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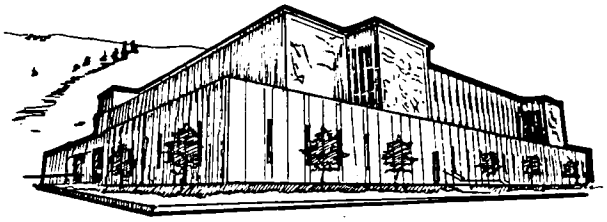

DETERMINATION OF CADMIUM TOXICITY AND THE RELATIONSHIP BETWEEN DOSE AND METALLOTHIONEIN LEVELS IN THE HONEY BEE, *Apis mellifera.*

By

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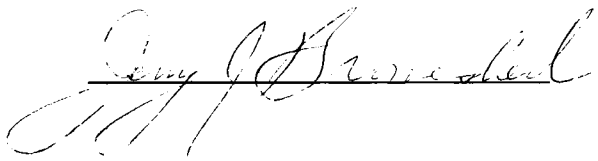

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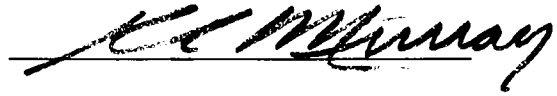

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Determination of Cadmium Toxicity and the Relationship Between Dose and Metallothionein Levels in the Honey Bee, *Apis mellifera*.

Director: Dr. Jerry J. Bromenshenk 

The purpose of this study is to investigate the toxicity of two cadmium salts to honey bees (Hymenoptera: *Apis mellifera* L.) and to investigate the association between oral dose with the accumulation of low molecular weight cadmium-binding proteins.

Honey bees were fed syrup containing cadmium as either cadmium chloride or cadmium sulfate. Bees were also injected with a saline solution containing cadmium chloride. A record was kept of the number of bees dead and syrup consumed at 1, 24, 48 and 96 hours, and probit analysis was used to calculate the median lethal dose (LD50) for cadmium salts at 48 and 96 hour intervals. Data suggests that cadmium is moderately to highly toxic to honey bees, depending upon the form used and length of exposure. LD50 values ranged from 3.51 μg Cd/bee with cadmium chloride and 2.34 μg Cd/bee for cadmium sulfate at 48 hour exposure, to 2.80 μg Cd/bee with cadmium chloride and 1.44 μg Cd/bee with cadmium sulfate at 96 hour exposures. Data from injection studies showed similar values for the 48 hour LD50 of cadmium chloride. At the end of each experiment, remaining honeybees were frozen at -80°C for protein analyses.

Whole honeybees were examined for low molecular weight cadmium-binding proteins by homogenization, heat treatment of the supernatant and size fractionation by Sephadex gel filtration. Cadmium-containing fractions between 14-4 kDa were pooled, concentrated and rechromatographed on anion exchange chromatography. Assays for purity included denaturing and non-denaturing gel electrophoresis, metal/protein ratios and UV-absorbance characteristics. Final characterization of this cadmium binding protein shows it to contain 11 cysteine amino acids, 3 gram atoms Cd and 1 gram atom Zn per molecule. The protein displays an apothionein weight of 4500 Daltons, giving a native molecular weight of 4900. This protein qualifies as a "metallothionein" under definitions outlined by the Second International Congress on Metallothioneins (1985).

A positive correlation was found between the actual dose administered and the level of whole body low molecular weight cadmium-binding protein. The level of tissue protein increase dramatically during days one and two, remaining elevated from days three through seven.

ABBREVIATIONS USED

AmS	ammonium sulfate
CdBP	cadmium binding protein
CBB	coomassie brilliant blue
Da	daltons
DTNB	5,5'-dithiobis(2-nitrobenzoic acid)
EtOH	ethanol
INEL	Idaho National Engineering Laboratory
IRC	Idaho Research Center
ß-ME	ß-mercaptoethanol
mol	mole, Avagadro's number
mRNA	messenger ribonucleic acid
p-CMB	para-chloromercuribenzoic acid
LD_{50}	median lethal dose
MTh	metallothionein
aM_r	apparent molecular weight
ppb	parts per billion
ppm	parts per million
PAGE	polyacrylamide gel electrophoresis
SDS	sodium dodecyl sulfate
Tris	tris(hydroxymethyl)aminomethane
V_e	elution volume of a protein
V_i	internal volume of a column
V_o	void volume of a column

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CHAPTER I

INTRODUCTION

Plant and animal responses to xenobiotic substances have been shown to correlate well with ambient concentrations of specific environmental pollutants for over 100 years (Nylander, 1866). Many anthropogenically-derived contaminants move across the physical boundaries of air, water and soil to become incorporated into biological tissues, often concentrated many times above the level found in abiotic sources (Jenkins, 1981). The study of how organisms interact with pollutants has been termed "bioassessment." These interactions are being extensively studied to gain an understanding of contaminant dispersion patterns, biological accumulation and the effects of exposure to a variety of toxicants.

The increasing acceptance of bioassessment in the fields of toxicology, ecology and analytical chemistry (Suter, 1982; Bromenshenk, 1985a) has prompted federal, state and private agencies to incorporate biomonitoring programs in order to predict the fate of pollutants of interest, or to study "barometers" of environmental quality. In the case of Department of Energy's Idaho National Engineering Laboratory in southeast Idaho, biomonitoring for trace elements, radionuclides and organic chemicals is extensive and geographically widespread, being performed by private contractors, universities and DOE research staff using organisms as varied as insects, mammals, birds and plants.

In the study of toxic trace element biomonitoring, current analytical techniques allow quantitation of these toxins to the sub-picogram level. This degree of sophistication in quantifying toxic metals starkly contrasts with our understanding of

the biological significance of many metals, or what effects continued exposure to low low levels may have. There is a lack of functional bioassays like those developed to measure the effects of organic chemical exposure, especially commercial pesticide impacts on insects. These assays, usually *in vitro* enzymatic reactions, have been used to assess normal or altered activities and quantities of microsomal oxygenases, epoxidases, transferases, esterases and other detoxification proteins (Yu, 1984). Recent studies have shown that natural resistance to many insecticides are accompanied with significantly elevated activity of these detoxification enzymes (Georghiou, 1980; Terriere, 1984).

Despite the abundance of biomonitoring data on "body burdens" of trace elements derived from insects (Anderson, 1977; Strojan, 1978; Bromenshenk, 1980; Bromenshenk, 1981; Dewart, 1984; Bromenshenk et. al., 1985b; Schmidt, 1986; Bromenshenk et. al., 1989), metal toxicity is a relatively unstudied aspect of insect biomonitoring. "Body burden" in this context refers to the amount (grams, curies, moles) of any specific contaminant an organism might carry per unit weight (grams). Of the 7.5 x 10^5 named insects (Wilson, 1985), metal toxicity tests have been performed on only a handful of common or economically-significant organisms. Less well defined are "dose-response" relationships where the "response" is linked to a physiological, genetic or enzymatic change rather than death of the organism. It is well understood that cadmium, as well as other metals, can exert important biochemical changes in homeostatic enzymes and proteins (Vallee, 1972), and many quantifiable changes may occur well below lethal metal intoxication. In order to

better understand the meaning of "body burden" data, metal toxicities need to be compared with quantitative bioassays that measure altered physiological or biochemical responses due to sub-lethal metal exposure.

Rationale: Honeybees have been well characterized as effective biomonitors for inorganic, organic and radionuclide contaminants (Bromenshenk, 1985a; Bromenshenk, 1985b; Morse et. al., 1987; Hakonson and Bostick, 1976). While metals such as arsenic appear to be an order of magnitude less toxic than some organic compounds, they can be markedly enriched in air soil and water around ore reduction and mining facilities (Page, 1973; Tetra-Tech, 1985; Fishbein, 1987). Since the turn of the century, significant bee mortalities have been reported to occur in close proximity to ore smelters (Root, 1907). These kills have often been labeled "arsenic poisoning" due to elevated levels of arsenic in dead bee tissue, the relative ease of As analysis and the well-researched oral LD_{50} of arsenical pesticides (Shaw, 1941: Atkins, 1981). This style of investigation ignores the myriad of elements often found in association with "arsenic-enriched" soils and their (often unknown) toxicity. Divalent group IB and IIB metals all exhibit high biochemical activity, and it is possible that highly toxic metals like cadmium, lead, mercury and nickel could compete with physiologically "native," less-toxic metals like copper, iron, manganese and zinc.

Honeybees have previously been shown to carry a wide variety of toxic metals as measured by body burden (Table 1). While data exists on the wide range of possible body burdens at metal-impacted sites, little to no documentation exists on the toxicity of heavy metals on honeybees, with the exception of arsenic which was used

in lead arsenate as a commercial pesticide until the 1950s. It is widely perceived that arsenic is the only heavy metal of concern in honeybee poisonings, as bee toxicity researcher E.L. Atkins stated that he expected cadmium to be "non-toxic to bees at doses as high as 500 µg Cd/ml" (1987, personal communication).

TABLE 1: Body burdens of trace elements found on honey bees (µg element per gram dry tissue).

Element	µg/g	Element	µg/g
Aluminum	39.6-100	Arsenic	0.10-18.5
Cadmium	0.10-6.90	Copper	19.1-61.3
Fluoride	4.3-405	Lead	4.30-299
Manganese	13.2-117	Nickel	0.30-484
Zinc	66.0-223		

(from Bromenshenk, unpublished data)

To date, target proteins that directly interact with toxic metals have not been identified in honey bees. While information on metal-binding proteins in invertebrate organisms is interesting at the level of metal balance, these proteins may also be used at a practical level as *in situ* markers of metal stress in natural populations of insects. With respect to honey bee biomonitoring, information on the presence of metal binding proteins could complement body burden data and information on biologically-available metals, providing needed insight to the relevence of metal levels encountered by the organism. This approach might be applied to metal-impacted ecosystems (such as the East Helena or Anaconda areas), providing information about the relevance of metal toxicty in bee kills, as well as impacts on other invertebrate and vertebrate animals. In situations that require remediation (Warm Springs Ponds or Milltown Dam), these metal binding proteins could potentially be used as a "biological effects"

monitor of metal exposure, providing timely information on the effectiveness of waste containment and isolation of waste from the surrounding environnment.

In order to gain an understanding of the importance of metal toxicity and metal binding protein mediation in honeybees, this study has focused on the following goals:

(1) **To quantify cadmium oral toxicity to honey bees as determined by LD_{50} analysis.** An LD_{50} has not yet been reported for honey bees exposed to cadmium, despite abundant toxicity data available for specific terrestrial and aquatic insects. While cadmium toxicity is of little or no importance in most regions, smelter activity in the Anaconda/Deer Lodge valleys and East Helena has enriched terrestrial environmental metal concentrations so that honey bees show 150X enrichment of Cd as compared to bees in Howe, Idaho, a predominantly agricultural area.

(2) **To examine cadmium-fed honey bees for the appearance and quantitation of target "cadmium-binding proteins".** The biochemical nature of the protein(s) was investigated in detail to determine if they are similar to those described from otherinsects, as well as other animals. Dose-Response relationships were investigated to see if these proteins increase in tissue with increasing cadmium dose and exposure. To date, cadmium binding proteins have been identified in only four insects, and the primary amino-acid sequence of only one form is known.

(3) **To investigate the potential of cadmium-binding protein(s) as "biomarkers" of *in situ* cadmium exposure of terrestrial insects.** It is hoped that by identifying, purifying and characterizing this protein that an understanding of how honey bees respond to significant doses of cadmium can be gained. These data could

be applied as a starting point for investigation into the toxicity of other heavy metals to honeybees, while screening the ability of those metals to induce metal binding protein production. These initial data could also be used as a comparison for future *in situ* cadmium "body-burden studies" that use honey bees or other insects.

CHAPTER II

LITERATURE REVIEW

In 1912, Bertrand postulated that (1) potentially every element has a biological function and, as such, organisms may show signs of a deficiency state (Vallee, 1972). The requirement for some elements may be so low that experimental production of the deficiency state may not be technically feasible; and (2) potentially, every element is a toxin when presented in high enough concentrations (Vallee, 1972).

To date, several toxic metals (including Pb, Cd, Hg, As, Ni and Be) have not demonstrated signs of a deficiency state, being highly toxic at low doses (Mehlman, 1987). Of the over 20 elements with known degrees of toxicity, cadmium has been listed by the EPA as one of the most important threats to environmental health, second only to lead (Jenkins, 1981). Cadmium is highly toxic to many forms of life, showing strong potential for biomagnification due to its biologically active nature and long physiological half life of up to 1240 days in some tissues (Shaikh, 1982). Since cadmium has only one common valence (+2), the element cannot be naturally transformed to a less toxic state, making it a persistent metal toxin that shows no attenuation over the course of decades (Page, 1973).

Cadmium: Natural and Anthropogenic Sources.

Cadmium is a widely distributed element that occurs at trace concentrations in soil, water and air (Page, 1973; Kay, 1985). In soils, it is often found in close association with zinc, commonly at levels under 0.1 mg Cd/kg. It can be elevated in some shales up to 30 mg/kg. Concentrations of Cd in water can vary from 0.01 µg/l

in sea water to around 1 μg/l in fresh water systems. In remote areas of the world, Cd concentrations in air are often below 0.001 $\mu g/m^3$ and can fluctuate with volcanic activity (Page, 1973).

While natural levels are often very low, cadmium concentrations can become significantly elevated due to anthropogenically-derived sources like ore mining and metal smelting, fossil fuel burning and refining operations (Kazantzis, 1987). Cadmium soil levels of 800 mg Cd/kg have been reported directly adjacent to lead smelters in England (Thornton, 1981). Standing and running water can also show enrichment, with drainage from Cd ore processing sites in Japan reaching 4130 μg Cd/l and nearby stream levels as high as 9 μg/l (Yamagata, 1970). Atmospheric releases have resulted in ambient air Cd concentrations up to a staggering 11.0 $\mu g/m^3$ in the vicinity of a secondary lead recovery plant in London (Muskett, 1979).

Regionally, smelting activities in the Anaconda-Deer Lodge areas and East Helena, MT have contaminated large geographic areas with cadmium through a combination of ore extraction, slag disposal and atmospheric releases (Tetra Tech, 1985). The most significant releases have been historical and associated with smelting of copper, lead and zinc ores over the course of 100 years. Today, significant releases still occur due to lead smelting in East Helena and decontamination processes associated with the Anaconda smelter and surrounding areas.

Samples of soil, water and air taken from both East Helena and Anaconda show significant enrichment of many elements, including cadmium. Soils downwind from the Asarco smelter in East Helena show Cd enrichment as high as 18 - 20 mg/kg on surficial soils (Bromenshenk et. al., 1989; Schlieman, 1990), with values ranging

from 9 to 85 mg/kg within two miles of the Anaconda smelter area (Tetra Tech, 1985). The Montana air quality bureau (1990) reports that flue dust from the Anaconda area contains up to 1,100 mg Cd/kg. Smelter activity in East Helena, MT has released several tons of elemental cadmium to the surrounding environment, giving atmospheric values as high as 3 μg Cd/m^3 reported as recently as 1988. In these areas, cadmium becomes a major component of the readily available divalent metals for microbial, plant and animal life.

Cadmium: Molecular mechanisms of toxicity.

In order to understand the mechanisms of Cd toxicity and detoxification, an understanding of the physical and chemical nature of cadmium is necessary. As a group IIB metal, cadmium shares similar properties with zinc and mercury including common oxidation states and electron configurations (Table 2). All group IIB metals show high biological activity, with Zn being recognized in the 1920s as both an acute toxin and an essential element. Zinc was first shown to play an important role in the enzyme catalysis of carbonic anhydrase (National Research Council, 1979), and is known as a necessary cofactor for many metalloenzymes, including dehydrogenases, proteases and polymerases. Metal replacement studies, where the native Zn atom has been removed and replaced with Cd show that the two metals, while chemically similar, demonstrate different activities in Zn-metalloenzymes (Table 3).

With this knowledge, a mechanism could be hypothesized where the toxic effect of cadmium could result from displacement of the native Zn atom in homeostatic metalloproteins. This would give the enzymes altered activity and possibly disrupt normal functions at the cellular level, ultimately resulting in the death

TABLE 2: Physical properties of the Group IIB metals; Zinc, Cadmium and Mercury.

	Zn	Cd	Hg
Outer electronic configuration	$(3d)^{10}(4s)^2$	$(4d)^{10}(5s)^2$	$(5d)^{10}(6s)^2$
Favored geometry	Octahedral >> Tetrahedral	Octahedral >> Tetrahedral	Linear > Tetrahedral

TABLE 3: Enyzmatic activities altered by <u>in</u> <u>vitro</u> cadmium substitution for native metals (from Vallee, 1972).

Enhanced	Inhibited
Acid Phosphatase (rat liver)	Acid Phosphatase (canine liver)
Adenosine Triphosphatase (fowl red blood cells)	Adenosine Triphosphatase (rat liver)
Alanyl Leucine dipeptidase	Aldolase
Alkaline Phosphatase (rabbit liver)	Alcohol dehydrogenase
δ-aminolevullinic acid dehydratase	Alkaline phosphatase (E. coli, (rat liver, kidney, testes)
Amylase (malt)	δ-ALA synthetase
Arginase	Amylase (bacterial)
Carboxypeptidase A, esterase	Aryl sulfatase
Carboxypeptidase B, esterase	Carboxypeptidase A, peptidase
Carnosinase	Carboxypeptidase B, peptidase
Cholinesterase (rat brain, heart)	Catalase
Cytochrome Oxidase (pigeon brain)	Cholinesterase (rat liver)
Glucose 6-phosphatase dehydrogenase (mouse liver, heart)	Cytochrome oxidase (rat liver)
Glutamate formimino transferase	ß-Fructofuranosidase
Histidine ammonia lyase	Gluconate dehydrogenase
Malic dehydrogenase	Glucose 6-phosphate dehydrogenase (rat liver)
Oxaloacetate decarboxylase	Glutamic oxaloacetic transaminase
Phosphopyruvate carboxylase	Glycerol phosphate dehydrogenase
Phosphopyruvate hydratase	Glycyl-glycine dipeptidase
Phosphorylase	Glycyl-serine dipeptidase
Pyruvate dehydrogenase	Isocitrate lyase
Pyruvate decarboxylase	Leucine aminopeptidase
Prolidase (swine kidney)	Lipase
Succinic dehydrogenase (pigeon liver; heart)	Lipoamide dehydrogenase
	Peptidase
	Phosphoribonuclease
	Plasmin
	Prolidase (swine kidney)
	Succinic dehydrogenase
	Tryptophan oxygenase

of cells, organs or the organism. Some experimental data do support this hypothesis of native metal substitution, as chronic Cd exposure in rats appears to cause a significant decrease in levels of Cu and Fe in liver when compared to non-treated controls (Friel, 1987). Similarly, biomonitoring studies using honey bees have shown trends where the natural levels of Cu, Zn and Mn were depressed as levels of Cd and Pb increased (Bromenshenk, unpublished results).

A proposed model by Pearson (1963) can help explain the selectivity of metalloprotein ligands for their specific metal atom. The "hard and soft acids and bases" theory (HSAB) groups metals according to their hardness or softness, which is defined by an element's affinity for halide ions. The term "acid" and "base" refer to lewis acids and bases, so cationic metal atoms would be considered acids. Hard acids (such as Al^{3+} or Ca^{2+}) show markedly different affinities for chelating groups as do intermediate acids (Co^{2+}, Fe^{2+} or Ni^{2+}) or soft acids (Ag^{+}, Cd^{2+} or Hg^{2+}).

Biologically, this theory demonstrates the affinity of different metals for specific ligands. In the case of proteins, such ligands can include carbonyl, carboxyl and hydroxyl oxygens, amine and imidazole nitrogens, and cysteine and methionine sulfur (Pearson, 1966). With nucleic acids, the ligands include purine and pyrimidine nitrogens and phosphate oxygens. The HSAB theory can, in effect, predict what type of amino acid functional groups are available for "metal displacement" changes. Explained simply, soft acids prefer to complex with soft bases and hard acids with hard bases. This theory has successfully predicted the relative toxicity of metals by their "softness" and shows good agreement with toxicity data derived from mouse

studies (Table 4; Jones, 1978).

According to HSAB theory, the group IIB metals are "soft metals", with Hg being the softest. The preferred ligands for Zn, Cd and Hg are considerably different (Table 4) with all metals binding well to sulfhydryl (cysteine) groups, but with zinc binding more efficiently to imidazole nitrogens (Vallee, 1984). This suggests that the "cadmium displacement" mechanism of toxicity that I proposed earlier is probably not a major factor with all ligands in metalloproteins. Experimental data shows that Zn atoms bound to histidyl nitrogens or carboxyl oxygens are not displaced by titration of cadmium ions (Vallee, 1972). Titration of sulfhydryl-bound Zn shows quite the opposite, however, as the affinity of Cd-S bonds is at least hundred-fold higher than Zn-S bonds (Kagi & Vallee, 1961). If a "displacement mechanism" of toxicity or detoxification is important physiologically, then it should be most readily observed with sulfur-containing proteins, or thiolenzymes that utilize cysteine in the active site.

TABLE 4: Ligand preference and toxicities of Group IIB Metals

	Zn	Cd	Hg
Softness Parameter (σ_p)	1.1	0.76	0.61
Mouse LD50 (I.P., mmol/kg)	10.0	0.33	0.19
Prefered Ligand	-SH, N,O	-SH >> N,O	-SH, S-S >> N,O

Low Molecular Weight Cadmium Binding Proteins.

While thiolenzymes have the potential to chelate cadmium, the most prominant molecules that bind Cd in vivo are the low molecular weight cadmium binding proteins, or "CdBP's." This class can include Γ-glutamyl peptides and

metallothioneins, both of which have molecular weights below 8000 Daltons. These molecules have the ability to bind approximately 3 to 8 mole equivalents of Cd per mole of protein, respectively, and can also bind Cu(I) and Zn(II). Both proteins utilize cysteinyl-bridges to coordinate the metal atom in a tetrahedral geometry, usually binding at a ratio of three sulfhydryls to one metal atom, which leaving one coordination site open to water.

The Γ-glutamyl peptides were recently characterized by Reese and Winge (1988) and were previously named "phytochelatin" or "cadystin." Several species of plants, fungi and possibly protists produce this cysteine-rich peptide in response to metal ion stress. Cells producing these peptides show tolerance to heavy metals at concentrations that are potentially cytotoxic. These peptide oligomers show a general structure of [(Γ-Glu-Cys)$_n$-Gly], with "n" varying between two to ten, four being the most common. The arrangement of cysteines in a "Cys-X-Cys" configuration (where "X" is glutamate in this case) allows for efficient metal chelation, as the n_4 peptides can sequester between two to three mole equivalents of Cd(II) or Cu(I) and often show traces of Zn(II). While Γ-glutamyl peptides appear to be important in confering metal tolerance and resistance to some species of plants and fungi, they may not play an important role in animals since they have yet to be reported.

The protein "metallothionein" characterized by Kagi and Vallee (1960) represents possibly the most studied and least understood type of CdBP. This class of proteins show a wide phylogenetic distribution and demonstrate highly conserved "Cys-X-Cys" regions necessary for cadmium binding. Metallothioneins (abbreviated "MTh") have been induced in fungi, plants and animals by exposure to Cd(II), Zn(II),

Cu(I) and Co(II), while demonstrating high binding affinities for Bi(II), Au(I), Hg(II), Pb(II) and Ag(I) in vitro (Pulido et. al., 1966). This protein has been implicated in roles ranging from natural metal balance and an antioxidant, to toxic metal sequestering and detoxification (Kagi, 1988). Metallothioneins have also been implicated as important causative agents in Cd-induced liver and kidney damage with mammals, as MTh accumulation in these organs is often associated with a high degree of tissue necrosis (Webb & Etiene, 1975), and administration of Cd-MTh shows 5 to 7 times greater toxicity than corresponding amounts of Cd^{2+} (Webb, 1977).

The name "metallothionein" reflects the proteins intrinsicly high concentrations of both metal (usually group IB or IIB) and cysteine. A commercially available cadmium-MTh from rabbit kidney shows a striking 90,000 µg Cd/g protein and 18,500 µg Zn/g protein - almost 10% metal by weight (Sigma Chemical product information). This protein is also composed by more than one-third of cysteine (22 of 62 amino acids), making the protein 7% elemental sulfur by weight.

A notable characteristic of all metallothioneins is their lack of aromatic amino acids. This protein shows no specific absorbance peaks between 268 and 278nm, demonstrating that Phe, Trp and Tyr are absent. The protein does, however, exhibit a cadmium-dependent absorbance "shoulder" at 254 nm which is due to the metal-thiolate charge transfer (Rupp, 1978). This absorbance shoulder can be abolished by removal of metals by H^+ titration (Buhler, 1974).

To date, all characterized metallothioneins show a high degree of primary sequence conservation, especially in the location of cysteine groups used to bind metals. The best characterized forms of MTh are mammalian, which contain between

61-62 amino acids per molecule. Most exist in multiple isoforms which arise from duplicated genes that differ in one or two amino acid coding sequences. In mammals, these proteins are usually expressed at very low basal level and can be induced by a variety of stimuli, including heavy metals, glucocorticoid hormones, inflammatory agents and α-interferon. Metallothioneins have been detected in many tissues including liver, kidney, intestine, muscle, heart, spleen, testes and brain (Searle, 1987).

Invertebrate metallothioneins are possibly the least-well characterized forms, while showing much greater variability than mammalian forms. The MTh from *Scylla serrata* (blue crab) is slightly smaller than mammalian forms, only 58 amino acids in length while containing 11 cysteines. *Drosophila melanogaster* metallothionein is a 40 amino acid protein containing 10 cysteines, all arranged in a "Cys-X-Cys" configuration. With this variation in metallothioneins, the Second Congress on Metallothionein (1985) proposed the following criteria for MTh proteins:

(1) Low molecular weight, below 7,000 amu.

(2) High group IB or IIB metal content, usually between 4 to 8 gram atoms per mole protein. If bound to Cu(I), this number could be as high as 10-12.

(3) High cysteine content (up to 20%) with most sulfhydryls reduced and participating in binding of metals.

(4) Contains no aromatic amino acids.

(5) Shows a high degree of similarity to mammalian metallothioneins, such as those characterized by Kagi and Vallee.

While little is know about the distribution or importance of metallothioneins in most invertebrates, existing information suggests that MTh could be well suited as a

protein-level marker of invertebrate metal stress. "Metallothionein-like" proteins have been shown to occur in several insects, including the fruitfly (*Drosophila melanogaster*; Maroni & Watson, 1985), the cockroach (*Blatella germanica*; Bouquegneau et. al., 1985), the waterflea (*Moina macrocopa*; Yamamura et. al., 1983) and the stonefly (*Eusthenia spectabilis*; Everard & Swain, 1983). All studies with these insects suggest that metal toxicity correlates well with cadmium binding protein accumulation in tissues.

To date, the best characterized insect metallothionein is that from *Drosophila melanogaster*. The amino acid sequence of this protein was determined indirectly by obtaining the sequence of *Drosophila* mRNA that bound to a mouse MT isoform I cDNA hybridization probe (Lastowski-Perry, 1985). This sequence codes for a 40 amino acid protein with an apothionein molecular weight of 4,000 daltons. Treatment of fruitfly larvae with 0.1 m$\underline{M}$ $CdCl_2$ showed that levels of MTh mRNA increased linearly from 0 to 30 hours, with the amount of mRNA unchanging from 30 to 72 hours. Nearly 80% of the MTh mRNA found in larvae was located in the alimentary canal, an organ which represents less than 10% of the larval weight. In treated larvae, MTh mRNA made up almost 12% of the total mRNA isolated from the alimentary canal. These data suggest that of the hundreds of proteins present in the gut of these larvae, one out of every ten being proteins produced were metallothionein!

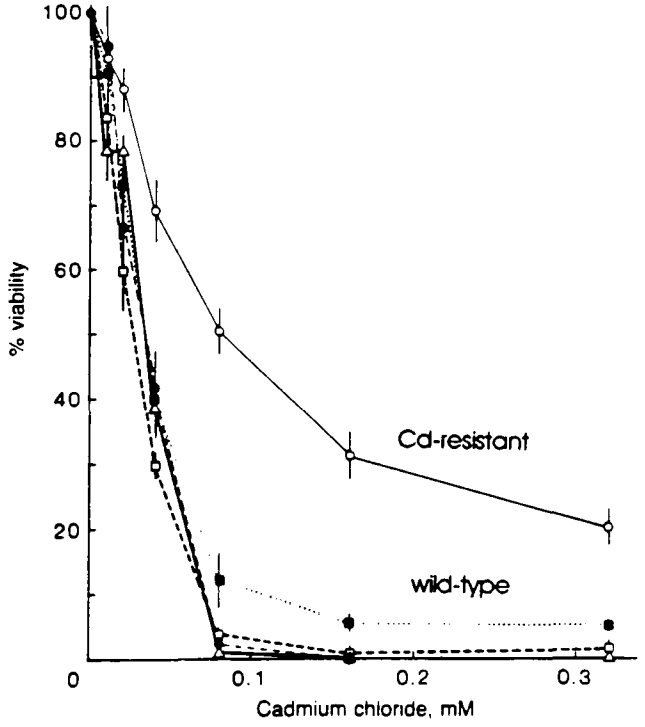
Molecular characterization of the fruitfly MTh gene shows that most populations contain a single MTh gene (Otto et. al., 1986; Maroni & Watson, 1986). Identification of a natural population of fruitfly with a single MTh gene duplication

showed that this strain could produce twice as much metallothionein mRNA after Cd exposure as compared to wild type strains (Otto, 1986). Most importantly, these gene duplication "mutants" showed a significant tolerance to Cd(II) and Cu(I), with viability in larvae 4 to 5 times as high as wild-type fruitflys at similar exposures. These data give strong support to the hypothesis that insect MTh plays an important role in detoxification of heavy metals.

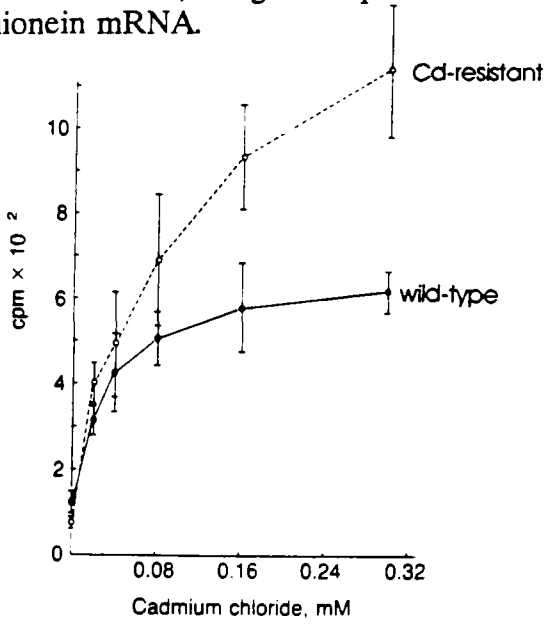
Cadmium toxicity studies also showed a clear relationship between the oral dose to wild-type larvae and both mortality and MTh gene expression. At low dose levels of 10 and 20 μM Cd^{2+}, the number of viable larvae decreased by 15 to 30% (Figure 1). These same exposures gave a corresponding increase in MTh mRNA of three to four-fold. While mRNA levels only imply an increase in protein concentrations, these data show that MTh protein is most likely produced at an accellerated rate when cadmium intake is sufficient to result in 10% mortality. Since no lower levels of Cd were investigated, it is not known if MTh mRNA (thus, MTh protein) is produced at a measurable rate when larvae are exposed to sub-lethal doses of Cd.

FIGURE 1: Relationship between MTh mRNA induction and oral cadmium dose in *Drosophila* larvae (from Otto, 1986).

(A) Viability of wild-type and Cd-resistant larvae after Cd treatment, 36 hours exposure. Note higher mortality in wild-type larvae, even at the lowest exposure level.



(B) Expression levels of MTh mRNA in wild-type and Cd-resistant larvae after Cd treatment, 36 hours exposure. Note that although mRNA induction begins at the lowest exposure level for both strains, the gene duplication mutant produces twice the amount of metallothionein mRNA.



CHAPTER III

MATERIALS AND METHODS

Cadmium Toxicity Studies:

From June 26, 1987 to October 28, 1988, thirteen oral dose and five subcutaneous injection tests on honeybees were conducted using $CdCl_2$ x $2.5H_20$ and $3CdSO_4$ x $8H_20$. Honeybees (Hymenoptera:*Apidae*) were collected from colonies located in an agricultural area three miles west of Mud Lake, ID (Figure 2). This site was chosen because of excellent food resources. Earlier biomonitoring studies of the surrounding areas showed Mud Lake to be relatively unimpacted by heavy metals released from facilities at the INEL (Bromenshenk, unpublished data).

Worker honeybees between the ages of 2 to 4 weeks old were collected in the afternoon by shaking a frame of young bees into a wooden box and covering them with a plastic screen. The bees were immediately placed in a large cooler with ice to prevent overheating and transported to the Idaho Research Center (IRC) in Idaho Falls, ID approximately 50 minutes away. Bees were collected in the afternoon at heavy foraging times to ensure a more homogeneous sampling of younger "house" bees. The older forager bees could introduce variability into the LD_{50} studies due to higher natural mortality as a consequence of flight activity.

At the IRC, bees were placed into screened feeding cages (Figure 3). Oral toxicity tests utilized an estimated 130-150 bees per cage and injection studies contained between 25 to 50 bees per cage. This number has been shown to be sufficient for allowing normal honeybee hoarding behavior and social interactions (Rinderer, 1978), yet low enough to provide a manageable sample size. Bees were

FIGURE 2: Location of colonies at the INEL used for cadmium toxicity and protein studies.

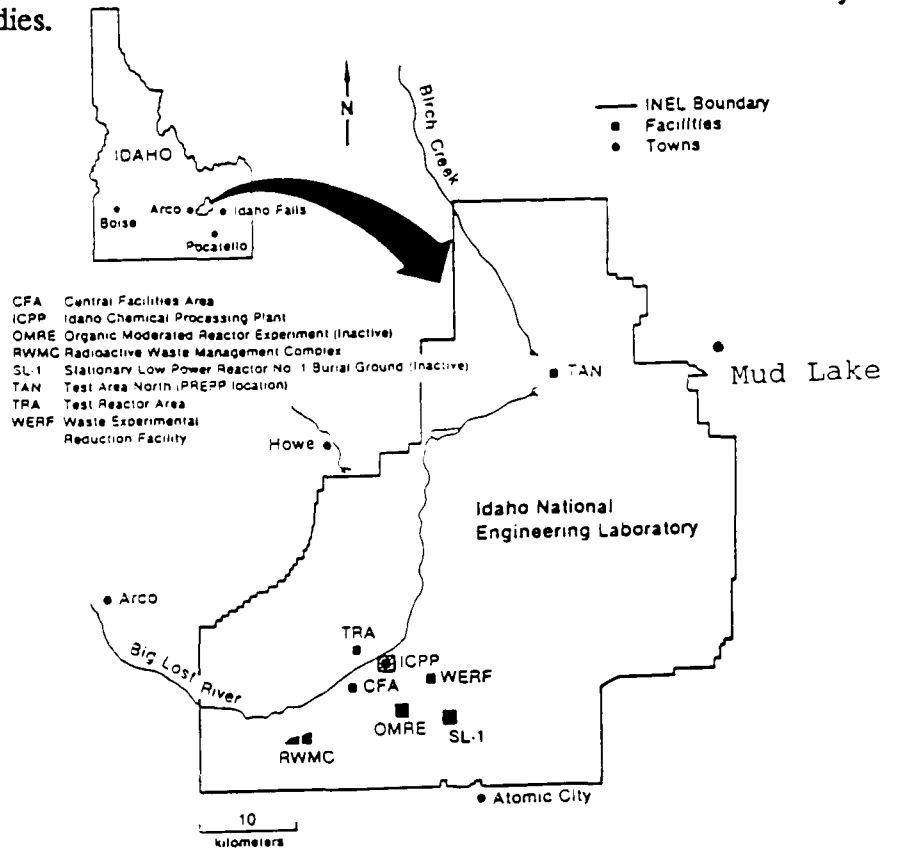
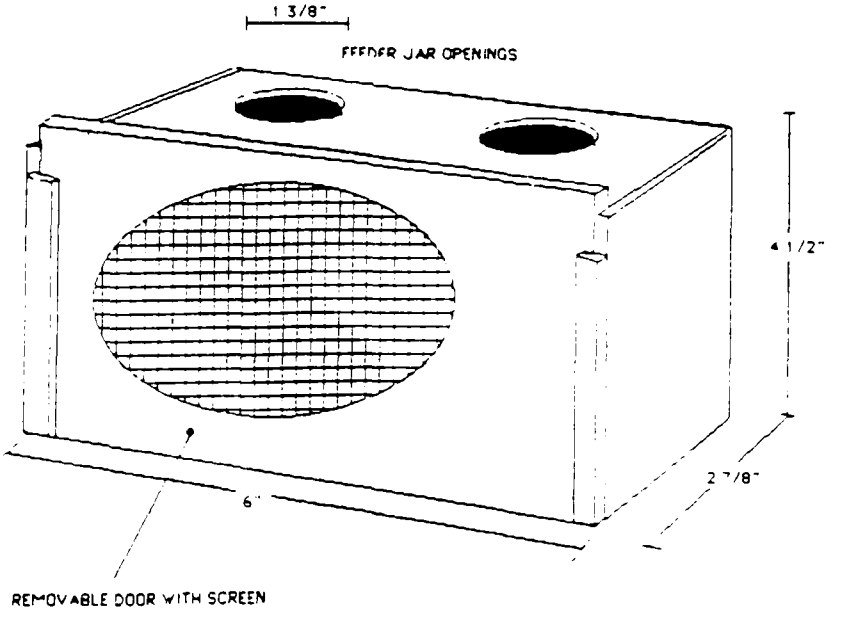


FIGURE 3: Diagram of feeding cages used for honey bee cadmium toxicity studies.



immediately fed a solution of 50% sucrose-30% glucose by a gravity feeder for 24 hours to determine mortality from handling. The bees were kept in a controlled environmental chamber that maintained a constant 22°C temperature and 12 hours of light (7am-7pm). After the 24 hour observation period, dead bees were removed from the cage, and the syrup was removed for approximately 12-16 hours to ensure the bees would consume the Cd-containing syrup.

