THE DYNAMICS OF CADMIUM AND METALLOTHIONEIN ACCUMULATION IN PERIPHERAL BLOOD CELLS AND SELECTED ORGANS OF RATS.

By

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ABSTRACT

Cadmium (Cd) is a naturally occurring metal with well documented toxic effects on humans and animals. MT is an endogenous protein that binds Cd and other metals; its role in relation to metabolism, homeostasis and detoxication of metals has yet to be fully elucidated. The dynamics of Cd and MT were studied in individual rats (3 per group). Each rat served as its own control with blood samples collected at -20, 0, 6, 12, 24, 48, 72, and 96h via a surgically inserted jugular cannula. Several cadmium chloride concentrations (2.5, 1.25, and 0.25 mg Cd/kg) or saline were investigated as short course (single IP injection at T=0h) or subchronic (multiple injections over 2 months) with 3 rats per dose. Feces, urine and whole blood were collected at each time point and blood was separated into whole blood, plasma, erythrocytes (RBC), polymorphonuclear leukocytes (PMN), lymphocytes (Ly) and monocytes (M). Liver, kidney, spleen and lung were collected at T=96h when the animals were euthanized. Quantitative determinations were made by electrothermal atomic absorption spectrometry (EAAS) for total Cd and by the Cd Saturation Assay (CSA) for MT. Cd and MT levels were elevated in a dose-response manner for all tissues and blood components except in the PMNs. The sequence of control (basal) blood MT concentrations was: plasma > RBC > M > Ly > PMN, compared to plasma > *M ≥ *Ly > RBC > PMN for the 1.25 mg/kg (medium) exposure group at T=12h. Similarly, the sequence of basal blood Cd concentrations was: whole blood > plasma > RBC ≥ Ly ≥ M ≥ PMN, compared to *whole blood > *RBC > > plasma > *M ≥ *Ly ≥ PMN for the medium exposure group at T = 12h. Significant differences (*$p<0.05$) were evident in longitudinal comparisons (concentration versus time) and between doses in both experimental protocols.

The evidence presented in this thesis clearly establishes Cd levels in RBCs and whole blood as reliable short- and long-term indices of exposure and possibly of body burden. MT levels in plasma and RBCs constitute similar markers. Further, Cd in lymphocytes shows promise as a long-term indicator and in monocytes as a short-term index of exposure. Post mortem examination of the subchronic organs did not support the contention of Cd-induced MT protection, but rather that of Cd accumulation and dose-dependent toxicity.

The *in vivo* jugular cannulation approach developed has both advantages and disadvantages. Body fluid samples can be collected over a 96-h time period from the same animal, thereby reducing inter-animal variation. The dynamics of Cd uptake and distribution and MT induction can thus be assessed in a single animal and be linked to the corresponding tissue results. The main disadvantage is that replicate sampling at a specific time point is limited by the volume of blood that can be drawn. However, this limitation is partially compensated by the ability to collect many samples over an extensive time period permitting longitudinal statistical comparisons with fewer animals. Another limitation is that the sample collection period is short compared to the time involved in chronic exposures.

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LIST OF ABBREVIATIONS

AA	Atomic Absorption
Ab°1	Primary antibody
Ab°2	Secondary antibody
A/C	Slope, absorbance (A) / concentration (C)
AF90	High dose, short course experimental animals A - F
Ag	Antigen
ANOVA	Analysis of Variance
BBS	Borate buffered saline
BSA	Bovine serum albumin
bwt	Body weight
CA	Number of cells analyzed
Ca	Calcium
CAB	Chromotrope Aniline Blue stain
CC	Coulter Counter cell number
Cd	Cadmium
^{109}Cd	Radioisotope of cadmium
$CdCl_2$	Cadmium chloride
CD90	High dose, subchronic experimental animals C - D
CDF	Cell dilution factor
CdMT	Cadmium metallothionein
C_L	Detection limit, concentration
cpm	Counts per minute
cpm_b	Counts per minute in the blank solution
cpm_s	Counts per minute in the test sample solution

cpm_t	Total counts per minute in the 1/10 dilution of the labelling solution used in the CSA
CSA	Cadmium saturation assay
Cu	Copper
CV	Coefficient of variation
D	Dalton(s)
DDW	Distilled deionized water, ultra - pure
DF	Dilution factor
dwt	Dry weight
ε	Molar extinction coefficient
EAAS	Electrothermal atomic absorption spectrometry
ECH	Environmental Health Criteria
EDTA	Etheylenediaminetetra-acetic acid (disodium salt)
FAO	Food Additive Organization
FCA	Freunds complete adjuvant
FIA	Freunds incomplete adjuvant
g	Gram(s)
GARG	Goat anti-rabbit IgG
GI	Gastrointestinal
GO91	Subchronic, low and medium dose experimental animals G - O
h	Hour(s)
Hb	Hemoglobin
Hg	Mercury
H&E	Hematoxylin and Eosin stain
HMW	High molecular weight
^{125}I	Radioisotope of iodine
IgG	Immunoglobulin G
IM	Intramuscular
IP	Intraperitoneal

IV	Intravenous
kD	Kilodaltons, apparent molecular weight
kg	Kilogram(s)
L	Liter(s)
LAPA	Leukocyte alkaline phosphatase activity
LD_{50}	Lethal dose in 50% of test animals
LGL	Large granular lymphocytes
LMW	Low molecular weight
L.S.	^{109}Cd labelling solution using in CSA
Ly	Lymphocytes
M	Monocytes
M	Molar
Mg	Magnesium
mg	Milligram(s)
mL	Milliliter(s)
MT 1	Metallothionein isoform 1
MT 2	Metallothionein isoform 2
N_2	Nitrogen
Ni	Nickel
NOAEL	No observable adverse effect level
NSHPP	N-hydroxysuccinimide-3-(4-hydroxyphenyl) propionate
PA91	Short course, low and medium dose experimental animals P-A
PAS	Periodic Acid Schiff's stain
PBS	Phosphate buffered saline
PCV	Packed cell volume
PE	Polyethylene
PMN	Polymorphonuclear leukocytes
ppb	Parts per billion
PPB	Perl's Prussian Blue stain

ppm	Parts per million
ppt	Precipitate
RACG	Rabbit anti-chicken IgG
RBC	Red blood cells, erythrocytes
RIA	Radioimmunoassay
RPMI	Roswell Park Memorial Institute culture medium
SC	Subcutaneous
SD	Standard deviation
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
TEMED	N,N,N',N'-tetramethylethylene diamine
TBS	Tris buffered saline
TWI	Tolerable weekly intake
ug	Microgram(s)
uL	Microliter(s)
uMol	Micromole(s)
UV	Ultra Violet
v	Volume
Vo	Void volume
Ve	Eluted volume
WB	Whole blood
WBC	White blood cells
WCV	Wet weight conversion factor
WHO	World Health Organization
wwt	Wet weight
x	arithmetic mean
Y_L	Absorbance limit
Zn	Zinc

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CHAPTER 1:

1. INTRODUCTION

1.1. Thesis Overview

This thesis provides the details of a study into the dynamics of cadmium (Cd) and metallothionein (MT) accumulation in male Sprague-Dawley rats. Chapter 1 summarizes the background of the occurrence, physical properties, health effects and biological importance of Cd and MT. Chapter 2 describes the experimental protocols (surgical, blood cell isolation, and injection regimes) used in the *in vivo* animal studies. Chapter 3 outlines the experimental methods, results and discussion for the determination of Cd in tissues, blood cells and body fluids, while Chapter 4 contains the corresponding information for the determination of MT in tissues, blood cells and body fluids. Chapter 5 describes the health status of the animals including (body and organ weights, differential white blood cell (WBC) counts, total WBC and erythrocyte (RBC) cell counts, leukocyte alkaline phosphatase activity (LAPA), packed cell volume (PCV)), and representative histomorphology of selected organs from representative animals. Chapter 6 features an integrated discussion of all the findings.

1.2. Chemical, Biological and Physical Properties of Cadmium

Cd is located in the Group IIB series of the Periodic Table and exists naturally as a divalent cation (Cd^{2+}). Cd has similar chemical properties to other Group II elements namely: calcium (Ca), and magnesium (Mg), which belong to Group IIA; zinc (Zn) and mercury (Hg) which are also members of Group IIB. Common chemical forms of Cd encountered occupationally include the chloride and sulphate salts, oxides, elemental dust and metal fumes. Solubility varies with the chemical form. Cd is soluble in acids, while the salts are generally

water soluble. The melting point for Cd is 320.9°C and boiling point is 765°C. Cd is a soft, silver white ductile metal with an atomic weight of 112.4, an atomic number of 48 and a specific gravity of 8.6 (Aylett, 1979; Sittig, 1985; Friberg et al., 1986a). In biological systems, Cd may exist as the free ion (Cd^{2+}), or is bound to an endogenous metal-binding protein, namely metallothionein (MT) (Kagi and Vallee, 1960). Cd^{2+} has a relatively long biological half life ($t_{1/2}$) of 10 - 30 years in humans (approximately 15 - 40% of the normal life span), and 200 - 700 days in the mouse or rat (approximately 20% of the life span) (Nordberg et al., 1985; Kjellstrom and Nordberg, 1985). By comparison, the CdMT complex has a relatively short *in vivo* turnover period of 1 to 3 days (Friberg et al., 1974; Neathery and Miller, 1975; Kotsonis and Klaassen, 1981; Friberg et al., 1986a). In addition, Cd interacts biologically with other divalent metal cations at the cellular level potentially altering absorption and metabolism of nutritionally important essential ions such as Ca^{2+}, Mg^{2+}, and Zn^{2+} (Friberg et al., 1974; Fox, 1976; Stonard and Webb, 1976; Underwood, 1977; Neathery, 1981; Friberg et al., 1986a).

1.3. Production and Uses of Cadmium

Mineralogically, Zn and Cd occur together; pure Cd ore deposits are nonexistent. Cd is considered a by-product of lead, copper and Zn mining, smelting and refining operations (Blejer, 1971; Neathery, 1981; Sittig, 1985; Friberg et al., 1986a). In 1980 alone, industrial world consumption of Cd reached 12,000 tons (Stubbs, 1982) and of that, little was recycled. Cd is used in a wide variety of industrial and commercial processes (see Table 1.1; adapted from Blejer, 1971) for which there are less toxic alternatives available (Friberg et al., 1986a). Iron products electroplated with Cd are more resistant to rust than the Zn-plated (galvanized) materials. Compared to the production of nickel-Cd (Ni-Cd) batteries, electroplating constitutes a minor use of Cd. Cd sulfate ($CdSO_4$), Cd iodide (CdI_2), Cd acetate ($Cd(C_2H_3O_2)_2$) and Cd cyanide

($Cd(CN)_2$) are some of the forms used in electroplating processes (Merck, 1976; Friberg et al., 1986a). The addition of Cd to metals yields Cd alloys, such as copper-Cd alloys, which improves stability and heat resistance, while Cd oxide (CdO) is used in the manufacture of silver alloys (Merck, 1976). Cd stearates are employed as stabilizers in plastics. Many of these Cd-dependent processes are utilized in the automobile industry.

Some segments of the general population may experience exposure to several forms of Cd such as $Cd(C_2H_3O_2)_2$), Cd chloride ($CdCl_2$), CdO, Cd sulfide (CdS) and Cd sulfoselenide (CdSSe) which are used as pigments in ceramic glazes, plastics and paints. Several forms of Cd such as Cd bromide ($CdBr_2$), $CdCl_2$, and Cd nitrate ($Cd(NO_3)_2$ are used in photographic emulsions; $CdBr_2$ and CdI_2 are used in lithography. X-ray screens contain Cd Tungstate(VI) (CdO_4W), and fluorescent screens contain CdS (Merck, 1976). Cd is also used in welding electrodes, silver solder, and Ni-Cd alkaline batteries (see Table 1.1) (Blejer, 1971; Merck, 1976; Friberg et al., 1986a; Kelecom, 1989). Cd has also been used therapeutically: as an antiseptic (Cd salicylate, $Cd(C_7H_5O_3)_2$), anthelmintic (CdO), fungicide (Cd succinate, $CdC_4H_4O_4$), and in dermatologic treatment CdS (Merck, 1976).

1.4. Health Effects of Cadmium

1.4.1. Acute Exposure

1.4.1.1 *Inhalation.*

Inhalation is considered the major route of exposure in the occupational setting (Friberg et al., 1974; Friberg et al., 1986a). Cd is used in a wide variety of occupations from traditional metal workers to artists (see Table 1.1., adapted from Blejer, 1971; NIOSH, 1986). Although many workers are not directly employed to work with Cd, they may be exposed to Cd dust or fumes. Cd fumes and vapors are more toxic than Cd dust, while CdO fumes constitutes a major

toxic form. Approximately 15-50 percent of the inhaled Cd dose is absorbed depending on the chemical form, with fumes and small particles being absorbed more readily than large particles (Hietanen, 1981; Friberg et al., 1986a; Goyer, 1991). Since Cd bioaccumulates, any exposure is considered significant. In acute Cd exposure, there is usually a delayed onset of 4 - 10 hours during which the individual may be asymptomatic. Depending on the exposure level, symptoms may resemble an acute infection of the upper respiratory system or a common cold, and are similar to 'metal fume fever' caused by zinc fumes which is considered reversible (Friberg et al., 1986a). The symptoms of acute Cd toxicity include all or some combination of the following: mild to severe bronchial and pulmonary irritation, pulmonary edema, sneezing, cough, chest pains, headache, dizziness, chills, diarrhea, nausea, vomiting, and exhaustion (Paterson, 1947; Tsuchiya, 1981; Elinder, 1985a,b; Friberg et al., 1986a). In some cases the symptoms may persist from days to years with development of pulmonary fibrosis (personal communication with Dr. M.P. Waalkes), if not fatal.

1.4.1.2 *Ingestion*

Acute Cd toxicity by ingestion of contaminated food or water has been documented in the past (Oak Ridge National Laboratory, 1973). Previously, Cd was used to plate some food and beverage containers. In the presence of acidic solutions, Cd was leached out of the containers resulting in varying degrees of Cd ingestion. Subsequently Cd has been banned by most countries for use in food-related products. Cd is found throughout the environment: in the soil, water, atmosphere, grain crops, vegetables, shellfish, food herds, cigarette smoke, and automobile exhaust to name a few and the concentrations have been increasing since the industrial revolution (Friberg et al., 1974; Fassett, 1975; Stoll et al., 1976; EPA, 1981; Elinder et al., 1983; Piscator, 1985; Friberg et al., 1986a; Goyer, 1991). The symptoms of acute oral Cd toxicity include some or all of the following depending on the dose ingested: rapid onset of acute gastroenteritis after ingestion, followed by severe nausea, vomiting and

cramps. Gastrointestinal absorption of Cd is estimated to be less than 10% (Tsuchiya, 1981; Hietanen, 1981), but is reported to increase in subjects with iron deficiency (Flanagan et al., 1978; Friberg et al., 1986a). Animal studies have indicated that diets low in calcium, iron, or protein, considerably increased the gastrointestinal absorption of Cd (Nordberg et al., 1985).

1.4.2. Chronic Exposure

Inhalation is considered the major route of chronic Cd exposure in the occupational setting. Chronic Cd inhalation and ingestion may also occur in the nonoccupational setting as a result of environmental contamination, diet, and cigarette smoke (Friberg et al., 1974; Elinder, 1985a,b; Piscator, 1985; Friberg et al., 1986a; Goyer, 1991). The health effects of chronic Cd exposure have been classified according to the major organs involved. Chronic exposure may go undetected for years until substantial tissue damage has occurred. The major areas of concern include: nephrotoxicity, impairment of the respiratory system, and bone damage (Tsuchiya, 1981; Elinder, 1985a,b; Friberg et al., 1986a; Goyer, 1991; EHC, 1992a; b). Other Cd-related toxic effects are cancer, hepatic function impairment, hepatic necrosis, teratogenicity and reproductive toxicity.

1.4.2.1. *Renal Effects*

The kidney is believed to be the critical target organ in Cd toxicity; it is the organ that exhibits the first signs of deleterious effects after low-dose Cd exposure (Friberg, 1950). The early signs of nephrotoxicity includes urinary excretion of plasma proteins in the molecular weight range 11 to 450 kD (Lauwerys, 1979; Lauwerys, 1983a; Lauwerys et al., 1984; Bernard and Lauwerys, 1984) and calciuria (Fowler et al., 1987a; Jin et al., 1987c). Specific proteins that reflect Cd-induced renal tubular dysfunction include: β_2-microglobulin (β_2-MG), retinol-binding protein (RBP), and metallothionein (MT) (Bernard and Lauwerys, 1982, Roels et al., 1982; Bernard and Lauwerys, 1984; Nogawa et al., 1986; Tohyama et al., 1986; Suzuki and Cherian 1987; Shaikh et

al., 1990; Glaven et al., 1991). Higher MW proteins such as albumin, transferrin, or IgG are diagnostic of glomerular dysfunction, while proteins of lower MW (≤ 40 kD) reflect tubular damage (Lauwerys, 1979; Bernard and Lauwerys, 1984; Cardenas et al., 1991). In animal studies, repetitive intraperitoneal (IP) Cd injections caused mixed or tubular type proteinuria, while prolonged oral Cd administration in rats resulted primarily in glomerular type proteinuria (Bernard et al., 1981; Lauwerys et al., 1984). These animal studies also indicated that tubular proteinuria was reversible on cessation of the IP injections of Cd, while the glomerular damage due to oral administration of Cd was not (Bernard et al., 1981; Lauwerys et al., 1984). Some concern was expressed in the oral study over the development of chronic progressive nephrosis which is associated with glomerular proteinuria (Gray, 1977; Bernard et al., 1981; Lauwerys et al., 1984). In human biological monitoring studies of Cd-induced renal dysfunction, calciuria and related effects have been well documented (Lauwerys, 1979; Buchet et al., 1980; Nogawa, 1981; Tsuchiya, 1981; Cherry, 1981; Jarup et al., 1983; Lauwerys, 1983a; Lauwerys, 1983b; Lauwerys et al., 1984; Bernard and Lauwerys, 1984; Nogawa, 1984; Piscator, 1985; Tohyama et al., 1986; Chung et al., 1986; Weening et al., 1987; Nieboer et al., 1987; Verschoor et al., 1987; Nieboer et al., 1988a; Pocock et al., 1988; Kido et al., 1988; Shaikh et al., 1990). Renal dysfunction in humans does not appear to be reversible and chronic nephropathy occurs after the critical level (200 ug Cd / g tissue) has been exceeded (Roels et al., 1979; Roels et al., 1981; Roels et al., 1982; Lauwerys, 1983a; Roels et al., 1983a; Lauwerys et al., 1984; Lauwerys and Malcolm, 1985; Goyer, 1985; Goyer, 1991). The correlation of Cd concentration in the kidney with urine levels found approximately 160 ug Cd/g kidney was equal to 10 ug Cd/g creatinine in urine (Lauwerys et al., 1979; Roels et al., 1979; Buchet et al., 1980; Roels et al., 1981; Lauwerys, et al., 1983a). Cd values found in non-occupationally exposed people were ≤ 1 ug Cd/g creatinine in urine (Bernard and Lauwerys, 1984). From these results, it was determined

that urinary Cd levels should not exceed 10 ug Cd/g creatinine in order to prevent renal dysfunction in occupationally exposed people (Lauwerys, 1983a).

The three major mechanisms involved in proteinuria are: (i) increased glomerular permeability, (ii) decreased tubular reabsorption (Lauwerys et al., 1984; Cardenas et al., 1991; Goyer, 1991), and (iii) a general membrane instability induced by Cd disturbance of normal calcium (Ca) metabolism (Jin et al., 1987c; Fowler et al., 1987a). The three major mechanisms involved in calciuria are: (i) impairment of Ca reabsorption by increased Cd concentrations, (ii) ionic interferences or changes in the electrochemical gradient across the luminal membrane as a result of Cd^{2+} ions being released from the CdMT complex, and (iii) replacement of Ca by Cd in bones and interference with Ca metabolism (Fowler et al., 1987a; Jin et al., 1987c).

1.4.2.2. *Respiratory Effects*

The respiratory effects of chronic low-dose exposure to Cd are chronic obstructive pulmonary disease and emphysema. The degree of toxicity is time- and dose-dependent (Friberg, 1950; Tsuchiya, 1981; NIOSH, 1986; Goyer, 1991). Alveolar macrophages are believed to be involved in the pathogenesis of the lung. The turnover of Cd^{2+} and macrophage necrosis results in the release of enzymes producing irreversible damage to the alveolar membranes and interstitial fibrosis occurs (Goyer, 1991). Emphysema has been associated with a genetic deficiency of alpha-1-antitrypsin activity. Inhalation of Cd dust or fumes has been linked with a inhibition of plasma alpha-1-antitrypsin activity (Chowdhury and Louria, 1976). Differentiation of plasma alpha-1-antitrypsin activity between Cd-exposed workers diagnosed with or without emphysema was non-conclusive (Lauwerys et al., 1979). Studies of chronic low-level Cd exposure in humans must take into account the life styles of the subjects. Cigarettes contain 1-2 ug Cd per cigarette, of which approximately 10% is absorbed (Elinder et al., 1983). Smoking a pack per day may increase the daily Cd intake by 2 to 4 ug (Elinder et al., 1983; Calabrese et al., 1985) and total

body burdens are generally double those of non-smokers (Friberg et al., 1974; Bernard and Lauwerys, 1984). Total Cd concentration in cigarettes is region specific depending on the area where the tobacco was grown (Friberg et al., 1974; Elinder et al., 1983; Friberg et al., 1986a). Studies have shown that cigarette smoking can have a profound effect on the outcome of human Cd monitoring studies (Smith et al., 1976; Lauwerys et al., 1979; Nieboer et al., 1987; Nieboer et al., 1988a).

1.4.2.3. *Effects on the Bone*

Cd interferes with calcium metabolism in normal physiological systems (see Section 1.3.2.1.) generating a general membrane instability (Jin et al., 1987c; Fowler et al., 1987a). The ionic interferences or changes in the electrochemical gradient across the luminal membrane occurs as a result of Cd^{2+} ions being released from the CdMT complex. The concurrent loss of calcium and phosphorus in the urine leads to bone pains and bone diseases, namely osteomalacia and osteoporosis (Nomiyama, 1980; Tsuchiya, 1981; Bernard and Lauwerys, 1984; Kjellstrom, 1985a; Klaassen, 1985; Friberg et al., 1986a; Goyer, 1991) as documented in the Itai-Itai syndrome, (Nogawa, 1981). Itai-Itai was first observed in Japan's Jinsu River basin in the Toyama Prefecture after World War II (Kono et al., 1956a-c; Kono et al., 1957a-b; Nogawa, 1981) and in other Cd-contaminated areas (Kjellstrom, 1979; Kjellstrom, 1985b). Gradual replacement of Ca by Cd in bones and metabolic reactions occurs with increasing Cd exposure (Fowler et al., 1987a; Jin et al., 1987c; Blazka and Shaikh, 1991). Cd also interferes with the metabolism of vitamin D (Feldman and Cousins, 1973; Friberg et al., 1986a) as in the case of chickens where Cd inhibits the hydroxylation of vitamin D in the renal tubular cells, reducing the concentration of the active form of vitamin D to the point of deficiency (Feldman and Cousins, 1973). Long-term administration of massive doses of vitamin D (maximum 138.8 million IU's) to Itai-Itai patients was successful in reducing the number of severe cases of the disease, as well as

reducing the associated bone symptoms and pain (Friberg et al., 1974; Nogawa, 1981; Friberg et al.,1986a).

1.4.2.4. *Cancer*

For the last decade, researchers have studied the relationship between Cd exposure and the development of lung and prostate cancers (Potts, 1965; Kjellstrom, 1979; Kjellstrom et al., 1979; Elinder and Kjellstrom, 1985; Elinder, 1985a, b; Thun et al., 1985; Friberg et al., 1986a; IARC, 1987). Respiratory cancers were found in workers from a U.S. Cd - recovery plant (Thun et al., 1985). Increased lung and prostate cancers were found in Swedish workers at a Cd-Ni and Cd-Cu alloy plant (Kjellstrom et al., 1979). Re-evaluation of previous cohort studies of occupationally exposed Cd workers in Sweden, U.S., and the U.K., confirmed the apparent increased incidence of lung and prostate cancers (Elinder and Kjellstrom, 1985; Elinder, 1985a,b; IARC, 1987). However, a definitive conclusion that long-term Cd exposure produced lung cancer in Cd workers was confounded by simultaneous exposures to other potential respiratory carcinogens (cigarette smoke), or other toxic agents (nickel, arsenic, lead) found in the occupational setting (IARC, 1987). The question of whether Cd induces prostate cancer in humans appears unresolved (Bernard and Lauwerys, 1984; Friberg et al., 1986a; IARC, 1987; Goyer, 1991), however IARC has determined that there is sufficient experimental evidence to correlate Cd exposure with other forms of cancer (IARC, 1987; Waalkes et al., 1992). The first significant animal study to determine the dose-response relationship between chronic Cd inhalation exposure and cancer was published by Takenaka et al., (1983). Wistar rats were exposed for 18 months in four separate $CdCl_2$ dose (0.0, 12.5, 25, and 50 ug/m^3 $CdCl_2$ aerosol) groups of 40 rats per group, followed by a 13 month follow-up period. The study showed a Cd dose-response increase in lung carcinomas (Takenaka et al., 1983). $CdCl_2$ as well as other forms of Cd (oxide, sulfide, sulfate, and powders) have been shown to produce local sarcomas after either IM or SC injection (Duval and

Grubb, 1986; Waalkes et al., 1989; Waalkes et al., 1991a; Waalkes et al., 1992). In a 90 week study, a single $CdCl_2$ (30 uMol/kg, SC) injection was shown to reduce the incidence of spontaneous large granular lymphocyte (LGL) leukemia in Fisher rats by week 54, while acute lymphonecrotic effects were observed in the same study at week 35 (Waalkes et al, 1991a). The evidence suggests that Cd cytotoxicity maybe related to the suppression of LGL leukemia (Waalkes et al, 1991a). In addition, Cd produces testicular interstitial cell tumors and prostatic adenomas in rodents (Waalkes et al., 1989; Waalkes et al, 1991a; Waalkes et al., 1992). While zinc, given in excess, had a protective role in the prevention of Cd-induced carcinogenicity (Waalkes et al., 1991b). Cd has been well established as a potent carcinogen in rodents and a potential carcinogen in humans (Waalkes et al., 1992). Recently, IARC has upgraded Cd to a Type 1 (known human) carcinogen.

1.4.2.5. *Miscellaneous Effects*

(i) Cardiovascular Disease, Hypertension and Blood

Animal studies have associated Cd with biochemical and electro-cardiographic changes in the myocardium and impairment of myocardial function (Kopp et al., 1983; Goyer, 1991). Rats receiving diet supplemented with Cu, Cd and selenium had reductions in heart cytosolic enzymes, specifically catalase, glutathione peroxidase and superoxide dismutase (Kopp et al., 1983; Goyer, 1991). Earlier animal studies found prolonged exposure to Cd in drinking water induced hypertension in rats (Kopp et al., 1982). Early human epidemiological studies found higher concentrations of Cd in the renal cortex of patients dying from hypertension than from other diseases (Schroeder, 1965; Nogawa, 1981). More recently, a study linked elevated systolic blood pressure with Cd exposure (Thun et al., 1989). The exact role of Cd in human hypertension remains controversial (Bernard and Lauwerys, 1984; Klaassen, 1985; Friberg et al., 1986a; Goyer, 1991). The suggested whole blood no-observed-adverse-effect-level (NOAEL) for long term Cd exposure level in

occupationally exposed humans is 10 ug Cd / L whole blood (Bernard et al., 1979; Bernard et al., 1980; Buchet et al., 1980; Lauwerys, 1983a). Cd values found in non-occupationally exposed people was $\leq$ 5 ug Cd/ L whole blood (Bernard and Lauwerys, 1984). Blood Cd concentrations represent short-term exposure levels (few months), however, if intermittent Cd exposure continues, then blood Cd levels also appear to be a reasonable indicator of body burden (Lauwerys, 1983a; Shaikh et al., 1990).

(ii) Liver Function

The liver is a major storage site for Cd in humans and animals. It is unclear as to the total effect of Cd on the liver. Several animal studies have documented changes in morphology and enzyme activities as a result of long-term Cd exposure (Friberg, 1950, Eaton et al., 1980; Muller and Stacey, 1988). Cd exposure was documented to decrease levels of hepatic enzymes: cytochrome c oxidase (Muller and Stacey, 1988), heme oxygenase, and cytochrome P-450 (Eaton et al., 1980), while Cd had little or no effect on glutathione levels (Eaton et al., 1980). Glutathione has been postulated as a first line of defense against initial Cd exposure before induction of MT occurs (Singhal et al., 1987). Recent studies have compared the protective roles of glutathione and MT in rats (Houghton and Cherian, 1991; Chan et al., 1992). However, liver damage in humans does not necessarily test positive using standard clinical diagnostic liver function tests (Friberg et al., 1986a). The liver is clearly a site of Cd storage and synthesis of the Cd-sequestering protein, metallothionein (MT). The ability to produce large concentrations of MT after a single exposure to Cd may afford protection from contact toxicity induced by Cd^{2+} ions (Nordberg et al., 1971a; Duval and Grubb, 1986; Friberg et al., 1986a; Goyer, 1991).

(iii) Teratogenicity

The evidence for Cd induced teratogenicity in the occupational setting is debatable with few human studies reported (Friberg et al., 1986a). Women

exposed to Cd in industry in what was previously known as the U.S.S.R. were reported to have low birth weights and several cases of rachitis (rickets) among neonates. However, there were no major malformations observed in the 1970 Cvetkova study (reviewed by Friberg et al., 1986b; Friberg et al., 1986a). Cytotoxicity caused by Cd accumulation in placenta may be responsible for the teratogenic effects seen in animals (Parizek, 1964), and perhaps also in humans (Wier et al., 1990; Goyer et al., 1992). This damage may reduce the Cd sequestering ability of the placenta resulting in increased fetal Cd burden and may also interfere with the transport of essential metals to the fetus (Goyer et al., 1992). Animal studies have demonstrated Cd-induced teratogenic effects (limb malformations, cleft palate and lip) in hamsters and rats after injections of 3 mg Cd/kg and higher doses (Ferm and Carpenter, 1968; Friberg et al., 1975; Ferm and Hanlon, 1983; Friberg et al., 1986a).

1.5. Metallothionein (MT)

1.5.1. Biological and Physical Properties of MT

Metallothionein (MT) is an endogenous protein found in virtually every species with a broad variation in the chemical structure, and has been subdivided into three classes (I, II, III) (Kagi et al., 1984; Fowler et al., 1987b; Kagi and Kojima, 1987). Class I MT refers to all polypeptides related in structure to equine renal MT. Class II represent forms which bear only limited similarity to mammalian MT such as in yeast (Winge et al., 1985) and sea urchin (Nemer et al., 1985) and class III refers to plant MTs which are atypical forms containing gamma-glutamylcysteinyl units (Grill et al., 1985). Other metal-rich proteins with low cysteine content have yet to be classified, for example those that occur in trout (Thomas et al., 1983).

Two major isoforms of class I MT have been characterized and are labelled as MT1 and MT2. The isoforms are further subdivided into subforms and denoted by MT1a, MT1b, MT2a, MT2b; other minor isoforms have been

reported (Kagi and Kojima, 1987). Class I MTs were first isolated and characterized from equine kidney (Margoshes and Vallee, 1957). Class I MTs have low molecular weight (6050 to 7000 D), are cysteine rich (30%), have a high sulfur content (11-13% depending on the source), with few aromatic amino acids. They avidly bind metals (Zn, Cd, Cu, and Hg), with seven atoms of Zn or Cd firmly bound by one molecule of MT (Margoshes and Vallee, 1957; Kagi and Vallee, 1960; Tohyama and Shaikh, 1978; Kotsonis and Klaassen, 1981; Kagi and Kojima, 1987; Mehra and Bremner, 1987). MT characteristically absorbs at 254 nm when bound to metals with virtually no absorbance at 280 nm because it lacks aromatic amino acids. The resulting UV absorbance ratio of 254 / 280 nm is virtually unique for MT (Margoshes and Vallee, 1957; Kagi and Vallee, 1961; Winge et al., 1975; Vander Mallie and Garvey, 1978; Kagi and Kojima, 1987). For further information see Section 4.1.1.

1.5.2. MT Protein Induction

MT is synthesized (induced) in response to drugs, exposure to metal ions, and stress (Oh et al., 1978; Onosaka and Cherian, 1981; Hager and Palmiter, 1981; Hamer, 1986; Lehman-McKeeman and Klaassen, 1987; Brzeznicka et al., 1987; Brady, 1991). The induction process is initiated by cellular uptake of the inducing agent (eg. Cd) which activates processes that turn on the MT gene. MT-mRNA is then synthesized in the nucleus from DNA. MT-mRNA acts as a cytosolic template for MT protein synthesis (Piscator, 1964; Ohi et al., 1981; Karin and Richards, 1984; Hamer, 1986; Grady et al., 1987; Hanke et al., 1988). MT induction capacities appear to vary between individuals (Harley et al., 1989). The half-life of MT-mRNA is about 10 - 12 hours (Darnell et al., 1986; Monia et al., 1986), while the half-life of CdMT protein is approximately 1 - 5 days and is tissue specific (Shaikh and Lucis, 1972; Kotsonis and Klaassen, 1981; Monia et al., 1986; Tanaka et al., 1987).

1.5.3. MT Studies in Animals and Humans

The effects, distribution and urinary elimination of Cd, MT, and CdMT

have been well documented in animal studies, both in *in vivo* studies (Tanaka et al., 1975; Cherian et al., 1976; Eaton et al., 1980; Templeton and Cherian, 1983; Petering et al., 1984; Sugihira et al., 1986; Duval and Grubb, 1986; Nath et al., 1987; Rehm and Waalkes, 1990; Konishi et al., 1990; Foulkes and Blanck, 1990; Min et al., 1991; Zalups and Cherian, 1992; Zalups et al., 1992), in *in vitro* preparations (Cherian, 1980; Din and Frazier, 1983; Frazier and Din, 1987; Huang et al., 1987; Konishi et al., 1990), and in blood cells (Hilderbrand and Cram 1979; Garty et al., 1981; Tanaka et al., 1985; Garty et al., 1986; Tanaka et al., 1986; Tanaka et al., 1987; Peavy and Fairchild, 1987; Koizumi et al., 1987). Details of these publications are provided in Sections 3.1 and 4.1.

Human studies of MT have been associated with biological monitoring of Cd exposure in occupational and non-occupational settings (Roels et al., 1983b; Lauwerys, 1983b; Bernard and Lauwerys, 1984; Lauwerys et al., 1984; Elinder, 1985a,b; Chung, et al., 1986; Nieboer et al., 1987; Nieboer et al., 1988a; Pocock, et al., 1988; Kido et al 1988; Harley et al., 1989; Shaikh et al., 1990; Saltzman et al., 1990; Glaven et al., 1991). Diet and life style affect the results obtained from human Cd and MT studies, for example: (i) cigarette smoking increases Cd intake and therefore MT induction and (ii) stress has been shown to increase MT production (Piscator, 1985; Friberg et al., 1986a; Nieboer et al., 1988a).

1.5.4. Biological Role of MT

The exact role of MT has yet to be fully elucidated, however MT has been implicated in the metabolism, homeostasis and detoxication of metals (Piscator, 1964; Nordberg et al., 1971a; Nordberg et al., 1971b; Eaton et al., 1980; Kotsonis and Klaassen, 1981; Cherian and Nordberg, 1983; Sarkar and Abdulwajid, 1983; Petering et al., 1984; Petering and Fowler, 1986; Durnam and Palmiter, 1987; Huang et al., 1987; Nordberg and Nordberg, 1987; Webb, 1987). MT has been postulated to afford protection against subsequent exposure to heavy metals (Nordberg et al., 1971a; Jin et al., 1987a; Jin et al.,

1987b; Frazier and Din, 1987; Huang et al., 1987; Nath et al., 1987); by contrast, other animal studies implicate MT as a potent nephrotoxin upon injection (Nordberg et al., 1975; Cherian et al., 1976; Suzuki and Cherian, 1987; Jin et al., 1987c; Fowler et al., 1987a; Elinder et al., 1987; Leffler et al., 1990; Zalups and Cherian, 1992; Zalups et al., 1992; Wang et al., 1993).

1.6. Protocols used in Previous Animal Studies

Traditionally, rodent studies into the toxic effects of Cd have involved sacrificing animals at specific time points after the administration of a toxic dose for pathological assessment. Repetitive collections of body fluids from the same animal are difficult to conduct due to the small volumes available, although, rabbits have been recently studied using cannulation (Foulkes and Blanck, 1990). Repetitive blood and urine collections are possible in studies with larger species, such as in dogs (Matsuno et al., 1991a; Matsuno et al., 1991b; Kodama et al., 1990; Hamada et al., 1991), monkeys (Paliwal et al., 1986), and rabbits (Duval and Grubb, 1986). Several chemical forms of Cd have been investigated (Min et al., 1986): $CdCl_2$ by parenteral (SC or IP) injections- (Bernard et al., 1981; Duval and Grubb, 1986; Konishi et al., 1990; Rehm and Waalkes, 1990), oral $CdCl_2$ administration (Bernard et al., 1981; Petering et al., 1984; Muller et al., 1986; Piotrowski et al., 1987; Scheuhammer 1988; Wicklund et al., 1988; Sorell and Graziano, 1990; ; Kodama et al., 1990; Min et al., 1991; Matsuno et al., 1991a; Matsuno et al., 1991b; Hamada et al., 1991), or CdMT injections (Cherian and Shaikh, 1975; Cherian et al., 1976; Templeton and Cherian, 1983; Suzuki and Cherian, 1987; Fowler et al., 1987a; Jin et al., 1987a-c). *In vitro* cell culture or incubation studies have been conducted with hepatocytes (Din and Frazier, 1983; Muller, 1986; Frazier and Din, 1987), intact mitochondria (Muller et al., 1988), testicular cells (Wahba et al., 1990), Chinese hamster ovary (CHO) cells (Huang et al., 1987) and blood cells (Hilderbrand

and Cram, 1979; Garty et al., 1981; Tanaka et al., 1985; Garty et al., 1986; Amoruso et al., 1987).

1.7. Research Goals

1). To develop improved analytical techniques for the determination of Cd and MT in tissues, blood cells, and body fluids which allow the use of small sample size or volume.

2). To employ a continuous blood sampling technique in rats which involves collection through a surgically inserted cannula.

3). To develop an experimental animal protocol that will allow the construction of a distribution and compartmentalization model comparing the accumulation of Cd and MT in the major organs (liver, kidney, spleen and lung) with that in peripheral blood cells (erythrocytes, lymphocytes, monocytes, polymorphonuclear leukocytes), body fluids (whole blood and plasma) and feces.

4). To evaluate the dependence on time of the differential accumulation of Cd and MT in peripheral blood cells.

5). To examine Cd-induced physiological and cellular changes as measures of animal health.

The rationale of the present research is to gain insight and understanding of the relationships between Cd and MT concentrations in the selected organs (at autopsy), peripheral blood cells, body fluids and feces in rats exposed to $CdCl_2$ by single or multiple injections using a continuous blood sampling protocol to follow changes with time. The specific hypotheses to be tested are: (1) Cd and MT concentrations in peripheral lymphocytes reflect the body burden of Cd (long-term exposure), while those in peripheral monocytes are an indication of short-term (current) exposure; (2) Cd and MT in peripheral blood cells reflect the corresponding levels in liver, kidney, spleen and lung.

Table 1.1 Occupations with Potential Exposure to Cadmium

Alloy makers	Jewelers
Aluminum solder makers	Lithographers
Auto mechanics	Lithopone makers
Battery makers, storage	Metalizers
Bearing makers	Paint makers
Braziers and solderers	Paint sprayers
Cable and trolley wire makers	Pesticide makers
Cd collecting-bag handlers	Pharmaceutical workers
Cd platers and smelters	Photoelectric cell makers
Cd vapour lamp makers	Pigment makers
Ceramics, pottery makers	Plastic product makers
Copper-Cd alloy makers	Sculptors, metal
Dental amalgam makers	Small arms ammunition makers
Electric instrument makers	Smoke bomb makers
Electrical condenser makers	Solder makers
Electroplaters	Textile printers
Engravers	Welders, Cd alloys
Glass makers	Welders, Cd-plated objects
Hobbyists, metal	Zinc mining, smelting and
Incandescent lamp makers	refining workers

CHAPTER 2:

2. EXPERIMENTAL PROTOCOLS FOR REPETITIVE BLOOD SAMPLING IN THE RAT AFTER CADMIUM EXPOSURE.

2.1. Introduction

The dynamics of cadmium (Cd) and metallothionein (MT) in peripheral blood cells, body fluids, and selected organs were examined using a jugular cannulation procedure. It is well documented that Cd exposure results in the induction (synthesis) of the protein MT by many tissues and blood cells in man, animals and plants (Kagi and Nordberg, 1979; Garty et al., 1981; Tanaka et al., 1985; Garty et al., 1986; Fowler, 1986; Kagi and Kojima, 1987; Klaassen and Waalkes, 1987; Koizumi et al 1987; Peavy and Fairchild, 1987; Suzuki, 1987; Harley et al., 1989). The current experimental animal model was developed to study the interdependence in an individual animal of Cd and MT concentrations in peripheral blood cells, body fluids and selected organs (liver, kidney, spleen and lung) over a preselected time period (Figure 2.1). The experiment was designed to collect multiple samples from groups of individual rats (3-5 rats per dosage group) exposed to various amounts of $CdCl_2$ (0.0, 0.25, 1.25 and 2.5 mg $CdCl_2$/kg body weight). These groups of rats were followed for a definite post-exposure time course (96 h); subsequently they were euthanized for assessment of the selected organs. The protocol described differs from the usual experimental design in which a number of animals (3 to 10) are sacrificed at each time point.

