in understanding all aspects of human health. Sea urchins have long been used to illustrate developmental processes. The fruit fly *Drosophila* has served as a model organism from the heyday of classical genetics through the advent of molecular biological techniques, where understanding of regulation of genes involved in development and differentiation have provided many clues to understanding their counterparts including man (Aquadro, 1992). Unraveling the elegant and complicated regulation of the cell cycle owes much to the study of the surf clam (*Spisula solidissima*) cyclin proteins (Aristarkhow *et al,* 1996). More recently, information obtained from the ciliate *Tetrahymena* led to the discovery of telomerase and the solution of the chromosomal end-replication problem (Greider and Blackburn, 1985) which has had a tremendous impact on the study of aging and cancer. In addition, one important view of the molecular mechanism underlying apoptosis has come from the study of the development of the nematode *Caenorhabditis elegans* (Yuan and Horvitz, 1995, 1992). These are but a small sampling of the contribution those invertebrate models other than planaria have made to our understanding of human health problems.

Reactive Oxygen Species and Cancer

A free radical is simply defined as any molecule or atom capable of

independent existence that contains one or more unpaired electrons; an unpaired electron is defined as an electron that is alone in an orbital. Common free radicals that are found in biological systems include such molecules as the superoxide molecule ($O_2^{\cdot -}$) which is an oxygen centered radical, the thiyl ($RS^{\cdot}$) and nitric oxide ($N0^{\cdot}$) radicals which are molecules that have an unpaired electron that is delocalized between both atoms. In this system of nomenclature the dot designates the presence of an unpaired electron in an orbital. Most molecules and atoms found *in vivo* are nonradicals, containing only paired electrons in their orbitals.

Radicals can react with other molecules in a number of different ways (Halliwell and Gutteridge, 1989). For example, if two radicals meet, they can combine their unpaired electrons and form a covalent bond in which they share a pair of electrons.

$$A + A \rightarrow A\text{–}A$$

In addition, a radical can donate its unpaired electron to another molecule. The radical might scavenge an electron from another molecule in order to pair electrons in an orbital. A feature of free radical reactions with nonradicals is that they usually proceed as chain reactions, where one radical in order to stabilize itself it removes an electron from another atom only to produce a different free radical. Only when two radicals meet, as exemplified by the

above equation, do they disappear in a termination type reaction that stops a radical induced type of chain reaction.

The oxygen molecule as it occurs naturally qualifies as a diradical because it has two unpaired electrons each located in different π^* antibonding orbitals. These two electrons have the same spin quantum number meaning that they have parallel spins. This is the most stable state of oxygen and is referred to as the ground state of oxygen. If oxidation is defined as the loss of electrons, i.e., conversion of a sodium atom to the Na^+ ion, then the oxygen atom is a good oxidizing agent because it easily gives up electrons to other atoms. In contrast, reduction is defined as the gain of electrons by an atom or molecule i.e., the conversion of a chlorine atom to the Cl^- ion. An oxidizing agent, therefore, is good at absorbing electrons from a molecule it oxidizes (as does the chlorine atom) whereas a reducing agent (such as the oxygen atom) is an effective electron donor. If oxygen attempts to oxidize another atom or molecule by accepting a pair of electrons from it, both of these electrons must be of antiparallel spin so that they fit into the vacant spaces in the π^* orbitals. A pair of electrons in an atomic or molecular orbital cannot meet this requirement because they are paired with parallel spins. This imposes a restriction on electron transfer to the oxygen molecule, which only allows the oxygen molecule to accept one electron at a time, causing oxygen

molecules to react sluggishly with many non-radicals and prevents spontaneous combustion reactions from occurring.

If a single electron is added to the ground state oxygen molecule (O_2), it must enter one of the π^* antibonding orbitals. The resulting product of the addition of a single electron to an oxygen atom is the superoxide radical ($O_2^{\cdot -}$). With only one unpaired electron in its outermost orbital, the superoxide ion is actually less of radical than is ground state oxygen, which has two unpaired electrons in its π^* antibonding orbitals. Addition of one additional electron to the superoxide radical results in the formation of the peroxide ion (O_2^{2-}) which by definition is not a radical because the two antibonding orbitals are full. Since the extra electrons in the $O_2^{\cdot -}$ and O_2^{2-} are entering antibonding orbitals, the strength of the oxygen–oxygen bond is decreasing. In ground state oxygen, the atoms are effectively bonded by two covalent bonds, but in $O_2^{\cdot}$ the bonding strength is only the equivalent of one and a half bonds because there is an extra electron in an antibonding orbital. With the peroxyl molecule the bond is a single covalent bond which effectively reduces the overall bonding strength. Addition of another two electrons to the O_2^{2-} molecule eliminates the bond entirely since the electrons would go into the two σ^* orbitals, resulting in the production of two O^{2-} species. In biological

systems the two-electron reduction product of oxygen is hydrogen peroxide (H_2O_2), and the four electron reduction product is water.

1) One electron reduction

$$O_2 \rightarrow O_2^{\cdot -}$$

2) Two-electron reduction (plus 2 H^+)

$$O_2 \rightarrow H_2O_2 \text{ (Protonated form of } O_2^{2-}\text{)}$$

3) Four electron reduction (plus four H^+)

$$O_2 \rightarrow 2H_2O \text{ (Protonated form of } O^{2-}\text{)}$$

It is now well established that free radicals, especially superoxide and other reactive oxygen species like hydrogen peroxide are continuously produced *in vivo*. As a consequence, aerobic organisms have evolved not only antioxidant defense systems to protect against reactive oxygen species but also repair systems to protect against the accumulation of oxidatively damaged molecules (Sies, 1981; Fridovich, 1989). The term reactive oxygen species is a collective one that includes not only oxygen centered radicals such as the superoxide and hydroxyl radicals but also some nonradical derivatives of oxygen such as hydrogen peroxide, singlet oxygen and hypochlorous acid (HOCl).

Hydroxyl radicals are produced in living organisms by at least two

mechanisms. The first mechanism is the reaction of a transition metal with hydrogen peroxide in a Fenton-type reaction known as the Haber-Weiss Reaction. Hydroxyl radicals are also produced by the homolytic fusion of water caused by exposure to ionizing radiation (VonSonntag, 1987). Hydroxyl radicals are especially reactive in biological systems where they have the ability to abstract hydrogen ions from several different biological molecules. The hydrogen atom has a single electron in its outer most shell, and thus qualifies as a radical by definition. Hence removal of a hydrogen atom from a biological molecule leaves behind an unpaired electron on the atom to which the hydrogen was originally bonded. Thus hydrogen ion abstraction from biological macromolecules can initiate a type of chain reaction that can be extremely destructive (Halliwell and Gutteridge, 1984).

Oxidative stress occurs when reactive oxygen species are not adequately removed from a biological system. This can happen if antioxidants are depleted and/or if the formation of reactive oxygen species is increased beyond the body's ability to cope with them (Sies, 1991). There are a number of different ways that oxidative stress can be induced in biologic systems. Experimentally induced oxidative stress can be imposed *in vitro* by simply incubating cells with elevated oxygen concentrations (Gille *et al,* 1989). In addition, cells can be incubated with enzymes that generate reactive

oxygen species such as xanthine oxidase. In combination with one of its substrates, xanthine or hypoxanthine, it increases the levels of oxidative stress in cells (Schraufstatter *et al,* 1988; Weitberg, 1989; Paul *et al,* 1989; Philips *et al,* 1984; Iwata *et al,* 1984; Hall *et al,* 1988; Carson *et al,* 1986). When hydrogen peroxide or hydroperoxides are added directly to a cell system, oxidative stress increases (Schraufstatter *et al,* 1988; Jonas *et al,* 1989: Kleiman *et al,* 1990; Prise *et al,* 1989; Ochi, 1989; Nakayama *et al,* 1986). Direct exposure to compounds like paraquat or menadione which increase intracellular generation of superoxide radicals and/or hydrogen peroxide when metabolized by cells, is another way oxidative stress can be generated in biological systems (Eklow-Lastbom *et al,* 1986; Orrenius *et al,* 1989). Inflammation with activated phagocytes also results in an increased oxidative stress (Chong *et al,* 1989; Weiberg *et al,* 1989; Shacter *et al,* 1988).

Subjecting cells to oxidative stress can result in severe metabolic dysfunctions, including peroxidation of membrane lipids, depletion of nictinamide nucleotides, increases in intracellular free calcium ions, cytoskeletal disruption and DNA damage. The latter is often measured as the formation of single-strand breaks, double strand breaks or chromosomal aberrations.

Biological sources of superoxide radicals

Most oxygen taken up by higher aerobes is reduced to form water by the operation of the mitochondrial electron transport chain. Thus, the most important sources of superoxide radicals in aerobic cells are the electron transport chains of the mitochondria and the endoplasmic reticulum. The most important function of mammalian mitochondria is the oxidation of NADH and $FADH_2$ produced during the Kreb's cycle, β-oxidation of fatty acids and other metabolic pathways. An electron transport chain is located in the inner mitochondrial membrane, where a series of oxidation reactions occur which oxidize the NADH and $FADH_2$ molecules to produce energy and water by an enzyme complex known as NADH-coenzyme Q reductase (Beyer *et al,* 1987). Energy released by these oxidation reactions is used by the mitochondria to drive ATP synthesis. In this system, for every four electrons fed into the cytochrome oxidase complex a molecule of oxygen is reduced to two molecules of water. Cytochrome oxidase has apparently evolved in such a way that all the radical intermediates produced are bound tightly to the protein complex and leakage of these radicals from the protein complex have never been demonstrated *in vitro* or *in vivo.* Unlike cytochrome oxidase, some other components of the electron transport chain appear to leak a few electrons onto oxygen atoms while passing the great bulk of them on to the next

component in the chain. As would be expected with oxygen chemistry, this leakage produces a univalent reduction of oxygen to yield the superoxide radical. The main site of leakage seems to be the NADH-coenzyme Q reductase complex, and the reduced forms of coenzyme Q itself (Beyer *et al*, 1987).

Mitochondria isolated from mammalian tissue have been shown to produce hydrogen peroxide *in vitro* (Beyer *et al*, 1987; Halliwell, 1990). Most, if not all, arises as a result of dismutation of the superoxide radical by mitochondrial SOD activity. Mitochondria possess MnSOD in the matrix and might have a little CuZnSOD in the space between the inner and outer membranes (Halliwell, 1990). As would be expected, the rate of superoxide production, and hence hydrogen peroxide production by mitochondria increases with increasing oxygen concentrations.

The endoplasmic reticulum of mammalian and some plant cells contain cytochromes known collectively as cytochrome P-450 enzymes. Cytochrome P-450 enzymes are involved in the oxidation of a wide range of substrates at the expense of molecular oxygen. In these reactions, one atom of the oxygen complex combines with the substrate and the other reacts with hydrogen to form water. The functioning cytochrome P-450 complex requires the presence of a reducing agent (RH2), and the overall reaction catalyzed can be

represented by the following reaction in which AH represents the substrate:

$$AH + O_2 + RH_2 \rightarrow A\text{-}OH + R + H_2O.$$

Usually the product of a P-450 induced reaction is a less toxic product than the parent product but that is not always the case. In some situations, the P-450 mediated reaction rather than producing a less toxic product, creates a metabolic product that is more reactive than the parent compound (Halliwell and Gutteridge, 1989).

In the liver, NADPH donates the electrons required by the P-450 system via a flavoprotein enzyme, NADPH-cytochrome P-450 reductase. Adrenal cortical mitochondria contain a cytochrome P-450 which is involved in the hydroxylation of cholesterol to produce the adrenal steriod hormones but the electrons it requires are donated by a non-heme-iron protein known as adrenodoxin. Reduced forms of adrenodoxin and/or NADPH-cytochrome P-450 reductase can donate single electrons to molecular oxygen, reducing it to give the superoxide radical. In addition, there is some evidence that oxygenated intermediates of cytochrome P-450 can decompose in a minor side reaction and release the superoxide radical (Halliwell and Gutteridge, 1989). Indeed, isolated microsomal fractions from various mammalian tissues have been shown to rapidly produce hydrogen peroxide *in vitro* in the presence of NADPH, presumably via dismutation of the superoxide radical

(Halliwell and Gutteridge, 1989). The membrane surrounding the cell nucleus also contains an electron transport chain, of unknown function, that can leak electrons to give the superoxide radical, at a rate increasing with oxygen concentration, in the presence of NADH or NADPH. It resembles the microsomal electron transport system and may be of special importance *in vivo* because the oxygen radicals it generates are close to the cell's DNA (Halliwell and Gutteridge, 1989).

Sources and reactions of superoxide

Superoxide radicals are far less reactive than hydroxyl radicals, but a number of biological targets are sensitive to the superoxide radical. The superoxide radical can react with nitric oxide ($NO^{\cdot}$), produced by a several cell types (especially phagocytes and vascular endothelial cells), to produce peroxynitrite (Saran *et al*, 1989). Nitric oxide (or a derivative) acts on smooth muscle cells in vessel walls to produce relaxation. By opposing its action, therefore, the superoxide radical can act as a vasoconstrictor, which may have deleterious effects in some clinical situations (Laurindo *et al*, 1991). There is considerable debate in the literature as to whether the interaction of the superoxide radical and nitric oxide might be damaging to cells (Radi *et al*, 1990) or even if it can occur physiologically (Saran *et al*, 1989; Halliwell, 1989; Bekman *et al*, 1990; Omar *et al*, 1991). It has been suggested by some that

peroxynitrite decomposes to form the hydroxyl radical in a reaction that is independent of transition metal ions (Radi et al. 1990; Beckman *et al*, 1990).

In bacteria, superoxide radicals can attack enzymes containing an iron-sulfur cluster, such as *Escherichia coli* dihydroxyacid dehydratase, aconitase and 6-phosphogluconate dehydratase (Fridovich, 1989; Imlay and Fridovich, 1991; Gardner and Fridovich, 1991). In eukaryotic cells thus far few enzymes have proven to be targets of superoxide radical attack. Superoxide, *in vitro*, can inactivate the NADH dehydrogenase complex of the mitochondrial electron transport chain (Zhang *et al*, 1990), but this phenomenon has not yet been demonstrated *in vivo*. The protonated form of the superoxide radical (HO_2) is more reactive than the superoxide radical *in vitro* but has not yet been shown to mediate damaging effects *in vivo*.

Some of the superoxide radical production that occurs *in vivo* appear to be a chemical accident that can be due to autoxidation reactions or the "leakage" of electrons from the electron transport chain onto oxygen molecules (Fridovich, 1989; Imlay and Fridovich, 1991). Other superoxide radicals are produced more deliberately by phagocyte activation (Babior and Woodman, 1990) and to a lesser extent by other cell types such as lymphocytes and fibroblasts (Murrell *et al*, 1990; Malay, 1990; Meier *et al*, 1990; Jones *et al*, 1991). Removal of excess superoxide by superoxide

dismutase (SOD) from the cell is an important defense mechanism in aerobic organisms (Fridovich, 1989; Imlay and Fridovich, 1991; Touati, 1989; Chang *et al,* 1991; White *et al,* 1991; Liochev and Fridovich, 1991).

Hydrogen peroxide and hydroxyl radical formation

In addition to serving as a source of hydroxyl radicals, the hydrogen peroxide might conceivably act as a metabolic signal under certain circumstances, possibly by oxidizing specific protein thiol groups and thereby triggering specific intracellular events depending on the protein that is oxidized. For example, hydrogen peroxide can cause expression of the human immunodeficeincy virus gene in infected cells, possibly through the oxidation of the nuclear regulatory protein NFKappa-B (Schreck *et al,* 1991; Roeder *et al,* 1991; Legrand-Poels *et al,* 1990). Molecular hydrogen peroxide easily crosses cell membranes and can therefore attack cellular targets directly. Because of this, high levels of hydrogen peroxide can inactivate the glycolytic enzyme, glyceraldehyde-3-phosphate dehydrogenase in mammalian cells (Hyslop et al, 1988; Baker *et al,* 1991) and the plant enzymes fructose bisphosphatase (Charles and Halliwell, 1981) and aconitase (Verniquet *et al,* 1991).

Overall, the superoxide radical and hydrogen peroxide molecule have limited chemical reactivity in biological systems. For example, increased

superoxide and hydrogen peroxide generation in cells often leads to DNA damage, but neither of these species have been shown to react directly with DNA (Halliwell and Amoma, 1991). As a result, interest has focused on the ability of the superoxide radical and hydrogen peroxide molecule to generate the more reactive species such as the highly reactive hydroxyl radical *in vivo* in the presence certain catalysts.

Several transition metal salts can reduce hydrogen peroxide to generate the hydroxyl radical *in vivo* and *in vitro*. The most important feature of transition metals from a radical point of view is their variable valency, which allows them to undergo changes in oxidation states involving the transfer of electrons. Transition metals are found in the d-block of the periodic table and all contain unpaired electrons in their outer orbitals, thus by definition they qualify as radicals, with the sole exception of the zinc atom. Copper, another metal found with the transition metals along with zinc, fits the definition of a transition metal as its 3d orbitals are full but the metal readily forms the Cu^{2+} ion. This is achieved through the loss of two electrons, one from its 4s- and one from its 3d-orbital. Zinc by contrast, has only one valence state (Zn^{+2}) and it does not promote radical reactions. In fact, It has been suggested that Zn ion may inhibit radical reactions *in vivo* by displacing other transition metals such as iron from the binding sites from which they promote oxidation

reactions.

In contrast to the reactions with some transition metals, the reactions of iron and copper salts with hydrogen peroxide are not simple. Evidence was slow in coming to prove that hydroxyl radicals were formed when Cu ions reacted *in vivo* with hydrogen peroxide (Jefcoate and Norman, 1968). This production of hydroxyl radicals was confirmed *in vivo* by examining the pattern of damage to purine and pyrimidine DNA bases following exposure to copper ions and hydrogen peroxide, and demonstrating that the pattern of damage was characteristic of hydroxyl radical attack (Aruoma *et al*, 1991).

Several transition metal salts react with hydrogen peroxide to form the hydroxyl radical. Although most transition metals will catalyze the reaction, the most attention has been paid to iron ions (Halliwell and Aruoma, 1991; Halliwell and Gutteridge, 1990b), although interest in copper has increased (Sutton and Winterbourn, 1989; Aruoma et al, 1991). The reaction of interest occurs when ferrous salts react with hydrogen peroxide to form hydroxyl radicals by the so-called Fenton reaction, which is usually written as:

$$Fe(II) + H_2O_2 \rightarrow OH^{\cdot} + OH^{-} + Fe(III)$$

Despite the simplicity of the above reaction, Fenton chemistry is far more complex than this simplistic formula implies (Koppenol, 1985; Aust *et al*, 1985; Sutton and Winterbourn, 1989; Croft *et al*, 1992). The initial product of

the above formula may be an oxo-iron complex, possibly the ferryl complex, which then decomposes to form the hydroxyl radical (Halliwell and Gutteridge, 1990b).

$$Fe\ (II) + H_2O_2 \rightarrow FeOH_3^{+}\ (\text{or } FeO_2^{+}) \rightarrow OH^{-} \rightarrow OH^{\cdot} + Fe(III)$$

Different ligands bound to the Fe(II) ion may stabilize the intermediate so that little hydroxyl radical is formed, whereas other bound ligands tend to destabilize the intermediate product in the above reaction. Although it is convenient to assume that only a single product is formed in the Fenton reaction, in reality there are several different reactive species that are probably generated when iron salts are combined with hydrogen peroxide *in vivo*. This helps to explain the periodic controversies that arise regarding the production of hydroxyl radicals by the Fenton reaction (Koppenol, 1985; Aust *et al*, 1985; Sutton and Winterbourn, 1989; Bielski, 1991; Croft *et al*, 1992). In addition to the hydroxyl radical, other reactive species are also generated which may damage biologic macromolecules (Bielski, 1991; Gutteridge et al, 1990).

The above Fenton chemistry reactions result in the generation of the Fe (III) ion. Ferric complexes react slowly, when compared to the ferrous complex reactions with the hydrogen peroxide molecule because reducing agents stimulate Fenton reactions. For example, the ascorbate molecule can

stimulate conversion of the conversion of the ferric ion to the ferrous ion by the following reaction:

$$\text{Fe (III)} + \text{ascorbate} \rightarrow \text{Fe (II)} + \text{semihydroascorbate}$$

which results in increased hydroxyl radical production when this reaction takes place in the presence of the hydrogen peroxide molecule (Buettner *et al*, 1987). It is assumed that other reducing agents *in vitro* promote the production of reactive oxygen species in a similar manner.

Superoxide can reduce certain ferric chelates. For example, the reaction of the Fe (III) ion with superoxide appears to proceed via a perferryl intermediate (Halliwell and Gutteridge, 1990b).

$$\text{Fe (III)} + O_2^{\cdot} \leftrightarrow [Fe^{3+} + O_2^{\cdot-} \leftrightarrow Fe^{2+}—O_2] \leftrightarrow Fe^{2+} + O_2$$

With the sum of the reactions ignoring the oxo-iron intermediate being:

$$O_2 + H_2O_2 \xrightarrow{Fe} O_2^{\cdot} + OH^- + O_2$$

This reaction is sometimes called the iron catalyzed Haber-Weiss reaction or perhaps more appropriately a superoxide-driven Fenton reaction. This reaction can account for at least part of the damage caused to living cells by excess generation of reactive oxygen species (Halliwell and Aruoma, 1991; Halliwell and Gutteridge, 1990; Kyle *et al*, 1988; Sakaida *et al*, 1991; Imlay and Linn, 1988; Halliwell, 1987; Orrenius *et al*, 1989; Gutteridge *et al*, 1979). Orrenius et al. (1989) suggest that, just as oxidative stress causes a rise in

intracellular concentrations of free calcium ions by interfering with the normal calcium-sequestering mechanisms, oxidative stress may also increase iron ion concentrations available within cells to catalyze free radical reactions. It should also be noted that the Haber-Weiss reaction can be inhibited by iron chelating agents such as deferoxamine (Gutteridge, 1991)

Biological antioxidants

Aerobes have evolved antioxidant defenses to protect themselves against the reactive oxygen species generated *in vivo*. These defenses include enzymes i.e., superoxide dismutase, catalase and glutathione peroxidase. In addition, low molecular weight agents such as α-tocopherol and ascorbic acid are protective as are some intracellular molecules like glutathione and metallothionein, which bind metal ions in forms that do not accelerate free radical reactions (Halliwell and Gutteridge, 1990a; Frei *et al*, 1989).

In 1968, McCord and Fridovich isolated a protein from erythrocytes that effectively removed superoxide radicals, which they identified as a superoxide dismutase (SOD) enzyme. Subsequent studies have shown that CuZn superoxide dismutases (CuZnSOD) are found in virtually all eukaryotic cells i.e., yeast, plants and animals. In most cases the CuZnSOD enzyme is not found in prokaryotic cells (Halliwell and Gutteridge, 1989). All CuZn SOD

enzymes isolated from eukaryotic cells have an approximate relative molecular weight of 32,000 daltons and contain two protein subunits, each of which bear an active site containing one copper and one zinc ion (Halliwell and Gutteridge, 1989). CuZnSODs catalyze a dismutation reaction which converts superoxide radicals to hydrogen peroxide and oxygen.

$$O_2^{\cdot -} + O_2^{\cdot -} \rightarrow H_2O_2 + O_2 \text{ (Ground-state)}.$$

The uncatalyzed dismutation reaction is highly pH dependent and at physiological pH it has a rate constant of $5X\ 10^5\ M^{-1}s^{-1}$. The presence of bovine erythrocyte CuZnSOD makes the reaction much more efficient because it is relatively independent of pH in the range of 5.3 to 9.5. When bovine CuZnSOD is present the rate constant for the dismutation reaction is increased to about $1.6 \times 10^9\ M^{-1}s^{-1}$ (Halliwell and Gutteridge, 1989).

The copper ions in CuZnSOD appear to function in the dismutation reaction (Corey *et al*, 1987; Osman and Basch, 1984) by undergoing alternate oxidation and reduction: i.e.,

$$\text{Enzyme-}Cu^{2+} + O_2^{\cdot -} \rightarrow \text{Enzyme} - Cu^{+} + O_2$$

$$\text{Enzyme-}Cu^{+} + O_2^{\cdot -} + 2H^{+} \rightarrow \text{Enzyme-}Cu^{2+} + H_2O_2$$

This results in the net reaction:

$$O_2^{\cdot -} + O_2^{\cdot -} \rightarrow H_2O_2 + O_2 \text{ (Ground-state)}.$$

However, at least one other mechanism of action is also feasible, in which the

first superoxide radical does not reduce the copper ion but forms a complex with it (Corey *et al*, 1987; Osman and Bash, 1984). Cyanide and diethyldithiolcarbamate are potent inhibitors of superoxide dismutase because they bind the copper ion at the active site (Hassan *et al*, 1980).

The Zn^{2+} ion does not function in the catalytic cycle but appears to act as a stabilizer for the enzyme. This conclusion was drawn from research projects that removed the respective metal ions from the CuZnSOD and replaced them with different metal ions. Manganese substitution for the copper ion on the SOD molecule failed to yield a functional enzyme. In general, ions of other transitional metals like cobalt, mercury and Cd ions were able to replace the Zn^{2+} ion and maintain enzyme stability (Halliwell and Gutteridge, 1989; Clare *et al*, 1984). Substitution of cobalt for the copper ion yielded a functional enzyme although the rate constant is much lower than that of the original enzyme (Oberly, 1982). Replacement of the copper ion with other transitional metals failed to yield a functional enzyme (Halliwell and Gutteridge, 1989; Clare *et al*, 1984).

Bacteria also contain a SOD but unlike the mammalian CuZnSOD, it is not inhibited by either cyanide or diethyldithiolcarbamate. It has a molecular mass of approximately 40,000 daltons and contains manganese at its active site rather than Cu. Both MnSODs and CuZnSOD's catalyze the same

dismutation reactions (Halliwell and Gutteridge, 1989). In addition, a different SOD is also found in some bacteria and fungi, which contains an iron ion rather than the Mn or Cu ions described previously. This enzyme catalyzes the same dismutation reaction as the other SODs discussed earlier (Parker and Blake, 1988).

Subcellular fractionation studies in rat liver have shown that most CuZnSOD is found primarily in the cellular cytosol with some activity noted in the lysosomes (Ekstrom *et al*, 1981). Additional activity was also present between the outer and inner mitochondrial membranes (Ekstrom *et al*, 1981). Minor activity was also noted in the nucleus (Ekstrom *et al*, 1981). MnSOD activity is found only in the mitochondrial matrix (Halliwell and Gutteridge, 1989).