To administer soluble cadmium, a pre-weighed bottle containing 25.0 ml of the desired test syrup (between 0 and 100 ppm Cd; recipe in Appendix E) was placed on each cage, and the cages were arranged randomly in the chamber using a random number table (Rohlf, 1981) to assign positions. Throughout the duration of the feeding test, dead bees were counted at 1, 24, 48 and 96 hour intervals, removed and frozen at -20°C in Whirl-pak bags. Measurements were also made on the volume of syrup consumed at each time interval by weighing the syrup bottle and calculating the volume remaining (using a value for syrup density obtained prior to the experiment for each batch of 30% glucose-50% sucrose syrup). Bee mortality due to handling and transferring was low, ranging from 0% to 0.9%. Control honeybees fed 0 ppm cadmium syrup never exceeded 2.3% mortality for the 96 hour tests, or 3% for the longer 10 day feeding experiments.

At the end of each 96 hour test, the remaining live bees were asphyxiated under N_2 and immediately frozen at -80°C to reduce protein denaturation and proteolysis. After each experiment, cages and screens were washed in hot soapy (Liquinox) water and rinsed with tap, deionized and glass distilled water. Feeding vials were washed in a similar manner with one acid-washing step (25% HCl) and one

more water rinse.

A concern was raised that starvation, and not cadmium toxicity, was responsible for mortality seen in feeding tests since bees consumed decreasing amounts of syrup with increasing cadmium concentrations. To answer this question, two studies were initiated: the first was an investigation into the effects of food deprivation for up to 96 hours; the second was to determine the toxicity of cadmium administered by injection.

Two tests were run to quantify honey bee mortality from starvation. These tests were set up nearly identical to oral toxicity experiments with the exception that bees were given only water, rather than sugar syrup, for the duration of the experiment. Dead bees were counted at 24 hour intervals up to 96 hours. The remaining live bees were fed syrup at the end of the experiment. The survivors weakened state appeared reversible within a day, and they were released back to Mud Lake.

A total of five tests were conducted where honey bees were injected subcutaneously with a physiological saline solution containing various concentrations of $CdCl_2$ to determine median lethal injected dose. For four of the five tests, honey bees were fed sucrose/glucose syrup ad libitum up to 12 hours prior and immediately after the injections. For each injection series, between 10 and 50 honeybees were secured with flat filter forceps by the back leg, and then gently grasped between thumb and forefinger. It was at this point during the first injection series that I chose to use a 12-hour syrup deprivation period: caged bees appear to gorge themselves, and upon grasping will regurgitate syrup. The effects didn't appear to be detrimental,

except that body "hairs" can become matted and sticky unless the syrup is removed quickly. Bees did best if they were placed back into a cage full of hungry bees so that they were cleaned quickly. Honey bees were injected using a 48-gauge needle with a 22-degree bevel attached to a calibrated Hamilton syringe (#805RN). Injection volumes consisted of 5 µl of a heat-sterilized insect physiological saline solution (Euphrussi and Beadle, 1936) that contained 128 mM NaCl, 4.7 mM KCl, 1.9 mM $CaCl_2$ and filter-sterilized Cd from an appropriate $CdCl_2$ stock (Table 5). This recipe was recommended by Dr. Marc Klouden, an insect physiologist from the University of Idaho's Department of Plant, Soil and Entomological Sciences. Honeybees were injected in the dorsal side of the abdomen between the 4th and 5th tergites. The intersegmental membrane becomes exposed as bees extend their abdomen (a natural response with attempted stinging), and can easily be pierced with a minimum of "bleeding." In worst cases, approximately 3 µl of hemolymph would weep from the point of injection. Normally, no bleeding could be observed as the membrane closed "accordion-like" to seal the hole. After the membrane was pierced, the needle was inserted approximately 2 mm and the contents were injected slowly. The syringe was rinsed with sterile physiological saline and wiped with isopropyl alcohol between injections.

To assure that the insect physiological saline showed no toxicity, the initial two tests included an extra set of "pin prick" controls that were stuck with the syringe needle alone. These were omitted in later tests, as it was demonstrated that there was no significant difference ($P \leq 0.05$) between "pin prick" treated bees and bees injected with physiological saline.

TABLE 5: Dilution directions used for cadmium chloride injection studies.

µg Cd/bee per 5 µl injection	Components Added		[Cd] in Saline, ppm
0	25 µl distilled H_20	+ 4.975 ml Saline	0.0
0.01	100 µl 100[a] ppm $CdCl_2$	+ 4.900 ml Saline	2.00
0.10	100 µl 1000 ppm $CdCl_2$	+ 4.900 ml Saline	20.0
0.25	250 µl 1000 ppm $CdCl_2$	+ 4.750 ml Saline	50.0
0.50	50 µl 10000[b] ppm $CdCl_2$	+ 4.950 ml Saline	100
1.00	100 µl 10000 ppm $CdCl_2$	+ 4.900 ml Saline	200
2.00	200 µl 10000 ppm $CdCl_2$	+ 4.800 ml Saline	400
2.50	250 µl 10000 ppm $CdCl_2$	+ 4.750 ml Saline	500
5.00	500 µl 10000 ppm $CdCl_2$	+ 4.500 ml Saline	1000
12.5	1250 µl 10000 ppm $CdCl_2$	+ 3.750 ml Saline	2500

* dilutions were made assuming $CdCl_2$ hemipentahydrate contains 49.23% cadmium by weight.

[a] 100 and 1000 ppm solutions were made from serial dilutions of 10000 ppm stock solution with sterile saline.

[b] 10000 ppm stock solution = 0.40637 grams per 20 ml sterile saline and 0.2 µm filter sterilized (actual concentration = 10,003 ppm).

After bees were injected, dead bees were counted at 1, 24, 48 and 96 hour intervals. Results were tallied as was done in oral toxicity tests, except that syrup consumption was not monitored. Mortality at 48 hours from handling and injecting ranged from 20-50 % in controls for the first injection experiment. Much of this high mortality was attributed to a set of leaky syrup bottles that were used for feeding during the experiment. When the volume of syrup in each feeder was reduced (from 25 to 5 ml) and the bottles were sealed with parafilm, 48 hour mortality for controls was reduced to a more manageable 8-16 % for the last four experiments. Due to the rigorous nature of these tests, LD_{50}'s were computed for only the 48 hour time interval. It is understood that these LD_{50}'s represent a crude estimation of median lethal dose, and as such little can be said about the comparison to oral LD_{50} data.

Statistical Methods:

Mortality response has been graphically depicted using two methods. The first set of plots show cumulative mortality (expressed as a % of total test bees) vs. time at several intervals (1, 24, 48 and 96 hours). The second set of plots show cadmium dose-mortality response relationships from two salts of cadmium at two time periods (48 and 96 hours).

Median lethal doses were calculated by the method of least-probit analysis as outlined by Finney (1952). To calculate 48 and 96-hour LD_{50}'s, the following procedure was used (see Figure 4 for example probit plot):

1. For each cadmium level examined, multiply the total volume of syrup consumed per time period by the concentration of cadmium used.

2. Calculate "dose per bee" (expressed as µg Cd) by dividing the result in step 1 by the total number of bees treated at that feeding level.

3. Calculate percentage mortality by dividing the number of dead bees per time interval by the total number treated at that feeding level, and multiply by 100.

4. Assign empirical probit values using probit transformation table from Finney (1952; also in Appendix F).

5. Plot the probit values as the linear ordinate values on semilog graph paper with "dosage per bee" on the logarithmic abscissa.

6. Using visual inspection and a straight-edge, draw the best fitting line.

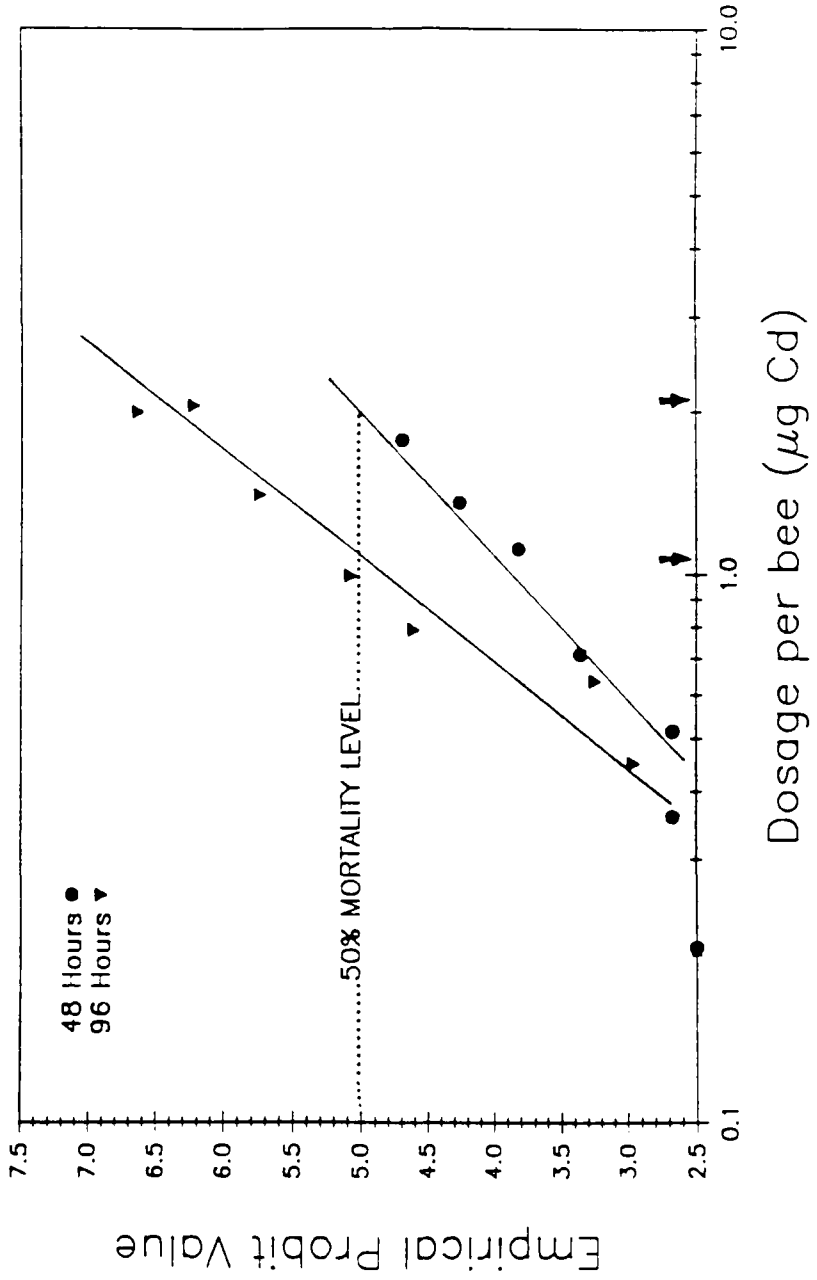
7. Draw a vertical line through the point where the dose-response curve crosses the probit = 5.0 line and note the point where the abscissa is intersected. This point is the estimated LD50.

The standard error was calculated on SPSS, the Statistical Package for the Social Sciences (Norusis, 1988) using the following formula: SE LD50 = 2S/ 2N, where 2S equals the difference between the values of x corresponding to probit 4.0 and 6.0, and N is the total number of test organisms in the groups included in the range of 3.5 to 6.5 probits. The 95% confidence intervals were also calculated on SPSS using the following equation: 95% CI = $X \pm (1.96*SE)$. Scattergram analyses, correlation coefficients, Student's t and Tukey's b analysis of variance (ANOVA) tests were all performed using SPSS with an alpha of less than or equal to 0.05 used as the significance level for all statistical inferences. Data was manipulated using the Quattro spreadsheet program and was graphically displayed using Quattro and SigmaPlot programs.

Protein Purification:

Several studies were initiated in order to examine for the appearance of low molecular weight cadmium binding proteins ("CdBP's"). For each study, these proteins were isolated using whole honey bees rather than specific tissues. Over the

FIGURE 4: An example probit plot using data from $CdSO_4$ Test 1 to determine the 48 and 96 hour LD50 (see previous page for discussion).



* From this probit graph, the experimental LD50 for 48 hour exposure would be approximately 2.2 µg Cd per bee, with 96 hour exposure at 1.0 µg Cd per bee.

course of the study, a total of four tests were conducted and are summarized below. Control experiments were also performed using rabbit metallothionein (Cd/Zn form; Sigma Chemical Company) as a standard. These experiments are summarized in appendix D. Unless otherwise specified, procedures used for all protein manipulations were described by Freifelder (1982).

TEST 1: Analysis of Cd-exposed honey bees for CdBP. Honey bees were fed $CdSO_4$ at 20 ppm for varying lengths of time from 6 hours to 144 hours. Remaining live bees at the end of each time period were pooled by treatment and and frozen at minus 80°C. Entire samples from each treatment were then homogenized and run separately on Sephadex G-50 using the method of Kagi (1961). The low molecular weight proteins (4 through 14 kDa) from all time-interval samples were pooled and rechromatographed using DEAE Sephadex A-25. The resolved CdBP were concentrated by lyophyllization and characterized for protein concentration and metal (Cd, Zn) content, absorbance characteristics, purity as determined by gel electrophoresis and sulfhydryl content.

TEST 2: Analysis for CdBP after variable cadmium ingestion. Remaining live honey bees from 96 hour $CdCl_2$ feeding studies were asphyxiated under N_2, frozen and weighed at the end of each test. Bees from each treatment level were homogenized independently from all other samples and stored at -80°C. After five oral toxicity studies, homogenates from each exposure level were pooled to give eight samples total: bees exposed to no cadmium, one, two, five, 10, 20, 50 and 100 ppm cadmium in syrup. Aliquots from each of the eight samples were analyzed for CdBP by the method of Winge (1986) to determine CdBP levels as "µg protein expressed per

gram bee tissue" after 96 hours exposure.

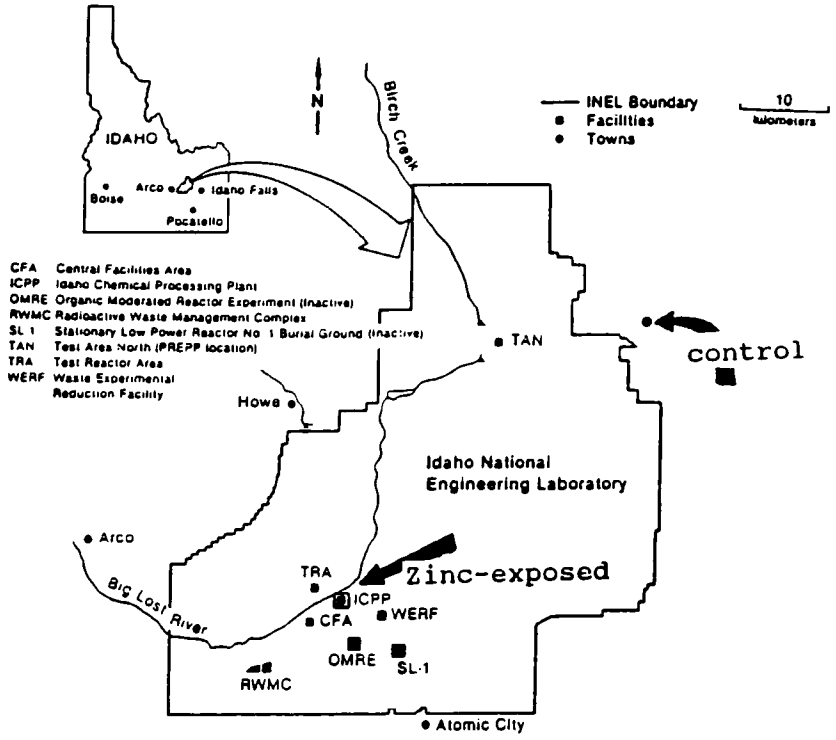
TEST 3: Analysis for CdBP after variable time exposure. Honey bees were fed two levels of $CdCl_2$ syrup (2 and 20 ppm Cd) for six time intervals (zero, one, 24, 48, 96 and 144 hours). Remaining live bees at each time interval were anaesthetized, frozen and weighed. Samples were homogenized independently and aliquots examined for CdBP by the method of Winge to determine CdBP levels as "µg protein expressed per gram bee tissue" after exposure to 2 and 20 ppm Cd over time.

TEST 4: Analysis for CdBP from in situ samples. Honey bee samples were collected from "metal poor" areas of Mud Lake, ID and "metal enriched" areas of the INEL in Idaho and Anaconda, Montana. The locations of sampled sites are shown on Figure 5. Samples were prepared and purified by the method of Winge to determine CdBP levels in tissues of honeybees exposed to cadmium from natural processes.

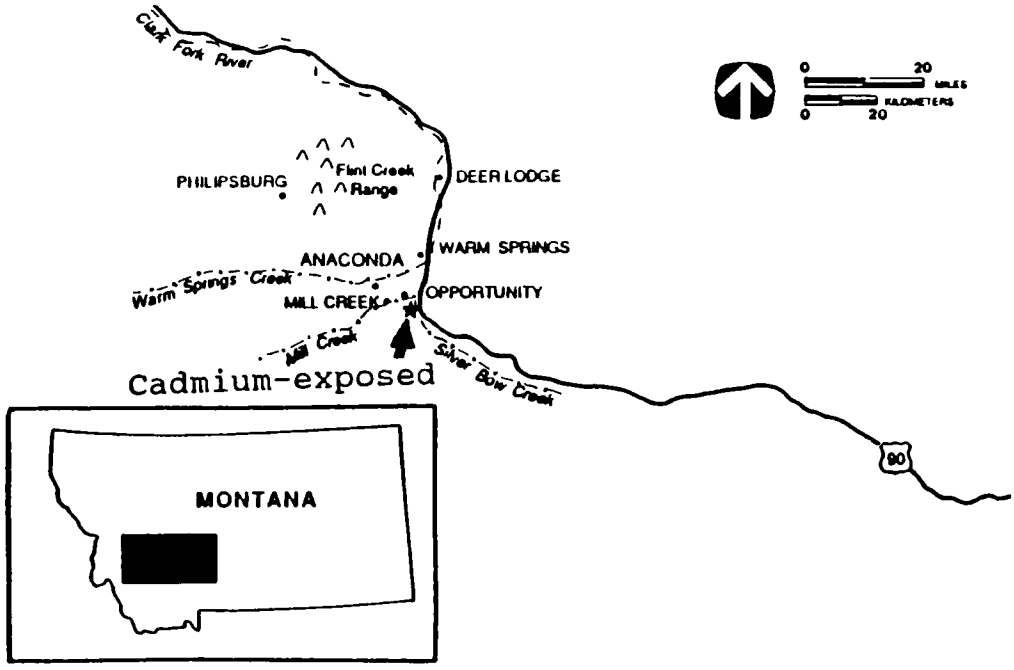
All honey bee samples were obtained by anaesthetizing cages of live bees (approx 20-100) with N_2, and then freezing at minus 80°C. The honey bees had to be gassed to prevent clustering at low temperature, a process that prevented bees from freezing for up to 15 minutes at these extremely low temperatures. The anaesthetization appeared to have no visible effect on honeybees, as they came out of their torpor within one minute after being removed from N_2, and resumed normal activity (fanning, flying, clustering) within three to four minutes. Once bees were frozen, they were immediately weighed and placed into 2 volumes of ice-cold buffer A (10mM Tris-HCl, pH 8.5, 10 mM DTT) containing 10% sucrose. The bees were then homogenized in a waring blender for 4 x 30 second pulses, and sonicated for 4 x 60

FIGURE 5: Location of field sampling sites for <u>in situ</u> honey bee CdBP accumulation.

(A) Idaho National Engineering Laboratory, for control (Mud Lake) and Zinc-exposed (ICPP) honey bee samples.



(B) Anaconda-Mill Creek area for Cadmium-exposed honey bee samples.



seconds on a Branson sonicator. This homogenate was then stored at -80°C until analyzed.

Sample preparation and purification was performed using two methods. The first method was adapted from Kagi and Vallee (1960; Figure 6) and was used for samples in Test 1. This method was first used to isolate equine Cd-MTh from renal cortex tissue and originally contained one chromatography step. A Sephadex G-50 fractionation step was added to remove higher molecular weight proteins, and DEAE Sephadex was used in place of DEAE Cellulose to take advantage of the higher flow rates of Sephadex. The second method was suggested by Dr. Dennis Winge (Figure 7) and was used to prepare subsequent samples. This method differs from the previous procedure in that samples were heated at 65°C for 10 minutes, as most MTh's have been shown to be heat-stable. Protein samples were concentrated by acetone precipitation rather than by tedious ammonium sulfate fractionation, and Tris-HCl was used in most steps rather than phosphate buffers.

Metal concentration was determined on a Perkin Elmer model 5100 atomic absorption spectrophotomer using either flame or electrothermal atomization spectroscopy (EAS or "graphite furnace") methods. The conditions used for each element examined are summarized in Table 6. The minimum detection limits (MDL) for cadmium using flame-AAS was approximately 0.03 ppm with a linear range of 0.05 to 2.0 ppm. Graphite furnace EAS was used with samples which contained small amounts of Cd or were hard to obtain. An MDL of 0.002 ppm was obtained with a linear range of 0.005 to 0.05 ppm. The definition of "MDL" was taken from the Perkin Elmer manual and defined as a level of metal which gives a signal to noise

FIGURE 6: Purification of low MWT-CdBP, Kagi method.

discard fractions — mainline fractions

Whole Honeybees

* homogenization in 50mM phosphate buffer, pH 8 containing 10mM 2-mercaptoethanol
* add 1.0 ml of 2000ppm Cd (as CdCl2)
* centrifugation, 30,000xg for 30 minutes

Residue I

Supernatant I

Precipitate I

* centrifuge 40000xg, 2 hours

Supernatant II

Precipitate II

* Add 1.2 parts EtOH/0.094 parts chloroform
* centrifuge 30000xg, 1 hour

Supernatant III

* dialyze, 50mM phosphate/10mM 2-mercaptoethanol.
* centrifuge 40000xg, 1 hour

Precipitate III

Supernatant IV

* fractionate on Sephadex G50
* pool 4-14kDa fractions

Low MWT CdBP I

* dialyze to 100% AmS saturation
* centrifuge 30000xg, 30 minutes

Supernatant V

Precipitate IV

* dialyze, 50mM phosphate/10mM 2-mercaptoethanol.
* fractionate on DEAE-A25, linear NaCl gradient 0 to 100 mM in phosphate buffer
* pool "MTh-like" proteins

Low-MWT CdBP II

FIGURE 7: Purification of low MWT-CdBP, Winge Method.

discard fractions — mainline fractions

Whole Honeybees

* homogenization in 10mM Tris-HCl buffer, pH 8.6 containing 10mM 2-mercaptoethanol
* centrifugation, 30,000xg for 30 minutes

Residue I

Supernatant I

* heat supe, 65°C for 15 minutes
* centrifuge 40000xg, 2 hours

Precipitate I

Supernatant II

* add 0.25 parts EtOH/0.15 parts chloroform, stir 15 minutes
* add 0.1 part H2O, stir 5 minutes
* centrifuge 30000xg, 1 hour

Precipitate II

Supernatant III

* add 2 volumes -20°C Acetone
* centrifuge 30000xg, 1 hour at -10°C.

Supernatant IV

Precipitate III

* resuspend in Tris/ß-ME buffer
* fractionate on Sephadex G50
* pool low-mwt (4-14kDa) fractions

Low MWT CdBP I

* dialyze against 5mM Tris/ß-ME
* fractionate sample on DEAE-A25, linear NaCl gradient 0 to 100 mM
* pool "MTh-like" proteins

Low MWT CdBP II

TABLE 6: Parameters for elemental quantitation using atomic absorption spectrophotometry (Perkin Elmer model 5100).

(A) Flame AAS determinations:

Element	Line (nm)	MDL, ug/mL	Linear Range, ug/mL	Interferences (nm)
Cd	228.8	0.03	0.05 - 2.0	As (228.8)
Cu	324.8	0.20	0.20 - 5.0	Ni (324.3)
Zn	360.1	2.00	2.00 - 120	none

* values were obtained using air-acetylene flame (10 and 2 liters per minute flow, respectively) and recommended lamp currents. Nebulizer flow rates were approximately 6 to 8 ml per minute.

(B) Graphite furnace AAS determinations:

Element	Line (nm)	MDL, pg	Interferences (nm)
Cd	228.8	0.003	As (228.8)
Cu	324.8	6.0	Ni (324.3)
Zn	213.9	1.0	Sr (214.1)

ratio of 2.00. All metal standards were made from 1000 ppm stock solutions (Fisher Scientific) and balanced with the appropriate buffer to reduce matrix effects.

Gel filtration columns were calibrated by the method of Andrews (1970) using globular proteins of defined molecular weight (Table 7). The V_o of each column was determined by measuring the elution volume of Blue Dextran (2,000 kDa) as determined by absorbance at 280 nm, and the V_i measured by the elution volume of Zn^{2+} (from $ZnCl_2$) as determined by flame AAS. The volume of each standard solution applied to the column was approximately 2% of the total internal volume of the column. Zinc was used rather than cadmium because of their similar valences, chemical behavior, and of the low toxicity of Zn. Column sizes varied depending on the step used. Initial protein fractionation on Sephadex G-50 required a large column (2.5 x 70 cm, 225 cm^3), while later runs utilized a smaller 1.6 x 30 cm column (37.5 cm^3). All analytical molecular weight estimations were made using a smaller column (1.0 x 60 cm, 47 cm^3). Initial DEAE Sephadex chromatography was performed on a 1.6 x 30cm column and later CdBP cleaning runs were made with 10cc "mini-columns." All chromatography was performed at 4°C.

Collected fractions were measured for three parameters: (1) UV absorbance, either the A_{254}/A_{280} ratio as determined by a flow-through cell or by A_{230} on a UV-Vis spectrophotometer, (2) soluble Cd determinations (or Zn in few instances) as determined by flame AAS at 228.8nm, and (3) soluble protein concentrations as determined by the CBB dye-binding method of Bradford using BSA as a standard.

After each chromatography step, CdBP proteins were pooled and taken to the next step. For Sephadex G-50 fractionation, CdBP were defined as those proteins

eluting at an approximate V_e/V_o ratio of 2.0 to 2.5 (corresponding to 4,000-14,000 daltons). For DEAE Sephadex chromatography, CdBP were defined as the fractions that had a high A_{254}/A_{280} ratio and contained soluble cadmium. Protein quantitation was performed using the coomassie brilliant blue (CBB) R-250 dye binding assay (Bradford, 1976) and integration of the cadmium peak.

CdBP samples used for physical characterizations were lyophyllized and stored at -20°C. To determine CdBP protein and metal concentrations, aliquots of this powder were dissolved into buffer A and quantified for protein spectrophotometrically at 595 nm using the CBB R-250 dye binding method of Bradford. This method, like the Lowry method (Stanesh, 1984), requires aromatic amino acids for maximal color development. Unlike the Lowry method, CBB also binds to basic amino acids and has been shown to yield more color than the Lowry method when using rabbit Cd/Zn metallothionein as a standard (Appendix D). Bovine serum albumin was used as the standard when determining the protein content of complex mixtures, and rabbit Cd/Zn MTh was used as a standard in more purified preparations.

Analysis of proteins by SDS-polyacrylamide and "native" gel electrophoresis was performed using the discontinuous method of Laemmli (1970; Pharmacia, 1980). Gels containing 0.1% SDS were run with 17.5% total acrylamide and a constant current of 25 mA. Calibration was made using SDS-PAGE low molecular weight markers (Sigma #MW-SDS-17) that include cleavage products of horse apomyoglobin from 16,950 to 2,510 daltons (Table 8). "Native" gels were actually run with 6 M urea-denatured proteins, so "native" in this instance only implies "no detergent." Initial gels were visualized by coomassie brilliant blue staining, but samples of

TABLE 7: Gel filtration molecular weight standards used.

Standard	Reported Mr, kDaltons	Reference
Blue dextran	2,000	Pharmacia, 1980
Bovine serum albumin	66.0	Andrews, 1970
Egg ovalbumin	42.9	Andrews, 1970
Horse myoglobin	17.4	Andrews, 1970
Rabbit metallothionein	9.6	Appendix D
Vitamin B12	1.5	Sigma Chemical Co.

TABLE 8: Electrophoresis molecular weight standards used.

Standard	Reported[a] Mr, Daltons
Myoglobin, peptide backbone	16,950
Myoglobin, fragment I + II	14,400
Myoglobin, fragment I	8,160
Myoglobin, fragment II	6,210
Myoglobin, fragment III	2,510

[a] Values for molecular weights were reported by Sigma Chemical Company (1986).

TABLE 9: Cysteine-containing protein standards and assay results.

Protein Used	mol cys per mol protein[a]	Observed mol cys per mol protein pCMB	% Recovery	DTNB	% Recovery
BSA	1	0.8	80	0.5	50
Egg lysozyme	8	7.8	98	5.5	69
Metallothionein	22	21.2	96	16.5	75

[a] Values for BSA were reported by Boyer (1954), lysozyme by Creighton (1980) and metallothionein by Sigma Chemical Co.

purified MTh bound the CBB stain so poorly that gels required destaining of the CBB and overstaining with silver nitrate (Merrill, 1981). This meticulous procedure could yield dark yellow gels with well defined black bands if CBB destaining went to completion. If CBB wasn't removed, the silver staining step would produce black gels with no detail. If destaining went too long (greater than 12 hours), these low molecular weight proteins would diffuse from the gel, leaving no bands for staining.

Samples used in spectral studies and sulfhydryl analysis utilized CdBP dialyzed against 5 mM phosphate buffer (pH 8.0) containing 1 mM $CdCl_2$ and no reducing agents. Reducing agents (ß-ME, dithiothreitol) were removed since Cd-mercaptides would interfere with both procedures, and $CdCl_2$ was added to provide protection for cys-metal groups in the protein. It is possible that the protein was changed from its native form, which contains Zn, to a "pure Cd" protein which wouldn't affect these studies. The final protein concentration of this solution was estimated to be approximately 800 µg/ml. Samples used to examine for cadmium-thiolate charge absorbance shoulder were diluted 1:3 in 50 mM phosphate buffer with pH ranging from 2 to 8.

Samples used for cysteine quantitation were diluted 1:4 in 100 mM phosphate buffer (pH 8.0) and titrated spectro-photometrically at 412 nm with [5, 5'-dithiobis(2-nitrobenzoic acid)] using procedures adapted from Ellman (1959) with bovine serum albumin, chick egg lysozyme and rabbit Cd-MTh as standards (Table 9). This procedure gave poor results, with cysteine yield of approximately 70% using egg lysozyme and rabbit metallothionein as standards. A second method using slightly basic para-chloromercuribenzoic acid (p-CMB) to titrate sulfhydryls was adapted from

Boyer (1954). While the extinction coefficient of the CMB-cysteine complex is approximately half that of DTNB, Kagi (1961) showed excellent results quantifying cysteine in equine MTh with p-CMB at pH 7.0, with nearly instantaneous displacement of Cd from Cd-thiolate bonds. Analysis of sulfhydryl recovery gave excellent results of 80-98% recovery with the standards used.

CHAPTER IV

RESULTS

Mortality Response: Feeding Studies.

Figures 8 and 9 show average percent honeybee mortality versus time for varying levels of cadmium chloride and cadmium sulfate, respectively. Mean mortality is also ranked for significance using Tukey's b test, as means followed by the same letter are not statistically different ($P \leq 0.05$). Data for these tests are summarized in tables 10 and 12, with accumulated statistics (mean, standard deviation, standard error and 95% confidence interval) listed in tables 11 and 13. Data from each individual test are listed in appendix A.

These plots show a consistent trend where populations are unaffected by all syrup doses at one hour, and increasing mortality with respect to the control ("0 ppm Cd") at 24, 48 and 96 hour intervals in the higher cadmium feeding levels. For the chloride salt, the 20, 50 and 100 ppm Cd feeding levels showed significant bee mortality as early as 24 hours, where bees fed 10 ppm Cd didn't show significant mortality versus controls until 96 hours (Tukey's b ANOVA; values on Tables 11 and 13). Mortality for all 0 ppm $CdCl_2$ controls never exceeded 2.5% over the 96 hour test period.

A higher toxicity of cadmium sulfate with respect to $CdCl_2$ appears to be evident in the mortality graphs. Mortality measurements at one, 24 and 48 hours showed similar responses to the $CdCl_2$ tests. Significant mortality was evident at 48 hours in the three highest (20, 50, 100 ppm) levels tested. By 96 hours, significant mortality was observed in five of the levels tested including 5, 10, 20, 50 and 100 ppm Cd. Bees exposed to lower cadmium sulfate concentrations (1 and 2 ppm) exhibited similar mortality as bees

FIGURE 8: Mean cumulative honey bee percent mortality, $CdCl_2$ feeding studies. Means sharing the same letter are not significantly different ($P \leq 0.05$; Tukey's b ANOVA).