The selected experimental design allowed blood, urine and feces collection over a short time-course (-20, 0, 6, 12, 24, 48, 72, and 96 h). Individual baseline values for all biomarkers were determined for each rat during

the post-operative recovery period (at times T = -20 and 0 h), which served as the control values for the 96 h observation period. Blood, urine and feces specimens were collected at time T = 0 h, whereupon the animals were administered either saline or $CdCl_2$ by intraperitoneal (IP) injection. At each time point for the duration of the experiment the rats were weighed then blood, urine, and feces samples were collected. At the end of the experiment, the rats were euthanized with sodium pentobarbital. The selected organs were examined for gross pathological changes and stored for Cd and MT analysis (Figure 2.1).

2.2. Experimental Details

2.2.1. Animal Husbandry

Male Sprague-Dawley rats weighing between 0.250 and 0.350 kg were used for the *in vivo* Cd studies. The rats were housed in the McMaster University Central Animal Facility (CAF) for a 3 - 5 day recovery period prior to experimentation to reduce stress, which has been documented to increase MT levels, during starvation (Bremner and Davies, 1975), exposures to cold environments (4°C) and strenuous exercise (Oh et al., 1978). The recovery period not only allowed the rats to recuperate from shipping stresses, but permitted them to acclimatize to their new surroundings. All animals were housed individually in either plastic rat cages or rack-style metabolism cages. All rats were allowed food (Purina Rodent Chow) and water *ad libitum* throughout the experiment. Body weight measurements were taken and health assessments were made for all rats upon arrival and throughout the experiment.

2.2.2. Cannulation Surgery

2.2.2.1. *Surgical Preparations*

The cannula were gas-sterilized prior to surgery. The complete cannula unit consisted of 3 major sections (Figure 2.2): (i) the internal cannula, corresponding to the actual section of cannula inserted into the external jugular vein; (ii) the external cannula corresponding to the section of cannula that

connects the internal cannula to the sampling tube at the back of the neck; (iii) the sampling tube, consisting of a 15 cm long polyethylene tubing (PE 90) connected to the external cannula at one end (using a cannula connector) and to a 4-way stopcock fitted with two syringes at the other end (Figure 2.2).

The internal cannula section was prepared from silastic tubing cut in 1.5-2 cm lengths. One of the open ends was cut at a 45° bevel, and two 1 mm v-shaped holes were cut into opposite sides of the beveled end. At the junction of the internal and external cannulae, a silastic ring was attached using Silastic Medical Adhesive (silicone type, Dow Corning #890). The external cannula was 2-2.5 cm long and was made of silastic tubing with a silastic ring attached 3-4 mm from the free end; the external cannula was plugged with a modified 23-gauge stainless-steel needle 3 mm long with one end crimped and both ends filed smooth. A cannula connector was constructed from a 23-gauge needle with both ends removed and filed smooth.

2.2.2.2. *Cannulation Surgery*

Prior to surgery, the rats were weighed and anesthetized with sodium pentobarbital (45 mg/kg bwt, IP). The rats were shaved along the dorsal and ventral neck midline and the area was washed with a 10% iodine antiseptic solution. A 5 mm longitudinal incision was made through the skin on the ventral right-hand side of the neck. Care must be exercised as the jugular veins are fairly superficial. Gentle teasing of the fatty tissue and muscle layer using curved hemostats and blunt-nosed scissors exposed the external jugular vein. The vessel was isolated by standard surgical procedures (Waynforth, 1980), permitting extended time sampling and a metal free environment (Ortner et al., 1981; Galvin et al., 1982; London et al., 1985). The isolated vessel was held in position by inserting a metal spatula beneath the vessel and above the underlying muscle tissue. The spatula supported the vessel during cannulation. Throughout the cannulation procedure, the vessel and surrounding tissue was kept moist with normal saline to prevent drying, tissue damage and internal

bleeding. The anterior section of the jugular vessel was tied off using 4(o)-surgical silk. The posterior section was temporarily ligated with a small vessel clamp. Near to the anterior ligation, a small 45° degree incision was made approximately two thirds of the way through the vessel using curved micro-scissors. The silastic cannula was inserted into the collapsed vein with the aid of micro-tweezers. Once the cannula was in the vein to a depth of 2 to 3 mm, the posterior vessel clamp was removed and the cannula was gently inserted up to the silastic ring (to a depth of approximately 1.5 - 2 cm). The posterior section of the vessel containing the cannula was gently tied off using surgical silk and the silastic ring of the cannula was sutured to the surrounding muscle tissue (Figure 2.3). The external section of the cannula was inserted between the acromiotrapezius neck muscles and the skin exiting through a small 1-2 mm incision at the dorsal midline of the neck between the ears. The external portion of the cannula was secured in place by suturing the external silastic ring to the surrounding tissue and closing the dorsal incision around the cannula. A cannula plug was inserted 2 mm into the end of the external cannula to close off the port when not in use. The sampling tube was connected to the external section using a modified 23-gauge needle and connected to the other end of the sampling tube was a 4-way stopcock with two syringes (Figure 2.2). One syringe contained 2 mL heparinized saline and the other a heparinized sample-collection syringe. At each blood sampling time point, the sampling tube and syringe unit were attached to the external cannula by removal of the cannula plug and inserting the modified needle. The 0.5 mL blood sample was collected and the same volume of heparinized saline (100 units/mL) was injected. This also served to flush the internal and external sections of the cannula with heparinized saline to prevent blood clots. The rats did not appear agitated by the 2 to 3 mm of exposed external cannula and plug at the back of the neck. After surgery all rats received a penicillin injection to prevent infection. All rats were observed for 20 hours after surgery, labelled as the post-operative

recovery period. In the event of a blood clot in the cannula, or when extensive handling of the animals was necessary, the rats were anesthetized for blood collection. If the clot did not dissolve, then the remaining samples were collected from the anesthetized rats by either cardiac puncture or tail vein bleeding. Rats were allowed to fully recover from the anesthetic prior to the next sample collection.

2.2.3. Administration of Dose, Injection Schedule and Animal Codes

2.2.3.1. *Time Course and Administration of Dose*

Following the acclimatization period, initial health assessment (see Section 2.2.5 and Figure 2.1), and jugular cannulation surgery, each rat was allowed a 20 h post-operative recovery period where blood, urine, and fecal samples were collected just after cannulation (T = -20 h) and again at (T = 0 h). These data points represent the individual baseline values prior to either saline or $CdCl_2$ injection. Immediately after T= 0 h, the rats were exposed to either saline or $CdCl_2$ by IP injection. The remainder of the experiment constitutes the post-exposure sampling period labelled as T = 6 through 96 h.

The animal experiments were conducted in two ways: *short course*, and *subchronic*. In the short course protocol, animals received a single IP injection immediately at T = 0 h; samples were subsequently collected for the 96 h post-exposure period. In the subchronic time course, the rats received multiple IP injections (one every 2.5-3 weeks) for a 1.5 - 2 month period prior to surgery, followed by a final IP injection at T = 0 h. Again blood, urine, and fecal samples were collected during the 96 h post-exposure period. Several $CdCl_2$ concentrations were investigated (0.00, 0.25, 1.25, and 2.5 mg $CdCl_2$/kg bwt), with 3 rats per dosage group and conducted as a double blind study. The injection solutions for each experiment were colour coded and sealed in envelopes by Glenn Fletcher, a non-participating research associate in Dr. Nieboer's laboratory. The codes were kept sealed until after the Cd and MT

analyses.

2.2.3.2. *Injection Schedule and Animal Codes*

Experiments AF90 and PA91 were short course $CdCl_2$ exposure studies in which all rats received a single IP injection after the T = 0 h samples were collected. Decoding revealed the following dosage information: rats B, E, T, U, Z, AA (0.00 mg $CdCl_2$/kg body wt); rats V, W, X, Y, ZZ (0.25 mg/kg); rats P, Q, R, S, YY (1.25 mg/kg); and rats A, F, D (2.5 mg/kg); see Table 2.1.

In the subchronic exposure experiments CD90, and GO91, each rat received a total of three IP injections with a 2.5-3 week interval between injections over a 1.5 - 2.0 month induction period. The final (third) injection was administered at T = 0 h of the 96 h post-exposure sampling time course. Decoding revealed the following dosage information (dose of each injection and cumulative total in parentheses): rats G, H, I (0.00 mg/kg = 0.00 mg/kg total); rats J, K, L (0.25 mg/kg each = 0.75 mg/kg total); and rats M, N, O (1.25 mg/kg = 3.75 mg/kg total); rat C (2.5 mg/kg = 7.5 mg/kg total); and rat D was a saline control with a single 2.5 mg/kg IP injection at time T= 0 h (Table 2.2).

2.2.4. Specimen Collection

2.2.4.1. *Blood*

For each whole blood (WB) sample collected, 5 - 6 slides were prepared in the dark for assessment of Cd related changes in leukocyte alkaline phosphatase activity (LAPA) and white blood cell (WBC) differential counts (see Sections 5.4 and 5.5). At each time pcint, 0.4 mL of the total 0.5 mL WB was separated into plasma (P) and blood cell pellet (see Figure 2.4 and Section 2.3). The volume of plasma removed from the blood cell pellet was recorded for subsequent pellet rehydration. Lymphocytes (Ly), monocytes (M), polymorphonuclear leukocytes (PMN), and erythrocytes (RBC), were isolated from the rehydrated blood cell pellet using a double Percoll density gradient (Figure 2.4 and Section 2.3). Aliquots of WB, plasma, and blood cell isolates were set aside for biochemical, Cd, and MT analyses. Total RBC and WBC

counts were determined using a Coulter Counter (Model #3805) for each time point in Experiments GO91 and PA91. Hematocrit measurements were monitored to detect changes in packed cell volume (PCV).

The miniaturization of Cd and MT determination procedures (Figure 2.4; Sections 3.2.3 and 4.2.6) for blood products (RBC, WBC, and plasma) and the revised cell isolation procedures (Section 2.3.1) allowed the blood volume required to be limited to 0.5 mL per time point (Appendix 1). This was important in the prevention of anemia during repetitive sampling. In spite of the small sample size, it was impossible to collect duplicate or triplicate blood samples.

2.2.4.2. *Tissue, Feces, and Urine*

As already indicated in Section 2.1, the rats were euthanized with sodium pentobarbital at the termination of the experiment for organ assessment and tissue collection. Representative rats from each dosage group were photographed and histological tissue samples collected from liver, kidney, lung, and spleen (see Section 2.4). The collected organs were rinsed in saline, lightly dried with kimwipes, and organ wet weights (wwt) recorded. These organs were immediately frozen in liquid nitrogen and lyophilized overnight. The organ dry weights (dwt) were recorded and precise tissue water weights were determined for all rats except those from which histological samples were obtained. Lyophilized organs were ground to a fine powder, stored individually in acid washed snap-top containers (25 to 50 mL size), and stored desiccated at -20^{0}C until analyzed.

Fresh fecal samples were collected and weighed at each time point and stored at -20°C until analyzed. Total urine volumes were also recorded at each time point for animals housed in metabolism cages. All observations and data collected were entered into computer spreadsheets for record keeping and analysis.

2.2.5. Animal Health Assessment

The rats were inspected upon arrival at the McMaster University CAF.

Generally, at this stage the rats appeared dehydrated from shipping. Body-weight measurements and visual inspection of the rats were performed daily. During the sample collection period of the experiment, packed cell volumes (PCV) were monitored as indices of the state of hydration, blood cell volume, and anemia (see Section 5.3.3). A dehydrated rat shows excessively high PCV numbers, while internal bleeding or anemia produce low PCV numbers. PCVs near 46% were considered ideal for normal rats (Baker et al., 1979). Body weights were monitored throughout the experiment as an index of health, physiological stress and cadmium toxicity. Body and organ weights are described in detail in Section 5.3.

2.3. Isolation of Blood Cells

2.3.1. Separation

After blood samples were collected, plasma was separated from blood cell pellets by centrifugation and stored at -20°C for later analysis (Figure 2.4). The blood cell pellets were rehydrated to their original whole blood volume with phosphate buffered saline (PBS) to correct for plasma removal and the cells separated by centrifugation over a Percoll gradient (Boyum, 1968; Pertoft et al., 1980; Davies and Lloyd, 1989). In Experiment AF90, cell isolations were performed using a single 60% Percoll gradient for lymphocytes and monocytes. Cell isolations for Experiments GO91 and PA91 were modified to incorporate PMN isolation using a double layer (58%/68%) Percoll gradient. In brief, first the Ly/M ring, followed by the PMN ring were separated by aspiration from the RBC pellet into separate microcentrifuge tubes. Each cell type was washed, resuspended in 1 mL of RPMI cell media (Ly/M) or PBS (PMN) and total cell counts were manually assessed using a hemocytometer; Wright's stain was used for cellular identification. PMNs were stored in 1 mL PBS at -20°C until analyzed. The Ly/M cell preparations were incubated in RPMI complete cell media at 37°C in 1.5 mL microcentrifuge tubes for a minimum of 1 h. Monocytes

were separated from lymphocytes by virtue of their ability to stick to plastic surfaces (Pennline, 1981). The monocytes were stored in 0.5 mL of 30 mM Tris HCl (pH 8.6) at -20°C until analyzed. The lymphocytes were washed and rehydrated with 1 mL PBS and the cells were recounted manually as above. The lymphocytes were centrifuged and resuspended in 0.5 mL of 30 mM Tris HCl (pH 8.6) and stored at -20°C until analyzed (Figure 2.4).

The erythrocyte (RBC) pellet which remained from the Percoll separation was washed and rehydrated to the original whole blood volume with PBS to correct for plasma removal. The rehydrated RBC solution was lysed by the addition of 0.7 mL of 30 mM Tris HCl (pH 8.6), frozen in liquid N_2 and thawed in an ultra-sonic bath. The freeze/thaw sonication steps were performed twice and RBC lysates were stored at -20°C until analyzed (Figure 2.4). The lysates were then treated according to the requirements of either electrothermal atomic absorption spectrometry (EAAS) for Cd concentrations or cadmium saturation assay (CSA) for MT determination (see Sections 3.2 and 4.2.6).

2.3.2. Cell Identification

The isolated cell preparations were checked for cell types using Wright's stain (Sandoz, 1973; Reith and Ross, 1977; McDonald et al., 1978; Hall and Malia, 1984). The stained cell preparations exhibit similar staining patterns to that observed in fixed slide differential cell counts. The individual cell isolate solutions were mixed with Wright's stain and observed with a Leitz microscope under 400x magnification. Lymphocytes and monocytes stain similarly with varying shades of blue cytoplasm with dark purple-black nuclear material. The size, nuclear shape, and cytoplasm to nucleus ratio aided in the distinction between lymphocyte and monocyte cells (Sandoz,1973; McDonald et al., 1978; Hall and Malia, 1984). The nuclear remains of megakaryocytes (platelet precursor cells that have ruptured and dispersed their platelets) stain similarly to small lymphocytes. WBC differential cell slides were stained with Wrights stain and 100 consecutive WBC were counted using a manual (5 cell type) counter,

100x objective and oil immersion. The data were expressed as percent of 100 cells. For differential identification, the surrounding cells must also be examined for evidence of recent platelet dispersal to help differentiate small lymphocytes from the megakaryocyte nucleus (Sandoz, 1973; McDonald et al., 1978; Hall and Malia, 1984). The PMN's are broken down into neutrophils (neutral staining cytoplasm, lobular nucleus and granules), basophils (basic blue black staining cytoplasm and granules) and eosinophils (acidic red staining cytoplasm and granules) (Sandoz, 1973; McDonald et al., 1978; Hall and Malia, 1984). The cell identification, viability and manual cell counts were performed simultaneously on a hemocytometer slide.

2.3.3. Cell Viability

The individual cell isolates were assessed for cell viability using Wright's stain and a hemocytometer. Preparations containing live cells exhibited intact cell membranes and were motile. Increased cellular debris, free granules, and non-motile cells indicated a loss of cell viability. Based on these criteria, all of the isolated cells were judged to be viable at the time of cell identification and counting, prior to the addition of 30 mM Tris HCl for cell lysis (Harley et al., 1989).

2.4. Tissue Histology

Prior to tissue collection, the internal organs of representative rats from each dosage group (0.0, 0.25, and 1.25 mg/kg) were photographed to document gross pathological abnormalities and to assess health status. Representative liver (transverse section of the left lobe), kidney (right transverse section; left longitudinal section), lung (not inflated), and spleen (longitudinal section) specimens from each dosage group (0.00, 0.25 and 1.25 mg/kg) were collected and fixed in a buffered 10% formalin mixture. All histological tissue slide preparations were performed by the Clinical Pathology Department staff at McMaster University Medical Center. In brief, the tissues were fixed, embedded

in paraffin, sliced, mounted, and stained with hematoxylin and eosin (H&E), periodic acid Schiff's (PAS), Perl's Prussian Blue (PPB), Trichrome, and Chromotrope Aniline Blue (CAB), for later histological examination (Reith and Ross, 1977; Copenhaver et al., 1978; Bivin et al., 1979). Representative histological slides from each group were photographed using a Leitz microscope with a 35 mm camera attachment (See Chapter 5). Additional examination of the slides was performed by Dr. D. deSa, Pathology Professor, McMaster University Medical Center.

2.5. Concluding Remarks

The cannulation technique described in Section 2.2 allowed specimens to be collected from a number of individual animals per dosage group over a 96-h time course. This approach significantly reduced the total animal requirement and permitted a continuous 'same animal' experimental design. The volume of blood collected was adequate to allow all of the analyses to be performed, although obtaining time-specific replicates was not possible. The exact volume of WB necessary for blood cell isolations in order to yield sufficient cell lysate volumes for all analyses were determined in earlier pilot studies (Appendix 1).

Implantation of the cannula unit was performed under anesthesia and caused little discomfort to the animals. Removal of the sampling tube and insertion of the cannula plug allowed free movement and did not interfere with normal behavior. The rats appeared to be in good health and free of infection (see Chapter 5). In a few cases (3), the external cannula was accidentally pulled out while grooming. The incidence of clot formation was higher in the Cd-exposed animals. In the earliest high-dose experiment (AF90), clotting resulted in the termination of the time course because of sampling difficulties. To overcome this problem in experiments PA91 and GO91, blood was drawn under anesthesia from a tail vein or by cardiac puncture in approximately 50 % of these animals.

Table 2.1. Short Course Experiments: Injection Schedule and Animal Codes.

Time Hours	Number of Rats per Dose Indicated (a,b) 2.5 mg/kg	1.25 mg/kg	0.25 mg/kg	0.0 mg/kg	TOTAL
-20	A, F, 2	P,Q,R,S,YY 5	V,W,X,Y, ZZ 5	B, E, T,U,Z,AA 6	18
0	A, F, 2	P,Q,R,S,YY 5	V,W,X,Y, ZZ 5	B, E, T,U,Z,AA 6	18
6	A, F, 2	P,Q,R,S,YY 5	V,W,X,Y, ZZ 5	B, E, T,U,Z,AA 6	18
12	A, F, 2	P,Q,R,S,YY 5	V,W,X, ZZ 4	B, E, T,U,AA 5	16
24	A, F, 2	P,Q,R,S,YY 5	V,W,X, ZZ 4	B, E, T,U, AA 5	16
48	-	P,Q,R,S,YY 5	V,W,X, ZZ 4	E, T,U, AA 4	13
72	-	P,Q,R,S,YY 5	V,W,X, ZZ 4	T,U, AA 3	12
96	A, 1	P,Q,R,S,YY 5	V,W,X, ZZ 4	B, T,U, AA 4	14

a. Capital letters represent individual rats.

b. A single IP injection was administered at T = 0 h (doses are indicated). Rats S, ZZ and YY did not receive a complete injection.

Table 2.2. Subchronic Experiments: Injection Schedule and Animal Codes.

Time Hours	Number of Rats per Dose Indicated (a,b) 2.5 mg/kg	1.25 mg/kg	0.25 mg/kg	0.0 mg/kg	TOTAL
-20	C, 1	M, N, O, 3	J, K, L, 3	G, H, I, D, 4	11
0	C, 1	M, N, O, 3	J, K, L, 3	G, H, I, D, 4	11
6	-	M, N, O, 3	J, K, L, 3	G, H, I, 3	9
12	C, -	M, N, O, 3	J, L, 2	G, H, I, 3	8
24	-	M, N, O, 3	J, L, 2	G, H, I, 3	8
48	-	M, N, O, 3	J, L, 2	G, H, I, 3	8
72	-	M, N, O, 3	J, 1	G, H, 2	6
96	-	M, O, 2	J, 1	G, H, 2	6

a. Capital letters represent individual rats.

b. Each rat received a total of three equal IP injections of CdCl2 (doses of each injection as indicated) over a 1.5-2 month period. The third injection was administered at T = 0 h. Cumulative doses: 0.0 mg/kg, 0.75 mg/kg, 3.75 mg/kg, and 7.5 mg/kg, respectively.

Figure 2.1 Experimental Outline

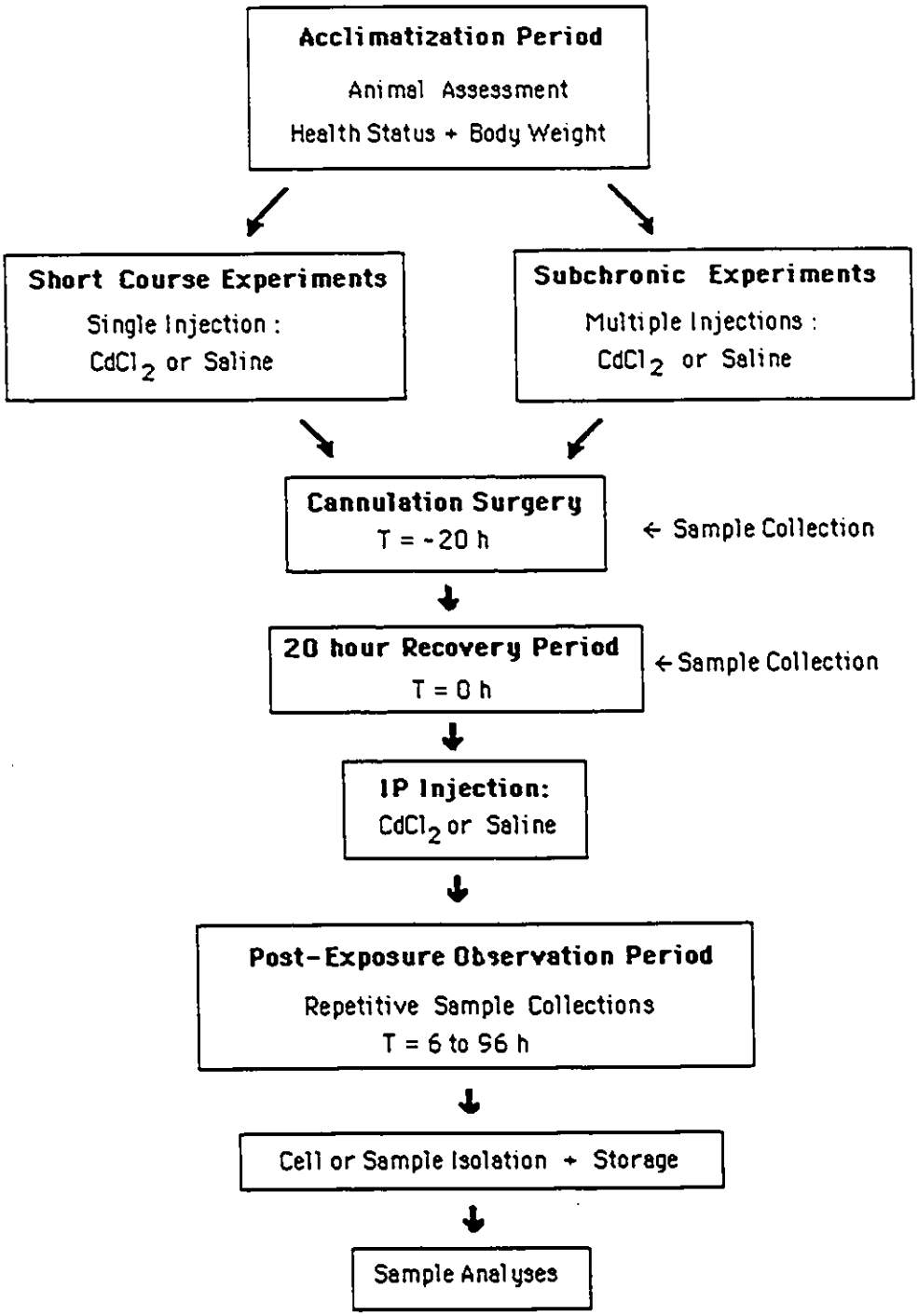
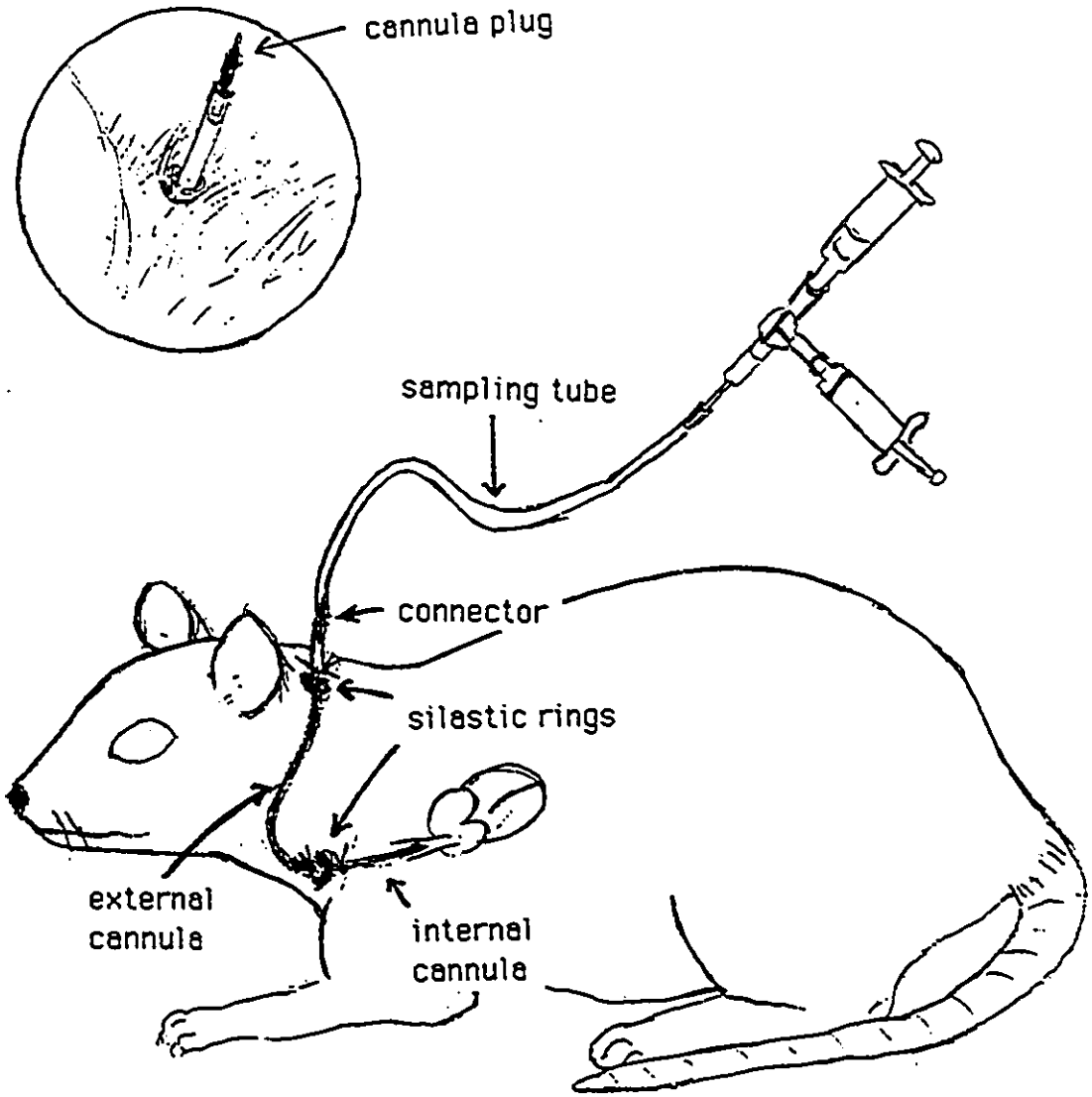


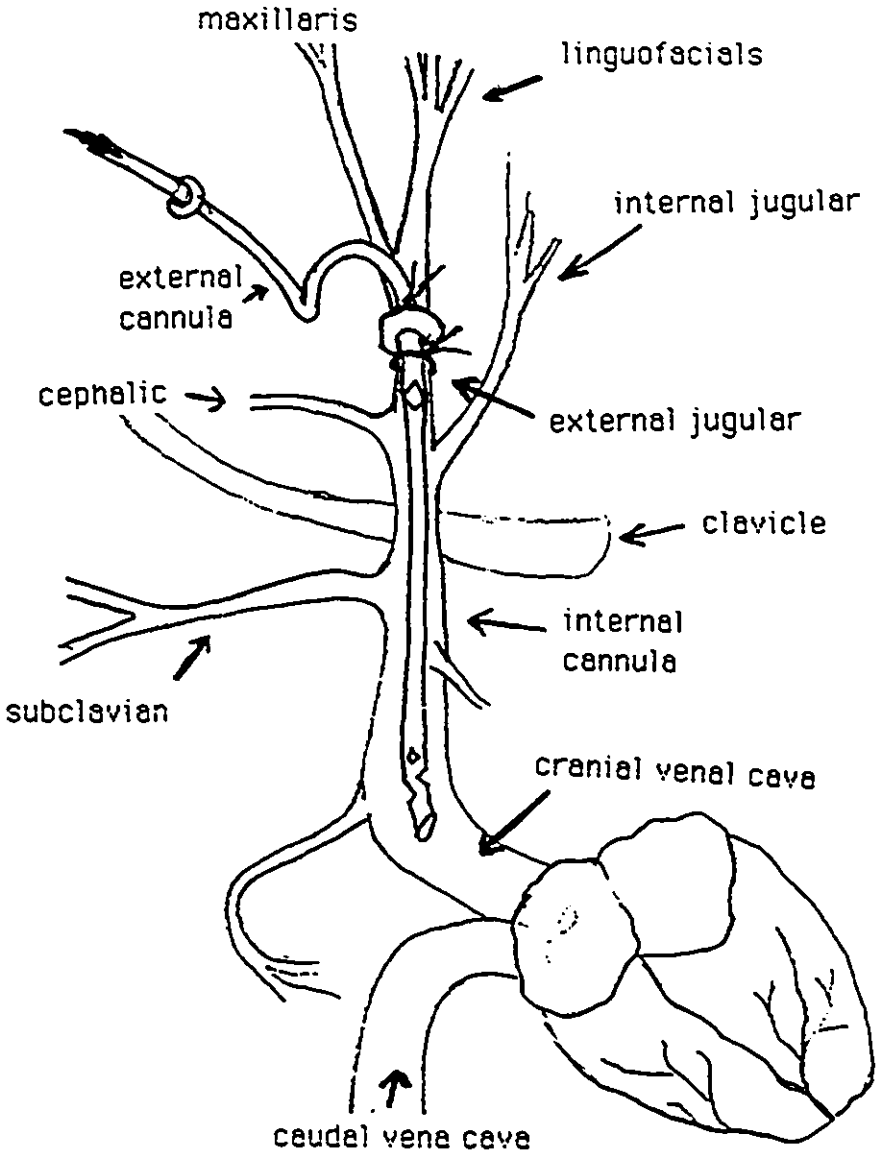
Figure 2.2. The Complete Cannula Unit in Situ.



Legend to figure:

The complete cannula unit consist of three sections: 1. the internal cannula; 2. the external cannula; and 3. the removable sampling tube. The circular insert shows a close-up of the external cannula with the plug in place. This is the normal set-up for the animal between sample collection times. The sample tube is attached to the external cannula with a connector for blood collection. For details see Section 2.2.2. in the text.

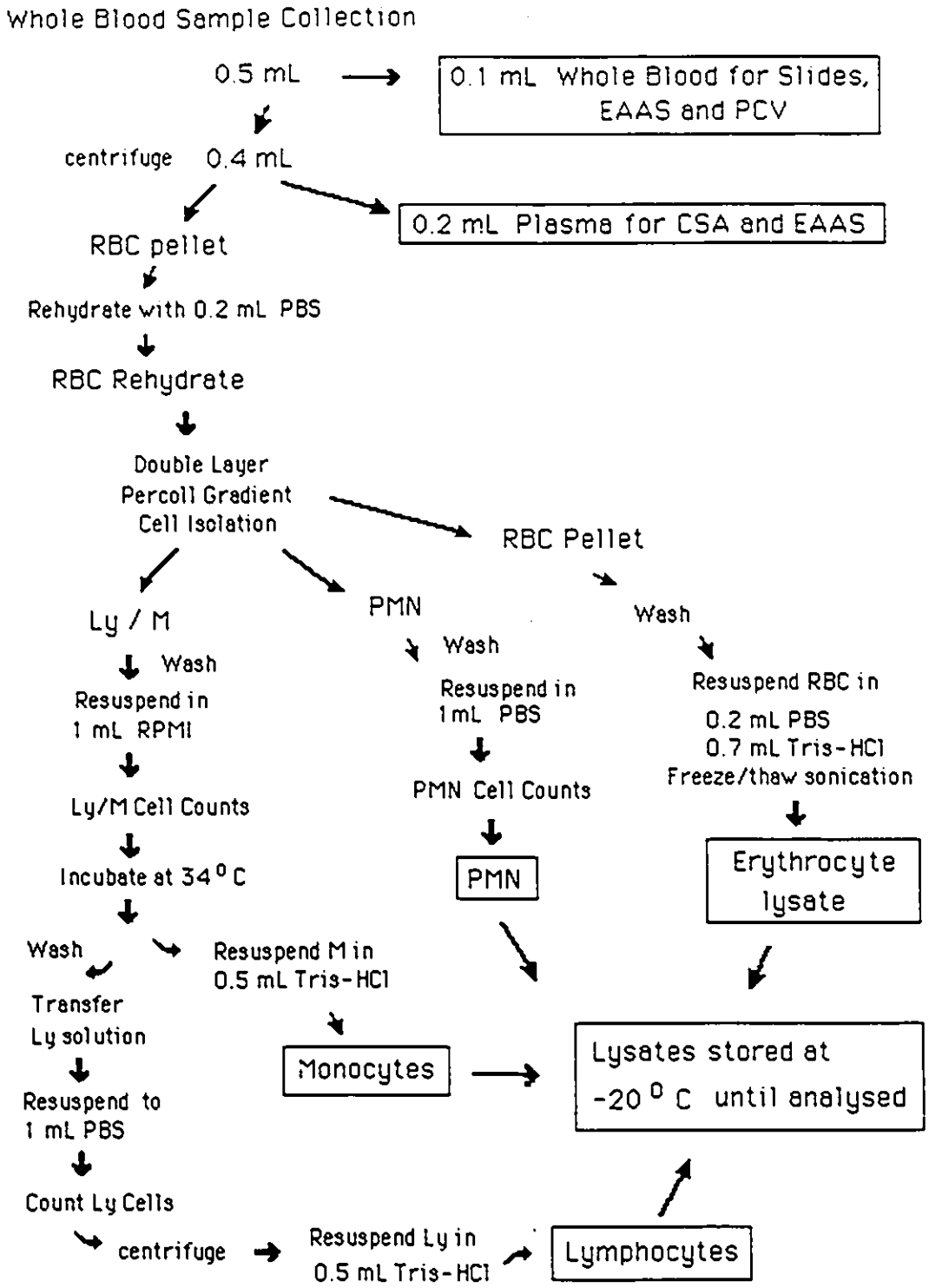
Figure 2.3. The Position of the Cannula in Relation to the Heart and Vessels of the Rat.



Legend to figure:

The complete cannula unit consist of three sections: 1. the internal cannula; 2. the external cannula; and 3. the removable sampling tube. The figure shows a close-up of the internal and external sections of the cannula in relation to the heart and major vessels. For details see Section 2.2.2.

Figure 2.4 Blood Cell Separation



CHAPTER 3:

3. CADMIUM (Cd) DETERMINATION IN SELECTED ORGANS, PERIPHERAL BLOOD CELLS AND BODY FLUIDS.

3.1. Introduction

The physical, chemical, and biological properties of Cd are described in detail in Section 1.1. Information regarding production and uses of Cd are discussed in Section 1.2., and the health effects of Cd are discussed in Section 1.3. Experimental design, general cell isolation and tissue preparations are described in detail in Chapter 2.

3.1.1. Acute Toxicity in Animals

The inhalation LD_{50} (the dose that produces mortality in 50% of the test animals) ranged between 500 to 15,000 mg/m^3min, which varied depending on species. The target organ was the respiratory system and death was caused by pulmonary edema (EHC, 1992a). Approximately 15 - 50% of the inhaled Cd dose is absorbed depending on the chemical form, with fumes and small particles being absorbed more readily than large particles (Hietanen, 1981; Friberg et al., 1986a; Goyer, 1991).

The oral LD_{50} values of Cd compounds vary depending on their water solubility. The oral LD_{50} values of $CdCl_2$ in mice and rats ranged from 75.5 to 111.9 mg/kg body weight (bwt), with the mean of 93.7 (EHC, 1992a). Gastrointestinal absorption of Cd was estimated to be less than 10% (Tsuchiya, 1981; Hietanen, 1981). The major effects of oral administration of Cd compounds include necrosis of the gastrointestinal mucosa, desquamation of the epithelial lining of the gastrointestinal tract, and dystrophy of the heart, and

reduced liver and kidney functions (EHC, 1992a).

The injection LD_{50} values of soluble Cd compounds ranged from 2.5 mg/kg bwt for IV administration to 25 mg/kg bwt for parenteral injection. The LD_{50} values vary with species, route of administration (IV, IP, IM, SC), and solubility (Friberg, 1950; EHC, 1992a). Parenteral injection LD_{50} values are generally 10 to 20 times lower than the oral LD_{50} values for Cd compounds (EHC, 1992a). Death was from liver damage (Dudley et al., 1982), although severe endothelial damage of the small blood vessels in the peripheral nervous system and testes have been observed (Parizek, 1957; Gabbiani, 1966; EHC, 1992a). The major target organ of acute Cd-induced toxicity from a single injection is the liver (Dudley et al., 1982, EHC, 1992a).

The *in vitro* LC_{50} (lethal concentration) values determined for blood cells exposed to Cd compounds varied according to cell type. Mononuclear leukocyte LC_{50} values ranged from 83 to 114 uM Cd, with a mean of 100 $\pm$ 8.6 uM Cd (Enger et al., 1983; Harley et al., 1989). The LC_{50} value for monocytes was 66 uM Cd, compared to 93 uM Cd for lymphocytes, and 128 uM Cd for polymorphonuclear leukocyte (PMNs) (Enger et al., 1983). The study also showed that a significant fraction of the PMN (16%) were resistant to cytotoxicity at 350 uM Cd (Enger et al., 1983). The erythrocytes (RBC) showed high resistance to the cytotoxic effects of Cd in culture tolerating concentrations as high as 500 uM Cd. Higher Cd concentrations were not examined due to the precipitation of the Cd salts (Garty et al., 1986).

3.1.2. Sub-Acute Studies

3.1.2.1. *Overview*

The kidney is the critical organ in humans who have been chronically exposed to Cd. Consequently, a large percentage of the animal and *in vitro* studies are concerned with the correlation of Cd exposure to kidney damage

namely, renal dysfunction (e.g. proteinuria, calciuria) and cytotoxicity (EHC, 1992a). Other important health concerns include the impairment of the respiratory system, and bone damage (Tsuchiya, 1981; Elinder, 1985a,b; Friberg et al., 1986a; Goyer, 1991; EHC, 1992a). Additional Cd-related ailments include contact cytotoxicity, cancer, hepatic function impairment, teratogenicity and reproductive toxicity (Friberg et al., 1986a; EHC, 1992a; see Section 1.4).