Two different enzyme systems have been shown to remove hydrogen peroxide from cells. One enzyme is catalase, which catalyzes the reaction that combines two hydrogen peroxide molecules to yield two molecules of water and one molecule of ground state oxygen. The other enzyme are the peroxidases which bring about the general reaction:

$$SH_2 + H_2O_2 \rightarrow S + 2H_2O$$

in which the SH_2 acts as a substrate that becomes oxidized (Halliwell and Gutteridge, 1989).

Most aerobic cells have catalase activity, although there are a few that do not. Most anaerobic bacteria do not exhibit catalase activity, although there are a few exceptions. In animals, catalase has been demonstrated in all of the major body organs, with especially high activity levels in hepatocytes and erythrocytes. The brain, heart, and skeletal muscie contain only small amounts of catalase, however, the activity varies vary between different muscle groups and even between different regions of the same muscle (Halliwell and Gutteridge, 1989b).

The catalase enzyme structure is made up by four protein subunits, each of which contain a heme (Fe (III)-protoporphyin) group bound to its active site. Each subunit also contains one molecule of NADPH bound to it, which appears to help stabilize the enzyme. Dissociation of catalase into its various subunits causes a loss of enzymatic activity (Halliwell and Gutteridge, 1989b). The catalase molecule is easily dissociated if the cellular environmental conditions change (Halliwell and Gutteridge, 1989).

The mechanism of action of the catalase enzyme appears to be a two-stage process during which an intermediate compound is formed. The exact nature of the intermediate compound (compound I) is uncertain, although it is theorized that iron is oxidized to a nominal valency of Fe (V). Extensive charge-delocalization occurring in the heme rings makes description of the

exact structure difficult (Halliwell and Gutteridge, 1989). It is now known that the catalase reaction requires the imput of two molecules of hydrogen peroxide at a single active site, which become less likely as hydrogen peroxide concentrations decrease (Turens *et al,* 1984). Hence for a given concentration of catalase, the initial rate of hydrogen peroxide removal becomes proportional to the hydrogen peroxide concentration (Kirkman *et al,* 1987).

Some nonspecific enzyme inhibitors, such as cyanide or azide can inhibit catalase activity (Morris and Albright, 1984). A more specific catalase inhibitor used in research is aminotriazole, which specifically inhibits catalase activity. Its inhibitory mechanism of action is exerted on the intermediate compound (Compound I), so it can only inhibit catalase if hydrogen peroxide is present to allow the intermediate's generation.

The catalase activity of both animal and plant cells is primarily located in the peroxisomes. Peroxisomes also contain some of the cellular hydrogen peroxide generating enzymes such as glycollate oxidase, urate oxidase and flavoprotein dehydrogenases that cause β-oxidation of fatty acids, part of a metabolic pathway found in both mammalian mitochondria and peroxisomes.

Hepatic mitochondria, chloroplasts and the endoplasmic reticulum do not contain significant catalase activity, so any hydrogen peroxide these cells

generate *in vivo* must be disposed of in a different way. G.C. Mills first discovered glutathione peroxidase in animal tissues in 1957. Glutathione peroxidase is not as a general rule found in bacteria and higher plants. This enzyme uses a low molecular weight thiol called glutathione as a substrate. Glutathione is found in mammalian and plant cells as well as some bacteria, often in millimolar concentrations (Kosower and kosower, 1978). Most free glutathione found *in vivo* is the reduced (GSH) form rather than the oxidized (GSSG) form. Interestingly, it has been shown that approximately one-third of the total cellular glutathione may be present as mixed disulfides which are compounds in which the sulfhydryl group on the glutathione molecule combines with other compounds that contain sulfhydryl groups, such as cysteine, coenzyme A or proteins (Brigelius *et al*, 1983; Keeping and Lyttle, 1984). As an enzyme, glutathione peroxidase catalyzes the oxidation of reduced glutathione (GSH) to oxidized glutathione (GSSG) at the expense of hydrogen peroxide:

$$H_2O_2 + 2GSH \rightarrow GSSG + 2H_2O$$

(Harman *et al*, 1986). The enzyme is found to be present at high levels in liver, moderate levels in heart, lung and brain and in low levels in muscle (Sies *et al*, 1978). The enzyme specifically uses the reduced glutathione molecule as a hydrogen donor and is capable of oxidizing other peroxides, in addition to

hydrogen peroxide. Glutathione peroxidase consists of four protein subunits, each of which contains one atom of selenium at its active site. Selenium is a group VI element in the periodic table and has properties intermediate between those of a metal and a non-metal. It is probably present at the active site as selenocysteine complex, the amino acid cysteine in which the normal sulfur atom has been replaced by a selenium ion (Igwe, 1986). The reduced glutathione molecule apparently reduces the selenium ion and the reduced form of the enzyme then reacts with hydrogen peroxide (Diplock, 1981).

Reduced glutathione/oxidized glutathione ratios (GSH/GSSG) in normal cells are kept high by the enzyme glutathione reductase which catalyzes the reaction:

$$GSSG + NADPH + H^{+} \rightarrow 2GSH + NADP^{+}$$

(Reed, 1986). Glutathione reductase also catalyzes the reduction of certain mixed disulfides, such as that which occurs between the glutathione molecule and coenzyme A. The oxidative pentose phosphate pathway, provides the NADPH that glutathione reductase requires to reduce the oxidized glutathione. The first enzymatic reaction in this pathway is the conversion of glucose 6-phosate dehydrogenase in the presence of $NADP^{+}$ to produce 6-phosphogluconate (Liochev and Fridovich, 1985).

The rate limiting step in the pentose phosphate pathway is the

conversion of glucose 6-phosphate dehydrogenase to 6-phosphogluconate and this reaction is controlled by the supply of $NADP^+$ (Reed, 1986). As glutathione reductase operates and lowers the NADPH/$NADP^+$ ratio, the pentose phosphate pathway speeds up to replace depleted NADPH (Meister, 1983). The glutathione reductase molecule contains two protein subunits, each with the flavin FAD at its active site. It appears that the NADPH reduces the FAD, which then passes its electrons on to a disulfide bridge between two cysteine residues in the protein (Winterbourn and Sutton, 1986). The two sulfhydryl groups so formed then interact with oxidized glutathione and reduce it to two glutathione (GSH) molecules, reforming the oxidized protein disulfide. Hepatocytes contain large concentrations of both catalase and glutathione peroxidase in different cellular substructures. Catalase is usually found in the peroxisomes. In comparison, large amounts of glutathione peroxidase are found in the cytosolic fraction with smaller amounts found in the mitochondrial matrix. The cellular distribution of glutathione is similar. In addition, tissue glutathione content also varies with tissue type as well as time of the day, and also time of the day. Thus, hydrogen peroxide produced by peroxosomal enzymes is disposed of by catalase, where as hydrogen peroxide produced in mitochondria, the endoplasmic reticulum or by cytosolic enzymes such as SOD is reduced by glutathione peroxidase (Wefers and Sies, 1983).

In addition to using enzymes and proteins to combat oxidative stress, biological systems also rely on several putative physiologic antioxidants to scavenge radicals to block oxidation of biological molecules. Antioxidants such as vitamin E (α-tocopherol) act by transferring a hydrogen atom along with its single electron to the lipid molecule, thus removing the peroxyl free radical faster than these radicals can react with membrane proteins or with acidic side chains on the membrane lipids.

$$\text{Lipid—O}_2 + \text{tocopherol—OH} \rightarrow \text{Lipid—O}_2\text{H} + \text{tocopherol—O}$$

However, when the tocopherol molecule loses the hydrogen ion, it leaves an unpaired electron on the oxygen atom and thus becomes a radical, fulfilling the rule that the only way to get rid of a radical is to combine two radicals. Despite the fact that the tocopherol molecule forms a radical, this product is relatively unreactive and can be removed by reacting with ascorbic acid, thereby regenerating the tocopherol molecule (Burton and Ingold, 1989; Tappel, 1968). It should be noted that this reaction has not been rigorously demonstrated *in vivo* (Burton *et al*, 1990). Another possible fate of tocopherol radicals in mitochondria is to react with ubiquinol (Kagan et al. 1990).

$$\text{tocopherol—O}^{\cdot} + \text{CoQH}_2 \rightarrow \text{tocopherol—OH} + \text{CoQH}^{\cdot}$$

In either case new radicals are generated. It should be noted that the ubiquinol radical can be reduced by the electron transport chain in

mitochondria (Kagan et al. 1990).

Pure ascorbic acid is a water-soluble vitamin that most animals and plants synthesize from glucose. Some species including man, primates, guinea pigs and fruit bats are unable to synthesize the compound because of the loss of one of the necessary enzymes during their evolution. Ascorbic acid has many antioxidant properties (Bendich *et al,* 1986; Frei *et al,* 1989; Halliwell 1990; Coassin *et al,* 1991). It is often considered to be one of the most important antioxidants in the body because it disappears faster than other antioxidants when plasma is exposed to reactive oxygen species. The ability of vitamin C to function as an antioxidant is related to its ability to quickly react with many reactive oxygen species, such as peroxyl radicals, with the production of the semidehydroxyascorbate radical (Bielski, 1991). Thus the ascorbate molecule acts as a reducing agent or electron donor. Donation of one electron by the ascorbate molecule produces the semidehydroascorbate radical, which can then be further oxidized to give dehydroascorbate (Bielski, 1991). The semidehydroascorbate radical is not particularly reactive and mainly undergoes a disproportionation reaction to produce ascorbate and dehydroascorbate. The dehydroascorbate is unstable and breaks down rapidly in a very complex way, eventually producing oxalic and L-threonic acids. The ascorbate molecule also scavenges singlet oxygen,

reduces thiyl radicals, and combines quickly with hypochlorous acid, a powerful oxidant generated at sites of inflammation (Bielski, 1991). Ascorbic acid concentrations can be quite high and in some cells such as lung cells may reach the millimolar range (Bielski, 1991).

In addition to being an antioxidant, ascorbate also shows prooxidant properties (Halliwell, 1990). Mixtures of copper or iron salts with ascorbic acid have been used to stimulate lipid peroxidation and the formation of hydroxyl radicals from hydrogen peroxide (Walling, 1982). Indeed, all reported prooxidant properties of ascorbate probably involve its interaction with transition metal ions (Halliwell, 1990). For example, ascorbate has been reported as being mutagenic to cells in culture, but metal ions added to or possibly contaminating the culture could easily account for this (Halliwell, 1990). The reported ability of ascorbate to degrade DNA and damage to various animal cells in culture, including cancer cells, can probably be attributed to the formation of the ascorbate radicals in the presence of trace of copper ions in the solution. Ascorbic acid $-Cu^{2+}$ mixtures also inactivate proteins, probably through the formation of hydroxyl radicals and or the Cu (III) species.

Glutathione is a tripeptide (γGlu-Cys-Gly) that can serve as a scavenger of the hydroxyl radical, a substrate for glutathione peroxidase to

remove hydrogen peroxide from the cell and a substrate for dehydroascorbate reductase. Since it is found in high concentration in many cells, it may help to protect them against free radicals. Glutathione also has been shown to reactivate some enzymes that have been inhibited by exposure to high oxygen concentrations. It has been suggested that exposure of the protein to an oxygen atom results in the oxidation of essential sulfhydryl groups on the enzyme, which are then regenerated by incubation with glutathione (Halliwell, 1990). The reaction of reduced glutathione with the hydroxyl radical produces thiyl radicals which can also be formed when glutathione reacts with peroxidases and/or with oxygen in the presence of transition metal ions such as Fe^{2+} or Cu^{2+}. It should be noted that the thiyl radical is less reactive than hydroxyl radicals and less likely to cause problems *in vivo*.

Glutathione serves as a cofactor for several enzymes in widely different metabolic pathways, such as glyoxylase, protaglandin endoperoxide isomerase and DDT dehydrochlorinase and it may be involved in the synthesis of thyroid hormones (Halliwell, 1990). It also plays a role in the degradation of insulin in mammalian tissues and functions in the metabolism and excretion of herbicides, pesticides and "foreign" compounds. The first stage in this metabolic process is the conjugation of the compound with glutathione by glutathione-S-transferase enzymes. As a general rule,

glutathione conjugates are excreted into bile, using the same transport mechanism that ejects oxidized glutathione when the liver is subjected to oxidative stress (Halliwell, 1990). If a tissue is exposed to a large flux of hydrogen peroxide and or the hydroxyl radical, a point might be reached at which the GSH/GSSG ratios cannot be maintained at normally high levels when oxidized glutathione accumulates faster than it can be reduced. Because oxidized glutathione can inactivate a number of enzymes (probably by forming mixed disulfides and inhibiting protein synthesis), excess oxidized glutathione is exported into the intracellular environment (Halliwell, 1990). This is an attempt by the cell to maintain cellular protection against oxidative stress.

Glutathione is important as a substrate for glutathione peroxidases, transferases and several other enzymes and as a general radical quencher in cells (Chance *et al.* 1979; Eaton, 1991; Maiorino *et al,* 1991). In addition, it also appears to participate in the recycling of tocopherol radicals *in vivo* as was discussed earlier (Sies and Murphy, 1991). In both cases, it appears to function by donating its hydrogen atom. However, loss of hydrogen from thiyl groups produces thiyl radicals or $GS^{\cdot}$ in the case of glutathione. Thiyl radicals have been reported to be capable of reacting with oxygen to give potentially damaging oxysulfur radicals although the exact chemistry of these reactions is

still unclear (Asmus, 1990; Sevilla *et al,* 1989).

Oxidative damage in biological systems

Oxidative stress is capable of causing major derangements in cell metabolism, including DNA strand breakage (Halliwell and Aruoma, 1991), rises in intracellular "free" calcium, damage to membrane ion transporters and other specific proteins and lipid peroxidation (Kyle *et al,* 1988; Sakaida *et al,* 1991; Imlay and Linn, 1988; Orrenius *et al,* 1989). The resulting cellular damage can be direct, (i.e., hydrogen peroxide oxidizes protein thiols or when hydroxyl radicals are produced in close association with DNA molecules), indirect or both. An excessive rise in intracellular calcium ions can activate proteases which attack can attack the cytoskeleton causing loss of cellular structure and death and/or nuclear damage with fragmentation of DNA molecules (Martin and Dean, 1991). In addition, some cell-surface receptors can respond to oxidative stress in ways that perturb cell metabolism (Halliwell and Gutteridge, 1989). Thus, a fine balance is required at sites of acute controlled inflammation between the regulatory and damaging properties of reactive oxygen species, depending on the extent and type of reactive oxygen species generated, the cellular target, the availability of antioxidant defenses and the presence or absence of transition metal ions.

Lipid peroxidation has been broadly defined as the oxidative

deterioration of polyunsaturated lipids, which are fatty acids that contain more than two carbon-carbon double bonds (Halliwell and Gutteridge, 1989). In a completely peroxide free lipid system, first-chain initiation of the peroxidation sequence in a membrane or polyunsaturated fatty acid refers to the attack of a species that has sufficient reactivity to abstract a single hydrogen atom from a methylene group (Halliwell and Gutteridge, 1989). Radiolysis of aqueous solutions, which produces the hydroxyl radical, stimulates the peroxidation of any nearby lipids (Halliwell and Gutteridge, 1989). The immediate presence of hydroxyl radical scavengers such as mannitol or formate inhibits this type of peroxidation reaction (Halliwell and Gutteridge, 1989). In contrast, the superoxide radical is not able to abstract hydrogen molecules from lipids (Halliwell and Gutteridge, 1989). In addition, the radical would not be expected to enter the hydrophobic interior of membranes because of its charged nature. In agreement with this, the superoxide radical does not readily cross biological membranes, the only exception to this rule being the erythrocyte membrane where it can travel via the anion channels, which normally carry chloride and bicarbonate ions (Halliwell and Gutteridge, 1989).

Protonated forms of the superoxide and peroxyl radicals are more reactive and appear to be capable of abstracting hydrogen atoms from some fatty acids. However, to date, the peroxyl radical has not been proven to

initiate peroxidation in the cell membrane, although it has been shown to occur *in vitro* with isolated low-density-lipoprotein fractions (Gebicki and Bielski, 1981). In addition, various iron-oxygen complexes may also be capable of abstracting hydrogen atoms and initiating peroxidation (Burcher *et al*, 1983; Aruoma *et al*, 1989; Minotti and Aust, 1986).

Because the hydrogen atom only has one electron, abstraction of hydrogen from a methylene group leaves behind an unpaired electron on the carbon. The presence of a carbon-carbon double bond in the fatty acid weakens the carbon-hydrogen bonds on the carbon atom adjacent to the double bond, which makes removal of the hydrogen atom easier. The resulting carbon radical tends to be stabilized by a molecular rearrangement that forms a conjugated diene radical (Halliwell and Gutteridge, 1989). This molecular instability can then lead to a number of different possible reactions. For example, if two conjugated dienes were to come into contact in a membrane, they could cross-link their respective fatty acid molecules. However, the most likely fate of conjugated diene radicals under aerobic conditions is to combine with an oxygen molecule, especially because oxygen is hydrophobic and tends to concentrate within the membrane interior. This reaction with oxygen results in the production of a lipid peroxyl radical. If oxygen concentrations are low in the biological system then carbon centered

radicals or conjugated proteins are more likely to be found, but as oxygen concentrations increase the peroxyl radicals tend become the dominant product. Peroxyl radical formation has been demonstrated in rat liver microsomes using spin-trapping methods (Goddard and Sweeney, 1986). The formation of the peroxyl radical is important because they are capable of abstracting hydrogen atoms from adjacent fatty-acid-side chains. This is what is referred to in the literature as the "propagation stage of lipid peroxidation". Carbon radicals so formed can then react another oxygen molecule to form another peroxyl radical and thus the chain reaction of lipid peroxidation continues. The peroxyl radical can also combine with the hydrogen atom that it abstracts to produce a lipid hydroperoxide, which is often called in the literature a "lipid peroxide". A probable alternative fate of the peroxyl radicals is the formation of cyclic peroxides. Cyclic peroxide formation can cause fragmentation of the unsaturated fatty acids to yield aldehydes which includes such products as malondialdehyde and polymerization products (Halliwell and Gutteridge, 1989).

The exact role of transition metal ions in the acceleration of lipid peroxidation is at present an area of great confusion. Catalase and/or hydroxyl scavengers do not inhibit iron-stimulated lipid peroxidation in most membrane systems (Aruoma *et al,* 1989; Braughler *et al,* 1986), even though

hydroxyl radicals are formed in these systems. This evidence strongly suggests that hydroxyl radical formation is not required for lipid peroxidation to occur. Some researchers have interpreted this to mean that the required hydroxyl formation must be a site-specific reaction, involving only iron ions bound to the membrane and not amenable to interference by hydroxyl radical scavengers (Aruoma *et al,* 1989; Braughler *et al,* 1986). However, a source of hydrogen peroxide would still be required and catalase should still inhibit or at least decrease the rate of lipid peroxidation. The fact that it does not has led researchers to suspect that first chain initiation of lipid peroxidation in membrane systems incubated with iron salts in the presence of oxygen is achieved by reactive species other than the hydroxyl radical (Aruoma *et al,* 1989; Braughler *et al,* 1986). However, it is known that a reduced iron complex can react with the lipid peroxide molecule to form an alkoxyl radical similar to way it reacts with the hydrogen peroxide molecule (Aruoma *et al,* 1989).

The reaction of transition metal ions with lipid peroxides generates a number of different products including epoxides, compounds containing carbonyl groups and hydrocarbon gases (Corongiu *et al,* 1986). As stated earlier, many of the carbonyl compounds produced by the decomposition of lipid peroxides are aldehydes. Malondialdehyde is formed in most tissues

during the peroxidation of cell membranes. In most membranes, the propagation reactions of lipid peroxidation do not proceed very far before they meet a protein molecule, which can then be attacked and damaged (Richter, 1987). Protein molecules are commonly attacked by alkoxy radicals, peroxyl radicals and to a lesser extent by carbon centered radicals (Richter, 1987). In addition, dialdehydes such as malondialdehyde also form DNA adducts in addition to being capable of attacking amino groups on protein molecules to form both intramolecular cross-links and also cross-links between different protein molecules (Gutteridge and Quinlan, 1983). Enzymes that require $-NH_2$ or –SH groups for their activity are usually inhibited during lipid peroxidation (Mak *et al,* 1983). Hydroxy-trans-nonenal and other low-molecular weight products of lipid peroxidation have also been shown to inhibit protein synthesis, form DNA adducts and interfere with growth of bacterial and mammalian cells in culture (Esterbauer *et al,* 1986; Hauptlorenz *et al,* 1985). Other products produced during peroxide decomposition include unsaturated aldehydes including hexanal and nonenal, saturated aldehydes like propanal, butanal and hexanal and a wide range of ketones like butanone, pentanone and octanone (Richter, 1987). In mammalian systems some of the aldehyde products may exert chemotactic actions upon neutrophils and several of the aldehydes have been shown to be mutagenic (Richter, 1987).

These complex reactions of radicals, aldehydes and other products of lipid peroxidation can also cause severe damage to membrane proteins. Peroxidation of liver or erythrocyte membranes has been shown to cause the formation of high-molecular mass protein aggregates within the membrane. Surface receptor molecules that allow cells to respond to hormones can be inactivated as a result of lipid peroxidation, as are enzymes such as glucose-6-phosphatase and the Na^+ K^+ATPases which are involved in maintaining the correct ion balance in cells (Richter, 1987). In general, the overall effects of lipid peroxidation are a decrease in membrane fluidity, increased permeability of the membrane to substances that do not normally cross it, like the calcium ion and to inactivate membrane-bound enzymes. Continued fragmentation of fatty-acid side chains will produce aldehydes and hydrocarbons such as pentane and eventually result in the complete loss of membrane integrity (Richter, 1987). Rupture of organelles such as lysosomes due to loss of membrane integrity can spill hydrolytic enzymes into the rest of the cell, which tends amplify the damage (Richter, 1987). Lipid peroxidation of erythrocyte membranes causes them to lose their ability to change shapes and squeeze through the smallest of capillaries and will eventually lead to hemolysis (Richter, 1987). In some bacteria where DNA is close to or attached to the cell membrane, DNA damage can result as a consequence of lipid

peroxidation (Richter, 1987).

A number of different DNA lesions can be produced by reactive oxygen species such as the hydroxyl radical. Reaction with deoxyribose results in fragmentation of the molecule with loss of the base and appearance of a strand break (Imlay and Linn, 1988; Teebor *et al*, 1988). Alternatively, reaction with thymine produces a variety of lesions removed by repair enzymes that similarly produce single strand breaks in DNA (Imlay and Linn, 1988; Teebor *et al*, 1988). Thus, the appearance of DNA single strand breaks is a common consequence of interaction of DNA with reactive oxygen species. Single-strand breaks have been reported to accumulate in both mammalian and bacterial cells following exposure to a number of different oxidative scenarios (Imlay and Linn, 1988; Teebor *et al*, 1988; Cantono *et al*, 1987; Hoffman *et al*, 1984; Imlay *et al*, 1988; Leandon *et al*, 1988; Schraufstatter *et al*, 1986).

In turn, oxidative DNA damage has been implicated as a cause of death in both bacterial and mammalian cells by reactive oxygen radicals. Mammalian cell lines exposed to hydrogen peroxide have been shown to exhibit a dose-dependent accumulation of DNA single-strand breaks and cell death (Hoffman *et al*, 1984). Superoxide dismutase decreased the number of thymine glycols produced by benzo(a)pyrene exposure in epithelial cells and

also increased cell survival rates following exposure to the chemical (Leandon *et al*, 1988). The EM9 Chinese hamster ovary cell line has been shown to be very sensitive to hydrogen peroxide toxicity. This sensitivity correlates well with a decreased ability of these cells to repair lesions in DNA and makes them extremely important tools to study oxidative stress *in vitro* (Cantono *et al*, 1987). Finally inhibition of poly (ADP-ribose) polymerase, an enzyme that is activated by DNA strand breaks, protected P388D1 cells from the toxicity of hydrogen peroxide (Schraufstatter *et al*, 1986). This result was interpreted as indicating that the metabolic responses to DNA damage may initiate a sequence of events that result in cell killing (Schraufstatter *et al*, 1986).

In addition to unsaturated fatty acids and DNA molecules serving as a target for reactive oxygen species, protein molecules also react with a number of different radicals including the relatively unstable oxygen species (Stadtman, 1993; Davies *et al*, 1987; Uchida and Kawakishi, 1990; Heinecke *et al*, 1993). The primary amino acids that serve as targets for reactive oxygen species are methionine, histidine, cysteine and tyrosine (Cance, 1979; Davies *et al*, 1987; Dizdaroglu, 1993; Stadtman, 1993; Halliwell, 1994). Metal catalyzed protein oxidation results in the introduction of carbonyl groups on amino acid residues including lysine, arginine, proline and/or threonine by a site-specific mechanism (Levine, 1983; Climent *et al*, 1989).

Reactive oxygen research in non-mammalian systems

Oxidative stress, imposed by a variety of different mechanisms as well as exposure to a number of different chemicals have been shown to be mutagenic to bacteria (Touati, 1989; Storz *et al,* 1987; Levin *et al,* 1992; Abril and Pueyo, 1990). Similar mutagenic effects have also been shown in mammalian cells subjected to oxidative stress and or chemical exposure (Weitzman and Stossel, 1984; Hsie *et al,* 1986). Moraes et al. (1989) demonstrated a pattern of mutations in a gene of shuttle plasmid-transfected simian cells exposed to hydrogen peroxide. This research demonstrated that both single base alterations and deletions could result in unrepaired DNA lesions that became mutagenic following mitosis. In another study, treatment of a plasmid with hydrogen peroxide before transfection did not produce an increase in the number of mutants unless iron ions were added (Moraes *et al,* 1989). These results suggest that the hydrogen peroxide molecule is unable to react directly with DNA. Similar results have been demonstrated with chemical studies that looked at the effect of the hydrogen peroxide molecule on DNA (Blakely *et al,* 1990).