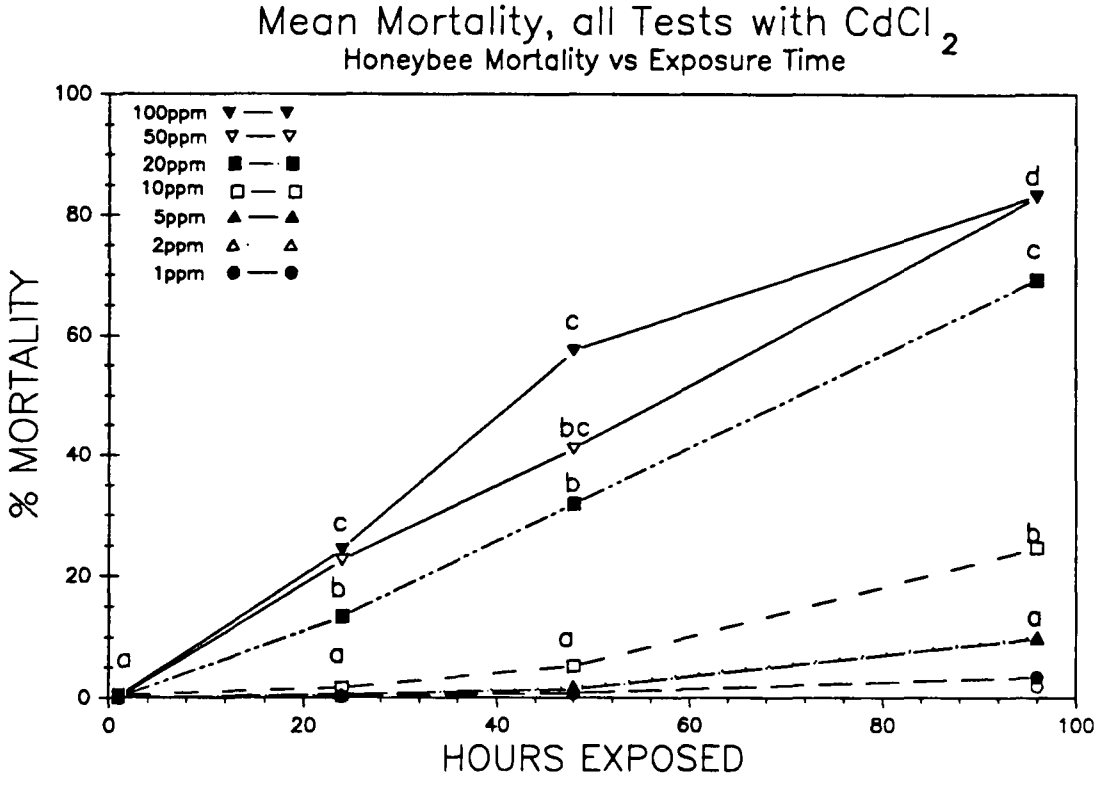


TABLE 10: Tabulated honey bee percent mortality data from $CdCl_2$ feeding studies.

$CdCl_2$,	24 Hours			48 Hours			96 Hours		
ppm	$\overline{X}$[a]	SD[b]	SE[c]	$\overline{X}$	SD	SE	$\overline{X}$	SD	SE
0	0.31	0.43	0.19	0.64	0.36	0.16	1.90	0.47	0.21
1	0.31	0.43	0.19	0.77	0.54	0.24	3.40	1.79	0.80
2	0.33	0.45	0.20	1.87	1.92	0.86	10.1	6.92	3.09
5	0.61	1.00	0.45	1.38	1.27	0.57	9.84	5.67	2.53
10	1.66	1.57	0.70	5.24	5.47	2.44	24.7	8.92	3.99
20	15.0	6.78	3.03	31.9	5.35	2.39	69.3	3.55	1.59
50	22.6	6.42	2.87	41.1	7.64	3.42	83.1	5.39	2.41
100	24.4	3.34	1.49	49.3	12.1	5.39	83.3	8.50	3.80

[a] Mean observed percent mortality for five feeding tests.
[b] Standard deviation of five observations.
[c] Standard error of the mean (95%CI = 1.96*SE).
[d] One-hour feeding data were omitted due to low mortality.

TABLE 11: Statistical values for Tukey's B comparisons - $CdCl_2$ mortality data.

24 Hour Percent Mortality Statistics

Source	DF	Sum of Squares	Mean of Squares	F Ratio Value	F Critical Value	F Prob.
between groups	7	4017.26	573.9	44.84	2.33	0.0000
within groups	32	409.57	12.8			
Σ -	39	4426.8				

* Ranges for 0.05 Level = 3.73, 4.03, 4.20, 4.33, 4.43, 4.51 and 4.58.
* The value actually compared with Mean(J)-Mean(I) is... 2.5297 * range * Sqrt[1/n(I) + 1/N(J)]

48 Hour Percent Mortality Statistics

Source	DF	Sum of Squares	Mean of Squares	F Ratio Value	F Critical Value	F Prob.
between groups	7	14917.0	2131.0	63.52	2.33	0.0000
within groups	32	1073.6	33.5			
Σ	39	15990.7				

* Ranges for 0.05 Level = 3.73, 4.03, 4.20, 4.33, 4.43, 4.51 and 4.58.
* The value actually compared with Mean(J)-Mean(I) is... 4.0957 * range * Sqrt[1/n(I) + 1/N(J)]

96 Hour Percent Mortality Statistics

Source	DF	Sum of Squares	Mean of Squares	F Ratio Value	F Critical Value	F Prob.
between groups	7	46348.4	6621.2	191.16	2.33	0.0000
within groups	32	1108.4	34.6			
Σ	39	47456.8				

* Ranges for 0.05 Level = 3.73, 4.03, 4.20, 4.33, 4.43, 4.51 and 4.58.
* The value actually compared with Mean(J)-Mean(I) is... 4.1615 * range * Sqrt[1/n(I) + 1/N(J)]

FIGURE 9: Mean cumulative honey bee percent mortality, $CdSO_4$ feeding studies. Means sharing the same letter are not significantly different ($P \leq 0.05$; Tukey's b ANOVA).

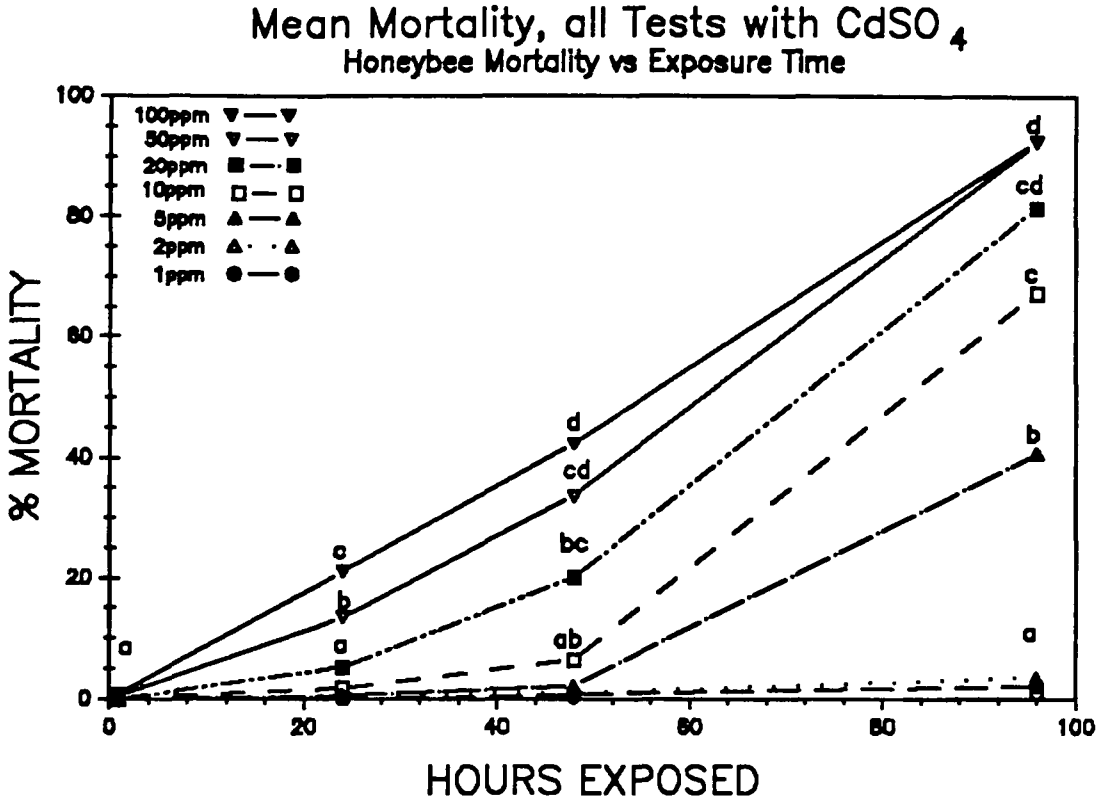


TABLE 12: Tabulated honey bee percent mortality data from $CdSO_4$ Feeding studies.

$CdSO_4$, ppm	24 Hours $\overline{X}$[a]	SD[b]	SE[c]	48 Hours $\overline{X}$	SD	SE	96 Hours $\overline{X}$	SD	SE
0	0.23	0.40	0.23	0.47	0.40	0.23	0.97	0.46	0.27
1	0.00	0.00	0.00	0.71	0.72	0.41	2.17	0.12	0.07
2	0.73	0.76	0.43	0.96	0.83	0.48	3.54	0.82	0.48
5	0.50	0.87	0.50	2.13	1.20	0.69	40.7	22.8	13.2
10	1.42	0.72	0.42	6.45	3.10	1.79	67.2	12.5	7.22
20	5.17	2.74	1.58	20.1	13.7	7.89	81.5	4.38	2.53
50	13.4	6.07	3.50	33.7	9.56	5.52	92.4	2.46	1.42
100	20.9	0.93	0.54	42.3	5.26	3.04	92.6	3.44	1.99

[a] Mean observed percent mortality for three feeding tests.
[b] Standard deviation of three observations.
[c] Standard error of the mean (95%CI = 1.96*SE).
[d] One-hour feeding data were omitted due to low mortality.

TABLE 13: Statistical Values for Tukey's B Comparisons - $CdSO_4$ Mortality Data.

24 Hour Percent Mortality Statistics

Source	DF	Sum of Squares	Mean of Squares	F Ratio Value	F Critical Value	F Prob.
between groups	7	1271.1	181.65	30.80	2.66	0.0000
within groups	16	94.3	5.89			
Σ	23	1365.9				

* Ranges for 0.05 Level = 3.95, 4.27, 4.47, 4.61, 4.73, 4.82 and 4.90.
* The value actually compared with Mean(J)-Mean(I) is... 1.7172 * range * Sqrt[1/n(I) + 1/N(J)]

48 Hour Percent Mortality Statistics

Source	DF	Sum of Squares	Mean of Squares	F Ratio Value	F Critical Value	F Prob.
between groups	7	5858.9	837.0	21.03	2.66	0.0000
within groups	16	636.9	39.8			
Σ -	23	6495.8				

* Ranges for 0.05 Level = 3.95, 4.27, 4.47, 4.61, 4.73, 4.82 and 4.90.
* The value actually compared with Mean(J)-Mean(I) is... 4.4614 * range * Sqrt[1/n(I) + 1/N(J)]

96 Hour Percent Mortality Statistics

Source	DF	Sum of Squares	Mean of Squares	F Ratio Value	F Critical Value	F Prob.
between groups	7	35377.9	5054.0	56.386	2.66	0.0000
within groups	16	1434.2	89.6			
Σ	23	36812.2				

* Ranges for 0.05 Level = 3.95, 4.27, 4.47, 4.61, 4.73, 4.82 and 4.90.
* The value actually compared with Mean(J)-Mean(I) is... 6.6948 * range * Sqrt[1/n(I) + 1/N(J)]

exposed to $CdCl_2$ for each time interval. Bees fed these low levels also showed no significant mortality as compared to controls at the 96 hour interval.

Overall, the cadmium chloride data appeared slightly more variable than the $CdSO_4$ tests, as observed by slightly greater standard error within a treatment level. This variability is surprising, as higher error was expected for $CdSO_4$ due to the lower number of test replications. This could due to seasonal differences in honeybee physiology, as chloride tests were run mid-summer (July to September), and sulfate tests were late-summer to early fall (September to October). Variability in the age structure of sampled bees could also be an important factor, as well as seasonal differences in brood rearing conditions, sperm source(s) for egg fertilization and food resources.

The mortality obtained with either $CdCl_2$ or $CdSO_4$ never exceeded 96% within 96 hours. This is surprising, as I expected to kill all bees within four days using 100 ppm Cd syrup. I offer two possible explanations for this phenomena: (1) that metal-resistant individuals make up a natural component of the populations tested; and (2) that all bees didn't consume equal amounts of the Cd-syrup. The second hypothesis is most probable, since honeybees routinely feed each other by regurgitation. I envision a small percentage of the population that prompted other individuals to feed them their uncontaminated gut contents early in the test, and that these bees just didn't consume much syrup from the bottle over the test period.

Analysis of syrup consumption data shows a significant cadmium effect, where bees consumed decreasing amounts of syrup with increasing levels of Cd salt. Figure 10 shows mean syrup consumption per bee per day versus ppm cadmium (as $CdCl_2$) for all treatment levels at the 96 hour test interval. Data were analyzed by Tukey's b ANOVA

test with test values summarized in Table 14. A similar plot, Figure 11, was composed for $CdSO_4$ feeding data. Controls for all tests consumed an average of 0.117 ml/bee/day (std. dev. = 0.013, n = 8 tests) over the 96 hour feeding studies. This value agrees well with that obtained by Fujii (1980), where control honeybees consumed approximately 0.20 ml/bee/day.

Consumption of syrup decreased to 0.011 ml/bee/day for the 100 ppm chloride salt, and the total volume of syrup consumed per bee over the 96 hour test showed a highly negative correlation coefficient when compared to ug cadmium per ml in syrup (-0.884). Consumption of syrup decreased to 0.007 ml/bee/day when syrup contained 100 ppm Cd, and consumption per bee over 96 hours also showed a highly negative correlation when compared to µg cadmium per ml in syrup (-0.900).

One common trend seen at all feeding levels irrespective of cadmium source was that syrup consumption appeared to be non-linear over the 96 hour study, with highest rate of syrup consumption occurring within the first 24 hours. It is assumed that this trend is a result of the short period of food deprivation used to get bees to consume cadmium syrup.

It was hypothesized early in the study that the increasing mortality seen with higher levels of cadmium in the syrup could have been due to starvation. To examine this possibility, two tests were run to determine the nature of "food-deprived" mortality for the 96 hour test period. Data plotted in Figure 12 show that mortality does not increase until the 72 and 96 hour periods, and the increases appear non-linear. This response is quite different than the response seen in bees fed higher cadmium levels, and it is hypothesized that honeybees show resistance to starvation either due to slowed

FIGURE 10: Mean syrup consumption per honey bee per day, $CdCl_2$ feeding studies. Bars denote means ± standard deviation. Means sharing the same letter are not significantly different ($P \leq 0.05$; Tukey's b ANOVA).

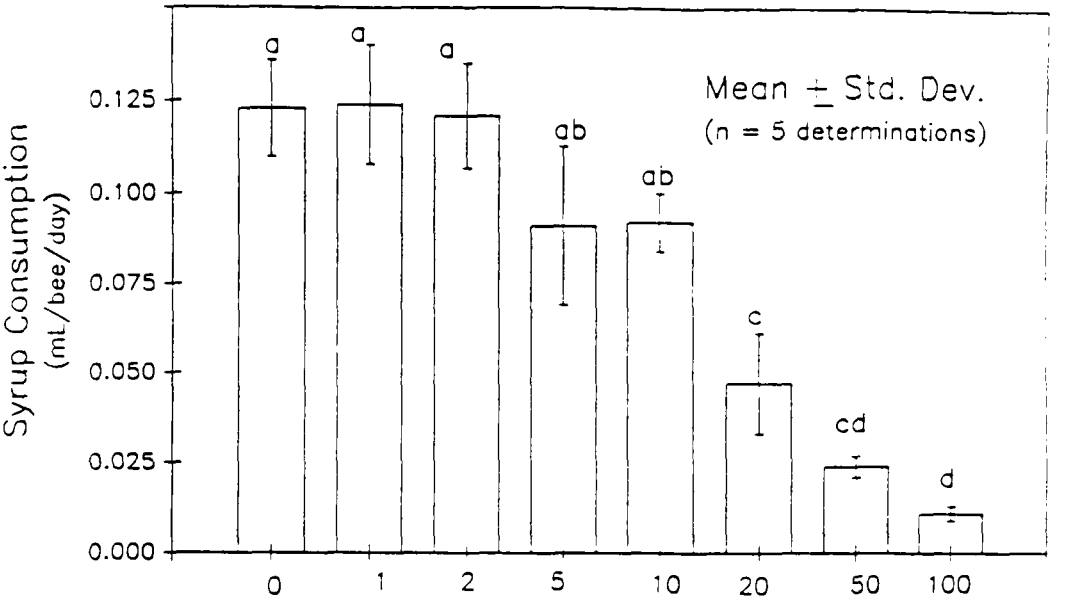


FIGURE 11: Mean syrup consumption per honey bee per day, $CdSO_4$ feeding studies. Bars denote means ± standard deviation. Means sharing the same letter are not significantly different ($P \leq 0.05$; Tukey's b ANOVA).

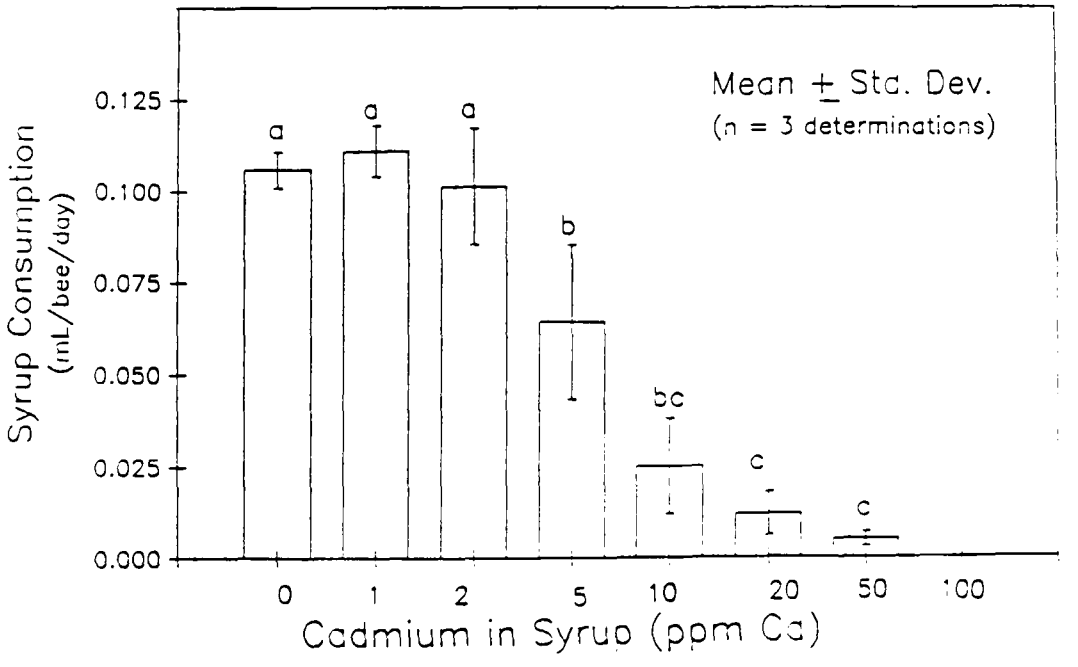


TABLE 14: Statistical values for Tukey's B comparisons - Syrup consumption for $CdCl_2$ and $CdSO_4$ feeding experiments.

CdCl2 Feeding Statistics: ml consumed vs ppm Cd

Source	DF	Sum of Squares	Mean of Squares	F Ratio Value	F Crit.	F Prob.
between groups	7	0.0737	0.0105	48.17	2.33	0.0000
within groups	32	0.0070	0.0002			
Σ	39	0.0806				

* Ranges for 0.05 Level = 3.73, 4.03, 4.20, 4.33, 4.43, 4.51 and 4.58.

* The value actually compared with Mean(J) - Mean(I) is... 0.0105 * range * Sqrt[1/n(I) + 1/N(J)]

CdSO4 Feeding Statistics: ml consumed vs ppm Cd

Source	DF	Sum of Squares	Mean of Squares	F Ratio Value	F Crit.	F Prob.
between groups	7	0.0392	0.0056	29.80	2.66	0.0000
within groups	16	0.0030	0.0020			
Σ	23	0.0422				

* Ranges for 0.05 Level = 3.95, 4.27, 4.47, 4.61, 4.73, 4.82 and 4.90.

* The value actually compared with Mean(J) - Mean(I) is... 0.0097 * range * Sqrt[1/n(I) + 1/N(J)]

physiological state (as many were visibly weakened by 96 hours), or through use of their proventriculus ("honey stomach") that can be used for short-term syrup storage.

A trend also observed and possibly related to syrup consumption is that honey bees average weight decreased with increasing cadmium chloride dose. While a decrease was not observed in bees fed 1 ppm Cd syrup, bees fed more than 2 ppm Cd syrup showed a significantly reduced weight per bee when compared to controls ($P \leq 0.02$). A summary of this weight decrease is shown in Figure 13, where bee weight is plotted versus ppm Cd in syrup.

The calculated median lethal dose for $CdCl_2$ and $CdSO_4$ are summarized in Table 15, with t-test values listed in Table 16. Cadmium chloride toxicity proved to be significantly less than cadmium sulfate at similar levels ($P \leq 0.01$), presumably due to a toxicity contribution of the sulfate anion. The LD50 of $CdCl_2$ ranged from 1.7 to 4.3 ug Cd per bee over a 48 hour period, to 2.1 to 3.7 ug Cd per bee over a 96 hour period. A significant difference was not seen ($P = 0.25$) between the 48 and 96 hour LD50 values, presumably due to the high standard error in 48 hour LD50s. For $CdSO_4$, the 48 hour LD50 ranged from 2.2 to 2.4 ug Cd per bee to 0.98 to 1.8 ug per bee for the 96 hour LD50. A significant difference was seen between the LD50 at these two time intervals. Using the classification proposed by the University of California-Davis Division of Agricultural Sciences, cadmium appears to fit well into the group of moderately-toxic to highly-toxic pesticides (Atkins, 1981).

FIGURE 12: Starvation-induced cumulative mortality in honey bees over a 96 hour period.

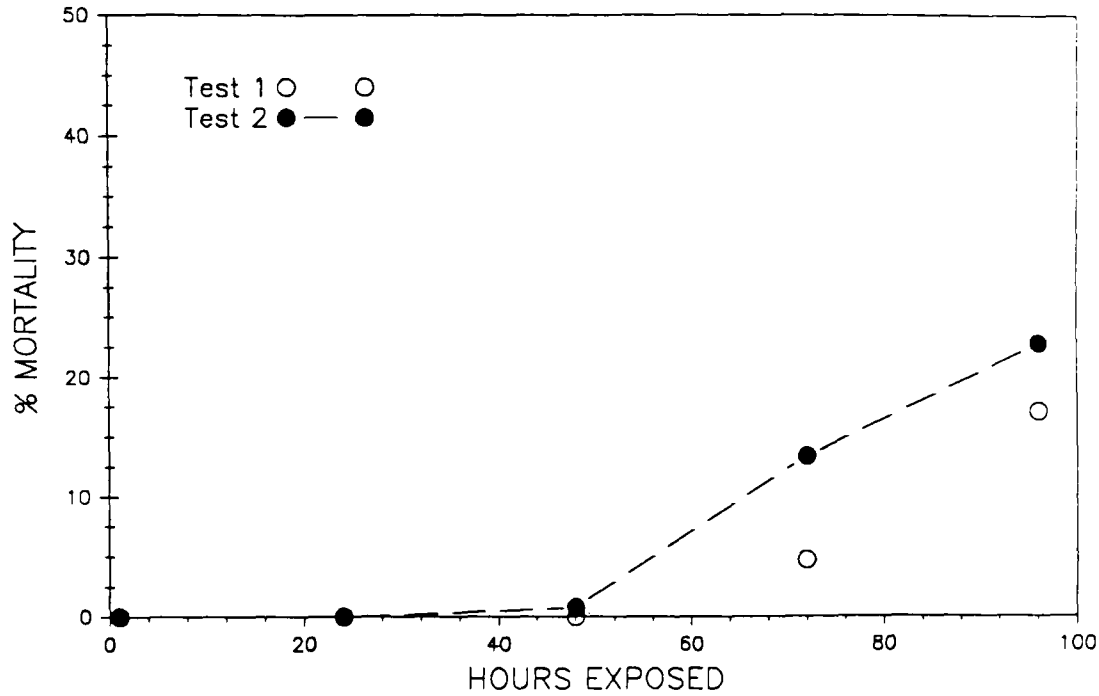


FIGURE 13: Effect of different concentrations of Cd syrup on bee weight. Bars denote mean ± standard deviation. Means sharing the same letter are not significantly different ($P \leq 0.05$; Tukey's b ANOVA).

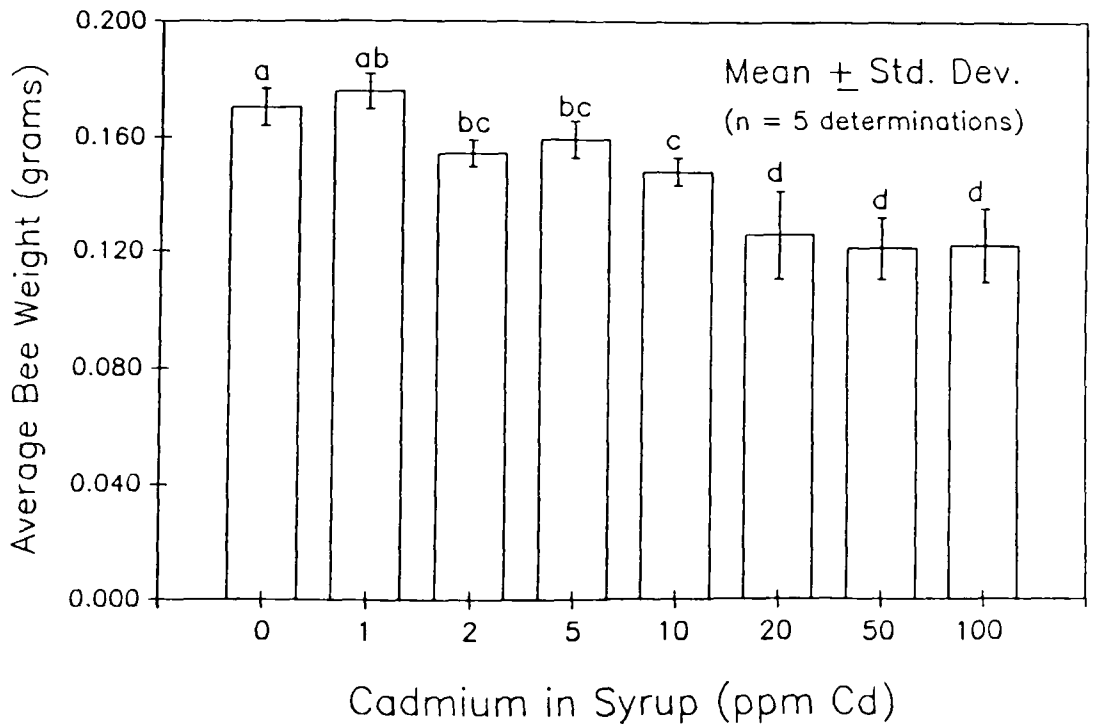


TABLE 15: Estimated median lethal oral dose (LD50, in μg) to honey bees using $CdCl_2$ and $CdSO_4$.

Test #	Cadmium source	48 Hr LD50	95% C.I.	Slope, probits	96 Hr LD50	95% C.I.	Slope, probits
1	CdCl2	1.65	± 0.224	2.07	2.05	± 0.290	2.59
2	CdCl2	4.10	± 0.362	2.27	3.12	± 0.235	2.69
3	CdCl2	4.41	± 0.263	2.14	3.25	± 0.232	2.82
4	CdCl2	3.01	± 0.204	2.14	2.68	± 0.128	2.78
5	CdCl2	4.38	± 0.303	1.54	2.91	± 0.102	2.54
6	CdSO4	2.21	± 0.196	2.45	0.98	± 0.053	5.52
7	CdSO4	2.43	± 0.198	2.37	1.58	± 0.058	4.95
8	CdSO4	2.39	± 0.173	2.36	1.75	± 0.072	4.71

* Mean $CdCl_2$ LD50s: 48h = 3.51 ± 1.04 96h = 2.80 ± 0.41 (μg/bee ± 95% C.I.)

* Mean $CdSO_4$ LD50s: 48h = 2.34 ± 0.14 96h = 1.44 ± 0.46 (μg/bee ± 95% C.I.)

TABLE 16: Statistical data[a] for t-tests on LD50 values.

CdCl2 Toxicity Statistics: 48 hr LD50 vs. 96 hr LD50

Group	N	$\bar{X}$	S.D.	S.E	t-Value	t-Crit	DF	Prob
48 Hr	5	3.51	1.19	0.530	1.24	2.57	4	0.250
96 Hr	5	2.80	0.47	0.211				

CdSO4 Toxicity Statistics: 48 hr LD50 vs. 96 hr LD50

Group	N	$\bar{X}$	S.D.	S.E.	t-Value	t-Crit	DF	Prob
48 Hr	3	2.34	0.12	0.071	3.70	3.18	4	0.021
96 Hr	3	1.44	0.41	0.234				

[a] Statistics computed using pooled variance estimates and two-tailed probability t-values.

Mortality Response: Injection Studies.

A series of five injection studies using $CdCl_2$ were performed on bees to determine the LD50 of Cd by injection. Figure 14 summarizes the mean cumulative bee mortality from the five experiments and the data from 48 hour intervals are summarized in Table 17.

These tests, by the nature of handling and injecting, were much more rigorous on the bees than passive feeding studies. This is reflected by the high honey bee control mortality of 50% in the first experiment. Since data at the 48 hour interval appeared the most consistent, this was the only time interval used to calculated an LD50 by injection. This is the time interval reported in almost all toxicity studies with honey bees (although these data certainly are no more relevant for predicting "non-laboratory" toxicities of any metal). It was determined that variability could be reduced somewhat by increasing the number of bees per dosage level from 25 to 50. Since one hour was required to inject 50 bees, the number of dose levels and the number of replicate tests were kept to a minimum.

As can be seen in Figure 14, the earliest injection experiment showed the highest mortality for controls. This was probably due to a combination of: (1) I was learning how to inject bees without getting stung; and (2) the tests were performed in November. These bees had been collected and caged two weeks prior to initiating the study and, as such, were older than bees used in feeding experiments (one to two months as opposed to one to two weeks old). Later tests were all performed during spring and summer months (May, June and August) with young bees and show much less mortality with controls. With this in mind, the data from the first injection experiment are omitted from

FIGURE 14: Mortality from $CdCl_2$ injection tests. Values plotted are the mean of four determinations, and error bars denote ± standard deviation.

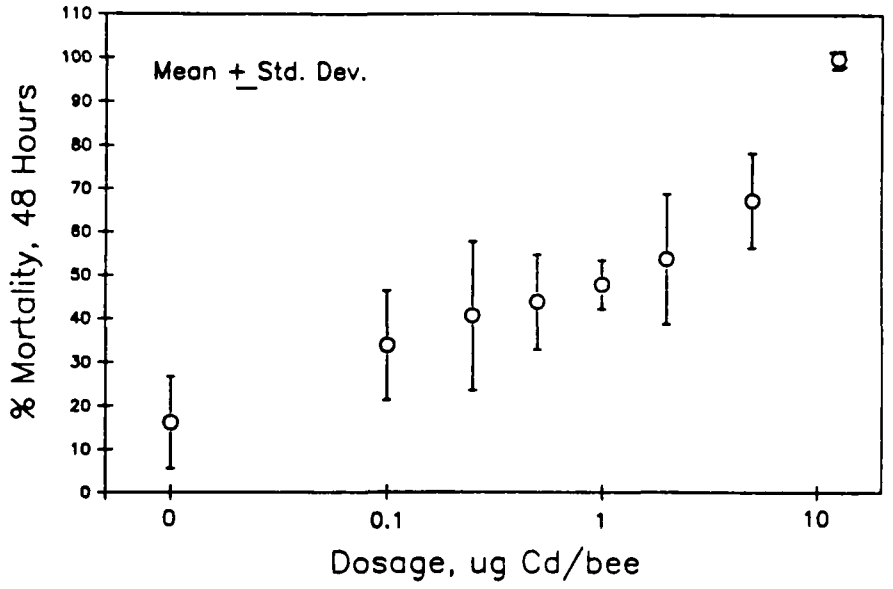


TABLE 17: Tabulated mortality data from $CdCl_2$ honey bee injection studies (48 hour interval).

Injected Cd, µg	Average % Mortality	Standard Deviation	Standard Error	95% C.I.
0.0	16.2	10.6	4.76	9.33
0.1	36.0	12.6	5.62	11.02
0.25	43.5	18.5	9.25	18.13
0.5	45.2	9.86	4.41	8.64
1.0	46.0	9.59	4.29	8.41
2.0	60.0	6.34	4.92	2.83
5.0	72.0	5.66	9.63	5.54
12.5	100	--	--	--

TABLE 18: Estimated median lethal dose (LD50), honey bee $CdCl_2$ injections.

Test Number	48 Hr LD50	Slope, probits	Standard Error	r2 of regression
1	2.08	0.081	0.347	0.24
2	1.62	0.960	0.335	0.79
3	1.65	0.560	0.195	0.80
4	2.32	0.324	0.201	0.55
5	2.39	0.837	0.379	0.70

* Mean LD50 (± SD) of $CdCl_2$ by injection = 1.99 ± 0.42 µg
* 95% Confidence Interval = 1.58 to 2.40 µg

cumulative statistics (mean, standard deviation) in Table 17.

The calculated LD50 for all tests are summarized in Table 18. A mean 48-hour LD50 of 1.95 ug Cd per bee was obtained with slopes ranging from 0.32 to 0.96 probits. These slopes are considerably less than those obtained in either the CdCl2 (1.54 to 2.82) or CdSO4 (2.36 to 5.52) feeding studies. Since the calculations for LD50 and slope are made after the background ("control") mortality is subtracted, this is not an artifact from the higher background mortality. In general, compounds with a shallow slope display a wide lethal dose range, requiring only small doses to be toxic (Ottoboni, 1984).

When comparing the mean 48 hour LD50 from injections and feeding studies, the values are very similar with the injection values appearing slightly lower. The mean of injection LD50 values were not different than the mean LD50 from CdSO4 feeding studies ($P = 0.20$), but were significantly lower than CdCl2 feeding studies. This difference could suggest that route of administration plays an important factor in metal toxicity. The LD50 values obtained from injection studies, while lower than feeding studies, appear similar enough to suggest that cadmium intoxication and poisoning, as opposed to starvation, led to the death of the bees in feeding studies.

Protein Studies.

PROTEIN TEST 1: Analysis of Cd-Exposed Honeybees for CdBP.

In this test, bees were fed CdSO4-containing syrup for time intervals ranging from 6 to 144 hours. At the end of each test, bees were homogenized by the method of Kagi with modifications that included addition of Cd^{2+} to the sample and a Sephadex G50 fractionation. The Cd addition step was a suggestion by Dr. David Quigley, as it has been demonstrated that purification in the presence of reducing agents as well as excess divalent group IIB metal (Zn, Cd) gives added protection against oxidation of sulfhydryl groups. This step was eliminated in later protein purifications (tests 2 through 4), as it created an artificial Cd distribution in the Sephadex chromatograms and probably changed the form of the CdBP's from a mixed Cd/Zn metalloprotein to a "pure Cd" form.

Separation of cadmium-containing peaks can be seen in chromatograms 1 through 6 (Figure 16) from Sephadex fractionation. The largest cadmium peak observed in all chromatograms was bound to molecules eluting at or near the void volume of the column (molecular weight $\geq$ 60,000 Daltons) between fractions 20 to 30. This peak displayed a high A254/A280 ratio (as determined by a flow-through UV cell), suggesting it was either high in cysteine-Cd complexes or nucleic acid. This cadmium peak was later determined to be artifactual and dependent upon addition of 2000 µg Cd early in the homogenization steps. The gram ratio of cadmium to protein appeared nearly constant throughout this peak, ranging from around 6 to 12 µg Cd per mg protein. This value is not much different from those found throughout the entire chromatogram of control bees fed 0 ppm Cd syrup, which leads me to believe that binding of Cd to protein was non-specific. As such, this large Cd peak only reflects the large amount of higher molecular weight

FIGURE 15: Sephadex Chromatograms of homogenized honey bees: cadmium fed from 0 through 48 hours (NOTE: large Cd peak eluting at the void is an artifact of adding soluble Cd^{2+}).