3.1.2.2. *Inhalation Studies*

In acute Cd exposure by inhalation (5 - 20 mg/m^3 for 1 - 2 h) the major target organ is the lung and respiratory tract, producing pulmonary edema in rodents (Hayes et al., 1976; Strauss et al, 1976; EHC, 1992a). Chronic or repeated inhalation exposure to a variety of Cd compounds (10 to 270 ug/m^3; $CdCl_2$ at 30 and 90 ug/m^3) produced interstitial fibrosis and alveolar hyperplasia in rats and golden hamsters (Heinrich et al., 1989). Lung fibrosis and emphysema were observed in rats exposed to CdO fumes (1.0 mg Cd/m^3) for 3 months (Yoshikawa et al., 1975). Emphysema was also observed in repeated 1 h exposures of $CdCl_2$ (10 mg/m^3) for 5 to 15 days (Snider et al., 1973). A long-term inhalation study of CdO dust (8 mg/m^3 for 3 h/day, 20 days/month for 8 months) in rabbits showed moderate proteinuria after 4 months; interstitial pneumonitis, emphysema, and leukocyte infiltration of the kidney interstitial spaces after 8 months (Friberg, 1950).

3.1.2.3. *Nephrotoxicity and Hepatic Toxicity*

The tissue distribution and associated nephrotoxicity was documented for several forms of Cd in a recent animal study (Min et al., 1986). Repeated IP injections of $CdCl_2$ in rats demonstrated a mixed or tubular type proteinuria which is characterized by urinary excretion of low molecular weight (LMW, ≤ 40,000 D) proteins namely, β_2 microglobulin (β_2MG), retinol-binding binding protein (RBP), and lysozyme which are normally reabsorbed in the proximal

tubules (Bernard et al., 1981). This short-term (2 months) rat study also demonstrated that tubular type proteinuria was reversible after suspension of the treatment (Bernard et al., 1981; Lauwerys et al., 1984). By contrast, a long-term (11 months) oral Cd exposure study in rats showed glomerular type proteinuria (characterized by excretion of high molecular weight (HMW) plasma proteins in the urine) was not reversible (Bernard et al., 1981; Lauwerys et al., 1984). Nephrotoxicity, in the form of acute renal lesions were demonstrated in Syrian hamsters, while not in mice and rats, after an acute $CdCl_2$ (30 to 50 uMol/kg) SC injection (Rehm and Waalkes, 1990). The hamsters did not develop extensive hepatocellular lesions, which are common in mice and rats at similar Cd doses (Rehm and Waalkes, 1990).

A study using repeated SC Cd injections in rabbits showed fibrotic changes in the liver (Friberg, 1950). This was supported by a later study where acute Cd-induced toxicity from a single injection was observed, and it was concluded that the liver was a critical target organ in animals (Dudley et al., 1982). The effects of repeated SC injections of $CdCl_2$ (0 to 30 uMol Cd/kg per week for 18 weeks) on the pancreas of Wistar and Fisher rats showed a high incidence of pancreatic metaplasia resulting in the formation of pancreatic hepatocytes (Konishi et al., 1990). The Cd-induced transformation was dose- and strain-dependent, with Wistar rats more susceptible (Konishi et al., 1990).

Tissue Cd accumulation was demonstrated to be a function of blood Cd concentration in rabbits exposed to $CdCl_2$ (0.15 or 1.5 mg Cd/day for 28 days) by SC implanted mini-pumps, which also showed that accumulation of Cd was highest in the target organs (liver and kidney) and blood cells, while plasma contained less than 10% of the total blood Cd (Duval and Grubb, 1986). In this study tissue necrosis was observed on the skin overlying the pumps in the high dose rabbits (Duval and Grubb, 1986).

A long-term (11 months) study in rats exposed to Cd (200 mg Cd/L) in

the drinking water demonstrated total proteinuria (mainly glomerular type) by 8 months with a kidney cortex Cd level of 200 mg/kg wwt (Bernard et al., 1981). Gastrointestinal absorption of Cd in humans has been estimated to be less than 10% (Tsuchiya, 1981; Hietanen, 1981), but is reported to be higher in subjects with iron deficiency (Flanagan et al., 1978; Friberg et al., 1986a). Animal studies indicate that diets low in calcium, iron, or protein, considerably increase the gastrointestinal absorption of Cd (Nordberg et al., 1985). Since Cd bioaccumulates, any absorption is considered significant. Other studies have shown histological and morphological changes in the kidney, liver, and bone after long-term oral exposure to Cd (reviewed in EHC,1992a; also see Chapter 5).

CdMT injections in animals were shown to cause acute renal toxicity and necrosis of the proximal renal tubular cells after 24 hours. CdMT injections resulted in Cd accumulation in the kidneys, while it did so in the liver after $CdCl_2$ injections (Cherian and Shaikh, 1975; Cherian et al., 1976). ^{109}Cd labeled CdMT, and $CdCl_2$ (7 ± 1 ug Cd/kg) administered to rats by IV injection, confirmed earlier reports that CdMT accumulated selectively in the kidneys, while $CdCl_2$ produced accumulation in the liver (Templeton and Cherian, 1983). A study of renal toxicity and enzymuria in rats after IP injections of CdMT (0.3 mg Cd/kg) showed renal proximal tubular damage and significant increases in the urinary excretion of enzymes, namely ALP, gamma-glutamyltranspeptidase (GGT) and N-acetyl-glucosaminidase (NAG), (Suzuki and Cherian, 1987). This study also showed that pretreatment with Zn salts 24 hours prior to Cd exposure reduced cellular damage and excretion of NAG, while, excretion of ALP and GGT were not altered (Suzuki and Cherian, 1987). IP administration of CdMT was shown to damage the proximal tubule cell lysosome system, producing tubular proteinuria which was similar to that found in chronic Cd exposure and was followed by calciuria (Fowler et al., 1987a). The study demonstrated that

calciuria occurred as a result of proteinuria, and not cell necrosis; further, Zn pretreatment prevented proteinuria and calciuria (Fowler et al., 1987a). In order to study the time frame of CdMT induced proteinuria and calciuria, a single SC injection of CdMT (0.0, 0.01, and 0.4 mg Cd/kg) was administered to rats and urine samples were collected for 14 days. A number of the rats were euthanized for membrane vesicle preparation 24 hours after exposure (Jin et al., 1987c). Urinary protein, Ca, and Cd exhibited different rates of elimination: protein excretion increased after 16 h, Ca excretion occurred after 8 h, and Cd excretion increased during the first 4 h. (Jin et al., 1987c). Ca uptake and binding decreased in the membrane vesicles with increasing Cd concentrations, and decreased Ca reabsorption was responsible for the calciuria (Jin et al., 1987c). Proteinuria depended on the administered dose of Cd. Little or no effect was observed at 0.01 to 0.05 mg Cd /kg, while proteinuria occurred rapidly at 0.4 mg Cd/kg (Jin et al., 1987 a-c).

3.1.2.4. *Distribution Studies*

The study of Cd, Zn, and Cu metabolism in rat kidney has shown the importance of MT in the binding of Cd at the expense of normal Zn and Cu binding, and that Cd-induced toxicity occurred when the quantities of MT were insufficient to bind the Cd upon cellular uptake or that from the biodegradation of CdMT (Petering et al., 1984). The absorption and distribution of Cd, Cu, and Zn in rats after oral exposures (25 ug Cd/day for 28 exposure days) to several forms of Cd (Cd-acetate, CdMT, and Cd-glutathione), demonstrated that Cd distribution was dependent on the chemical form of Cd administered and that the normal distribution of Cu and Zn was altered (Muller et al., 1986). Intestinal MT was shown to increase the intestinal Cd absorption and preferential distribution of Cd to the kidneys were observed after oral administration of $^{109}CdCl_2$ (0.05 to 20 mg Cd/10 uCi/kg) to Zn-pretreated rats (Min et al., 1991). In another rat study of the intestinal absorption of Cd showed no major involvement of MT (Piotrowski et al., 1987). Recently, a study of oral Cd

exposure in pregnant rats showed higher maternal tissue Cd accumulation compared to the fetus, and maternal Zn concentrations were significantly increased compared to the depressed fetal Zn levels (Sorell and Graziano, 1990).

A long-term (8 years) study of oral Cd exposure (1 to 100 mg Cd/day in the feed) in Beagles showed no changes in biological functions measured by serum alkaline phosphatase (ALP), glutamic pyruvic transaminase (GPT), and glutamic oxalacetic transaminase (GOT) in the 1 to 10 mg Cd/day exposure groups. Blood and urinary Cd levels in these animals continuously increased until a steady state was observed (Kodama et al., 1990; Matsuno et al., 1991a). The higher exposure groups (50 to 100 mg Cd/day), showed significantly higher blood and urinary Cd levels; renal dysfunction characterized by increased urine output, proteinuria, and decreased tubular reabsorption of phosphate was observed after 100 weeks (Kodama et al., 1990; Matsuno et al., 1991a). The study also showed that when tissue Cd reached an equilibrium, the newly absorbed Cd was excreted in the urine (Matsuno et al., 1991a). The body weights were depressed in the high dose group for both male and female dogs (a recognized sign of Cd-induced toxicity), while, osteomalacia was not observed (Kodama et al., 1990). A two-compartment model was used to describe the kinetic behavior of Cd in the study (Matsuno et al., 1991b). The histopathological results showed significant renal atrophy in the 50 and 100 mg Cd/kg/day exposure group, yet bone lesions were not detected (Hamada et al., 1991).

3.1.2.5. *In Vitro Studies*

In addition to *in vivo* animal studies, *in vitro* studies have been well documented using a variety of cell types or cell components. Studies of Cd exposure on rat hepatocytes showed that Zn-pretreatment had no effect on the uptake of Cd (Din and Frazier, 1983). Later studies showed that induction of intracellular ZnMT reduced Cd-induced toxicity (Frazier and Din, 1987). *In vitro*

studies of rat mitochondria exposed to Cd showed that low levels of Cd (0.005 to 0.05 uM Cd) disturbed the mitochondrial membrane integrity and impaired the cellular energy (ATP, ADP) supply; higher Cd levels (10 to 100 uM Cd) decreased ATP/ADP ratios, yet increased lipid peroxidation (Muller, 1986). In a similar study, oral Cd acetate (25 ug Cd/kg, given 5 times per week for 6 weeks) was shown to reduce by 50% the levels of liver mitochondrial cytochrome c oxidase; partial protection was observed by Zn-pretreatment. Another enzyme, namely renal adenosine triphosphatase (ATPase), was inhibited by Cd and Zn, while hepatic ATPase was not (Muller et al., 1988). Studies of testicular cells receiving *in vivo* pretreatment with low levels of Cd (3.0 umol/kg, SC) demonstrated testicular cell tolerance to later administration of testopathic (20 umol/kg, SC) doses of Cd (Wahba et al., 1990). The absolute mechanism of pretreatment tolerance has yet to be fully elucidated, since induction of LMW testicular cell binding protein was not significantly elevated (Wahba et al., 1990).

Studies of human peripheral blood cells exposed to Cd using *in vitro* cell culture methodology, demonstrated that lymphocytes accumulated significantly higher levels of Cd than the mature RBC expressed on a per cell basis (Hilderbrand and Cram, 1979; Enger et al., 1983). The determination of the cellular LD_{50} values in blood cells indicated that the monocytes were the most sensitive to Cd-induced cytotoxicity, followed by lymphocytes, and PMNs (Enger et al., 1983). This work was supported by other studies of leukocyte Cd-induced cytotoxicity (Koizumi et al., 1987; Harley et al., 1989). The sensitivity of both *in vitro* human and mouse lymphocytes (T- and B-cells) to Cd exposure has demonstrated that T-cells appear more sensitive to Cd toxicity than B-cells (Koizumi et al., 1987). Studies of peripheral blood cells isolated from rats and mice exposed to $CdCl_2$ by IV injection (Garty et al., 1981) and SC injection (Tanaka et al., 1985) demonstrated a significant increase in Cd accumulation in the RBC with time. Since mature RBCs did not incorporate large concentrations

of Cd *in vitro* (Hildebrand and Cram, 1979), the mechanism of Cd uptake in RBCs was studied and showed that Cd entered the cells by passive transport (Garty et al., 1986). Cd was also shown to interact with the sulfhydryl functional groups in RBC membranes; it may also bind to other membrane binding sites, including the amino, carboxyl and phosphate functional groups (Garty et al., 1986; Amoruso et al., 1987). Other effects of Cd on RBCs include decreased membrane lipid fluidity and membrane integrity, which may result in abnormal cell function and cell death (Amoruso et al., 1987).

Many of these Cd studies also examined the induction of MT as a result of Cd exposure (see Chapter 4). In brief, the induction of MT in RBCs occurred only in the erythroblast (immature RBC) which still contains a nucleus, and the increases in RBC MT levels follows a pattern similar to that observed for Cd accumulation in the RBCs (Tanaka et al., 1985; Tanaka et al., 1987). In addition, monocytes and lymphocytes have been shown to synthesize MT, while PMNs do not (Hildebrand and Cram, 1979; Enger at al., 1983; Peavy and Fairchild, 1987; Koizumi et al., 1987; Harley et al., 1989).

3.1.3. Relevant Human Studies

Human studies of Cd exposure are restricted to biological monitoring of occupational and environmentally exposed volunteers (see Section 1.4). Diet and life style (cigarette smoking, stress) affect the results obtained for blood and urinary Cd level (Piscator, 1985; Friberg et al., 1986; Nieboer et al., 1988a; Kreis et al., 1992; Benedetti et al., 1992). Recently, it has been suggested that the provisional tolerable weekly intake (TWI) of 400 - 500 ug Cd/week per person set by FAO/WHO (EHC, 1992a) is too high (Kreis et al., 1992). Earlier human studies reported that the critical Cd level for kidney damage was approximately 200 ug Cd/g wwt tissue, (ppm) which corresponds to approximately 30 ug Cd/g wwt in liver (Roels et al., 1981; Lauwerys, 1983a). Correlations of Cd concentrations in kidney to urinary levels found approximately 160 ug Cd/g wwt kidney was equal to 10 ug Cd/g creatinine in

urine (Lauwerys et al., 1979; Roels et al., 1979; Buchet et al., 1980; Roels et al., 1981; Lauwerys, et al., 1983a). From these results, it was determined that urinary Cd levels were a reliable index of body burden and should not exceed 10 ug Cd/g creatinine in order to prevent renal dysfunction in occupationally exposed people (Lauwerys, 1983a). The suggested whole blood NOAEL for long term Cd exposure in occupationally exposed humans is 10 ug Cd/L whole blood (Bernard et al., 1979; Bernard et al., 1980; Buchet et al., 1980; Lauwerys, 1983a). Cd values found in non-occupationally exposed people were ≤ 5 ug Cd/L whole blood, and ≤ 1 ug Cd/g creatinine in urine (Bernard and Lauwerys, 1984). As indicated, cigarette smoking has been shown to interfere with the results obtained in human monitoring studies and should be included in the data analysis (Elinder et al., 1983; Nieboer et al., 1988a; EHC, 1992a). Previously, blood Cd concentrations were believed to represent short-term exposure levels (few months); however, if intermittent Cd exposure continues, then blood Cd levels also appear to be a reasonable indicator of body burden (Lauwerys, 1983a; Shaikh et al., 1990).

3.1.4. Specific Research Objectives

1). To develop improved analytical techniques for the determination of Cd in tissues, blood cells, and body fluids which allow the use of small sample size or volume.

2). To employ a continuous blood sampling technique in rats which involves collection through a surgically inserted cannula.

3). To develop an experimental animal protocol that will allow the construction of a Cd-distribution and Cd-compartmentalization model comparing the accumulation of Cd in the selected major organs (liver, kidney, spleen and lung), with that in peripheral blood cells (erythrocytes, lymphocytes, monocytes, polymorphonuclear leukocytes), body fluids (whole blood and plasma) and feces.

4). To evaluate the dependence on time of the differential accumulation

of Cd in peripheral blood cells.

3.2. Analytical Methods

3.2.1. Instrumentation

The samples were analyzed by graphite furnace, electrothermal atomic absorption spectrometry (EAAS) using a Perkin-Elmer (PE) Model 703 equipped with a HGA 500 furnace, AS-1 autosampler, deuterium background corrector, PE-56 chart recorder, PRS-10 printer and a Cd hollow cathode lamp. Pyrolytically coated L'vov platform graphite tubes were used for all EAAS analyses. Standard curves were generated for each analysis session. The detection limits (C_L) were determined from the reciprocal of the calibration curve slope (A/C), multiplied by a constant (k) (set at 3) and the standard deviation of the absorbance of the sample blank (SD blank) according to the expression: $(C/A)(k)(SD_{blank})$, (Welz, 1985). The corresponding absorbance limit (Y_L) was calculated as $(k)(SD_{blank})$, (Welz, 1985). The experimental Cd concentrations (ng/ml or ug/L) were manually calculated from the calibration curves of average absorbance values (minimum n = 2) *versus* concentration (Welz, 1985). The general spectrometer operating parameters and time/temperature programme summarized in Table 3.1 were used for Cd analysis, with minor adjustments when necessary.

3.2.2. Reagents Employed and Cleaning of Laboratory Wares

All chemical reagents employed in the Cd analysis were of high quality in order to limit contamination of the samples (see Table 3.2). All laboratory wares (plastic and glassware) used for the collection, storage and analysis of tissues and body fluids were acid washed using the general guidelines for cleaning laboratory wares (Nieboer and Jusys, 1983; Jusys et al., 1988). Specifically, the labware was soaked in 2 M HNO_3 (see Table 3.2) for 1 - 5 days in snap-top polyolefin containers. After acid soaking, the labware was drained,

rinsed thoroughly with distilled deionized water (DDW) and soaked overnight in DDW. Afterwards, the labware was again drained, rinsed with DDW, and dried in an oven at 50 - 60°C. Items such as beakers, volumetric pipettes, and volumetric flasks were sealed with parafilm after cleaning to prevent accidental contamination. Acid-washed plasticware (pipette tips, test tubes and AA cups) and glass test tubes were stored in snap-top containers. The acid-bath solution was recycled 3 - 5 times and frequently checked by EAAS for Cd contamination. Rinse water was not recycled. Glassware which contained Cd standards or samples containing high Cd levels were pre-soaked or rinsed separately with 2 M HNO_3 (acid discarded after use) prior to the general soap wash and subsequent acid-washing procedure.

3.2.3. Sample Digestion and Preparation

3.2.3.1. *Tissues and Feces*

Tissues, namely liver, kidney, spleen, lung; and feces were acid-digested using the following procedure. Quantities of powdered tissue (0.15g dry weight liver, kidney, 0.06-0.10g spleen, 0.10-0.12g lung), and feces (0.16-0.40g wet weight) were first partially digested in 1 mL of concentrated ultra pure nitric acid (see Table 3.2) for 1 h in acid washed Pyrex test tubes (see Section 3.2.2). Subsequently, 2 mL of ultra pure concentrated acid mixture (Table 3.2) consisting of nitric, sulfuric, and perchloric acids in a 3:1:1 volume ratio was employed to complete the digestion. The sample digestion mixture was heated in specially designed aluminum blocks for two, 12 hour periods, at 180-200°C in a stainless-steel back-washed perchloric fume hood. Digestion continued until a residual volume of 0.4 mL sulfuric acid remained. In Experiment AF90, the digested samples were transferred to 10 mL volumetric flasks and diluted in DDW (Dilution Factor (DF1)). In Experiments GO91 and PA91, the digested samples were diluted in the Pyrex test tubes with DDW using 10 mL volumetric pipettes (DF1). Further dilution with 1% nitric acid was necessary (DF2) for the 0.0 and 0.25 mg Cd/kg dosage groups. The 1.25 and 2.5 mg Cd/kg dosage

groups required a third (DF3) or fourth (DF4) dilution step to bring the analyte into the calibration range of the atomic absorption spectrometer.

3.2.3.2. *Blood Lysates*

The analytical method described by Stoeppler and Brandt, (1980) for plasma and whole blood Cd determination was adopted and adapted for Cd in blood cell lysates. Generally speaking, blood cell lysates were acid deproteinized employing ultrapure nitric acid solutions (DF1), followed by centrifugation at 3000 rpm for 10 min. Additional details are provided below for each specimen and blood cell type. Deproteinized supernatants were diluted appropriately with a nitric acid solution (DF2) and subsequently analyzed by EAAS. As described in Section 2.3.1, a cell lysate dilution factor (CDF) also needed to be applied.

3.2.3.3. *Erythrocytes*

Rehydrated erythrocyte (RBC) lysates consisted of ruptured cell walls and cellular contents. An aliquot of rehydrated RBC lysates (250 uL) were deproteinized with 750 uL 1 M nitric acid (DF1 = 1:3.84; see below). Deproteinized RBC supernatants were diluted further 1:5 (DF2) with 1 M nitric acid prior to analysis by EAAS. In whole blood, the cell water averages 0.84 kg/L (Beutler, 1975; Tietz, 1976; Sunderman et al., 1984; Jusys et al., 1988), such that a 1 mL blood sample is equivalent to 0.84 mL whole blood cell water and the rest is cellular proteins. Thus for the Cd analysis, the deproteinized RBC supernatants (0.84 mL blood water + 3.0 mL HNO_3) yields a total volume of 3.84 mL protein-free analyte; the corresponding dilution factor (DF1), is 1:3.84 (Beutler, 1975; Tietz, 1976; Sunderman et al., 1984; Jusys et al., 1988).

3.2.3.4. *Polymorphonuclear Leukocytes*

Rehydrated polymorphonuclear leukocytes (PMN) were lysed by freeze/thaw treatments. Aliquots of the rehydrated PMN lysate (300 uL) were deproteinized with 100 uL 10% nitric acid (DF1 = 1:1.33). The deproteinized PMN supernatants (300 uL) were diluted prior to EAAS analysis with 100 uL

10% nitric acid (DF2 = 1:1.33).

3.2.3.5. *Lymphocytes and Monocytes*

Rehydrated lymphocytes (Ly) and monocytes (M) were lysed and aliquots (200 uL) were deproteinized with 100 uL 10% nitric acid (DF1 = 1:1.5). The deproteinized supernatants (200 uL) were diluted with 200 uL 10% nitric acid (DF2 = 1:2) prior to analysis.

3.2.3.6. *Whole Blood*

Whole Blood (WB) samples for Experiments GO91 and PA91 were observed to have ccagulated prior to analysis. Therefore, the initial whole blood volumes were estimated by comparison with known volumes in identical containers. It was necessary to digest (with heating) the coagulated WB in 1 mL ultra pure HNO_3 (DF1). The heat-digested samples were diluted four-fold (1:3.84) for the control and short-course animals, six-fold (1:5.76) for the 0.25 mg/kg subchronic, or eight-fold (1:7.68) for the 1.25 mg/kg subchronic animals with DDW (DF2). Whole blood water volumes were taken into account when calculating DF2 (Beutler, 1975; Tietz, 1976; Sunderman et al., 1984; Jusys et al., 1988). More concentrated samples were diluted further as required (1:5 to 1:16; DF3) with 1% nitric acid.

Whole blood samples (250 uL) from Experiment AF90 were acid deproteinized with 750 uL 1 M nitric acid (DF1 = 1:3.84). The deproteinized WB supernatants were diluted 1:5 (DF2) with 1 M nitric acid before EAAS analysis. As required, more concentrated samples were diluted further.

3.2.3.7. *Plasma*

Plasma samples showed signs of precipitation and coagulation to various degrees after storage and were sonicated prior to sampling. The plasma samples (75 uL) were acid deproteinized with 150 uL of 10% nitric acid (DF1 = 1:3), and centrifuged at 3000 rpm for 10 minutes. The supernatants were diluted further 1:2 (DF2) in 10% nitric acid.

3.2.3.8. *Urine*

Urine samples required no digestion or deproteinization prior to dilution with 1% nitric acid. However, a matrix modifier had to be added (prepared in a 1:1:1 volume ratio of 1086 ppm $PdCl_2$, 0.2 M $Mg(NO_3)_2$ and 0.1% Triton), (Yin et al., 1987; Jusys et al., 1988). Urine samples from Experiment AF90 were collected manually, EAAS performed using the matrix modifier detailed above. The spectrometer conditions are summarized in Table 3.1. The urine samples from Experiments GO91 and PA91 were collected in metabolism cages and when analyzed employing the matrix modifier exhibited a residual matrix effect. Several different furnace temperature programmes and five different matrix modifiers were investigated with little success. Briefly, several concentrations of the above $PdCl_2$ - $Mg(NO_3)_2$ matrix modifier were tested (Jusys et al., 1988), as well as a modifier composed of $NH_4H_2PO_4$, and $(NH_4)_2HPO_4$ in HNO_3 (Pruszkowska et al., 1983); the modifiers Pd + NH_4NO_3 and NH_4NO_3 were also tried (Yin et al., 1987). In further studies, several urine samples were predigested in concentrated nitric acid and diluted in 1% nitric acid with the original $PdCl_2$ - $Mg(NO_3)_2$ matrix modifier prior to analysis. The results still were not satisfactory. Tissue digestion failed to overcome this interference (see Section 3.2.3.1).

3.2.4. Calculation of Cd Concentration

The basic calculation for tissue Cd concentration involved multiplication of all dilution factors and graph value (derived from the calibration curve); the number was then divided by the tissue weight in kilograms dry weight (kg dwt) as summarized in Equation 1.

[1]. (DF1)(DF2)...(DFn)(Graph Value) / kg dwt.

The general equation employed for calculating the Cd concentration per cell is summarized in Equation 2.

[2]. (DF1)(DF2)...(DFn)(Graph Value) / (#cells analyzed) = ng Cd/cell.

To normalize the data to a constant number of cells, the results of

Equation 2 are multiplied by either 10^6 (for Ly, M, PMN) or 10^9 (for RBC), as illustrated in Equation 3. Normalized concentrations are reported in all tables and figures, except in the case of the mass balance results (see Equation 4).

[3]. (ng Cd/cell) (10^6 or 10^9) = ug Cd / L normalized.

For the mass balance calculations, the results of Equation 2 were multiplied by the cell dilution factor (CDF) and (Actual Cell Number) summarized in Equation 4. The actual cell numbers were determined by manual cell counting for Ly, M, PMN; and by Coulter Counter for RBC.

[4]. (ng Cd/cell) (CDF) (Actual Cell Number) = ug Cd / L actual.

Whole blood, plasma and urine Cd concentrations were calculated by multiplying the values obtained from the calibration curve by the appropriate dilution factors. The results were expressed as ug Cd / L, as summarized in Equation 5.

[5]. (DF1)....(DFn) (Graph Value) = ug Cd / L.

3.2.5. Statistical Analysis of Cd Results

A combination of three different statistical tests were applied to the data; the two sample unpaired Student's t test, the longitudinal paired t test, and One-way ANOVA (level of significance set at 95%) (Rosner, 1986). The unpaired t test was applied to the time course and organ data to test for significant differences in the results between the different exposure groups and the control animals at each time point, also known as cross-sectional data analysis. The longitudinal paired t test was used to analyze the way individual animals responded to Cd exposure throughout the time course, also known as a follow-up study. In this statistical test, the control time points (T= -20 and 0 h) were averaged and compared to each time point using the paired t test to reflect significant changes from the individuals' baseline (initial value) with the passage of time (-20 to 96 h). The One-way ANOVA was applied to the organ and inter-compartment comparison data to test for the variance between the different groups and experiments. The data are assumed to follow a normal distribution.

The calculated p-values are reported and indicated on the figures. The pooled standard deviations were calculated from the summed standard deviations (SD) divided by the total number of SD per time course (n) using the following equation: $\Sigma(SD)/n$ (Rosner, 1986). The pooled SD are reported on the time course figures and the mean $\pm$ SD are reported in the tables. The inter-animal coefficient of variation is equal to the standard deviation divided by the mean times 100, (%CV = (SD/x)(100)). The CV was calculated for each tissue type within a dosage group.

3.3. Experimental Results

Cd concentrations were determined for samples collected at each time point: whole blood (WB), plasma, lymphocyte (Ly), monocyte (M), polymorphonuclear leukocytes (PMN), erythrocytes (RBC), urine (when possible) and feces. Tissue Cd levels were determined at the termination of the experiments. Missing data points are denoted by a 'dash' in the tables and entered as 'zeros' in the graphs. Experiments AF90 and PA91 employed the short course protocol (single IP injection) while Experiments CD90 and GO91 employed a multiple IP injection subchronic experimental protocol (see Section 2.2). Typical reported EAAS detection limits for blood and urine ranged from 0.1 to 0.3 ug Cd/L (EHC, 1992a). The overall EAAS detection limit for Cd in the present study was 0.10 $\pm$ 0.05 ug Cd/L (or ug Cd/kg). The corresponding overall absorbance limit in this study was 0.011 $\pm$ 0.005.

3.3.1. Cd Concentration in Tissues

The EAAS detection limit (C_L) for the dried tissues ranged from 0.05 to 0.11 ug Cd/kg (see Section 3.2.1). The tissue Cd results were analyzed by the two-sample unpaired t test to determine if the control and Cd-induced rats were significantly different ($p \leq 0.05$). A Cd dose-response was observed in all tissue types analyzed for both experimental protocols (Figures 3.1 - 3.11, Tables 3.3 - 3.4). Tissue Cd concentrations within an exposure group were higher in animals

receiving the multiple injection subchronic protocol compared to the single injection short course protocol (Figures 3.1 - 3.11, Tables 3.3 - 3.4). The inter-animal CV for the administered Cd doses ranged from 4 to 59% while in the controls it was 12 to 67% (Tables 3.3 - 3.4). The liver Cd concentration data in Table 3.5 illustrates that the EAAS analytical results were reproducible upon repeat analysis. The inter-run CV values ranged from 4 to 30%.

In the two-sample unpaired t test analysis of the data from the short course experiments, the liver Cd concentrations for all dosage groups (low, 8688 $\pm$ 1037; medium, 26200 $\pm$ 2196; and high, 109611 $\pm$ 9882 ug Cd/kg dwt tissue) were significantly higher ($p \leq 0.05$) than the baseline levels (19 $\pm$ 12 ug Cd/ kg dwt) determined in the controls (Figures 3.1 and 3.5, Table 3.3). Similarly, the Cd concentrations determined in the kidney, spleen and lung samples for all dosage groups were significantly elevated ($p \leq 0.05$) compared to their respective control groups (Figures 3.2 - 3.7, and Table 3.3). The analysis of the tissue Cd concentrations determined in the subchronic protocol yields the same significant dose-response ($p \leq 0.05$) observed in the short course experiment. However, the Cd levels were higher for all organs analyzed and reflect tissue specific bioaccumulation of Cd (Figures 3.5 - 3.11, and Table 3.4).

In addition to the unpaired t test, the tissue Cd data were also analyzed by the One-way ANOVA (significance level set at 95%). The control (baseline) Cd levels between the different tissue types revealed kidney levels were the highest (57 $\pm$ 38 ug Cd/kg dwt tissue), followed by spleen (43 $\pm$ 29 ug Cd/kg dwt), liver (19 $\pm$ 12 ug Cd/kg dwt), with the lowest levels found in the lung (3 $\pm$ 1 ug Cd/kg dwt). The ANOVA comparisons of the organs from the short course animals showed the order of Cd concentrations were: *kidney > *spleen > liver > lung, where kidney and spleen Cd levels were significantly higher than those in liver and lung (* $p \leq 0.05$). The kidney and spleen Cd concentrations were not significantly different from each other; neither were the liver and lung. The Cd

concentrations found in the controls for the subchronic experiment were highest in the kidneys (193 ± 22 ug Cd/kg dwt), followed by liver (84 ± 19 ug Cd/kg dwt), spleen (41 ± 5 ug Cd/kg dwt), and lung (13 ± 6 ug Cd/kg dwt),(Tables 3.3 and 3.4). The ANOVA comparisons of the organs from the subchronic control animals showed the order of Cd concentrations were: *kidney >> *liver > spleen > lung, with the kidney Cd levels significantly higher than in all other organs; the liver levels were also significantly higher than the spleen and lung, while those in the spleen and lung were not significantly different (* $p \leq 0.05$). By contrast, the order of Cd accumulation in the organs from the short course Cd-exposed animals were: *liver >> *kidney >>> spleen > lung, with liver Cd levels significantly elevated relative to all other organs at all dose levels (* $p \leq 0.05$). Kidney Cd levels were significantly higher than spleen and lung, except in the high dose group for which spleen and kidney levels were not significantly different. There were no significant differences in Cd levels detected by the ANOVA for the spleen and lung in any of the exposure groups. By comparison the order of Cd accumulation in the organs from the subchronic Cd-exposed animals were: *liver > *kidney > *spleen > lung, with liver Cd levels significantly higher compared to all organs (* $p \leq 0.05$). Kidney Cd levels were significantly higher than those in spleen and lung, while spleen Cd was significantly higher than in lung, except in the medium dose group.

ANOVA analysis of the Cd levels in a specific organ across all dosage groups in both experimental protocols was also conducted. Liver Cd concentrations in the low Cd exposure group were significantly higher ($p \leq 0.05$) than in controls of the subchronic, but not in the short course experiment. Cd concentrations in the medium and high exposure groups were significantly higher than the control and the low exposure groups in both the short course and subchronic experiments ($p \leq 0.05$). Similarly, the liver Cd levels were significantly elevated in the high dose group compared to the medium group in both experimental protocols. ANOVA comparisons of the short course versus

the subchronic experiments revealed liver Cd levels were significantly higher in the subchronic, medium and high Cd exposure groups ($p \leq 0.05$), compared to their respective short-course exposure groups; the control and low dose groups were not significantly different.

By contrast to the liver results, the kidney Cd levels in the low dose were not significantly different from the controls in either the short course or subchronic experiments. The medium and high exposure groups were significantly elevated above the controls in both experimental protocols ($p \leq 0.05$). Similarly, the kidney ANOVA comparisons revealed that the Cd concentrations were significantly higher in the subchronic compared to the short course experiments within the medium and high exposure groups ($p \leq 0.05$), while the control and low exposure groups were not significantly higher. Interestingly, the short course medium exposure group was not significantly different from the subchronic low exposure group in total accumulated Cd.

The ANOVA comparison of spleen Cd data revealed a different trend, one that was intermediate with respect to the results determined in the other tissues. The control spleen Cd levels were not significantly lower than the low and medium exposure groups in either the short course or subchronic experiments. One exception was noted in the subchronic medium dosage group, for which Cd levels were significantly higher even with an extremely large standard deviation ($p \leq 0.05$). By contrast to all other organs, no significant differences were observed in spleen Cd concentrations between exposure groups in the short course experiments. In the subchronic experiment, the control and low dose spleen results were not different, which is similar to the trend observed in the kidney ANOVA analysis. Cd accumulation in both the control and low dose groups was significantly lower than in the medium dose ($p \leq 0.05$), a trend which was observed in all tissue Cd ANOVA results. By comparison to other organs, the spleen tissue from the subchronic high Cd group was morphologically damaged (deteriorated) when inspected at autopsy,

making sampling impossible (see Chapter 5). As for liver and kidney, Cd concentrations in the subchronic medium exposure group were significantly higher than in the respective short course exposure group ($p \leq 0.05$), while the control and low dose groups were not different.

In lung, the ANOVA results for Cd concentration followed the trend observed in the liver for both the short course and subchronic experiments. Comparisons of the exposed and control groups, showed that Cd levels were significantly higher in both experimental protocols with one noted exception, namely in the short course low-dose group. The analysis revealed that Cd accumulation in the medium exposure groups was significantly higher than the low dose groups. This pattern was observed in both experimental protocols. ANOVA comparisons showed that lung Cd levels were not significantly different in the subchronic control and low dose groups compared to their respective short course exposure groups, while the medium exposure group was significantly higher ($p \leq 0.05$).

3.3.2. Cd Concentration in Whole Blood

The detection limit for whole blood (WB) was 0.07 ug Cd/L in experiment AF90, and 0.12 ug Cd/L in experiments GO91 and PA91. Baseline WB Cd concentrations at or below 5.7 ug Cd/L were observed during the recovery period (T = -20 and 0 h) for all short course animals. WB Cd levels in all Cd exposure groups were significantly higher than the control groups from T = 6 h onward ($p \leq 0.05$), as determined by the unpaired t test. Maximum Cd responses of 24.3 ± 1.5 ug Cd/L for the low dose, 90.6 ± 6.6 for the medium dose, and 53 ± 28 ug Cd/L for the high Cd exposure group were observed at T = 6 h. Thereafter, the levels dropped to 15.7 ± 2, and 30.0 ± 7.8 in the low and medium groups respectively (Tables 3.6 and 3.8). The WB Cd levels gradually increased with time (Figure 3.12 and Tables 3.6 and 3.8). The control animals exhibited baseline Cd levels throughout the time course. In each exposure group, the data from each time point for individual animals were compared to

their corresponding initial values (T = -20 and 0 h) using the longitudinal paired t test (Rosner, 1986). The control rats did not show significant increases in WB Cd. By contrast, both the low and medium Cd exposure groups exhibited significantly elevated whole blood Cd by T = 6 h onwards, when compared to their own initial values ($p \leq 0.05$, paired t test). In the high dose group, only point T = 24 h was significantly higher than their initial values ($p \leq 0.05$, paired t test, Figure 3.12).

The trend observed for the subchronic WB Cd levels was different from the short course results from the first time point (T = -20 h) onwards. A dose-response pattern was evident (Figure 3.13 and Tables 3.7 - 3.8). The subchronic WB Cd concentrations were significantly higher than the control rats throughout the time course ($p \leq 0.05$ to 0.0005 unpaired t test, Figure 3.13). The maximum levels were 57.5 $\pm$ 3.9 ug Cd/L in the low dose, 131.3 $\pm$ 2.7 in the medium, and 155.5 ug Cd/L in the high Cd exposure group (Tables 3.7 - 3.8). The control animals exhibited baseline Cd levels below 8 ug Cd/L at all sampling times. The longitudinal analysis of the subchronic WB Cd results showed significantly higher levels at T = 6 h for the medium dose and T = 48 h for the low dose, while all other time points were not different from their individual initial values ($p \leq 0.05$). The subchronic longitudinal results showed little or no significant differences from their initial values, while the short course longitudinal results were significantly different ($p \leq 0.05$) from their initial values from T = 6 h onwards (Figures 3.12 and 3.13).

3.3.3. Cd Concentration in Plasma

The detection limit for plasma was 0.11 ug Cd/L in experiment AF90, and 0.16 ug Cd/L in experiments GO91 and PA91. Baseline plasma Cd levels were determined for the recovery period (T = -20 and 0 h) for all dosage groups and were not statistically different from the control group, with one noted exception, the subchronic medium dose group ($p \leq 0.05$) was significantly higher (Figures 3.14 - 3.15). The cross-sectional analysis (unpaired t test) of the

short course plasma Cd data shows significantly higher levels in all dosage groups at T = 6 h onwards ($p \leq 0.05$ to 0.0005) compared to controls, with the exception of the high exposure group from T = 12 h onwards, where no statistical significance was determined due to high standard deviations (Figure 3.14). Maximum plasma Cd concentrations were 1.0 $\pm$ 0.3 ug Cd/L in the controls, 4.9 $\pm$ 0.5 in the low, 8.7 $\pm$ 1.1 in the medium, and 8.0 $\pm$ 1.3 ug Cd/L in the high dose group at T = 6 h (Figure 3.14 and Tables 3.9 and 3.11). The high group also had a single rat at T = 96 h with 22.2 ug Cd/L (Table 3.11) which appeared to be an artifact. Longitudinal analysis of the plasma Cd concentrations in the short course Cd-exposed animals showed significantly elevated Cd levels compared to their initial values ($p \leq 0.05$ to 0.005) in all samples, except the T = 72 h medium dose group and the high dose group from T = 12 h onward (Figure 3.14).

The trend observed for plasma Cd levels in the short course experiments were also observed for the subchronic experimental results (Figure 3.15 and Tables 3.10 and 3.11). An exception were the Cd levels in the subchronic medium dose group, which were significantly higher ($p \leq 0.05$, unpaired t test) than the controls during the recovery period, T = -20 and 0 h (Figure 3.15).

The plasma Cd levels returned to control values in both experimental protocols and therefore did not follow the same pattern of Cd accumulation than that observed in the whole blood (Figures 3.12 - 3.15).

3.3.4. Cd Concentration in Erythrocytes

The detection limit for erythrocyte (RBC) was 0.21 ug Cd/L in experiments GO91 and PA91. The RBC Cd concentration was not part of the experimental design in the earlier high dose AF90 experiment. Once again, baseline Cd values (ranging from 0.06 to 1.5 ug Cd/L) were observed during the recovery period for the short course experiments. A dose-dependent increase in RBC Cd levels was apparent with a maximum concentration of 2.4 $\pm$ 0.5 ug

Cd/L in the low dose detected at T = 48 h, and 11.5 ± 0.8 ug Cd/L in the medium dose group at T = 72 h. Baseline Cd levels below 1.9 ug Cd/L were determined in the control animals (Figure 3.16 and Table 3.12). Cross-sectional analysis of the short course medium exposure group revealed significantly elevated RBC Cd levels compared to the controls from T = 6 h onwards ($p \leq 0.05$ to 0.0005). A time-dependent increase of Cd in RBCs (Cd accumulation) was observed in the short course experiment for both the low and medium exposure groups. The Cd levels in the low dose group were significantly higher than the controls at T = 48 h onwards. A noticeable time lag in Cd accumulation was observed in RBCs between the two exposure groups (Figure 3.16). This effect was also determined to be statistically significant by the longitudinal analysis (Figure 3.16).