Carcinogenic transformation and the reactive oxygen radical

There are many steps that have to occur before an apparently healthy cell becomes a malignant tumor. Three stages have been identified by cancer

biologists in the transformation process. During the initiation stage, DNA is damaged and that damage is not repaired prior to mitosis. Thus, the damage to the genetic material becomes a permanent, irreversible part of that cell's genetics. It must be noted that an unrepaired DNA lesion must be by-passed by the DNA repair mechanisms for the cell to become mutated and or transformed. Once cellular DNA damage has occurred and the cell has undergone initiation, the cell can then be influenced by a number of genetic factors (oncogenes), which can then give preferential growth opportunities to the transformed cell. Mechanisms related to these proto-oncogenes selectively stimulate the mutated cell to continue to divide and undergo further changes in gene expression and give specific growth advantages to the mutant (Ziegler-Skylakakis and Andrea, 1987; Yamashina *et al,* 1986). This is refered to as the progression stage of carcinogenesis and it establishes a distinct cell line that evolved from a single mutant cell. Increased expression of such proto-oncogenes as c-fos and c-myc have been shown in several mammalian cell lines exposed to oxidative stress and a number of different toxic metals including Cd (Sherman *et al,* 1990; Shibanuma *et al,* 1988; Cerutti *et al,* 1989; Takeda *et al,* 1994; Abshire, 1996). In addition, several tumor promoters, such as phorbol ester TPA and acetate, are also powerful activators of superoxide and hydrogen peroxide production by phagocytes

(Cerutti, 1985). The third step in the carcinogenic process is one of progression in which the promoted cell line then undergo further changes in their DNA makeup leading to the eventual production of a grossly detectable malignant tumor.

DNA damage resulting from oxidative stress, metal or chemical exposure doesn't necessarily cause cancer (Dreher and Junod, 1998). The production of an initiated cell appears to be associated with the degree of damage and length of exposure to the suspected carcinogen by the DNA molecule. Low levels of damage may be efficiently repaired with minimal risk of error (Halliwell and Gutteridge, 1990b). High levels of DNA damage caused by an initial high level exposure or low level exposure for a prolonged period of time may eventually lead to DNA damage that causes cell death so that initiated cells do not remain in the organism. Thus, intermediate levels of damage usually caused by chronic exposure scenarios are most likely to predispose cellular DNA damage sufficient to produce initiated cells which may then if not removed from the system by the DNA repair mechanisms to progress on to produce a malignant tumor (Stohs and Bagchi, 1995; Kowaltowski and Vercesi, 1999).

Repair systems have evolved to remove most DNA lesions that result from attack on the DNA molecule by hydroxyl radicals and other radicals

formed by metal and chemical interactions (Breimer, 1991). Single strand breaks are quickly repaired and are in fact generated as intermediates in the repair of other lesions. 8-Hydroxyguanine, a type of DNA adduct caused by xenobiotic exposure is an example of a type of DNA defect that is slowly removed from cellular DNA and has been used by a number of researchers to demonstrate changes in cellular DNA (Boifeux and Radicella, 1999). Several lesions, including changes to the thymine moiety, are probably removed by the action of a DNA glycosylase, which cuts the base-deoxyribose bond to give an abasic site (Yu. Z. *et al*, 1999). This site is recognized by endonuclease activity, which nicks the damaged strand at the abasic site. The damaged part of the strand is then removed, followed by resynthesis of the DNA molecule and re-joining of the strand by a DNA ligase enzyme. In addition, glycosylases that recognize hydroxymethyluracil and opened-ring purines lesions in DNA have been described in mammalian cells (Doetsch *et al*, 1990).

Superoxide and hydrogen peroxide do not react directly with DNA unless transition metal ions are present to allow the hydroxyl radical to form (Aruoma *et al*, 1989; Blakely *et al*, 1990). This is because the hydroxyl radical is so reactive that it attacks what ever it is near once it is formed. Unless it is formed next to the DNA molecule it will not survive long enough to be carried

to the molecule but if it is near the DNA molecule it can attack all of its molecular structure (Steenken, 1989). Thus the hydroxyl radical is capable of abstracting hydrogen atoms from deoxyribose, leaving sugar radicals that can fragment in various ways. Reactions of deoxyribose-derived radicals can lead to the release of purine and pryimidine bases from the DNA molecule producing strand breaks and abasic sites. Some of the altered sugars that remain attached to DNA can be split to produce strand breaks by incubation with alkaline solutions, the so-called alkali-labile sites" (Breesk *et al,* 1976).

Peroxidizing lipids have also been reported to damage the DNA molecule (Brambilla *et al,* 1989; Hruszkewewycz and Bertgold, 1990) but peroxidizing lipids produce a wide range of reactive oxygen species including the hydroxyl radical, hydrogen peroxide, peroxyl radicals and alkoxyl radicals (discussed earlier in the peroxidation section) and the contributions of each of these compounds to the carcinogenic process needs to be evaluated (Frankel *et al,* 1987). Lipid peroxides also decompose to give a huge range of products including carbonyl compounds such as malondialdehyde and the unsaturated aldehyde 4-hydroxy-2-trans nonenal both of which have been shown to be mutagenic to mammalian cells (Frankel *et al,* 1987). If these compounds are generated in the vicinity of a DNA molecule, they may then combine with it to form a number of distinctive products that may result in the

initiation of the carcinogenic process.

Chapter 3

Characterization of a Cadmium Binding Protein in the Planarian, *Dugesia dorotocephala*

Introduction

Metallothionein-like proteins were first isolated from the renal cortex of equine kidney (Margoshes and Vallee, 1957). These metal-binding proteins are characterized as low molecular weight macromolecules that have a high sulfur content (primarily in the form of cysteine). Metallothionein (MT) expression can be induced by exposure to metals (Cherian *et al,* 1994) and the protein selectively binds the group II metals including zinc (Zn) and cadmium (Cd) (Waalkes *et al.* 1992). The inducible nature of this protein suggests that MT may play an important role in the detoxification and regulation of toxic metal ions (Shaikh and Lucis, 1971; Cherian and Nordberg, 1983). This protein may also function as a scavenger of oxygen free radicals and, in some species, MT expression can be induced by oxidative stress (Hamer, 1986; Dunn *et al,* 1987). Metallothionein-like proteins have been isolated from a wide variety of eucaryotic species including vertebrates, invertebrates, microorganisms and plants (Dunn *et al,* 1987; Highman *et al,* 1986; Imagawa *et al,* 1991; Kagi and Schaffer, 1988; slice *et al,* 1990). Recent work by Roesijadi (1992) documented that

metallothioneins are found in a large number of aquatic species.

The freshwater planarian, *Dugesia dorotocephala*, has been shown by previous researchers to be capable of modeling many important aspects of the anatomical, physiological and biochemical organization of higher animals (Best, 1972; Best and Morita, 1982; Deither, 1964, Lenntz, 1968; Best and Morita, 1984). Despite the broad scope of planarian research, MT-like proteins have neither been identified nor characterized in planarian tissue. Our interest in the possible existence of such a protein was stimulated by previous work that showed that asexual *Dugesia dorotocephala* exposed to Cd and 12-O-tetradecanoylphorbol-13-acetate (TPA) developed a high incidence of post-pharyngeal tumors (Hall *et al*, 1986). The malignant nature of these tumors was confirmed by transplantation studies (Hall *et al*, 1986).

This study was designed to test the hypothesis that the planarian, *Dugesia dorotocephala*, contains a MT-like protein that is inducible by metal exposure or oxidative stress and is capable of binding Cd and other metals like mammalian MT.

Materials and methods

TRIS-(hydroxymethyl)aminomethane (TRIS), 5,5'-dithiobis (2-nitrobenzoic acid (DTNB), phenylmethylsulfonyl fluoride (PMSF), and Coomassie Brilliant Blue, were obtained from Research Organics, Inc. (Cleveland, OH).

Benzoyl peroxide, iminodiacetic acid (Chelex 100 resin), reduced glutathione, calf thymus DNA and bis-benzamide (Hoechst 33258) were obtained from Sigma Chemical Company (St. Louis, MO). Zinc sulfate, (ethylenedinitrilo)-tetraacetic acid disodium salt (EDTA), sodium phosphate, sodium chloride, sodium bicarbonate, calcium sulfate, nitric acid and magnesium sulfate (all analytical reagent grade) were obtained from Mallinckrodt Specialty Chemical Company (Paris, KY). Cadmium chloride was from Merck and Company (Rawhay, NJ), and 2-mercaptoethanol (2-ME) was obtained from Amresco (Solon, OH).

Both asexual and sexual strains of the planarian, *Dugesia dorotocephala*, were used in this research as sources for protein extraction. Stock laboratory cultures of both strains were maintained in aerated tap water at 20^0 C ($\pm$ 2^0 C) with a 12-hour light and dark cycle. All laboratory stock was fed raw beef liver twice weekly.

Three days before metal or chemical exposure, uniformly sized planaria with normal anatomical morphology were segregated from stock cultures into glass vessels (10.5-cm diameter) containing soft reconstituted water. Soft reconstituted water was made freshly, on a weekly basis using 3 liters of type I water and adding 144 mg sodium bicarbonate, 90 mg calcium sulfate, 90 mg magnesium sulfate and 6 mg potassium chloride (ATSM, 1992). After preparation, the soft reconstituted water was aerated for 12 hours prior to use in

the planarian cultures.

Immediately after segregation and isolation in the soft reconstituted water, the planaria were fed raw liver. Five hours later the worms were transferred to fresh soft reconstituted water and then treated with the test compounds. Segregated planaria were then maintained in a fasting state for the remainder of the exposure period. Culture water was changed every 24 hours during exposure periods. Each culture dish contained either 10 asexual or sexual worms in 50 ml of exposure solution.

A minimum of three replicates were performed for each experimental treatment. The minimum number of each strain of planaria needed to yield measurable levels of protein was based on protein assays run on different numbers of worms. For purposes of consistency in this study, all replicates consisted of either 10 sexual planaria or 30 asexual planaria. Controls were maintained for each experimental exposure with planaria taken from the same stock culture and handled in the same manner as the exposed planaria with the exception of exposure to the metal or chemical of interest.

Dose levels for the different metals and chemical exposures used in these experiments were chosen from range finding experiments with *D. dorotocephala* conducted in our laboratory. Briefly, planaria were isolated in soft reconstituted water and then exposed to varying concentrations of the twst chemical or metal.

The exposed planaria were observed for five days for mortality and abnormal behavior. Experimental dose levels in the reported experiments were based on the maximum exposure level from the range finding experiments that produced minimal planarian mortality. Dose levels were determined for Cd, Zn, mercury, copper, chromium, arsenic, nickel and benzoyl peroxide (Bp). The dose levels for metal exposures in both strains of *D. dorotocephala* for Cd and Zn were 10 and 500 μg/L respectively. A benzoyl peroxide (Bp) concentration of 1 μg/L was used.

Concentrated stock solutions for both Cd and Zn were prepared and maintained in soft reconstituted water and stored at 4^0C. Exposure dilutions in soft reconstituted water were made from stock solutions on a daily basis. Stock metal solutions were routinely evaluated using an atomic absorption spectrophotometer to verify metal concentrations. Fresh Bp solutions were made daily. Exposures to Bp were conducted over a 96-hour time period and the planaria evaluated every 24 hours for mortality and abnormal behavior. Metal exposures were conducted for a maximum of 10 consecutive days.

At the end of the exposure period, planaria were rinsed with soft reconstituted water and then homogenized in a buffer containing 50 mM TRIS, 0.2 mM EDTA, 20 mM 2-ME and 0.05 mM PMSF (pH 8.0) (buffer A). Homogenization was accomplished using an ultrasonic cell disrupter (Sonifier

Cell Disrupter, Heat Systems Company) at a power setting of 4 utilizing three consecutive 10-second bursts. After isolation and during the homogenization procedure, planaria and planarian homogenates were maintained on ice. The resulting homogenate was centrifuged at 10,000 x *g* for 15 minutes to precipitate cellular debris. All centrifugation procedures were carried out at a temperature of 4^0 C. The supernatant was centrifuged at 100,000 x *g* for 100 minutes to separate the cytosolic and microsomal fractions. The cytosolic fraction was heat treated in a boiling water bath for 20 minutes and then centrifuged at 16,000 x *g* for an additional 10 minutes to remove the heat denatured protein fraction. Protein content of the heat-stable protein fraction was determined using the Bradford dye binding protein assay (Bradford, 1976), using bovine albumin to construct standard curves. The resulting heat-treated cytosol was then removed in 1 ml aliquots with a pipette and stored at -80^0C.

An alternative method to denature unwanted cytosolic proteins described by Winge and Brower (1986) utilizes solvents rather than heat. This method was evaluated to determine if there was a difference in the yield of the desired cytosolic protein. There was no difference in the quantities of the planarian protein that bound cadmium isolated by the chloroform-ethanol extraction followed by acetone precipitation compared to the heat treatment procedure. The heat treatment method was preferred, because of the ease of handling the

sample.

Heat-treated cytosol samples were thawed and then treated with 0.02 mg Cd per mg of protein to saturate metal binding sites prior to fractionation. Excess cadmium in the sample was removed using Chelex 100 resin (iminodiacetic acid) as described by Bartsch *et al.* (1990). The Cd-saturated cytosolic fraction was then loaded on a 90 cm x 2 cm column packed with Bio-Gel P-100 gel (BioRad Laboratories, Hercules, CA) equilibrated with buffer A. Eluate was collected in 2.5 ml fractions. The absorbance of eluted fractions was measured at 254 and 280 nm using a Hitachi U3000 spectrophotometer. Fractions were analyzed for Cd content using an atomic absorption (AA) spectrophotometer (Varian Spectr AA5; cadmium lamp at 228.8 nm; air/acetylene flame). Prior to AA analysis, samples were acidified using analytical grade concentrated nitric acid to bring the final sample acid concentration to 1% (v/v).

Gel filtration column elution was calibrated using a mixture of protein standards trypsinogen (24,000 D), trypsin inhibitor (20,000 D), cytochrome C (12,384 D) and rabbit metallothionein (6,000 D). The standard protein solution was saturated with 0.02 mg Cd per mg of protein and then treated with Chelex 100 resin prior to fractionation. Eluted fractions from the protein standards were evaluated using the same techniques described above for the exposed and control planarian protein fractions. The apparent molecular weight of the

planarian protein was calculated by comparing the unknown protein peak elution time with that of the known protein standards (Andrews *et al,* 1964).

Fractions binding Cd eluted from the gel filtration column were pooled and concentrated using Centricon 3 concentrator tubes (Amicon, Inc. Beverly, MA). The concentrator tubes were centrifuged in a fixed angle centrifuge at 7,500 x g for three hours to concentrate a 2 ml sample. A 50 µl fraction of the concentrated protein sample was then injected onto a DEAE ion exchange column (HEMA-IEC BIO 1000 DEAE 10 u column, 50 mm X 4.6 mm, Alltech Associates, Inc. Deerfield IL) eluted with a flow rate of 0.5 ml/min measuring absorbance at 254 nm (Hitachi L6200 pump and an Hitachi L 4200 UV-VIS detector). The mobile phase employed a concentration gradient that went from 20 mM TRIS-phosphate (pH 8.45) to 400 mM TRIS-phosphate (pH 8.45) in 1 hour. Fractions that demonstrated absorbance at 254 nm were collected, pooled and concentrated using Centricon 3 concentrator tubes. Protein samples that had high 254 nm and low 280 nm absorbance were then assayed for Cd and sulfhydryl content. The peak fraction with a high sulfhydryl content and bound Cd were pooled and the protein precipitated with methyl alcohol and chloroform (Wessel and Flugge, 1984). The purification process for protein samples submitted for amino acid analysis was to first separate the homogenate proteins by size on a Bio-Gel P-100 gel and then in a DEAER ion exchange column. The

purification of the protein was further evaluated using electrophoretic techniques tricine gel. A single protein band on the tricine gel from the planaria that separated at the same site as mammalian MT was cut from the gel, extracted and precipitated. The precipitated protein sample was then submitted to the Protein Chemistry Facility, W. Alton Jones Cell Science Center, Lake Placid, NY, for amino acid analysis. The method described by Hirs (1967) was used to prepare the sample, which was then analyzed by HPLC using cation exchange chromatography (Klapper, 1982).

Protein sulfhydryl content in gel filtration column fractions was determined using the method described by Ellman (1959). An aliquot of each sample (25 μL) was diluted with 150 μL of 0.1 M phosphate buffer (pH 8.0) in a 96 well-plate. Twenty-five μL of 1 mM DTNB in a 0.1 M phosphate buffer (pH 7.0) was added to the mixture and incubated for five minutes. Absorbance was measured at 405 nm. Absorbance obtained with buffer alone was subtracted from the value of each fraction assayed. Because of the high sulfhydryl content of the column elution buffer, a standard curve was prepared using reduced glutathione diluted in the same buffer.

Routine analysis of the planarian Cd binding protein was performed on samples homogenized in buffer A as described earlier. Following centrifugation at 10,000 *x g* for 15 minutes, the supernatant was pipetted off and treated in a

boiling water bath for 20 minutes and the pellet was reserved for DNA analysis. The heat-treated sample was then centrifuged for 10 minutes at 10,000 x g to precipitate heat denatured protein. Heat stable protein in the supernatant was assayed using the Bradford dye binding protein assay using bovine serum albumin as a standard (Bradford, 1976). Excess Cd was added to the sample at a concentration of 0.02 mg Cd/ mg cytosolic protein and the sample mixed for 10 minutes. Chelex 100 resin was then added to the sample as described by Bartsch *et al* (1990) followed by mixing for an additional 15 minutes. Chelex 100 resin was removed from the protein sample by centrifugation at 8,000 *x g* for 5 minutes. Analysis by atomic absorption spectrometry was used to determine the metal content of samples following acidification with nitric acid. For planaria treated with copper or mercury, the respective metal was substituted for Cd in the metal saturation step and was the analyte in atomic absorption analysis.

Planarian DNA content was used to normalize results because of the size variation between the different strains of planaria and the difficulty of making accurate body weight measurements. In addition, planaria normally produce large amounts of mucus protein when handled and this precluded normalization on a protein content basis. DNA content from exposed planaria was determined in the homogenized cell debris fraction obtained in the first centrifugation step described earlier. Cellular debris was suspended in a phosphate-saline buffer

(0.005 M sodium phosphate, 2.0 M sodium chloride and 3 mM EDTA, pH 7.4). Aliquots (100 µl) were mixed with the phosphate buffer containing 1 µg/ml of bis-benzamide (Hoechst 33258) per ml (Labraca and Pagen, 1980). Fluorescence was measured using a fluorescence spectrophotometer (Hitachi F2000) measuring emission at 492 nm with excitation at 356 nm. A standard curve was prepared using calf thymus DNA.

Results

Gel filtration chromatography was used to fractionate samples of heat-stable planarian cytosolic protein (Figures 3-1 and 3-2). The absorbance of eluted fractions was determined at 254 and 280 nm and Cd concentrations were evaluated by AA analysis. Exposure to 10 µg/L Cd appeared to increase the amount of the cadmium binding protein present in the heat-treated cytosolic from both asexual (Figure 3-3) and sexual (Figure 3-4) *D. dorotocephala*.

The apparent molecular weight of the heat stable protein that bound Cd was estimated by comparing its elution from the gel filtration column with a standard protein mixture run under the same conditions (Figure 3-5). A plot of elution volume for each eluted peak of the known standards against the log of the molecular weight of the standards yielded an approximate molecular weight for the planarian Cd-binding protein of 7400 daltons.

The protein fraction from the gel filtration column that contained Cd had a

higher sulfhydryl content than did the other protein peaks collected from the cytosol when evaluated using the DTNB sulfhydryl assay (Figure 3-6). The heat stable planarian protein was shown by this assay to contain 0.21 μmoles of sulfhydryl groups per μmole of heat-treated cytosolic planarian protein.

Fractions collected from the gel-filtration column that had both a high sulfhydryl content and bound Cd were concentrated and analyzed on an HPLC-DEAE ion exchange column (Figure 3-5). This analysis revealed the presence of two protein peaks. Fractions were collected from elution of the DEAE ion exchange column for further analysis. The protein that eluted at 32.6 minutes (Figure 3-7) was shown by AA spectrophotmetry to contain bound Cd whereas the protein eluted at 17.3 minutes did not. Sulfhydryl analysis of the eluted fractions demonstrated that the protein eluted at 32.6 minutes had an eight-fold higher sulfhydryl content than the earlier eluting protein peaks.

The protein fraction that eluted at 32.6 minutes contained 0.233 μg of protein/ml after concentration. When assayed using the AA spectrophotmetry, this concentrated protein solution contained 24.8 μg/L Cd. Thus, this protein was found to bind seven μmoles of cadmium per μmole of protein based on the estimated molecular mass of the protein as 7400 daltons.

The Cd-binding protein collected from the DEAE ion exchange chromatography column showed a single band when electrophoresed on a

tricine gel in a single band. The band was cut from the tricine gel, extracted and the protein precipitated. The protein wass then submitted for amino acid analysis. The results revealed that approximately 28% of the amino acid residues present were cysteine (Table 3-1). Threonine, lysine, serine and aspartic acid were the next most common amino acids identified in the cadmium binding protein. A low amount of phenylalanine was found but tyrosine and tryptophan were not determined.

In order to allow more rapid and convenient quantitative determination of the planarian cadmium binding protein, a saturation assay was developed, similar to methods published previously for analysis of mammalian metallothionein (Vallee, 1991). After exposure of planaria to the test compound, they were then homogenized, centrifuged and heat-treated to obtain a heat stable protein fraction. This sample was then saturated with Cd followed by Chelex 100-resin treatment to remove unbound metal. Gel filtration chromatography of samples prepared in this manner demonstrated that over 95 % of the Cd co-eluted with the protein peak characterized previously. Based on the stoichiometry of Cd binding 7 μmoles of Cd/μmole of protein, the amount of Cd found in a sample was used to calculate the amount of Cd-binding protein present. Both negative (unexposed planarian cytosol) and positive controls (rabbit metallothionein) were used to validate the assay. Good correlation was

found between controls and exposed planarian cytosol using Cd levels to estimate the Cd-binding protein levels (data not shown). Rabbit metallothionein, which was used as a control, was treated in a manner identical to that used to isolate the heat stable Cd-binding protein from planarian homogenate.

Studies were carried out on both sexual and asexual planaria to evaluate the effects of prolonged exposure to Zn or Cd on the levels of Cd-binding protein found in the planaria (Table 3-2). Asexual *D. dorotocephala* exposed to Cd at a concentration of 10 µg/L for 10 days had a heat-stable protein fraction containing 403 pg Cd /pg of DNA compared to unexposed asexual planaria which bound 48.1 pg Cd/ pg DNA in the Cd-binding protein fraction (Table 3-2). This represents a nine-fold increase in the amount of Cd-binding protein over control levels. Sexual *D. dorotocephala* exposed to Cd at 10 µg/L demonstrated an eight-fold rise in the Cd-binding protein content when compared to unexposed sexual planarian controls. When exposed to zinc at 500 µg/L for 10 days, asexual planaria demonstrated an increase in Cd binding capacity that increased from a mean of 48.01 pg Cd/ pg DNA in unexposed planaria to 397.11 pg Cd/ pg of planarian DNA. This represents greater than a 7.5 -fold increase in Cd-binding protein in zinc-exposed asexual planaria. Similar results were observed with Zn exposure in sexual *D. dorotocephala* (Table 3-2).

Protein-bound metal content was measured with AA spectrophotometry

following exposure of asexual *D. dorotocephala* to a number of different metals over a range of concentrations for 5 days (Figure 3-8). Among the metals tested, Cd had the highest potency for causing induction of the Cd-binding protein (Figure 3-8). Comparably low concentrations of Hg were also effective in increasing its concentrations but the peak level achieved without appreciable toxicity was lower. Copper treatment also increased the levels of Cd-binding protein but at a concentration approximately one order of magnitude higher than Cd. Zinc was also capable of increasing Cd-binding protein content, albeit at higher concentrations, with a dose response slope and maximal levels comparable to Cd and Hg. In contrast, Cr had induction potency similar to zinc, but with a much shallower dose response curve and lower peak level. Although arsenic increased Cd-binding protein content, concentrations two orders of magnitude higher than Cd were required to do so. Nickel at high concentrations had a positive dose relationship with Cd-binding protein content but none of the values were significantly higher than control levels

Benzoyl peroxide treatment was used as a model of oxidative stress to evaluate whether it could cause increases in Cd-binding protein levels. Asexual *D. dorotocephala* exposed to 1 mg/L benzoyl peroxide demonstrated a 4.3-fold increase in Cd-binding protein levels after a 24 hour exposure (Table 3-3). After 96 hours of exposure, the Cd-binding protein levels rose 6.4-fold higher than

levels measured for controls.

Discussion

The results of these studies demonstrate that the planarian *D. dorotocephala* contain a metal-binding protein with properties consistent with its characterization as a metallothionein. Besides binding Cd, this protein is heat stable and has a relatively high cysteine content (Table 3-1). In addition, the levels of this protein in planaria treated with metals or an oxidant respond in a manner expected for a MT.

The protein is present in the cytosolic fraction prepared from D. dorotocephala like mammalian MT. It remains soluble following heating or treatment with chloroform-ethanol extraction followed by acetone precipitation. Both procedures have been used to isolate MT and MT-like proteins from mammalian and invertebrate systems (Winge and Brower, 1986; Everard and Swain, 1982).The estimated molecular mass (7400 d) is in the range of previously isolated MTs (Hamer, 1986).

The protein exhibited lower absorbance at 280 nm than at 254 nm. This is consistent with a low content of aromatic amino acids (Figure 3-1). Other invertebrate MT's have been shown to contain aromatic amino acid residues, in contrast with the mammalian proteins, which are devoid of aromatic side chains (Hamer, 1986; Everard and Swain, 1983). The planarian protein has a relatively

high content of sulfhydryl groups, as assessed with Ellman's reagent (Figure 3-6).

The amino acid content determined for the purified protein confirms the observation of a relatively high sulfhydryl content (Table 3-1). Cysteine comprises over a fourth of the protein. The small amount of phenylalanine was found would account for some of the 280 nm absorbance detected. The Cd-binding protein was shown to bind seven μmoles of Cd per μmole of protein. This is in agreement with the stoichiometry of Cd binding observed in previous studies of mammalian MT and MT-like proteins in invertebrates (Hamer, 1986).

All of this evidence is consistent with a conclusion that the protein we isolated from the planarian, *D. dorotocephala*, is a metallothionein. Every effort was made to ensure that we isolated a single protein including sephadex gel filtration, DEAE ion exchange chromatography and tricine gel electrophoresis with a single band cut from the electrophoresed gel. Like mammalian MT, the planarian Cd-binding protein has been shown to be a heat-stable, low molecular weight protein with a high cysteine content (Hamer, 1986). Unlike mammalian MT the planarian Cd-binding protein contains some aromatic amino acids but it should be noted that MT and MT-like proteins from other phyla typically contain small amounts of aromatic amino acids (Hamer, 1986).

Treatment of *D. dorotocephala* with Cd (10 μg/L) for 10 days resulted in a

nine-fold increase in the specific content of MT (Table 3-2). Similar results were obtained for both the sexual and asexual strains. Other metals were also capable of causing an increase in the MT content of planaria, notably Zn and Hg (Figure 8). This is characteristic of metallothionein-like proteins found in other invertebrates (Roesijadi, 1992; Mac Kay *et al,* 1993; Lerch *et al,* 1982; Nemer *et al,* 1994). Metallothionein induction by cadmium has previously been demonstrated in mammals and birds (Saikh and Lucis, 1971; Weser *et al,* 1973; Olafson *et al,* 19791).