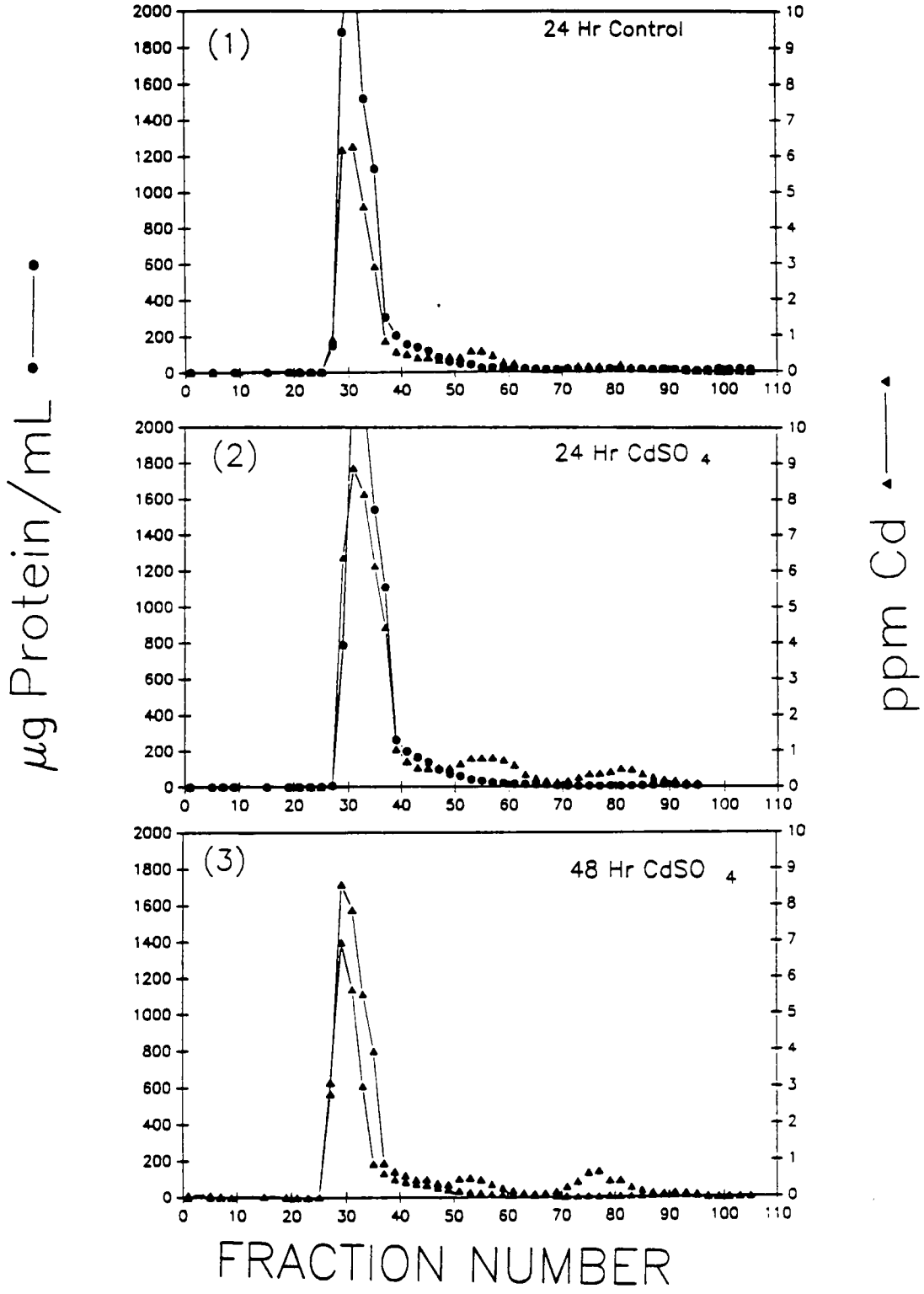
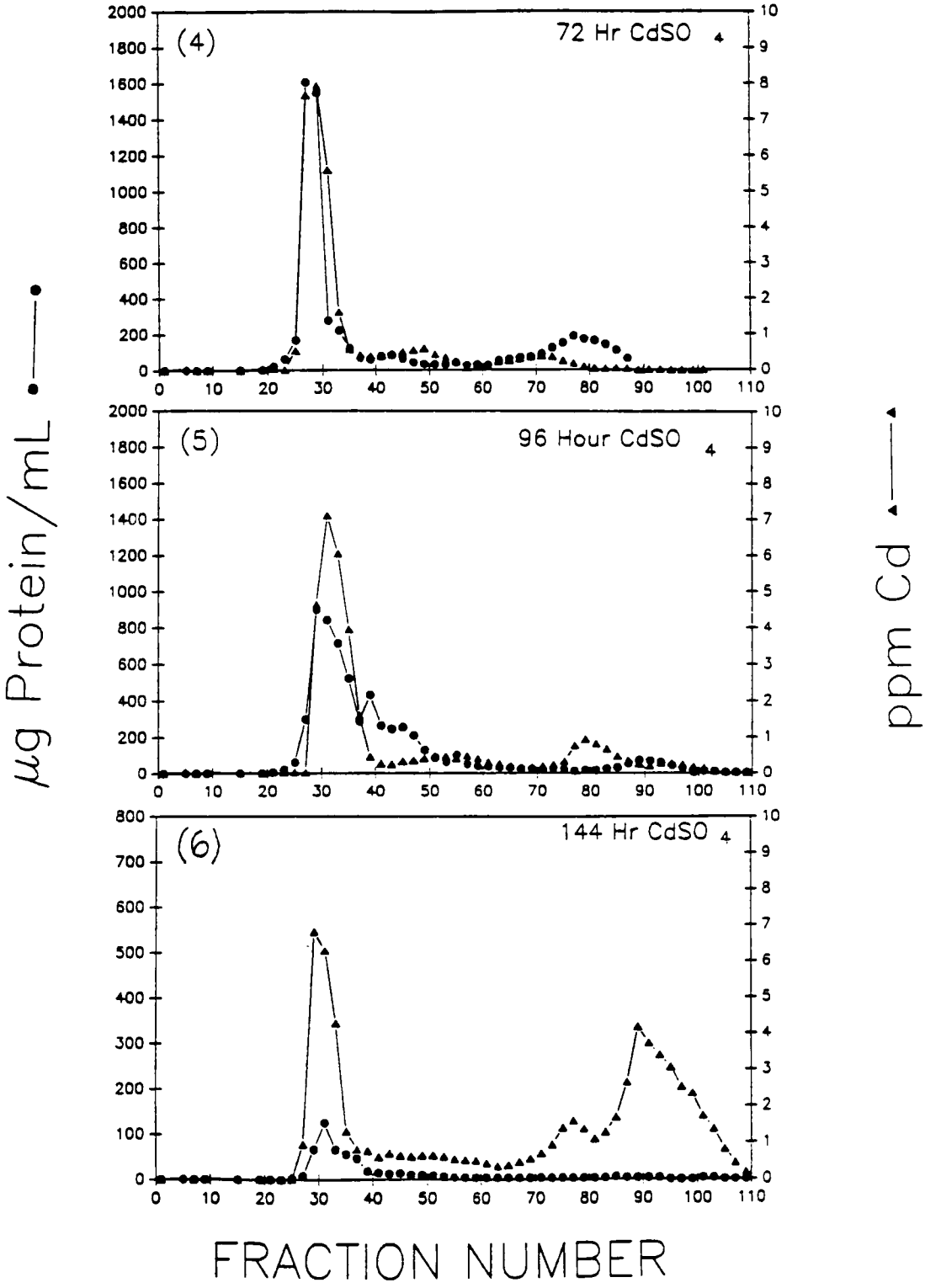


FIGURE 16: Sephadex Chromatograms of homogenized honey bees: cadmium fed from 72 through 144 hours (NOTE: large Cd peak eluting at the void is an artifact of adding soluble Cd^{2+}).



proteins and residual nucleic acids in the sample.

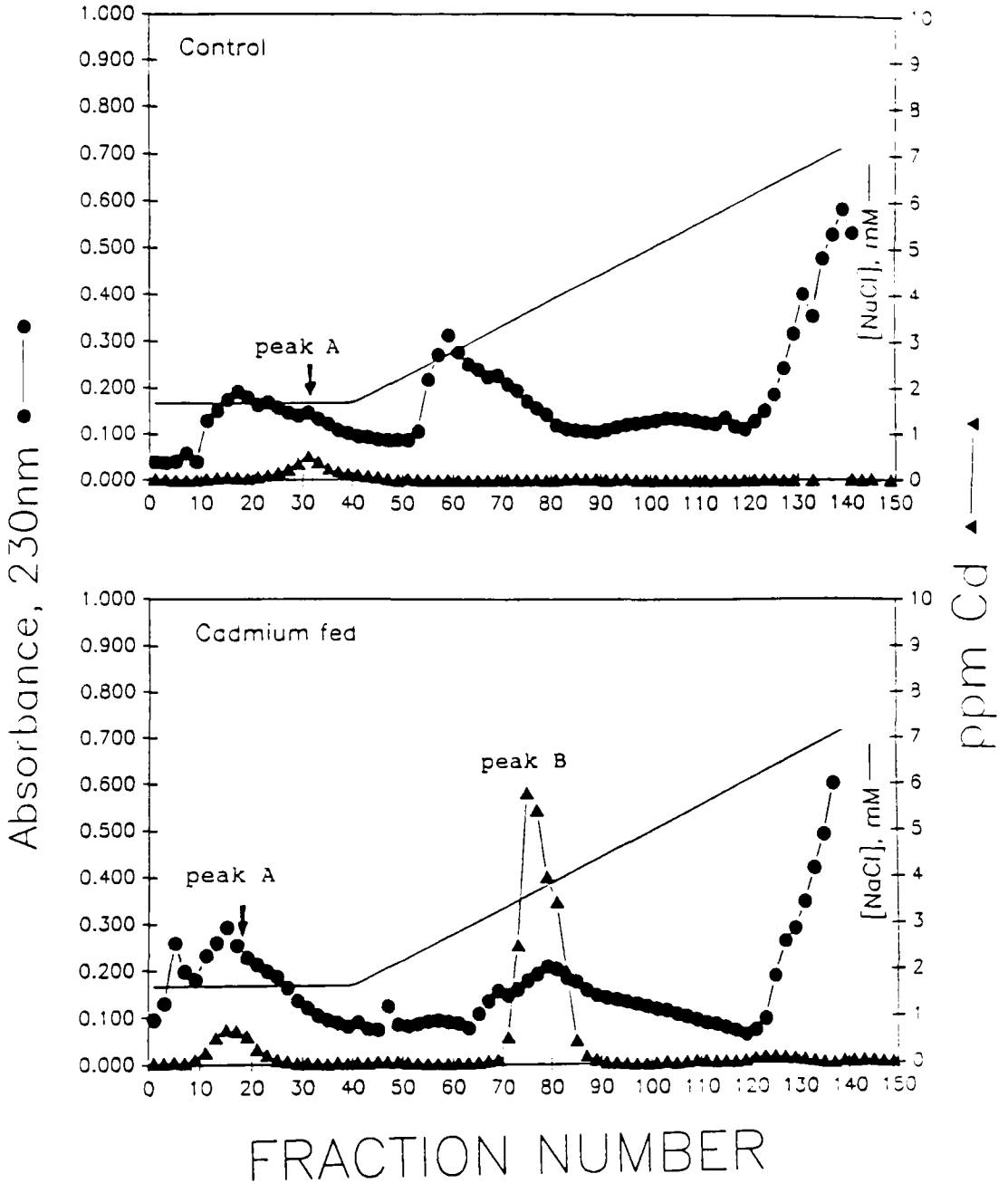
A cadmium "shoulder" was observed eluting after the void volume peak at an approximate Ve/Vo ratio of 1.8 (fractions 40 to 50). This shoulder corresponds to a protein of molecular weight around 25 to 35 kDa. This protein differed from the void volume peak in that it bound cadmium at a slightly higher ratio, ranging from 12 to 20 µg Cd/mg protein. Data suggests that this group of proteins may preferentially bind cadmium from solution, but this was not studied in detail as the protein appeared to be present in the control bees fed 0 ppm Cd syrup. The nature of this protein peak was not studied, but it most likely contains high sulfhydryl content, either in the reduced (-SH) form, or bound to zinc which allows cadmium substitution.

With increasing exposure to cadmium, a Cd-rich peak appeared as early as 24 hours, and persisted in all samples with an elution ratio of Ve/Vo of 2.5 (fractions 65-90). This protein peak corresponded to an apparent molecular weight of 7,000 to 10,000 Daltons. The cadmium binding capacity of this protein peak appeared to increase with respect to time, ranging from 10-15 µg Cd/mg protein in early samples (Figure 16, graphs 2-4) to as high as 50-100 in the 96 and 144 hour bee samples (graphs 5, 6). This peak showed a high A254/A280nm ratio, which could be attributed to Cd-thiolate bonds, yet it eluted well before the V_t where Cd-mercaptide complexes (cadmium mercaptoethanol) eluted. It is this peak that was tentatively identified as a "HB-CdBP", and fractions were pooled from all samples and were lyophyllized to concentrate the sample. This yellow-tan powder was dissolved in distilled water containing 5 mM ß-ME, centrifuged at 40,000 x g for 30 minutes and dialyzed overnight against 5 mM Tris-HCl containing 5 mM ß-ME (pH 8.6).

A 10 ml sample from the pooled cadmium treatments was rechromatographed on DEAE-Sephadex A25 (Figure 17). A similarly treated "control" was also run using Sephadex fractionated proteins from a sample of bees fed no cadmium. Columns were eluted using a linear gradient from 0 to 250 mM NaCl in buffer A. An initial peak containing a significant portion (5%) of cadmium eluted between fractions 10 through 30, even before the salt gradient was started (note "peak A," Figure 17). This protein failed to bind to DEAE Sephadex under the conditions used. I feel that this cadmium peak is due to one of three possibilities: the elution of free Cd2+, a cadmium-mercaptide complex, or non-specifically complexed Cd-protein mixture. Since a similar peak is also seen in the control chromatogram between fractions 25-35, it is most likely not a specific cadmium-binding protein induced from feeding studies.

A second major peak ("peak B," Figure 17) containing approximately 95% of total cadmium applied eluted at approximately 55 mM NaCl (fractions 70-85), binding weakly to DEAE Sephadex. This protein fraction eluted at a NaCl concentration slightly lower than that used to elute rabbit metallothionein, suggesting the honey bee protein has a different charge (more positive) than that of rabbit MTh. This protein peak bound cadmium in a ratio of approximately 62 µg Cd per mg protein, suggesting that it was a CdBP inducible from cadmium feeding. The majority of the total protein applied to the column began eluting at approximately 100 mM NaCl and continued until approximately 250 mM (not shown on graph). None of the fractions beyond approximately 75 mM NaCl contained soluble cadmium above background levels. The pooled sample from the "CdBP-peak" was dialyzed against 5 mM Tris-HCl (pH 8.6) with 5 mM ß-ME and lyophyllized to dryness. This sample was then resuspended in 10 ml of distilled water

FIGURE 17: DEAE-sephadex chromatograms of CdBP samples; untreated control and cadmium fed honey bee samples.



containing 5 mM ß-ME, centrifuged and examined for protein and cadmium concentration. Aliquots were saved for re-chromatography for molecular weight determination and absorbance scans in the UV range. A summary of purification of this cadmium binding protein is listed in Table 19, with a SDS-PAGE profile of samples from the purification process in Figure 18.

When analyzed on SDS-PAGE, this cadmium-binding protein sample demonstrated approximately 6-7 bands after visualization with silver staining (Figure 19). The molecular weights for these bands are graphically displayed in Figure 20. A "smearing" band can be observed in the region from 4,300 to 5,200 molecular weight region of the gel, presumably due to incomplete denaturation of the CdBP after heating for 10 minutes at 85°C. The greatest intensity is seen at around 4.60 kDa, which was taken as the apparent denatured molecular weight (or aM_r). This information suggests that the CdBP purified with one ion-exchange chromatography step is only partially purified.

To make a second estimate of this proteins molecular size, an aliquot of this honey bee CdBP was applied to a pre-calibrated analytical Sephadex G-50 column, and fractions were examined for soluble Cd and absorbance at 230 nm. A minor cadmium-containing peak eluted at a Ve/Vo of around 1.8 (30-40 kDa), but comprised less than 1% of the total cadmium. The major cadmium-containing peak eluted at a Ve/Vo of 2.6, corresponding to an aM_r approximately 7.80 kDa (Figure 21). This value does not agree with values obtained from SDS-PAGE, but agrees with the reported nature of mammalian MTh which displays a molecular weight of 10,000 using gel filtration, yet shows a denaturing gel electrophoresis aM_r 6,500 (Hamer, 1986). It is believed that metal atoms bridge sulfhydryl groups to preserve a rigid beta-sheet structure that gives MTh a non-

TABLE 19: Purification table of honey bee CdBP as illustrated by soluble cadmium/protein ratios.

SAMPLE	volume	[Cd], µg/ml	total µg Cd	[protein], µg/ml	total µg protein	µg Cd per mg Protein
Crude Bee Homogenate	750	13.3	10000	ND[a]	ND	ND
Sephadex G50 Purified CdBP	15	7.5	113	300	4500	25.1
DEAE-Sephadex Purified CdBP	10	1.91	19.1	32.5	325	58.7

[a] ND = Not Determined. Values for "µg Cd/mg Protein" would show artificial elevation due to addition of $CdCl_2$ after homogenization step.

FIGURE 18: SDS-PAGE analysis of honey bee CdBP samples from the purification scheme (gel was coomassie brilliant blue stained and silver over-stained, hence the overdevelopment of crude samples).

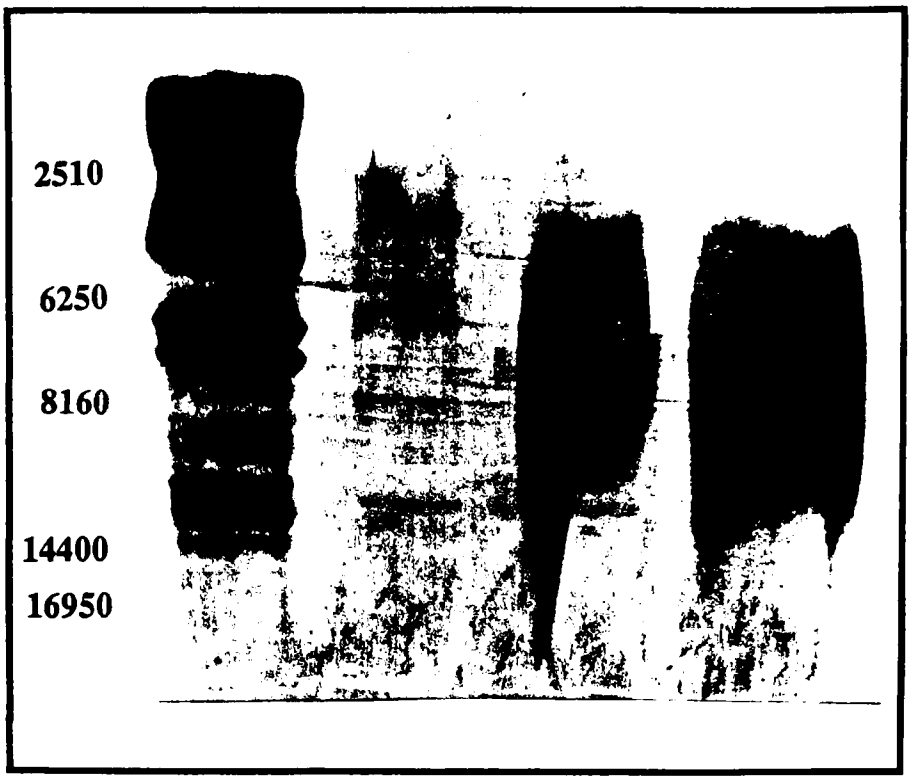


FIGURE 19: SDS-PAGE Analysis of Purified honey bee CdBP. Note that the most intense band migrates with an approximate mass of 4500 Daltons.

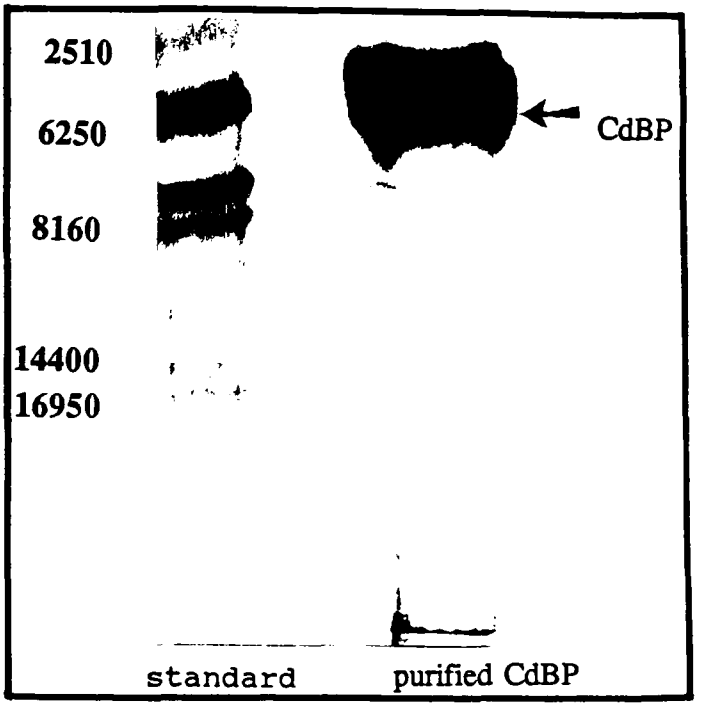
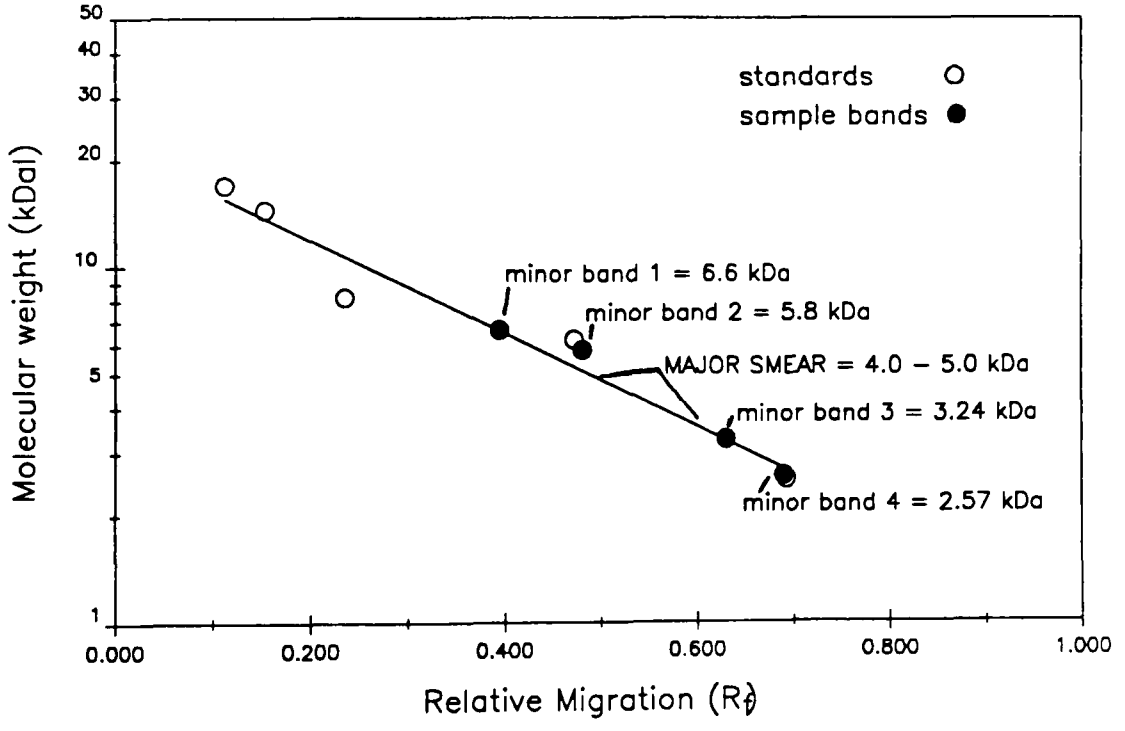


FIGURE 20: Determination of honey bee CdBP molecular weight: Plot of CdBP migration versus standards.

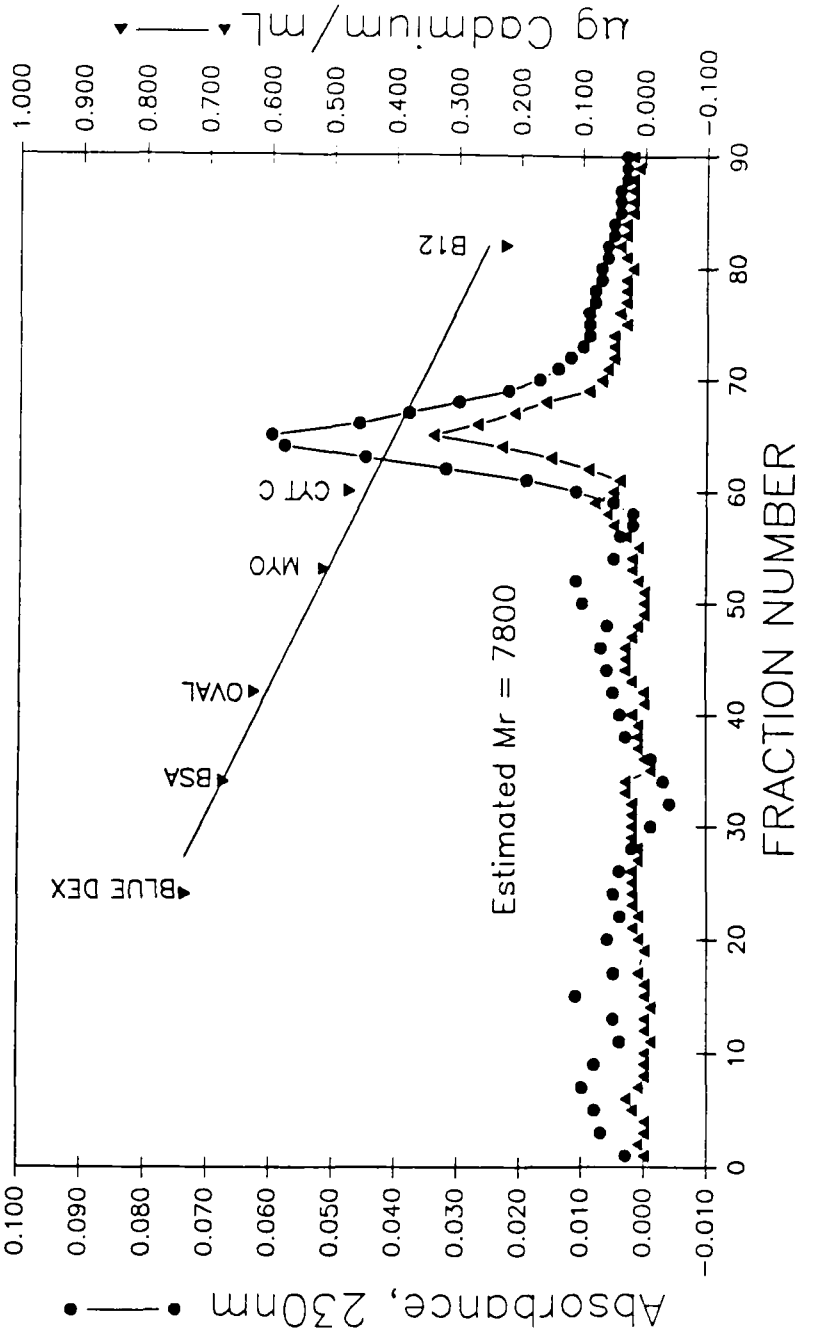


ellipsoid shape. This "distorts" the shape of the protein, making it appear larger than it actually is. For calculations requiring a molecular weight, the SDS-PAGE apoprotein value of 4,600 (or 4.60 kDa) was used rather than the higher (and less accurate) gel filtration estimate.

An aliquot was lyophyllized and resuspended in a reduced (1:4) volume of 33 mM phosphate buffer, pH 7.0 without reducing agents but containing 1 mM $CdCl_2$ to keep sulfhydryls saturated. The sample was dialyzed overnight against two changes of buffer, and unbound cadmium was dialyzed away with one change of buffer. The sample was titrated with slightly basic (NaOH) para-chloromercuribenzoic acid for sulfhydryl quantitation. Ellman's reagent (5,5'-Dithiobis[2-nitrobenzoic acid]) and 2,2'-dithiopyridine were not used since they yielded unsatisfactory results with sulfhydryl standards. Using "thionein" (or apoprotein) molecular weight of 4600, the CdBP contained 10.7 mol cysteine per mole protein. This value is substantially less than the 21.0 mol cysteine per mol protein reported for mammalian metallothioneins.

When examined for protein, the sample purified from DEAE chromatography contained 32.5 μg protein per ml (using rabbit Cd-MTh as a standard). Using an estimated honey bee CdBP molecular weight of 4.60 kDa, I calculated the sample to contain approximately 7.14 nmol CdBP per ml. Metal concentrations ranged from 3.26 μg/ml for Cd (29.1 nmol Cd per ml) to 0.09 μg/ml for Zn (1.38 nmol Zn per ml). These data suggest that the CdBP contained 0.20 mol of Zn per mol protein and 4.1 mol of Cd per mol of protein, or 102 μg Cd per mg total protein. If the gel filtration molecular weight (7,800 Daltons) of honey bee CdBP is substituted for these calculations, a metal content of 6.9 mol Cd and 0.34 mol Zn per mol CdBP is calculated, an unreasonably high

FIGURE 21: Chromatogram of sephadex molecular weight determination for honey bee CdBP. Standards are listed with elution volume.

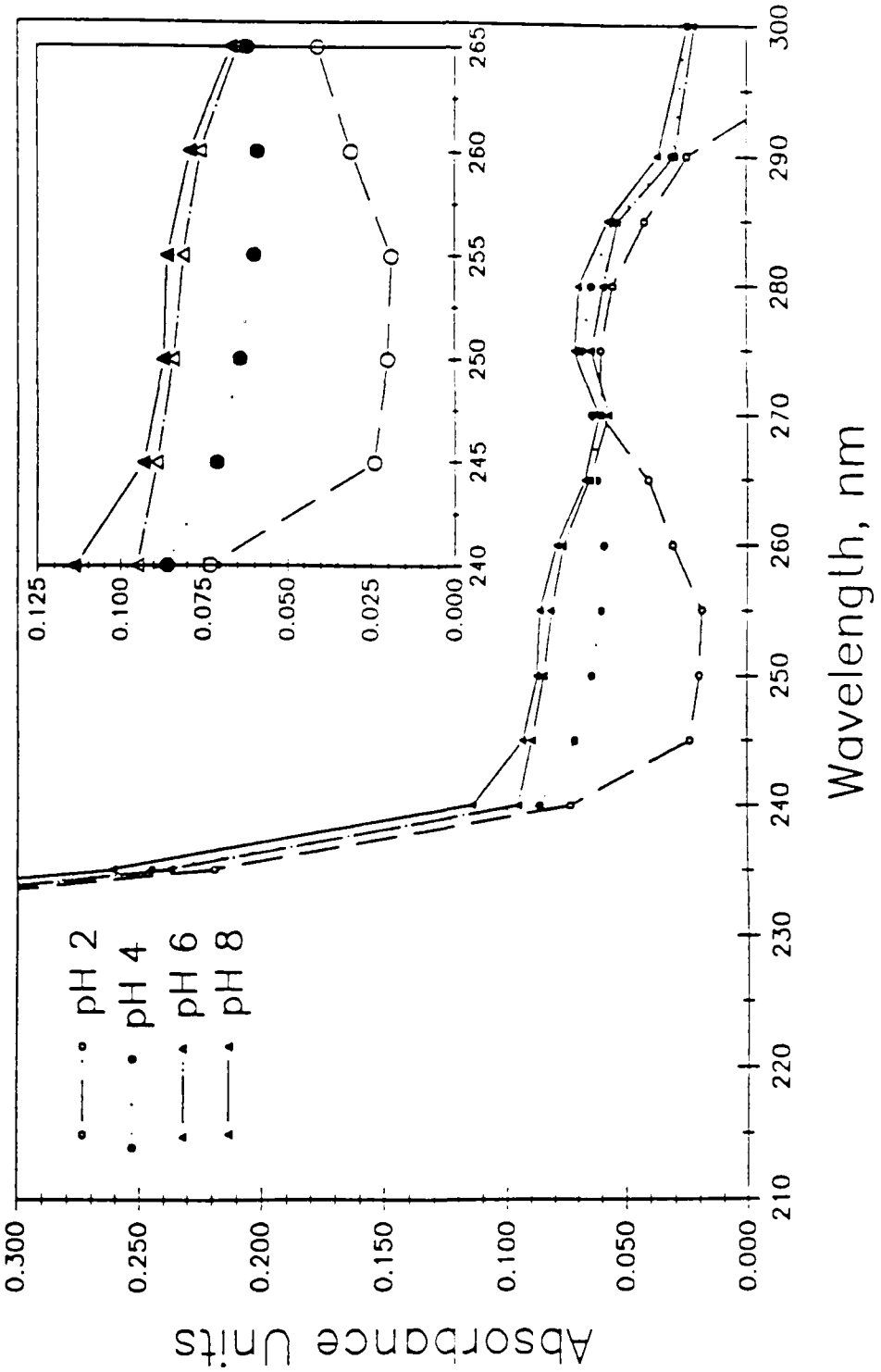


value considering the molecular weight and sulfhydryl content of this CdBP.

In order to examine the absorbance characteristics of this honey bee CdBP, the remainder of the sample (approx. 300 µg protein) was dialyzed against distilled water containing 2 mM CdCl2. This sample was lyophilized to dryness and resuspended in 1.0 ml distilled water. Equal 250 µl aliquots were divided and diluted with 250 µl of 10 mM Tris-HCl buffer at pH 2.0, 4.0, 6.0 and 8.0 to give four samples of 500 µl each. These dilutions were scanned at 10 nm intervals between 300 and 220 nm for absorbance (Figure 22). All scans showed a broad absorbance peak at 280 nm, possibly due to aromatic amino acids present in the CdBP or contaminating proteins. Of most interest is the absorbance shoulder in the 250-260 nm range (see inset), which was diminished at pH 4.0 and abolished at pH 2.0. This absorbance shoulder is similar to that first characterized by Kagi (1961) using equine Cd-MTh, where the absorbance was attributed to the cadmium-thiolate charge transfer.

Based on these data, it appears that honeybees demonstrate an accumulation of a soluble sulfhydryl-rich cadmium binding protein with a low molecular weight. Under the homogenization conditions used, the protein showed a preferential affinity for cadmium versus zinc at a binding ratio of 20:1. The presence of an absorbance shoulder at 254 nm suggested that the Cd and Zn metal ions could be coordinated to cysteine sulfhydryls, a coordination which was disrupted by titration with H+ ion (lowered pH) as demonstrated by the abolishment of the absorbance shoulder at 254 nm. This protein contained 10.7 mol cysteine and 4.1 mol Cd, quite different than that observed in mammalian metallothioneins but sufficient to be classified as a "metallothionein" under the guidelines suggested by the 2nd Congress on Metallothioneins (Table 20). This honey bee CdBP

FIGURE 22: Absorbance characteristics of purified honey bee CdBP.



closely resembles that reported from the genetic sequence of cDm51, the metallothionein cDNA isolated from *Drosophila melanogaster* (Lastowski-Perry, 1985), although no cadmium binding ratio was proposed for the *D. melanogaster* protein.

TABLE 20: Summary of Honeybee Cadmium Binding Protein (CdBP) Characteristics.

Criteria for Metallothioneins	Rabbit Cd-MTh	Drosophila Cd-MTh	Honeybee CdBP
Cadmium-rich.	6 - 7 mol per mol MTh	N.D.[a]	3.7 - 4.1 mol per mol
Low Zinc content	1 - 2 mol per mol MTh	N.D.	0.2 - 0.9 mol per mol
High -SH content	21 mol per mol MTh	10 mol per mol MTh	10.5 -10.7 mol per mol
Low Molecular Weight	6,600 g/mol	4,000 g/mol	4,500g/mol (SDS-PAGE)
Low Aromatic content.	no Phe, Tyr or Trp	no Phe, Tyr or Trp	N.D.[b]

[a] N.D. = not determined

[b] While direct measurements were not made, spectral evidence suggests that the protein has a low aromatic composition by the low A280 and high A254/A280 ratio.

PROTEIN TEST 2: Analysis for CdBP after varied Cd ingestion.