By contrast to the short course RBC Cd data, the subchronic results were significantly higher ($p \leq 0.05$ to 0.0005) than the controls for all Cd exposure groups from the onset of the experimental time course, T = -20 h (Figure 3.17). The subchronic RBC Cd data followed the same trend observed for Cd concentration in whole blood (Figures 3.13 and 3.17). The controls exhibited levels below 1.0 ug Cd/L. The RBC Cd levels showed a plateau with Cd concentrations around 4.0 ± 1.7 ug Cd/L in the low, and 19.2 ± 3.2 ug Cd/L in the medium exposure group (Figure 3.17 and Table 3.13). Longitudinal analysis of the subchronic RBC Cd data revealed that the levels were not statistically different from their initial values (Figure 3.17). A statistically significant dose-dependent accumulation of Cd in erythrocytes was observed in both experimental protocols with a progression from the short term to the long term experimental levels (Figures 3.16 - 3.17, and Tables 3.12 - 3.13).

3.3.5. Cd Concentration in Polymorphonuclear Leukocytes

The detection limit for polymorphonuclear leukocytes (PMN) was 0.15 ug Cd/L in experiments GO91 and PA91. The PMN Cd concentration was not part of the experimental design in the earlier high-dose AF90 experiment. The

PMN Cd concentrations determined in the short course samples showed no significant differences detected by the cross-sectional analysis between the exposed and non-exposed groups (Figure 3.18 and Table 3.14). However, differences were detected by longitudinal analysis at T = 12 h in the medium dose and T = 24 to 48 h in the low dose compared to their corresponding initial values (Figure 3.18). The subchronic PMN Cd levels followed a similar trend with no significant differences determined between the exposed and control groups, except at T = 48 h where the levels were significantly higher ($p \leq 0.05$, unpaired t test) in the medium dose group compared to the control group (Table 3.15 and Figure 3.19). The longitudinal analysis of the PMN Cd levels from the subchronic rats showed significantly lower Cd levels at T = 0, 6, 24, and 72h in the medium dose group and at T = 48 h in the control group ($p \leq 0.05$) compared to their initial values (Figure 3.19). The PMN Cd levels were generally at or below the baseline levels determined in the control RBCs (1.2 $\pm$ 0.5 ug Cd/L) in both experimental protocols (Figures 3.16 - 3.19, and Tables 3.12 - 3.15).

3.3.6. Cd Concentration in Lymphocytes

The detection limit for lymphocytes was 0.09 ug Cd/L in all experiments. The lymphocyte Cd levels were significantly higher ($p \leq 0.05$, unpaired t test) than in the control group at T = 6 h for the medium, and at T = 24 h for all short course Cd exposure groups (Figure 3.20). These results were in contrast to the pattern observed for the PMNs. The maximum Cd concentrations observed in the short course lymphocytes were: 0.7 $\pm$ 0.4 ug Cd/L (controls), 2.3 $\pm$ 0.6 (low dose), 1.5 $\pm$ 0.2 (medium dose), and 0.5 $\pm$ 0.2 ug Cd/L in the high dose (Tables 3.16 and 3.18, also Figure 3.20). Longitudinal analysis of the lymphocyte Cd data from the short course rats revealed the same time points were significantly different from the corresponding initial values ($p \leq 0.05$) in all Cd exposure groups except the high dose (Figure 3.20). The control lymphocytes showed baseline values below 0.7 $\pm$ 0.4 ug Cd/L and were not statistically different

throughout the experimental time course.

A similar trend was observed in the subchronic lymphocyte Cd concentration in that the low exposure group exhibited the highest accumulation of Cd (Figure 3.21 and Table 3.17). By contrast, the subchronic dose-dependent accumulation pattern for the lymphocytes was a plateau which differed from the maximum peak observed in the short course results (Figures 3.21 and 3.20). The Cd concentrations in the lymphocytes for the low dose group were significantly higher than the control group ($p \leq 0.05$ to 0.005, unpaired t test) at T = 0, 6, and 24 h (Figure 3.21), while those for the remaining groups were not statistically different. The Cd levels in the subchronic groups were comparable to the Cd concentrations in the corresponding short course group (Figures 3.20 - 3.21, also Tables 3.16 - 3.18).

3.3.7. Cd Concentration in Monocytes

The detection limit for monocytes was 0.05 ug Cd/L in experiment AF90, and 0.082 ug Cd/L in experiments GO91 and PA91. During the recovery period, baseline Cd concentrations of 0.7 ± 0.3 ug Cd/L and below were determined in the monocytes for both experimental protocols (Tables 3.19 - 3.20). In the short course experiment, Cd levels in the medium dose group were significantly elevated above the controls at T = 6 and 24 h ($p \leq 0.05$, unpaired t test). By contrast, the low dose was not statistically different from the control group (Figure 3.22 and Table 3.19). The maximum Cd concentrations determined in the short course monocytes were: 0.8 ± 0.4 ug Cd/L (controls), 1.3 ± 0.3 (low dose), 2.1 ± 0.1 (medium dose), and 2.4 ± 1.6 ug Cd/L (high dose) (Tables 3.19 - 3.21, also Figure 3.22). These results were in contrast to the pattern of Cd accumulation observed in the lymphocytes. The longitudinal analysis of the monocyte Cd data from the short course rats showed the low dose group had higher Cd levels at T = 24 and 48 h ($p \leq 0.05$, paired t test). The medium group had elevated monocyte Cd at T = 6 and 24 h ($p \leq 0.05$). Although the control group exhibited baseline Cd levels during the time course, the levels were

significantly lower at T = 72 h than their initial values ($p \leq 0.005$, paired t test).

By contrast, in the subchronic experiment, all exposure groups exhibited fluctuations in monocyte Cd levels and the range of concentrations observed were considerably lower compared to the short course monocyte Cd data. There were few detectable differences between the subchronic treatment groups. The Cd levels were significantly lower ($p \leq 0.05$, unpaired t test) than the control group at T = -20 h in the medium dose group and at T = 48 h in the low dose group (Figure 3.23 and Table 3.20). The longitudinal analysis of the monocyte Cd data from the subchronic rats showed that the levels in the medium dose group were significantly higher at T = 0 h, followed by significantly lower levels at T = 12 h, compared to their initial values ($p \leq 0.05$, paired t test). The remaining time points and other dosage groups were not significantly different from their initial values, due in part to high standard deviations (Figure 3.23). The data from the high dose experiments qualitatively support these trends (see Table 3.21), although no statistical significance was determined due in part to high standard deviations.

3.3.8. Cd Concentration in Feces

The detection limit for fecal Cd was 0.05 ug Cd/kg in experiment AF90, and 0.06 ug Cd/kg in experiments GO91 and PA91. The fecal Cd data (Figures 3.24 and 3.25) illustrates that Cd appeared in the feces in a dose-dependent pattern during the period ranging from 6 to 48 h after administration (Tables 3.22 - 3.24). The maximum Cd concentrations found in the short course feces were: 149 $\pm$ 103 ug Cd / kg wwt (controls), 563 $\pm$ 135 (low dose), 1688 $\pm$ 1048 (medium dose) and 4804 $\pm$ 4243 ug Cd / kg wwt (high dose). The maximum Cd levels in the subchronic feces were: 228 $\pm$ 107 ug Cd/ kg wwt (controls), 496 $\pm$ 125 (low dose), and 1023 $\pm$ 232 ug Cd / kg wwt (medium dose). Inter-animal differences in the excretion patterns (dependence on time) of fecal Cd were observed in the medium and high dose groups resulting in high standard deviations and loss of statistical significance. The control animals exhibited

baseline Cd levels throughout the time course (Figures 3.24 - 3.25, also Tables 3.22 - 3.24). The cross-sectional analysis of the fecal Cd data showed significantly higher Cd concentration in the low dose compared to the control group for the short course and subchronic experiments at T = 24 h ($p \leq 0.05$, unpaired t test). However, only the subchronic medium dose group was significantly higher than the controls at T = 12 and 24 h ($p \leq 0.05$, unpaired t test). In comparing the fecal Cd levels between the experiments, the levels were generally lower in the subchronic exposure groups (Figures 3.24 - 3.25, and Tables 3.22 - 3.24).

3.3.9. Cd Concentration in Urine

The detection limit for urine was 0.17 ug Cd/L in experiment AF90. The urine samples for Experiments PA91 and GO91 were collected in metabolism cages. These urine samples exhibited severe analytical matrix interferences and could not be analyzed for Cd content by the EAAS system available. However, urine samples from Experiments AF90 were collected manually in beakers at each time point and showed increased Cd levels at T = 6 h, onwards. The short course urine Cd levels were significantly higher than controls at T = 24 h ($p \leq 0.05$, unpaired t test) and returned to background levels of 1.0 ± 0.5 ug Cd/L at T = 72 h (Figures 3.26). The subchronic urine Cd levels ranged from 79 to 93 ug Cd/L and were higher than the controls (1.0 ± 0.5 ug Cd/L) in all available samples (Figure 3.27 and Table 3.25).

3.3.10. Inter-Compartment Comparison and Mass Balance

3.3.10.1. *Inter-Compartment Comparison*

The overall EAAS detection limit (average of all C_L assessments) for Cd was 0.10 ± 0.05 ug Cd/L (or ug Cd/kg). Comparisons were made between measured whole blood Cd and that calculated from the Cd levels found in the individual blood cell compartments (Ly, M, PMN, and RBC) for Experiments PA91 (Figures 3.28 - 3.30) and GO91 (Figures 3.31 - 3.33), while only Ly, and M could be compared for Experiment AF90. The Cd concentrations found in the

individual blood cell compartments in exposed animals were well above the individual detection limits (reported in each section, 3.3.2 - 3.3.9). In some instances, the control and initial values were at or below the upper end of the overall limit (0.15 ug Cd/L). At specific time points, the inter-compartment Cd results were compared using the ANOVA (significance level set at 95%).

Baseline Cd levels averaged 0.34 $\pm$ 0.21 ug Cd/L in lymphocytes to a maximum of 1.5 $\pm$ 0.17 ug Cd/L in PMNs (Figure 3.28). Initially, the general order in the control blood cell compartments was *RBC > PMN $\geq$ M > Ly, where RBC Cd levels were significantly higher than all other cell compartments as analyzed by the ANOVA (* $p \leq 0.05$). The order changed to *PMN > RBC $\geq$ M $\geq$ Ly at T = 12 and 24 h. At T = 96 h the final order was *RBC $\geq$ *M > Ly > PMN, with the RBC and monocyte levels not significantly different from each other, but were significantly higher than those in the lymphocytes and PMNs (* $p \leq 0.05$).

In the short course low-dose group, the minimum Cd level averaged 0.50 $\pm$ 0.20 ug Cd/L in the monocytes to a maximum of 2.4 $\pm$ 0.50 ug Cd/L in the RBCs (Figure 3.29). Initially, the order of compartmentalization in the low dose was *PMN $\geq$ RBC $\geq$ Ly $\geq$ M from T = -20 to 12 h, where the PMN Cd levels were significantly higher than the lymphocyte and monocyte levels (*$p \leq 0.05$); the PMN and RBC levels were not different. Thereafter, changes in cell order occurred in a time-dependent manner. At T = 24 h the order was *Ly >> RBC $\geq$ PMN = M, with lymphocyte Cd levels significantly higher than in all other cells (*$p \leq 0.05$). This order changed to *RBC >> *M > Ly $\geq$ PMN at T = 48 h, with the RBC Cd levels significantly higher than in all other cells. Monocyte Cd levels were significantly higher than those in lymphocytes and PMNs (*$p \leq 0.05$). The final ranking at T = 96 h of the low dose blood cell compartments was *RBC >> PMN $\geq$ M $\geq$ Ly (* $p \leq 0.05$).

In the medium dose group, the Cd levels increased dramatically with minimum Cd levels of 0.33 $\pm$ 0.03 ug Cd/L detected in PMNs to a maximum of 11.5 $\pm$ 0.8 ug Cd/L in the RBCs (Figure 3.30). As seen in the low dose group, a

time-dependent change was observed in the ranking of the blood cell compartments. Initially, the order was *RBC ≥ M > Ly ≥ PMN, with RBC Cd levels significantly higher than in lymphocytes and PMNs (* $p \leq 0.05$), but not so in monocytes (including the time point T = 6 h). The leukocyte order changed at T = 12 h to *RBC >> PMN ≥ M > Ly, with RBC Cd levels again significantly higher than in all other cell compartments (* $p \leq 0.05$). By T = 24 h, the order reverted to the initial ranking of *RBC >> *M ≥ *Ly > PMN, with RBC Cd levels significantly higher than in all other cell types; levels in monocytes and lymphocytes were significantly higher than the PMNs, yet they were not different from each other (* $p \leq 0.05$). The Cd level in the RBC compartment increased with time, while that in leukocytes peaked at or below 2.5 ug Cd/L from T = 12 to 24 h and then returned to baseline values.

In the high dose animals, only lymphocyte and monocyte Cd levels were determined. The minimum level averaged 0.10 ± 0.08 ug Cd/L in lymphocytes to a maximum of 2.4 ± 2.1 ug Cd/L in monocytes. The order of Cd compartmentalization was M > Ly, with no significant differences detected at T = 6 and 24 h by the ANOVA.

In the subchronic experiments, the Cd levels in the control group were similar to the short course levels; with minimum Cd levels averaging 0.1 ± 0.08 ug Cd/L in the RBC and monocytes to a maximum of 1.6 ± 0.5 ug Cd/L in the PMNs (Figure 3.31). The general compartment ranking at T = -20 h was *PMN ≥ *Ly ≥ M > RBC, with PMN Cd levels significantly higher than in monocytes and RBCs, but not significantly different from lymphocytes (* $p \leq 0.05$). Cd concentrations in lymphocytes were significantly higher than in RBCs, but not in monocytes. At T = 24 h, the order changed to PMN ≥ Ly = M = RBC, and at T = 48 h to Ly ≥ M ≥ RBC ≥ PMN, with no significant differences detected by the ANOVA. At T = 96 h, the order of Cd levels in the cells reverted back to the initial sequence of *PMN ≥ *Ly > M > RBC (* $p \leq 0.05$).

In the subchronic low dose group, the minimum Cd concentrations

averaged 0.15 ± 0.2 ug Cd/L in the monocytes to a maximum level of 4.0 ± 1.7 ug Cd/L in the RBCs (Figure 3.32). The initial order of Cd concentration in the blood cell compartments for the low dose was *RBC >> *PMN ≥ Ly ≥ M, with the RBC Cd levels significantly higher than in all other cells; those in PMNs were significantly higher than in monocytes but not in lymphocytes (* $p \leq 0.05$). The compartment order changed to RBC > Ly > PMN > M from T = 12 h onwards, with no significant differences detectable by ANOVA.

In the subchronic medium dose group, the minimum Cd concentrations averaged 0.13 ± 0.11 ug Cd/L in the monocytes to a maximum of 19.2 ± 3.2 ug Cd/L in the RBCs (Figure 3.33). The initial order of Cd concentration in the subchronic medium dose was *RBC >>> Ly ≥ *PMN > M , with the RBC Cd levels significantly higher than in all other cell compartments. The lymphocyte and PMN Cd levels were significantly higher than in monocytes (* $p \leq 0.05$), although they were not different from each other. At T = 48 h, the cell order was *RBC >>> Ly ≥ PMN > M, with the RBC Cd levels significantly higher than in all other cell compartments (* $p \leq 0.05$).

In the high dose subchronic group, the minimum Cd concentration observed was 0.05 ug Cd/L in the lymphocytes and the maximum level was 1.7 ug Cd/L in the monocytes. The order of Cd compartmentalization was M > Ly, however no significant differences were detected.

3.3.10.2. *Mass Balance*

A second comparison involved summing the multiplication products of the normalized Cd concentrations (see Section 3.2.4.) for all blood compartments (Ly, M, PMN, plasma, and RBC) and the standard blood cell distribution numbers for the rat (Baker et al., 1979; see Table 5.1). These calculated levels were compared with values derived from the measured Cd concentrations and actual measured cell counts, as well as with the measured whole blood Cd levels. The calculated totals and the observed whole blood Cd levels were not significantly different between these assessments (Figures 3.34

and 3.35). The WB Cd concentration determined for the short course medium-dose group at T = 6 h may have been an artifact or experimental error (Figure 3.34, see Section 3.2.3.6). Since the RBC and PMN Cd determinations were not part of the experimental design in the earlier high-dose AF90 experiment, the mass balance calculations were not possible.

3.4. Discussion

The salient features of the Cd concentrations determined in the different tissues and blood cells from the *in vivo* time-course experiments are detailed in this section (3.4.1), which is followed by an interpretation section (3.4.2). In this and subsequent sections, Figures 6.1, 6.3, 6.5, and 6.7 have been consulted. The data in Chapter 3 were combined in these figures. Since Cd exposure and MT induction are closely interrelated, a model of Cd and MT dynamics is presented and discussed in Chapter 6.

3.4.1. Salient Features of Cd Accumulation

3.4.1.1. *Cd Concentration in Tissues*

• A dose-response was observed for the tissue Cd levels in the exposed rats (Figures 3.1 - 3.11, see also Section 3.3.1). Significantly higher Cd accumulation occurred in the *liver and *kidney (* $p \leq 0.05$) compared to spleen and lung for all Cd exposure groups in both experimental protocols (see Tables 3.3 - 3.4).

• There were differences in the Cd accumulation patterns seen in the two exposure protocols. With repeated Cd exposure, the proportion of Cd in the liver compartment was somewhat reduced (high dose) compared to the proportion of Cd in the kidney, spleen and to a lesser extent, the lung.

• Overall, Cd concentrations were higher in the subchronic time course animals than the short course.

• The basal Cd concentrations in the short-course control rats were significantly higher in the kidney (57 ug Cd/kg dwt) and spleen (43 ug Cd/kg

dwt; $p \leq 0.05$), compared to basal levels in liver (19 ug Cd/kg dwt) and lung (3 ug Cd/kg dwt). By comparison, subchronic basal Cd levels were significantly higher in kidney (193 ug Cd/kg dwt) and liver (84 ug Cd/kg dwt; $p \leq 0.05$) compared to spleen (41 ug Cd/kg dwt) and lung (13 ug Cd/kg dwt; see Section 3.3.1).

3.4.1.2. *Cd Concentration in WB and RBCs*

• In this and subsequent sections, the data for the subchronic high-dose (2.5 mg/kg; rat C) are excluded from the general discussion because there was only one rat which only survived till T = 12 h; visual inspection at necropsy did, however, provide clear evidence of acute Cd toxicity (see Chapter 5).

• In the short course experiments, the Cd accumulation in WB resembled a saturation curve with a suggestion of transient peaking around T = 6 h (Figure 3.12). By contrast, in the subchronic experiments there was little evidence of increased loading in the WB compartment in response to the Cd injection at T = 0 h (Figure 3.13). Nevertheless, the WB Cd levels were elevated at 'steady state' levels equal to or exceeding those observed at the end of the short course experiment.

• The Cd accumulation pattern in RBCs was very similar to that described for WB in both experimental protocols (Figures 3.16 - 3.17).

3.4.1.3. *Cd Concentration in Plasma*

• A perusal of the data summarized in Figures 3.14 - 3.15 clearly indicates that in both experimental protocols Cd reached maximum concentrations in the plasma compartment at T = 6 h.

• Plasma clearance was very rapid in the subchronic experiment, returning to or just above initial values by T = 24 h. The clearance in the short course experiment was slower, never returning completely to initial values.

3.4.1.4. *Cd Concentration in PMNs*

• A significant Cd dose-response was not evident in the PMNs from either the short course or subchronic treatment groups (Figures 3.18 - 3.19).

3.4.1.5. *Cd Concentration in Lymphocytes*

• It is clear from the lymphocyte data in Figures 3.20 - 3.21 that patterns similar to WB and RBC 'steady state' levels were observed in the subchronic group, with an indication of some transient elevations of Cd in the short course between T = 6 - 24 h.

• By contrast to the RBCs, the level of Cd in the lymphocytes at the medium dose (1.25 mg/kg), while still elevated, was lower than the 0.25 mg/kg treatment group in both protocols.

3.4.1.6. *Cd Concentration in Monocytes*

• The monocyte response did not resemble those observed for RBCs and lymphocytes. In the subchronic experiments, there was no evidence of Cd elevation for any of the administered doses. By contrast, in the short course groups there was some evidence for a transient elevation of Cd with time between T = 6 - 48 h (Figures 3.22 - 3.23).

3.4.1.7. *Cd Concentration in Feces and Urine*

• In both experimental groups, significant amounts of Cd was excreted between T = 6 - 72 h. A comparison of the data summarized in Figures 3.24 - 3.25 indicated that the relative amounts of Cd was greater in the feces of the short course animals.

• Although the urinary Cd data are very limited, the subchronic levels appeared higher by about a factor of approximately 1.5 as shown in Figures 3.26 - 3.27.

3.4.1.8. *Inter-Compartment Comparison and Mass Balance*

• The inter-compartment concentration comparisons on a 'per cell basis' and expressed as ug Cd/L showed the Cd levels were the highest in RBCs for both single and multiple exposures (Figures 3.29 - 3.30, and 3.31 - 3.33).

• Perusal of all the data related to leukocytes (Figures 3.18 - 3.23) and RBCs (Figures 3.16 - 3.17) demonstrated transient responses in the leukocytes, while the RBCs showed a gradual increase toward a systemic 'steady state'

response. Subchronic RBCs and lymphocytes also showed 'steady state' Cd levels.

• The total Cd concentrations in the WB compartment calculated from the expected cell distribution and from measured cell numbers (i.e., mass balance) were within reasonable agreement.

3.4.2. Interpretation of the Observed Cd Levels

3.4.2.1. *Cd Concentration in Tissues*

Immediately after a single IP injection of $CdCl_2$, the liver and kidney appear to be the important tissue compartments for Cd accumulation while the spleen and lung appear to be secondary. With repeated exposure, the kidney compartment appears to becomes a more important depot of Cd, followed by the spleen and then lung, supporting earlier conclusions (Lauwerys 1983a; Friberg et al., 1986a; Goyer, 1991; EHC, 1992a). The data suggest that the expected transfer of Cd from liver to kidney takes place over a time period longer than the present time course.

3.4.2.2. *Cd Concentration in Whole Blood*

Cd levels in WB reached a maximum at T = 6 h in both experimental protocols, no doubt reflecting absorption of the injected $CdCl_2$. Presumably this is followed by a redistribution of Cd in the next 6 - 12 h. In addition, the subchronic WB Cd levels were already elevated at 'steady state' levels at T = 0 h equal to or exceeding those observed at the end of the short course experiment. The short course animals showed a gradual increase in WB Cd concentration. These findings support earlier studies in rats and mice (Nordberg et al., 1971b; Garty et al., 1981; Tanaka et al., 1985; Tanaka et al., 1987). The subchronic longitudinal Cd concentrations did not rise significantly above initial Cd levels at T = 0 h, which suggests that a 'steady state' condition had been established with the earlier doses (Nordberg et al., 1971b; Lauwerys 1983a; Shaikh et al., 1990).

3.4.2.3. *Cd Concentration in Plasma*

Evidence of a sustained or 'steady state' plasma Cd level was not observed in either protocol. Rapid clearance of Cd from the plasma compartment occurred in the subchronic animals, while the single-exposure animals showed evidence of a more prolonged Cd distribution/redistribution period, supporting the role of tissue (liver) MT in the removal of Cd from plasma (see Chapters 4 and 6). These results are in agreement with previous studies (Nordberg et al., 1971a; Garty et al., 1981; Shaikh and Tohyama, 1984; Tanaka et al., 1985; Duval and Grubb, 1986; Bremner et al., 1987; Wang et al., 1993).

3.4.2.4. *Cd Concentration in RBCs*

The initial Cd peak observed in the WB, which is interpreted to reflect a rapid absorption of the administered Cd dose into the blood stream, was not seen in the RBC compartment. However, the RBCs did show a significant, time-dependent Cd accumulation and dose-response in both the short course and subchronic exposure groups, similar to that seen for WB. Increased RBC Cd levels were observed between 1 to 4 days after the single exposure and support earlier animal studies (Nordberg et al., 1971b; Garty et al., 1981; Tanaka et al., 1985; Tanaka et al., 1987). Although the exact mechanism by which Cd enters the cells was not investigated in the present study, earlier work has demonstrated that Cd enters the RBCs primarily by passive transport, at least *in vitro* (Garty et al., 1986). The delayed Cd increase observed is interpreted to reflect gradual incorporation into erythroblasts, with subsequent MT induction (see Section 4.3.4.3; Garty et al., 1981; Tanaka et al., 1985).

The data reported supports the conclusion that the RBC compartment is a reliable index of Cd accumulation, reflecting both current and chronic exposures (Garty et al., 1981; Lauwerys 1983a; Shaikh et al., 1990). Since the life span of RBCs in humans is 120 days (Hall and Malia, 1984) and 84 days in the rat (Ringler and Dabich, 1979), a gradual decline in RBC Cd concentration after cessation of exposure may be expected and is observed (Tanaka et al., 1985).

3.4.2.5. *Cd Concentration in PMNs*

The transient and 'steady state' trends observed in the WB were not observed in the PMN compartment. Overall, PMN Cd concentrations were at or below the control levels determined in the RBCs. Significant differences were not observed between the Cd exposed and non-exposed groups. The PMN results appear, judging by the poor reproducibility, to represent general background scatter in both the short course and subchronic experiments. It appears that the Cd levels in PMNs were too close to the detection limit (see Section 3.3.5.). Perhaps the short residence time of PMNs in blood prevents uptake, although limitations in cell separation or cell lysis cannot be ruled out as contributing factors.

For the reasons cited, PMN Cd levels do not appear to serve as a reliable index of Cd accumulation or body burden. However, the rise in PMN cell numbers appear to reflect Cd cytotoxicity (see Section 5.4.2; Enger et al., 1983). Our findings do not support an earlier study showing significant elevations of PMN Cd levels in cell culture. This differential response likely reflects differences in the *in vitro* and *in vivo* PMN responses to Cd (Enger et al., 1983).

3.4.2.6. *Cd Concentration in Lymphocytes*

In both experimental protocols, a significant dose-response was observed for Cd accumulation in the lymphocytes, in agreement with earlier findings (Hildebrand and Cram, 1979; Garty et al., 1981; Harley et al., 1989). The lymphocyte results differed from the other blood cells in that Cd accumulation for the medium dose (1.25 mg/kg), while still elevated, was lower than in the 0.25 mg/kg treatment group in both protocols. Cd-induced cytotoxicity may have occurred, as seen in cell cultures (Harley et al., 1989). The lymphocyte LD_{50} has been determined to be 93 uM Cd, which was lower than that for PMNs and higher than monocytes (Enger et al., 1983; see Section 3.1.1.). Studies of human and mouse lymphocyte subpopulations (T- and B-

cells) have demonstrated that T-cells were more sensitive to the effects of Cd toxicity then B-cells (Koizumi et al., 1987). In normal rat whole blood, 60 - 70% of small lymphocytes are T-cells, 30 - 40% are B-cells (Carter and Bazin, 1979). Since our study did not separate T and B lymphocytes, we must accept that a portion of the total circulating lymphocytes may have succumbed to Cd toxicity in the medium and high dose groups.

As for PMNs, the relatively poor reproducibility observed for Cd in lymphocytes in the short course experiments is due to the relatively low levels compared to the detection limit, as well as cell losses. The drop in lymphocyte numbers (see Section 5.4.2) may signify direct cytotoxicity, although their dispatch in response to tissue damage is more likely (Carter and Bazin, 1979; Hall and Malia, 1984). Injections of $CdCl_2$ have been documented to cause cell injury (Duval and Grubb, 1986; Waalkes et al., 1989; 1991a), which would likely initiate lymphocyte diapedesis and recirculation, and thus remove lymphocytes from peripheral circulation (Carter and Bazin, 1979; Hall and Malia, 1984).

In conclusion lymphocyte Cd levels do reflect both short-term and long-term exposure, but appear most suitable as an index to the latter.

3.4.2.7. *Cd Concentration in Monocytes*

Only in the short course experiments did monocyte Cd levels show a measurable dose-response, reflecting the relatively short residence time in the blood compartment. Differentiation into macrophages in response to Cd as a foreign agent in the tissues may have contributed to an even shorter residency period (Carter and Bazin, 1979; Hall and Malia, 1984). As for PMNs (both protocols) and lymphocytes (short-course), the absence of clear trends in the subchronic data likely reflects both analytical and biological limitations. Of the WBCs, monocytes appear to be the most sensitive to the toxicity of Cd with an LD_{50} of 66 uM (see Section 3.1.1). This may also be a cause of short-term variability. These findings add support to the cytotoxicity observed in mononuclear leukocytes studies (Enger et al., 1983; Harley et al., 1989).

In conclusion, monocytes appear to serve as an index to short-term exposure. However, the sampling times chosen after exposure appear critical.

3.4.2.8. *Cd Concentration in Feces*

The results reported demonstrate that animals without prior exposure excreted larger quantities of Cd in the feces than those receiving repeated Cd doses (Figures 3.24 - 3.25). This is consistent with earlier studies in which Cd is initially eliminated via the bile and feces as free Cd. When CdMT forms, the route of elimination shifts away from bile toward urine (Klaassen and Kotsonis, 1977; Klaassen, 1985; Friberg et al., 1986a). The critical sampling time for Cd elimination via the feces was 12 to 24 hours post exposure, although elevated levels were persistent for the period T = 12 to 72 h.

3.4.2.9. *Cd concentration in Urine*

In both experimental protocols the low and medium dose samples could not be analyzed due to matrix effects, thus limiting the available data. Nevertheless, the data suggest that urinary elimination of Cd was increasingly more important in animals receiving repeated Cd exposure in agreement with earlier reports that have shown urinary Cd concentration to be a good measure of body burden (Lauwerys, 1983a; Lauwerys, 1983b; Klaassen, 1985; Friberg et al., 1986a).

3.4.2.10. *Inter-Compartment Comparison*

Overall, the RBCs alone showed evidence of significant Cd accumulation (both protocols) followed by lymphocytes (subchronic) and monocytes (short course). While on a 'per cell' basis the leukocytes may exhibit higher Cd concentrations than the RBCs, the total proportion of leukocyte Cd in the blood is insignificant in comparison to the total Cd contribution by the RBCs, in agreement with earlier reports (Hildebrand and Cram, 1979; Garty et al., 1981; EHC, 1992a). The data clearly indicated that there is a critical (optimum) sampling time associated with each cell type for the determination of Cd as a biomarker of exposure (see Section 3.5).

3.4.2.11. *Mass Balance*

The mass balance calculations served to validate that the sum total of the Cd concentrations found in the individual blood compartments corresponded to that determined in WB. The estimate derived from the normalized concentrations and the accepted cell distribution pattern was higher than that based on actual cell numbers. Presumably, this reflects the limitations of using assumed cell numbers. However, both methods of calculating the total Cd concentrations in the WB compartments were in reasonable agreement with the actual WB Cd levels. The peak WB Cd concentration at T = 6 h was not reproduced by the calculated estimates. Some other deviations were also noted in the medium dose groups, perhaps reflecting errors in the estimation of WB volume as altered by coagulation (see Section 3.2.3.6), in the cell isolation process, or in the analytical step.

3.5. Conclusions

A dose-response was observed for Cd accumulation in the selected major tissues (liver, kidney, spleen, and lung), WB, and RBCs from the short course and subchronic time course rats. Sustained levels of Cd were not evident in the plasma, PMNs, and monocytes. Cd elimination was higher in the feces of the single versus multiple exposure animals, while urinary excretion was higher for multiple doses compared to the single injection.

An integral part of Cd dynamics in the selected organs, blood cells and body fluids is the induction of MT. This is addressed in Chapter 4.

The data suggest that optimum sampling times are associated with each blood compartment for the determination of Cd as an indicator of exposure. In the short course experiments, the most suitable sampling times were: 12 h for PMN, 12 - 24 h for lymphocytes, 24 - 48 h for monocytes, and 6 - 48 h post exposure for RBCs. Since a 'steady state' plateau was observed for WB, RBCs, and lymphocytes in the subchronic protocol, sampling times were not critical.

The evidence presented in this chapter clearly establishes Cd in RBCs and WB as reliable indices of Cd accumulation and possible body burden for short- and long-term exposures. Cd concentration in lymphocytes show promise as an index to long-term exposure (and presumably body burden), while that in monocytes shows promise as an indicator of short-term exposure. However, the analytical challenges for these two cell types are more demanding.

Table 3.1. Instrumental Parameters for Cd Analysis by EAAS.

A. AA Spectrometer Parameters

1. Hollow Cathode Lamp Current	6 mW
2. Wavelength	228.8 nm
3. Slit Width	7 (0.4 nm)
4. Deuterium Background Corrector	On
5. Peak Integration Time	5s
6. Signal	Absorbance
7. Mode	Peak Area
8. Internal Argon Gas Flow[a]	300 mL/min
9. Graphite Tube	L'vov platform, pyrolytic

B. Automatic Sampler

1. Sample Size	20 uL

C. Graphite Furnace Parameters

Step	Temp(°C)	Ramp(s)	Hold(s)	Read/Rec	Baseline
1. Drying	130	40	0	off	off
2. Charring	350	30	10	off	at 34s
3. Atomization	1600	0	5	0 ,-1 rec,	off
4. Cleaning	2300	1	2	off	off
5. Cooling	20	10	10	on	off

a. Internal argon flow was 0 mL/min during Step 3.

Table 3.2. Chemical Reagents Employed in Cd Analysis by EAAS.

Chemical Reagent	Source	Grade	Comments
Acid mixture	J.T. Baker	Ultrex	3:1:1 mixture of c HNO_3, $HClO_4$, and H_2SO_4 acids used for tissue digestions.
Cd standard	Fisher	Certified AAS	1000 ug Cd/mL in dilute HNO_3 used for cadmium standards.
HNO_3	J.T. Baker,	Ultrex II	70% (w/v) solution, for tissue digestions; 1 and 10 % used for standards and sample dilutions.
HNO_3	BDH	Analytical	2 M used for washing glassware.
$HClO_4$	J.T. Baker,	Ultrex	60% (w/v) solution, for tissue digestions.
H_2SO_4	J.T. Baker,	Ultrex	60% (w/v) solution, for tissue digestions.
$Mg(NO_3)_2$	BDH	AnalaR	0.2 M used as matrix modifier.
Pd powder	Alfa Products	Certified	0.5-0.7% Pd as matrix modifier.
Triton X-110	BioRad	Electrophoresis purity	0.1% used for atomic absorption spectrometry, matrix modifier.
Water (DDW)	McMaster	Ultra-pure	Prepared by demineralizing laboratory distilled water using a Corning LD-2a demineralizer and Corning Mega-Pure still.

TABLE 3.3. Cadmium Concentrations in Tissues, Short Course Experiments.

Tissue	Administered Dose (mg/kg)		Individual Concentrations (ug Cd/kg dwt) [a,b]					Mean (minimum n=3)	± SD	CV (%) [c]
Liver	0.00	mg/kg	19	34	25	7	8	19	12	62
	0.25	mg/kg	7516	9065	9484	-	-	8688	1037	12
	1.25	mg/kg	24742	28726	25133	-	-	26200	2196	8
	2.50	mg/kg	98560	117600	112672	-	-	109611	9882	9
Kidney	0.00	mg/kg	67	96	87	21	14	57	38	67
	0.25	mg/kg	4902	4402	2059	-	-	3788	1518	40
	1.25	mg/kg	10951	12830	17722	-	-	13834	3495	25
	2.50	mg/kg	42739	20138	21420	-	-	28099	12695	45
Spleen	0.00	mg/kg	43	42	89	35	8	43	29	67
	0.25	mg/kg	466	858	487	-	-	604	221	37
	1.25	mg/kg	3250	3800	3833	-	-	3628	327	9
	2.50	mg/kg	4025	3081	2707	-	-	3271	679	21
Lung	0.00	mg/kg	2	3	4	-	-	3	1	33
	0.25	mg/kg	288	139	289	-	-	239	86	36
	1.25	mg/kg	1300	1019	1034	-	-	1118	158	14

a. Values for liver, 0.25 mg/kg spleen and kidney samples are reported as averages of duplicate determinations; remaining values are for single determinations.
b. For rat codes see Table 2.1: 0.0 mg/kg = T, U, AA, B, E, from left to right respectively; 0.25 mg/kg= V, W, X; 1.25 mg/kg = P, Q, R; 2.5 mg/kg = A, D, F.
c. Inter-animal coefficient of variation (CV %).

TABLE 3.4. Cadmium Concentration in Tissues, Subchronic Experiments.

Tissue	Administered Dose (mg/kg)	Individual Concentration[a,b] (ug Cd / kg dwt)			Mean n = 3	± SD	CV[c] (%)
Liver	0.00 mg/kg	63	90	99	84	19	23
	0.25 mg/kg	12500	12016	12917	12478	451	4
	1.25 mg/kg	58466	78758	82847	73357	13057	18
	2.50 mg/kg	283920	-	-	-	-	-
Kidney	0.00 mg/kg	188	218	174	193	22	12
	0.25 mg/kg	7059	6242	7176	6826	509	7
	1.25 mg/kg	39384	39557	65260	48067	14890	31
	2.50 mg/kg	125821	-	-	-	-	-
Spleen	0.00 mg/kg	45	35	43	41	5	12
	0.25 mg/kg	3426	3562	3765	3584	171	5
	1.25 mg/kg	6944	24755	13861	15187	8979	59
Lung	0.00 mg/kg	18	7	13	13	6	43
	0.25 mg/kg	499	653	652	601	88	15
	1.25 mg/kg	1781	2956	2972	2570	683	27

a. Each rat received a total of three IP injections of the indicated CdCl2 doses over a 1.5-2 month period. The third injection was administered at T = 0 h. For rat codes see Table 2.2: 0.0 mg/kg = G,H,I, respectively from right to left; 0.25 mg/kg = J,K,L; 1.25 mg/kg = M,N,O; 2.5 mg/kg = C.

b. Values for liver, 0.25 mg/kg spleen and kidney samples are reported as averages of duplicate digestions, remaining values are for single digestion determinations.

c. Inter-animal coefficient of variation (% CV)

TABLE 3.5. Cadmium Concentration in Duplicate Digested Liver Samples.

Administered Dose: mg/kg	Rat Code [a]	Concentration (ug Cd/kg dwt) [b] Repeat 1	Repeat 2	Average	Mean ± SD [c]	CV (%) [d]
Short Course Experiment:						
0.00 mg/kg	T	26	13	19	24	46
	U	42	26	34	11	
	Z	19	16	18		
	AA	13	38	25		
0.25 mg/kg	V	7226	7806	7516	8484	16
	W	8258	9871	9065	1382	
	X	11226	7742	9484		
	Y	7355	8387	7871		
1.25 mg/kg	P	24194	25290	24742	26200	8
	Q	29839	27613	28726	2185	
	R	26000	24267	25133		
Subchronic Experiment:						
0.00 mg/kg	G	52	75	63	84	33
	H	58	121	90	28	
	I	110	88	99		
0.25 mg/kg	J	11928	13072	12500	12478	6
	K	11774	12258	12016	736	
	L	12167	13667	12917		
1.25 mg/kg	M	52000	67900	59950	73852	17
	N	74194	83323	78758	12496	
	O	80000	85694	82847		

a. Capital letters denote individual rats (see Tables 2.1 and 2.2).
b. Dates samples were analysed: Repeat 1 on 12-10-91, Repeat 2 on 2-10-92.
c. Mean ± standard deviation; short course 0.0, 0.25 mg/kg (n = 8); 1.25 mg/kg, subchronic (n = 6).
d. Inter-animal coeficient of variation (CV %).

Table 3.6. Cadmium Concentration in Whole Blood, Short Course Experiment PA91.

Time (h)	Cadmium Concentration (ug Cd/L)[a]			Mean ± SD[b]		CV (%)[c]
Administered Dose: 0.00 mg/kg				minimum (n=3)		
	T	U	AA			
-20	1.4	0.6	6.5	2.8	3.2	113
0	1.5	0.0	6.8	2.8	3.6	128
6	4.2	0.9	5.8	3.6	2.5	68
12	4.0	1.5	8.1	4.5	3.3	73
24	4.9	3.1	7.7	5.2	2.3	44
48	0.0	1.6	7.2	3.0	3.8	128
72	0.0	2.7	6.8	3.2	3.4	108
96	1.9	0.6	7.9	3.5	3.9	112
Administered Dose: 0.25 mg/kg						
	V	W	X			
-20	5.8	5.0	5.2	5.3	0.4	7.3
0	4.8	4.3	4.6	4.6	0.2	5.3
6	25.9	23.0	24.0	24.3	1.5	6.0
12	17.7	15.8	13.7	15.7	2.0	12.6
24	11.1	11.1	9.6	10.6	0.9	8.0
48	15.3	13.6	13.6	14.2	1.0	6.9
72	14.9	18.2	15.3	16.2	1.8	11.3
96	19.2	18.7	20.4	19.4	0.9	4.4
Administered Dose: 1.25 mg/kg						
	P	Q	R			
-20	2.7	0.5	2.9	2.1	1.3	63.6
0	2.6	2.6	2.6	2.6	0.0	0.1
6	82.9	95.0	93.8	90.6	6.6	7.3
12	37.4	30.7	21.8	30.0	7.8	26.1
24	49.2	23.8	31.3	34.8	13.0	37.4
48	48.7	46.1	32.0	42.3	9.0	21.2
72	31.5	39.5	40.5	37.2	4.9	13.3
96	63.1	50.1	50.1	54.4	7.5	13.8

a. Capital letters denote individual rats (see Table 2.1).
b. Mean ± standard deviation (minimum n = 3).
c. Inter-animal coefficient of variation (% CV).