Planaria subjected to oxidative stress also exhibited elevated levels of MT (Table 3-3). The six-fold increase in Cd-binding protein levels per pg of planarian DNA demonstrated in this experiment is similar to increases in levels of cellular MT in other studies that have used reactive oxygen radicals in the form of organic hydroperoxides in mammalian cell culture systems (Ochi and Mitaurura, 1989; Bauman *et al,* 1991).

In this research project, *D. dorotocephala* were exposed to Cd concentrations of 10 μg/L. These concentrations were found by previous experiments in our laboratory, to be non-lethal for *D. dorotocephala* (unpublished dose observations). In experiments conducted by Hall (1986), the concentrations that were required to produce tumors in asexual *D. dorotocephala*, were 30-fold higher than those required to increase MT content.

It is possible that the high Cd levels that Hall used in his work with the planarian, *D. dorotocephala*, were necessary because the planarian MT effectively bound the Cd ions at lower dosages and prevented the amount of DNA damage necessary to cause the malignant tumors he demonstrated.

The Cd-binding protein we isolated from *D. dorotocephala* thus has characteristics of mammalian MT and demonstrates responses to toxic metals expected for a mammalian MT. It is a cytosolic protein that has a low molecular weight and is heat stable. Amino acid analysis showed it to have a high cysteine content and it increases in content when the organism is exposed to metals and oxidative stress the same as mammalian MT. It has also been shown to sequester metal ions when exposed to metals and thus would be expected to reduce DNA damage associated with Cd exposure as has been demonstrated with mammalian MT. Cloning and sequence analysis of the gene coding this protein would allow confirmation of its homology with vertebrate metallothionein.

Table 3-1. Amino acid composition of a Cd-binding protein isolated from D. dorotocephala

Amino Acid	Composition Mole %[a]
Cysteine	28.23
Aspartic Acid	8.05
Glycine	4.61
Proline	3.64
Histidine	1.40
Threonine	10.40
Serine	8.46
Glutamine	6.10
Alanine	3.97
Valine	1.20
Methionine	0.91
Isoleucine	0.99
Leucine	0.91
Lysine	8.67
Arginine	0.31
Asparagine	5.88
Phenylanine	2.58
Glutamic Acid	3.68
Tryptophan	ND[b]
Tyrosine	ND

[a] Values are percent of total amino acid composition

[b] Not Determined

Table 3-2. Planarian cadmium binding protein and bound cadmium content in *D. dorotocephala* exposed to metals.

Treatment	pg bound Cd / pg DNA	pg PCBP / pg DNA[b]
Asexual *Dugesia dorotocephala*		
No Treatment	48.0 ± 1.5[a]	7.4 ± 0.2
Cd[c]	403.0 ± 13.7[d]	67.2 ± 2.3[d]
Zn[e]	397.1 ± 10.1[d]	66.2 ± 1.9[d]
Sexual *Dugesia dorotocephala*		
No Treatment	86.9 ± 3.8	14.4 ± 0.4
Cd[c]	662.6 ± 28.2[d]	110.4 ± 4.7[d]
Zn[e]	670.0 ± 43.9[d]	111.6 ± 43.0[d]

[a] Data expressed as mean ± standard error, There were 4 individual groups of planaria made up of either 30 asexual or 10 asexual *Dugesia dorotocephala*.

[b] Specific planarian MT content was calculated based on a Cd content of 7 µmole of Cadmium per µmole of protein.

[c] Exposed to 10 µg/L Cd for 10 days.

[d] Significantly different from untreated group of same strain of *D. dorotocephala* using ANOVA $p < 0.05$.

[e] Exposed to 500 µg/L Zn for 10 days.

Table 3-3. Planarian cadmium binding protein and bound cadmium content in D. dorotocephala exposed to benzoyl peroxide (1 g/L).

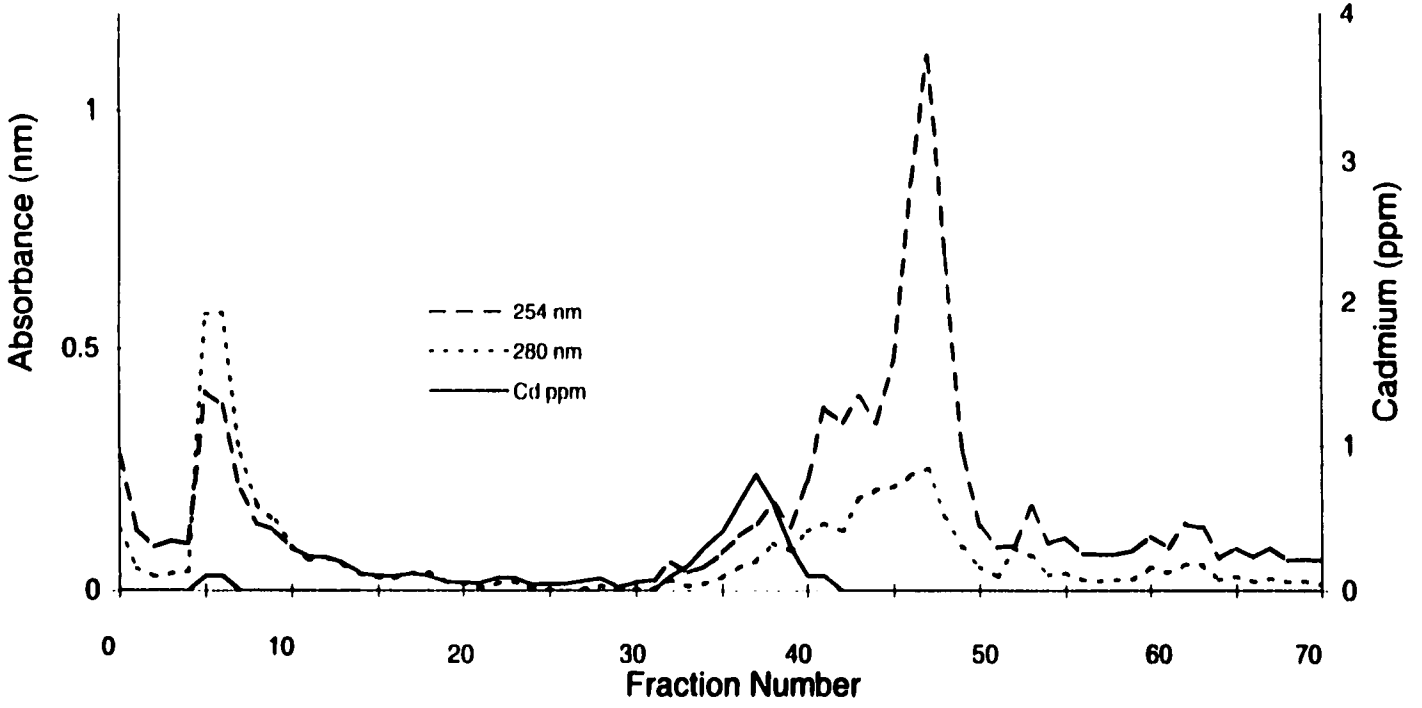
Treatment	pg Bound Cd / pg DNA	pg PCBP / pg DNA[b]
No Treatment	45.6 ± 1.1 [a]	7.6 ± 0.2
24 Hours	195.6 ± 5.9[c]	32.6 ± 1.0[c]
48 Hours	228.8 ± 5.6[c]	38.1 ± 1.0[c]
72 Hours	256.1 ± 5.6[c]	42.7 ± 1.1[c]
96 Hours	290.4 ± 7.1[c]	48.4 ± 1.1[c]

[a] Data expressed as mean ± standard error, n = 4 pooled groups of either 30 asexual or 10 sexual *Dugesia dorotocephala*.

[b] Exposed to 10 µg/L Cd for 10 days.

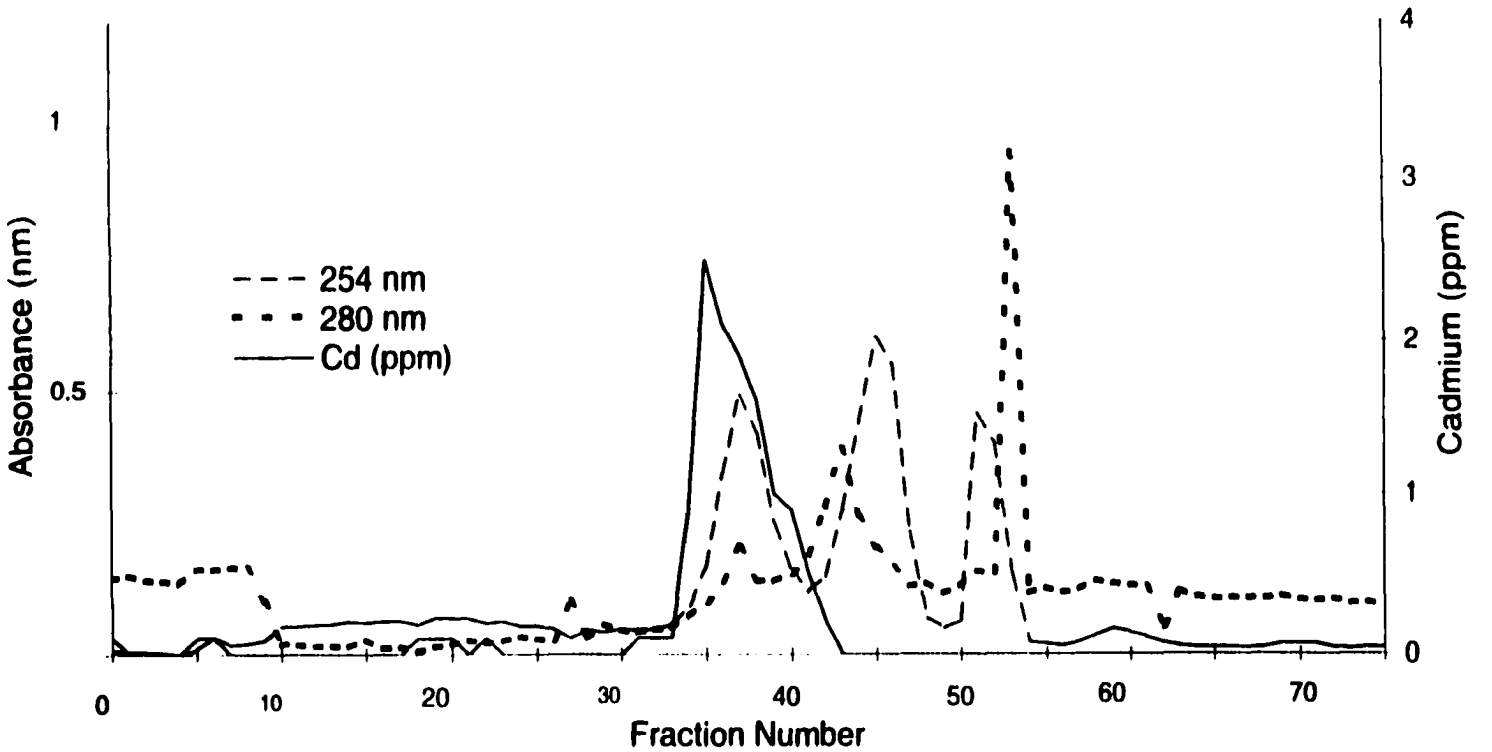
[c] Significantly different from untreated group of same strain of *D. dorotocephala* using ANOVA $p < 0.05$.

Figure 3-1. Gel filtration chromatography of heat treated cytosol from asexual *D. dorotocephala*.



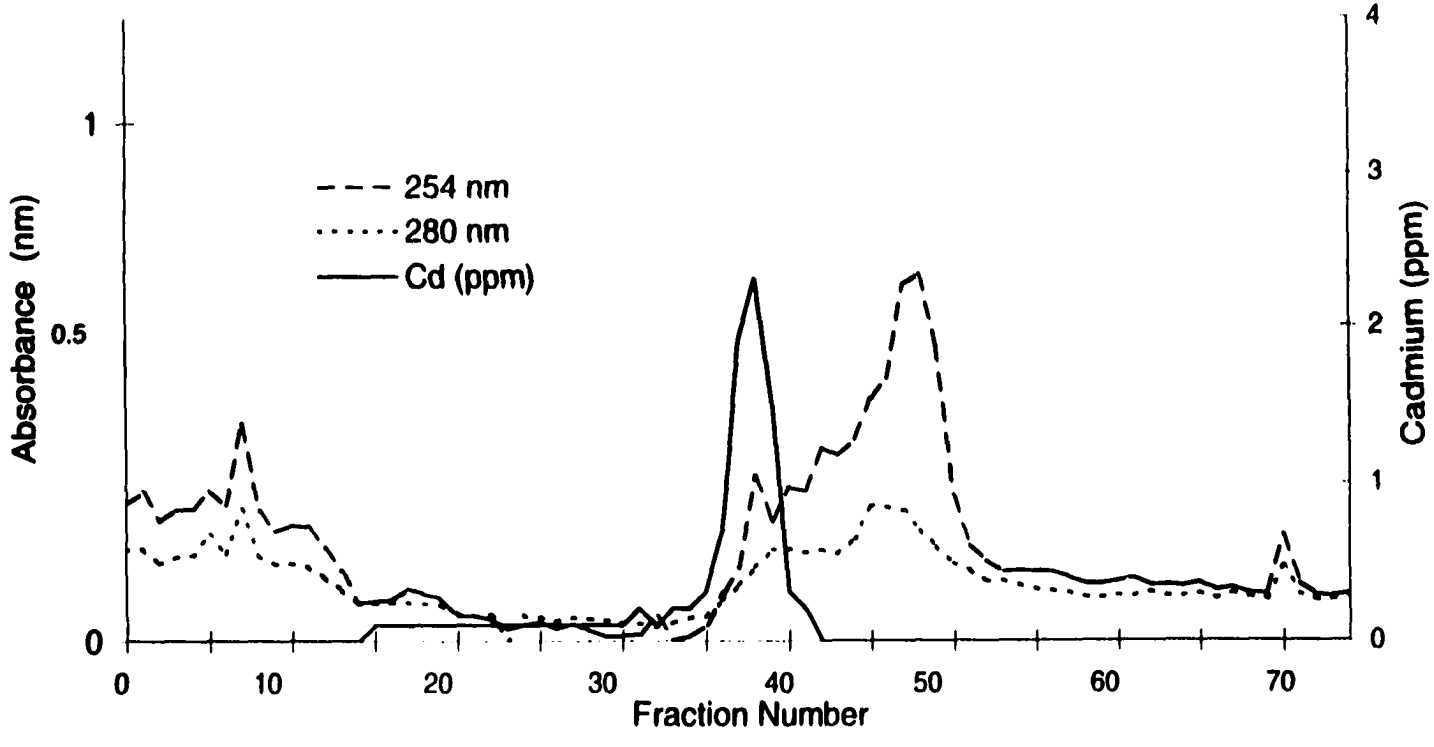
The heat stable fraction of cytosol (2 mg) was treated with cadmium chloride (0.02 mg/mg protein), followed by Chelex 100 resin to remove unbound Cd. The sample was applied to a Bio-Gel P-100 gel column (90X2 cm) equlibrated with buffer A (50 mM TRIS, 0.2 mM EDTA, 20 mM 2-MEand 0.05 mM PMSF, pH 8.0). It was eluted with the same buffer and fractions collected for measurment of absorbance at 254 and 280 nm. Cadmium contentwas measured by AA specrtophotometric analysis. Heat treated , Cd saturated cytosol from asexual *D. dorotocephala* (200 worms).

Figure 3-2 Gel filtration chromatography of heat treated sexual *D. dorotocephala*



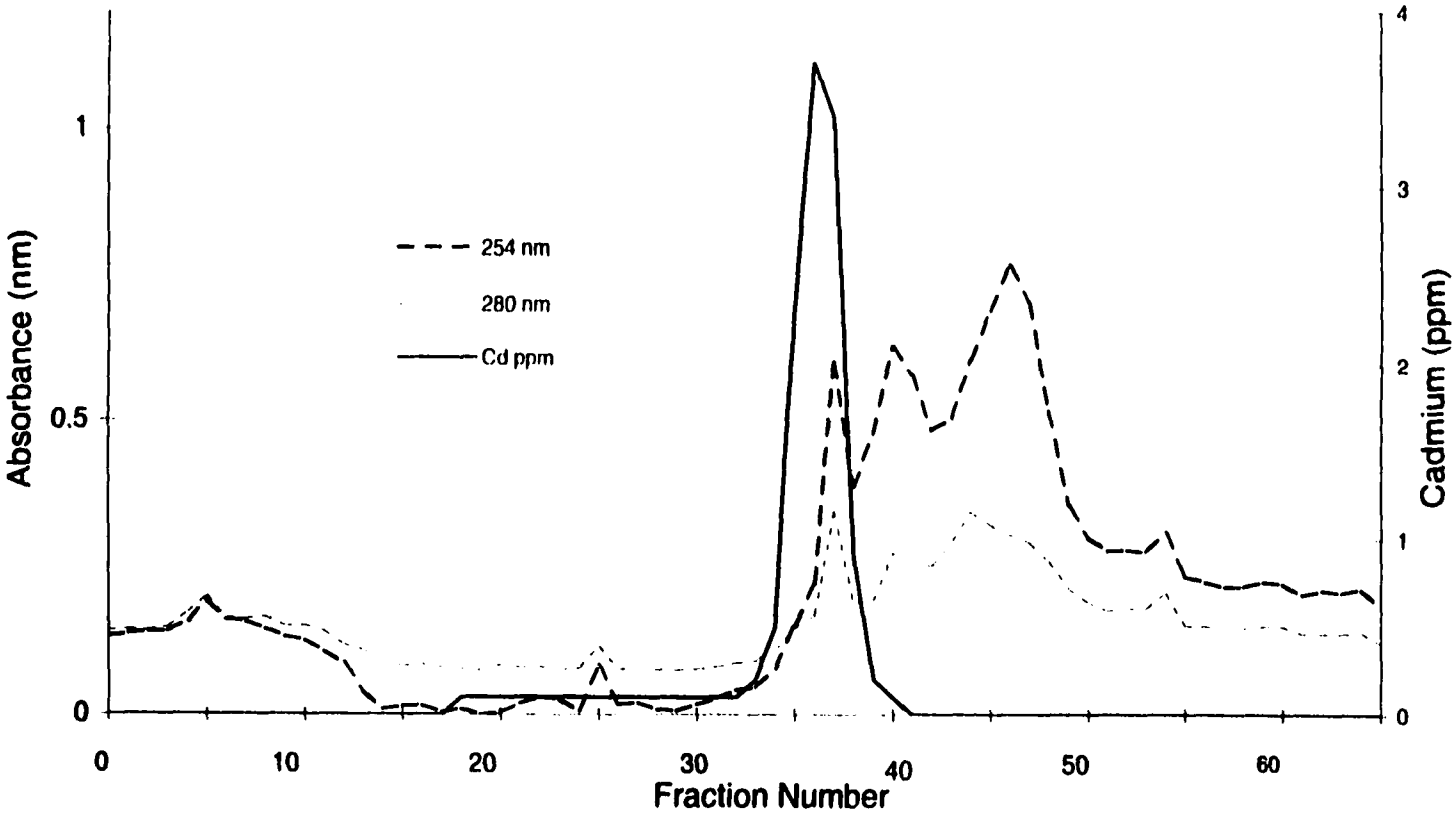
Sample treatment, chromatography and analyses were done as described in the legend for figure 3-1. Heat treated, Cd saturated cytosol from sexual *D. dorotocephala* (60 worms).

Figure 3-3. Heat-treated and Cd saturated cytosol from asexual *D. dorotocephala* (200 worms) exposed to 10 ug/L Cd for 10 days.



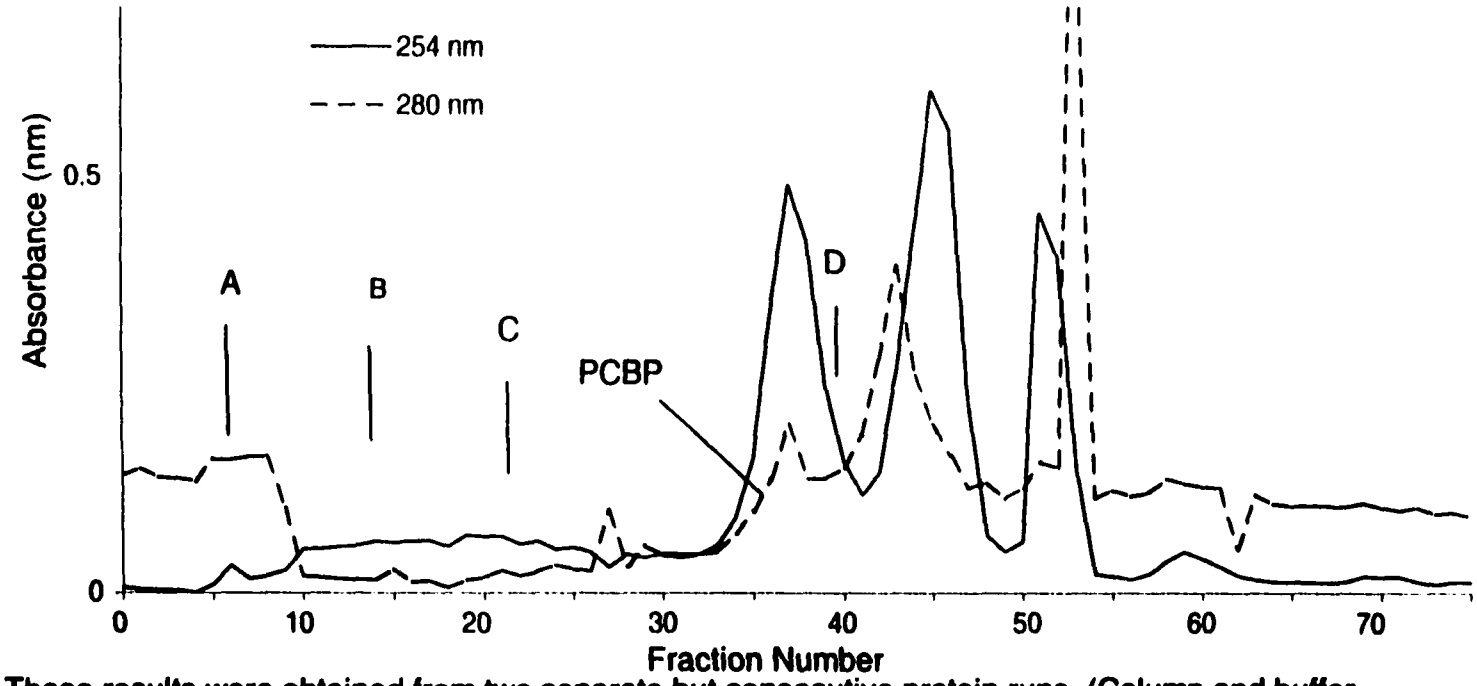
Sample treatment with the exception that these worms were exposed to 10 ug/L Cd for 10 days before cytosoloic collection and the worms in 3-1 were not exposed to Cd was the same as with planaria in 3-1 chromatography and sample analyses were performed as described in figure 3-1.

Figure 3-4. Heat treated, Cd saturated cytosol from sexual *D. dorotocephala* (60 worms) exposed to 10 ug/L Cd for 10 days.



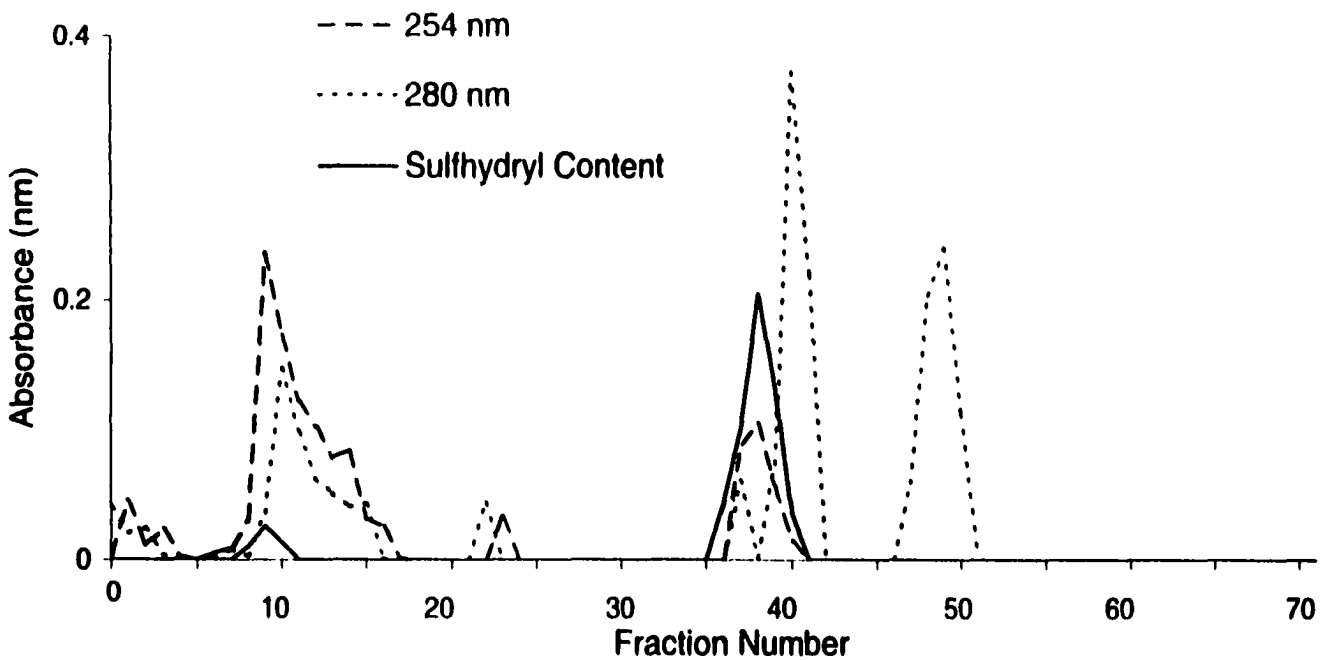
Sample treatment, chromatography and analyses were done as described in figure 3-1 with the exception that the sexual planaria were exposed to 10 ug/L Cd for 10 days before cytosol collection.