In order to measure the levels of CdBP induced after of varying cadmium dose, all remaining live bees from the 96 hour $CdCl_2$ toxicity tests were immediately anaesthetized, and stored frozen at -80°C. These samples were later thawed and all bees from similar treatments were pooled. This was necessitated by the small number of live bees remaining at the end of each experiment at the higher Cd doses.

Bees were then homogenized using a slightly different method (Winge, 1986) at a ratio of 1 gram tissue to 15 ml total volume of buffer and bees, or roughly 1 volume bees to 2 volumes buffer. After heat treatment and centrifugation steps, a 75 ml sample (or one-half of the total) of each homogenate was frozen and lyophyllized to dryness. This sample was resuspended overnight in 10mL buffer A at ice temperatures to make a viscous, near-saturated protein solution. This solution was then centrifuged for 30 minutes at 10,000 rpm to remove insoluble material. Protein assays of these supernatants yielded between 23.3 to 34.8 mg of protein (BSA Units) per ml sample. With this homogenization ratio, a one ml aliquot of the solution was equivalent to approximately 0.5 grams of bee tissue. Using this extraction procedure, between 46 to 69 mg of soluble protein per gram honey bee tissue could be recovered.

A sample size of five ml (or a tissue equivalent of 2.5 grams) was applied to a Sephadex G-50 column for size exclusion chromatography as described in Test 1. In order to conserve sample, individual fractions were only monitored for A254/A280 absorbance ratio with a flow through cell and not soluble protein or metal. Samples elution at a Ve/Vo ratio of 2.0-2.5 (approximately 14.0 to 2.0 kDa) were saved and pooled. This sample was applied to a DEAE-Sephadex column and eluted with a linear

NaCl gradient as in Test 1. Fractions were monitored for A254/A280 ratio and analyzed for soluble Cd by flame AAS, a more sensitive and accurate method of quantitation than protein assays. Soluble cadmium from CdBP was determined by integration of the cadmium peak corresponding to CdBP elution volume, and converted to gram units by the value 101.9 µg Cd per milligram protein (determined on page 64).

This purified CdBP appeared quite similar to that obtained with the purification procedure of Kagi, except that there were fewer bands on SDS-PAGE in the higher molecular weight region (Figure 23). There appeared to be approximately three contaminating protein bands, with the CdBP migrating in a "smear" as seen with the prior batch of CdBP. The protein sample contained 3.9 mol Cd and 10.5 mol of sulfhydryl sulfur per mole protein. This suggests that the same protein was isolated at a slightly better purity, probably due to the homogenate heating step.

The quantities of CdBP isolated from these samples are summarized in Figure 24 and plotted as the amount of CdBP (in µg) isolated per gram tissue versus the ppm Cd in syrup. This unit was chosen rather than the amount of CdBP per mg total protein in order to make data relevant at an organismal level, and to keep it independent of extraction efficiency.

CdBP was observed in all samples from cadmium-fed bees, appearing with as low of a dose as 1 ppm Cd in syrup. Honey bees fed between 2 to 50 ppm Cd syrup produced an almost constant amount of CdBP, with averages ranging from 6.0 to 9.0 µg CdBP per gram tissue. A general trend was seen where CdBP/gram tissue increased nearly linearly to a maximum when fed 20 ppm Cd syrup; bees fed concentrations greater than, or equal to, 50 ppm Cd showed decreasing amounts of CdBP, although probably not

FIGURE 23: SDS-PAGE analysis of purified CdBP using the Winge method. Notice the relative absence of contaminating proteins bands.

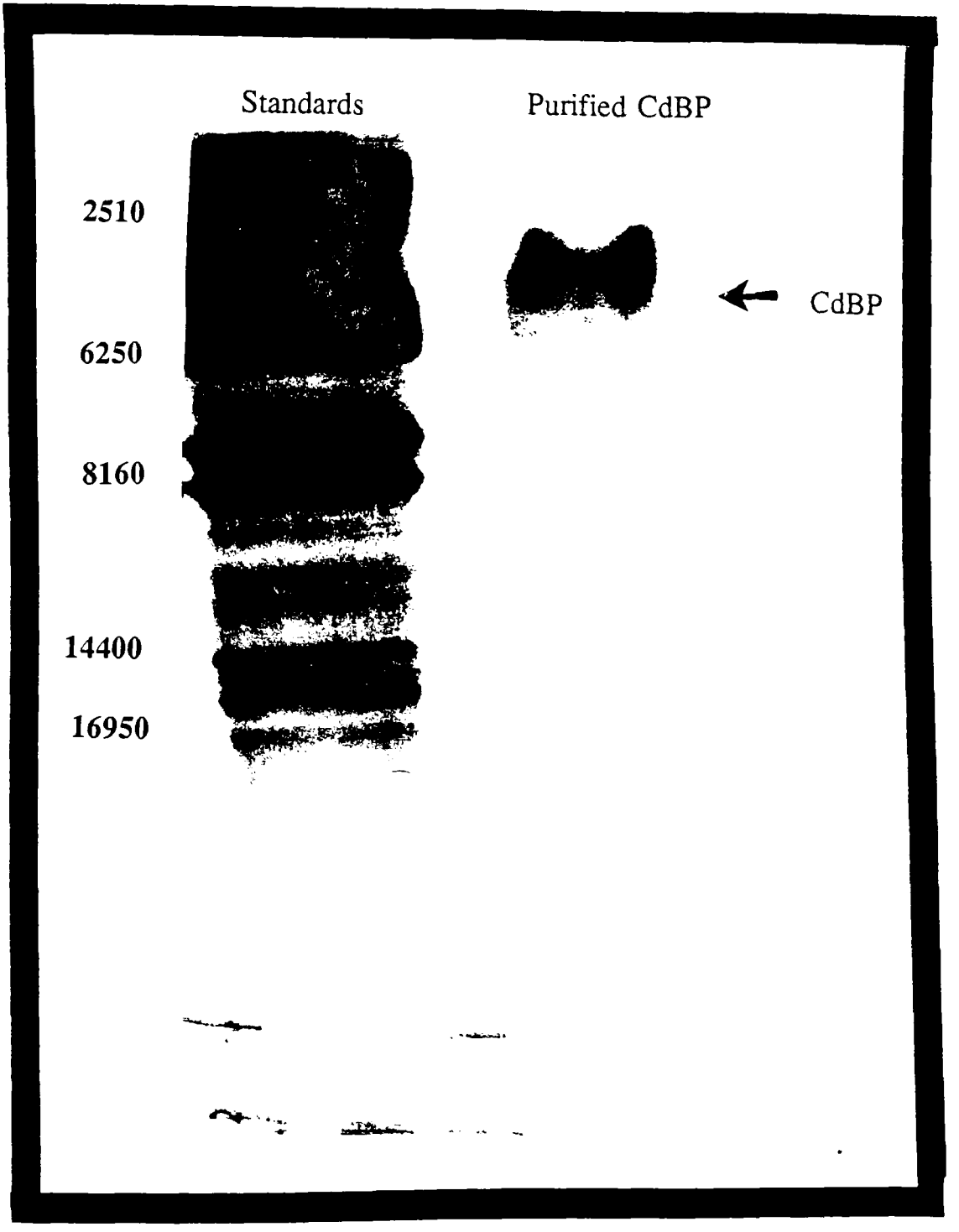


FIGURE 24: Accumulation of CdBP per gram of honey bees fed cadmium containing syrup. Plot shows μg CdBP isolated per gram tissue versus Cd concentration in syrup.

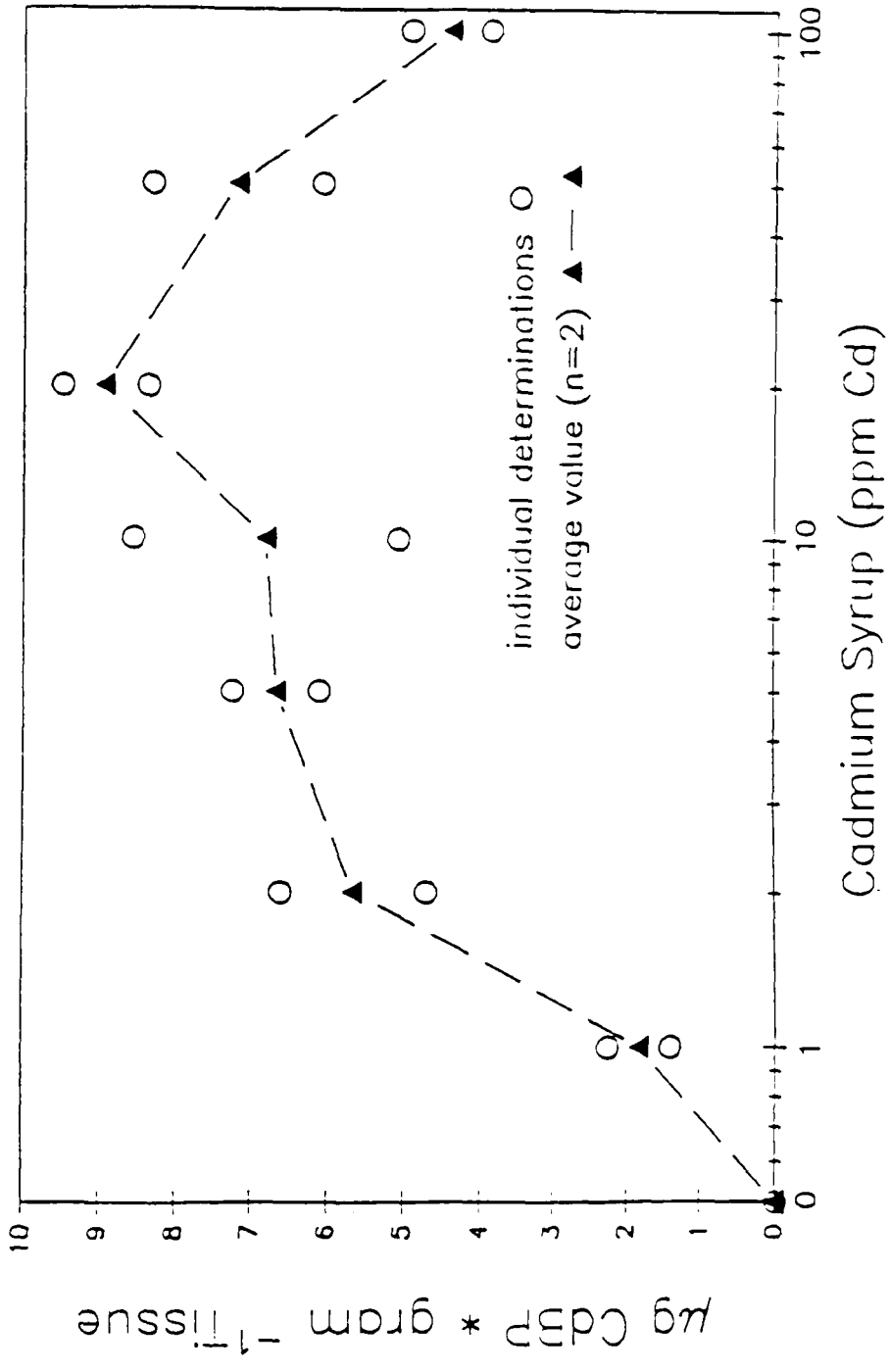


TABLE 21: CdBP isolated from $CdCl_2$ feeding studies: Cumulative data.

µg/mL Cd in Syrup	average µg Cd injested per bee, 96 hours	average µg Cd consumed per 1 gram bees	µg Cd as CdBP expressed per 1 gram bees	µg CdBP expressed per bee	% of Cd bound by CdBP
0	0.00	0.00	0.00	0.00	-
1	0.49	8.43	0.39	0.27	4.7
2	0.96	16.4	1.23	0.83	7.5
5	1.82	30.9	1.45	0.99	4.7
10	3.68	62.6	1.48	1.00	2.4
20	3.76	63.9	1.94	1.30	3.0
50	4.80	81.6	1.57	1.10	1.9
100	4.40	74.8	0.96	0.65	1.3

* Values for converting "grams tissue" to "number of bees" were obtained from Figure 13 under Cadmium Oral Dose Toxicity studies using the mean of n = 5 determinations. Briefly, they are:

0ppm Cd = 0.172 g/bee 1ppm Cd = 0.175 g/bee 2ppm = 0.154 g/bee
5ppm Cd = 0.159 g/bee 10ppm Cd = 0.148 g/bee 20ppm Cd = 0.126 g/bee
50ppm Cd = 0.121 g/bee 100ppm Cd = 0.122 g/bee

TABLE 21: CdBP isolated from CdCl2 feeding studies: Cumulative data.

a statistically significant amount. Since these samples required an excessive amount of time to process for a single data point, they were only run in duplicate; the significance of this decrease cannot be tested.

In order to compare oral dose to CdBP recovered, consumption data and body weights were utilized from the cadmium feeding experiments. These are summarized in Table 21. An important note to make is that the average body weight per bee decreased significantly with dose, with the largest drop occurring with doses greater than or equal to 10ppm Cd syrup. CdBP concentrations in tissues ranged from 0.27 to 1.3 μg protein per bee, with samples between five and 50 ppm Cd displaying up to 75% of the maximum CdBP observed in all samples.

The amount of CdBP isolated per bee appeared to increase with increasing dose until 2.0 μg/bee, where the amount of CdBP became variable. This created a poor linear response over the entire dose range (line equation: $y = x\ (0.02) + 0.717$, $r = 0.172$). It is also possible that this response is biphasic, with a linear increase of CdBP for lower Cd doses and a tapering off effect at higher doses, although this is extrapolation well beyond what the data can show. With honey bees that had consumed up to 2.0 μg Cd during the test period, CdBP levels increased linearly as a first order response (line equation: $y = x\ (1.93) + 0.137$, $r = 0.895$). CdBP levels in bees that consumed greater than 2.0 μg Cd were more variable, although CdBP production appeared to display more of a zero order response with no increase of CdBP with increasing dose (line equation: $y = X\ (-0.054) + 1.253$, $r = 0.796$).

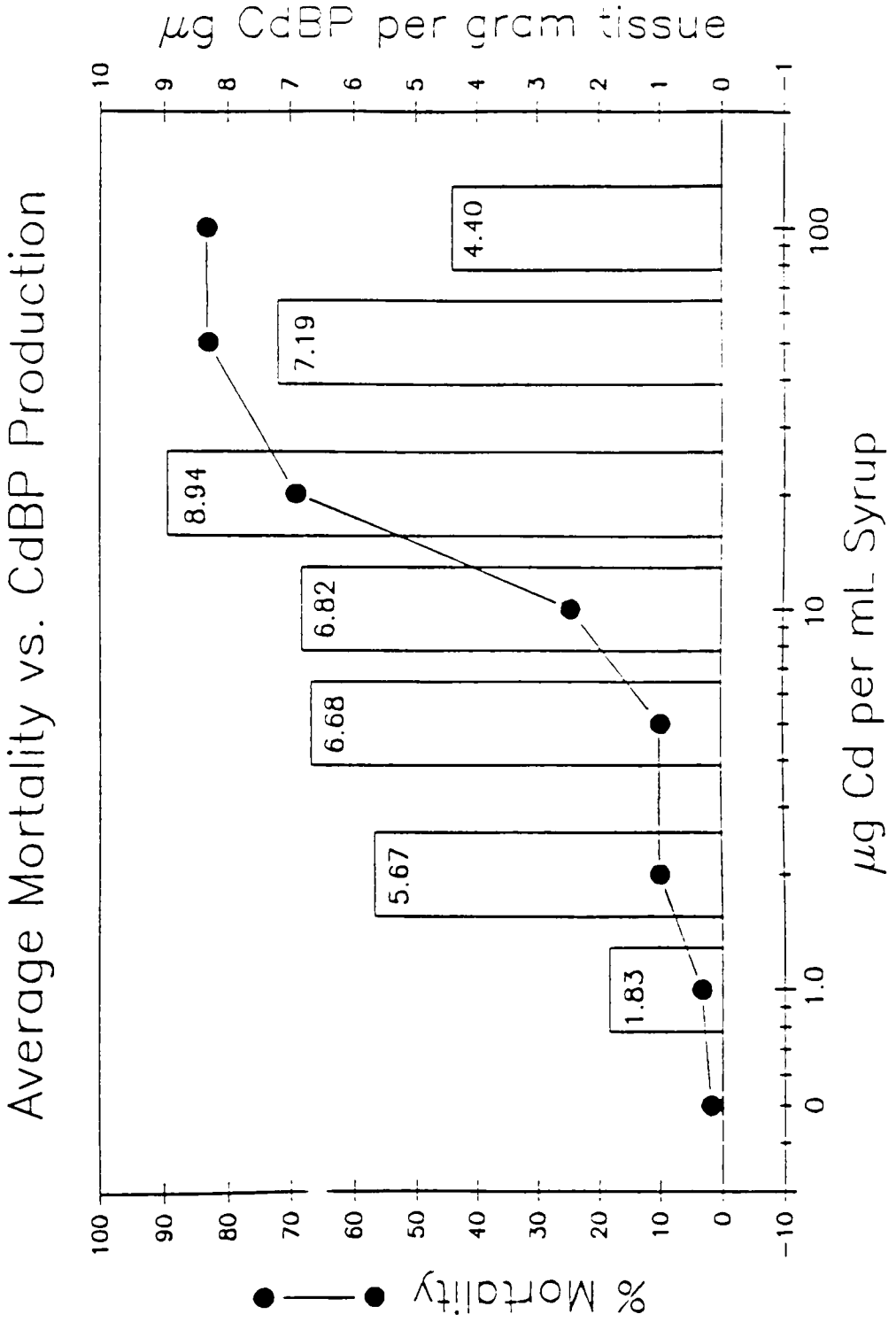
These data would imply that the maximum amount of CdBP produced per bee could be between 1.0 to 1.5 μg, although this hypothesis cannot be supported without

further study. If the maximum amount of CdBP produced per bee is in fact 1.0 to 1.5 μg, the maximum cadmium-sequestering potential per bee is 0.09 to 0.14 μg Cd per bee, or approximately 0.5 to 0.8 μg Cd per gram wet bee tissue. This CdBP leveling off phenomena may also be due to the nature of CdBPs or the isolation procedure used. Other CdBPs induced that have intrinsically different solubility, heat stability, or even CdBP that aggregate to a higher molecular weight than the cutoff point used would be selectively eliminated and not taken into account.

In order to directly compare the accumulation of honey bee CdBP with mortality data, the average CdBP isolated per sample (reported as "μg CdBP/g tissue") was compared to the average honey bee mortality for each dose tested (Figure 25). An interesting trend is seen where the accumulation of CdBP in bee tissue becomes measurable at the lowest oral dose (1 ppm Cd), a feeding level that did not show significantly different mortality from non-dosed controls. The accumulation of CdBP increases dramatically between 2 and 5 ppm Cd feeding levels, which also showed no significant mortality versus controls. The accumulation of CdBP in tissue continued to increase up to an oral dose of 20 ppm Cd and then appears to decrease with increasing dose.

The data in Table 21 and Figure 25 suggest that honey bee CdBP begins to accumulate after 96 hours exposure to syrup containing as little as 1 ppm Cd (approximately 0.5 μg Cd per bee). This accumulation appears to occur well below the cadmium dosage level required to show a significant mortality in treated bees, yet appears to decrease with increasing doses above 20 ppm Cd (approximately 3.7 μg Cd per bee; Table 21). To explain this decrease in CdBP accumulation, it is possible that the

FIGURE 25: CdBP Accumulation per gram Honey bee tissue and 96 Hour bee Mortality versus Cd-syrup consumed.



remaining live bees used for these protein analyses did not consume the syrup, or consumed very little. These bees would have an actual dose much lower than the "average consumption" data would suggest. The lower dose would then be reflected by a lower CdBP accumulation.

PROTEIN TEST 3: Analysis for CdBP after variable time exposure.

In order to measure the levels of CdBP induced after exposure to cadmium for different time periods, a second $CdCl_2$ oral dose experiment was set up similar to earlier oral toxicity tests. A total of 11 cages were used, each containing between 100 to 130 bees and representing one treatment level (Table 22). Bees were syrup deprived for 12 hours prior to the start of the experiment and then fed Cd-containing syrup for one, 24, 48, 96 and 144 hours. Samples were treated as in protein Test 2 with bees anaesthetized, frozen and weighed before homogenization.

TABLE 22: Treatments for caged bees in protein Test 3.

Exposure Time (hours)	Syrup Concentration of $CdCl_2$ 2 ppm	20 ppm
0 time zero controls	Cage 11	
1	Cage 1	Cage 2
24	Cage 3	Cage 4
48	Cage 5	Cage 6
96	Cage 7	Cage 8
144	Cage 9	Cage 10

After the appropriate time period, honey bees were homogenized and purified using the method of Winge, as in Test 2, with minor modifications. Earlier chromatography runs showed that the DEAE-Sephadex purification step was required to remove a contaminating Cd peak from the honey bee CdBP. This peak appeared to be free Cd^{2+}, either from loosely associated metal-protein complexes or from soluble cadmium eluting in the 4-14kDa pooled fraction range on Sephadex G50 chromatography.

When this soluble Cd^{2+} was removed from the sample, honey bee cadmium binding protein accounted for greater than 98% of all cadmium eluting later in the chromatogram.

This DEAE-Sephadex chromatography step was altered by running 10 cm^3 "mini-columns" instead of the larger columns, and substituting the gradient elution for a one-step isocratic elution using high salt buffer. Columns (10cc syringes plugged with a fritted disk and glass wool) were poured with DEAE-Sephadex slurry as before and equilibrated with 0 mM NaCl buffer. Pooled honey bee "CdBP-like" fractions collected from Sephadex G50 were applied to the DEAE mini-columns and washed with two column volumes (20 ml) of 0 mM NaCl buffer. Proteins were eluted using 30 ml of 200 mM NaCl buffer and 2 ml fractions were collected. After the sample eluted, the column was washed with 500 mM NaCl buffer (10 ml) and re-equilibrated with a minimum of five volumes of 0 mM NaCl buffer. This procedure greatly facilitated the processing of large numbers of samples by speeding the chromatography time. Cadmium was measured in all fractions, and CdBP was quantified by flame AAS using a value of 101.9 μg Cd per mg protein.

The quantities of CdBP isolated from these samples are summarized in Table 23 and graphically displayed in Figure 26 as the amount of CdBP (μg) isolated per gram tissue versus hours exposured. Triplicate analysis of homogenates were performed and showed coefficients of variation no greater than 23 %, with highest CVs observed with samples containing lower amounts of soluble CdBP. These samples contained soluble cadmium near the detection limits of flame AAS, where small variations can be multiplied to a large difference at the "CdBP" level. I believe that maximum C.V. values of 23% represent a reasonable value considering the dilutional nature of liquid chromatography

TABLE 23: CdBP Isolated from Variable Time Studies: Cumulative Data.

Hours Exposed, CdCl2	µg Cd per mL	Mean µg CdBP per gram bees	Std. Dev.	95% Confidence Interval
0	-	0.0	-	-
	-	0.0	-	-
1	2	0.0	-	-
	20	0.0	-	-
24	2	1.99	0.46	0.52
	20	5.62	1.03	1.16
48	2	4.69	0.23	0.26
	20	18.2	1.79	2.03
96	2	8.76	1.04	1.17
	20	14.7	1.38	1.56
144	2	3.64	0.79	0.90
	20	15.12	1.09	1.23

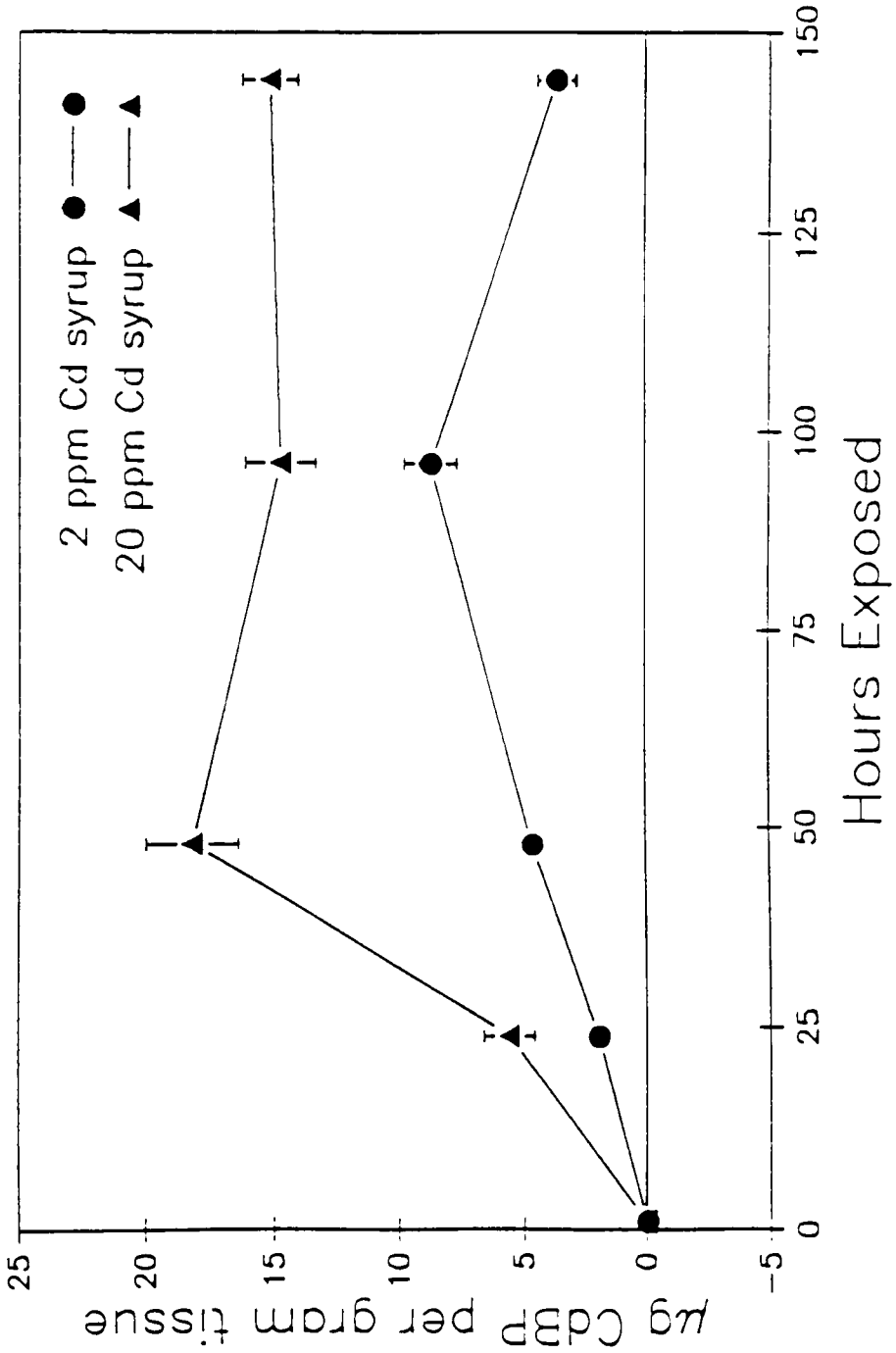
and the number of steps in the purification.

Cadmium binding protein was not detected in either the control sample or one-hour cadmium exposed samples. By the 24 hour interval, CdBP levels had increased to 2.0 μg per gram tissue in honey bees fed 2 ppm Cd-syrup and 5.6 μg per gram tissue in bees fed 20 ppm Cd-syrup. Honey bees fed 2 ppm Cd showed steady increases in tissue CdBP levels at the 48 and 96 hour intervals, but showed a decrease of CdBP in the 144 hour sample. Homogenates from bees fed 20 ppm Cd syrup showed steady increases of CdBP at 24 and 48 hours, with a nearly constant amount of CdBP isolated at the 96 and 144 hour intervals.

Data from this experiment show that honey bees can accumulate low molecular weight cadmium-binding protein as rapidly as 24 hours after oral exposure to $CdCl_2$. After approximately six days of exposure to cadmium feed, honey bee samples still showed 42% (2 ppm Cd) to 83% (20 ppm Cd) of the maximum amount of CdBP measured for the duration of the test. This suggests that CdBP is persistant in honey bee tissues and is either produced at an appreciable rate after exposure to cadmium or has a long biological half life.

Between days four and six, homogenates from honey bees fed either 2 or 20 ppm Cd showed decreases in the amount of CdBP isolated from tissue, with bees fed 2 ppm Cd showing the most noticable drop. This could be an artifact due to variations in daily cadmium doses, although trends very similar to this (i.e., initial rise in tissue CdBP, then leveling off or actual decrease) have been reported to occur with tissue metallothionein levels in cultured insect cells (Debec, 1985), and mammalian organisms (Nordberg, 1972) after chronic exposed to low levels of cadmium.

FIGURE 26: CdBP Accumulation per gram Honey bee tissue versus time - 2 and 20 ppm Cd syrup.



Honey bees fed 20 ppm Cd from 48 to 144 hours gave very similar recoveries of CdBP, suggesting that a maximum accumulation was reached. The maximum accumulation of CdBP determined for these honey bee samples corresponds to approximately 1.7 to 2.1 μg CdBP per individual bee, considerably higher than the values obtained in Test 2 (between 1.0 to 1.3 μg CdBP per bee).

PROTEIN TEST 4: Analysis of CdBP from in situ samples.

In order to investigate the occurance of CdBP from the tissue of bees in metal-contaminated environments, honey bees were collected from the Anaconda-Mill Creek area of Montana, the Idaho Chemical Processing Plant (ICPP) area of the INEL, and Mud Lake, Idaho. Samples were collected by aspiration of young forager bees from the entrance of the hives (approx. 200 bees per sample), immediately frozen at dry ice temperatures, and transferred to -80°C within 36 hours. Homogenization of bees and CdBP purification procedures used in this test were the same as those described in Test 2. This lengthy separation was used to assure reasonable purity of isolated CdBP's, as the DEAE mini-column separation in Test 3 allows "contaminating" proteins to co-elute with CdBP. Final low molecular weight protein (or "metal binding protein") samples were analyzed for soluble protein, cadmium, zinc and sulfhydryl content.

The sampling sites chosen have been well characterized for trace metal enrichment in local soils and biological materials. Soils from the Mill Creek site ("cadmium impacted") contain between 5.0 to 11.0 µg Cd per gram dry weight, an enrichment of 20X over soils in Townsend, MT (Tetra-Tech, 1985). The concentrations of the following metals are also higher than surrounding control areas: arsenic (110 µg/g, 10X enrichment), copper (500 µg/g, 25X enrichment), lead (230 µg/g, 10X enrichment) and zinc (500 µg/g, 8X enrichment). Tissue concentrations of trace elements in honey bees sampled from this area are summarized in Table 24. Soils from the ICPP at the INEL ("zinc impacted") contain up to 120 µg Zn per gram soil, an enrichment of approximately 2-3 fold above distant control sites in Lemhi, ID (Rope, in press).

TABLE 24: Honey bee sampling locations and contamination levels.

Site	Soil Contamination (μg element/g)	Bee Tissue Levels (μg element/g)
Mill Creek, MT	Cd = 5.0 to 11.0 Zn = 500 As = 110 Cu = 500 Pb = 230	0.3 to 0.6
ICPP, ID	Cd = trace Zn = 120	trace 200 to 250
Mud Lake, ID	Cd = trace Zn = 35	trace

Biological materials (big sagebrush and "grasses") tested in an earlier study showed significant enrichment of zinc, while cadmium levels were at or near minimum detection limits. Honey bees from this location contain approximately 200 μg Zn per gram dry bee tissue, an increase of almost three fold as compared to distant control sites (Table 24).

The Mud Lake, ID colonies were chosen as a control site since soil and atmospheric monitoring show this area to be sufficiently distant from the INEL to be unaffected by releases from the Idaho Chemical Processing Plant. Four years of honeybee biomonitoring studies confirm these data, with only one element (fluoride) showing slight elevation in a sample from a commercial colony.

The results from these protein isolation samples are listed in Table 25. Control samples from Mud Lake and the "Zn-enriched" INEL Chemical Processing plant showed very low levels of Cd and Zn in the Sephadex G50/Sephadex DEAE fractionated pool, with sulfhydryl content too low to quantitate. These data imply that metal binding proteins in these homogenates were present in quantities too low to detect.

TABLE 25: CdBP Isolated from *in situ* samples: Cumulative data.

Bee Sample	Site	µg protein per ml	µg Cd per ml	µg Cd per mg Protein	µg Zn per ml	µg Zn per mg Protein
MDLK1	MUD LAKE	9.4	< 0.01	< 1.0	< 0.05	< 5
CPP1	CHEM PLANT	7.0	< 0.01	< 1.0	< 0.05	< 5
CPP2	CHEM PLANT	9.8	< 0.01	< 1.0	< 0.05	< 5
DL1765	MILL CREEK	25.2	2.13	84.5	0.30	11.9
DL1766	MILL CREEK	29.9	3.05	102	0.06	2.0

TABLE 26: CdBP Isolated from *in situ* samples: Quantities and physical characteristics.

Bee Sample	g-atoms Cd[b] per mol CdBP[a]	g-atoms Zn[c] per mol CdBP	mol SH per mol CdBP	µg CdBP per gram tissue
Mud Lake	< 0.01	0.09	0.62	< 0.1
Chem Plant	< 0.01	0.20	0.57	< 0.1
DL1765	3.4	0.89	10.5	0.72
DL1766	4.2	0.15	10.7	1.05

[a] Molar value used for CdBP was the 4,500 amu apothionein weight.
[b] Cadmium values were determined by flame AAS.
[c] Zinc values were determined by graphite furnace AAS.

Suprisingly, samples DL1765 and DL1766 from the Mill Creek area both yielded a low-molecular weight metal binding protein that displayed a high sulfhydryl content. While these samples were taken from nearly adjacent colonies, a difference was observed in the metal content of the proteins isolated. Both samples contain similar mole ratios of sulfhydryl to protein, yet the two isolated proteins differ in the ratios of bound cadmium and zinc. Sample DL1765 shows highly elevated zinc levels, sufficient to account for approximately 1 mole Zn per mole protein (Table 26). The total metal and sulfhydryl content of these isolated proteins were nearly identical to the protein isolated in the laboratory Cd-feeding tests, suggesting that they are the "cadmium binding protein" characterized in tests 1, 2 and 3.

This cursory study suggests that metal binding proteins ("metallothionein-like") exist in honeybees that are exposed to cadmium in the environment, either through air, water or soil. While honeybee tissue levels of cadmium from biomonitoring studies would suggest that Cd is not abundant from this site (up to 0.6 μg Cd per gram dry bee tissue), these data show that exposure is sufficient to induce production of this CdBP. The accumulated level of this metal binding protein ranged from 0.72 to 1.05 μg protein per gram wet bee tissue, or between 40 to 60% of the level accumulated after feeding with 1 ppm cadmium syrup for 96 hours.

CHAPTER V

DISCUSSION

This study has focused upon the toxicity of cadmium salts to honey bees in a controlled environment. The median lethal dose of cadmium to this organism, which had not previously been reported, has been determined for oral administration as well as injection for one salt. I hope that these data illustrate that arsenic isn't the only concern in metal impacted areas, and that it's toxicity is most likely altered (antagonistically and synergistically) by other metals associated with it in the environment.

I have also characterized a specific cadmium-binding protein that accumulates after short (24 hours) or long (144 hours) exposure to cadmium. This protein was isolated from laboratory experiments using caged honey bees that were fed soluble cadmium orally, and from free-flying bees from a cadmium-enriched area near the Anaconda smelter area.

The measured median lethal oral dose of both cadmium salts examined shows cadmium to be a potent toxin, with LD_{50} values similar to that of several arsenic derivatives. The average LD_{50} of cadmium chloride was 3.51 μg Cd/bee for 48 hours and 2.34 μg for 96 hours. The average LD_{50} of cadmium sulfate was 2.80 μg Cd/bee for 48 hours and 1.44 μg for 96 hours.