Table 3.7. Cadmium Concentration in Whole Blood, Subchronic Experiment G091.

Time (h)	Cadmium Concentration (ug Cd/L)[a]			Mean ± SD[b]		CV (%)[c]
Administered Dose: 0.00 mg/kg				(minimum n=2)		
	G	H	I			
-20	5.3	8.2	6.5	6.7	1.4	21.7
0	8.6	8.4	5.9	7.7	1.5	20.0
6	6.9	7.5	8.3	7.6	0.7	8.9
12	5.9	6.5	8.6	7.0	1.4	20.5
24	8.0	7.5	7.2	7.6	0.4	5.4
48	7.4	7.5	7.1	7.3	0.2	2.3
72	6.5	8.2	-	7.4	1.2	15.9
96	8.2	8.1	-	8.1	0.04	0.5
Administered Dose: 0.25 mg/kg						
	J	K	L			
-20	18.2	31.7	20.4	23.4	7.2	30.8
0	20.4	25.6	24.2	23.4	2.7	11.7
6	44.2	35.3	46.5	42.0	5.9	14.0
12	28.2	-	25.0	26.6	2.3	8.5
24	60.5	-	54.9	57.7	3.9	6.8
48	29.7	-	32.0	30.8	1.7	5.4
72	20.7	-	-	-	-	-
96	27.3	-	-	-	-	-
Administered Dose: 1.25 mg/kg						
	M	N	O			
-20	107.9	108.1	96.3	104.1	6.8	6.5
0	108.3	110.7	100.9	106.6	5.2	4.8
6	128.9	130.7	134.2	131.3	2.7	2.1
12	97.4	97.9	102.9	99.4	3.1	3.1
24	86.4	90.2	104.1	93.6	9.3	9.9
48	110.9	110.5	115.3	112.2	2.6	2.3
72	91.4	104.3	105.7	100.5	7.9	7.9
96	92.2	-	91.7	91.9	0.4	0.4

a. Capital letters denote individual rats (see Table 2.2).
b. Mean ± standard deviation (minimum n = 2).
c. Inter-animal coefficient of variation (% CV).

Table 3.8. Cadmium Concentration in Whole Blood, Short Course AF90 and Subchronic CD90 Experiments.

Time (h)	Cadmium Concentration (ug Cd/L)[a]			Mean ± SD[b]		CV (%)[c]
Short Course:						
Administered Dose: 0.00 mg/kg				minimum (n=2)		
	B	E				
-20	0.8	0.3		0.6	0.4	73
0	1.3	0.4		0.8	0.6	78
6	1.2	0.3		0.7	0.6	88
12	1.2	0.5		0.8	0.5	59
24	0.1	0.0		0.06	0.04	64
34	0.2	0.1		0.17	0.07	42
48	-	0.5		-	-	-
96	0.5	-		-	-	-
Administered Dose: 2.50 mg/kg						
	A	F	D			
-20	0.5	0.4	0.2	0.36	0.17	47
0	2.4	0.8	0.4	1.19	1.07	90
6	73.0	32.6	-	52.8	28.5	54
12	68.3	27.1	-	47.7	29.2	61
24	74.5	35.3	45.1	51.6	20.4	39
34	-	39.4	-	-	-	-
96	91.2	-	-	-	-	-
Subchronic:						
Administered Dose: 2.50 mg/kg						
	C					
-20	46.3			-	-	-
-12	39.9			-	-	-
0	47.0			-	-	-
12	155.5			-	-	-

a. Capital letters denote individual rats (see Tables 2.1 and 2.2).
b. Mean ± standard deviation (minimum n = 2).
c. Inter-animal coefficient of variation (% CV).

Table 3.9. Cadmium Concentration in Plasma, Short Course Experiment PA91.

Time (h)	Cadmium Concentration (ug Cd/L)[a]			Mean ± SD[b]		CV (%)[c]
Administered Dose: 0.00 mg/kg				minimum (n=3)		
	T	U	AA			
-20	1.0	0.7	0.8	0.8	0.2	18.4
0	1.0	0.8	1.0	1.0	0.1	12.8
6	0.8	0.5	0.9	0.7	0.2	30.0
12	0.8	0.8	1.1	0.9	0.2	20.0
24	0.7	0.6	0.7	0.7	0.1	9.1
48	0.8	0.8	0.8	0.8	0.02	2.1
72	0.8	0.8	1.4	1.0	0.3	32.9
96	1.0	0.5	1.3	0.9	0.4	45.8
Administered Dose: 0.25 mg/kg						
	V	W	X			
-20	0.7	0.8	0.5	0.7	0.1	20.2
0	1.1	0.8	0.8	0.9	0.2	22.4
6	5.5	4.6	4.6	4.9	0.5	9.9
12	4.8	4.4	3.4	4.2	0.7	17.0
24	2.4	1.9	2.3	2.2	0.2	11.4
48	1.3	1.4	1.3	1.3	0.03	2.6
72	1.7	2.0	1.5	1.7	0.2	14.1
96	1.7	1.5	1.9	1.7	0.2	12.3
Administered Dose: 1.25 mg/kg						
	P	Q	R			
-20	1.5	1.1	1.2	1.3	0.2	17.2
0	0.9	0.8	1.1	0.9	0.1	13.3
6	9.3	7.4	9.4	8.7	1.1	12.5
12	6.1	4.4	4.4	4.9	1.0	19.6
24	5.3	3.7	3.5	4.2	1.0	24.7
48	2.3	1.8	1.9	2.0	0.3	14.8
72	2.5	1.9	1.5	2.0	0.5	24.6
96	3.5	2.8	3.1	3.1	0.4	12.6

a. Capital letters denote individual rats (see Table 2.1).
b. Mean ± standard deviation (minimum n = 3).
c. Inter-animal coefficient of variation (% CV).

Table 3.10. Cadmium Concentration in Plasma, Subchronic Experiment G091.

Time (h)	Cadmium Concentration (ug Cd/L) [a]			Mean ± SD [b]		CV (%) [c]
Administered Dose: 0.00 mg/kg				(minimum n=2)		
	G	H	I			
-20	1.4	2.0	1.1	1.5	0.4	29.3
0	1.1	1.5	1.3	1.3	0.2	15.2
6	1.4	2.2	1.4	1.7	0.5	28.6
12	1.9	1.9	1.9	1.9	0.03	1.8
24	1.7	2.0	2.0	1.9	0.1	6.4
48	1.4	2.0	1.4	1.6	0.3	22.0
72	2.1	1.1	-	1.6	0.7	41.9
96	2.0	0.9	-	1.4	0.8	53.0
Administered Dose: 0.25 mg/kg						
	J	K	L			
-20	1.3	1.4	2.0	1.5	0.4	25.0
0	1.4	1.4	1.7	1.5	0.2	10.6
6	7.4	5.3	7.2	6.6	1.2	17.6
12	2.5	-	3.8	3.1	0.9	27.7
24	2.2	-	2.6	2.4	0.3	10.6
48	1.8	-	1.7	1.7	0.1	3.7
72	3.2	-	-	-	-	-
96	1.8	-	-	-	-	-
Administered Dose: 1.25 mg/kg						
	M	N	O			
-20	2.7	3.6	3.2	3.2	0.5	14.2
0	3.2	2.6	2.3	2.7	0.5	18.0
6	9.9	11.5	10.4	10.6	0.8	7.8
12	4.8	6.7	6.9	6.1	1.2	19.0
24	2.3	2.2	2.2	2.2	0.1	3.9
48	2.3	3.4	2.5	2.7	0.6	21.9
72	1.9	5.0	3.2	3.4	1.5	46.0
96	2.4	-	6.1	4.2	2.6	61.2

a. Capital letters denote individual rats (see Table 2.2).
b. Mean ± standard deviation (minimum n = 2).
c. Inter-animal coefficient of variation (% CV).

Table 3.11. Cadmium Concentration in Plasma, Short Course AF90 and Subchronic CD90 Experiments.

Time (h)	Cadmium Concentration (ug Cd/L)[a]			Mean ± SD[b]		CV (%)[c]
Short Course:						
Administered Dose: 0.00 mg/kg				minimum (n=2)		
	B	E				
-20	0.01	0.4		0.2	0.3	135
0	0.6	0.1		0.3	0.4	120
6	-	0.4		-	-	-
12	0.0	0.9		0.5	0.6	138
24	0.2	0.0		0.10	0.12	127
34	-	1.1		-	-	-
48	-	0.2		-	-	-
96	2.2	-		-	-	-
Administered Dose: 2.50 mg/kg						
	A	F	D			
-20	0.02	0.3	1.6	0.6	0.9	132
0	1.5	0.7	1.5	1.2	0.5	40
6	7.1	8.9	-	8.0	1.3	16
12	9.0	2.9	-	6.0	4.3	72
24	5.6	1.6	10.7	5.9	4.6	77
34	-	5.0	-	-	-	-
96	22.2	-	-	-	-	-
Subchronic:						
Administered Dose: 2.50 mg/kg						
	C					
-20	0.7			-	-	-
-12	1.3			-	-	-
0	2.1			-	-	-
12	40.2			-	-	-

a. Capital letters denote individual rats (see Tables 2.1 and 2.2).
b. Mean ± standard deviation (minimum n = 2).
c. Inter-animal coefficient of variation (% CV).

Table 3.12. Cadmium Concentration in Erythrocytes, Short Course Experiment PA91.

Time (h)	Cadmium Concentration (ug Cd/L)[a]			Mean ± SD[b]		CV (%)[c]
Administered Dose: 0.00 mg/kg				(minimum n=3)		
	T	U	AA			
-20	1.2	0.9	0.6	0.9	0.3	34
0	1.4	1.5	0.6	1.2	0.5	39
6	1.4	1.0	0.5	1.0	0.4	44
12	1.0	0.8	0.6	0.8	0.2	25
24	1.2	1.1	0.6	1.0	0.4	38
48	1.0	0.7	0.5	0.7	0.3	36
72	1.5	0.7	0.4	0.9	0.6	68
96	1.9	1.2	0.5	1.2	0.7	57
Administered Dose: 0.25 mg/kg						
	V	W	X			
-20	1.1	0.6	0.6	0.8	0.3	36.9
0	1.0	0.7	0.6	0.8	0.2	28.1
6	0.8	1.4	1.1	1.1	0.3	28.6
12	1.1	1.5	1.3	1.3	0.2	15.9
24	1.4	1.1	1.0	1.2	0.2	20.1
48	2.9	2.4	2.0	2.4	0.5	18.7
72	2.7	2.7	1.8	2.4	0.5	20.9
96	2.5	2.2	2.4	2.4	0.2	6.3
Administered Dose: 1.25 mg/kg						
	P	Q	R			
-20	0.6	1.0	1.2	0.9	0.3	35.3
0	1.0	1.6	1.1	1.2	0.3	25.4
6	6.1	4.2	3.6	4.6	1.4	29.2
12	4.7	4.5	4.7	4.6	0.1	2.6
24	5.9	4.6	5.7	5.4	0.7	13.5
48	7.7	8.5	8.1	8.1	0.4	4.9
72	10.5	11.8	12.1	11.5	0.8	7.0
96	8.7	11.3	9.6	9.9	1.3	13.5

a. Capital letters denote individual rats (see Table 2.1).
b. Mean ± standard deviation (minimum n = 3).
c. Inter-animal coefficient of variation (% CV).

Table 3.13. Cadmium Concentration in Erythrocytes, Subchronic Experiment G091.

Time (h)	Cadmium Concentration (ug Cd/L)[a]			Mean ± SD[b]		CV (%)[c]
Administered Dose: 0.00 mg/kg				(minimum n=2)		
	G	H	I			
-20	0.5	0.3	0.4	0.4	0.1	19
0	0.1	0.0	0.3	0.12	0.14	119
6	0.0	0.1	0.2	0.11	0.09	77
12	0.1	0.1	0.4	0.20	0.18	89
24	0.2	0.0	0.9	0.4	0.5	136
48	0.0	0.2	1.1	0.5	0.6	130
72	0.5	0.5	-	0.5	0.03	5.0
96	0.4	0.1	-	0.24	0.19	78
Administered Dose: 0.25 mg/kg						
	J	K	L			
-20	2.8	4.0	2.9	3.2	0.7	20
0	2.4	4.0	3.0	3.1	0.8	27
6	3.5	4.3	3.5	3.8	0.5	12
12	2.8	-	3.6	3.2	0.6	18
24	5.3	-	2.8	4.0	1.7	43
48	4.0	-	4.2	4.1	0.1	3.1
72	3.9	-	-	-	-	-
96	4.7	-	-	-	-	-
Administered Dose: 1.25 mg/kg						
	M	N	O			
-20	19.7	18.1	17.6	18.5	1.1	5.8
0	20.1	16.0	15.6	17.2	2.5	14
6	18.5	17.5	18.6	18.2	0.6	3.5
12	16.2	16.4	17.0	16.5	0.4	2.6
24	17.7	17.9	15.2	16.9	1.5	9.0
48	16.8	20.2	18.4	18.5	1.7	9.1
72	15.5	21.3	20.8	19.2	3.2	17
96	15.9	-	18.3	17.1	1.7	9.9

a. Capital letters denote individual rats (see Table 2.2).
b. Mean ± standard deviation (minimum n = 2).
c. Inter-animal coefficient of variation (% CV).

Table 3.14. Cadmium Concentration in Polymorphonuclear Leukocytes, Short Course Experiments PA91.

Time (h)	Cadmium Concentration (ug Cd/L) [a]			Mean ± SD [b]		CV (%) [c]
Administered Dose: 0.00 mg/kg				minimum (n=3)		
	T	U	AA			
-20	0.6	0.2	1.7	0.8	0.7	90
0	0.2	0.9	0.3	0.5	0.4	82
6	0.6	1.5	0.8	1.0	0.5	50
12	1.6	1.3	1.6	1.5	0.2	11
24	0.4	1.9	0.9	1.1	0.8	72
48	0.2	0.3	0.9	0.5	0.4	76
72	0.9	0.4	0.6	0.6	0.3	47
96	0.3	0.1	0.5	0.3	0.2	56
Administered Dose: 0.25 mg/kg						
	V	W	X			
-20	2.1	1.8	2.2	2.0	0.2	8.5
0	1.3	1.3	0.6	1.0	0.4	40
6	1.2	0.8	1.8	1.3	0.5	36
12	1.6	1.6	2.7	2.0	0.6	33
24	0.9	0.8	0.9	0.9	0.1	9.5
48	0.8	0.5	0.6	0.6	0.2	26
72	1.0	1.3	0.5	0.9	0.4	44
96	1.3	1.1	0.5	1.0	0.4	42
Administered Dose: 1.25 mg/kg						
	P	Q	R			
-20	0.6	0.3	0.4	0.4	0.1	32
0	0.3	0.3	0.4	0.3	0.02	7.5
6	0.7	0.5	1.1	0.8	0.3	38
12	2.5	0.7	0.9	1.4	1.0	72
24	0.8	0.3	1.0	0.7	0.4	52
48	1.4	0.3	0.7	0.8	0.6	77
72	0.5	0.3	1.0	0.6	0.3	53
96	0.5	0.6	0.9	0.6	0.2	30

a. Capital letters denote individual rats (see Table 2.1).
b. Mean ± standard deviation (minimum n = 3).
c. Inter-animal coefficient of variation (% CV).

Table 3.15. Cadmium Concentration in Polymorphonuclear Leukocytes, Subchronic Experiment GO91.

Time (h)	Cadmium Concentration (ug Cd/L) [a]			Mean ± SD [b]		CV (%) [c]
Administered Dose: 0.00 mg/kg				(minimum n=2)		
	G	H	I			
-20	2.1	1.1	1.3	1.5	0.5	35
0	0.8	1.2	0.7	0.9	0.3	32
6	0.4	0.9	0.6	0.6	0.2	37
12	0.7	0.9	0.9	0.8	0.1	15
24	2.3	0.7	0.7	1.2	0.9	76
48	0.7	0.1	0.3	0.4	0.3	87
72	1.0	0.8	-	0.9	0.1	13
96	2.0	1.3	-	1.6	0.5	31
Administered Dose: 0.25 mg/kg						
	J	K	L			
-20	0.5	1.6	1.9	1.3	0.7	57
0	0.6	2.7	3.4	2.2	1.5	67
6	0.3	2.1	1.8	1.4	0.9	67
12	0.6	-	1.2	0.9	0.4	45
24	0.3	-	1.5	0.9	0.9	93
48	0.4	-	1.1	0.7	0.5	68
72	0.5	-	-	-	-	-
96	1.9	-	-	-	-	-
Administered Dose: 1.25 mg/kg						
	M	N	O			
-20	1.7	1.7	1.0	1.5	0.4	25
0	1.2	1.3	0.9	1.1	0.2	21
6	1.2	1.1	0.7	1.0	0.3	29
12	1.3	0.8	1.8	1.3	0.5	39
24	1.1	1.3	0.8	1.1	0.3	26
48	1.2	1.1	1.2	1.2	0.04	3.6
72	0.7	0.5	0.5	0.6	0.1	19
96	0.7	-	0.9	0.8	0.2	21

a. Capital letters denote individual rats (see Table 2.2).
b. Mean ± standard deviation (minimum n = 2).
c. Inter-animal coefficient of variation (% CV).

Table 3.16. Cadmium Concentration in Lymphocytes, Short Course Experiment PA91.

Time (h)	Cadmium Concentration (ug Cd/L)[a]			Mean ± SD[b]		CV (%)[c]
Administered Dose: 0.00 mg/kg				(minimum n=3)		
	T	U	AA			
-20	0.8	0.4	0.9	0.7	0.3	42
0	0.6	0.1	0.3	0.3	0.2	63
6	0.6	0.5	0.4	0.5	0.1	20
12	0.8	0.7	0.8	0.8	0.1	11
24	0.8	1.0	0.6	0.8	0.2	23
48	0.9	0.7	0.4	0.7	0.3	44
72	0.9	0.5	0.5	0.6	0.2	36
96	1.1	0.3	0.5	0.7	0.4	61
Administered Dose: 0.25 mg/kg						
	V	W	X			
-20	0.9	0.5	1.0	0.8	0.2	29
0	1.2	0.3	0.4	0.6	0.5	81
6	0.5	0.4	0.6	0.5	0.1	20
12	0.6	1.0	0.6	0.7	0.2	28
24	2.4	1.7	2.9	2.3	0.6	24
48	0.7	0.8	0.6	0.7	0.1	19
72	1.0	0.5	0.6	0.7	0.3	40
96	0.5	0.7	0.9	0.7	0.2	22
Administered Dose: 1.25 mg/kg						
	P	Q	R			
-20	0.3	0.7	0.4	0.5	0.2	44
0	0.3	0.3	0.5	0.4	0.1	32
6	0.9	1.0	1.3	1.1	0.2	19
12	0.9	0.7	1.1	0.9	0.2	24
24	1.4	1.4	1.7	1.5	0.2	14
48	0.3	0.6	1.2	0.7	0.4	59
72	1.4	0.9	0.9	1.1	0.3	25
96	0.9	1.5	1.9	1.5	0.5	34

a. Capital letters denote individual rats (see Table 2.1).
b. Mean ± standard deviation (minimum n = 3).
c. Inter-animal coefficient of variation (% CV).

Table 3.17. Cadmium Concentration in Lymphocytes, Subchronic Experiment G091.

Time (h)	Cadmium Concentration (ug Cd/L)[a]			Mean ± SD[b]		CV (%)[c]
Administered Dose: 0.00 mg/kg				(minimum n=2)		
	G	H	I			
-20	1.2	0.5	1.3	1.0	0.4	39
0	0.4	0.7	1.4	0.9	0.5	58
6	1.0	0.3	0.3	0.5	0.4	75
12	1.0	0.8	0.5	0.8	0.3	32
24	0.4	0.5	0.3	0.4	0.1	26
48	1.0	0.4	0.3	0.6	0.4	62
72	0.5	0.7	-	0.6	0.2	31
96	1.0	0.6	-	0.8	0.3	34
Administered Dose: 0.25 mg/kg						
	J	K	L			
-20	1.3	1.0	0.7	1.0	0.3	33
0	1.9	2.1	2.4	2.1	0.2	12
6	1.2	1.8	1.5	1.5	0.3	22
12	0.8	-	2.8	1.8	1.4	80
24	1.6	-	1.7	1.6	0.1	5
48	2.6	-	1.1	1.8	1.0	54
72	1.5	-	-	-	-	-
96	1.2	-	-	-	-	-
Administered Dose: 1.25 mg/kg						
	M	N	O			
-20	2.4	1.4	1.6	1.8	0.5	31
0	1.0	2.4	1.1	1.5	0.8	52
6	0.8	1.3	1.0	1.0	0.3	25
12	1.7	1.2	1.0	1.3	0.3	27
24	0.5	0.9	1.7	1.0	0.6	60
48	0.9	1.4	1.0	1.1	0.3	24
72	0.5	0.9	0.8	0.8	0.2	28
96	0.7	-	1.1	0.9	0.2	26

a. Capital letters denote individual rats (see Table 2.2).
b. Mean ± standard deviation (minimum n = 2).
c. Inter-animal coefficient of variation (% CV).

Table 3.18. Cadmium Concentration in Lymphocytes, Short Course AF90 and Subchronic CD90 Experiments.

Time (h)	Cadmium Concentration (ug Cd/L)[a]			Mean ± SD[b]		CV (%)[c]
Short Course:						
Administered Dose: 0.00 mg/kg				(minimum n=2)		
	B	E				
-20	0.4	0.1		0.2	0.16	64
0	0.0	0.2		0.1	0.09	83
6	0.2	0.1		0.2	0.05	28
12	0.4	0.1		0.3	0.21	79
24	0.1	0.1		0.1	0.03	36
34	-	0.1		-	-	-
48	-	-		-	-	-
96	0.3	-		-	-	-
Administered Dose: 2.50 mg/kg						
	A	F	D			
-20	0.2	0.1	0.1	0.1	0.08	78
0	0.1	0.2	0.1	0.2	0.07	41
6	0.6	0.3	-	0.5	0.19	40
12	0.4	0.5	-	0.5	0.07	14
24	0.3	0.2	0.2	0.2	0.05	24
34	-	0.3	-	-	-	-
96	0.2	-	-	-	-	-
Subchronic:						
Administered Dose: 2.50 mg/kg						
	C					
-20	0.09			-	-	-
0	0.05			-	-	-
12	0.09			-	-	-

a. Capital letters denote individual rats (see Tables 2.1 and 2.2).
b. Mean ± standard deviation (minimum n=2).
c. Inter-animal coefficient of variation (% CV).

Table 3.19. Cadmium Concentration in Monocytes, Short Course Experiment PA91.

Time (h)	Cadmium Concentration (ug Cd/L)[a]			Mean ± SD[b]		CV (%)[c]
Administered Dose: 0.00 mg/kg				(minimum n=3)		
	T	U	AA			
-20	0.9	0.8	0.4	0.7	0.3	37
0	0.7	0.6	0.1	0.5	0.3	64
6	0.9	0.6	0.2	0.6	0.4	62
12	1.1	0.9	0.4	0.8	0.4	44
24	0.8	1.0	0.6	0.8	0.2	25
48	0.4	1.1	0.1	0.6	0.5	89
72	0.7	0.6	0.2	0.5	0.3	55
96	1.1	0.8	0.6	0.8	0.2	28
Administered Dose: 0.25 mg/kg						
	V	W	X			
-20	0.4	0.7	0.6	0.6	0.1	25
0	0.3	0.6	0.6	0.5	0.1	29
6	0.4	0.7	0.4	0.5	0.2	41
12	0.4	0.6	0.9	0.7	0.2	35
24	0.8	0.9	1.1	0.9	0.2	19
48	1.5	1.5	0.9	1.3	0.3	23
72	0.8	1.0	0.7	0.8	0.2	22
96	0.6	1.4	0.5	0.8	0.5	60
Administered Dose: 1.25 mg/kg						
	P	Q	R			
-20	0.6	0.5	0.9	0.7	0.2	29
0	0.7	0.8	0.8	0.8	0.1	8.7
6	1.2	1.2	1.3	1.2	0.1	6.2
12	1.4	1.2	1.0	1.2	0.2	15
24	2.2	2.1	2.0	2.1	0.1	3.9
48	1.1	1.2	1.8	1.4	0.4	28
72	1.0	0.7	1.0	0.9	0.2	22
96	1.2	0.9	0.9	1.0	0.2	18

a. Capital letters denote individual rats (see Table 2.1).
b. Mean ± standard deviation (minimum n = 3).
c. Inter-animal coefficient of variation (% CV).

Table 3.20. Cadmium Concentration in Monocytes, Subchronic Experiment G091.

Time (h)	Cadmium Concentration (ug Cd/L)[a]			Mean ± SD[b]		CV (%)[c]
Administered Dose: 0.00 mg/kg				(minimum n=2)		
	G	H	I			
-20	0.6	0.4	0.5	0.5	0.1	21
0	0.6	0.8	0.6	0.6	0.1	16
6	0.5	0.0	0.5	0.3	0.3	87
12	0.0	0.6	0.7	0.4	0.4	88
24	0.7	0.4	0.0	0.4	0.4	94
48	0.5	0.4	0.6	0.5	0.1	19
72	0.1	0.2	-	0.13	0.08	61
96	0.3	0.2	-	0.2	0.06	26
Administered Dose: 0.25 mg/kg						
	J	K	L			
-20	0.1	0.4	0.0	0.15	0.20	132
0	0.2	1.4	1.2	0.9	0.7	74
6	0.0	0.0	0.8	0.3	0.5	173
12	1.3	-	0.0	0.7	1.0	141
24	0.4	-	0.0	0.2	0.3	141
48	0.0	-	0.0	0.0	0.0	-
72	0.0	-	-	-	-	-
96	0.1	-	-	-	-	-
Administered Dose: 1.25 mg/kg						
	M	N	O			
-20	0.0	0.3	0.2	0.15	0.13	91
0	0.4	0.5	0.5	0.5	0.1	15
6	0.0	0.2	0.2	0.13	0.11	89
12	0.0	0.5	0.4	0.3	0.2	88
24	0.0	1.1	1.2	0.8	0.7	87
48	0.1	0.5	0.7	0.4	0.3	82
72	0.0	0.7	0.6	0.4	0.4	87
96	0.3	-	0.3	0.3	0.03	10

a. Capital letters denote individual rats (see Table 2.2).
b. Mean ± standard deviation (minimum n = 2).
c. Inter-animal coefficient of variation (% CV).

Table 3.21. Cadmium Concentration in Monocytes, Short Course AF90 and Subchronic CD90 Experiments.

Time (h)	Cadmium Concentration (ug Cd/L)[a]			Mean ± SD[b]		CV (%)[c]
Short Course:						
Administered Dose: 0.00 mg/kg				(minimum n=2)		
	B	E				
-20	0.1	2.3		1.2	1.5	125
0	0.1	1.2		0.7	0.8	120
6	1.3	1.7		1.5	0.3	18
12	0.7	1.7		1.2	0.7	62
24	0.3	10.7		5.5	7.3	133
34	-	6.1		-	-	-
48	-	-		-	-	-
96	0.8	-		-	-	-
Administered Dose: 2.50 mg/kg						
	A	F	D			
-20	0.0	0.5	0.4	0.30	0.27	89
0	0.0	3.2	4.0	2.4	2.1	88
6	1.3	3.5	-	2.4	1.6	67
12	0.8	1.0	-	0.9	0.2	18
24	1.2	1.5	2.0	1.6	0.4	27
34	-	2.1	-	-	-	-
96	0.2	-	-	-	-	-
Subchronic:						
Administered Dose: 2.50 mg/kg						
	C					
-20	1.7			-	-	-
0	0.3			-	-	-
12	0.3			-	-	-

a. Capital letters denote individual rats (see Tables 2.1 and 2.2).
b. Mean ± standard deviation (minimum n=2).
c. Inter-animal coefficient of variation (% CV).

Table 3.22. Cadmium Concentration in Feces, Short Course Experiment PA91.

Time (h)	Cadmium Concentration (ug Cd/kg wwt) [a]			Mean ± SD [b]		CV (%) [c]
Administered Dose: 0.00 mg/kg				(minimum n=3)		
	T	U	AA			
-20	225	150	170	182	39	21
0	104	96	191	130	53	41
6	93	86	212	130	71	54
12	73	109	267	149	103	69
24	60	71	230	120	95	79
48	112	193	150	151	41	27
72	176	109	145	143	34	23
96	168	192	135	165	28	17
Administered Dose: 0.25 mg/kg						
	V	W	X			
-20	220	242	180	214	32	15
0	193	283	135	204	74	36
6	183	265	268	239	48	20
12	244	229	627	367	225	61
24	450	712	527	563	135	24
48	300	278	358	312	41	13
72	261	323	305	296	32	11
96	237	292	216	248	39	16
Administered Dose: 1.25 mg/kg						
	P	Q	R			
-20	97	117	175	130	41	31
0	90	67	115	91	24	27
6	100	73	83	85	14	16
12	161	126	1712	666	906	136
24	2640	1860	565	1688	1048	62
48	727	411	258	465	239	51
72	309	181	221	237	65	28
96	118	184	122	141	37	26

a. Capital letters denote individual rats (see Table 2.1).
b. Mean ± standard deviation (minimum n = 3).
c. Inter-animal coefficient of variation (% CV).

Table 3.23. Cadmium Concentration in Feces, Subchronic Experiment G091.

Time (h)	Cadmium Concentration (ug Cd/kg wwt) [a]			Mean ± SD [b]		CV (%) [c]
Administered Dose: 0.00 mg/kg				(minimum n=2)		
	G	H	I			
-20	225	213	158	199	36	18
0	295	283	105	228	107	47
6	191	154	130	158	30	19
12	244	223	211	226	17	7.4
24	296	183	210	230	59	26
48	168	173	167	169	3	1.9
72	130	162	-	146	22	15
96	193	217	-	205	18	8.6
Administered Dose: 0.25 mg/kg						
	J	K	L			
-20	195	163	144	167	26	15
0	162	219	154	178	36	20
6	120	180	127	142	33	23
12	237	-	218	227	13	5.7
24	408	-	584	496	125	25
48	180	-	222	201	30	15
72	173	-	-	-	-	-
96	141	-	-	-	-	-
Administered Dose: 1.25 mg/kg						
	M	N	O			
-20	184	216	186	195	18	9.0
0	275	246	196	239	40	17
6	232	296	205	244	47	19
12	-	710	728	719	13	1.7
24	-	1187	858	1023	232	23
48	378	1186	642	735	412	56
72	248	425	467	380	116	30
96	367	-	392	379	18	4.8

a. Capital letters denote individual rats (see Table 2.2).
b. Mean ± standard deviation (minimum n = 2).
c. Inter-animal coefficient of variation (% CV).

Table 3.24. Cadmium Concentration in Feces, Short Course AF90 and Subchronic CD90 Experiments.

Time (h)	Cadmium Concentration (ug Cd/kg wwt) [a]			Mean ± SD [b]		CV (%) [c]
Short Course:						
Administered Dose: 0.00 mg/kg				(minimum n=2)		
	B	E				
-20	130	-		-	-	-
0	140	109		125	22	17
6	179	92		136	62	45
12	286	109		197	125	63
24	101	88		94	9	10
34	227	100		164	90	55
72	821	-		-	-	-
96	115	-		-	-	-
Administered Dose: 2.50 mg/kg						
	A	F	D			
-20	107	154	62	108	46	43
0	223	167	180	190	29	16
6	166	145	102	137	32	24
12	-	151	61	106	63	60
24	2811	-	1608	2210	851	38
34	4779	9059	573	4804	4243	88
72	1268	-	-	-	-	-
96	384	-	-	-	-	-
Subchronic:						
Administered Dose: 2.50 mg/kg						
	C					
-20	150			-	-	-
0	142			-	-	-
12	-			-	-	-

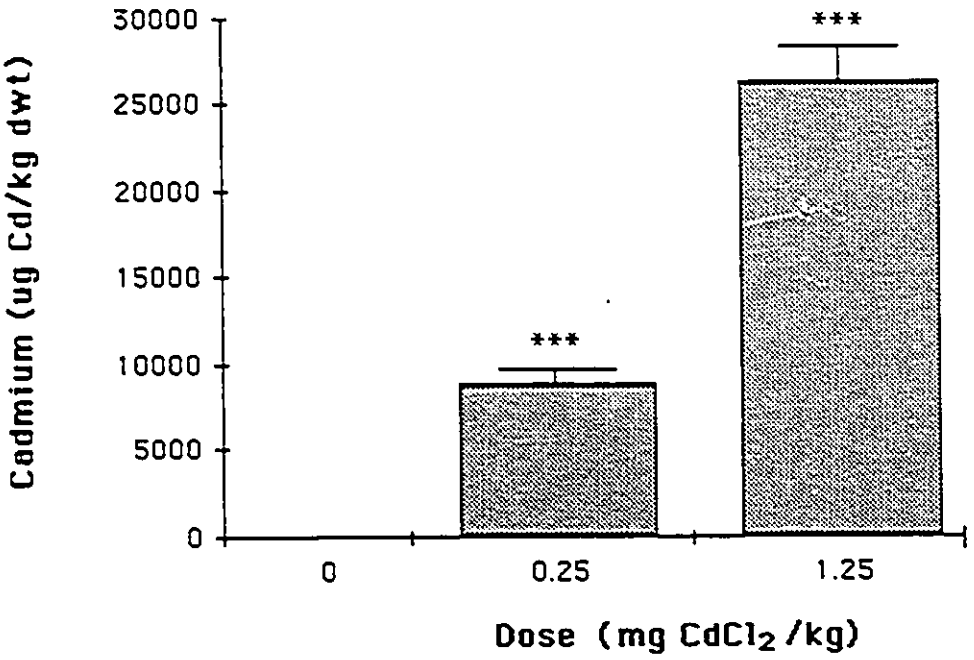
a. Capital letters denote individual rats (see Tables 2.1 and 2.2).
b. Mean ± standard deviation (minimum n = 2).
c. Inter-animal coefficient of variation (% CV).

Table 3.25. Cadmium Concentration in Urine, Short Course AF90 and Subchronic CD90 Experiments.

Time (h)	Cadmium Concentration (ug Cd/L) [a]			Mean ± SD [b]		CV (%) [c]
Short Course:						
Administered Dose: 0.00 mg/kg				(minimum n=2)		
	B	E				
-20	1.0	-		-	-	-
0	0.7	1.4		1.0	0.5	50
6	0.3	1.0		0.7	0.5	81
12	0.0	-		-	-	-
24	0.8	0.5		0.6	0.2	33
34	1.0	1.2		1.1	0.1	12
72	0.4	-		-	-	-
96	1.1	-		-	-	-
Administered Dose: 2.50 mg/kg						
	A	F	D			
-20	0.2	-	-	-	-	-
0	-	0.1	1.4	0.7	1.0	130
6	29.0	11.9	-	20.5	12.1	59
12	7.6	14.9	-	11.2	5.2	46
24	83.3	54.3	5.0	47.5	39.6	83
34	60.5	29.1	-	44.8	-	-
72	2.0	-	-	-	-	-
96	1.8	-	-	-	-	-
Subchronic:						
Administered Dose: 2.50 mg/kg						
	C					
-20	-			-	-	-
0	78.8			-	-	-
12	92.5			-	-	-

a. Capital letters denote individual rats (see Tables 2.1 and 2.2).
b. Mean ± standard deviation (minimum n = 2).
c. Inter-animal coefficient of variation (% CV).

Figure 3.1. Cadmium Concentration in Liver, Short Course Experiment PA91.

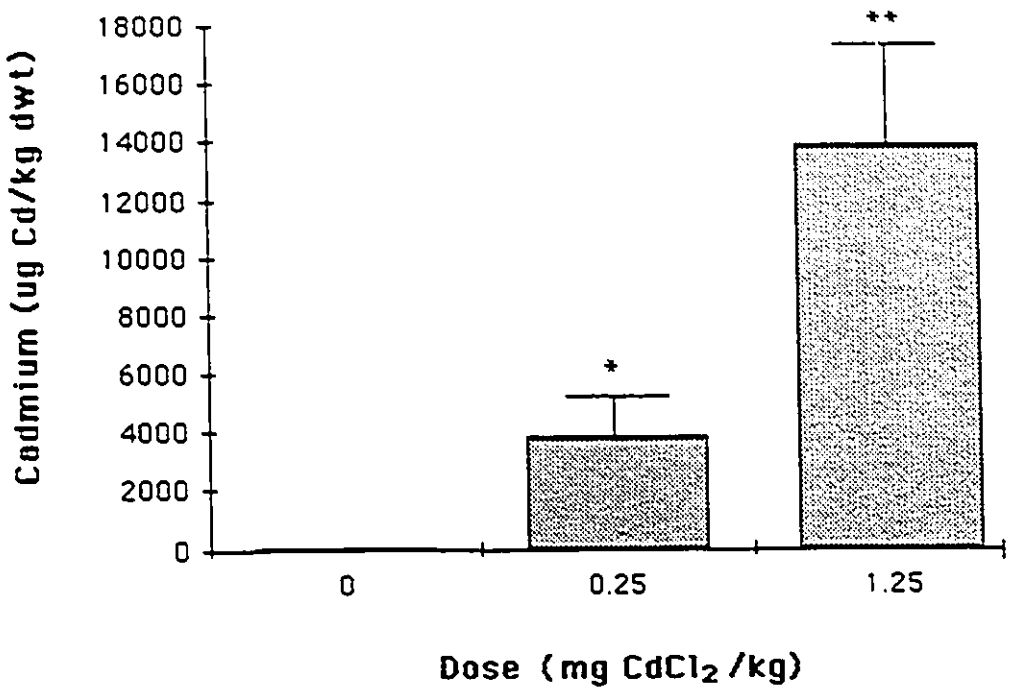


Legend to figure (administered dose):

0.0 mg/kg (T,U,AA); 0.25 mg/kg (V,W,X); 1.25 mg/kg (P,Q,R);

Each rat received a single IP injection at T = 0 h. The bar graphs represent the mean ± standard deviation (n=3),*** p<0.0005. See Table 2.1 for rat identification and Table 3.3 for Cd concentrations.

Figure 3.2. Cadmium Concentration in Kidney, Short Course Experiment PA91.

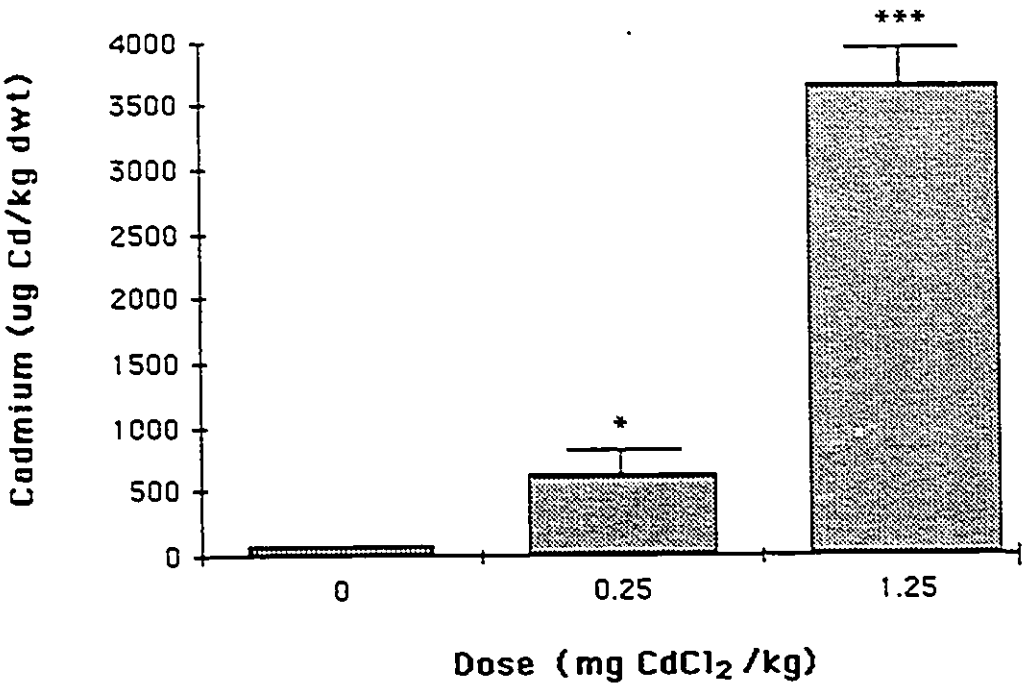


Legend to figure (administered dose):

0.0 mg/kg (T,U,AA); 0.25 mg/kg (V,W,X); 1.25 mg/kg (P,Q,R);

Each rat received a single IP injection at T = 0 h. The bar graphs represent the mean ± standard deviation (n=3). *p<0.05, **p<0.005. See Table 2.1 for rat identification and Table 3.3 for Cd concentrations.