Figure 3-5. Gel filtration chromatography of heat treated planarian cytosol and standard proteins to determine the molecular weight of planarian MT



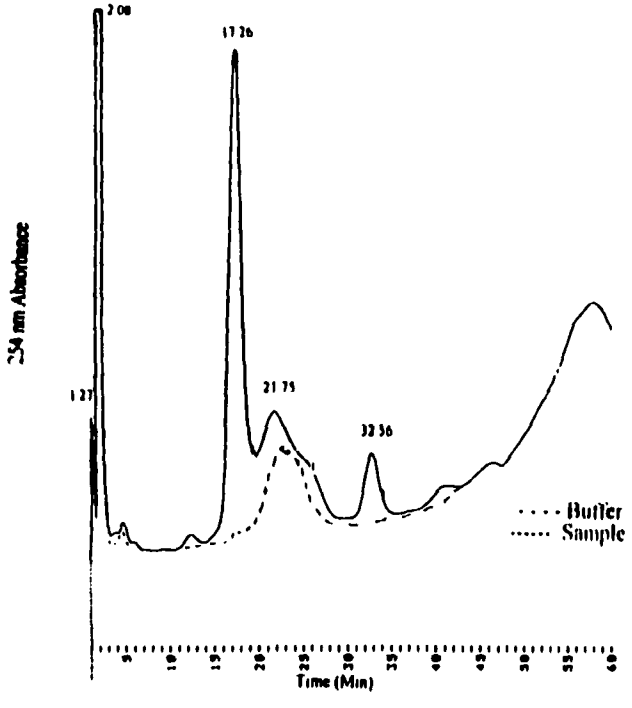
These results were obtained from two separate but consecutive protein runs. (Column and buffer described Figure 3-1). The first run was made with heat treated cytosol and the second with a mixture of known protein standards. Both samples were treated with excess Cd and Chelex to remove unbound Cd before loading on the column. The protein standards loaded were A) trypsinogen, B) Trysin inhibitor, C) cytochrome C and D) rabbit metallothionein. Cd was detectable in the cadmium binding peak (PCBP) and peak D with the use of AA spectrophotometry.

Figure 3-6. Gel filtration chromatography of heat-treated cytosol from asexual D.dorotocephala evaluating sulfhydryl content.



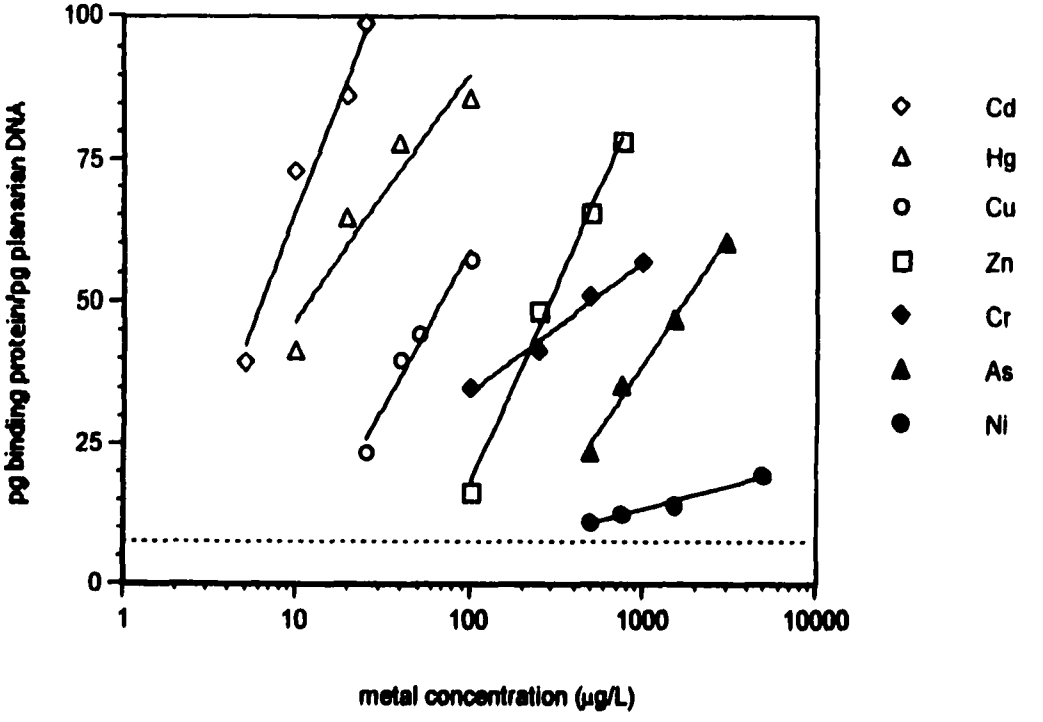
Two hundred asexual planaria were exposed to 10 ug/L Cd for 10 days prior to homogenization. The subsequent cytosol form the exposed planaria was heat-treated. The sulfhydryl content of the eluted fractions was assayed by reaction with DTNB and absorbance was measured at 370 nm.

Figure 3-7. An HPLC DEAE ion exchange separation of Cd binding protein collected from a sephadex gel filtration column.



The sephadex gel filtration column is described in figure 1. Fractions that bound Cd and demonstrated high sulfhydryl content were pooled and concentrated using Centricon 3 concentrator tubes, the concentrated samples were then injected on the column, (HEMMA-IEC BIO 1000 DEAE 10µ, 50 mm X 4.6 mm), and eluted with a linear gradient of 400 mM to 20 mM Tris (pH 8.45) over 60 minutes at a flow rate of 0.5 ml/min. Only the peak at 32.5 min bound Cd and had a high sulfhydryl content.

Figure 3-8. Planarian cadmium binding protein bound metal content following exposure of asexual D. dorotocephala to different metal for five days.



Planaria were exposed to different metals at varying concentrations for five consecutive days. Protein bound metal levels were measured using AA spectroscopy of acidified, heat-treated planarian cytosol isolated as described in figure 1. All protein bound metals were replaced by saturation with 0.02 mg Cd/mg protein with the exception of mercury and copper, which were saturated with the respective metal. Unbound metal ions were removed with Chelex 100 resin and centrifugation. The dotted line indicates the level of Cd-binding protein found in control samples

Chapter 4

Planarian Metallothionein and DNA Damage

Introduction

Cadmium (Cd) is a nonessential, environmentally ubiquitous metal that has been widely used in the galvanizing, electroplating and battery industry. In addition to environmental exposure, consumption of contaminated food products and cigarette smoking are major sources of Cd exposure in humans. Results from previous research suggest that Cd is a carcinogen in experimental animals causing tumors of the prostate, testes, lungs, at injection sites and in the mammalian hematopoetic system (Kazantzis and Handbury, 1966; Poirier *et al,* 1983; Hadley *et al,* 1979; Takenaka *et al,* 1983; Walkes *et al,* 1988; Waalkes *et al,* 1992; Waalkes and Oberdorster, 1990) . Epidemiological studies with human cancer endpoints link an increased risk of tumor development with either occupational or environmental exposure to Cd (Flanders, 1984; Bako *et al,* 1982; Lemen *et al,* 1976; Boffetta, 1992; Kazantzis *et al,* 1992). The development of malignant tumors has been demonstrated in the planarian, *Dugesia dorotocephala*, exposed to Cd, a process markedly enhanced by co-treatment with a phorbol ester (Hall *et al,* 1986a).

Cadmium induced genotoxicity and carcinogenicity are complex

processes, because the metal can interact with proteins, DNA, or both. Cadmium interactions with nucleic acids favor the bases over the phosphate groups in the DNA molecule (Koizumi and Waalkes, 1990; Langlais *et al*, 1990). Literature reports also indicate that Cd can inhibit DNA replication and decrease DNA polymerase fidelity (Zakour et al., 1981).

Metallothioneins (MTs) are small (MW ~ 6,000 Da) stress responsive, metal-binding proteins that appear to protect cells against the toxic effects of metals (Hamer, 1986), genotoxic agents (Lazo and Pitt, 1995) and oxygen- and nitrogen-based reactive species (Lazlo and Pitt, 1995; Schwartz *et al*, 1995; Tamai *et al*, 1993). Metallothionein induction can occur as a result of metal exposure (Fieldman *et al*, 1978; Suzuki *et al*, 1990), various forms of stress (Kazantzis and Hanbury, 1966) or radiation exposure (Matsubara, 1988). In mammalian systems the MT protein has been shown to be the most important metal-binding protein associated with cellular resistance to Cd-genotoxicity (Masters *et al*, 1994; Waalkes and Goering, 1990; Imagawa *et al*, 1991). The primary structure of all mammalian MTs is highly conserved and MT and MT-like proteins have been isolated from a wide variety of eukaryotic species including vertebrates (Hamer, 1986), invertebrates (Imagawa *et al*, 1991; Slice *et al*, 1990) microorganisms (Highman *et al*, 1986) and plants. Metallothionein-like proteins appear to have functions similar to those of mammalian MTs in their respective species. Recently we reported the isolation and characterization of a MT-like protein from the planarian, *D. dorotocephala* with properties similar to

mammalian MT.

Exposure to hydroxyl radicals produced by the interaction between Cd and hydrogen peroxide *in vitro* under physiological conditions detected using paramagnetic resonance can cause DNA mutations (Ochi *et al*, 1984). Thornalley and Vasak demonstrated in 1985 that the MT molecule was an efficient hydroxyl radical scavenger (Thornalley and Vasak, 1985). Coogan *et al*, (1992) demonstrated a reduced number of DNA single strand breaks in cells with induced MT expression prior to exposure to Cd compared to non-induced controls. Glutathione (GSH) provides cellular protection against oxidative damage (Meister and Anderson, 1983). Several metals, including Cd, form complexes with GSH which is thought have a protective role against metal toxicity (Perrin and Watt, 1971). Thus, there is a potential for interaction between protective effects of MT and GSH. Depletion of GSH by treatment with buthionine sulfoximine (BSO) and diethylmaleate (DEM) can provide evidence for the involvement of GSH in antioxidant action (Meister, 1983; Griffith, 1982).

Measurment of DNA strand breakage using agarose gel electrophoresis has been applied in mammalian cell research (Wlodek *et al*, 1991) and in environmental monitoring studies (Theodorakis *et al*, 1992) to determine the genotoxicity of individual chemicals and chemical mixtures. Alkaline agarose gel electrophoresis involves subjecting partially purified DNA to electrophoresis to detect DNA fragmentation. Both single and double DNA strand breaks will result in the generation of fragments with greater electrophoretic mobility than intact

DNA.

In this study, we tested the hypothesis that planarian MT is protective against DNA strand breaks resulting from exposure to cadmium chloride. Planarian MT was induced by exposing the worms to zinc (500 µg/L) for 5 days prior to the experimental Cd treatment. We also evaluated the effectiveness of planarian MT in protecting against DNA fragmentation in the asexual planarian, *Dugesia dorotocephala*, with MT induction prior to glutathione depletion with either BSO or DEM.

Materials and methods

Chemicals

Bis-benzamide, calf thymus DNA, buthionine sulfoximine (BSO), diethylmaleate (DEM) and Chelex 100 were obtained from Sigma Chemical Company (St Louis, MO). Reagent grade nitric acid was obtained from Mallinckrodt (Phillipsburg, NJ). All other reagents including buffers and salts were reagent grade chemicals obtained from commercial vendors.

Animals

Laboratory stock cultures of asexual *Dugesia dorotocephala* were maintained in aerated tap water at 20^0 C ($\pm 2^0$ C) with a 12 hour light and dark cycle. Laboratory stock was fed raw beef liver twice weekly. Three days before treatment with zinc or Cd exposure, uniformly sized asexual planaria were segregated from stock cultures into round glass vessels (10.5 X 4.45 cm) containing soft reconstituted water. Soft reconstituted water was made fresh on a

weekly basis using 3 liters of ultra-pure water to which was added 144 mg sodium bicarbonate, 90 mg calcium sulfate, 90 mg magnesium sulfate and 6 mg potassium chloride (ATSM, 1992). After preparation, the soft reconstituted water was aerated for 12 hours prior to use with the planarian cultures.

Immediately after isolation, segregated planaria were fed raw beef liver and five hours later the test subjects were transferred to fresh soft reconstituted water. Isolated planaria were then maintained in a fasting state for the remainder of the experimental period. The water was changed in the culture dishes every 24 hours.

A minimum of three replicates were utilized for each experimental protocol. Replicates of 30 asexual planaria were used for MT level determination. An individual planarian was used for each DNA lane that was evaluated by the alkaline agarose gel electrophoretic technique.

Treatments

Five days before Cd exposure planaria were either pre-exposed to zinc (500 µg/L) in soft reconstituted water or the reconstituted water without Zn. They were then exposed to Cd at 0, 100, 300 or 2500 µg/L for periods ranging from 1 to 48 hours. For planaria treated for more than one day, the exposure solution was changed after the first 24 hours. Control planaria were maintained in the reconstituted water during the exposure period.

To deplete glutathione, planaria were treated with a combination of BSO (5 g/l) and DEM (2.35 g/L) in soft reconstituted water for 5 days with daily

renewal of the solution. Additional groups were also treated with zinc (500 µg/L) concurrently with the BSO/DEM mixture. Following these pre-treatments, the planaria were then exposed to Cd as described previously.

Other planaria were exposed to benzoyl peroxide (3 mg/L) with or without zinc pre-treatment as described above. Benzoyl peroxide exposures were conducted for different time periods up to 36 hours.

At the end of each exposure period, one set of worms and an appropriate set of controls were immediately prepared and analyzed for DNA single strand breaks. Another group of thirty worms per replicate with three replicates per exposure was used to determine MT levels.

Planarian MT Assay

Each replicate of planaria (n = 30) was isolated on ice and then homogenized in 500 µL of homogenization buffer containing 50 mM TRIS, 0.2 mM EDTA, 20 mM 2-mercaptoethanol and 0.05 mM phenylmethylsulfonyl fluoride (pH 8.0). Homogenization was accomplished using an ultrasonic cell disrupter (Sonifier Cell Disrupter, Heat Systems Company; power setting 4) using three 10 second bursts while keeping the tube on ice. The resulting homogenate was then centrifuged at 10,000 x g for 15 minutes to precipitate cellular debris. The pellet was retained for determination of planarian DNA content. The supernatant was removed with a pipette and heat treated in a boiling water bath for 20 minutes and then centrifuged at 16,000 x g for 10 minutes to precipitate heat denatured protein. The heat stable supernatant was then collected and the

protein content of the heat stable fraction determined using the Bradford dye binding protein assay (Bradford, 1976) using bovine serum albumin to construct standard curves. Aliquots of the heat treated cytosolic protein were stored at -80° C prior to the assay.

An aliquot of heat-treated planarian cytosol was thawed at room temperature and then treated with 0.02 mg Cd per mg protein. After 10 minutes, excess Cd in the sample was removed using Chelex 100 resin (iminodiacetic acid) as described by Bartsch *et al* (1990). The samples were then diluted with ultra-pure water and acidified using analytical grade concentrated nitric acid to bring the final sample acid concentration to 1% (v/v). The samples were then analyzed for Cd content using atomic absorption spectrophotmetry. Planarian MT was shown by earlier work to be the primary Cd binding protein found in the heat stable cytosolic fraction treated by this method. Based on the knowledge that 1 planarian MT molecule binds 7 molecules of cadmium (reported earlier), the amount of MT was then calculated and reported as pg MT/ pg planarian DNA.

DNA content determination

Because of the large and variable amounts of mucus produced by planaria when handled, accurate and reproducible body weight measurements are difficult. Data were normalized to planarian DNA content to avoid misleading values. Planarian DNA content from each replicate was measured from the homogenate pellet obtained as described above using a modification of the

method described by Labarca and Paigen (1980). Briefly, the sample was suspended in a phosphate-saline buffer (0.05 M sodium phosphate. 2.0 M sodium chloride and 3 mM EDTA, pH 7.4). Aliquots (100 µL) of the mixture were mixed with phosphate buffer (0.05 M sodium phosphate, 2.0 M sodium chloride, pH 7.4) containing 1 µg of bis-benzamide (Hoechst 33258) per ml. Fluorescence was measured using a fluorescence spectrophotometer (Hitachi F2000) measuring emission at 492 nm with excitation at 356 nm. A standard curve was prepared using calf thymus DNA (Sigma Chemical Company). Planarian DNA content for the alkaline gel elution assay was determined by the same protocol.

Alkaline agarose gel assay

The alkaline agarose gel assay was a modification of the method described by Theodorakis to evaluate DNA strand breakage in fish red blood cells (Theodorakis, 1994). Following timed exposure to varying doses of Cd, individual planaria were immediately placed into clean reconstituted soft water and then immersed in 100 µL of 0.5 N sodium hydroxide in a micro-centrifuge tube. The planarian was then minced with a sharpened spatula to facilitate digestion by the sodium hydroxide solution and incubated at room temperature for 2 hours. DNA content of the digested sample was quantified using bis-benzamide fluorescence as described above.

Planarian DNA (17 µg) was loaded in wells in a 1.5% agarose gel prepared in 50 mM sodium chloride and 4 mM EDTA. Wells were sealed with a

1% low melting point agarose gel and allowed to harden. The loaded agarose gels were then immersed in 600 ml of freshly prepared running buffer (30 mM sodium hydroxide and 30 mM EDTA) and allowed to incubate at 4^0 C for 1.5 hours. The gel was then electrophoresed at 7.5 volts per cm at 4^0 C for 1.5 hours. Following electrophoresis, the gel was neutralized in a 200 mM TRIS buffer (pH 7.0) for forty-five minutes. The neutralized gel was then stained with ethidium bromide (3 µg/ml) for 13 minutes, followed by destaining in deionized water for 24 hours. Gels were photographed on a UV illuminator and analyzed using a densitometer to determine the mean migration distance of the DNA in each lane of the gel on the photographic negative.

The mean migration distance (mm) for DNA that migrated out of the sample loading well was derived using densitometric measurements of the entire DNA lane (Photometrics 1D, full version). The software was used to integrate both intensity and area to determine the mean migration distance of the DNA in each lane. The average mean migration distance from the three replicates was calculated for each Cd dose and exposure period. A Hind III digestion of λ-phage DNA that consists of various DNA fragments of known molecular length was used as an internal standard and quality control for gel-to-gel variation. DNA from control planaria did not migrate out of the gel wells. Statistical analysis was performed using a two way ANOVA and the Bonferroni method for multiple comparisons to test for statistical significance.

Glutathione assay

For five days prior to determination of glutathione content, isolated planaria were exposed to 5 g/L BSO and 2.35 g/L DEM in 100 ml of soft reconstituted water, changing solutions every 24 hours. Glutathione was measured using a modification of the assay described by Martin and White (1991), which utilizes a NH_2 column and fluorescence detection to determine GSH levels (both reduced and oxidized) in deproteinized samples derivitized with iodoacetic acid and dansyl chloride. Standards of reduced glutathione were derivatized and analyzed under the same conditions to provide a standard curve.

Results

Agarose gel electrophoresis of DNA extracts from control planaria under alkaline conditions allowed assessment of the extent of DNA fragmentation (strand break frequency) in samples of individual planaria. Figure 4-1 is a photograph representative of the alkaline agarose gels used to quantify DNA strand breaks by densitometric analysis. Control planarian DNA produced a distinct band that did not migrate out of the gel well (Figure 4-1, lanes 1-3). There was a dose-related increase in the amount of DNA migration in samples from planaria not pre-treated with Zn (500 μg/L for 5 days) prior to being exposed to Cd at doses ranging from 100 to 2,500 μg/L for 12 hours (Figure 4-1, lanes 4-12). The extent of electrophoretic migration is directly related to the sizes of the DNA strand breaks. As a molecular size standard, Hind III digestion of λ-phage markers were included (lane 13, Figure 4-1). These markers contain DNA

fragments that range from approximately 6,600 to 23,00 base pairs. Pretreatment of the planaria with zinc (500 μg/L for 5 days) prior to being exposed to Cd doses that ranged from 100 to 2,500 μg/L for 12 hours attenuated DNA fragmentation (Figure 4-1, lanes 14-22). Planaria pre-treated only with Zn and not exposed to Cd (Figure 4-1, lanes 23-25) produced DNA that did not migrate out of the gel well indicating that as with the control DNA (Figure 4-1, lanes 1-3) there was no observable DNA fragmentation.

Planarian MT levels in worms pre-treated with zinc for 5 days increased from 10.1 pg MT/pg planarian DNA to 65.2 pg MT/ pg planarian DNA, which represents a 6.5 fold increase in protein levels (Table 4-1). Worms pre-exposed to Zn (500 μg/L for 5 days) and then exposed to Cd (100 to 2,500 μg/L) were shown to have an additional 37 to 49 percent increase in MT levels that appear to be related to the Cd exposure alone (Table 4-1). Planaria exposed only to Cd (with no Zn pre-treatment) demonstrated greater increases in MT levels, ranging from 4.1- to 4.7-fold higher after 48 hours of Cd treatment (Table 4-1).

Exposure to Cd at 100, 300 and 2,500 μg/L for 1 hour resulted in mean migration distances of 7.7, 7.8 and 9.6 mm for planarian DNA from planaria not pre-treated with Zn, respectively. In contrast, mean migration distances of Zn pre-treated planarian DNA were 3.4, 5.2 and 5.9 mm, respectively (Figures 4-2, 4-3 and 4-4). Planarian MT levels in the non-pretreated planaria were 10.2, 13.3 and 16.3 pg planarian MT/pg planarian DNA, compared to 66.6, 73.61 and 74.7 pg planarian MT/pg planarian DNA in the Zn pre-treated worms (Table 4-1).

The DNA from control planaria exposed only to soft reconstituted water and the DNA from worms pre-exposed to Zn but not Cd did not migrate of their respective agarose gel wells with the alkaline agarose gel electrophoresis assay (Figure 4-1). DNA from planaria given the control pre-treatment followed by exposure to Cd at 100, 300 or 2,500 µg/L for 12 hours had mean migration distances of 3.6, 5.6 and 5.9 mm respectively. In comparison, the mean migration distance for planarian DNA from Zn-pretreated worms exposed to the same Cd concentrations for 12 hours had mean migration distances of 1.6, 1.8 and 2.43 mm respectively (Figures 4-2, 4-3 and 4-4). These differences were accompanied by increases in MT levels in the zinc pretreated groups of 4.5-, 3.8 and 3.4-fold for the 100, 300 and 2,500 µg/L Cd treatment groups, respectively, compared with their control pretreatment groups (Table 4-1). Similar differences in MT content after 1 hr or 48 hr of Cd treatment were observed between control and zinc pretreated planaria (Table 4-1). There were increased amounts of DNA damage associated with Cd exposure at 100, 300 and 2,500 µg/L in planarian not pre-treated with zinc over that measured in planarian DNA pretreated with zinc (Figures 4-2,4-3,4-4).

The mean migration distance for DNA from planaria not pre-exposed to Zn but exposed to Cd for 48 hours at 100, 300 or 2,500 µg/L was 3.4, 3.9 and 3.6 mm, respectively, compared to mean migration distances of 2.5, 3.3 and 3.4 mm for DNA isolated from Zn pre-treated worms exposed to the same Cd doses (Figures 4-2, 4-3 and 4-4). Planarian MT levels in the non-pretreated planaria

measured at 48 hours ranged from 26.8 to 30.7 pg planarian MT/pg planarian DNA for worms exposed to 100 and 2,500 μg/L Cd, respectively (Table 4-1). The MT content of planaria pre-exposed to Zn prior to exposure to 100 or 2,500 μg/L Cd was found to range from 88.5 to 96.1 pg planarian MT/pg planarian DNA.

DNA from worms not pre-treated with Zn showed 2.3-, 3.1- and 2.4-fold increases in DNA damage relative to DNA from planaria pretreated with Zn following a 12 hour exposure to 100, 300 or 2,500 μg Cd/L, respectively (Figure 4-5). Accompanying this decrease in DNA damage there were corresponding 4.2-, 3.8- and 3.4 fold increases in planarian MT levels (Table 4-1). At 48 hours the only significant difference in DNA fragmentation was observed at 100 μg Cd/L (Figure 4-6). It should be noted that there are significant increases in the levels of the planarian MT levels after 48 hours of Cd exposure, regardless of the Zn treatment status (Table 4-1).

Exposure to the BSO/DEM mixture (5.0 and 2.35 g/L, respectively) for 5 days lowered GSH levels to 20% of that found in control worms. The BSO/DEM treatment was associated with an increase in MT levels from 6.6 to 8.6 pg planarian MT/ pg planarian DNA (Tables 4-1 and 4-2). All planaria died when pre-treated with the BSO/DEM mixture and then exposed to Cd at a concentration of 2,500 μg/L for more than 36 hours. Survival was good at this Cd concentration in planaria pretreated with BSO/DEM if Cd exposures lasted no longer than 24 hours. No mortality was observed in BSO/DEM treated worms

that were exposed to Cd concentrations less than 2500 µg/L for up to 48 hours.

Glutathione depletion by the BSO/DEM mixture followed by exposure to 100 and 300 µg Cd/L for up to 48 hours resulted in a significant increase in DNA damage at all time points measured (Figures 4-7and 4-8). When exposed to 2500 µg/L Cd, the amount of DNA damage did not increase significantly as a result of glutathione depletion until 24 hours of exposure (Figure 4-9). The BSO/DEM pretreatment resulted in 3.0-and a 2.2-fold increases in MT levels, respectively, after 48 hours of exposure of planaria exposed to 100 and 300 µg/L Cd (Table 4-2).

Samples of DNA from planaria pre-treated with the BSO/DEM mixture but not pretreated with zinc exposed to 100, 300 or 2,500 µg Cd/L for 12 hours had mean migration distances of 2.6, 4.3 and 5.9 mm compared to the mean migration distances of 1.7, 1.9 and 2.7 mm , respectively, of DNA from planaria depleted of GSH activity, and pre-treated with Zn (500 µg/L for 5 days) prior to Cd exposure for 12 hours (Figures 4-10,4-11 and 4-12). There was a significant decrease in the mean migration distance of DNA from planaria with depleted GSH activity, pre-treated with Zn prior to Cd exposure when compared to the mean migration distance for DNA from GSH depleted planaria not pre-treated with Zn at all time intervals and Cd levels measured except at the 36 hour 100 µg Cd/L level (Figures 4-10, 4-11 and 4-12). At the 12 hour exposure period, MT levels in planaria exposed to 100, 300 and 2,500 µg Cd/L depleted of GSH activity, and pre-treated with Zn were 74.1, 80.2 and 89.0, respectively,

compared to planarian MT levels of 18.9, 21.9 and 25.7 pg planarian MT/pg planarian DNA in GSH depleted, non Zn pre-treated planaria exposed to the same Cd concentrations (Table 4-2).