The data from these cadmium toxicity tests show striking similarity with reported values for 96 hour arsenic feeding LD50's in honeybees. Fujii (1980) reported that bees fed syrup containing between 0.30 to 50.0 ppm As (sodium arsenite, $NaHAsO_3$, and arsenic trioxide, As_2O_3) consumed as little as 0.030 ml syrup per bee per day, much lower

than the observed 0.200 ml/bee/day in controls fed no As. The average range of 96 hour LD_{50} for these compounds were 0.544 $\pm$ 0.006 μg elemental As per bee for Sodium Arsenite and 2.30 $\pm$ 0.05 μg As/bee for arsenic trioxide. Slopes reported were similar to those found in this study with Cd salts. Values obtained by others (Beard, 1949; Bertholf, 1941) are in agreement with the arsenic toxicity data reported by Fujii, except for Atkins (1981), who reports "arsenical pesticides" were at least an order of magnitude less toxic at 27.15 μg elemental As per bee (slope = 1.22 probits). It is not known what compounds were designated as "arsenicals" for Aktins tests.

While little information exists on free metal-toxicity to honeybees, cadmium appears to be nearly as toxic as sodium arsenite and is much more toxic than metal-containing fungicides (Table 27). Using the classification proposed by the University of California-Davis Division of Agricultural Sciences, cadmium appears to fit well into the group of moderately-toxic to highly-toxic pesticides, with a higher toxicity than that reported of arsenical pesticides by E.L. Atkins.

TABLE 27: Reported toxicity of metals and metal-containing pesticides to honey bees.

Highly Toxic: 48 Hour LD50 < 1.99 μg per bee.
- Sodium Arsenate (Fujii, 1980)
- Calcium Arsenite (Bertholf, 1941)
- Arsenic Trioxide (Troup, 1918)

Moderately Toxic: LD50 = 2.00 to 10.99 μg per bee.
- Arsenic Trioxide (Fujii, 1980)

Relatively Non-Toxic: LD50 > 11.0 μg per bee.
- Arsenic Acid (Dessicant L-10; Atkins, 1979)
- Arsenic Trioxide (Atkins, 1954)
- Calcium Arsenate (Atkins, 1979)
- Copper Sulfate (Atkins, 1979)
- Cupric Oxide (Atkins, 1979)
- Copper Oxychloride Sulfate (Atkins, 1979)
- Copper 8-quinolinolate (Atkins, 1979)
- Cupric Hydroxide (Kocide; Atkins, 1979)

In order to examine the effect of a different route of exposure, the median lethal dose of cadmium chloride by injection was also investigated. The average 48 hour LD_{50} from these studies was 1.95 μg Cd/bee, a value significantly less than those obtained by feeding studies. These data suggest that either the route of exposure or the short dose period were responsible for higher mortality.

These studies show that cadmium could pose as important a toxicity threat to honey bees as does arsenic. While never used commercially as a pesticide, cadmium can be significantly enriched close to smelters like those of Anaconda and East Helena, Montana. While arsenic and other metals may be more abundant in the Anaconda valley, injury from cadmium exposure could be significant and an important factor in colony fitness. In East Helena where the amount of environmental cadmium is greater than the amount of arsenic, cadmium toxicity may play a major role in bee kills.

Oral doses of cadmium result in the accumulation of a low molecular weight cadmium binding protein in honey bees. This protein contains 4.1 mol Cd, 0.2 mol Zn, and 10.7 mol cysteine per mol CdBP, suggesting that this protein could be a "metallothionein." Metal content may vary with exposure conditions, as a sample obtained from the Mill Creek area showed almost 1 gram atom of Zn. Accumulation of the protein was induced by feeding honeybees as little as 0.5 μg Cd over 4 days, a level that does not show increased mortality versus controls. Tissue levels of this protein appear to reach a maximum within 72 hours and do not appear to decrease significantly over the course of the next 72 hours.

It appears that this cadmium binding protein could be used in field studies of honey bee biomonitoring. Protein investigations would complement body burden studies,

helping provide an answer to the amount of metal(s) an insect encounters, and if the organism shows physiological stress from exposure to the metal(s).

Obviously, the techniques used in this study are not well suited for screening large numbers of samples due to the number of steps involved and the potentially poor recovery due to dilutional processes such as liquid chromatography. Several techniques, however, could streamline isolation and detection of not only honey bee cadmium binding proteins, but many invertebrate metallothioneins. I'll summarize these methods briefly.

(1) Tissue Specific Studies.

Work by Maroni (1985), Bouquegneau (1985) and Lastowski-Perry (1985) show that the alimentary canal (midgut) of insects is the major tissue of accumulation for many group IB and IIB metals. Working with only these tissues homogenized in a small volume of buffer would be much easier than the "bucket" biochemical separations I've used, yet could provide similar results.

(2) Improved Analytical Techniques.

Several techniques could help improve detection of metal binding proteins. Substituting graphite-furnace for flame AAS can lower the detection limit 50-fold for many metals, while auto sampling will simplify analysis. Chromatographic techniques using reversed-phase (Suzuki, 1983) or anion exchange (Lehman, 1986) high-pressure liquid chromatography give improved separation of contaminating proteins and better yield. Quantitative electrophoresis techniques like electroblotting samples to nitrocellulose after PAGE separation could improve

detection to the picomole range (Mazen, 1988). Lastly, fluorescent or radiolabeled modifying agents could be attached to the sulfhydryl groups of these proteins, allowing quantitation by fluorescence or autoradiography. These could include iodo-derivatives (iodoacetamide, iodoacetate) or maleimide derivatives.

(3) Measurments of metallothionein mRNA.

The work by Lastowski-Perry (1985) on *Drosophila melanogaster* metallothionein shows that insect metallothionein mRNA accumulates with increasing Cd dose. More importantly, an increased capacity of MTh mRNA induction corellated well with metal tolerance. These data suggest that metallothionein could be used as a molecular marker of metal exposure. Considering the high degree of conservation in all characterized metallothioneins (especially in "Cys-X-Cys" regions), this technique could be applied to all insects, terrestrial or aquatic!

Since the *Drosophila* metallothionein mRNA has been well characterized, the labeled hybridization probes used to quantify fruit fly mRNA could be used to investigate metallothionein induction in other insects. By purifying total RNA from insect target tissues, mRNA can be quantified by hybridization to labeled cDNA and quantified by autoradiography or conventional ^{32}P counting. Most exciting are the detection techniques that incorporate non-radioactive labels (biotin or digoxigenin-UTP) into hybridization probes. These have detection limits to 1 pg of RNA or DNA, similar to ^{32}P labeling and sufficient to quantify mRNA from a small tissue sample. This technique has the advantages of radiolabeling without the concerns of disposal or supporting isotope producers. These techniques are reviewed by Adams (1986) and detailed in the "Handbook of Molecular Biology and Cloning" (1988).

CHAPTER VI

SUMMARY

* Cadmium salts are moderate to severe toxins to honey bees, with LD_{50} values similar to the most toxic forms of Arsenic. The LD_{50} values obtained in this study were: **ORAL** $CdCl_2$ = 3.51 μg/bee for 48 hours
2.34 μg/bee for 96 hours

$CdSO_4$ = 2.80 μg/bee for 48 hours
1.44 μg/bee for 96 hours

INJECTION $CdCl_2$ = 1.99 μg/bee for 48 hours

* Within 24 hours after consuming cadmium-syrup (as low as 1 ppm Cd), honey bees accumulate a low molecular weight cadmium binding protein. The amount of this protein accumulated per honey bee increases with increasing cadmium dose and exposure time.

* The protein isolated from cadmium-fed honey bees qualifies as a "metallothionein-like" protein. This protein was cadmium-rich (4 mol Cd per mol CdBP), low in zinc and high in cysteine (10-11 mol per mol CdBP). The protein exhibited a low molecular weight of 4,500 g/mol on SDS-PAGE.

* Cadmium binding proteins were found in honey bees living in the cadmium-impacted region by Mill Creek, MT. These proteins were not detected in areas that showed trace levels of cadmium pollution, suggesting that CdBPs either do not accumulate in unexposed bees, or accumulate at levels well below the analytical detection limits.

CHAPTER VII

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APPENDIX A

CUMULATIVE PERCENT MORTALITY GRAPHS: HONEYBEE FEEDING STUDIES

This appendix includes plots for all tests performed used to calculate the 48 and 96 hour LD50 values. Plots are arranged according to date of test, with honey bee mortality (expressed as percent) plotted versus hours exposed to Cd-containing syrup.

To summarize honey bee mortality from each cadmium salt, a graph preceeds individual tests that shows the mean of all tests for that compound. These data were tested for significance using Tukey's b ANOVA and means were assigned into significantly ($\alpha = 0.05$) different groups. Means that are not significantly different for a given dose will share the same letter.

Figure A1: Mean percent honey bee mortality for all $CdCl_2$ tests (n = 5). Plot shows bee mortality versus exposure time.

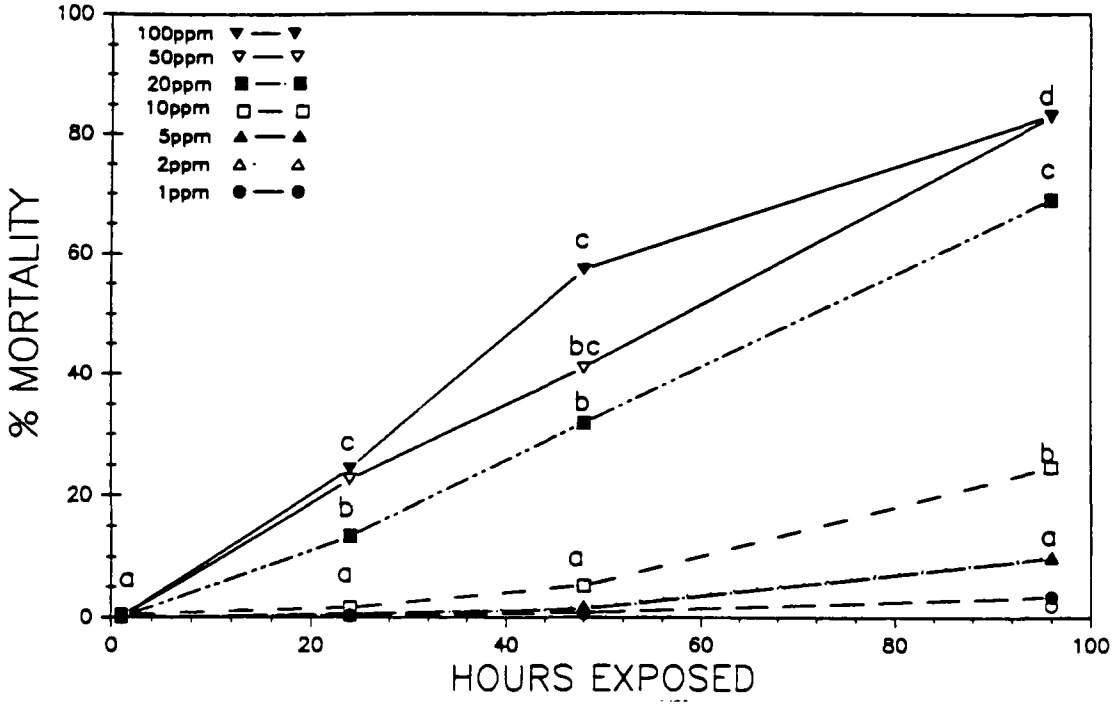


Figure A2: Percent honey bee mortality for $CdCl_2$ test one. Plot shows mortality versus exposure time for all syrup doses.

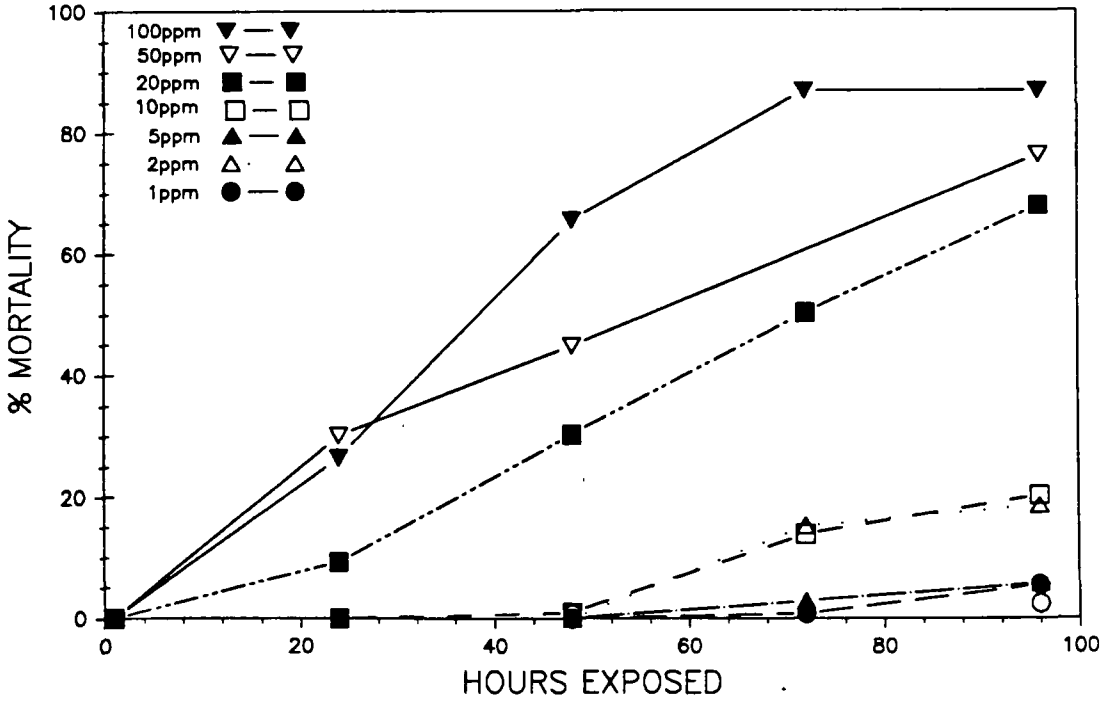


Figure A3: Percent honey bee mortality for $CdCl_2$ test two. Plot shows mortality versus exposure time for all syrup doses.

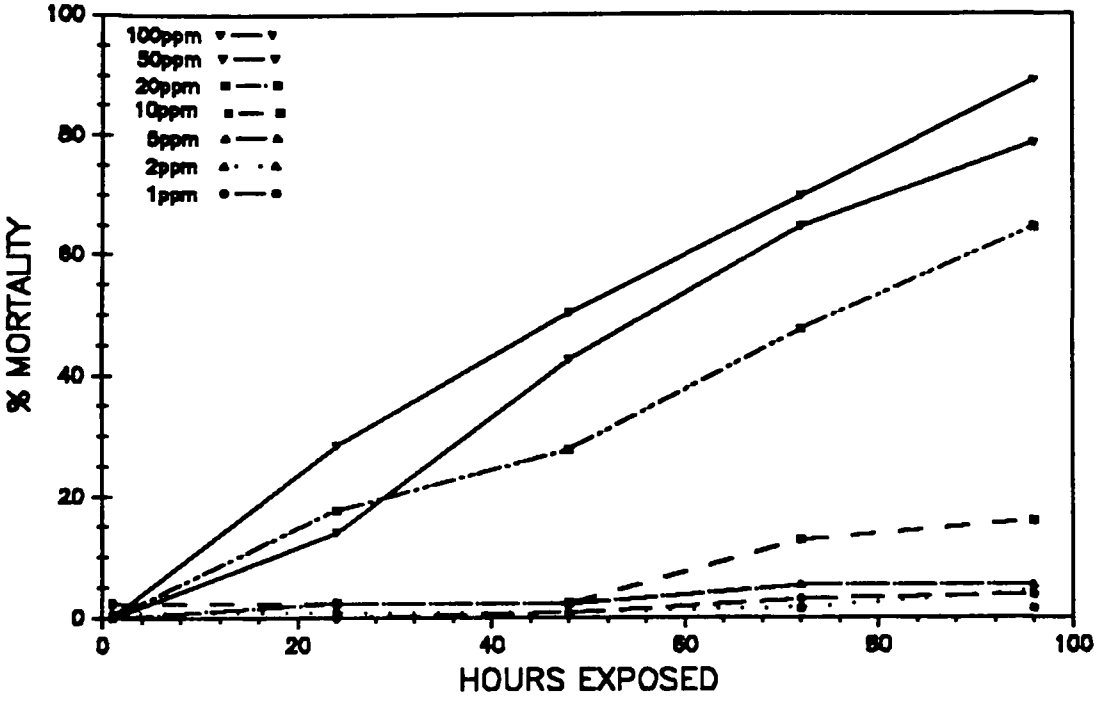


Figure A4: Percent honey bee mortality for $CdCl_2$ test three. Plot shows mortality versus exposure time for all syrup doses.

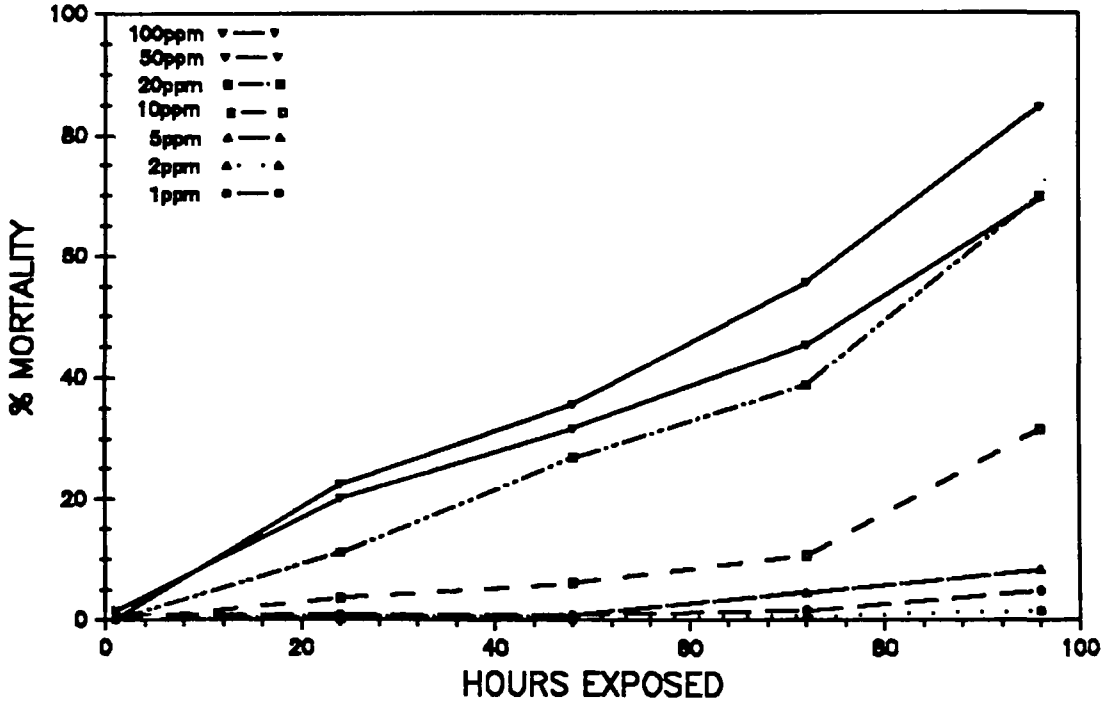


Figure A5: Percent honey bee mortality for $CdCl_2$ test four. Plot shows mortality versus exposure time for all syrup doses.

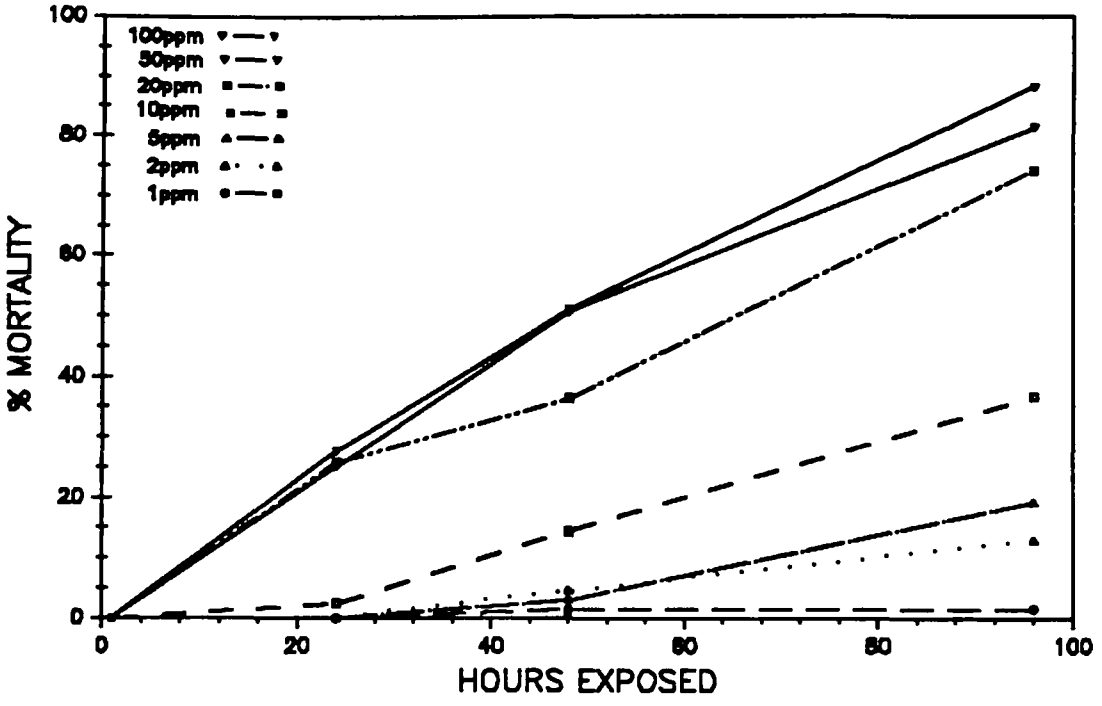


Figure A6: Percent honey bee mortality for $CdCl_2$ test five. Plot shows mortality versus exposure time for all syrup doses.

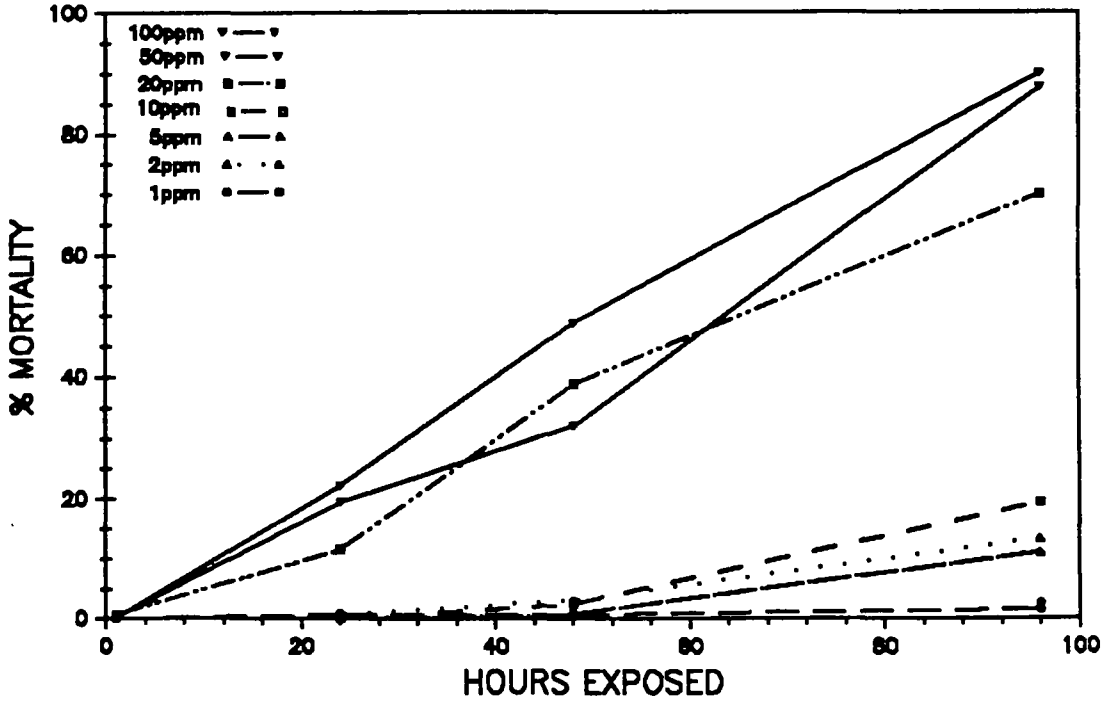


Figure A7: Mean percent honey bee mortality for all $CdSO_4$ tests (n = 3). Plot shows bee mortality versus exposure time.

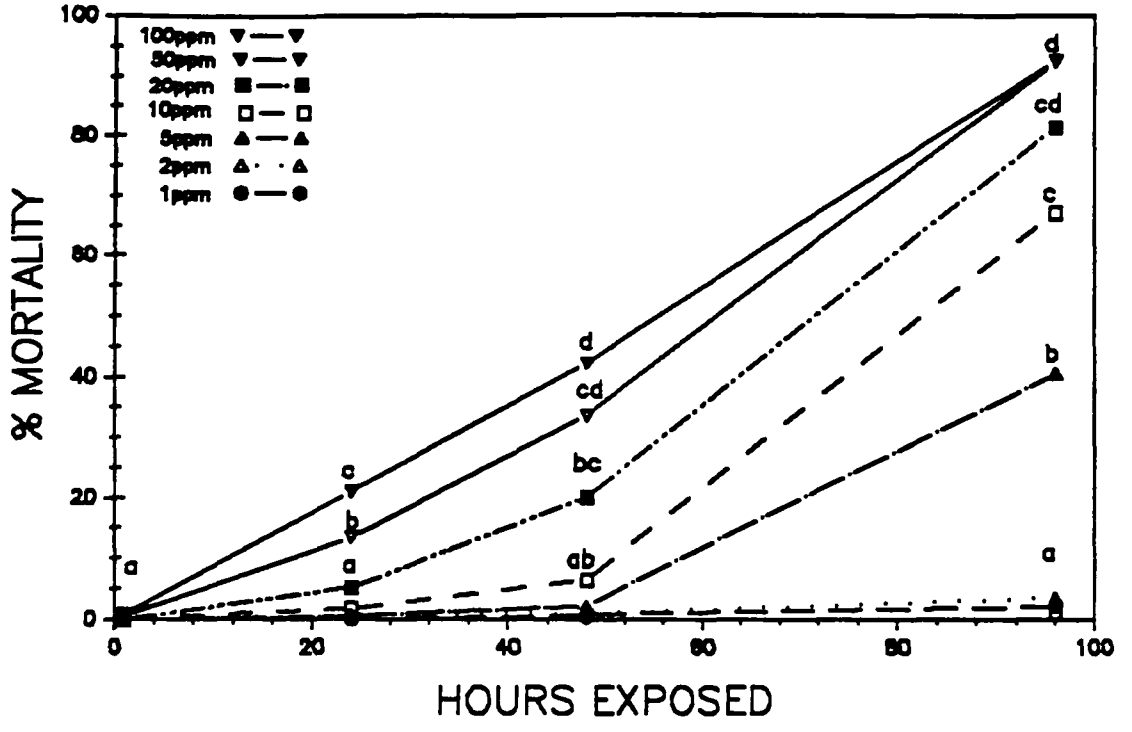


Figure A8: Percent honey bee mortality for $CdSO_4$ test one. Plot shows mortality versus exposure time for all syrup doses.

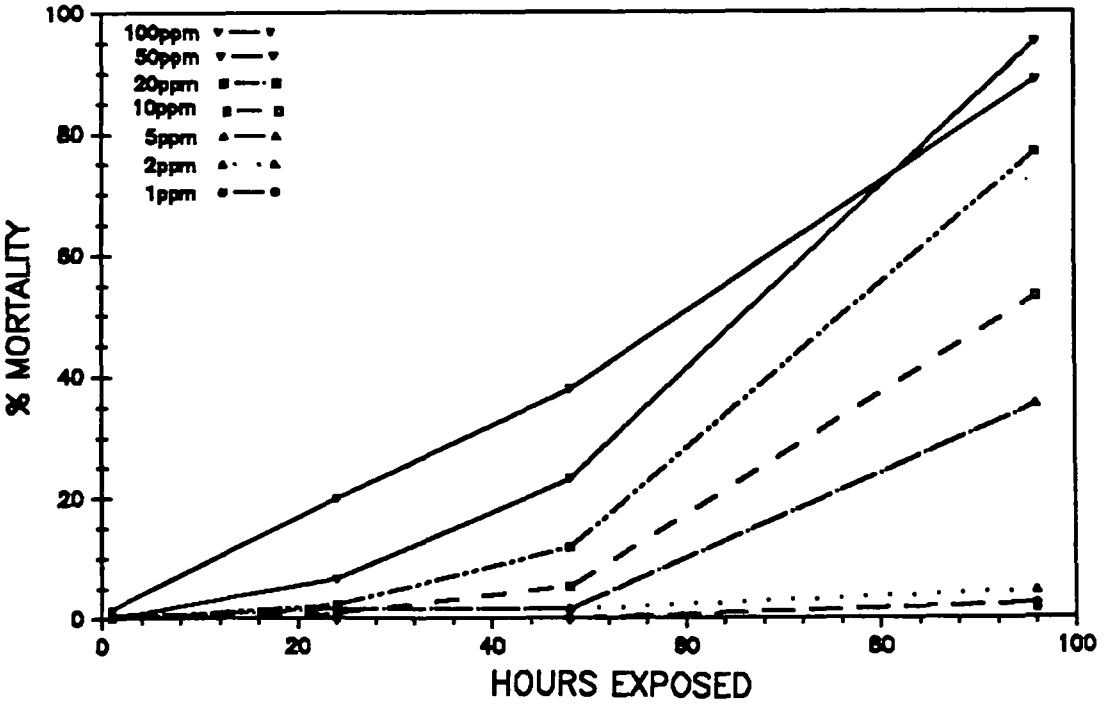


Figure A9: Percent honey bee mortality for $CdSO_4$ test two. Plot shows mortality versus exposure time for all syrup doses.

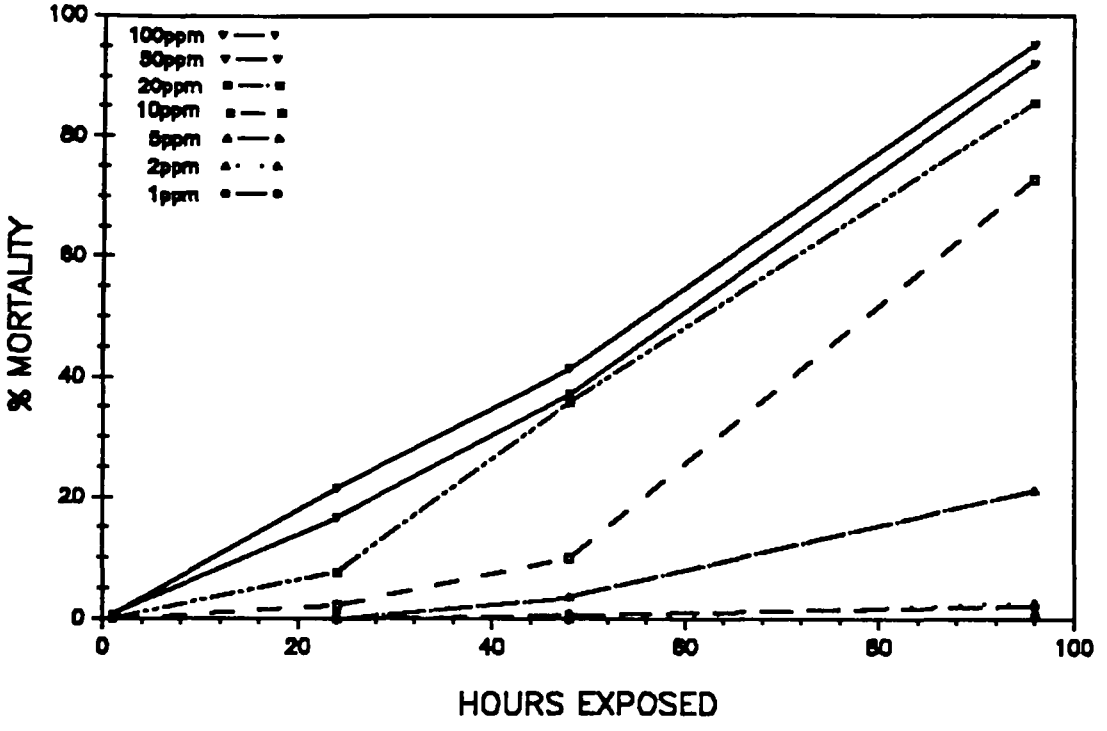
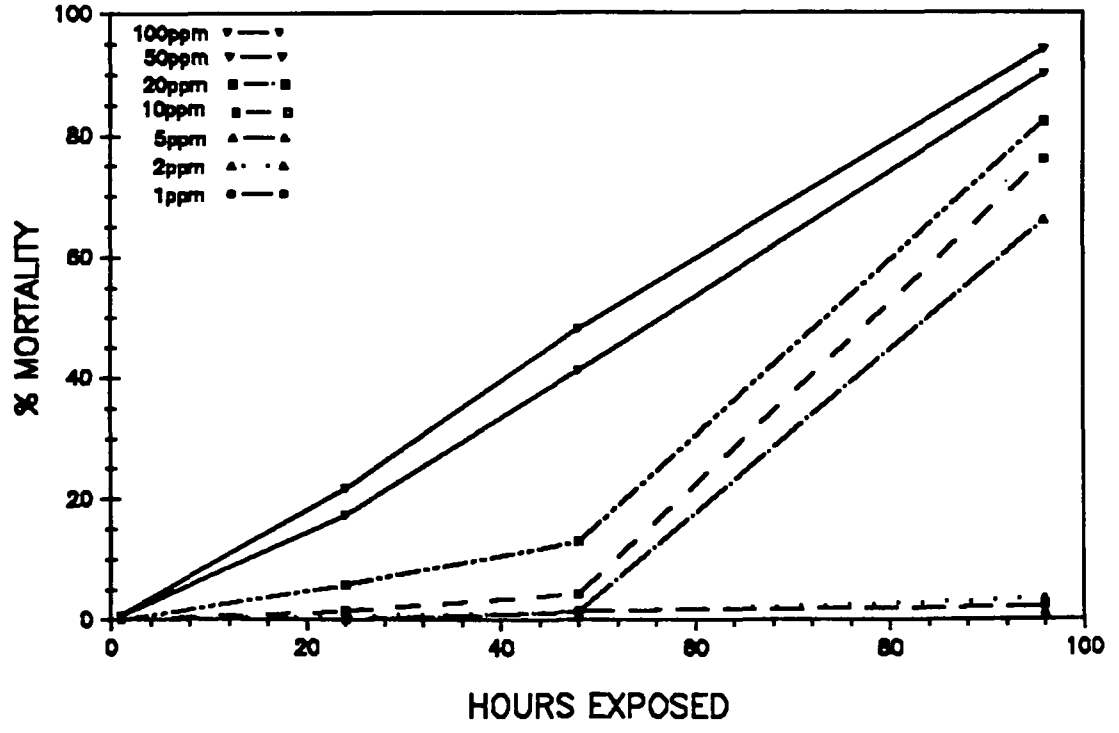


Figure A10: Percent honey bee mortality for $CdSO_4$ test three. Plot shows mortality versus exposure time for all syrup doses.



APPENDIX B

MEDIAN LETHAL DOSE AND PROBIT ANALYSIS: FEEDING STUDIES

This appendix includes only plots used for 48 and 96 hour cadmium LD50 determinations. Plots were made by two methods: by hand on two cycle semi-log paper and on the SigmaPlot plotting program. Values for slopes, intercepts and correllation coefficients were determined with the SPSS-PC statistical program. Raw mortality data were expressed as "percent mortality," transformed to probit values (Appendix G) and used as the dependent variable. The actual dose values were transformed to "dosage" values ($\log_{10}$ dose) and used as the independent variable.

KEY FOR APPENDIX B PLOTS:

● = 48 hour probit plots

▲ = 96 hour probit plots

Figure B1: Honey bee 48 and 96 hour LD_{50} estimates for $CdCl_2$ oral exposure; feeding study one.