Figure 3.3. Cadmium Concentration in Spleen, Short Course Experiment PA91.

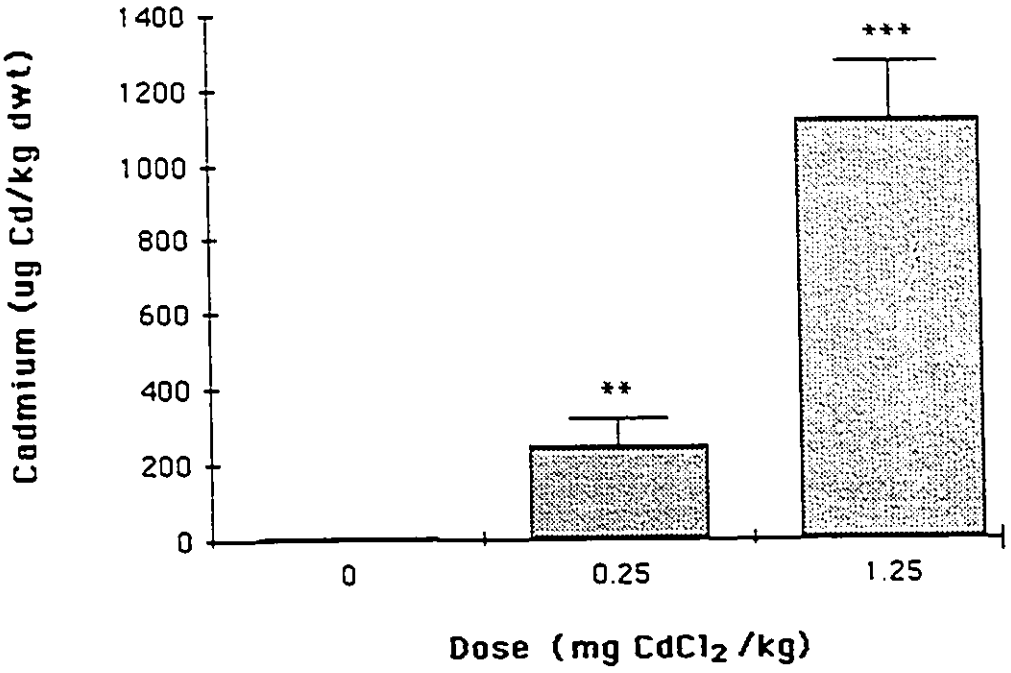


Legend to figure (administered dose):

0.0 mg/kg (T,U,AA); 0.25 mg/kg (V,W,X); 1.25 mg/kg (P,Q,R);

Each rat received a single IP injection at T = 0 h. The bar graphs represent the mean ± standard deviation (n=3), *p<0.05, *** p<0.0005. See Table 2.1 for rat identification and Table 3.3 for Cd concentrations.

Figure 3.4. Cadmium Concentration in Lung, Short Course Experiment PA91.



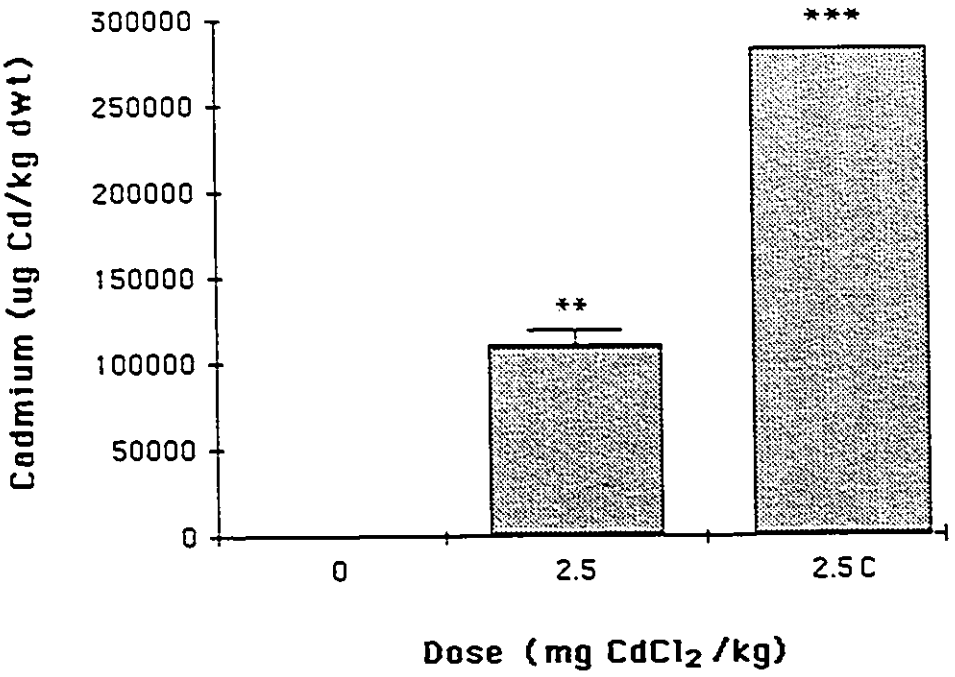
Legend to figure (administered dose):

0.0 mg/kg (T,U,AA); 0.25 mg/kg (V,W,X); 1.25 mg/kg (P,Q,R);

Each rat received a single IP injection at T = 0 h. The bar graphs represent the mean ± standard deviation (n=3), **p<0.005, *** p<0.0005.

See Table 2.1 for rat identification and Table 3.3 for Cd concentrations.

Figure 3.5. Cadmium Concentration in Liver, Short Course AF90 and Subchronic CD90 Experiments.



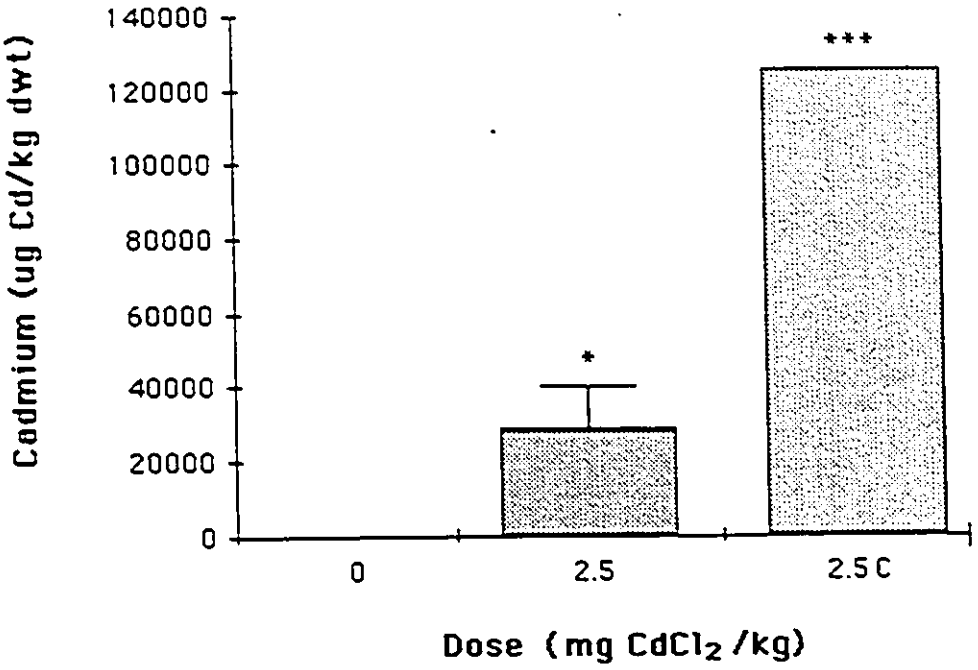
Legend to figure (administered dose):

Short Course Dose: 0.0 mg/kg (B,E); 2.5 mg/kg (A,F,D). Each rat received a single IP injection at T = 0 h. See Tables 2.1 and 3.3.

Subchronic Dose: 0.0 mg/kg (D); 2.5C mg/kg (C); Each rat received three IP injections in total of the indicated doses over 2 month period. The third injection (2.5 mg/kg) was administered to both rats at T = 0 h. See Tables 2.2 and 3.4.

The bar graphs represent the mean ± standard deviation, ** $p<0.005$, *** $p<0.0005$.

Figure 3.6. Cadmium Concentration in Kidney, Short Course AF90 and Subchronic CD90 Experiments.



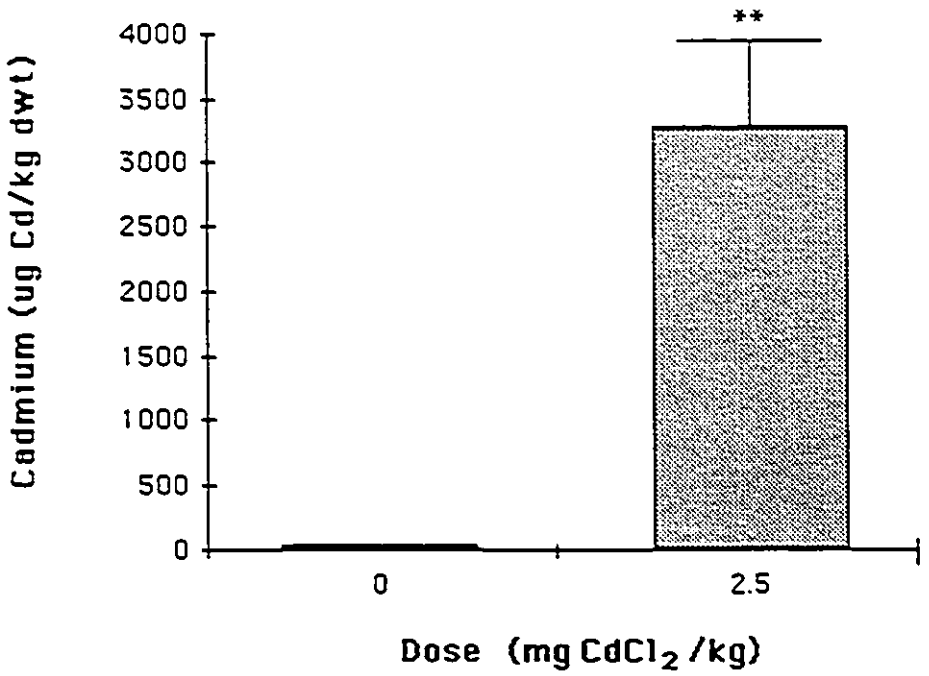
Legend to figure (administered dose):

Short Course Dose: 0.0 mg/kg (B,E); 2.5 mg/kg (A,F,D). Each rat received a single IP injection at T = 0 h. See Tables 2.1 and 3.3.

Subchronic Dose: 0.0 mg/kg (D); 2.5C mg/kg (C); Each rat received three IP injections in total of the indicated doses over 2 month period. The third injection (2.5 mg/kg) was administered to both rats at T = 0 h. See Tables 2.2 and 3.4.

The bar graphs represent the mean ± standard deviation, * $p<0.05$, *** $p<0.0005$.

Figure 3.7. Cadmium Concentration in Spleen, Short Course AF90 and Subchronic CD90 Experiments.



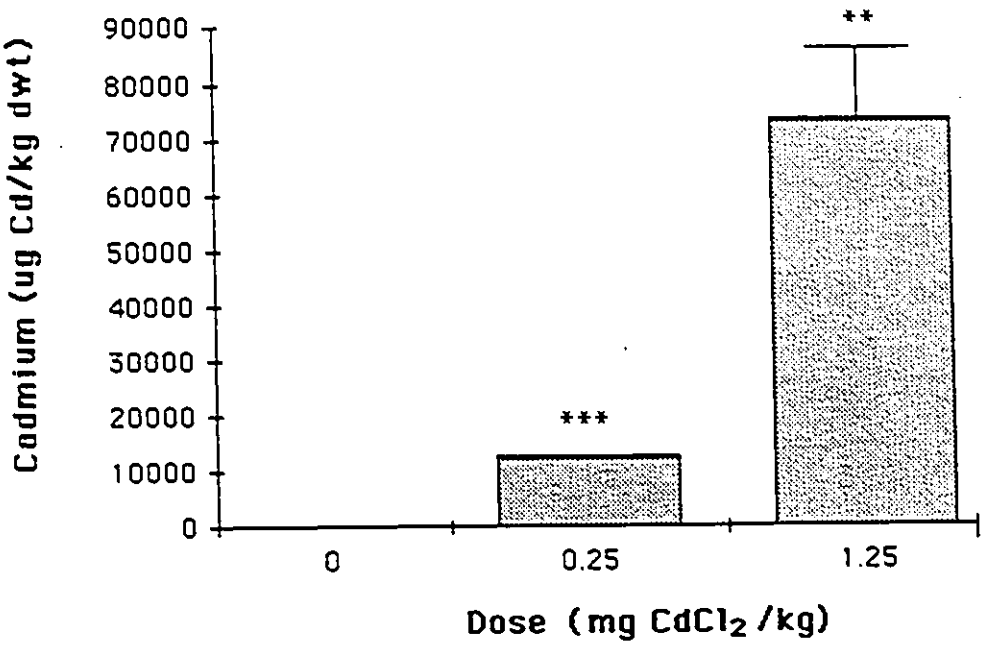
Legend to figure (administered dose):

Short Course Dose: 0.0 mg/kg (B,E); 2.5 mg/kg (A,F,D). Each rat received a single IP injection at T = 0 h. See Tables 2.1 and 3.3.

Subchronic Dose: 0.0 mg/kg (D); 2.5C mg/kg (C); Each rat received three IP injections in total of the indicated doses over 2 month period. The third injection (2.5 mg/kg) was administered to both rats at T = 0 h. See Tables 2.2 and 3.4.

The bar graphs represent the mean ± standard deviation, ** $p<0.005$. Spleen sample was not available for rat C, see Chapter 5 in the text for details.

Figure 3.8. Cadmium Concentration in Liver, Subchronic Experiment G091.

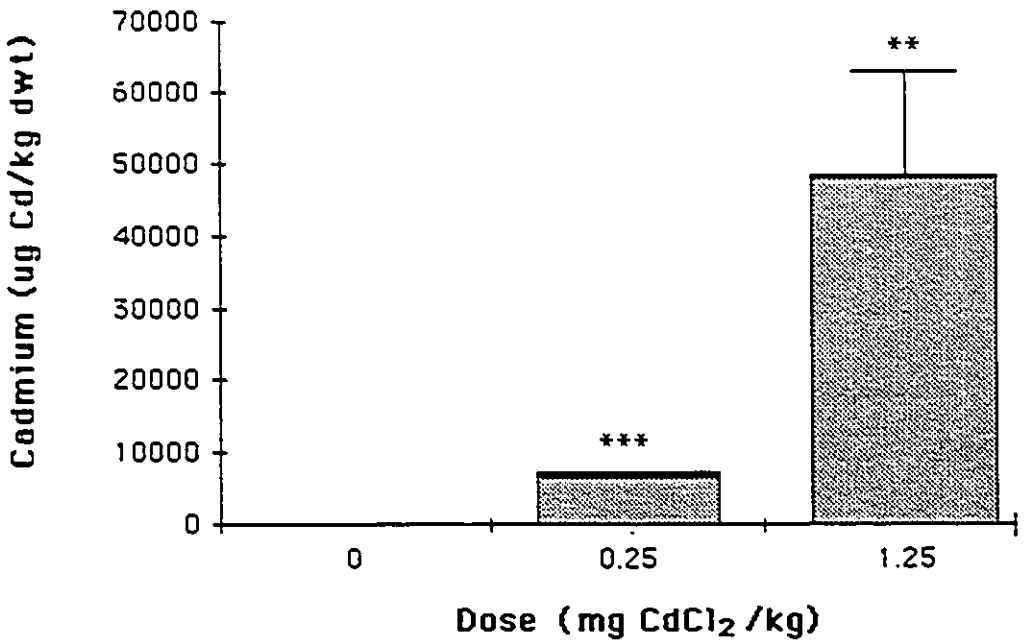


Legend to figure (administered dose):

0.0 mg/kg (G,H,I); 0.25 mg/kg (J,K,L); 1.25 mg/kg (M,N,O);

Each rat received a total of three IP injections of the indicated doses over a 1.5 - 2.0 month period, (except M had 0.5 months). The third injection was administered at T = 0 h. The following rats died as the experiment progressed; K after 6 h, I and L after 48 h, and N after 72 h. The bar graphs represent the mean ± standard deviation (n=3), ** $p<0.001$, *** $p<0.0005$. See Table 2.2 for rat identification and Table 3.4 for Cd concentration.

Figure 3.9. Cadmium Concentration in Kidney, Subchronic Experiment G091.

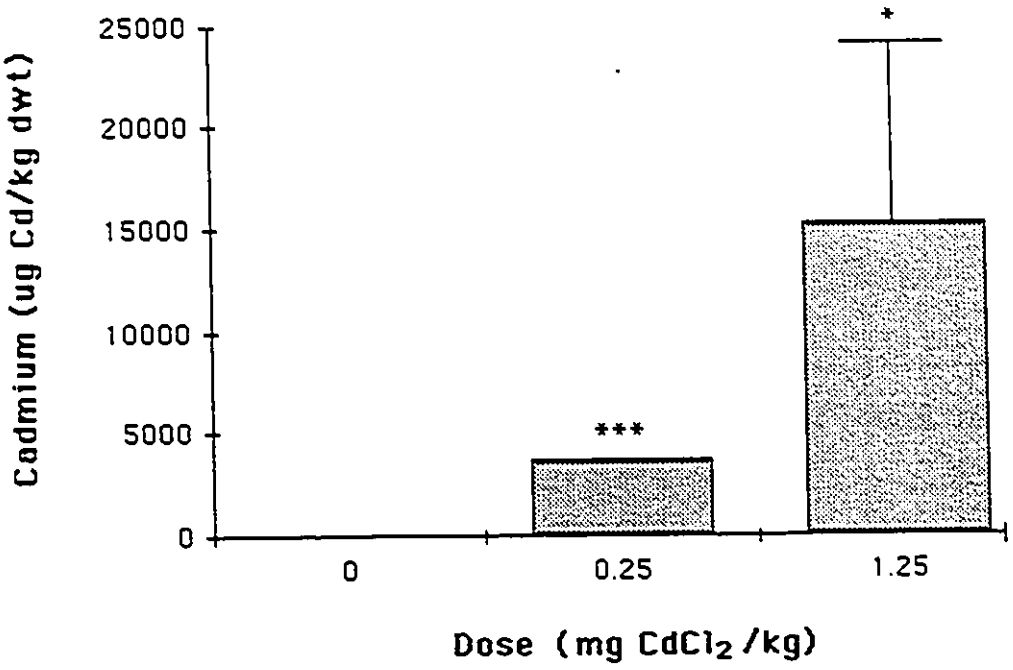


Legend to figure (administered dose):

0.0 mg/kg (G,H,I); 0.25 mg/kg (J,K,L); 1.25 mg/kg (M,N,O);

Each rat received a total of three IP injections of the indicated doses over a 1.5 - 2.0 month period, (except M had 0.5 months). The third injection was administered at T = 0 h. The following rats died as the experiment progressed; K after 6 h, I and L after 48 h, and N after 72 h. The bar graphs represent the mean ± standard deviation (n=3), ** $p<0.005$, *** $p<0.0005$. See Table 2.2 for rat identification and Table 3.4 for Cd concentration.

Figure 3.10. Cadmium Concentration in Spleen, Subchronic Experiment G091.

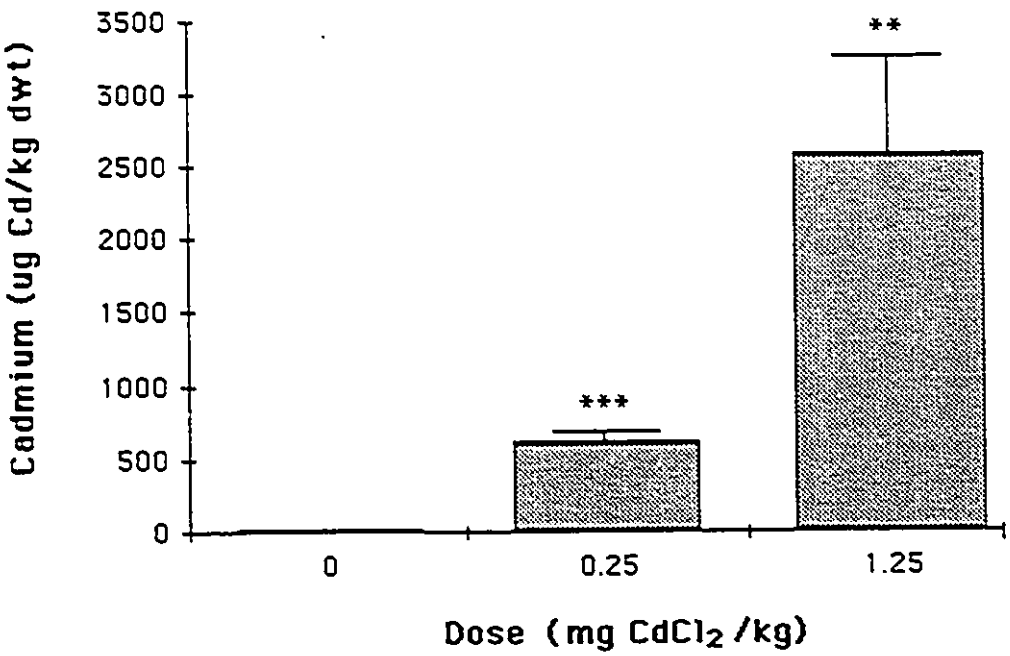


Legend to figure (administered dose):

0.0 mg/kg (G,H,I); 0.25 mg/kg (J,K,L); 1.25 mg/kg (M,N,O);

Each rat received a total of three IP injections of the indicated doses over a 1.5 - 2.0 month period, (except M had 0.5 months). The third injection was administered at T = 0 h. The following rats died as the experiment progressed; K after 6 h, I and L after 48 h, and N after 72 h. The bar graphs represent the mean ± standard deviation (n=3), * $p<0.05$,*** $p<0.0005$. See Table 2.2 for rat identification and Table 3.4 for Cd concentration.

Figure 3.11. Cadmium Concentration in Lung, Subchronic Experiment G091.

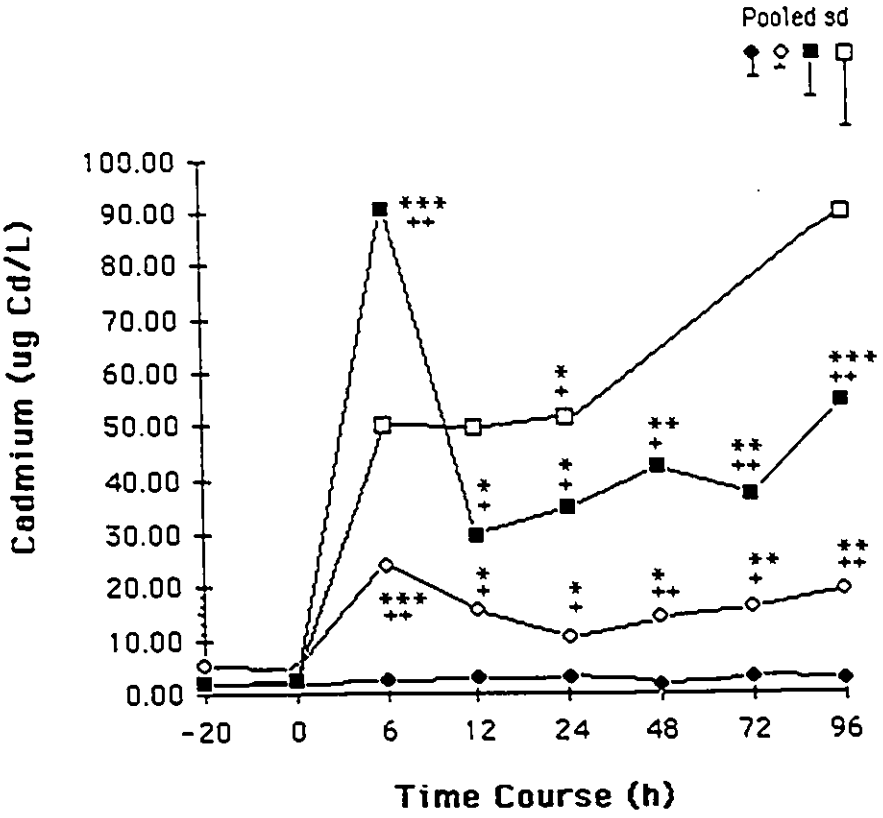


Legend to figure (administered dose):

0.0 mg/kg (G,H,I); 0.25 mg/kg (J,K,L); 1.25 mg/kg (M,N,O);

Each rat received a total of three IP injections of the indicated doses over a 1.5 - 2.0 month period, (except M had 0.5 months). The third injection was administered at T = 0 h. The following rats died as the experiment progressed; K after 6 h, I and L after 48 h, and N after 72 h. The bar graphs represent the mean ± standard deviation (n=3), ** $p<0.005$, *** $p<0.0005$. See Table 2.2 for rat identification and Table 3.4 for Cd concentration.

Figure 3.12. Cadmium Concentration in Whole Blood, Short Course Experiments.

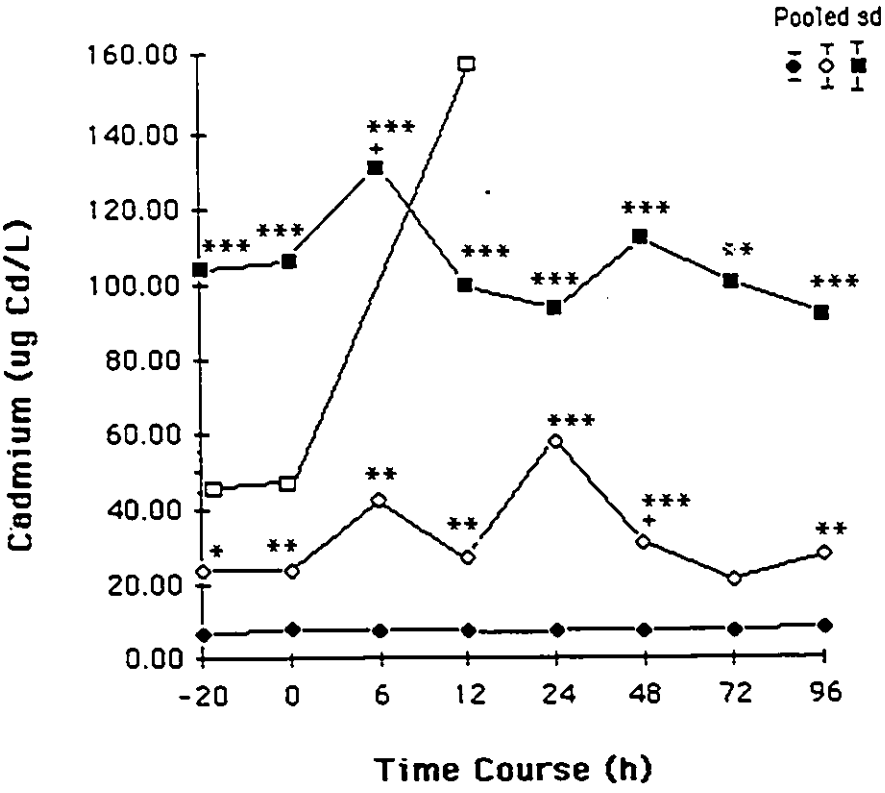


Legend to figure (administered dose):

-◆- 0.0 mg/kg; -○- 0.25 mg/kg; -■- 1.25 mg/kg; -□- 2.5 mg/kg;

Each rat received a single IP injection at T = 0 h. The data points represent the mean (n = 3). The pooled standard deviations are; -◆- (3.3), -○- (1.1), -■- (6.3) and -□- (13.2). *p<0.05, **p<0.005, ***p<0.0005 (unpaired t-Test); +p<0.05, ++p<0.005 (longitudinal paired t-Test). See Tables 3.6. and 3.8. for individual Cd concentrations.

Figure 3.13. Cadmium Concentration in Whole Blood, Subchronic Experiments.

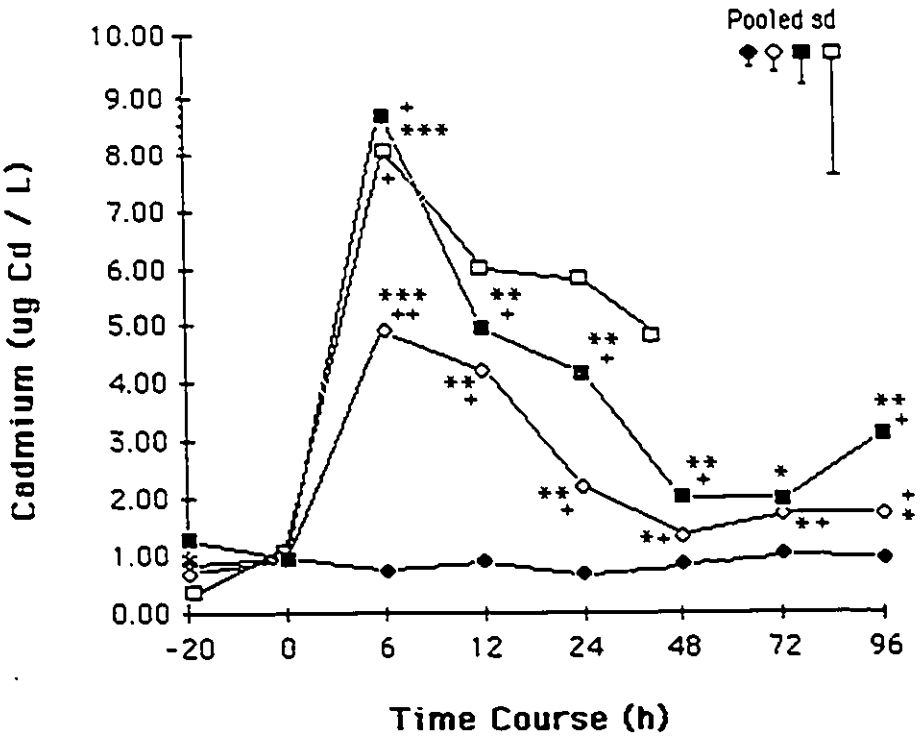


Legend to figure (administered dose):

-◆- 0.0 mg/kg; -◇- 0.25 mg/kg; -■- 1.25 mg/kg; -□- 2.5 mg/kg;

Each rat received a total of three IP injections of the indicated doses over a 1.5 - 2.0 month period. The third injection was administered at T = 0 h. The data points represent the mean (n=3), pooled standard deviations are; -◆- (1), -◇- (4), and -■- (5). * p<0.05, ** p<0.005, ***p<0.0005 (unpaired t-Test) +p<0.05 (longitudinal paired t-Test). See Tables 3.7. and 3.8. for individual Cd concentration.

Figure 3.14. Cadmium Concentration in Plasma, Short Course Experiments.

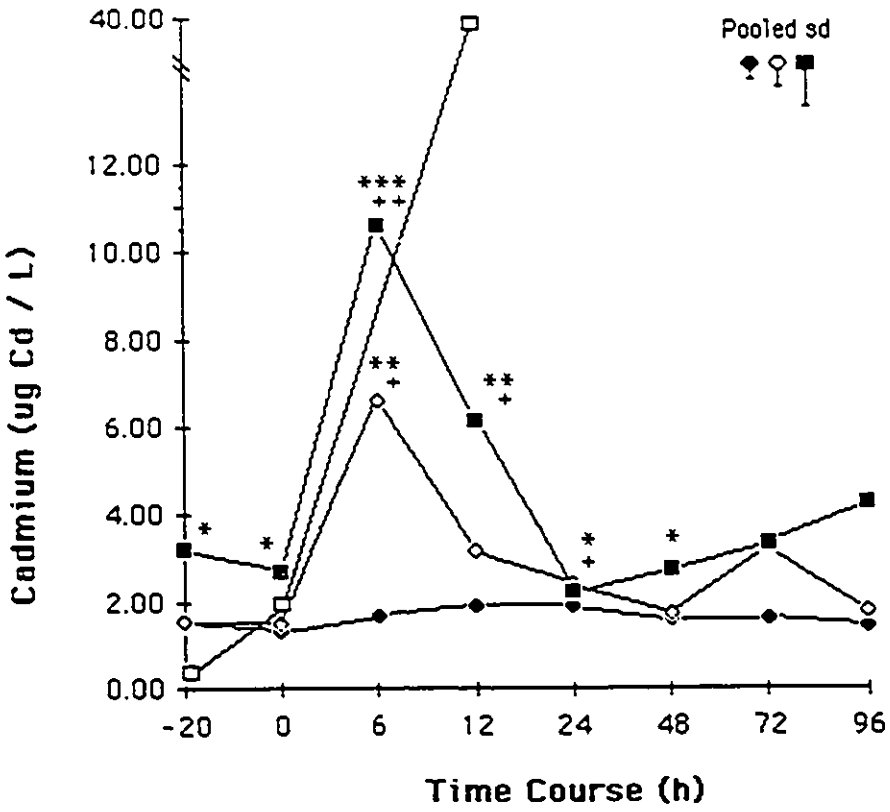


Legend to figure (administered dose):

-◆- 0.0 mg/kg ; -○- 0.25 mg/kg ; -■- 1.25 mg/kg ; -□- 2.5 mg/kg;

Each rat received a single IP injection at T = 0 h. The data points represent the mean (n = 3). The pooled standard deviations are; -◆- (0.19), -○- (0.29), -■- (0.57) and -□-(2.29). *p<0.05, **p<0.005, ***p<0.0005 (unpaired t-Test) +p<0.05, ++ p<0.005 (longitudinal paired t-Test). See Tables 3.9. and 3.11. for individual Cd concentrations.

Figure 3.15. Cadmium Concentration in Plasma, Subchronic Experiments.

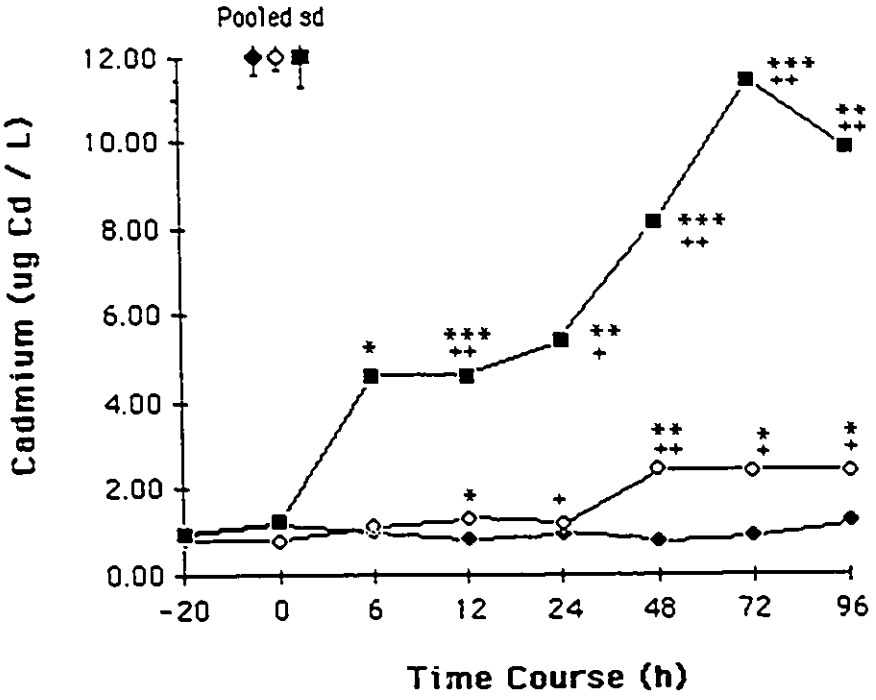


Legend to figure (administered dose):

-◆- 0.0 mg/kg ; -◇- 0.25 mg/kg ; -■- 1.25 mg/kg ; -□- 2.5 mg/kg;

Each rat received a total of three IP injections of the indicated doses over a 1.5 - 2.0 month period, (except M had 0.5 months). The third injection was administered at T = 0 h. The following rats died as the experiment progressed: K after 6 h, I and L after 48 h, and N after 72 h. The data points represent the mean (n=3), pooled standard deviations are; -◆-(0.4), -◇-(0.5), and -■-(1.0). * $p<0.05$, ** $p<0.005$, *** $p<0.0005$ (unpaired t-Test). + $p<0.05$, ++ $p<0.005$ (longitudinal paired t-Test). See Tables 3.10. and 3.11. for individual Cd concentrations.

Figure 3.16. Cadmium Concentration in Red Blood Cells, Short Course Experiment PA91.

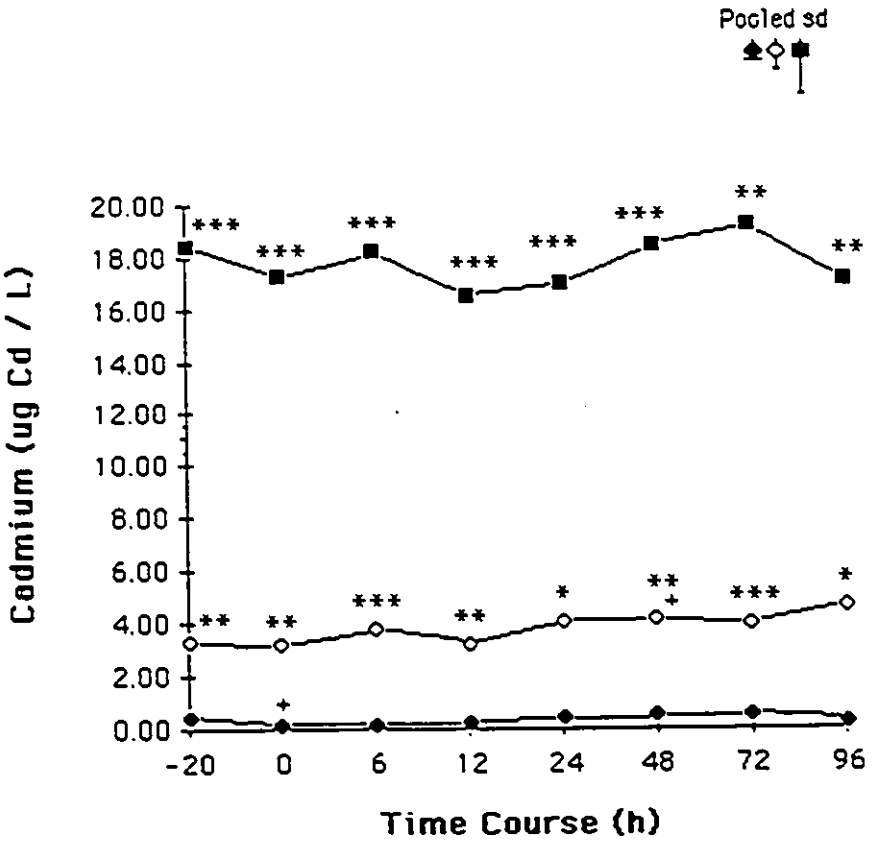


Legend to figure (administered dose):

·◆- 0.0 mg/kg (T,U,AA); ·◇- 0.25 mg/kg (V,W,X); ·■- 1.25 mg/kg (P,Q,R);

Each rat received a single IP injection at T = 0 h. The data points represent the mean (n = 3). The pooled standard deviations are; ·◆- (0.4), ·◇- (0.3), and ·■- (0.7). *p<0.05, **p<0.005, ***p<0.0005 (unpaired t-Test); +p<0.05, ++ p<0.005 (longitudinal paired t-Test). See Table 3.12 for individual Cd concentrations.

Figure 3.17. Cadmium Concentration in Red Blood Cells, Subchronic Experiment G091.

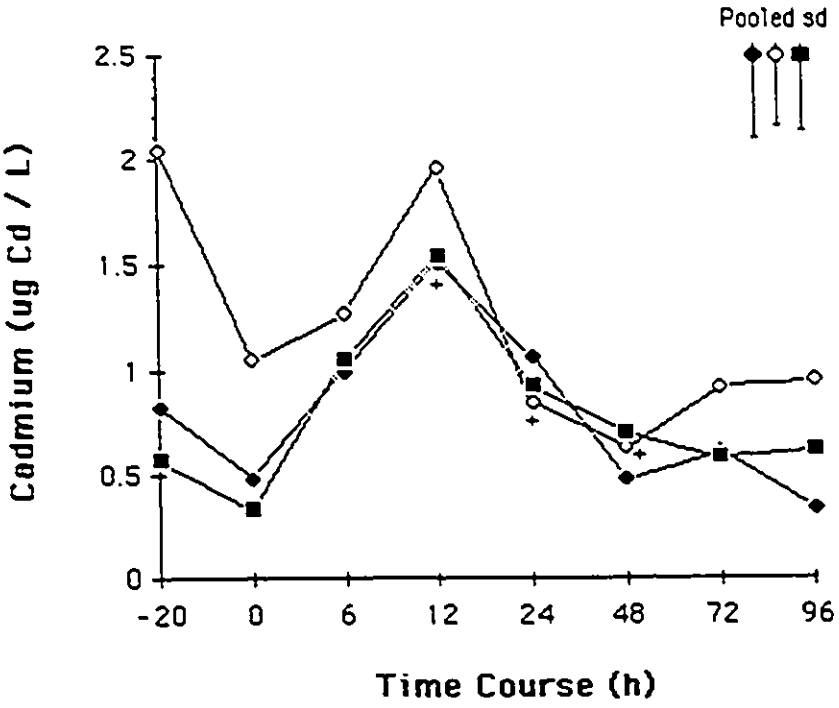


Legend to figure (administered dose):

◆ 0.0 mg/kg (G,H,I); ◇ 0.25 mg/kg (J,K,L); ■ 1.25 mg/kg (M,N,O);

Each rat received a total of three IP injections of the indicated doses over a 1.5 - 2.0 month period, (except M had 0.5 months). The third injection was administered at T = 0 h. The following rats died as the experiment progressed: K after 6 h, I and L after 48 h, and N after 72 h. The data points represent the mean (n=3), pooled standard deviations are; ◆ (0.2), ◇ (0.7), and ■ (1.6). * $p<0.05$, ** $p<0.005$, *** $p<0.0005$ (unpaired t-Test), + $p<0.05$ (longitudinal paired t-Test). See Table 3.13 for individual Cd concentrations.