Comparison of the DNA fragmentation dose response curves for the different levels of Cd exposure at 12 hours for GSH depleted, Zn pre-treated planaria demonstrates a significant decrease in DNA damage at all levels of exposure compared to GSH depleted planaria without Zn pre-treatment (Figure 4-13). Accompanying this decrease in DNA damage was a significant increase in planarian MT levels (Table 4-2).

Benzoyl peroxide was used to induce DNA strand breaks in planaria with and without zinc pretreatment (Figure 4-14). Exposure to 3 mg/L benzoyl peroxide caused DNA strand breaks with a frequency comparable that reported for 300 µg Cd/L (Figure 4-3). The protective effect of Zn pre-treatment was similar to that reported to that reported for planaria treated with Cd at 12 hours of exposure to the oxidant; the mean migration distance for DNA from planaria not pre-treated with Zn (500 μg/L for 5 days) was 5.5 mm compared to 2.3 mm in DNA from worms pre-treated with Zn. The zinc pre-treatment increased the planarian MT from the non-pre-treatment levels of 10.2 pg planarian MT/pg planarian DNA to 75.31 pg planarian MT/pg planarian DNA after 12 hours of exposuree to the oxidant, benzoyl peroxide. There were significantly higher levels of DNA fragmentation at all the times measured except at the 36 hour evaluation (Figure 4-14).

Discussion

Although there is evidence that MT is protective against Cd toxicity in mammalian systems (Hamer, 1986; Coogan *et al*, 1992), a similar role for planarian MT has not previously been demonstrated. Earlier research with *Dugesia dorotocephala* demonstrated that Cd exposure resulted in a high incidence of malignant carcinomas (Hall *et al*, 1986). Although the mechanisms of Cd carcinogenesis are unknown, possible pathways may involve either direct or indirect interactions between Cd and the DNA molecule. The genotoxic potential of Cd has been well documented in cultured cells and mammalian systems (Swierenga *et al*, 1987). Available evidence suggests that DNA damage appears to result from either free radical generation (Ochi *et al*, 1983; Snyder, 1988) or possibly the result of a direct reaction between the DNA molecule and the Cd ion. The purpose of the present study was to determine if Cd exposure causes DNA strand breaks in asexual planaria and if increased expression of the recently characterized planarian MT would reduce Cd-induced DNA fragmentation.

Results of the present study indicate that induction of the planarian MT with zinc (Table 4-1) was associated with reduced DNA damage due to Cd exposure (Figures 4-2 through 4-6). Coogan *et al* (1992) showed that prior induction of MT in TRL-1215 cells (derived from the livers of 10-day old Fischer 344 rats) reduced the number of DNA strand breaks in cells exposed to Cd compared to non-induced cells.

As with mammalian MT, the protective role of planarian MT may involve factors other than the simple sequestration of available Cd. Earlier research assessing the role of MT as a radical scavenger (Thornalley and Vasak ,1985) showed that the MT protein was an efficient hydroxyl radical and superoxide anion radical scavenger. Abel and DeRuiter (1989) demonstrated that MT has the ability to inhibit hydroxyl radical generated DNA damage. In addition, when looking at the DNA damage caused by the Cd ion, Ochi *et al,* (1983) and Snyder (1988) both suggested a role for active oxygen species in Cd induced DNA strand damage. Metallothionein has been detected in the cell nucleus, in addition to being found in the cytosol (Nartley *et al,* 1987), which may help explain the decrease in DNA damage noted in this research. Further work by Ochi (1985) implies that DNA strand breaks induced by Cd were suppressed by scavengers of reactive oxygen species, suggesting that reactive oxygen species mediate Cd induced genotoxicity.

Planarian MT appears to be capable of acting as a reactive oxygen scavenger as well. The extent of DNA strand breaks in planaria treated with benzoyl peroxide was diminished by prior treatment with zinc (Figure 4-14). The increase in MT levels observed was consistent with the ability of planarian MT to protect against oxidative DNA damage caused by this oxidant Tables 4-1 and 4-2).

Previous investigations have demonstrated that exposure to small doses of Cd or zinc result in the induction of MT in mammalian systems (Waalkes *et al,*

1998; Goering and Klassen, 1984), which appears to correlate directly with a reduction in both acute and chronic Cd toxicity. Like mammalian MT, levels of the planarian MT are also increased by exposure to zinc and are associated with attenuated effects of Cd toxicity. It is possible that the loss of this effect with longer periods of Cd exposure may be due to the ability of the planarian to remove damaged cells and regenerate severed parts. In order to be able to accomplish this process there are a large number of stem cells in the system and it is possible that cells that were damaged by Cd and/or the oxidant exposure are removed and replaced within the 48 hour time period. It is also possible that planarian DNA repair may also have a role in this process.

Glutathione is known to participate in a number of cellular processes including the protection of cells against certain toxic compounds, oxidative damage and radiation (Miester and Anderson, 1983). In this research, depletion of GSH with the use of BSO and DEM allowed us to further evaluate the effect of the planarian MT in protecting planarian DNA against fragmentation caused by Cd exposure (Figures 4-7 through 4-9). Depletion of GSH in planaria treated with BSO and DEM was associated with elevated levels of DNA strand breaks caused by Cd exposure. This increase in DNA damage was attenuated by zinc pre-treatment. Again, the protective effect was associated with increased levels of MT in the planaria (Tables 4-1 and 4-2), suggesting that planarian MT is capable of substituting for the DNA-protective effect of GSH. In earlier research when Chinese hamster V79 cells were exposed to sub-lethal doses of Cd they

also exhibited elevated MT levels and fewer DNA strand breaks (Mitchel and Russo, 1987). Similar results were also shown when Chinese hamster V79 cells were treated with BSO to deplete GSH activity (Mitchel and Russo, 1987). One hundred percent mortality was observed when *D. dorotocephala* were treated with the BSO/DEM mixture and then exposed to Cd at 2500 µg/L for more than 36 hours. This is consistent with earlier research that showed that depletion of GSH by exposure to BSO rendered Chinese hamster V79 cells more susceptible to Cd exposure (Ochi *et al*, 1988).

In conclusion, this research demonstrated that pretreatment with zinc prior to Cd exposure for varying periods of time up to 48 hours significantly reduced the frequency of strand breaks in planarian DNA. This protective effect was associated with significant increases in the level of planarian MT. Our results cannot distinguish between the possible DNA-protective roles of planarian MT as a Cd binding molecule or as an antioxidant. It would appear to have both capabilities, as indicated by its ability to protect against DNA damage caused by benzoyl peroxide as well as the affinity for Cd demonstrated previously (Chapter 3). These observations suggest that planarian MT has protective activities analogous to mammalian metallothioneins. Thus, *Dugesia dorotocephala* may represent a useful model for the investigation of Cd carcinogenesis.

Table 4-1. Comparison of planarian MT levels (pg MT/pg planarian DNA) in asexual *Dugesia dorotocephala* exposed to cadmium at 100 μg/L, 300 μg/L or 2500 μg/L for time periods ranging from 1 to 48 hours.

	Exposure Period		
Cd Treatment	1 Hour Exposure	12 Hour Exposure	48 Hour Exposure
Controls (no Zn pretreatment)			
Control values no Zn pretreatment or Cd exposure 6.6 ± 0.1 [a]			
100 μg/L Cd	10.2 ± 0.8	17.6 ± 0.8 [b]	26.8 ± 1.1 [b]
300 μg/L Cd	13.3 ± 0.2	20.8 ± 0.6 [b]	29.8 ± 0.7 [b]
2500 μg/L Cd	16.3 ± 0.7 [b]	23.9 ± 0.6 [b]	30.7 ± 0.9 [b]
Pre-treated with Zinc [c]			
Zn pretreatment control (no Cd exposure) 64.4 ± 0.7			
100 μg/L Cd	66.6 ± 1.6	73.4 ± 0.8 [b]	88.5 ± 0.3 [b]
300 μg/L Cd	73.6 ± 0.6	79.5 ± 0.8 [b]	92.2 ± 1.3 [b]
2500 μg/L Cd	74.4 ± 3.5	81.7 ± 0.2 [b]	96.1 ± 0.9 [b]

[a] Expressed as pg MT/pg planarian DNA. Specific MT content was calculated based on a Cd content of 7 μmole of cadmium per μmole of the protein. Data expressed as mean ± standard error. There were 7 individual groups of 30 asexual *Dugesia dorotocephala* used to calculate each reported value.

[b] Significantly different from respective controls of asexual *D. dorotocephala* using ANOVA $p>0.05$ and the Bonferroni method for multiple comparisons.

[c] Exposed to zinc at a dose of 500 μg/L for 5 days before exposure to different Cd dosages for time periods up to 48 hours.

Table 4-2. Effects of Zn pre-treatment on MT concentrations in asexual *D. dorotocephala* pre-treated with BSO and DEM prior to Cd exposure.

	Exposure Period		
Cd Treatment	1 Hour Exposure	12 Hour Exposure	48 Hour Exposure
Pre-treated with BSO/DEM[a] but not with Zn[c] (No Cd exposure)			
Control values following BSO/DEM pre-treatment 8.6 ± 0.6[b]			
100 µg/L Cd	9.0 ± 0.9	18.9 ± 0.4[d]	26.9 ± 0.4[d]
300 µg/L Cd	13.8 ± 1.2	21.9 ± 1.5[d]	30.8 ± 0.7[d]
2,500 µg/L Cd	16.3 ± 1.0	25.7 ± 0.8[d]	All worms died
Pre-treated with BSO/DEM[a] and Zn[b] (No Cd exposure)			
Control values following BSO/DEM and Zn pre-treatment 62.3 ± 0.2[d]			
100 µg/L Cd	65.7 ± 1.6	74.1 ± 1.8[d]	90.1 ± 1.1[d]
300 µg/L Cd	72.3 ± 0.7	80.2 ± 0.8[d]	93.5 ± 1.3[d]
2,500 µg/L Cd	79.1 ± 0.8	89.0 ± 1.0[d]	All worms died

[a] Pre-treated with BSO (5 g/L) and DEM (2.35 g/L) for 5 days before exposing the worms to the different Cd dosages for time periods up to 48 hours.

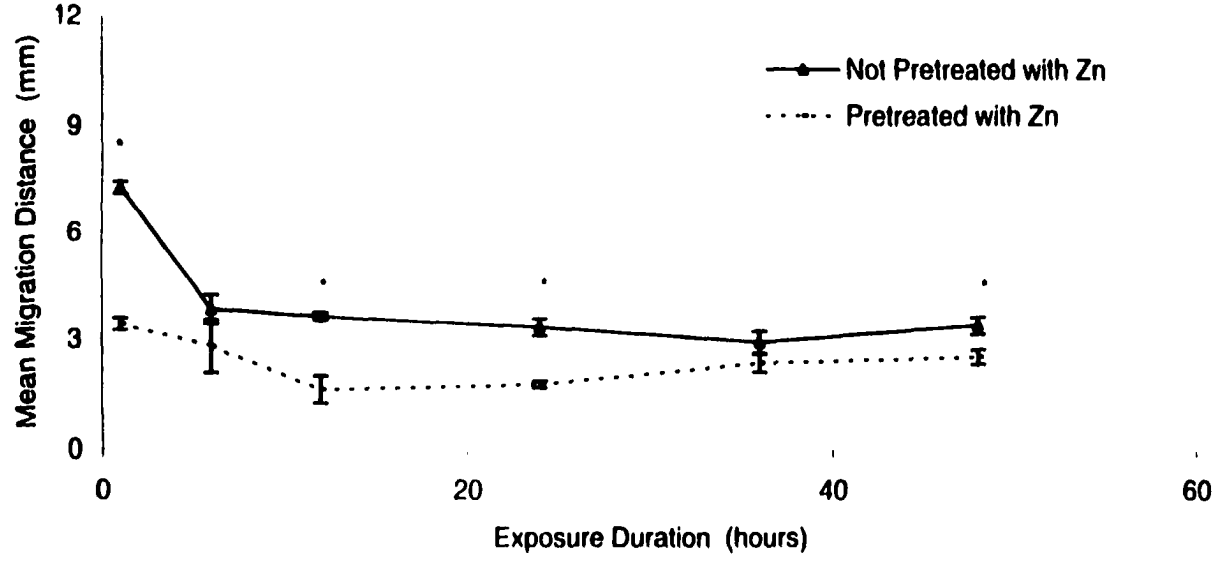
[b] Expressed as pg planarian MT/pg planarian DNA. Specific planarian MT content was calculated based on a CD content of 7 µmole of CD per µmole of the planarian protein. Data expressed as mean ± standard error. There were 7 individual groups of 30 asexual Dugesia dorotocephala used to calculate each reported value.

[c] Pre-treated with zinc at a dose of 500 µg/L for 5 days before exposure to Cd.

[d] Significantly different from controls depleted of glutathione activity both pre-treated and non pre-treated with zinc using ANOVA $p < 0.05$ and the Bonferroni method for multiple comparisons.

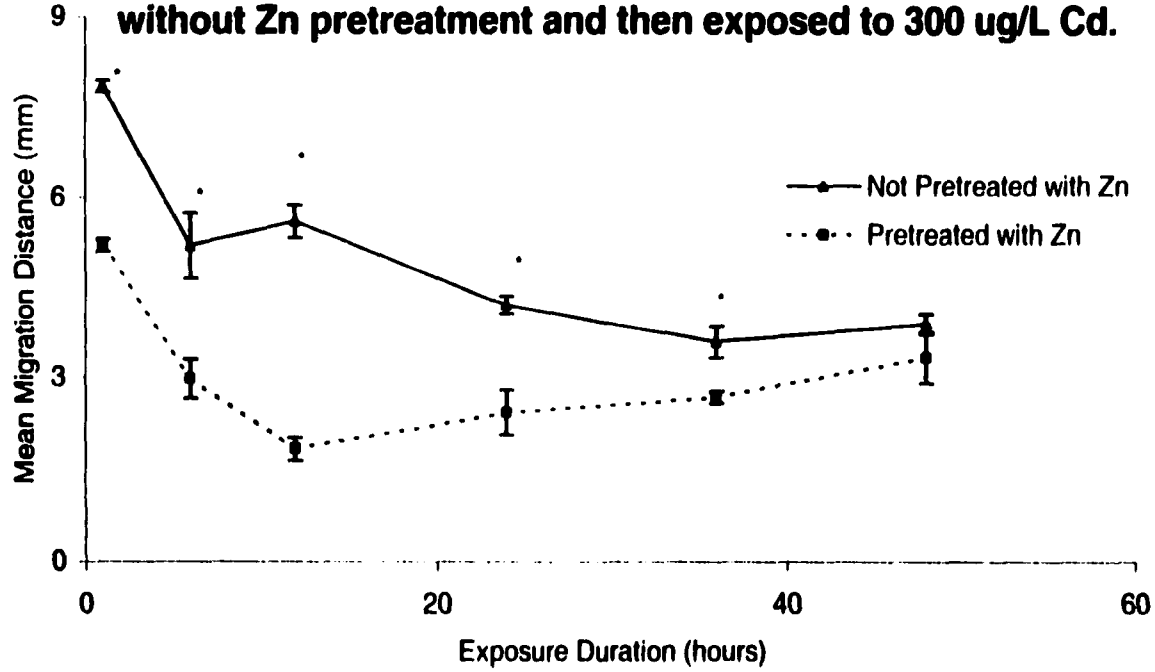
Figure 4-1. Photograph of planarian DNA in an alkaline agarose gel stained with ethidium bromide. The DNA in each lane was obtained from an individual D. dorotocephala. The photograph shows the effects of treatment with different Cd concentrations over a 12-hour exposure period in untreated worms (lanes 4-12) and planaria pretreated with Zn (500 μg/L) for 5 days (lanes 14-22). Controls without Zn or Cd exposure are found in lanes (1-3) and planaria treated with Zn but not Cd in lanes (23-25). The DNA in lane 13 is from a molecular marker (Hind III digest of λ-phage DNA). Planaria were exposed for 12 hours to 2500 μg/L Cd (lanes 4-6 and 14-16), to 300 μg/L Cd (lanes 7-9 and 17-19) or 100 μg/L Cd (lanes 10-12 and 20-22).

Figure 4-2. Fragmentation of planarian DNA from worms with or without Zn pretreatment and then exposed to 100 ug/L Cd.



Time course of DNA fragmentation in asexual *D. dorotocephala* exposed to 100 ug/L Cd. Planaria were treated with 100 ug/L Cd for up to 48 hr with (induced) or without (non-induced) pretreatment with 500 ug/L Zn for 5 days. DNA fragmentation was assayed using alkaline agarose electrophoresis and reported as the average mean migration distance (mm) of planarian DNA. Control DNA samples did not migrate out of their respective gel wells.
* Significantly different from the other treatment group at the same time point, $p < 0.05$, ANOVA and the Bonferroni method for multiple comparisons.

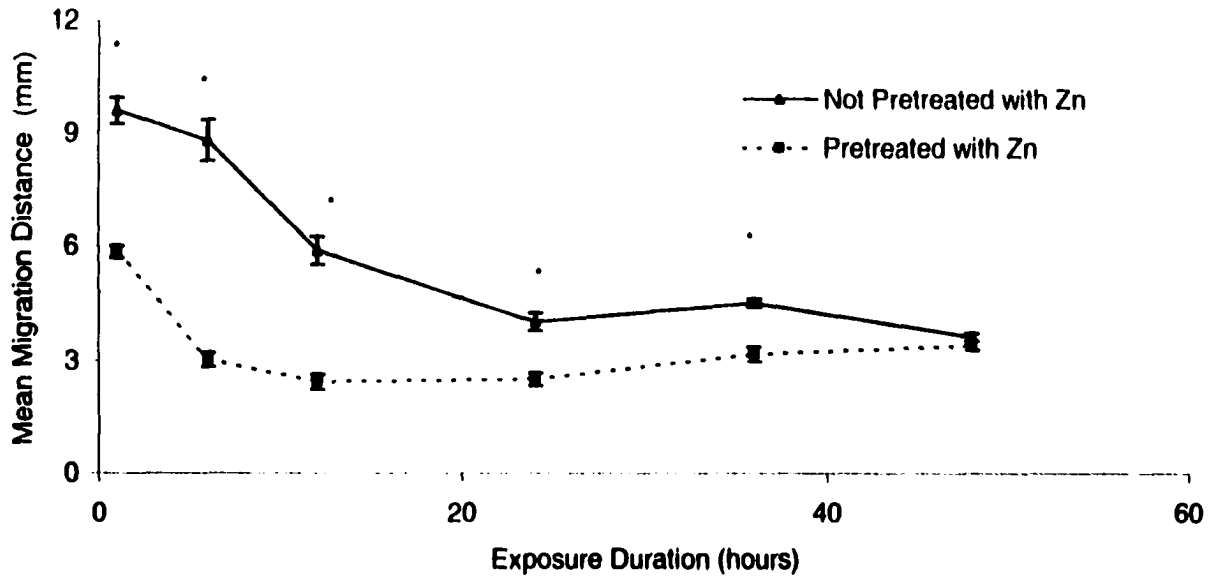
Figure 4-3. Fragmentation of planarian DNA from worms with and without Zn pretreatment and then exposed to 300 ug/L Cd.



Time course of DNA fragmentation in asexual *D. dorotocephala* exposed to 300 ug/L Cd. Planaria were treated with 300 ug/L Cd for up to 48 hr with (induced) or without (non-induced) pretreatment with 500 ug/L Zn for 5 days. DNA fragmentation was assayed with alkaline agarose gel electrophoresis and reported as the average mean migration distance (mm) of planarian DNA. DNA controls did not migrate out of their respective gel wells.

* Significantly different from the other treatment group at the same time point, $p < 0.05$, ANOVA and the Bonferroni method for multiple comparisons.

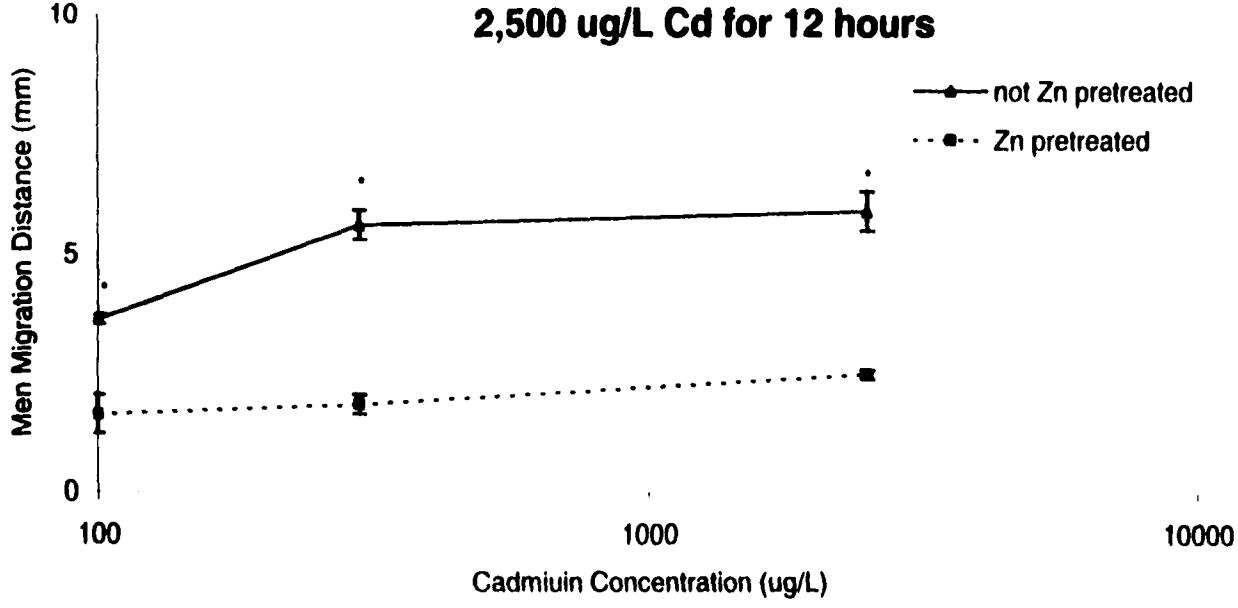
Figure 4-4. Fragmentation of planarian DNA from worms with and without Zn pretreatment and then exposed to 2500 ug/L Cd.



Time course of DNA fragmentation in asexual *D. dorotocephala* exposed to 2500 ug/L Cd. Planaria were treated with 2500 ug/L Cd for up to 48 hr with (induced) or without (non-induced) pretreatment with 500 ug/L Zn for 5 days. DNA fragmentation was assayed using alkaline agarose gel electrophoresis and reported as the average mean migration distance (mm) of planarian DNA. Control DNA samples did not migrate out of their respective gel wells.

* Significantly different from the other treatment group at the same time point, $p < 0.05$, ANOVA and the Bonferroni method for multiple comparisons.

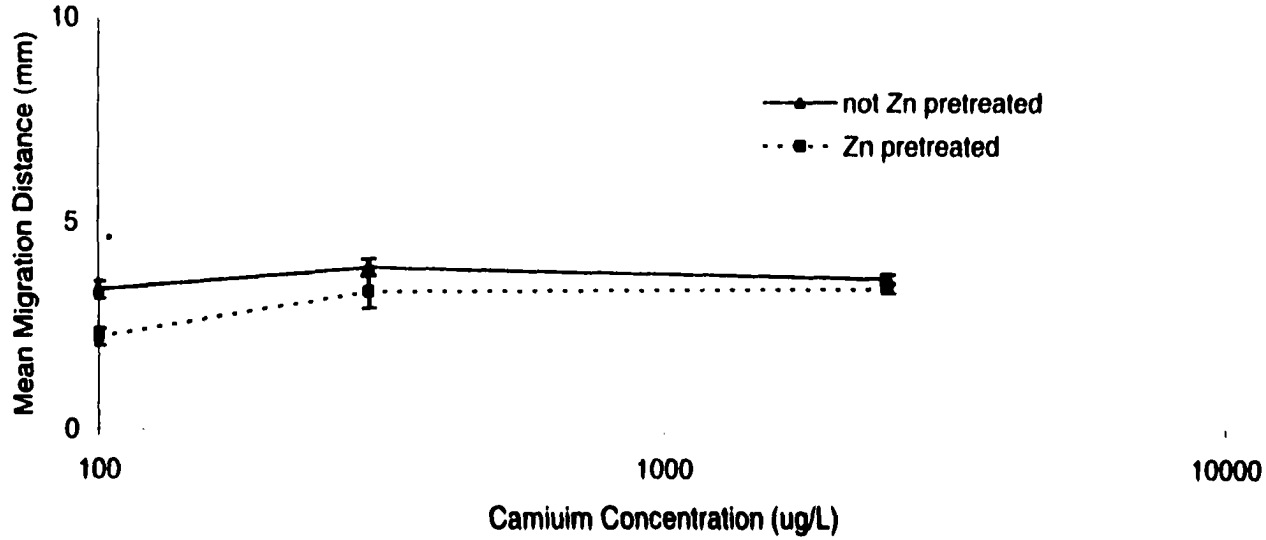
Figure 4-5 Comparison of the mean migration distance of DNA from planaria with and without Zn pre-treatment exposed to 100, 300 and 2,500 ug/L Cd for 12 hours



DNA fragmentation resulting from exposure to 100, 300 and 2,500 ug/L Cd for 12 hours in asexual *Dugesia dorotocephala* with and without Zn pre-treatment (500 ug, 5 days) after which they were exposed to 100, 300 or 2,500 ug Cd for 12 hours. DNA damage was evaluated by alkaline gel electrophoresis measuring the mean migration distance (mm) of planarian DNA. Control DNA samples did not migrate out of their respective gel wells.