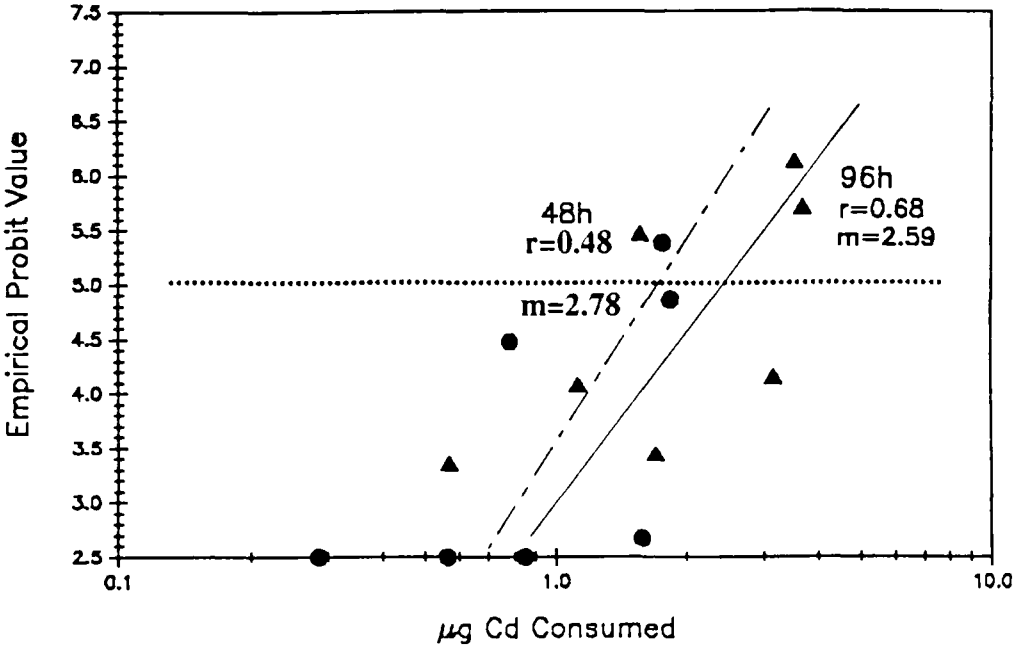


Figure B2: Honey bee 48 and 96 hour LD_{50} estimates for $CdCl_2$ oral exposure; feeding study two.

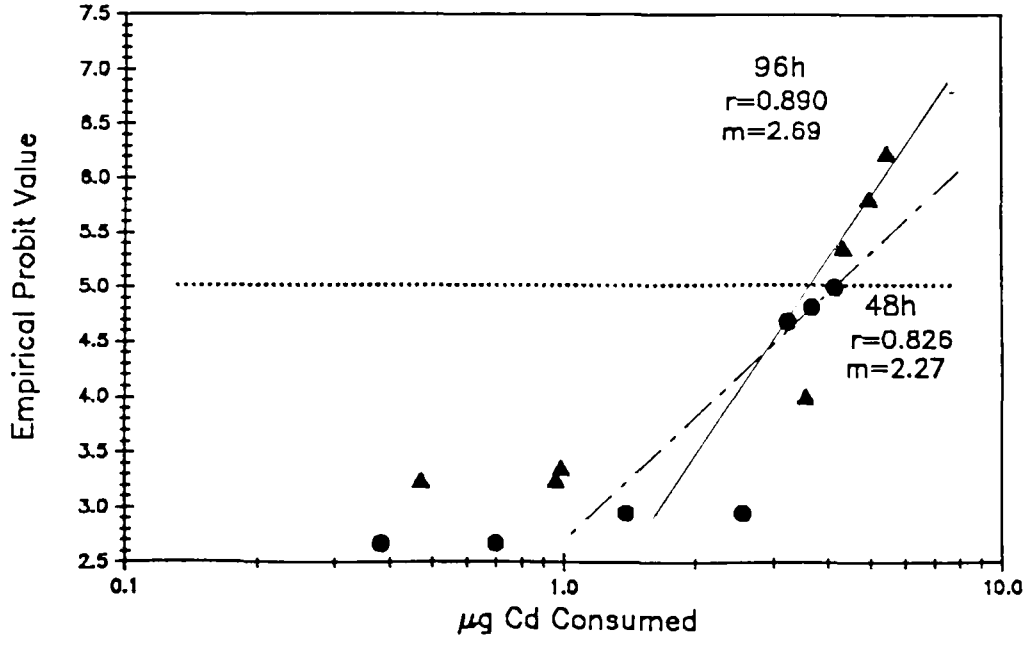


Figure B3: Honey bee 48 and 96 hour LD_{50} estimates for $CdCl_2$ oral exposure; feeding study three.

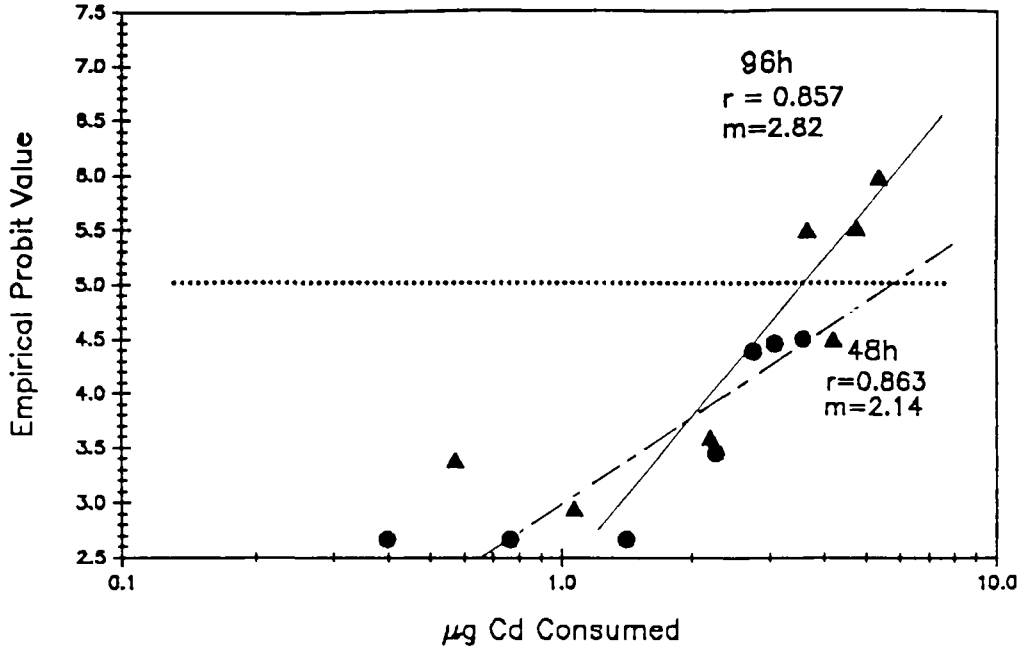


Figure B4: Honey bee 48 and 96 hour LD_{50} estimates for $CdCl_2$ oral exposure; feeding study four.

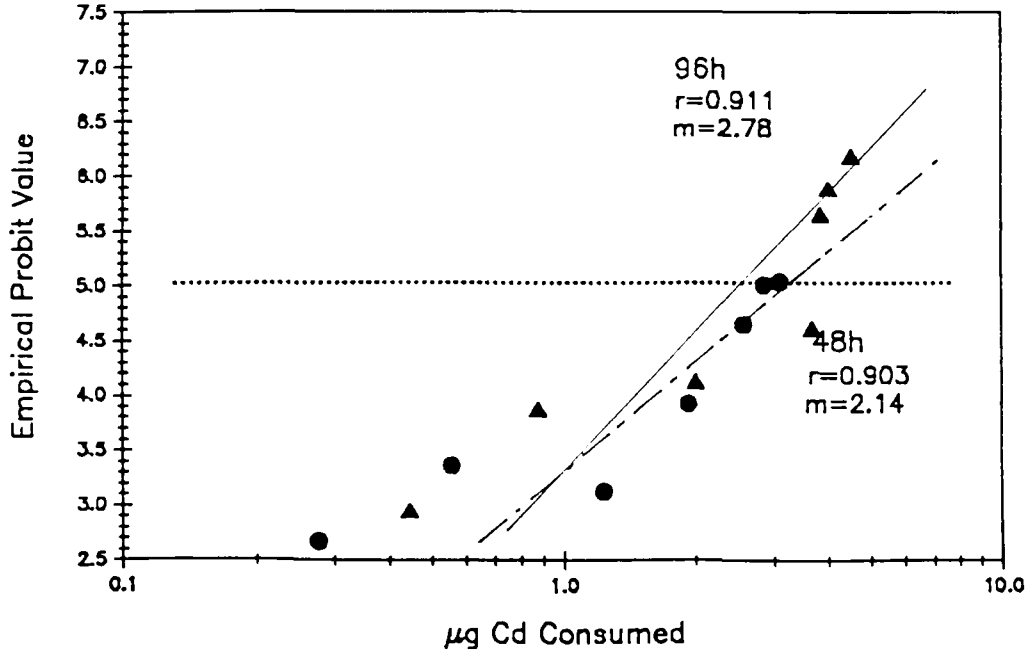


Figure B5: Honey bee 48 and 96 hour LD_{50} estimates for $CdCl_2$ oral exposure; feeding study five.

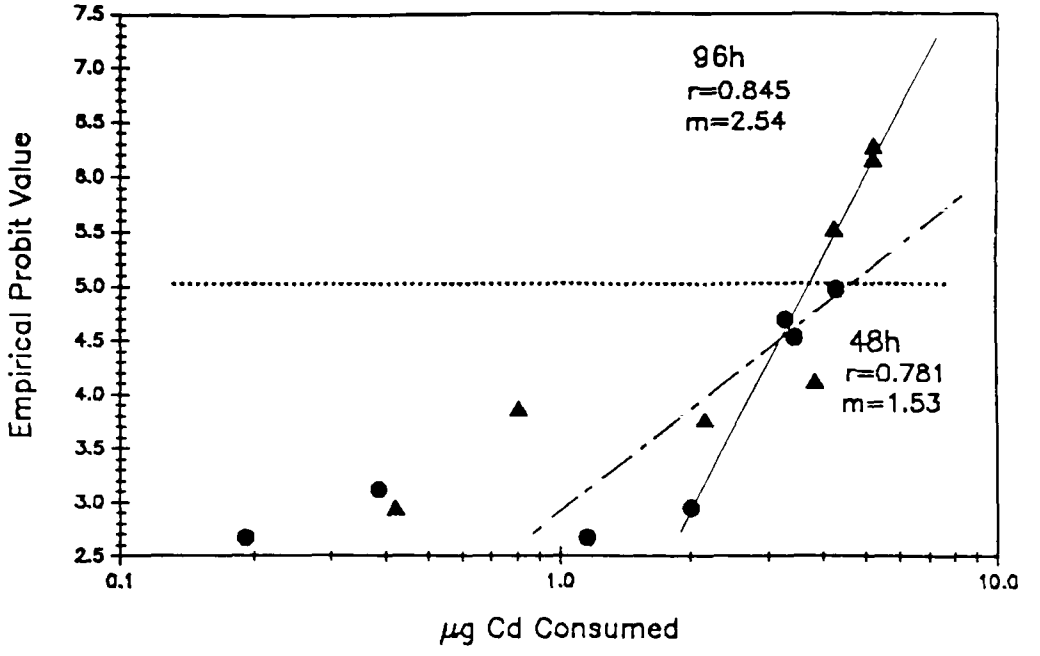


Figure B6: Honey bee 48 and 96 hour LD_{50} estimates for $CdSO_4$ oral exposure; feeding study one.

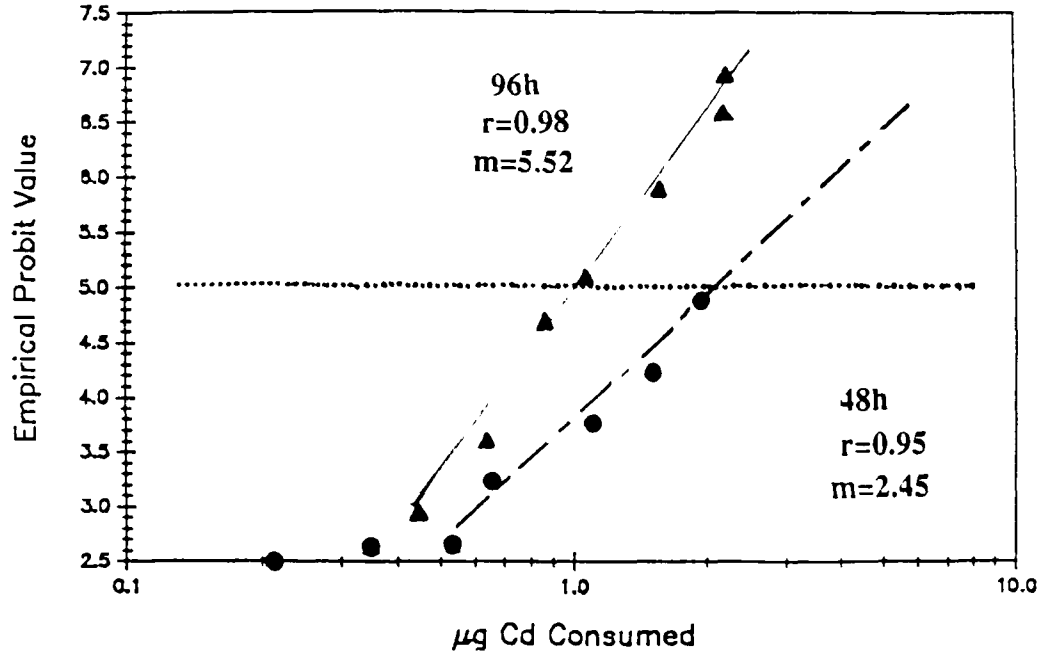


Figure B7: Honey bee 48 and 96 hour LD_{50} estimates for $CdSO_4$ oral exposure; feeding study two.

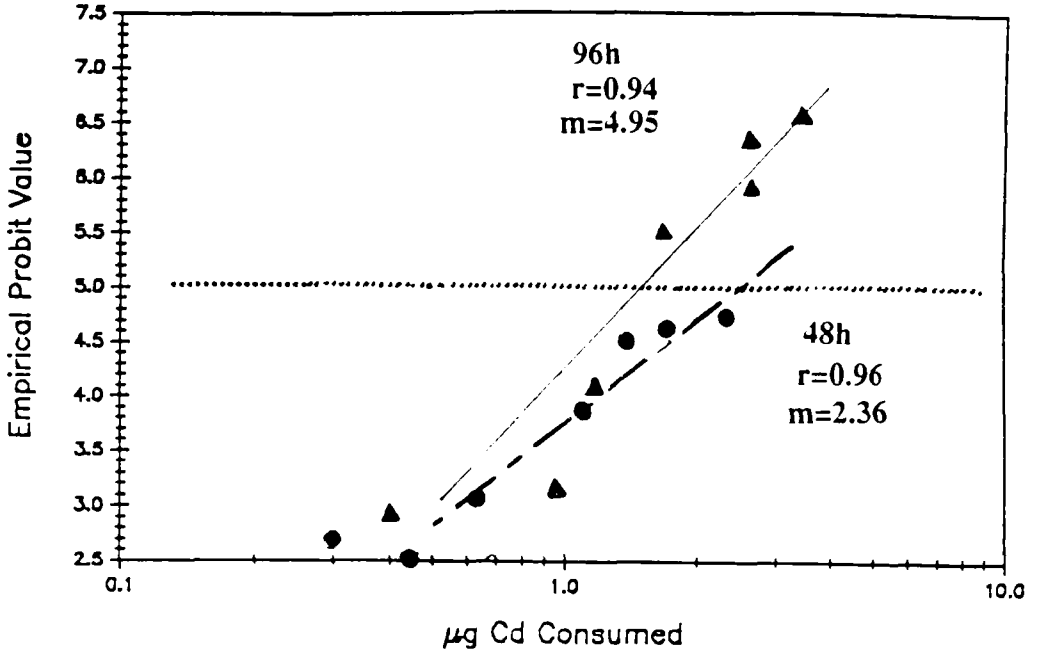
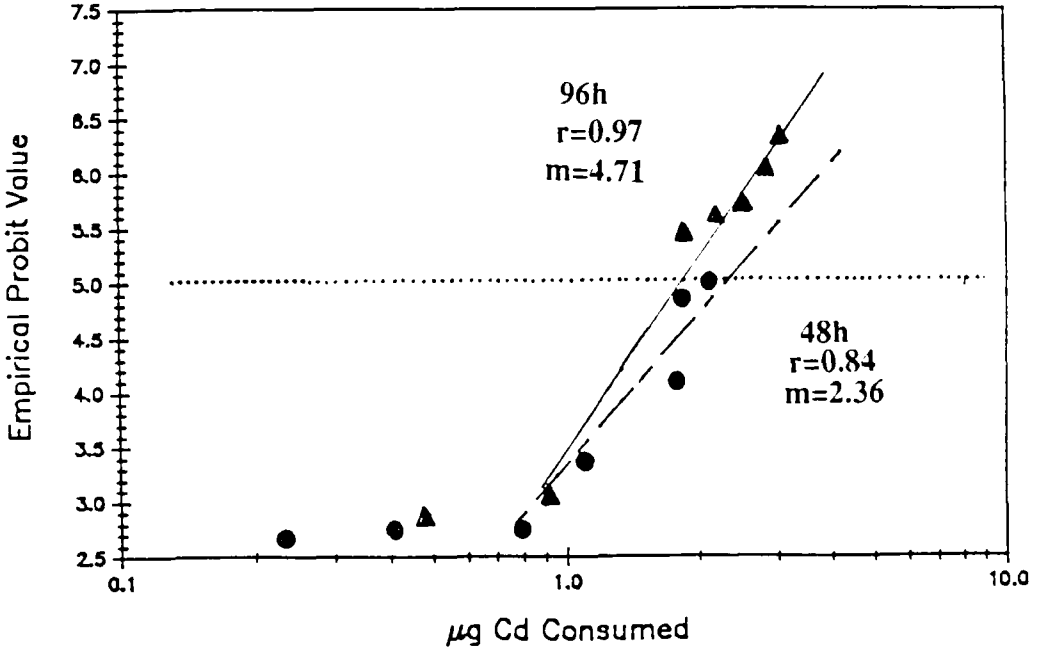


Figure B8: Honey bee 48 and 96 hour LD_{50} estimates for $CdSO_4$ oral exposure; feeding study three.



APPENDIX C

MEDIAN LETHAL DOSE AND PROBIT ANALYSIS: INJECTION STUDIES

* This appendix includes only the plots used for 48 hour cadmium injection LD50 values. Plots were made by two methods: by hand on two-cycle semilog paper and on the SigmaPlot plotting program. Values for slopes, intercepts and correllation coefficients were determined with the SPSS-PC statistical program. Raw mortality data were expressed as "percent mortality," transformed to probit values (Appendix G) and used as the dependent variable. The actual dose values were transformed to dosage values ($\log_{10}$ dose) and used as the independent variable.

Figure C1: Honey bee 48 hour LD_{50} estimate for $CdCl_2$ exposure; injection study one.

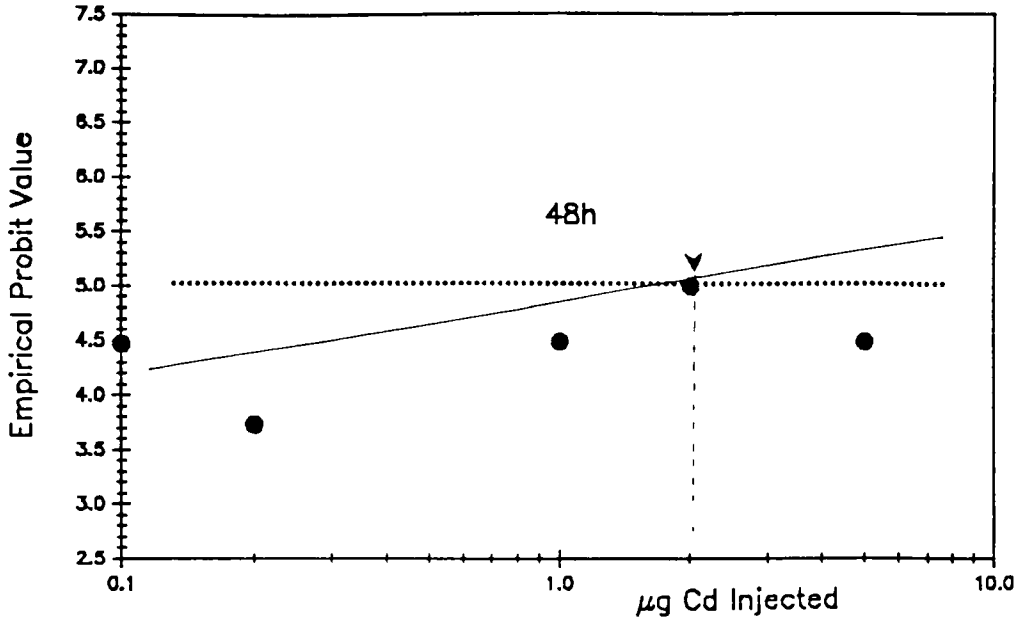


Figure C2: Honey bee 48 hour LD_{50} estimate for $CdCl_2$ exposure; injection study two.

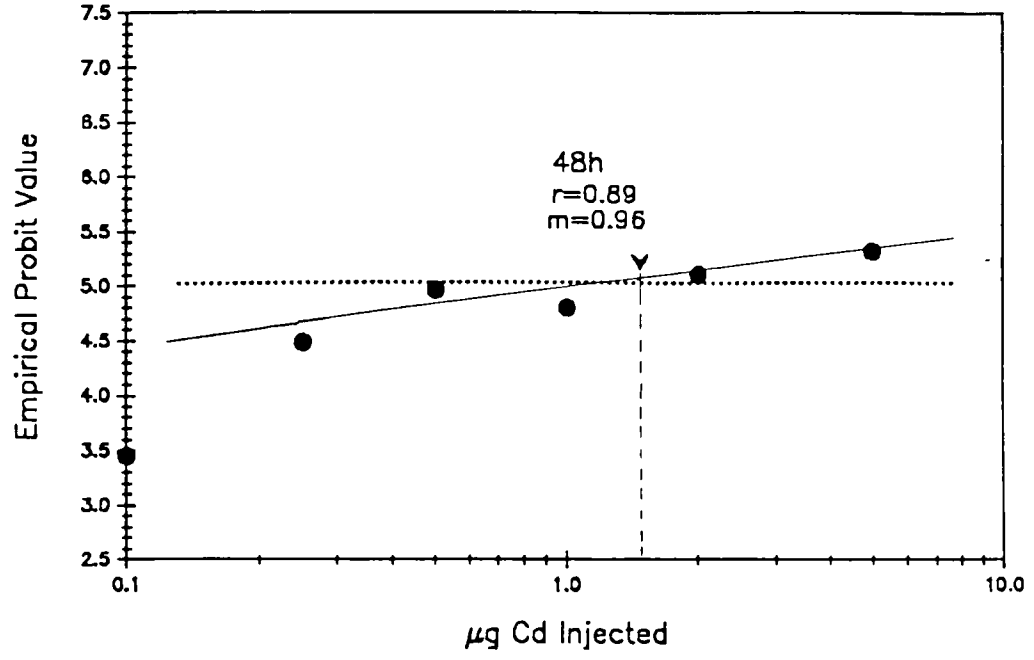


Figure C3: Honey bee 48 hour LD_{50} estimate for $CdCl_2$ exposure; injection study three.

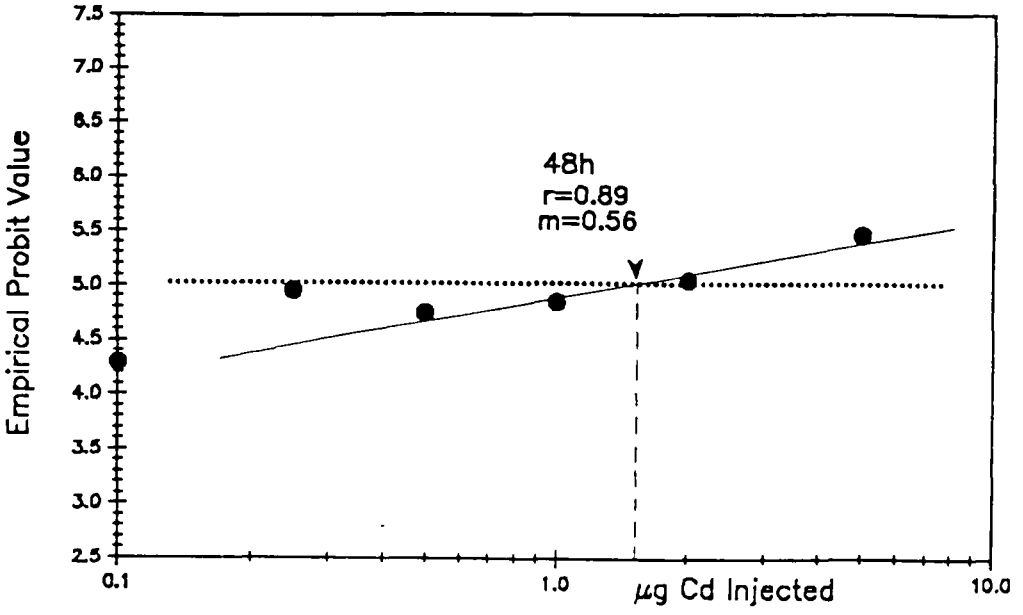


Figure C4: Honey bee 48 hour LD_{50} estimate for $CdCl_2$ exposure; injection study four.

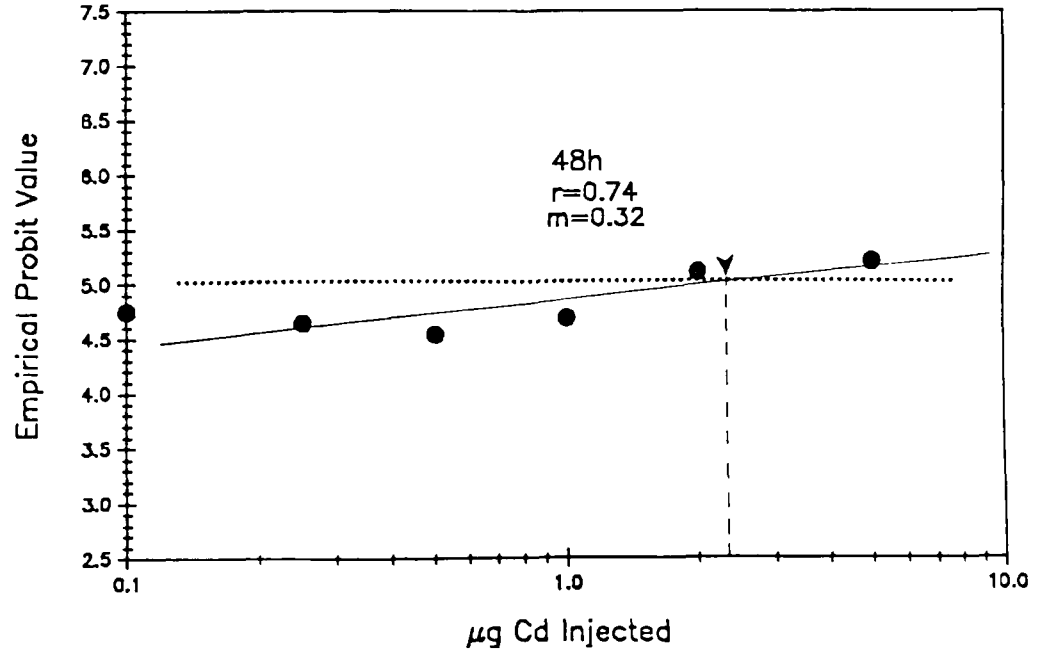
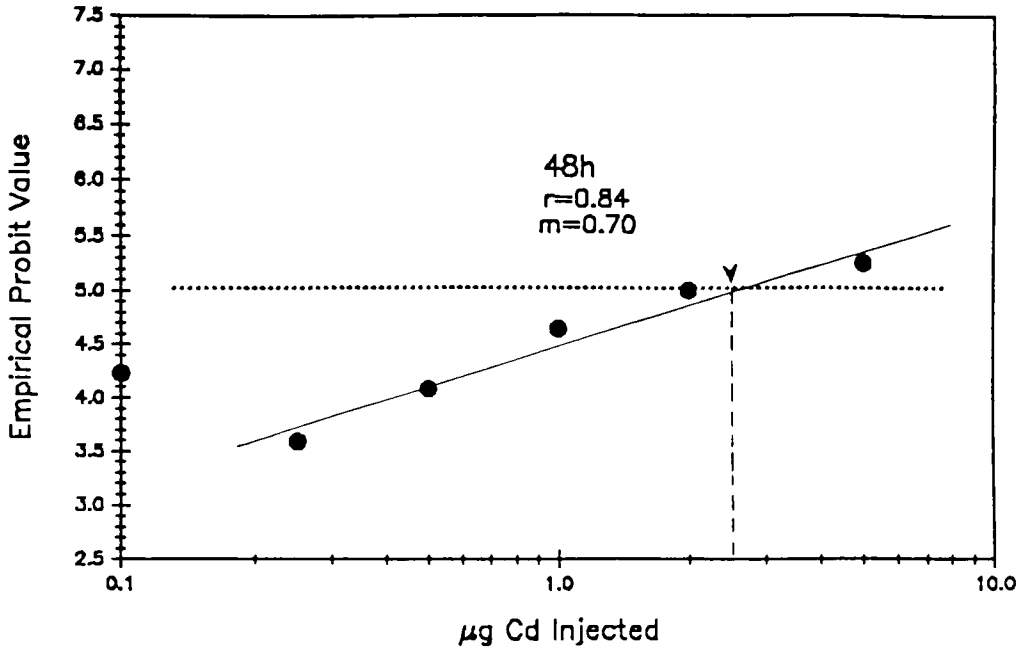


Figure C5: Honey bee 48 hour LD_{50} estimates for $CdCl_2$ exposure; injection study five.



APPENDIX D

RABBIT CADMIUM METALLOTHIONEIN CONTROL EXPERIMENTS

* This appendix includes data from several experiments conducted to evaluate isolation, purification and detection methods for metallothionein proteins. These experiments utilized SIGMA Rabbit cadmium metallothionein (# M7641), a protein that exists as two isoforms with molecular weights of approximately 6,620. This protein contains no aromatic amino acids, 94.0 µg and 18.5 µg Zn per mg protein and has 22 cysteines per molecule (data from supplier).

The following experiments were run:

* Protein assays: comparison of Rabbit Cd/Zn MTh to bovine serum albumin standards using method of Lowry (Stanesh, 1984) and the BioRad coomassie R-250 dye binding protein assay.

* Gel Filtration Molecular Weight: analysis of estimated molecular weight of Rabbit Cd/Zn MTh using Sephadex G50.

* DEAE Sephadex Purification: optimization of pH and NaCl elution parameters for Sephadex A25 anion exchange chromatography.

* Atomic Absorption Spectrophotometry of Cd-MT: analysis for minimum detection limits for Rabbit Cd/Zn MTh and examination of matrix effects and interferences.

PROTEIN ASSAYS

The coomassie brilliant blue R-250 (or "CBB") dye binding assay of Bradford has been shown a precise method of protein determination for complex protein mixtures, exhibiting far greater sensitivity than A230 or A280 methods and similar precision and accuracy as the method of Lowry (Bradford, 1976; Davis, 1988). A disadvantage with all protein assays is that they yield varying results with different purified proteins due to differences in amino acid content. Two experiments was run to determine which of the two colorimetric methods worked best in quantifying Rabbit Cd/Zn MTh, and the comparative color development of metallothionein versus BSA as a standard.

Both protein assays run in triplicate showed that purified MTh yielded less color than BSA diluted at a similar concentration. This was expected, as both methods rely on aromatic amino acids for color enhancement and Rabbit Cd/Zn MTh by definition contains no aromatic amino acids (BSA contains Phe, Try and Trp). Least squares analysis of the mean of Lowry assay triplicate runs showed a slope of 0.0015 for Rabbit Cd-MT ($r^2 = 0.980$, Figure D1), where the slope for BSA was much greater at 0.029 ($r^2 = 0.988$). A scattergram plotting the absorbance of Rabbit Cd/Zn MTh versus BSA was generated to determine the correlation between values (Figure D2). A reasonable fit resulted, giving a correlation coefficient of 0.981. These

Figure D1: Lowry assay determinations for soluble protein: Results using BSA and rabbit metallothionein.

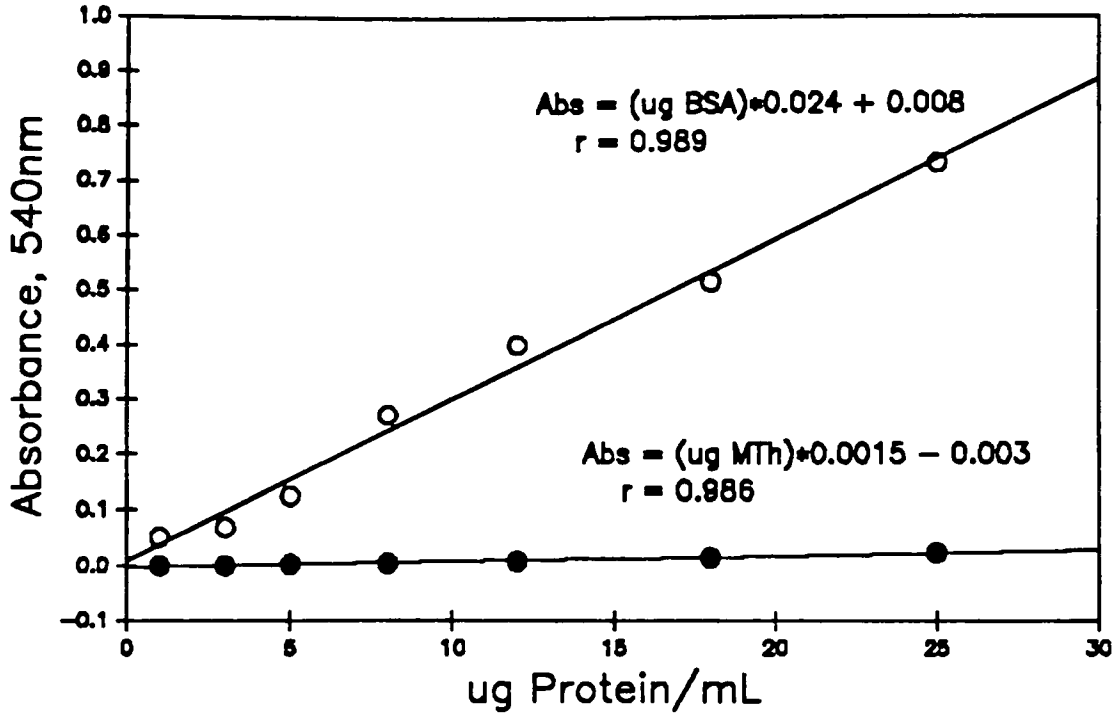


Figure D2: Replot of BSA absorbance values versus rabbit MTh values for test of linearity using the Lowry assay.

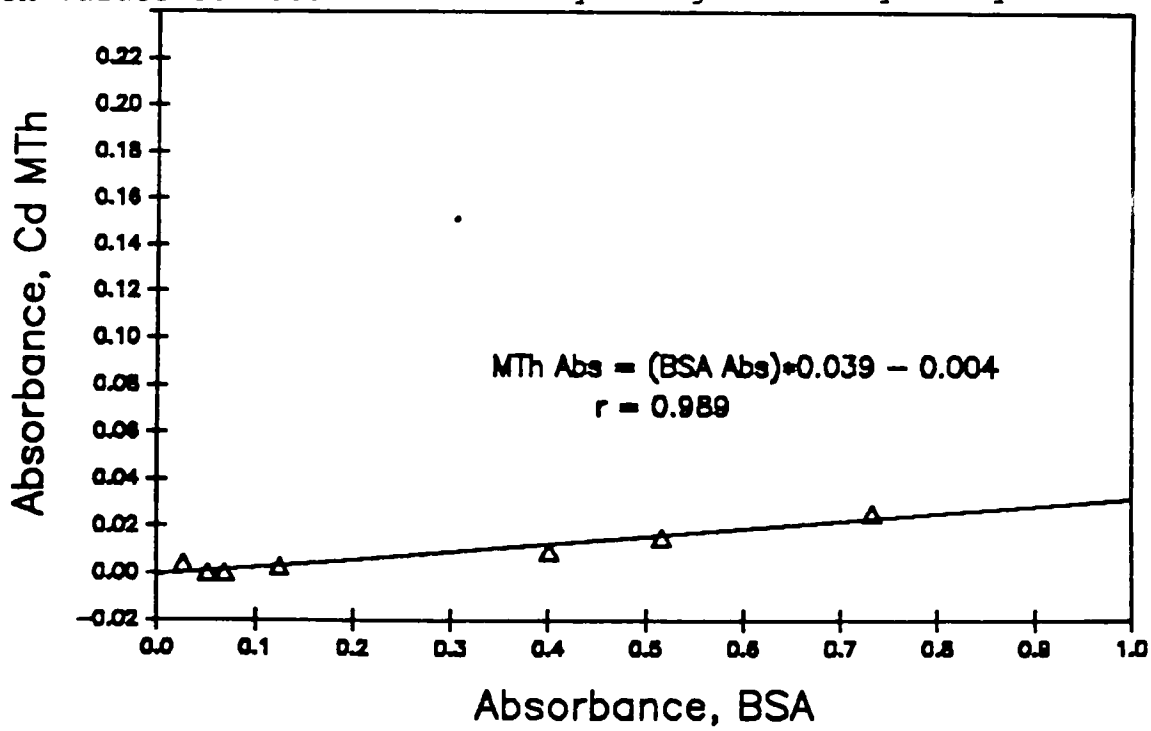


Figure D3: Bradford assay determinations for soluble protein: Results for BSA and rabbit metallothionein.