Figure 3.18. Cadmium Concentration in Polymorphonuclear Leukocytes, Short Course Experiment PA91.

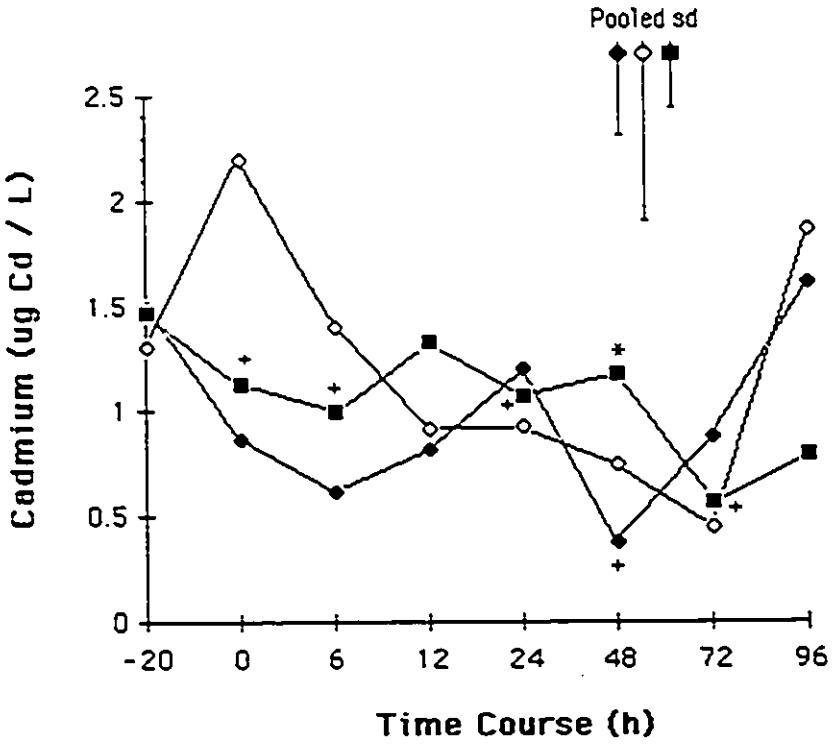


Legend to figure (administered dose):

-◆- 0.0 mg/kg (T,U,AA); -◇- 0.25 mg/kg (V,W,X); -■- 1.25 mg/kg (P,Q,R);

Each rat received a single IP injection at T = 0 h. The data points represent the mean (n = 3). The pooled standard deviations are; -◆- (0.43), -◇- (0.34), and -■- (0.36). +p<0.05 (longitudinal paired t-Test). See Table 3.14 for individual Cd concentrations.

Figure 3.19. Cadmium Concentration in Polymorphonuclear Leukocytes, Subchronic Experiment G091.

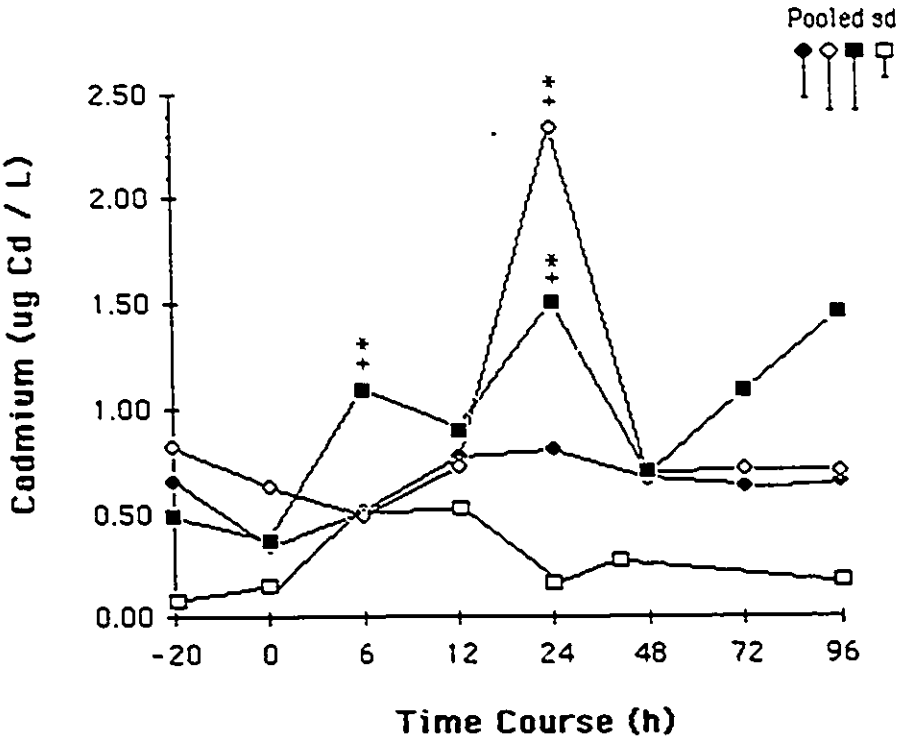


Legend to figure (administered dose):

·◆· 0.0 mg/kg (G,H,I); ·◇· 0.25 mg/kg (J,K,L); ·■· 1.25 mg/kg (M,N,O);

Each rat received a total of three IP injections of the indicated doses over a 1.5 - 2.0 month period, (except M had 0.5 months). The third injection was administered at T = 0 h. The following rats died as the experiment progressed: K after 6 h, I and L after 48 h, and N after 72 h. The data points represent the mean (n=3), pooled standard deviations are; ·◆· (0.38), ·◇· (0.82), and ·■· (0.25). * $p<0.05$ (unpaired t-Test), + $p<0.05$ (longitudinal paired t-Test). See Table 3.15 for individual Cd concentrations.

Figure 3.20. Cadmium Concentration in Lymphocytes, Short Course Experiments.

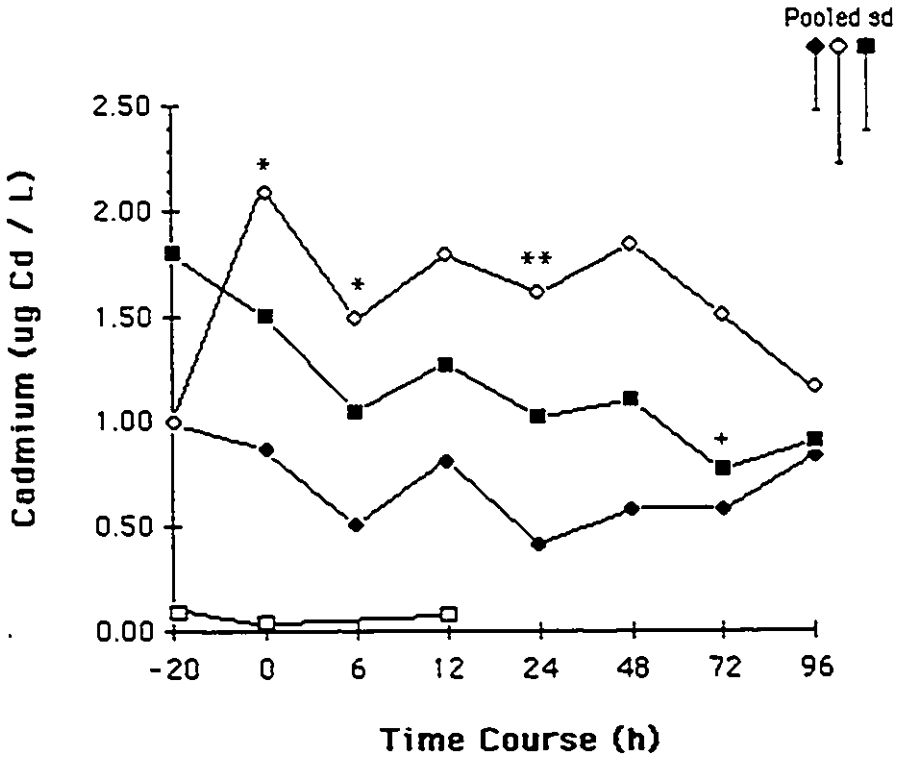


Legend to figure (administered dose):

◆- 0.0 mg/kg ; ◇- 0.25 mg/kg ; ■- 1.25 mg/kg ; □- 2.5 mg/kg;

Each rat received a single IP injection at T = 0 h. The data points represent the mean (n = 3). The pooled standard deviations are; ◆- (0.22), ◇- (0.27), ■- (0.27) and □- (0.09). *p<0.05 (unpaired t-Test), +p<0.05 (longitudinal paired t-Test). See Tables 3.16. and 3.18. for individual Cd concentrations.

Figure 3.21. Cadmium Concentration in Lymphocytes, Subchronic Experiments.

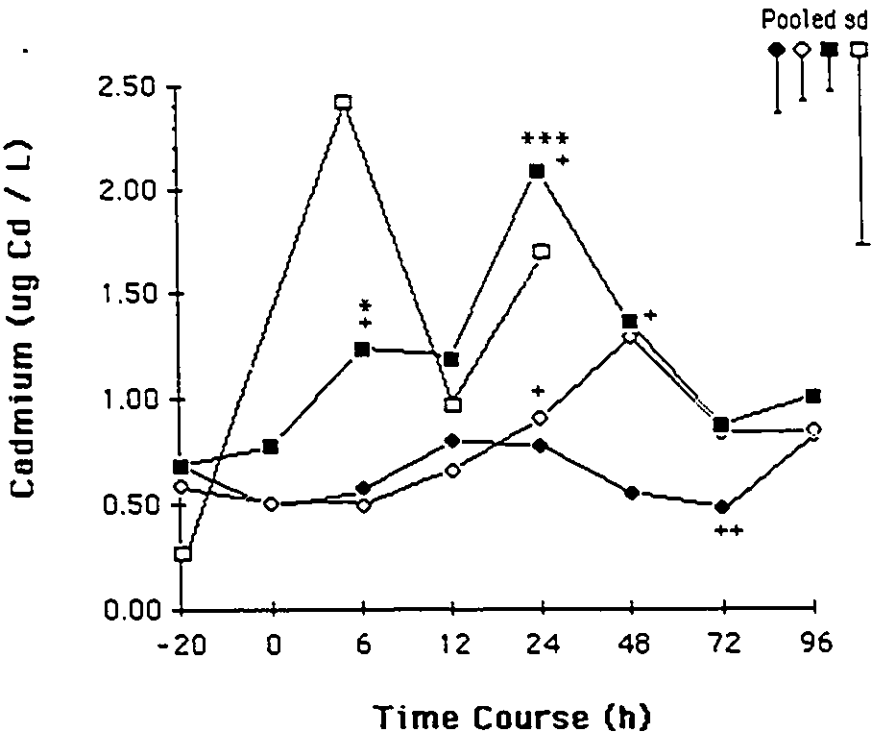


Legend to figure (administered dose):

-◆- 0.0 mg/kg; -○- 0.25 mg/kg; -■- 1.25 mg/kg; -□- 2.5 mg/kg;

Each rat received a total of three IP injections of the indicated doses over a 1.5 - 2.0 month period, (except M had 0.5 months). The third injection was administered at T = 0 h. The following rats died as the experiment progressed: K after 6 h, I and L after 48 h, and N after 72 h. The data points represent the mean (n=3), pooled standard deviations are; -◆-(0.3), -○-(0.6), and -■-(0.4). * $p<0.05$, ** $p<0.005$ (unpaired t-Test), + $p<0.05$ (longitudinal paired t-Test). See Tables 3.17. and 3.18. for individual Cd concentrations.

Figure 3.22. Cadmium Concentration in Monocytes, Short Course Experiments.

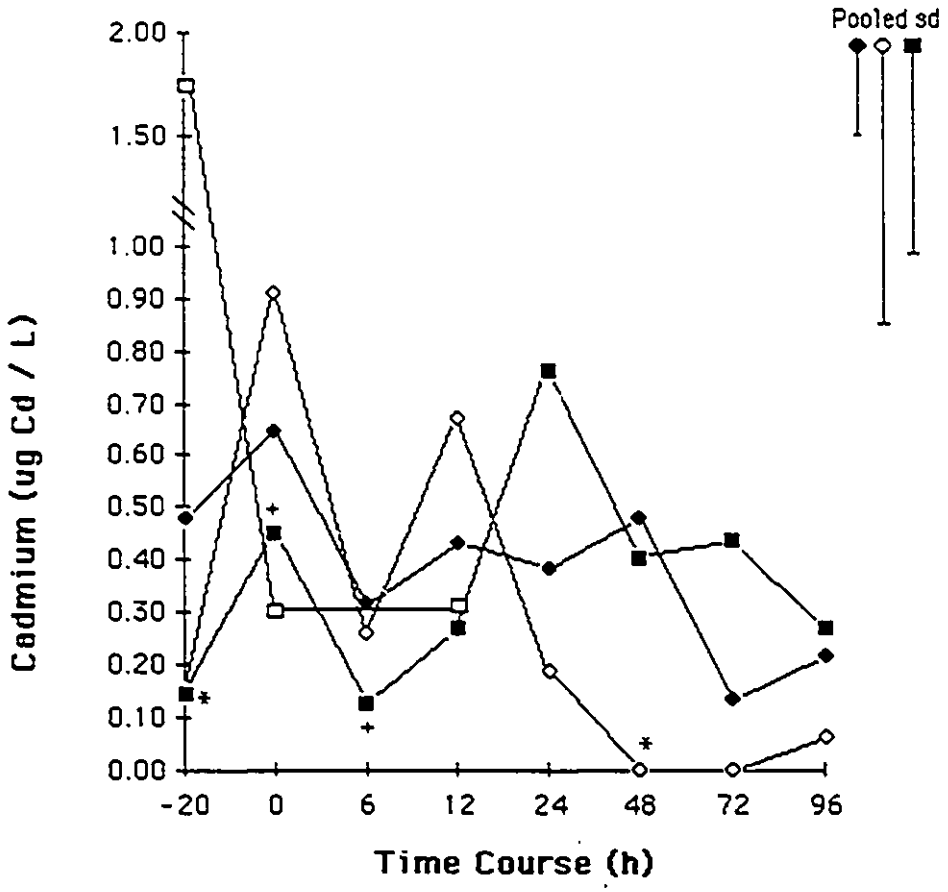


Legend to figure (administered dose):

-◆- 0.0 mg/kg; -○- 0.25 mg/kg; -■- 1.25 mg/kg; -□- 2.5 mg/kg;

Each rat received a single IP injection at T = 0 h. The data points represent the mean (n = 3). The pooled standard deviations are; -◆- (0.31), -○- (0.24), -■- (0.18) and -□-(0.9). *p<0.05, ***p<0.0005 (unpaired t-Test); +p<0.05, ++ p<0.005 (longitudinal paired t-Test). See Tables 3.19. and 3.21. for individual Cd concentrations.

Figure 3.23. Cadmium Concentration in Monocytes, Subchronic Experiments.

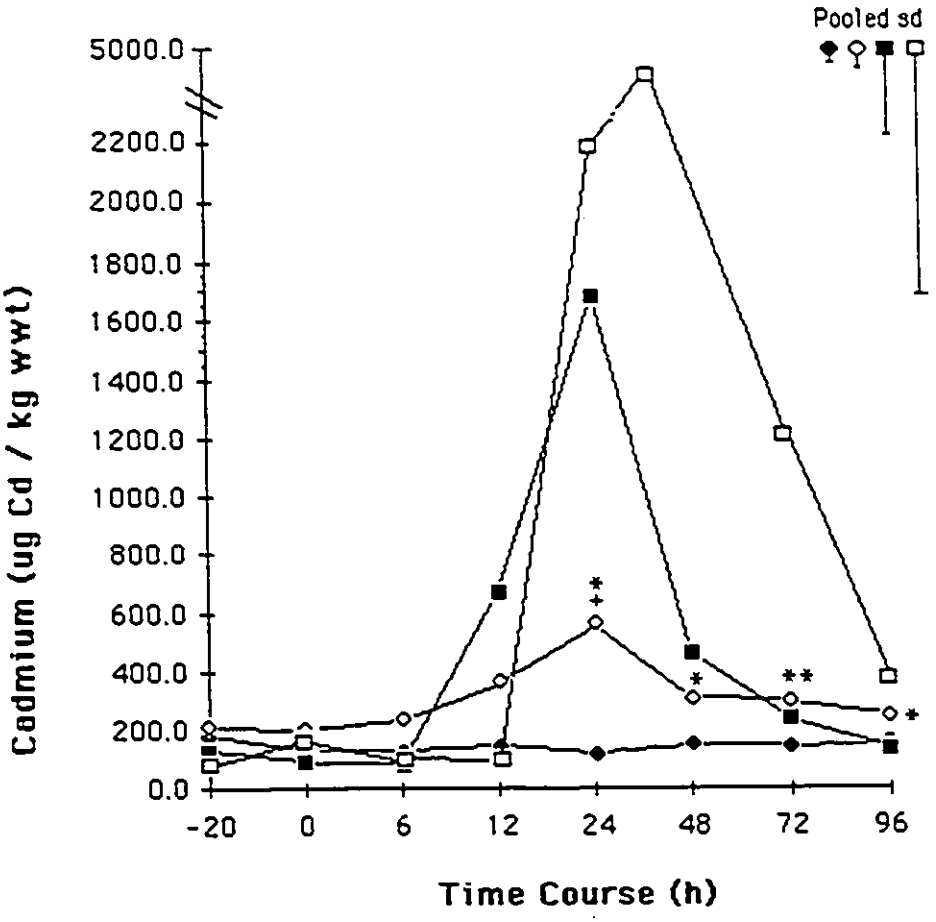


Legend to figure (administered dose):

-◆- 0.0 mg/kg; -◇- 0.25 mg/kg; -■- 1.25 mg/kg; -□- 2.5 mg/kg;

Each rat received a total of three IP injections of the indicated doses over a 1.5 - 2.0 month period, (except M had 0.5 months). The third injection was administered at T = 0 h. The following rats died as the experiment progressed: K after 6 h, I and L after 48 h, and N after 72 h. The data points represent the mean (n=3), pooled standard deviations are; -◆-(0.18), -◇-(0.57), and -■-(0.25). * $p<0.05$ (unpaired t-Test), + $p<0.05$ (longitudinal paired t-Test). See Tables 3.20. and 3.21. for individual Cd concentrations.

Figure 3.24. Cadmium Concentration in Feces, Short Course Experiments.

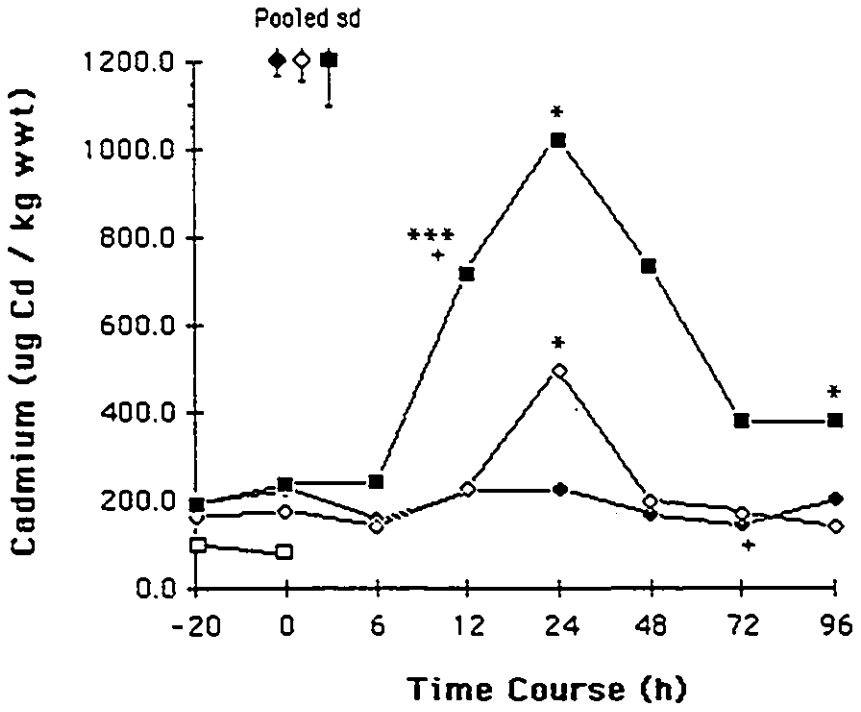


Legend to figure (administered dose):

◆- 0.0 mg/kg ; ◇- 0.25 mg/kg ; ■- 1.25 mg/kg ; □ 2.5 mg/kg;

Each rat received a single IP injection at T = 0 h. The data points represent the mean (n = 3). The pooled standard deviations are; ◆- (58), ◇- (78), ■- (297) and □ (877). *p<0.05, **p<0.005 (unpaired t-Test); +p<0.05 (longitudinal paired t-Test). See Tables 3.22. amd 3.24. for individual Cd concentrations.

Figure 3.25. Cadmium Concentration in Feces, Subchronic Experiments.

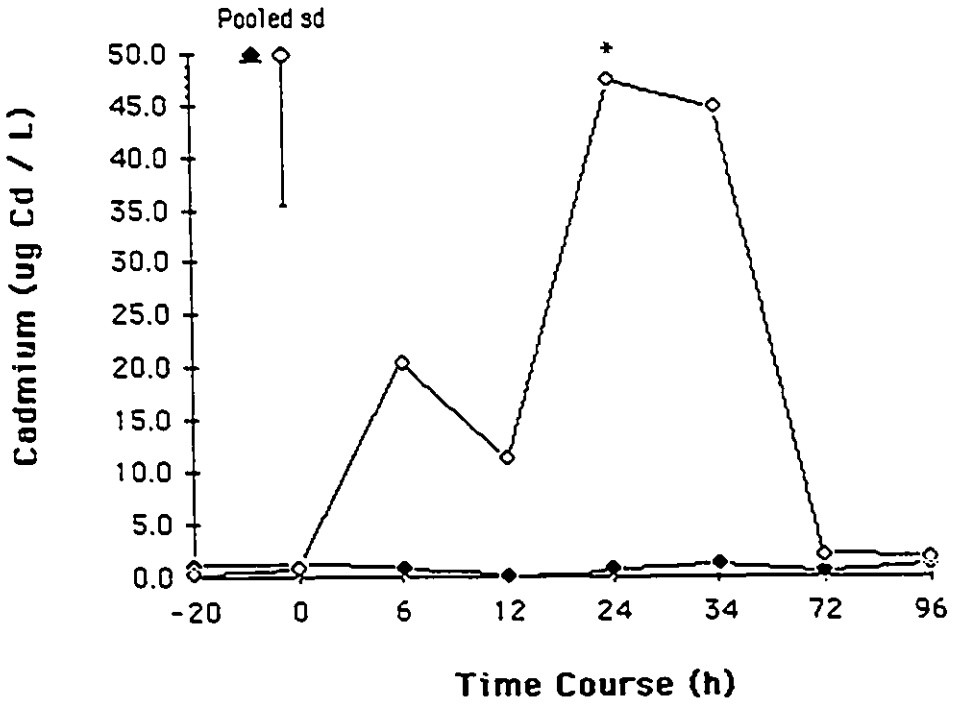


Legend to figure (administered dose):

-◆- 0.0 mg/kg; -◇- 0.25 mg/kg; -■- 1.25 mg/kg; -□- 2.5 mg/kg;

Each rat received a total of three IP injections of the indicated doses over a 1.5 - 2.0 month period, (except M had 0.5 months). The third injection was administered at T = 0 h. The following rats died as the experiment progressed: K after 6 h, I and L after 48 h, and N after 72 h. The data points represent the mean (n=3), pooled standard deviations are; -◆-(37), -◇-(44), and -■-(112). * $p<0.05$, *** $p<0.0005$ (unpaired t-Test), + $p<0.05$ (longitudinal paired t-Test). See Tables 3.23. and 3.24. for individual Cd concentrations.

Figure 3.26. Cadmium Concentration in Urine, Short Course Experiment AF90.

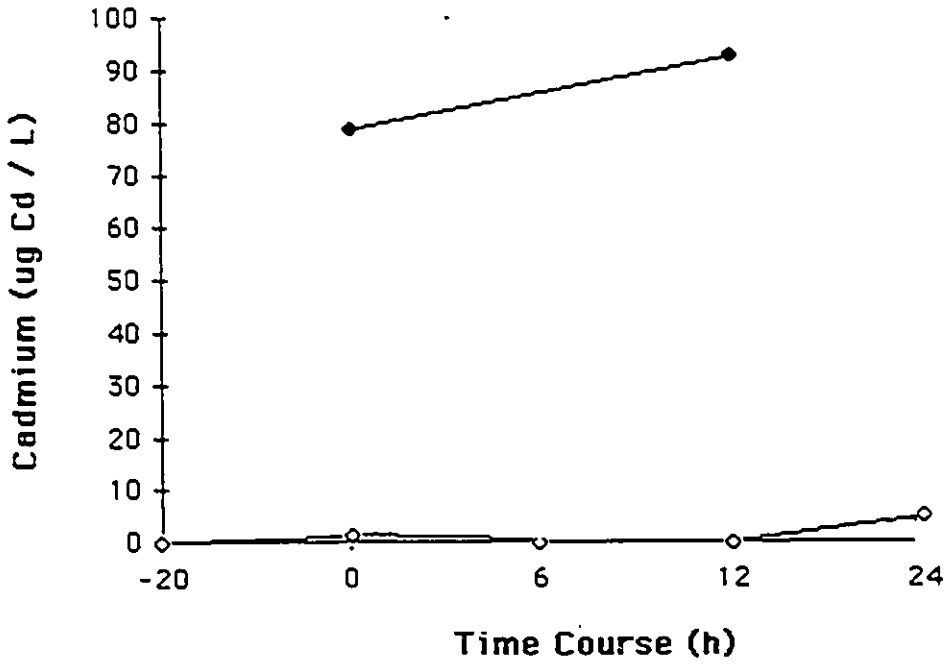


Legend to figure (administered dose):

-◆- 0.0 mg/kg (B,E); -◇- 2.5 mg/kg (A,F,D);

Each rat received a single IP injection at T = 0 h. The data points represent the mean, pooled standard deviations are; -◆-(0.35), and -◇-(14.5). *p<0.05 (unpaired t-Test). See Table 3.25 for individual Cd concentrations.

Figure 3.27. Cadmium Concentration in Urine, Subchronic Experiment CD90.

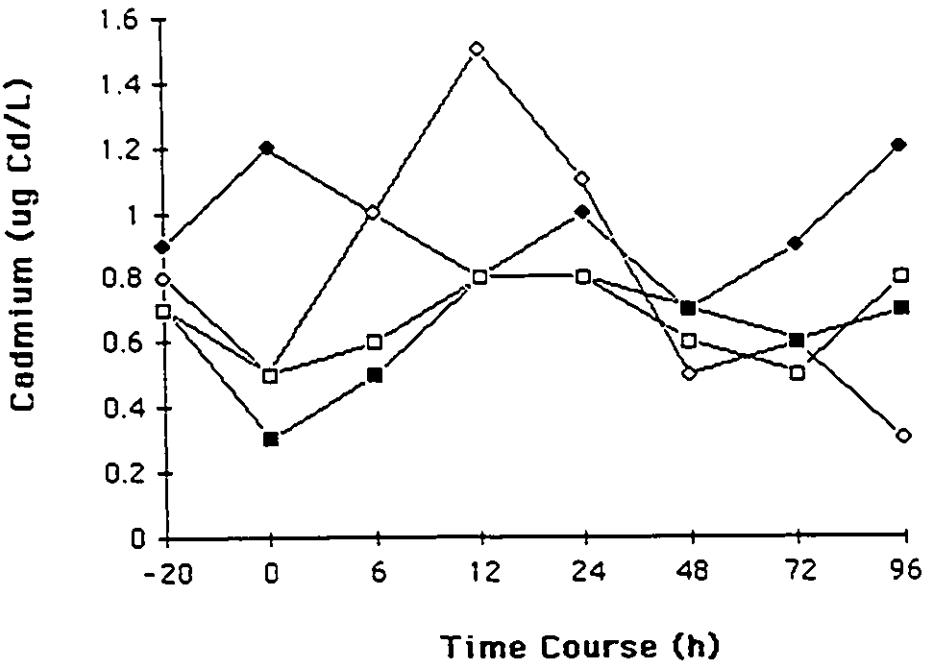


Legend to figure (administered dose):

◆- C (2.5 mg/kg); ◇- D (0.0 and 2.5 mg/kg)

Each rat received a total of three IP injections at the indicated doses over a 2.0 month period; C (2.5 mg/kg), D (0.0 mg/kg). The third injection (2.5 mg/kg for both rats) was administered at T = 0 h. (note that rat D received 2 doses at 0.0 mg/kg and at T = 0 h received one dose at 2.5 mg/kg). See Table 3.25 for individual Cd concentrations.

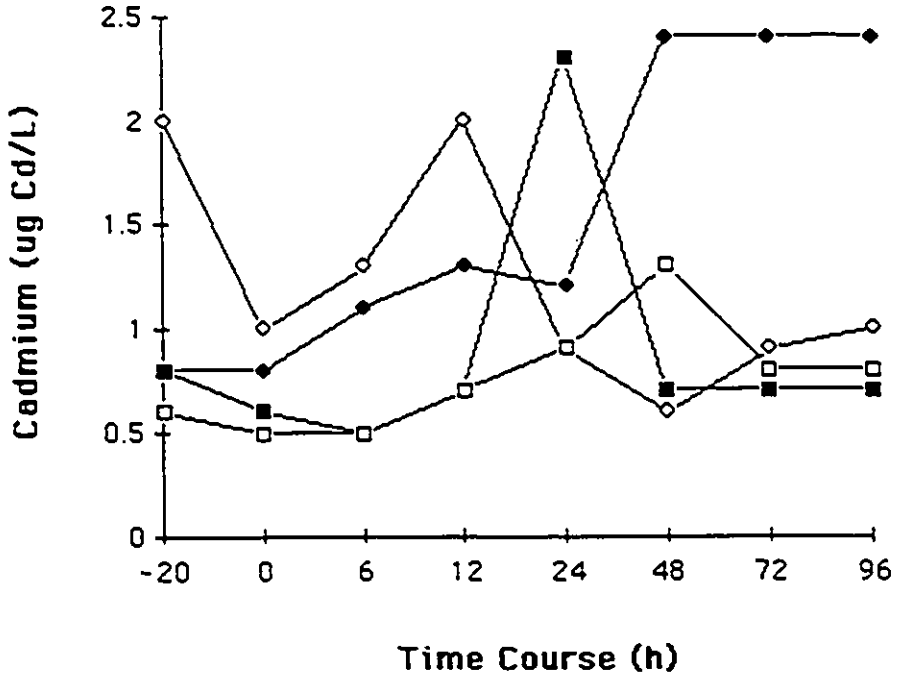
Figure 3.28. Comparison of Individual Control Blood Cell Compartments for Cd Concentration, Short Course Experiment PA91.



Legend to figure:

Each rat received a single IP injection at T = 0 h. The data points represent the mean (n=3). The individual blood cell compartments are: (-◆-) RBC, (-○-) PMN, (-■-) Lymphocytes, and (-□-) Monocytes for the control group.

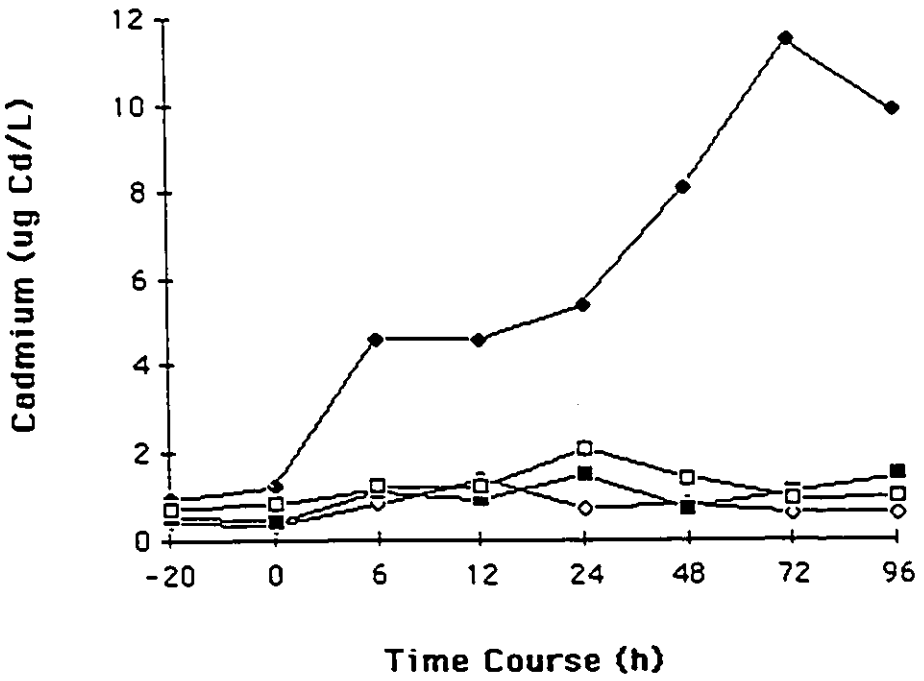
Figure 3.29. Comparison of Individual Low Dose Blood Cell Compartments for Cd Concentration, Short Course Experiment PA91.



Legend to figure:

Each rat received a single IP injection at T = 0 h. The data points represent the mean (n=3). The individual blood cell compartments are: (-◆-) RBC, (-○-) PMN, (-■-) Lymphocytes, and (-□-) Monocytes for the low dose (0.25 mg Cd/kg) group.

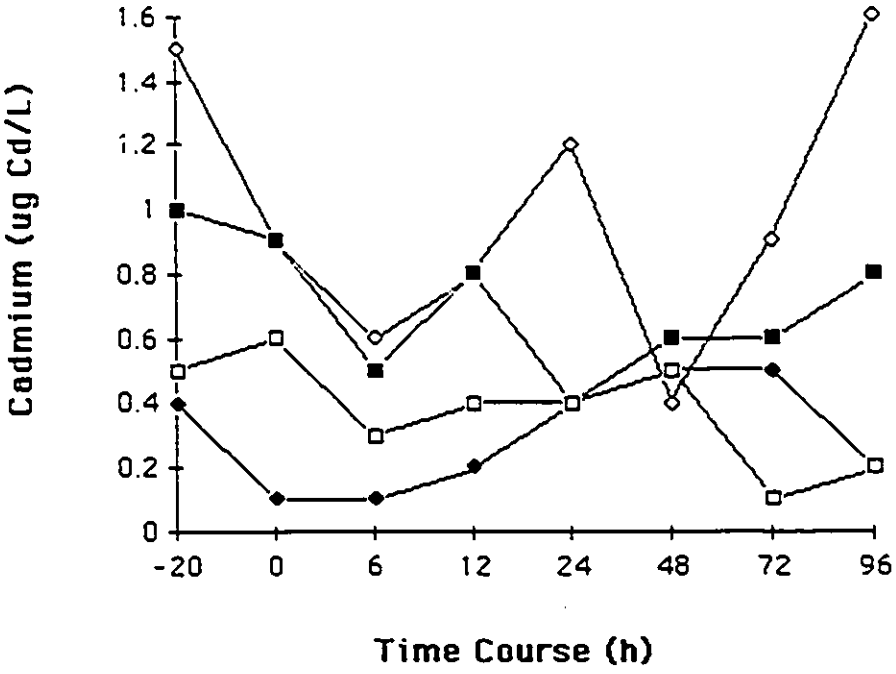
Figure 3.30. Comparison of Individual Medium Dose Blood Cell Compartments for Cd Concentration, Short Course Experiment PA91.



Legend to figure:

Each rat received a single IP injection at T = 0 h. The data points represent the mean (n=3). The individual blood cell compartments are: (-◆-) RBC, (-○-) PMN, (-■-) Lymphocytes, and (-□-) Monocytes for the medium dose (1.25 mg Cd/kg) group.

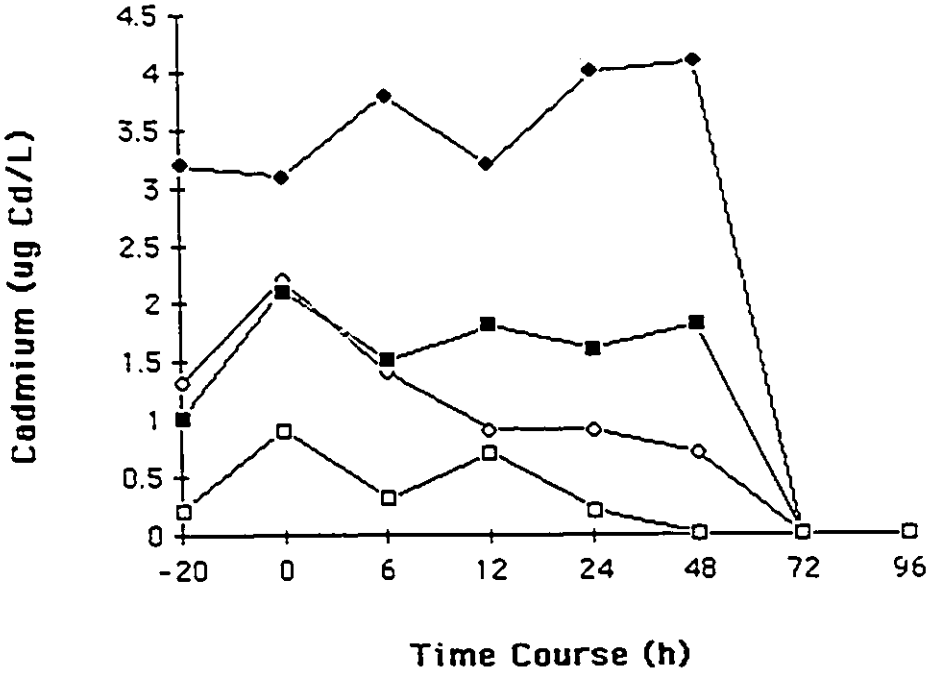
Figure 3.31. Comparison of Individual Control Blood Cell Compartments for Cd Concentration, Subchronic Experiment G091.



Legend to figure:

Each rat received a total of three IP injections of the indicated doses over a 1.5 - 2.0 month period. The third injection was administered at T = 0 h. The data points represent the mean (n=3). The individual blood cell compartments are: (-◆-) RBC, (-○-) PMN, (-■-) Lymphocytes, and (-□-) Monocytes for the control group.

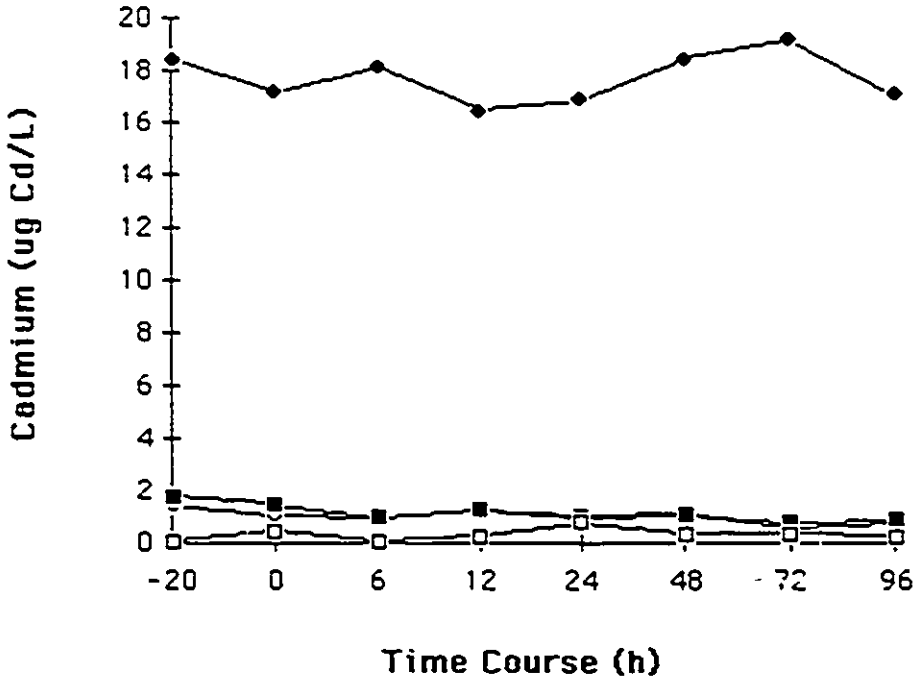
Figure 3.32. Comparison of Individual Low Dose Blood Cell Compartments for Cd Concentration, Subchronic Experiment G091.