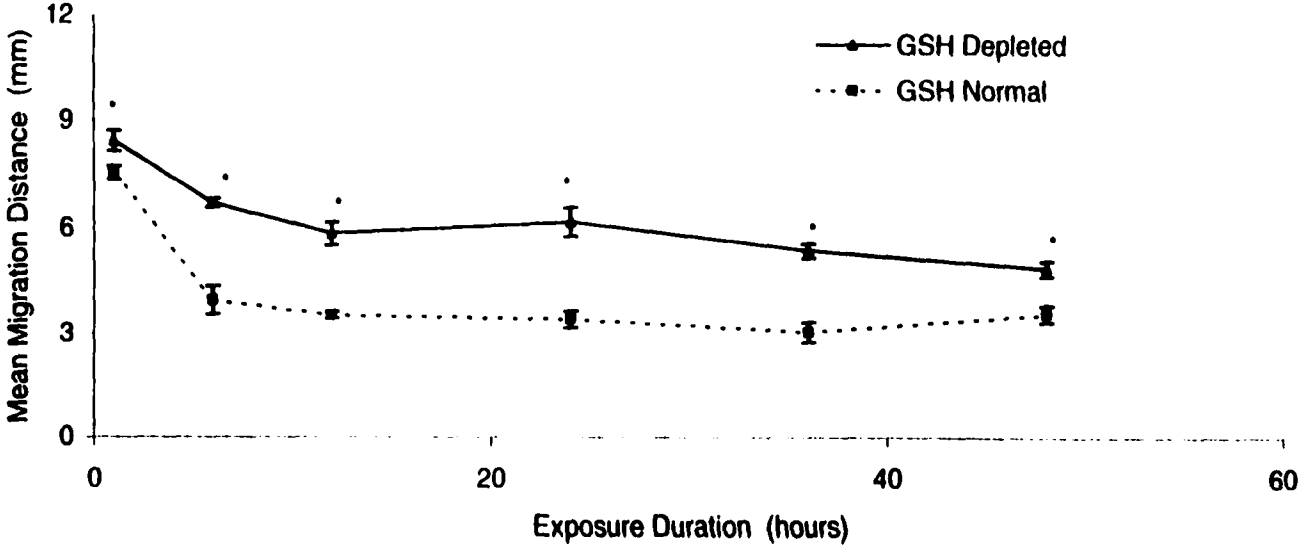
* Significantly different from the other treatment group at the same Cd treatment concentration, $p < 0.05$, ANOVA and the Bonferroni method for multiple comparisons.

Figure 4-6 Comparison of the mean migration distance of DNA from planaria with and without Zn pre-treatment exposed to 100, 300 and 2,500 ug/L Cd for 48 hours



DNA fragmentation resulting from exposure to 100, 300 and 2,500 ug/L Cd for 12 hours in asexual Dugesia dorotocephala with and without Zn pre-treatment (500 ug, 5 days) after which they were exposed to 100, 300 or 2,500 ug Cd for 48 hours. DNA damage was evaluated by alkaline gel electrophoresis measuring the mean migration distance (mm) of planarian DNA . Control DNA samples did not migrate out of their respective gel wells. *Significantly different from the other treatment group at the same Cd treatment concentration, $p < 0.05$, ANOVA and the Bonferroni method for multiple comparisons.

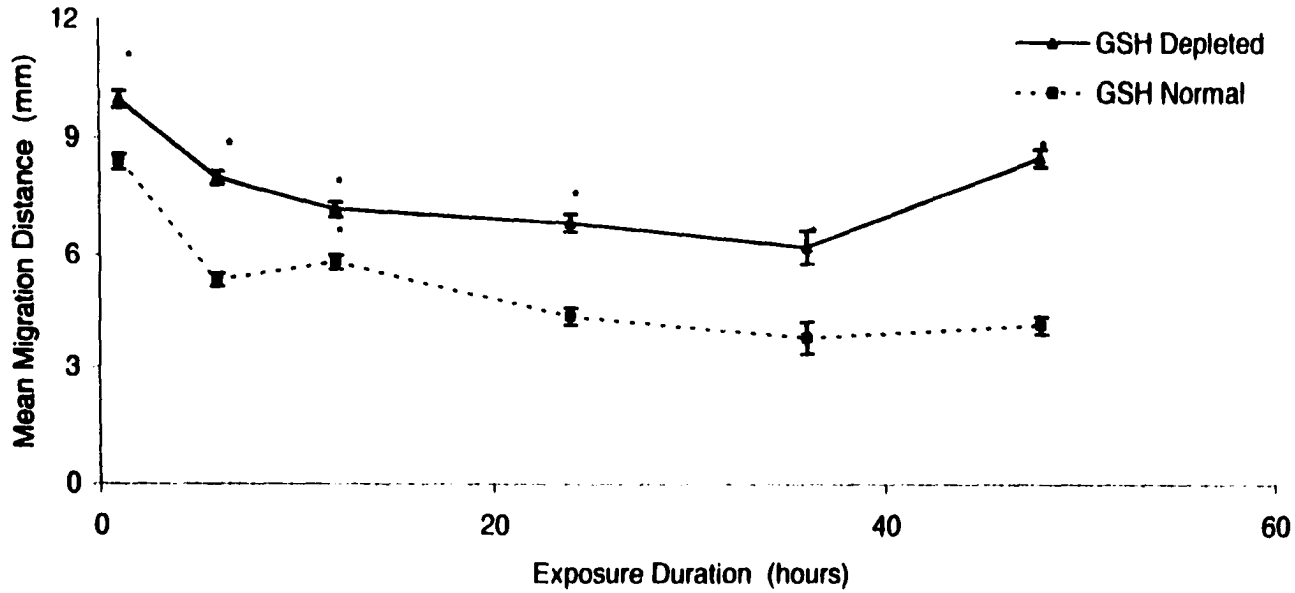
Figure 4-7. Fragmentation of planarian DNA from worms with and without BSO/DEM pretreatment and then exposed to 100 ug/L Cd.



DNA fragmentation resulting from exposure to Cd 100 ug/L in asexual *D. dorotocephala* with depleted glutathione levels. Planarian glutathione levels were depleted by pretreatment for 5 days to a BSO/DEM mixture (5.0 and 2.35 g/L, respectively) following which the worms were exposed to 100 ug/L Cd for time periods up to 48 hours. DNA damage was evaluated by alkaline gel electrophoresis measuring the mean migration distance (mm) of planarian DNA. Control DNA samples did not migrate out of their respective gel wells.

* Significantly different from the other treatment group at the same time point, $p < 0.05$, ANOVA and the Bonferroni method for multiple comparisons.

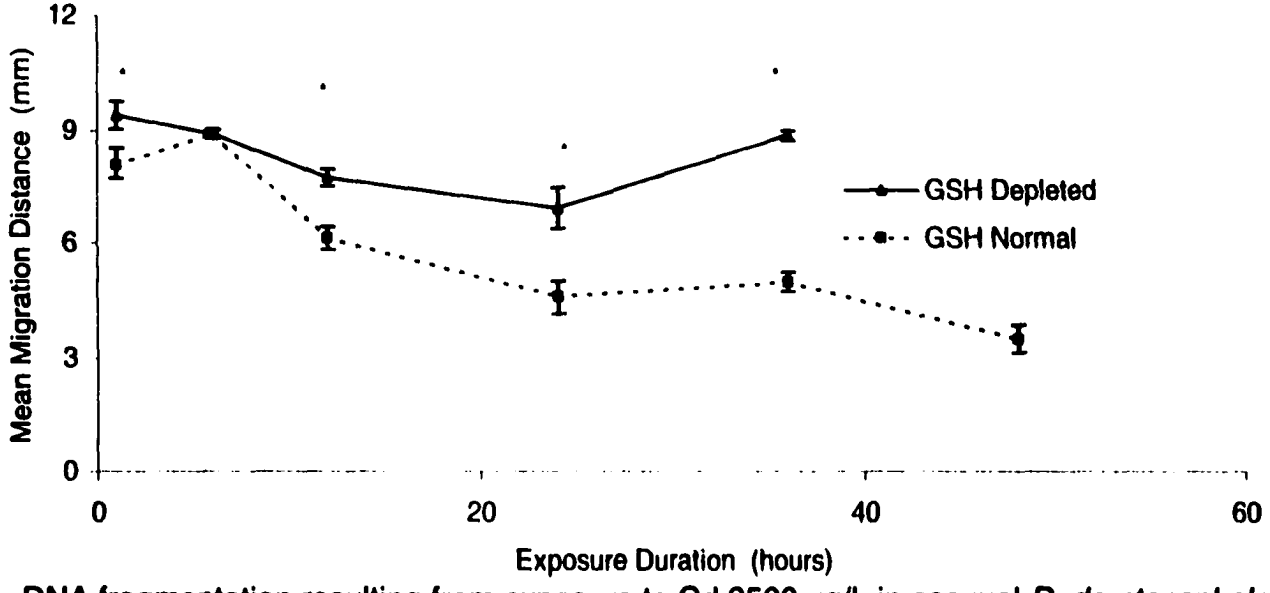
Figure 4-8. Fragmentation of planarian DNA from worms with and without BSO/DEM pretreatment and then exposed to 300 ug/L Cd.



DNA fragmentation resulting from exposure to Cd 300 ug/L in asexual *D. dorotocephala* with depleted glutathione levels. Planarian glutathione levels were depleted by pretreatment for 5 days to a BSO/DEM mixture (5.0 and 2.35 g/L, respectively) following which the worms were exposed to 300 ug/L Cd for time periods up to 48 hours. DNA damage was evaluated by alkaline gel electrophoresis measuring the mean migration distance (mm) of planarian DNA. Controll DNA samples did not migrate out of their respective gel wells.

* Significantly different from the other treatment group at the same time point, $p < 0.05$, ANOVA and the Bonferroni method for multiple comparisons.

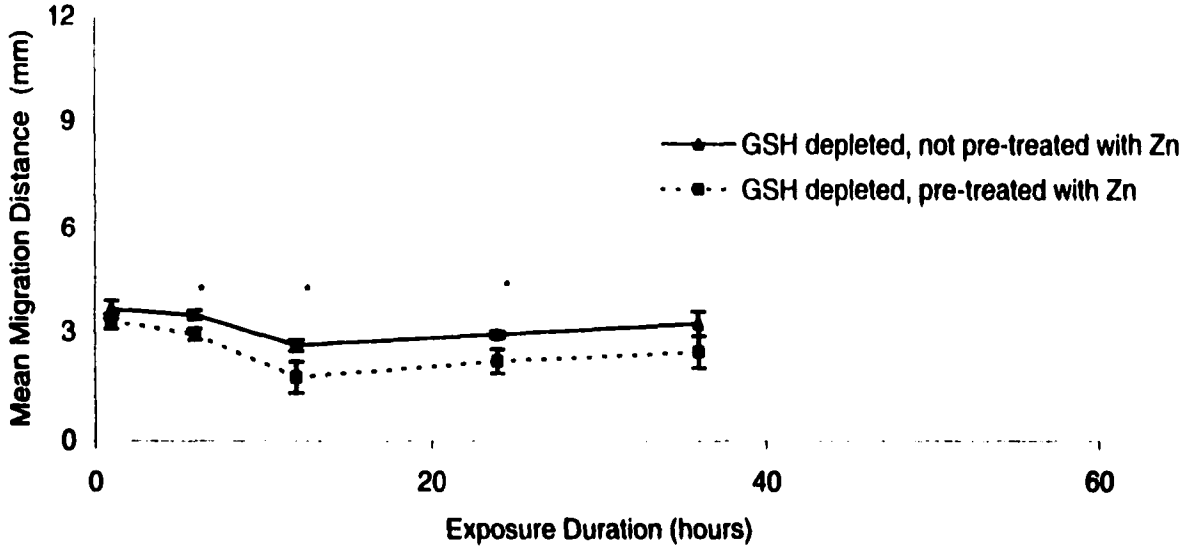
Figure 4-9. Fragmentation of planarian DNA from worms with and without BSO/DEM pretreatment and then exposed to 2500 ug/L Cd.



DNA fragmentation resulting from exposure to Cd 2500 ug/L in asexual *D. dorotocephala* with depleted glutathione levels. Planarian glutathione levels were depleted by pretreatment for 5 days to a BSO/DEM mixture, 5.0 and 2.35 g/L, respectively, following which the worms were exposed to 2500 ug/L Cd for time periods up to 48 hours. DNA damage was evaluated by alkaline gel electrophoresis measuring the mean migration distance (mm) of planarian DNA. Controll DNA samples did not migrate out of their respective gel wells.

* Significantly different from the other treatment group at the same time point, $p < 0.05$, ANOVA and the Bonferroni method for multiple comparisons.

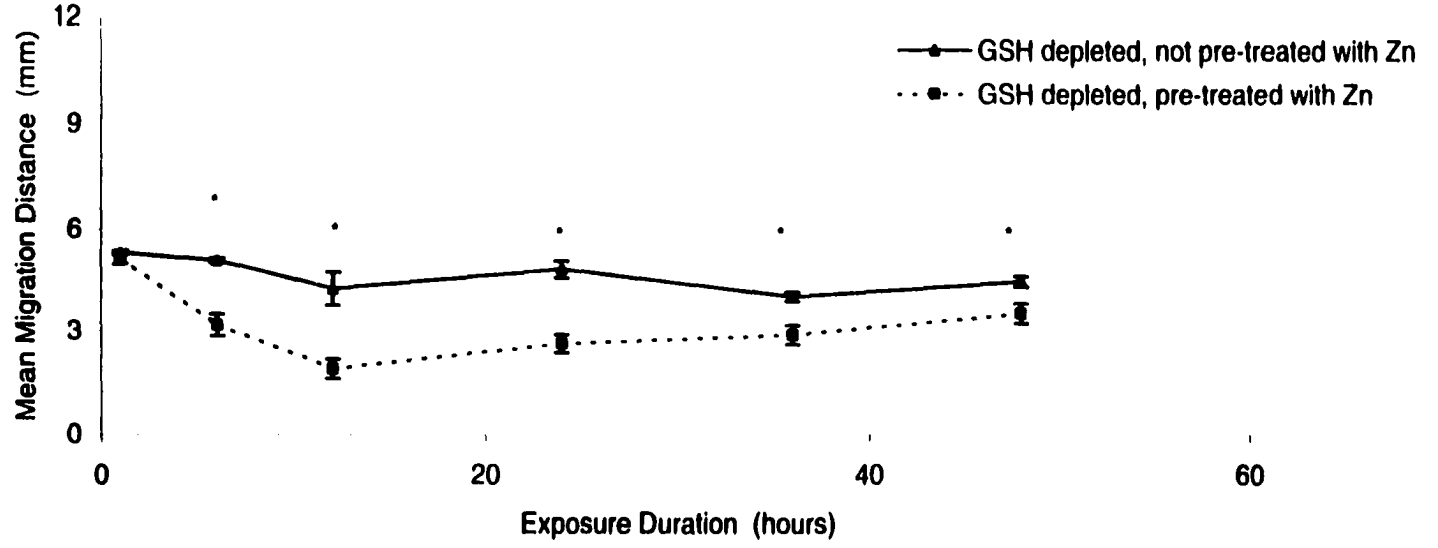
Figure 4-10. Fragmentation of planarian DNA from worms pretreated with BSO/DEM with and without Zn pretreatment then exposed to 100 ug/L Cd.



DNA fragmentation resulting from exposure to Cd at 100 ug/L in asexual *Dugesia dorotocephala* with BSO/DEM pretreatment (5.0 and 2.35 g/L, respectively for 5 days) with and without Zn pre-treatment (500 ug/L, 5 days) after which they were exposed to Cd over a 36 hour time frame. DNA damage was evaluated by alkaline gel electrophoresis measuring the mean migration distance (mm) of planarian DNA . Control DNA samples did not migrate out of their respective gel wells.

* Significantly different from the other treatment group at the same time point, $p < 0.05$, ANOVA and the Bonferroni method for multiple comparisons.

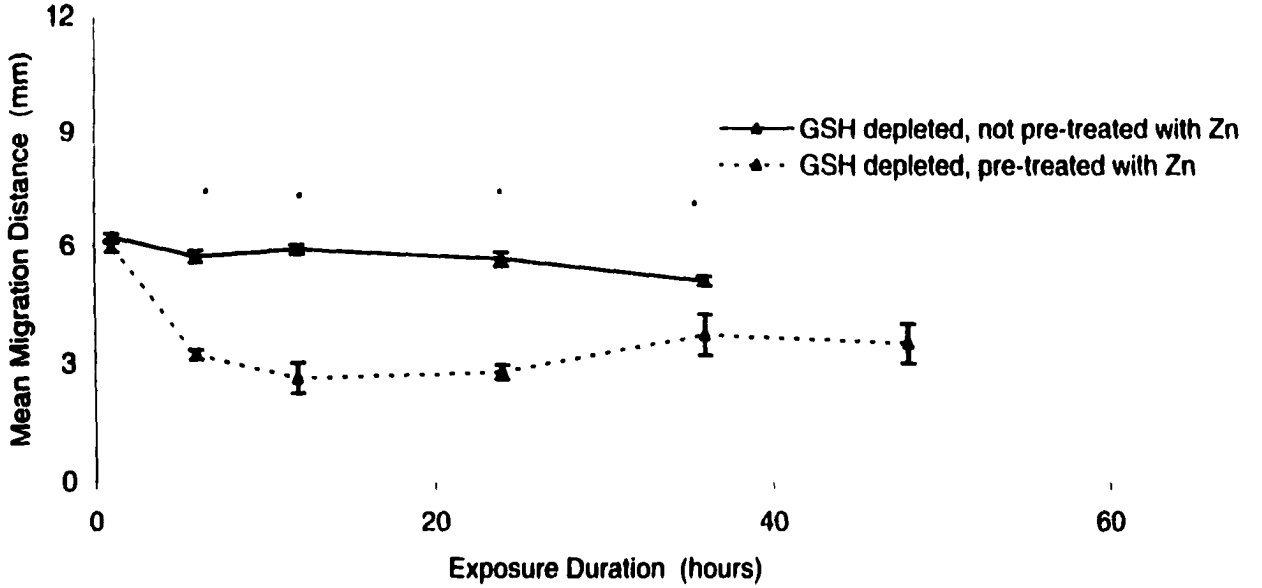
Figure 4-11. Fragmentation of planarain DNA from worms pretreated with BSO/DEM, with and without Zn pretreatment then exposed to 300 ug/L Cd.



DNA fragmentation resulting from exposure to Cd at 300 ug/L in asexual Dugesia dorotocephala with BSO/DEM pretreatment (5.0 and 2.35 g/L, respectively for 5 days) with and without Zn pre-treatment (500 ug, 5 days) after which they were exposed to Cd over a 36 hour time frame. DNA damage was evaluated by alkaline gel electrophoresis measuring the mean migration distance (mm) of planarian DNA . Control DNA samples did not migrate out of their respective gel wells.

* Significantly different from the other treatment group at the same time point, $p < 0.05$, ANOVA and the Bonferroni method for multiple comparisons.

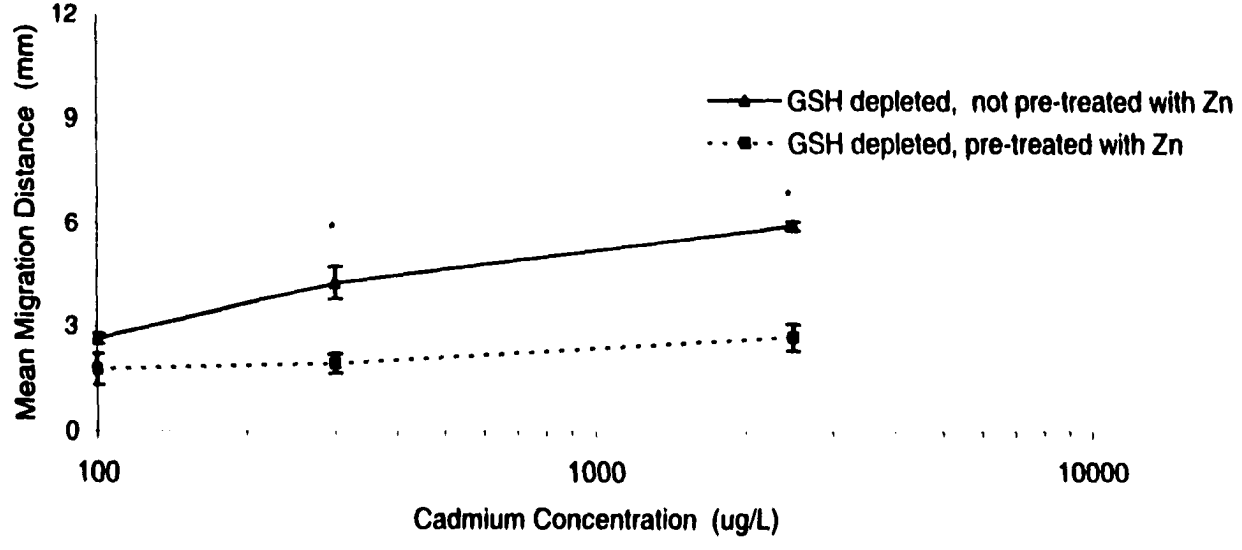
Figure 4-12. Fragmentation of planarian DNA from worms pretreated with BSO/DEM with and without Zn pretreatment and then exposed to 2,500 ug/L Cd.



DNA fragmentation resulting from exposure to Cd at 2,500 ug/L in asexual Dugesia dorotocephala with BSO/DEM pretreatment (5.0 and 2.35 g/L, respectively for 5 days) with and without Zn pre-treatment (500 ug/L, 5 days) after which they were exposed to Cd over a 36 hour time frame. DNA damage was evaluated by alkaline gel electrophoresis measuring the mean migration distance (mm) of planarian DNA . Control DNA samples did not migrate out of their respective gel wells.

* Significantly different from the other treatment group at the same time point, $p < 0.05$, ANOVA and the Bonferroni method for multiple comparisons.

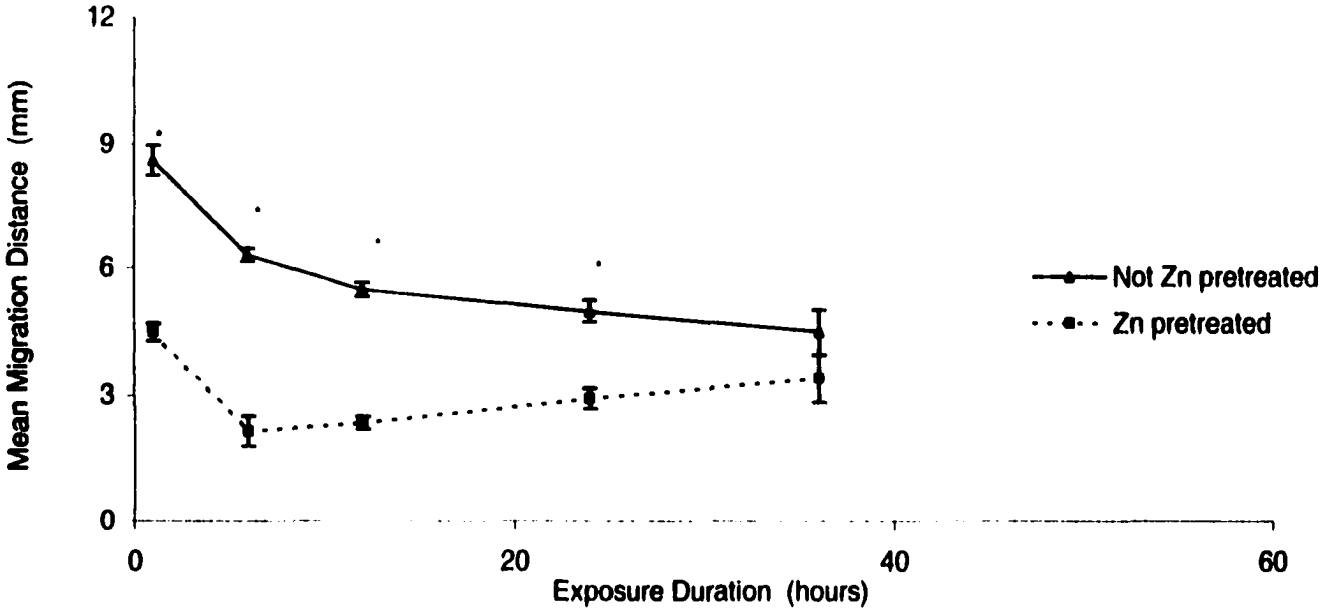
Figure 4-13 Comparison of the mean migration distance of DNA from planaria pretreated with BSO/DEM with and without Zn pre-treatment and then exposed to 100, 300 and 2,500 ug/L Cd for 12 hours



DNA fragmentation resulting from exposure to 100, 300 and 2,500 ug/L Cd for 12 hours in asexual *Dugesia dorotocephala* with and without Zn (500 ug/L, respectively for 5 days) but pretreated with BSO/DEM (5.0 and 2.35 g/L, 5 days) after which they were exposed to 100, 300 or 2,500 ug Cd for 12 hours. DNA damage was evaluated by alkaline gel electrophoresis measuring the mean migration distance (mm) of planarian DNA . Control DNA did not migrate out of their respective gel wells.

* Significantly different from the other treatment group at the same Cd treatment concentration, $p < 0.05$, ANOVA and the Bonferroni method for multiple comparisons.

Figure 4-14. Fragmentation of planarian DNA from worms with and with out Zn pretreatment and then exposed to 3 mg/L Benzoyl peroxide.



Time course of DNA fragmentation in asexual *Dugesia dorotocephala* exposed to benzoyl peroxide at a concentration of 3 mg/L. Planaria were treated with benzoyl peroxide at a concentration of 3 mg/L for 1 to 48 hours with (induced) or without (non-induced) pretreatment (500 ug/L zinc for 5 days). DNA fragmentation was assayed using alkaline agarose gel electrophoresis and reported as the average mean migration distance (mm) of planarian DNA. Planarian DNA controlls did not migrate out of their respective gel wells.

* Significantly different from the other treatment group at the same time point, $p < 0.05$, ANOVA and the Bonferroni method for multiple comparisons.

Chapter 5

Ionizing Radiation Induces Metallothionein and Protects Against DNA Fragmentation in the Sexual Planarian, *Dugesia dorotocephala*

Introduction

Ionizing radiation produces alterations in critical cell targets that may result in adverse biological effects through the formation of reactive oxygen species (ROS). DNA is an important target for radiation damage to mammalian cells. Free radical mediated radiation damage, if allowed to accumulate unrepaired, may lead to chromosomal aberrations, mutations or other genetic defects resulting in acute or chronic toxic consequences including cell death and cancer (Goodhead, 1989; 1994).

Metallothionein (MT) is a small (MW~6,000 Da) sulfhydryl rich, stress responsive, metal-binding protein that appears to protect cells against metals (Hamer, 1986), genotoxic agents (Lazo and Pitt, 1995), and oxygen- and nitrogen-based reactive species (Schwartz *et al,* 1994, 1995; Tamai *et al,* 1995). Metallothionein induction has been shown to occur as a result of exposure to metals (Fieldman *et al,* 1978; Suzuki *et al,* 1990), stress (Hamer, 1986), irradiation (Matsubara, 1988) and glucocorticoids (Filmus *et al,* 1992; Schmidt

and Hamer, 1986; Sun, 1996). Metallothionein and MT-like proteins have been isolated from a wide variety of eukaryotic species including vertebrates (Hamer, 1986), invertebrates (Imagawa *et al,*1991; Slice *et al,* 1990), microorganisms (Highman, *et al,* 1986) and plants. Roesijadi (1992) documented MT or MT-like proteins in a large number of aquatic species. Recently, we have reported the isolation and characterization of a cadmium binding protein from the planarian, *Dugesia dorotocephala,* that has properties similar to mammalian MT (Chapter 3).