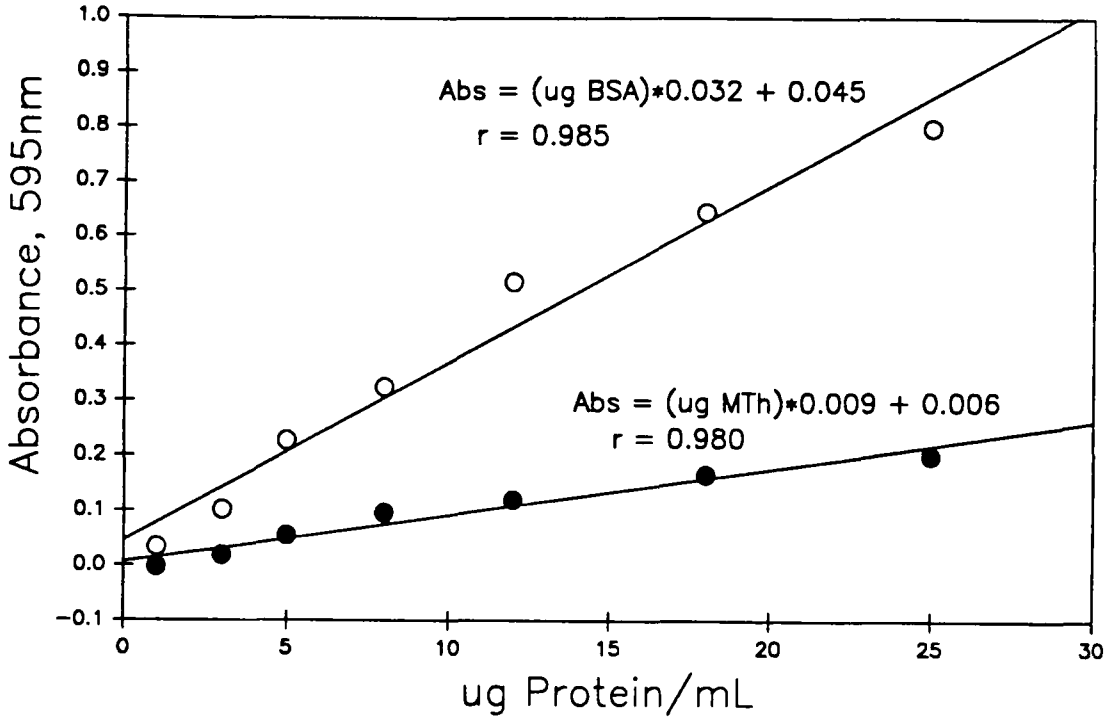
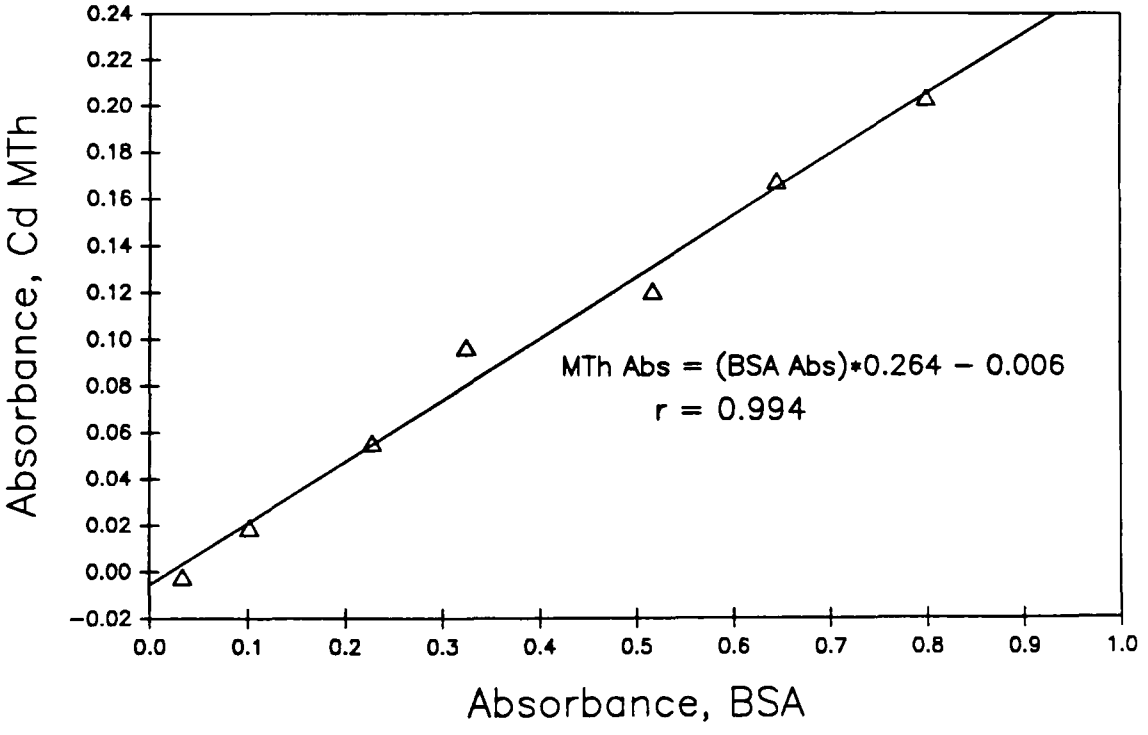


Figure D4: Replot of BSA absorbance values versus rabbit MTh values for test of linearity using the Bradford assay.



data indicate that the Lowry protein assay is a good indicator for BSA, but poor for quantitation of Rabbit MTh with an approximate minimum detection limit of 8 µg/ml.

Least squares analysis of the mean of BioRad CBB assay triplicate runs showed a slope of 0.0086 for Rabbit Cd/Zn MTh (r2 = 0.959, Figure D3), where the slope for BSA was much greater at 0.032 (r2 = 0.970). A scattergram plotting the absorbance of Rabbit MTh versus BSA was generated to determine the correlation between values (Figure D4). A good fit resulted, giving a correlation coefficient of 0.988. These data indicate that the BioRad CBB protein assay is a better indicator of Rabbit Cd/Zn metallothionein concentration than the Lowry method, but still does not develop color to the degree as seen with BSA. All subsequent assays on complex samples (i.e., yielded several bands on SDS-PAGE) utilized the CBB method using BSA as a standard, while purified samples of honey bee cadmium binding proteins were quantified using Sigma Rabbit Cd/Zn metallothionein as a standard. This assumes that honey bee CdBP also contains low aromatic content, an assumption that holds true for *Drosophila* metallothionein.

MINIMUM DETECTION LIMIT OF CADMIUM METALLOTHIONEIN

A short experiment was run to determine the minimum detection limits of Rabbit Cd/Zn metallothionein by Cd atomic absorption spectroscopy. From a stock solution of 1 mg/mL MTh in buffer A, dilutions of 0.01, 0.05, 0.10, 1.0,

5.0, 10 and 50 µg MTh per ml were made with buffer A. Triplicate determinations were made using flame AAS at the 228.8 nm wavelength, an absorbance line that generally gives a linear response from 0 to 2.5 ppm Cd/ml (data from Perkin-Elmer). The data summarized in Table D1 show that a minimum detection limit of between 0.01 to 0.05 µg MTh could be obtained, corresponding to absorbance readings of 0.02 and 0.06 units respectively. The response was linear to 50 µg MTh/mL and showed no effect from increasing protein concentrations. While a minimum detection limit was not investigated with graphite furnace EAS, the 50-fold lower detection limit for cadmium could give a theoretical detection limit of 0.001 to 0.0002 µg MTh. These data suggest that flame Cd-AAS shows less variability than the data derived from either Lowry or coomassie brilliant blue protein assays. With the greater sensitivity, flame (and graphite) AAS are the preferred method of detection for these cadmium-metalloproteins.

GEL FILTRATION MOLECULAR WEIGHT

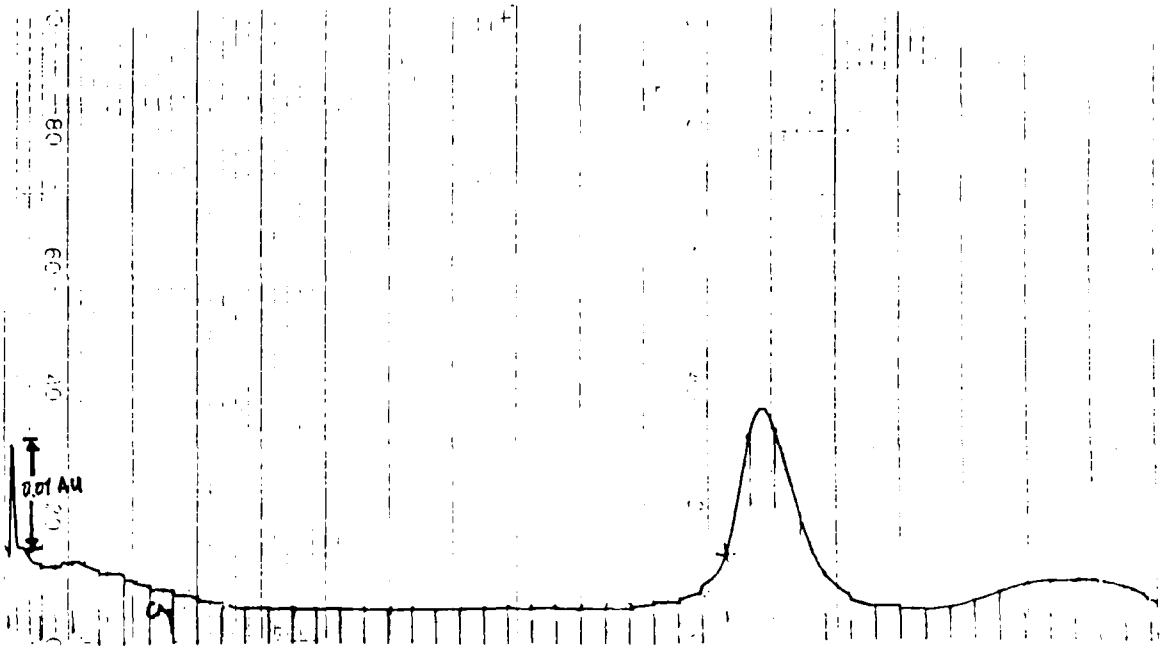
Andrews (1970) has reported that proteins which assume a near globular shape in solvent will migrate linearly as a function of the log of their molecular weights in gel filtration media. An analytical column (1.0 x 60 cm) was packed with Sephadex G50 pre-equilibrated in buffer A and calibrated using standard proteins of known molecular weight (Figure D5). An operating pressure of 40 cm buffer was

Table D1: Minimum detection limits of Cd-MTh by flame atomic absorption.

[MTh], µg/ml	$\bar{X}$* Sample Absorbance	Sample Cd, µg per ml	Theoretical Cd, µg/ml	% Cd recovery
0.01	0.000	-0.013	0.0010	-1350
0.05	0.002	0.003	0.0048	72.6
0.10	0.005	0.007	0.0096	72.9
1.0	0.011	0.096	0.0956	101
5.0	0.052	0.504	0.478	105
10	0.104	1.02	0.956	107
50	0.420	4.07	4.78	85.1

* number of determinations = 3

Figure D5: Chromatogram of Sephadex G-50 molecular weight determination for rabbit Cd-metallothionein.



used to give a flow rate of approximately 0.75 ml/min., and all column chromatography was performed at 4°C.

Rabbit Cd/Zn metallothionein was applied to the column (250µl of 1 mg/ml, or 23.5 µg Cd total) and fractions (0.5ml) were collected and analyzed for Cd by flame AAS at 228.8 nm. The estimated molecular weight was determined to be 9,600 Daltons. This estimate, while different from the actual molecular weight of Rabbit Cd/Zn MTh, is well within reported values for its' apparent gel filtration molecular weight, as the prolate-elipsoid shape of mammalian MTh shows irregular migration (Webb, 1977).

DEAE-SEPHADEX PURIFICATION

As a final purification step, most cadmium metallothioneins are often bound to weak anion exchangers and eluted differentially using NaCl gradients. The binding of protein to the exchanger is pH dependent, as proteins are amphoteric and exhibit different charges at different pH. This experiment examined the conditions necessary to bind Rabbit Cd/Zn MTh to Sephadex A25 DEAE, and the conditions needed to release the protein from the exchanger.

Effect of pH was examined by adding 25µg of Cd/Zn MTh diluted in 1 m<u>M</u> Tris-HCl (pH 7.0) to 50 mg of Sephadex A25 equilibrated in 0.975 ml of 20 m<u>M</u> Tris-HCl ranging in pH from 6.0 to 9.0. Triplicate samples were shaken gently for 30 minutes on ice, centrifuged at 5,000 rpm for 5 minutes, and the supernatant analyzed for soluble Cd (or "unbound

Figure D6: Binding affinity of Cd/Zn metallothionein for DEAE Sephadex A25; pH effects.

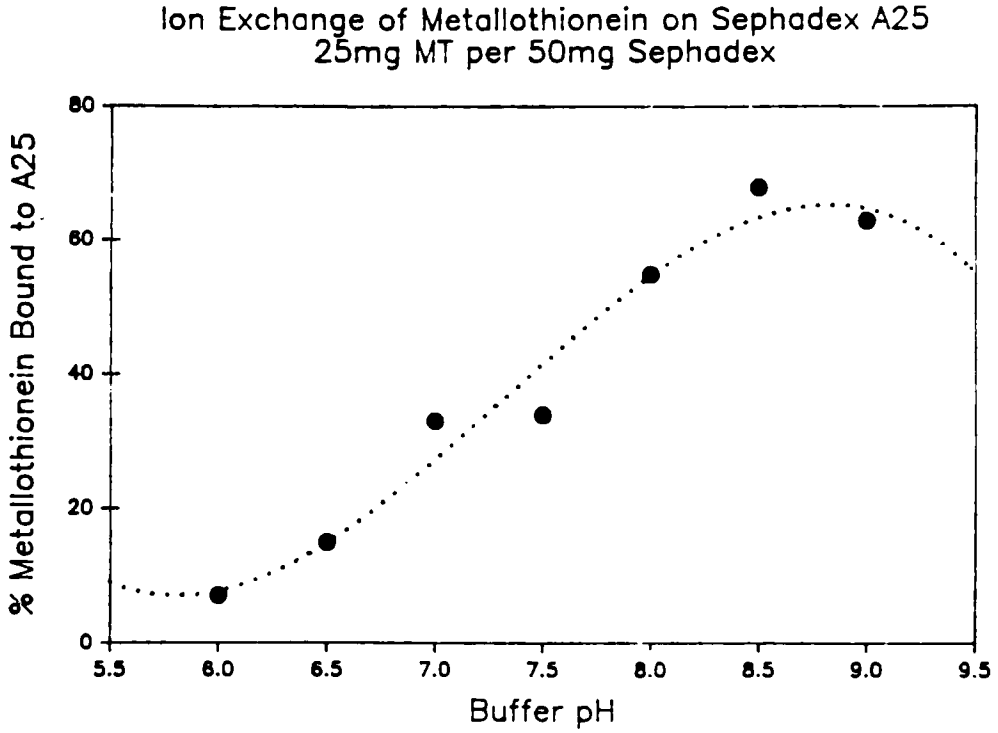
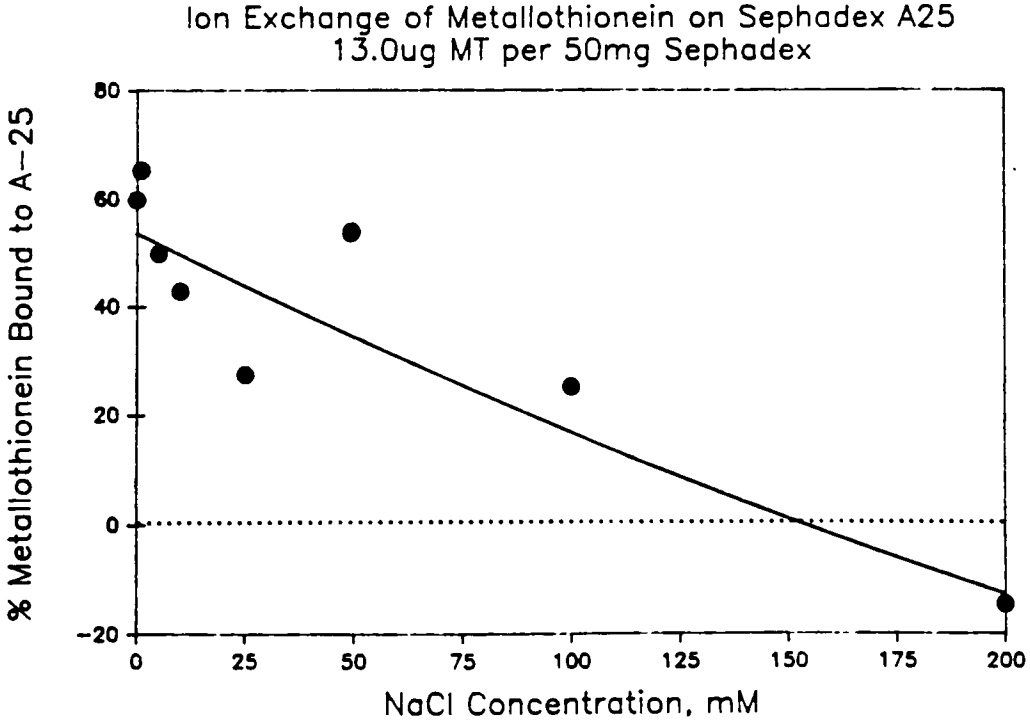


Figure D7: Binding affinity of Cd/Zn metallothionein for DEAE Sephadex A25; salt effects.

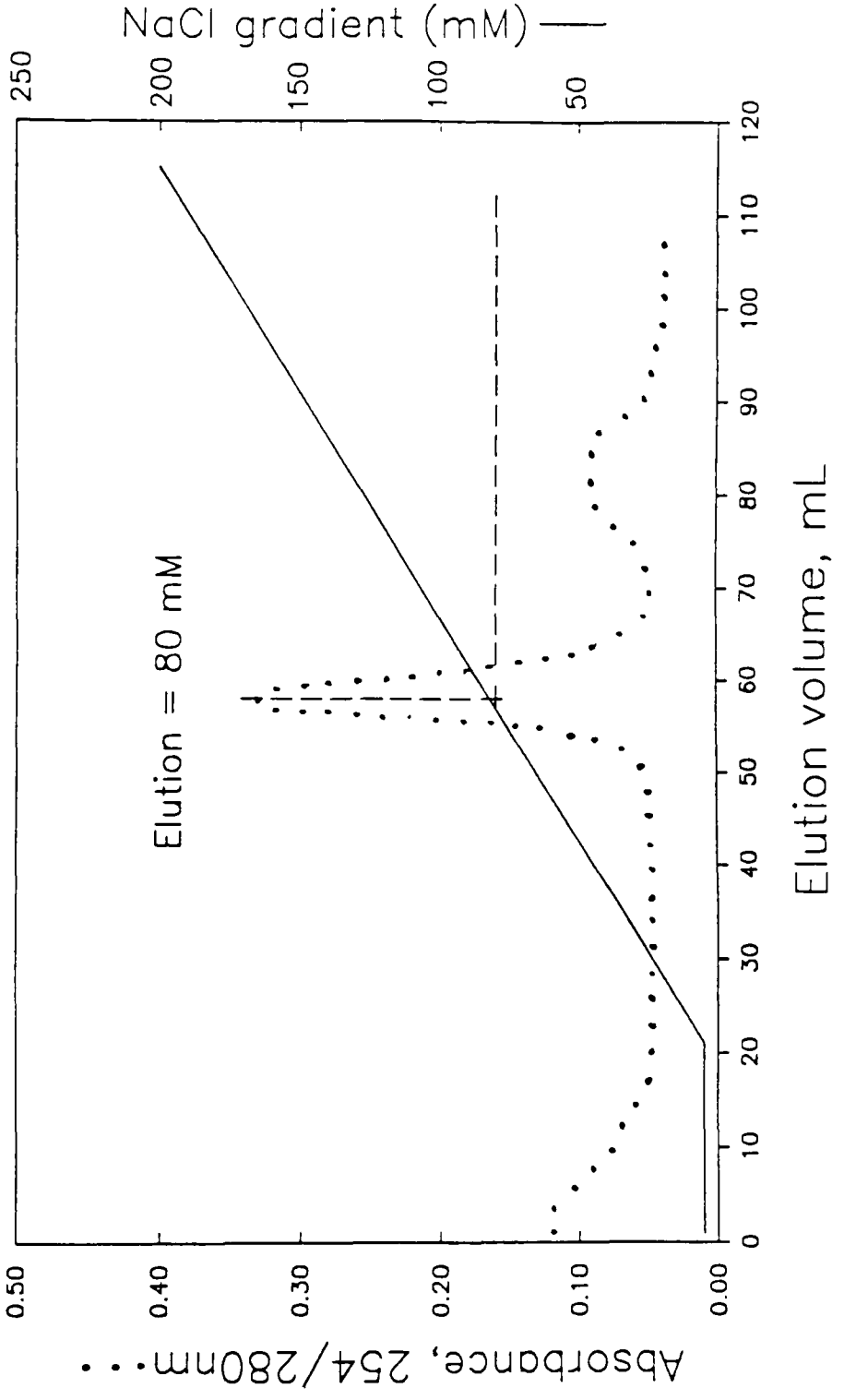


Cd-MT"). Triplicate controls were also run at pH 6.0 and 9.0 containing no Sephadex A25 for assurance of Cd recovery. Soluble Cd appeared greatest at pH 6.0 suggesting that less than 10% of Cd MTh had bound to the exchanger (Figure D6). The lowest soluble Cd was seen at pH 8.5, corresponding to approximately 75 % of the protein bound.

Effect of NaCl on elution was examined by adding 25 µg of the same Cd/Zn MTh solution to 50 mg Sephadex A25 equilibrated in 1.975 ml of Tris-Cl, pH 8.6 ranging from 0 to 200 m<u>M</u> NaCl. Triplicate samples were shaken and spun as above and examined for soluble cadmium. Triplicate controls were also run containing 0, 10, 50 and 200 m<u>M</u> NaCl for assurance of Cd recovery. Soluble Cd appeared to increase with the addition of as little as 5mM NaCl, and reached over 100 % recovery by 200 m<u>M</u> NaCl (Figure D7). The binding profile suggested that up to 80 % of the bound Cd MTh would release between 25-50 m<u>M</u>, but the final 20 % could be difficult to elute.

A final examination was made to observe the elution behavior of Rabbit Cd-MT on a 1.0 x 10 cm column of Sephadex A25. The Sephadex was prepared as described earlier and packed under 15cm H20 pressure to give approximately 0.6 ml/min flow rate. Rabbit Cd/Zn MTh (500 µg in 0.5 ml 1.0 m<u>M</u> Tris-Cl, pH 8.6 with ß-mercaptoethanol) was applied to the column, washed with 10 ml of buffer B containing 0 m<u>M</u> NaCl, then eluted with a 40 ml linear gradient of buffer B

Figure D8: Chromatogram of Cd-MTh elution profile on DEAE Sephadex A25.



containing 0 to 200 mM NaCl. A single peak eluted at approximately 45 mM NaCl (Figure D8), suggesting the isoforms of Rabbit Cd-MT could not be resolved on Sephadex A25.

ATOMIC ABSORPTION SPECTROPHOTOMETRY OF Cd-MT

Flame atomic absorption spectroscopy data can be greatly affected by factors that influence sample viscosity and aspiration. For quantitation of biomolecules, the major factor in sample viscosity is protein concentration. To see what effect protein concentration had upon detection of soluble cadmium in solution, I combined several levels of cadmium (from 0.01 to 5 ug/mL) with soluble proteins including ovalbumin (0 to 2,000 ug/mL) and crude honey bee homogenates free of cadmium contamination (determined to be 4,010 ug/mL by protein assays). These were run under the same conditions described in the "minimum detection limit" experiments and all samples were run in triplicate.

Concentrations of Ovalbumin as high as 2,000 ug/mL had no significant effect on the absorbance of cadmium under the condtions used (Figure D9). The recovery of cadmium at the highest level tested was not as good as seen in lower levels, with only 85% of the cadmium accounted for. This was most likely an artifact of the calibration procedure, as the 5.00 ppm Cd standard even gave a reading of 4.16 ug/mL.

Dilutions of honey bee homogenates gave similar results as the Ovalbumin with levels at or below 2,000 ug

protein/mL, but showed a significantly increased recovery at the 4,000 ug protein/mL levels (Figure D10). This would suggest that samples containing more than 2 mg protein per ml could interfere with cadmium determinations, giving values higher than the sample actually contained. This would show the greatest effect on crude homogenates (which often contain several mg soluble protein per ml) and with fractions from the "void volume" region of sephadex chromatography. For routine analysis of dilute protein mixtures, no matrix corrections were required.

Figure D9: Cadmium standard addition recovery in the presence of high ovalbumin concentrations.

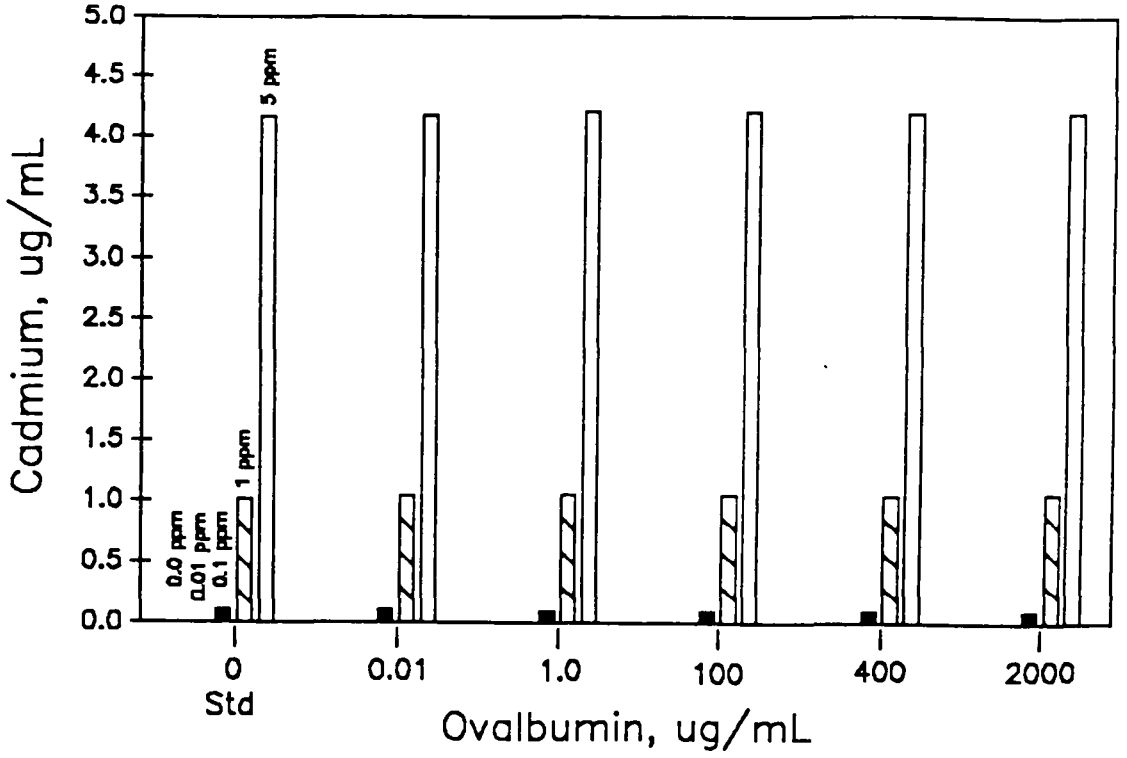
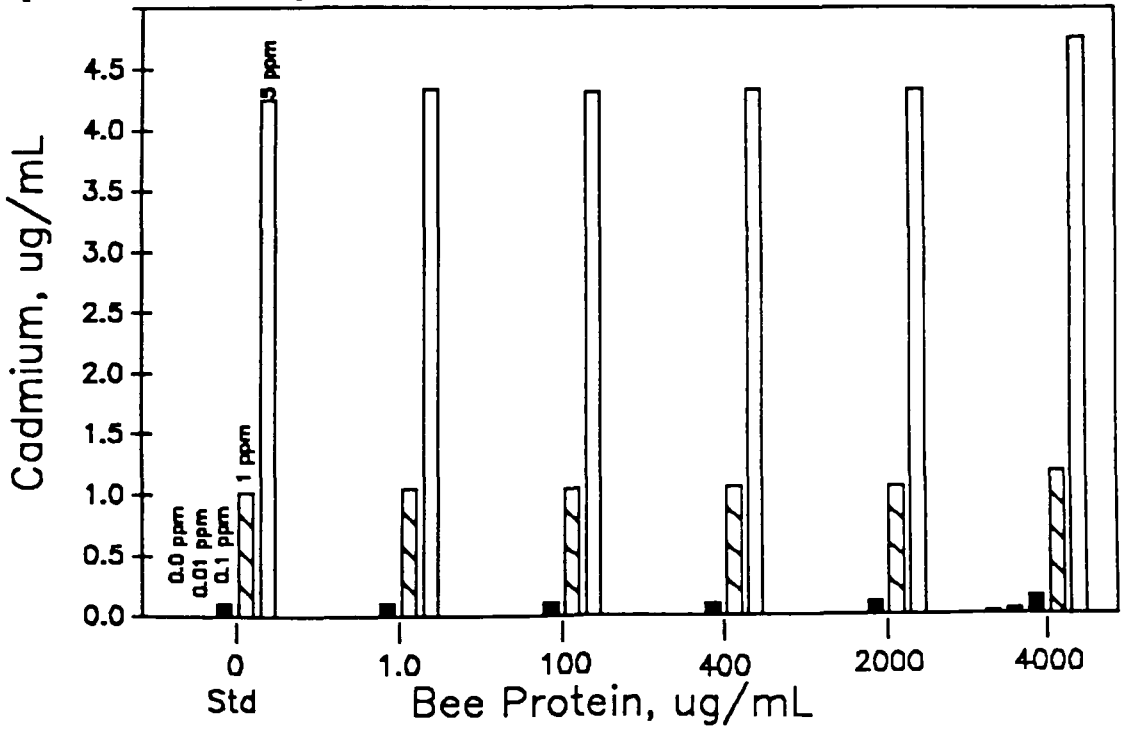


Figure D10: Cadmium standard addition recovery in the presence of high bee protein concentrations.



APPENDIX E

HONEYBEE SYRUP RECIPES

SUCROSE/GLUCOSE SYRUP: Approximately 250 g sucrose and 150 g D-glucose were slowly diluted to 500 ml with hot glass distilled water and stirred overnight until dissolved. This solution was briefly autoclaved for 15 minutes (20 psi, 121°C) to retard fungal growth.

CADMIUM SYRUP: Cadmium (from either $CdCl_2 \cdot 2.5H_2O$ or $CdSO_4.8H_2O$) was obtained from 10,000 ppm, 1,000 ppm and 100 ppm stock solutions. The appropriate amount of Cd Stock was pipetted into a clean, acid-washed 250 ml flask and sucrose/glucose syrup was added slowly while stirring. This solution was allowed to stir for approximately 10 minutes, then was transferred to individual, pre-weighed feeding vials with a 25.00 ml volumetric pipet (a very slow process!). A final weight was recorded, then vials were parafilmed and stored at 25°C until needed. Unused feeding solutions were pooled and disposed as hazardous waste.

[Cd] in syrup	Stock [Cd]	ml Stock added	ml Syrup added	Final Volume
1ppm	100ppm	1.000	99.0	100mL
2ppm	1,000	0.200	99.8	100mL
5ppm	1,000	0.500	99.5	100mL
10ppm	1,000	1.000	99.0	100mL
20ppm	10,000	0.125	49.8	50mL
50ppm	10,000	0.250	49.7	50mL
100ppm	10,000	0.500	49.5	50mL

CADMIUM STOCKS: Cadmium Chloride (as CdCl2.2.5H2O) and Cadmium Sulfate (as 3CdSO4.8H2O) were both obtained through Fisher Scientific. Cadmium Chloride hemipentahydrate was dried at 38°C, as it easily loses the waters of hydration, and all calculations were made with the anhydrous molecular weight (183.31 g/mol). Assuming a Cadmium content of 61.32 % by weight, 0.4077 g dried Cadmium Chloride was dissolved into glass distilled water and diluted to a final volume of 25.0 ml to give a concentration of 10,000 ugCd/mL (10,000 ppm). The 1,000 ppm and 100 ppm Cd stock solutions were made from serial dilutions of the 10,000 ppm stock by volumetric dilution of 1.000 ml Cd solution to 10.00 ml with distilled water. All stock solutions were stored tightly sealed at 4°C in acid-washed polypropylene containers.

Cadmium Sulfate was made using the hydrated molecular weight of 256.45 g/mol CdSO4. Assuming 43.67 % Cd by weight, 0.2290 g Cadmium Sulfate octahydrate was dissolved as above to give a 10,000 ppm stock, and dilutions were made to 1,000 ppm and 100 ppm. Stock solutions were stored as above.

APPENDIX F

PROBIT TRANSFORMATION TABLE

Reference: Finney, D.J. (1952). "Probit Analysis: A Statistical Treatment of the Sigmoid Response Curve," Second Edition. Cambridge University Press.

Response rate	0·00	0·01	0·02	0·03	0·04	0·05	0·06	0·07	0·08	0·09
0·00	—	2·67	2·95	3·12	3·25	3·36	3·45	3·52	3·59	3·66
0·10	3·72	3·77	3·82	3·87	3·92	3·96	4·01	4·05	4·08	4·12
0·20	4·16	4·19	4·23	4·26	4·29	4·33	4·36	4·39	4·42	4·45
0·30	4·48	4·50	4·53	4·56	4·59	4·61	4·64	4·67	4·69	4·72
0·40	4·75	4·77	4·80	4·82	4·85	4·87	4·90	4·92	4·95	4·97
0·50	5·00	5·03	5·05	5·08	5·10	5·13	5·15	5·18	5·20	5·23
0·60	5·25	5·28	5·31	5·33	5·36	5·39	5·41	5·44	5·47	5·50
0·70	5·52	5·55	5·58	5·61	5·64	5·67	5·71	5·74	5·77	5·81
0·80	5·84	5·88	5·92	5·95	5·99	6·04	6·08	6·13	6·18	6·23
0·90	6·28	6·34	6·41	6·48	6·55	6·64	6·75	6·88	7·05	7·33

Response rate	0·000	0·001	0·002	0·003	0·004	0·005	0·006	0·007	0·008	0·009
0·97	6·88	6·90	6·91	6·93	6·94	6·96	6·98	7·00	7·01	7·03
0·98	7·05	7·07	7·10	7·12	7·14	7·17	7·20	7·23	7·26	7·29
0·99	7·33	7·37	7·41	7·46	7·51	7·58	7·65	7·75	7·88	8·09