Work by Matsubara (1988) demonstrated that MT induction in liver cells prior to radiation exposure significantly decreased the incidence of cell death compared to exposed, non-induced cells (Slice *et al,* 1990). Thus, we sought to determine if expression of planarian MT was induced by radiation exposure. In addition, the role of the planarian MT in protection of cellular DNA in *Dugesia dorotocephala* against radiation-induced fragmentation was examined.

Materials and methods

Stock laboratory cultures of sexual *Dugesia dorotocephala* were maintained in aerated tap water maintained at 20^0 C.($\pm 2^0$ C) with a 12 hour light and dark cycle. Laboratory stock was fed raw beef liver twice weekly. Three days before radiation exposure, uniformly sized sexual planaria were segregated from stock cultures into glass vessels (10.5 X 4.45 cm) containing soft reconstituted water. Soft reconstituted water was made fresh weekly using 3 liters of ultra-pure water to which was added 144 mg sodium bicarbonate, 90 mg

calcium sulfate, 90 mg magnesium sulfate and 6 mg potassium chloride (ATSM, 1992). After preparation, the soft reconstituted water was aerated for 12 hours prior to use in the planarian cultures. Immediately after isolation the planaria were fed beef liver and five hours later the test subjects were transferred to fresh soft reconstituted water. Isolated planaria were then maintained in a fasting state for the remainder of the experimental period. Soft reconstituted water was changed in the culture dishes every 24 hours. A minimum of three replicates were exposed for each experiment. Replicates of 10 sexual planaria were used for MT content determinations. Individual planaria were used for DNA damage evaluation by an alkaline agarose gel electrophoretic technique. Controls were maintained for each experimental exposure with sexual planaria taken from the same stock culture and handled the same as exposed planaria but treated only with the soft reconstituted water isolation.

Radiation exposure

Sexual planaria were irradiated with a 22.2 TBq cesium 137 radiation source, J.L. Shepherd model 81-14. The focal distance from the radiation source to the planaria was 32.5 cm. Irradiation was delivered at a rate of 0.6 Gy per minute. On day one, ten groups of planaria with three replicates for each exposure level were irradiated with 1, 2, 4, or 6 Gy or maintained as unexposed controls. Following the initial radiation exposure, one set of planaria exposed to each level of radiation including controls were immediately processed and analyzed for DNA single strand breaks using an alkaline gel electrophoresis

assay. Additional groups of planaria with a minimum of ten planaria per group were irradiated and processed to determine MT levels. Three days later, groups of worms previously exposed to 4 or 6 Gy were again exposed again to the radiation source and analyzed for DNA fragmentation and MT levels along with appropriate non-exposed controls.

Planarian metallothionein assay

The technique used to assay planarian MT content was a modification of an assay described by Bartsch *et al* (1990). Briefly, following radiation exposure each replicate of exposed sexual planaria was isolated in 500 μL of homogenization buffer (50 mM TRIS, 0.2 mM EDTA, 20 mM 2-mercaptoethanol and 0.05 mM phenylmethylsulfonyl fluoride (pH 8.0) and homogenized with an ultrasonic cell disrupter (Sonifier Cell Disrupter, Heat Systems Company; power setting 4 using three - 10 second bursts). The planarian homogenate was then centrifuged at 10,000 x *g* for 15 minutes to precipitate cellular debris. The pellet was retained and used to determine DNA content for each experimental replicate. The supernatant was transferred to a clean tube, heat treated in a boiling water bath for 20 minutes and then re-centrifuged at 16,000 x *g* for 10 minutes to precipitate heat denatured protein. The protein content of the heat stable protein supernatant was determined using the Bradford dye binding protein assay utilizing bovine serum albumin to construct standard curves. Aliquots of the heat-treated cytosolic protein were stored at -80^0 C prior to measurement of MT levels. The solution was treated with 0.02 mg Cd^{++} per mg

protein. Following Cd saturation, excess Cd was removed using Chelex 100 resin (iminodiacetic acid). Cytosolic samples were then diluted with ultra-pure water and acidified using analytical grade concentrated nitric acid to bring the acid concentration to 1% (v/v). Cd content was analyzed using atomic absorption spectrophotmetry (Varian Spectr AA5; cadmium lamp at 228.8 nm; air/acetylene flame). Planarian MT content was calculated using the relationship that each MT molecule binds 7 molecules of Cd (Chapter 3) and reported as pg planarian MT/ pg planarian DNA. Because planaria produce variable amounts of mucus when handled, accurate reproducible body weight measurement was difficult. Because of this phenomenon, data were normalized to planarian DNA content to avoid reporting misleading values.

DNA content determination

DNA content of planarian samples was measured using a bis-benzamide assay (Labarca and Paigen, 1980). Samples were suspended in a phosphate-saline buffer (0.005 M sodium phosphate. 2.0 M sodium chloride and 3 mM EDTA, pH 7.4). Aliquots (100 µL) of the mixture were mixed with phosphate buffer (0.05 M sodium phosphate, 2.0 M sodium chloride, pH 7.4) containing 1 µg of bis-benzamide (Hoechst 33258) per ml. Fluorescence was measured at 492 nm with excitation at 356 nm using a fluorescence spectrophotometer (Hitachi F2000). Calf thymus DNA (Sigma Chemical Company) was used to prepare a standard curve.

DNA fragmentation assay

The alkaline gel assay used was a modification of a method used by Theodorakis to evaluate DNA strand breakage in fish red blood cells. Following exposure of sexual planaria to varying doses of irradiation, individual worms were immersed directly into 100 µl of 0.5 N sodium hydroxide in a micro-centrifuge tube. In order to facilitate digestion, the planaria were minced with a sharpened spatula. The planarian/sodium hydroxide mixture was allowed to digest for 2 hours at room temperature after which the DNA content was measured using bis-benzamide fluorescence as described above. Seventeen micrograms of planarian DNA from each sample were loaded into individual agarose gel wells for electrophoresis. A 1.5% agarose gel containing 50 mM sodium chloride and 4 mM EDTA was used. Following sample loading, wells were sealed with a 1% low melting point agarose and the gel immersed in 600 ml of freshly prepared running buffer (30 mM sodium hydroxide and 30 mM EDTA). The gel was allowed to incubate in the running buffer at 4^0 C for 1.5 hours after which the gel was electrophoresed at 7.5 volts per cm for 1.5 hours at 4^0 C. Following electrophoresis, the gel was neutralized in a 200 mM TRIS buffer (pH 7.0) for forty-five minutes at room temperature and then stained with ethidium bromide (3 µg/ml) for 13 minutes. Destaining was accomplished by immersing the gel in deionized water for 24 hours at room temperature. Gels were then photographed on a UV illuminator and the mean migration distance for the DNA was quantified from the negative using a densitometric analysis of a scanned

image.

The mean migration distance (mm) was derived using densitometric measurements made through the entire DNA lane for each sample (Photometrics ID full version). The average mean migration distance from the three replicates was calculated for each Cd dose and exposure period. A Hind III digest of λ-phage DNA, consisting of DNA fragments of known molecular size was used as a standard and quality control for gel-to-gel variation. DNA from control planaria did not migrate out of the gel wells. Statistical analysis was performed using a two way ANOVA and the Bonferroni method for multiple comparisons to test for statistical significance.

Results

Agarose gel electrophoresis of DNA extracts from control planaria under alkaline conditions allowed assessment of the extent of DNA fragmentation (strand break frequency) in samples of individual planaria. Figure 5-1 is a photograph representative of two different alkaline agarose gels used to quantify DNA strand breaks by densitometric analysis. Control planarian DNA produced a distinct band that did not migrate out of the gel well (Figure 5-1, lanes 7-9 and 17-9). Planarian DNA exposed to 1 and 2 Gy (lanes 14-16 and 11-13, respectively) had no observable migration out of the agarose gel wells, as was seen with control DNA, suggesting that detectable DNA fragmentation did not occur. There was a dose-related increase in the amount of DNA migration in planaria exposed to 4 Gy (lanes 4-6) and 6 Gy (lanes 1-3). The extent of

electrophoretic migration is directly related to the sizes of the fragmentated DNA strands. As a molecular size standard, Hind III digestion (of λ-phage markers were included (lane 13, Figure 5-1). These markers contain DNA fragments that range from approximately 6,600 to 23,00 base pairs.

Exposure of sexual *Dugesia dorotocephala* to varying doses of radiation caused a significant increase in the expression of planarian MT all doses measured 3 days following initial exposure (Figure 5-2). Treatment with 1.0 Gy increased protein levels from 14.5 to 114.5 pg MT/pg planarian DNA, which represents nearly a 8-fold increase over control levels. When exposed to 6.0 Gy the MT levels increased to 209.9 pg MT/pg planarian DNA, more than a 14-fold increase.

DNA from sexual planaria exposed to 1.0 and 2.0 Gy of radiation, evaluated for fragmentation using alkaline gel electrophoresis following treatment, did not migrate a significant distance out of the gel wells, similar to results obtained for DNA from control planaria not exposed to the radiation source (Figure 5-1). When exposed to either 4.0 or 6.0 Gy, there was a significant increase in the amount of fragmentation in the planarian DNA immediately following exposure compared to that demonstrated in control DNA (Figure 5-3). Evaluation of the mean migration distance in planarian DNA exposed to either 4.0 or 6.0 Gy showed significantly increased mean migration distances from the gel wells of 6.1 and 6.7 mm compared to control DNA that did not migrate out of the gel wells (Figure 5-3). There was no significant difference in the amount of DNA

damage caused either the 4.0 or the 6.0 Gy radiation dosages with this assay. Planarian MT levels in these groups of worms was 14.5 pg planarian MT/pg planarian MT (Figure 5-2).

A comparison of the amount of DNA fragmentation from planaria initially exposed to 4.0 or 6.0 Gy on day one and then re-exposed to the same dose on day three demonstrated a significant reduction in the amount of DNA damage (mean migration distance) over that measured on day one from 6.1 and 6.7 to 4.2 and 4.6 mm on day three, respectively (Figure 5-3). When compared to the amount of DNA damage remaining in worms exposed on day one to 4.0 or 6.0 Gy and not re-exposed on day three, there was a significant increase in the amount of damage from the second radiation exposure but not as great as that seen initially on day one (Figure 5-3). This decrease in the mean migration distance of planarian DNA was accompanied by significant increases in the planarian MT levels to 181.6 and 209.9 pg/pg planarian DNA, in worms exposed to 4.0 and 6.0 Gy, respectively compared to the control levels of 14.5 pg planarian MT/pg planarian DNA measured in unirradiated planaria.

Three days after the initial radiation exposure, when evaluated for DNA fragmentation without additional radiation exposure, there was a significant decrease in the mean migration distance of DNA from previously treated planaria compared with DNA evaluated immediately following radiation exposure (Figure 5-3). The DNA from planaria previously exposed to either 4.0 or 6.0 Gy had mean migration distances of 1.3 and 1.7 mm, respectively upon repeated

irradiation on day 3.

These organisms were not visibly affected in an adverse manner by the different doses of ionizing radiation. Three replicates of sexual planaria isolated from stock cultures were exposed to ionizing radiation (1.0 to 6.0 Gy). One group was irradiated once on day one and the other group exposed twice with a three-day period between exposures. The groups were maintained for a period of six months following irradiation and no observable differences with regard to body size or the number of egg cocoons produced was observed when compared to control groups not exposed to radiation (data not shown).

Discussion

The intracellular formation of reactive oxygen species is thought to be the major mechanism by which ionizing radiation causes cell damage and death (Von Sonntag, 1987). Much of the damage to cells from radiation is the result of either direct macromolecule ionization or indirect attack by the hydroxyl radical produced by the radiolysis of water. However, secondary hydroxyl radicals are produced by metal mediated reduction of hydrogen peroxide via the Fenton reaction. In addition, the sequential two-electron reduction of molecular oxygen is also thought to be involved in radiation induced cell damage (Von Sonntag, 1987; Gutteridge and Halliwell, 1994).

We hypothesized that exposure of planaria to varying doses of ionizing radiation would result in increased expression of MT and that the elevated protein levels would show a dose response effect similar to that seen with

mammalian MT in cells exposed to ionizing radiation (Hamer, 1986). The 7- to 14-fold increases in planarian MT levels are consistent with the results of experiments conducted with mammalian cells where ultraviolet radiation was found to induce the synthesis of MT (Lieberman *et al*, 1983). Matsubara (1988) reported a significant increase in MT expression in mouse liver cells following exposure to ionizing radiation. Similar results have been reported by Coogan (1992) using rat liver cells.

The elevated MT levels appear to be associated with a reduction in DNA fragmentation in planaria exposed to ionizing radiation compared to non-irradiated controls (Figures 5-2 and 5-3). Greenstock *et al* (1987) demonstrated that MT effectively reduced the amount of DNA damage in V-79 Chinese hamster cells exposed to varying doses of ionizing radiation. Likewise, induction of MT may play a role in reducing the sensitivity of tumors to radiation therapy (Shibuya *et al*, 1977).

The mean migration distance of planarian DNA in alkaline agarose gel electrophoresis pre-irradiated on day one was significantly less than that measured following irradiation on day three (Figure 5-3) The reduced DNA fragmentation was accompanied by a significant increase in planarian MT levels (Figure 5-2). There was also a significant reduction in the amount of DNA damage in worms 3 days after the initial dose of radiation at both dose levels (4.0 and 6.0 Gy) (Figure 5-3). Thus, planarian DNA strand breaks caused by the radiation exposure appear to be repaired rather quickly. But it was also noted

that there was a significant reduction in the amount of DNA damage in planaria that were treated with a dose of radiation 2 days before the second dose of ionizing radiation was administered. DNA strand break repair may contribute to the reduction in DNA fragmentation observed in planaria pre-treated with radiation and retreated 3 days later. Because DNA fragmentation was measured immediately upon completion of the second radiation treatment, it is more likely that the pretreatment results in the formation of fewer strand breaks.

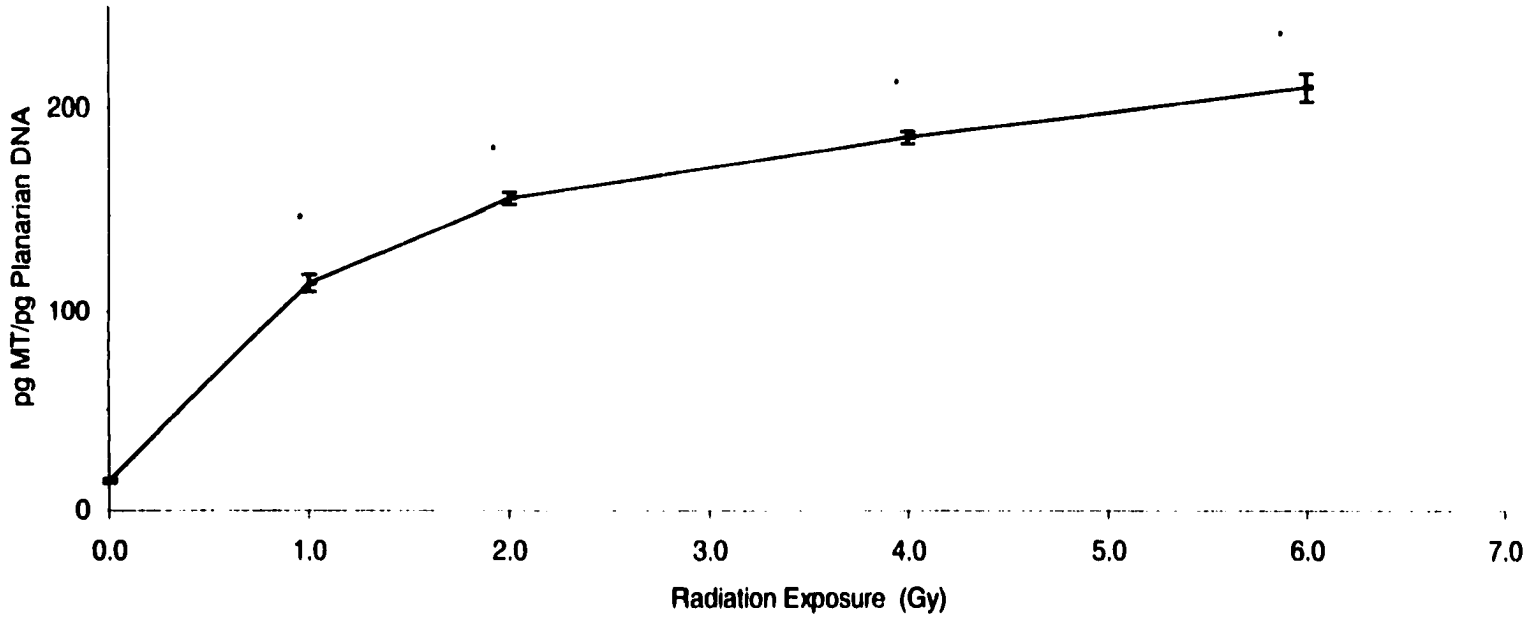
Much of the damage to DNA caused by ionizing radiation is generated by hydroxyl radicals produced by the radiolysis of water in cells (Alper, 1979). Chemical analysis has revealed that the MT molecule appears to protect effectively against radiation induced damage to the sugar-phosphate DNA backbone (Von Sonntag, 1987). Because of its high thiol content, MT may scavenge a variety of free radicals including superoxide, hydroxyl and organic radicals (Sato and Bremner, 1993). In addition, MT can prevent formation of damaging radical species by interaction with certain metals such as copper. A recent report indicated that addition of MT *in vitro* can protect against free radical induced damage to calf thymus DNA by sequestering copper and preventing its participation in redox reactions (Cai *et al,* 1995). Thornalley and Vasak (1985) demonstrated that the MT molecule was extremely efficient hydroxyl radical scavenger. Thus, the increased levels of MT observed in irradiated planaria are plausibly involved in protection of DNA against strand breakage.

In summary, this research has shown that expression of MT in the sexual

planarian, *Dugesia dorotocephala*, is increased by exposure to ionizing radiation. In addition, induction of MT was associated with a reduction in DNA damage upon subsequent radiation exposure.

Figure 5-1. Alkaline agarose gel electrophoresis of DNA from *D. dorotocephala* exposed to ionizing radiation. The DNA in each lane was obtained from an individual *D. dorotocephala* and was visualized by staining with ethidium bromide. Planarian DNA control (not exposed to ionizing radiation) is shown in lanes 7-9 and 17-19. DNA from planaria exposed to 6 Gy (lanes 1-3) and 4 Gy (lanes 4-6) showed fragmentation, which varied in a dose related manner. DNA from worms exposed to 1 and 2 Gy (lanes 14 – 16 and 11 –13, respectively) had no observable fragmentation when compared to non-treated controls. The DNA in lanes 10 and 20 represents the molecular marker (Hind III digest of λ-phage DNA) used a standard on each gel.

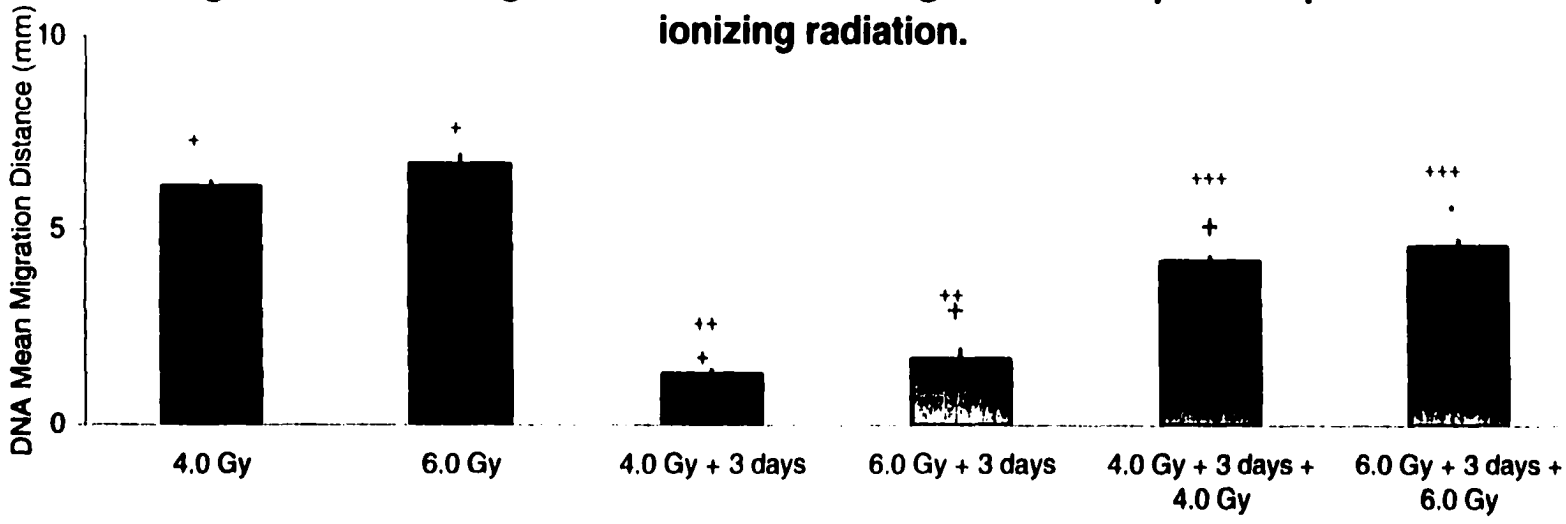
Figure 5-2. Effect of ionizing radiation on the expression of planarian metallothionein in sexual *D. dorotocephala*.



Sexual *Dugesia dorotocephala* were exposed varying doses of ionizing radiation (1.0,2.0, 4.0 and 6.0 Gy) from a cesium 137 radiation source. The planarian MT protein was assayed using a Cd saturation method three days after initial exposure. Values are reported as pg MT/pg planarian DNA.

* Statistically different from unirradated control, $p<0.05$, ANOVA and the Bonferroni method for multiple comparisons.

Figure 5-3. DNA fragmentation in sexual *Dugesia dortocephala* exposed to ionizing radiation.



Samples from sexual *D.dorotocephala* exposed to either 4.0 or 6.0 Gy from a cesium 137 radiation source. Planarian MT expression was stimulated with either 4.0 or 6.0 Gy three days prior to the analysis. Control DNA from unexposed planaria not exposed to the radiation source did not migrate out of their respective gel wells.

* Significantly different from their respective controls $p < 0.05$, ANOVA and the Bonferroni method for multiple comparisons.

++ Significantly different from the day one samples $p < 0.05$, ANOVA and the Bonferroni method for multiple comparisons.

+++ Significantly different from the day one and day 3 samples (irridated only on day 1) $p < 0.05$, ANOVA and the Bonferroni method for multiple comparisons.

Chapter 6

Conclusion

In 1986, Hall reported a high incidence of malignant postpharyngeal tumors in the planarian, *Dugesia dorotocephala*, initially exposed to Cd and then to the phorbol ester TPA. This report linking Cd exposure to a high incidence of malignant tumors paved the way for us to ask the question if planarian cells handle metal exposures like mammalian cells, which when exposed to Cd respond by producing increased amounts of metallothionein. This protein is a heat stable, low molecular weight compound that binds Cd and is induced by exposure to metals, radiation and/or oxidative stress. Mammalian MT has been shown to significantly reduce Cd induced DNA damage apparently by binding metal ions to the cysteine amino acid which comprises a large portion of mammalian MT.

The results of this research demonstrate that the protein isolated and characterized from planarian preparations has the characteristics of mammalian MT. This is based on the fact that it is a heat stable, low molecular weight protein that binds Cd, has a high cysteine content and is inducible by exposing the organism to a variety of different metals, oxidative stress caused by benzoyl peroxide and irradiation. In addition, it appears to reduce DNA fragmentation

caused by Cd and ionizing radiation, as does mammalian MT.

Because planaria develop a high incidence of malignant post-pharyngeal tumors in a short time frame following exposure to Cd and subsequent treatment with the phorbol ester TPA, the organism is an interesting model to study certain types of carcinogenesis. One of the problems prior to this work was the lack of information regarding how the organism responded to metal exposures. Using an organism that that has a large population of stem cells (neoblasts) (Hall, 1986) makes it an ideal model to study both the molecular biology of cancer and also to evaluate pharmaceutical interventions against tumor cells. The isolation of the MT protein from the planarian, *Dugesia dorotocephala*, is useful because it helps to show that planaria respond to metal exposures in a manner similar to mammalian cells. The next question that needs to be answered is whether planarian MT will reduce the incidence of Cd induced, TPA-promoted malignant postpharyngeal tumors in the planarian, *D. dorotocephala.* It would also be interesting to know if the low doses of Cd used in this research project, rather than the high doses Hall (1986) reported, will cause a significant increase in the number of Cd induced tumors and what happens to the levels of the MT protein in this scenario.

In this research project, tumors were never observed, possibly because of the shorter duration of the studies and the fact that worms were not treated with the tumor promoter, TPA. Another possibility is that the low doses of Cd used were insufficient to cause tumors, unlike the high levels Hall (1986)

reported using.

Another feature that makes the planarian an ideal non-mammalian model to study carcinogenesis is the fact that it is possible to split the organism and have it regenerate the missing portion of its body. One then wonders that if the organism were split down the midline and the organism were to regenerate, then the ideal control for experimental research would be produced, an exact replica or clone of the exposed organism. This could then provide a powerful research tool that could help to expand our knowledge of cancer and avoid some potential pitfalls that limit the ability to tease out minute differences that are often observed between controls and exposure subjects.

Because the organism is commonly found in most aquatic environments the ability of planarian MT to sequester metal ions may make the species an excellent aquatic biomonitor for metal exposure. It should be noted that they are relatively intolerant to metals, intoduction into a metal contaminated environment would permit evaluation of the bioavability of the metal ion in question. Current monitoring protocols for aquatic exposure utilize use chemical residue analysis to determine pollution levels. Use of a biological monitor like the planarian using the assays developed for this research project could allow the for a true assessment of hazard using the actual bioavailability of Cd (and possibly other metal ions) in the aquatic environment. Chemical analyses simply report metal concentration in the aqueous phase at the time of sampling. This ignores the fact that many metal ions (like Cd ions) bind to sediment and organic particles

assessment of hazard using the actual bioavailability of Cd (and possibly other metal ions) in the aquatic environment. Chemical analyses simply report metal concentration in the aqueous phase at the time of sampling. This ignores the fact that many metal ions (like Cd ions) bind to sediment and organic particles suspended in the water. These bound metal ions have reduced bioavailability and thus may not be able to damage biological systems. In addition, the biological response of planaria to metals can integrate exposures over a longer period of time. Thus, the use of a biomonitor like the planarian will help researchers and regulators to assess the true biological risk.

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