Legend to figure:

Each rat received a total of three IP injections of the indicated doses over a 1.5 - 2.0 month period. The third injection was administered at T = 0 h. The data points represent the mean (n=3). The individual blood cell compartments are: (-◆-) RBC, (-○-) PMN, (-■-) Lymphocytes, and (-□-) Monocytes for the low dose (0.25 mg Cd/kg) group.

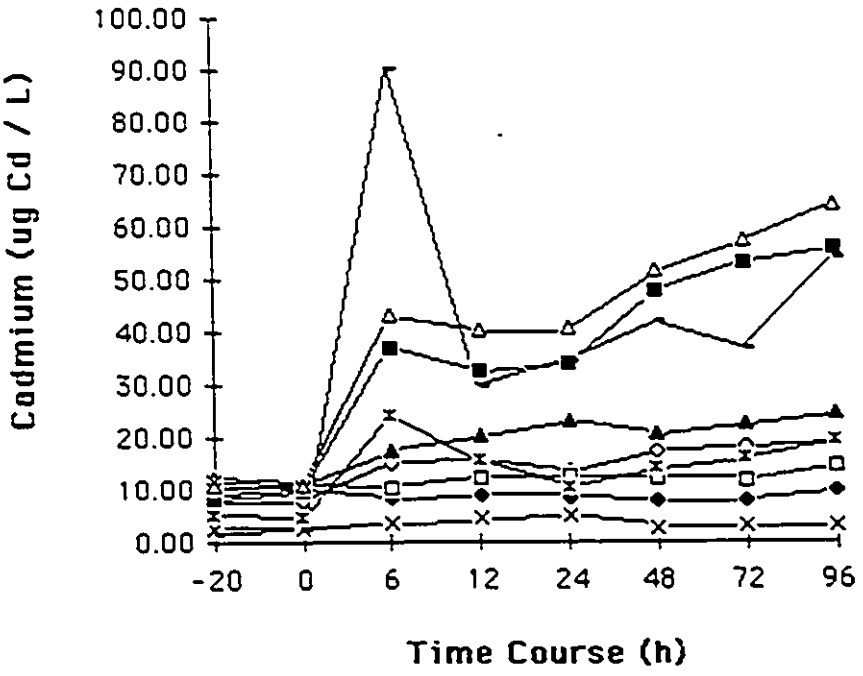
Figure 3.33. Comparison of Individual Medium Dose Blood Cell Compartments for Cd Concentration, Subchronic Experiment 6091.



Legend to figure:

Each rat received a total of three IP injections of the indicated doses over a 1.5 -2.0 month period. The third injection was administered at T = 0 h. The data points represent the mean (n=3). The individual blood cell compartments are: (-◆-) RBC, (-○-) PMN, (-■-) Lymphocytes, and (-□-) Monocytes for the medium dose (1.25mg Cd/kg) group.

Figure 3.34. Cadmium Concentration Mass Balance Comparisons in Whole Blood, Short Course Experiment PA91.



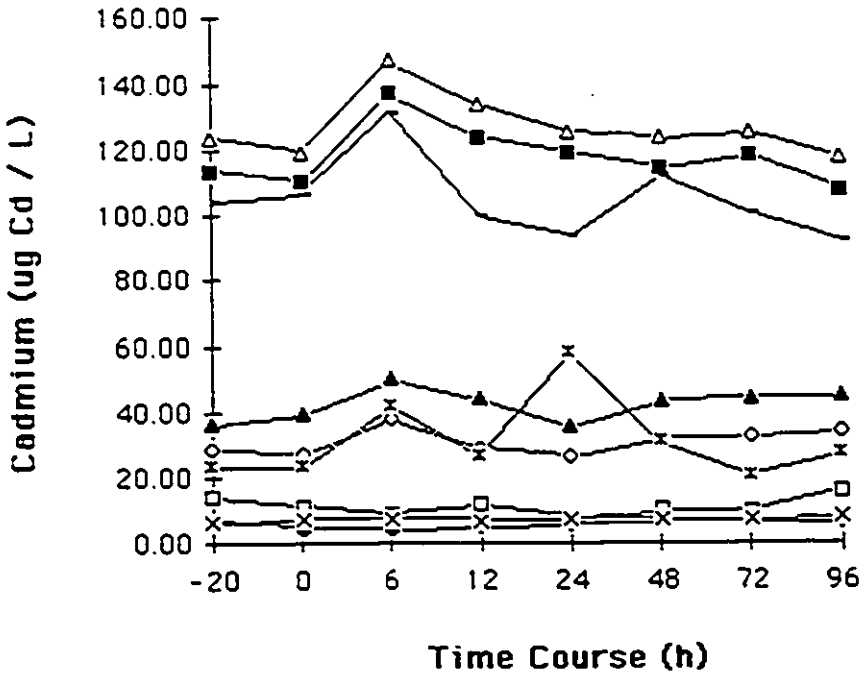
Legend to figure:

Each rat received a single IP injection at T = 0 h ; 0.0 mg/kg (T,U,AA); 0.25 mg/kg (V,W,X); 1.25 mg/kg (P,Q,R);

A 0.0	**A** 0.25	**A** 1.25
P 0.0	**P** 0.25	**P** 1.25
WB 0.0	**WB** 0.25	**WB** 1.25

A = Sum of total Cd concentration in all blood compartments (RBC, PMN, Ly, M, and plasma) using actual measured cell numbers. **P** = Sum of total Cd levels in all blood compartments using normalized concentrations and standard (typical) blood cell distribution numbers for the rat. **WB** = Total Cd concentrations determined in whole blood. The data points represent the mean (n=3).

Figure 3.35. Cadmium Concentration Mass Balance Comparisons in Whole Blood, Subchronic Experiment G091.



Legend to figure:

Each rat received three IP injections, the third was administered at T = 0 h; 0.0 mg/kg (G,H,I); 0.25 mg/kg (J,K,L); 1.25 mg/kg (M,N,O).

A 0.0	**A** 0.25	**A** 1.25
P 0.0	**P** 0.25	**P** 1.25
WB 0.0	**WB** 0.25	**WB** 1.25

A = Sum of total Cd concentration in all blood compartments (RBC, PMN, Ly, M, and plasma) using actual measured cell numbers. **P** = Sum of total Cd levels in all blood compartments using normalized concentrations and standard (typical) blood cell distribution numbers for the rat. **WB** = Total Cd concentrations determined in whole blood. The data points represent the mean (n=3).

CHAPTER 4:

4. METALLOTHIONEIN (MT) DETERMINATION IN SELECTED ORGANS, PERIPHERAL BLOOD CELLS AND BODY FLUIDS.

4.1. Introduction

4.1.1. Metallothionein (MT)

MT was first isolated and characterized in equine kidney (Margoshes and Vallee, 1957). MT proteins are low molecular weight, are rich in cysteine (30%) and lysine (13%), have few aromatic amino acids, and avidly bind metals (Zn, Cd, Cu, and Hg) (Margoshes and Vallee, 1957; Kagi and Vallee, 1960; Tohyama and Shaikh, 1978; Kotsonis and Klaassen, 1981; Kagi and Kojima, 1987; Mehra and Bremner, 1987; Klaassen and Waalkes, 1987). The molecular weight of MT is approximately 6 - 7 kD, although MWs have been reported as high as 10 kD (Margoshes and Vallee, 1957; Cherian, 1974; Winge et al., 1975; Onosaka and Cherian, 1981). More recently, an Ion Spray mass spectrometry study of equine renal MT1 determined the molecular weight to be 6,143.9 D with a standard deviation of 0.7 D (Pleasance et al., 1990). However, it is generally believed that MT forms polymers of approximately 14, 21, and 30 kD MW as a result of sulphydryl binding with other MT molecules (Kagi and Kojima, 1987). MT characteristically absorbs at 254 nm when bound to metals and virtually no absorbance occurs at 280 nm because it lacks aromatic amino acids. The resulting UV absorbance ratio of 254/280 nm is virtually unique for MT (Margoshes and Vallee, 1957; Kagi and Vallee, 1961; Winge et al., 1975; Vander Mallie and Garvey, 1978). Two major isoforms of MT have been characterized in virtually every species tested and labelled MT1 and MT2 based on their DEAE ion-exchange elution pattern (Vander Mallie and Garvey, 1978; Nordberg and Kojima, 1979; Hamer, 1986; Fowler, 1986; Paliwal et al., 1986;

Suzuki, 1987; Kagi and Kojima, 1987; Klaassen and Waalkes, 1987). MT isoforms are further characterized as subforms and denoted by lower case letters, ie. MT 1a, MT 1b, MT 2a, MT 2b, etc. (Fowler et al., 1987b; Hunziker and Kagi, 1987). The structure of MT has been elucidated as two metal-thiolate clusters labelled cluster A and B (Figure 4.1, adapted from Hunt et al., 1984). Cluster A binds 4 metal atoms and cluster B binds 3 metal atoms for a total of 7 metal atoms per molecule of MT (Kagi and Vallee, 1961; Nordberg and Kojima, 1979; Otvos and Armitage, 1980; Boulanger et al., 1983; Hunt et al., 1984; Kagi et al., 1984; Winge and Nielson, 1984; Bernhard et al., 1987; Kagi and Kojima, 1987).

The exact physiologic role of MT has yet to be fully elucidated, although MT has been implicated in the metabolism, homeostasis and detoxication of metals (Piscator, 1964; Nordberg et al., 1971a,b; Kotsonis and Klaassen, 1981; Eaton et al., 1980; Cherian and Nordberg, 1983; Sarkar and Abdulwajid, 1983; Petering et al., 1984; Petering and Fowler, 1986; Huang et al., 1987; Nordberg and Nordberg, 1987; Webb, 1987). MT is synthesized (induced) in response to drugs, exposure to metal ions, and stress (Oh et al., 1978; Hager and Palmiter, 1981; Hamer, 1986; Lehman-Mckeeman and Klaassen, 1987; Brzeznicka et al., 1987; Brady, 1991). The induction process is initiated by cellular uptake of the inducing agent (Cd) which initiated processes that turn on the MT gene. MT mRNA is then synthesized in the nucleus from DNA. MT mRNA acts as a cytosolic template for MT protein synthesis (Piscator, 1964; Ohi et al., 1981; Hamer, 1986; Grady et al., 1987; Hanke et al., 1988). MT-induction capacities in humans vary between individuals (Harley et al., 1989). The half-life of MT mRNA is about 10 - 12 hours (Darnell et al., 1986; Monia et al., 1986), while the half-life of CdMT protein is approximately 1 - 5 days and is tissue specific (Shaikh and Lucis, 1972; Kotsonis and Klaassen, 1981; Monia et al., 1986; Tanaka et al., 1987). MT has been postulated to afford protection against exposure to heavy metals (Piscator, 1964; Nordberg et al., 1971a; Jin et al

1987a,b; Frazier and Din, 1987); animal and tissue culture studies also implicate MT itself as a potent cytotoxin (Nordberg et al., 1975; Cherian et al., 1976; Suzuki and Cherian, 1987; Jin et al., 1987c; Fowler et al., 1987a; Elinder et al., 1987; Leffler et al., 1990).

4.1.2. Determination of MT

In the last 30 years several different methodologies have been developed for MT determination in tissues, blood cells and body fluids. In addition to the principal methods summarized in Table 4.1, gel filtration, atomic absorption spectrometry (AAS), high performance liquid chromatography (HPLC), sulphydryl assay (SH), sodium dodecyl sulphate polyacrylamide electrophoresis (SDS-PAGE), autoradiography and optical spectroscopy have been used to determine MT (Suzuki, 1980; Eaton and Toal, 1982; Aoki et al 1986; Lehman and Klaassen, 1986; Dieter et al., 1987; Stillman et al 1987; Andersen and Daae, 1988; Stillman and Gasyna, 1991). Several of the procedures are not used widely due to a requirement of expensive instrumentation, technical skills, and time (Eaton and Toal, 1982). The general principle employed for MT determination in metal saturation assays utilizes measurement of the metal binding capacity to indirectly estimate total MT concentration. The excess or non-bound metal is precipitated (as a complex of hemoglobin) and the supernatant containing the metal bound MT proteins are measured by a gamma counter or AAS. In the radioactive (^{109}Cd) cadmium saturation assay (CSA), also called Cd radioassay and Cd/Heme assay, MT protein concentration is calculated from the ^{109}Cd binding ratio of 7 mole Cd per mole MT (Chen and Ganther, 1975; Eaton and Toal, 1982; Nolan and Shaikh, 1986). A similar ratio of 6 or 7 mole Cd per mole MT was used in the non-radioactive Cd saturation assays with cold $CdCl_2$ used in conjunction with AAS (Onosaka and Cherian, 1982; Onosaka et al., 1984; Dieter et al., 1987). The mercury saturation assay or Hg/TCA assay calculates MT from Hg(II) binding instead of Cd(II) (Piotrowski et al., 1973; Kotsonis and Klaassen, 1977).

Other methods for assessing MT have been pursued, specifically polarographic, gel filtration column chromatography coupled with AAS and radioimmunologic methods. Polarography measures the concentration of heat-stable sulphydryl groups electrochemically to determine the MT protein levels in the test sample (Olafson and Sim, 1979; Onosaka and Cherian, 1981; Onosaka and Cherian, 1982; Olafson, 1987). G-75 Sephadex gel filtration column chromatography is used to isolate the MT containing fractions and AAS to determine the metal content. In the above mentioned procedures, MT levels were calculated from the metal content of the sample and assuming a MW of 6050-7000 D (Onosaka and Cherian, 1981; Nolan and Shaikh, 1986; Dieter et al., 1987). The radioimmunoassay (RIA) is a competitive binding assay using ^{125}I -MT as the radioisotopic label and either a MT specific double antibody complex (Brady and Kafka, 1979; Vander Mallie and Garvey, 1979; Garvey et al 1982; Bremner et al., 1987; Dieter et al., 1987; Shaikh, 1991), or ammonium sulfate (Nolan and Shaikh, 1986; Shaikh and Nolan, 1987; Shaikh, 1991) to precipitate the MT-Ab complex. The quantity of ^{125}I -MT present in the MT-Ab complex is inversely proportional to the amount of unlabelled MT in the sample. The double antibody complex of the RIA requires the isolation and purification of rat MT in large quantities and the production of rabbit anti-rat MT antibody, neither of which are available commercially. More recently, the enzyme-linked immunosorbent assay (ELISA) directly measures the absorbance of a suitable product generated by the enzyme while bound to a double antibody complex (Thomas et al., 1986; Garvey et al., 1987; Cousins, 1991).

All of the MT determination methods described above rely on non-specific or indirect measurements to estimate the MT concentration, except the RIA and ELISA which involve direct protein measurements. In comparison studies, the Cd and Hg radioassays gave similar results in one study (Waalkes et al., 1985), while overestimation of MT by the Hg assay was reported in another interlaboratory comparison (Dieter et al., 1987). The Cd radioassay

(CSA) is the most widely used assay to quantitate MT and was determined to be more advantageous for routine screening and analysis of tissue MT with a detection limit >10 ug/g wet weight (wwt) (Nolan and Shaikh, 1986; Shaikh and Nolan, 1987). An earlier study established a detection limit of 0.8 ug MT/g wwt (Eaton and Toal, 1982). The improvement in sensitivity was due to an increase in the specific activity of the ^{109}Cd label, achieved by lowering the concentration of unlabelled Cd (Eaton and Toal, 1982). Although the RIA is more complicated and time consuming than other assays, it is more specific and has been considered the method of choice for detection of MT in low concentrations (<10 ng/g) (Dieter et al., 1987; Shaikh and Nolan, 1987). However, because MT is an ubiquitous and highly conserved protein in most species (Kagi and Kojima, 1987), and exhibits rather low antigenic properties, production of MT antibodies used in the RIA is rather costly and time consuming (Vander Mallie and Garvey, 1978; Eaton and Toal, 1982). The Hg radioassay, while rapid and relatively easy, lacks sensitivity and the endogenous LMW Hg-binding substances like glutathione cause interferences in the assay (Zelazowski and Piotrowski, 1977; Waalkes et al., 1985). The G-75 gel filtration column chromatography method is time consuming and poses problems due to sample dilution or contamination (Eaton and Toal, 1982; Nolan and Shaikh, 1986; Dieter et al., 1987). The overall rating of the five methods mentioned (ie, the CSA, Hg radioassay, RIA, SH assay, and G-75 gel filtration) in an interlaboratory comparison study (Dieter et al., 1987), ranked the RIA and CSA as the most suitable for MT determination. The Hg radioassay showed overestimation of the protein, while the G-75 and SH assays lead to underestimation of MT concentration in addition studies. In a comparison study between CSA and polarographic methods, the polarographic determinations produced slightly higher values than the CSA (Onosaka and Cherian, 1982). Variability observed in the MT assays has been documented previously by several research groups (Tohyama and Shaikh, 1978; Eaton and Toal, 1982; Eaton, 1985; Waalkes et al., 1985; Nolan and Shaikh, 1986; Dieter

et al., 1987; Shaikh and Nolan, 1987).

In order to reduce interlaboratory differences the concentration of MT protein should be reported in a standardized form, specifically ug MT/g, or uMol MT/g tissue (Onosaka and Cherian, 1982; Nolan and Shaikh, 1986). In the present project values are reported as uMol MT/kg dry weight (dwt) for tissues and uMol MT/L for fluids in order to correlate these results more closely with human studies (Nieboer et al., 1988a, b). However, standardization of the MT concentration does not eliminate interlaboratory discrepancies and the lack of a standardized procedure adds to the problem (Nolan and Shaikh, 1986). In addition, endogenous substances (albumin, hemoglobin, mercaptalbumin, or transferrin) have been documented to bind Cd. In addition, metals (Cu, Ag, or Hg) compete for binding sites and as a result interfere with several MT assays, including the RIA and CSA (Eaton and Toal, 1982; Eaton, 1985; Suzuki et al., 1986; Nolan and Shaikh, 1986). Interferences may also occur from improper sample handling, MT oxidation and polymerization upon storage, proteolytic degradation of MT, aging of the hemolysate preparation and the binding of Cd to heat-stable fragments of Hb or other LMW substances all contribute to a reported 25% inter-assay variability; by comparison, an 8 % intra-assay variability has been reported (Minkel et al., 1980; Templeton and Cherian, 1984; Nolan and Shaikh, 1986; Mehra and Bremner, 1987).

4.1.3. Specific Research Objectives

The component of the research described in the present chapter involved an assessment of the distribution and concentration of MT in the major organs, peripheral blood cells and body fluids after single and multiple IP injections of $CdCl_2$ in rats according to the protocols described in Chapter 2.

Both RIA and CSA were investigated in detail for the routine quantitation of MT. Problems were encountered with both assays. In order to optimize the RIA, the efficiency of the N-succinimidyl-3-(4-hydroxyphenyl)propionate-MT (NSHPP-MT) adduct formation and subsequent ^{125}I labelling reactions were

studied along with antibody production in rabbits and chickens. Several parameters were systematically investigated to optimize the CSA specifically: tissue isolation, sample interferences, hemoglobin concentration, incubation temperature, buffer concentration, and the cold Cd concentration added to the radiolabeling solution.

4.2. Experimental Procedures

4.2.1. MT Isolation, Purification and Characterization

Male Sprague Dawley rats received multiple IP injections of $CdCl_2$ (2.5 mg $CdCl_2$/kg) to induce synthesis of metallothionein (MT) protein (see Chapter 2). The purified liver MT proteins were characterized by SDS-Page and G75 gel filtration prior to use in antibody production and RIA tests. $CdCl_2$ induced rat livers were homogenized in cold 0.1 M ammonium formate buffer (20% w/v), centrifuged at 37,000g for 30 minutes (Figure 4.2 and Table 4.2) (Vander Mallie and Garvey, 1978). The supernatant received alternate heat (80°C for 5 min) and ice treatments, and was centrifuged at 20,000g for 30 minutes. The supernatant was lyophilized and applied to a G-75 Sephadex gel-filtration column (2.6 x 70 cm, 1 mL/min flow rate) and eluted with 0.01 M ammonium formate buffer. The UV absorbance of the fractions (5 mL/tube) were monitored at 254 and 280 nm. Fractions with high 254/280 absorbance ratios were pooled and lyophilized. The lyophilized sample was applied to a DEAE A25 ion-exchange column (2.6 x 30 cm, 0.5 mL/min flow rate). Two MT isoforms were eluted with a 0.005 - 0.50 M Tris buffer gradient and fractions of 5 mL/tube were collected. The fractions were monitored as above, pooled and lyophilized employing the protocol of Vander Mallie and Garvey, (1978).

Each isoform was characterized as follows: SDS-PAGE to determine molecular weight and purity, the CSA for MT content, the Bio-Rad (Bradford, 1976) for protein content, molar extinction coefficients to determine MT

concentration (see Results, Section 4.3), and electrothermal atomic absorption spectrometry (EAAS) for Cd content. SDS-PAGE analysis using dissociating conditions was employed (Laemmli,1970; Dhamankar et al., 1986; Aoki et al., 1986; Andersen and Daae, 1988; Bio-Rad Protean II Slab Gel Instruction Manual). The protein solutions were dissociated with 10% SDS, beta-mercaptoethanol and heating, then resolved on 1.5 mm slab gels using a 4% stacking and 12% separating gels. The gels were stained with either Coomassie Blue R-250 or Bio-Rad silver stain. The MT concentrations in selected DEAE ion-exchange fractions, containing the MT isoforms, were determined by the micro-CSA method described in detail below (also see Figure 4.3). In the Bio-Rad protein assay, BSA, lysozyme and MT (Sigma) were used as reference proteins. This assay is based on the extent of color change of negatively charged Coomassie Brilliant Blue G-250 dye from red (465 nm) to blue (595 nm) when bound to positive charges on the protein (Bradford, 1976; Bio-Rad Protein Assay Instruction Manual; Robyt and White, 1990). Molar extinction coefficients (ε) were determined using a rearrangement of Beer's Law: $\varepsilon = A/bc$; where A = absorbance at 250 nm, b = 1 cm path length, c = molar concentration of the test solution. The experimental ε values were determined for rat MT isoforms (prepared by L.M.J. Smith) as well as rabbit and horse MT isoforms (Sigma).

4.2.2. MT Antibody Production

Rat hepatic MT1 and MT2 isoforms were mixed and polymerized for polyclonal antibody production in rabbits employing the protocols of Garvey et al. (1982), Harlow and Lane (1988) and in laying hens adapting the protocol of Polson et al. (1980, 1985). These antibodies were produced for MT quantification in various cells and body fluids using RIA. In brief, MT protein was isolated and purified by column chromatography from rat livers. The purified rat MT isoforms were polymerized using 2.5% glutaraldehyde (Habeeb and Hiramoto, 1968). The glutaraldehyde-MT solution was allowed to react for

several hours. The reaction was quenched by addition of 0.1 M lysine. The polymerized MT protein was separated from free lysine by Sephadex G-75 column chromatography. The polymerized rat MT was checked for purity by SDS-PAGE. A 1:3 mixture of MT with Freund's Adjuvant (employing a 1:9 ratio of complete to incomplete Freund's Adjuvant) were prepared prior to the injections. Four female New Zealand white rabbits initially weighed between 3.3 - 3.9 kg were housed individually in the Central Animal Facility from 7/89 to 6/90. Each rabbit received multiple IM injections in the hip region over 40 weeks. The rabbits received several IV injections of polymerized MT without Freund's Adjuvant via the marginal ear vein. These IV injections followed IM injections of benadryl (10 mg/mL) to prevent anaphylactic shock. At appropriate times after MT injection, sera were collected for antibody detection using the Ouchterlony plate immunodiffusion (OID) according to the method used by Garvey et al. (1982). In the OID tests, the center well contained 5 uL of 2 mg rat MT/mL. The six outer wells contained 10 uL of rabbit anti-rat MT sera diluted as: position 1 (12 o'clock) undiluted serum; position 2 (2 o'clock) 50 % serum dilution; position 3, 75 %; position 4 (6 o'clock) 87 %; position 5, 94 %; and position 6, contained a 97 % serum dilution in PBS (Figure 4.9). Rabbit #L-2 was euthanized with sodium pentobarbital and serum collected after the results of the sixth OID were obtained.

Three white Leghorn laying hens were housed in individual cages in the Central Animal Facility. Each chicken received similar IM injections of the MT Freund's Adjuvant mixture in the thigh region once a week for a total of four weeks with periodic boosters as necessary. Eggs were collected and antibodies were isolated from the yolks using several polyethylene glycol (PEG) separations, a cold ethanol precipitation of the antibody, followed by dialysis against phosphate buffer for 24 hours at 4°C. Development of antibodies were monitored by OID according to the procedure of Polson et al. (1985).

4.2.3. Radioimmunoassay

The radioimmunological assay (RIA) procedures of Vander Mallie and Garvey (1979) and Garvey et al. (1982) using antibodies generated in rabbits against rat MT (Vander Mallie and Garvey, 1978) had been modified and employed in this laboratory (Nieboer et al., 1988b) to detect MT in plasma, and urine. The present study examined the feasibility of extending this protocol to include tissue homogenates and blood cell lysates. The MT-RIA method was examined as an independent verification of the CSA protocol performance.

MT protein is not easily labelled with ^{125}I due to the high cysteine and low aromatic amino acid content (Kagi and Kojima, 1987). The reaction of Bolton and Hunter (1973) was adopted for adduct formation and iodination of MT as modified by Vander Mallie and Garvey (1978). Purified MT was mixed with NSHPP. The reaction product (NSHPP-MT) was separated from free NSHPP over a G-25 Sephadex column and fractions were monitored for UV absorbance at 220, 240, 254, 265 and 280 nm. The fractions containing high 254/280 absorbance ratios were pooled. Aliquots containing 4 - 5 ug NSHPP-MT were dispensed into Eppendorf tubes, lyophilized and stored at -20°C until iodinated by the chloramine-T reaction. NSHPP-MT was iodinated with a mixture of 1 mCi ^{125}I in 10 uL NaOH (pH 9), 20 uL 0.25 M phosphate buffer (pH 7.5) and the reaction started with the addition of 20 uL chloramine-T (2.5 mg/mL). The solution was vortexed for 45 seconds and the reaction quenched with 100 uL sodium metabisulphite (1.2 mg/mL). The reaction products were separated over either a G-50 or G-25 Sephadex gel filtration column equilibrated with 0.05 M TGB (0.05 M Tris, 0.1 % gelatin, 0.02 % NaN_3, pH 8.0) and 25 fractions of 1 mL were collected. Aliquots of 5 uL of each fraction were diluted to 500 uL with TGB and 5 uL of the diluted fractions were counted in a NE1612 gamma counter. The ^{125}I - MT label was diluted to contain 50,000 cpm / 50 uL and stored at 4°C. The label was used in the RIA for 1 - 2 weeks (Nieboer et al., 1988b). After each iodination reaction, an antibody titration curve assessed the antibody binding capacity for the MT antigen. In the antibody titration curve,

several dilutions of the 1°Ab (rabbit anti-rat MT or chicken anti-rat MT) were tested for its capacity to precipitate ^{125}I-MT labelled antigen (Table 4.8) using the MT specific double-antibody competitive-binding assay, (RIA-MT). Details of the RIA protocol employed (Nieboer et al., 1988b) are tabulated in Table 4.4 and the reagents used are presented in Table 4.3. At time 0 h, controls, test samples and MT standards were diluted with TGB buffer to 400 uL. To each tube, 50 uL of 1° Ab (rabbit anti-rat MT or chicken anti-rat) was added, vortexed, and incubated at 4°C for 24 hours. After the 24 h incubation period, 50 uL of ^{125}I-MT label (containing 50,000 cpm) was dispensed to each tube using an Eppendorf repeater pipette. The tubes were then mixed, and incubated for a further 6 hours at 4°C. At time 30 h, 100 uL of 1:20 diluted 2°Ab (goat anti-rabbit IgG (GARGG) or rabbit anti-chicken IgG (RACGG)) and 1.00 mL 4% polyethylene glycol (PEG) were added to the assay mixture, vortexed, allowed to equilibrate for 30 minutes at room temperature and then centrifuged at 3000 rpm for 30 minutes. The supernatant was decanted in a radioactive labelled sink with running water. The tubes were inverted to drain using a special locking test tube rack on an absorbant pad overnight. The tubes containing the pellet were counted twice for 60 seconds in a NE1612 gamma counter using the ^{125}I isotope detection mode (Nieboer et al., 1988b). MT concentration in the sample was determined from the standard curve (% bound versus log MT concentration).

4.2.4. Cadmium Saturation Assay (CSA), General Procedure

The CSA methodology used in the present study was adopted from Nolan and Shaikh (1986), Eaton and Toal (1982), Onosaka and Cherian, (1982) and adapted for small sample volumes (100 uL). Hemoglobin was employed instead of RBC hemolysate (Eaton and Toal, 1982). A summary of the approach to the CSA taken by other workers is provided in this section for comparison with the optimized mini-CSA (Table 4.6) and micro-CSA (Figure 4.3) developed in the present study (Section 4.2.6). In general, aliquots (200 - 500 uL) of semi-

purified test sample and blank solutions were mixed with (90 - 200 uL) ^{109}Cd radiolabel and incubated at room temperature with intermittent mixing for 10 - 15 minutes (Figure 4.3). Aliquots (50 - 200 uL) of 2% Hb or RBC hemolysate solution were added to each test tube, capped, vortex, and incubated at 80 - 100°C for 1 - 3 minutes. The tubes were placed on ice for 1 - 3 minutes and centrifuged at (1000 - 10,000 g) for 2 - 5 minutes. Supernatant aliquots (100 - 500 uL) were transferred to clean microcentrifuge tubes. The Hb precipitation, cooling and centrifugation steps were repeated twice. After the final centrifugation step, the analyte was transferred to appropriate test tubes for gamma counting (in the present study, 12 x 75 mm plastic tubes were used for the LKB Wallac 1275 in-line gamma counter and 12 x 75 mm glass tubes for the 12 well NE1612 gamma counter). Total counts were determined from a 1/10 dilution of the radiolabel in normal saline.

4.2.5. Parameters Investigated to Optimize the CSA

A systematic study of several parameters and conditions were investigated to optimize the CSA and eliminate assay variability. The following aspects were examined: 1. tissue isolation procedures; 2. CSA interferences and sample purity; 3. hemoglobin concentration; 4. incubation temperature; 5. buffer concentration; 6. concentration of cold Cd; and 7. column chromatography of the samples.

4.2.5.1. *Tissue Isolation Procedures*

The approach to sample preparation in the CSA versus MT isolation procedures is quite different (Figure 4.2). For liver and kidney preparations, the CSA uses a 10% (wwt/v) homogenate solution and the MT isolation uses a 20% homogenate solution. The sample preparation steps in both procedures include: centrifugation, protein precipitation in a near boiling water bath, followed by cooling in an ice bath and a second centrifugation step. In the CSA, the supernatant is centrifuged for a shorter time at lower speeds.

4.2.5.2. *CSA Interferences and Sample Purity*

Sample and reagent purity were checked by SDS-PAGE before and after CSA determination. Several different sample types (plasma, RBC, leukocyte lysate, urine, and RPMIc cell media) were examined by SDS-PAGE for impurities that could cause interferences in the CSA. EDTA and heparin were examined by CSA for possible interferences in the binding and precipitation of the ^{109}Cd label.

4.2.5.3. *Hemoglobin Concentration*

In several reported procedures, excess ^{109}Cd or $CdCl_2$ was removed by binding with hemolysate prepared from whole blood treated with KCl (Onosaka and Cherian, 1982; Nolan and Shaikh, 1986). The Hb portion of the hemolysate binds the excess ^{109}Cd or $CdCl_2$. Hemolysate solution may contain sources of contamination leading to increased total counts (Eaton and Toal, 1982; Eaton,1985). In the initial studies, the percent of ^{109}Cd precipitated from liver supernatant by 2 and 4 % Hb solutions (Sigma) were examined. Further tests examined Tris buffered saline (TBS) blank solutions and ^{109}Cd precipitation by 1 and 2% Hb solutions. Aliquots from each of the Hb precipitation (Hb ppt) steps were counted on a gamma counter for residual radioactive counts. For routine work, 3 Hb precipitation steps were found to be adequate to remove the excess radiolabel, although up to 6 Hb precipitation steps were investigated in this study.

4.2.5.4. *Incubation Temperature*

The incubation temperature was determined by monitoring the water bath temperature, color and physical changes of the CSA analyte/Hb mixture. The small assay volume in the 1.5 mL plastic Eppendorf tubes allowed the test samples to reach incubation temperature in 1 to 2 minutes. The reaction was carried out for 3 minutes. Blank solutions (30 and 50 mM TBS) with 2 % Hb solutions, were tested using 80 and 100°C water bath temperatures and the CSA was carried out to 6 Hb ppt steps as before.

4.2.5.5. *Buffer Concentration*

Several buffer concentrations were examined (5, 15, 30, 50, 100 mM TBS) with 2 % Hb ppt solutions and incubated at 100°C. The CSA analyte from each Hb ppt step was analyzed by SDS-PAGE in order to determine the degree of protein contamination. In order to hold the buffer concentration constant for the analyte/Hb mixture, aliquots of 0.5 mM TBS were added to each Hb precipitation step. Buffer adjusted and non-adjusted protocols were compared for effectiveness.

4.2.5.6. *Concentration of Cold $CdCl_2$*

The concentration of cold $CdCl_2$ added to the radiolabelling solution may interfere with the ^{109}Cd binding in the test solution (Eaton, 1985). Previously in Dr. Nieboer's laboratory, studies using CSA to determine MT concentration in lymphocyte and monocyte lysates used 10 ug/mL $CdCl_2$ in the ^{109}Cd radiolabel (Harley et al., 1989) in order to increase the sensitivity of the assay and improve the detection limit to 0.80 ug MT/mL or 0.11 uMol MT/L (Eaton and Toal, 1982). For tissues, 100 ug cold $CdCl_2$ has been added similarly to attain a detection limit of 10 ug MT/g wwt or 0.48 uMol MT/kg dwt (Nolan and Shaikh, 1986; Shaikh and Nolan, 1987). In the present study, three concentrations of cold $CdCl_2$ (9, 90, 900 ug $CdCl_2$) were examined. A total of 90 uL of the radiolabel was added to each test sample (300 uL). The concentration and specific activity of 1 uL of each labelling solution (L.S.) for the CSA optimization studies were: (i) in the 9 ug L.S. there was 0.013 uCi ^{109}Cd and 0.100 ug $CdCl_2$ with specific activity of 0.13 u Ci/ug/uL; (ii) in the 90 ug L.S. there was 0.013 uCi ^{109}Cd and 1 ug $CdCl_2$ with specific activity of 0.013 uCi/ug/uL; (iii) in the 900 ug L.S. there was 0.013 uCi ^{109}Cd and 10 ug $CdCl_2$ with a specific activity of 0.0013 uCi/ug/uL. Samples tested included 50 mM TBS and liver supernatants from control and Cd exposed rats. The CSA protocol used incorporated the optimized parameters: 2% Hb solution; an incubation temperature of 100°C; and a

constant buffer concentration of 50 mM TBS.

4.2.5.7. *Column Chromatography*

Column chromatography has been previously investigated for the use in sample clean up prior to the CSA (Shaikh and Nolan, 1987). Liver supernatant was purified using several different grades of Sephadex (G-25 , G-50f and a G-25/G-75 stacked) gel filtration mini-columns (1x10 cm). For each fraction (#1-12), the UV absorbance at 254 and 280 nm were measured and MT content was determined by CSA. Those fractions that exhibited the highest cpm in the CSA were passed over a second mini-column (G-50f); UV absorbances at 254 and 280 nm and total cpm were again monitored. The columns were calibrated using dextran blue to determine the void volume (Vo = fraction #4); albumin (66 kD, fraction #4) and cytochrome C (12.4 kD, fraction #6) were employed as MW markers. Sephadex G-50f gel filtration column chromatography and UV absorbance measurements were performed on the CSA analyte derived from the following samples; 50 mM TBS blank, purified liver supernatant, rat MT1 and MT2 prepared by L.M.J. Smith, and rabbit MT1 and MT2 purchased from Sigma (Table 4.9 and Figures 4.16 to 4.20). The optimized parameters and conditions for both CSA protocols are summarized in Table 4.6. The micro-CSA used in the rat time course experiments incorporated all of the optimized conditions investigated except column chromatography.

4.2.6. The Micro-CSA Procedure

The reagents employed in the micro-CSA (Table 4.5) and microcentrifuge tubes were prepared and labelled ahead of time. For personal safety, double gloves (latex) were worn with frequent changes, lab coat, radiation badge and eye protection were worn at all times during the assay. The radioactive labelling solutions were prepared in a fume hood. Suitable disposal containers for radioactive and biological waste were within easy access at all times. All CSAs were performed on the laboratory bench using disposable absorbent pads. The area was properly labelled for radioactive use according to

Health and Safety guidelines. The micro-CSA (Figure 4.3) was scaled up by a factor of 3 for all mini-CSA used in the optimization tests in order to have enough analyte to test at each of the Hb precipitation steps. The test sample volume of the mini-CSA was 300 uL, and 100 uL for the micro-CSA.

The micro-CSA consisted of the following steps.

Step 1. 100 uL of semi-purified test sample or blank (50 mM TBS) and 30 uL labelling solution were mixed in a 1.5 mL screw cap microcentrifuge tube and incubated at room temperature with intermittent mixing for 15 minutes (Figure 4.3). Step 2. To each tube, 10 uL 0.5 M TBS and 50 uL 2 % Hb solution was added, capped, vortexed, and boiled at 100°C for 3 minutes. Step 3. The tubes were placed on ice for 3 minutes and microcentrifuged at 10,000 g for 4 minutes. Step 4. 100 uL of the supernatant was carefully transferred to a clean microcentrifuge tube (incomplete separation of the supernatant from the Hb pellet was a constant problem). The surface detritus was removed with a pipette tip and the supernatant was transferred using a clean tip. Step 5. Steps 2 through 4 were repeated twice. Step 6. 100 uL of the analyte was transferred to the appropriate test tubes for gamma counting (in this study, 12 x 75 mm plastic tubes were used for the LKB Wallac 1275 in-line gamma counter and 12 x 75 mm glass tubes for the 12 well NE1612 gamma counter). Step 7. A 1/10 dilution of the labelling solution was prepared in normal saline and 20 uL was counted in a gamma counter.

Step 8. Calculation of MT Concentrations.

The calculation of MT concentrations was based on the activity of the labelling solution and conversion factors. To convert the measured CPM to MT concentration (uMol MT/L or uMol MT/kg) the following relationships apply: The labelling solution used in the CSA contained 0.1 ug/uL $CdCl_2$ • 2 1/2 H_2O (MW 228.35) for cell lysates for a total of 3 ug cold Cd per sample, and 1 ug/uL $CdCl_2$ • 2 1/2 H_2O for tissues for a total of 30 ug cold Cd per sample. The labelling

solution was diluted (0.1) and 20 uL of the diluted label was counted. The MW of MT was 7000 D and the assumption that 1 mol MT binds 7 mol Cd were used in the calculations. The following abbreviations or parameters were employed in the calculations summarized in Equations [1a] to [2b]: (cpm_t) = the total counts determined from 20 uL of a 1/10 dilution of the labelling solution in saline; (cpm_s) = counts per minute of the test sample; (cpm_b) = counts per minute of the blank solution; (DFs) = dilution of sample, calculated as uL of original remaining in the counted solution; (CA) = number of cells analyzed in 100 uL of CSA lysate; (10^9 cells)* = normalization to 1 L for Ly, M, and PMN cells; (10^{12} cells)* = normalization to 1 L for RBCs; (TBS) = sample in Tris buffered saline; and (WCV) = wet weight conversion to actual tissue water, (WCV) = (100/% tissue). For liver, the average water content observed was 71 $\pm$ 1% and consequently the WCV (100/29) = 3.45.

[1a] Calculation of (uMol MT/cpm) for cell lysates from experiments GO91.

$$cpm_t = \frac{(20\ uL \times 0.1)\ (0.1\ ug/uL\ CdCl_2 \cdot 2\ 1/2\ H_2O)}{(228.35 \times 7)}\ uMol\ MT$$

$$9259\ cpm_t = 1.251 \times 10^{-4}\ uMol\ MT$$

$$1\ cpm = 1.352 \times 10^{-8}\ uMol\ MT$$

[1b] Final calculation step for cell lysates (Ly, M, and PMN).

$$= cpm_s - cpm_b \times (1.352 \times 10^{-8}\ uMol\ MT/cpm) \times \frac{(10^9\ cells)^*}{(DFs)\ (CA)}$$

$$= uMol\ MT / 10^9\ cells;\ (normalized\ as\ uMol\ MT / L).$$

[2a] Calculation of (uMol MT/cpm) for tissues of experiments GO91 and PA91.

$$cpm_t = \frac{(20\ uL \times 0.1)\ (1\ ug/uL\ CdCl_2 \cdot 2\ 1/2\ H_2O)}{}\ uMol\ MT$$

$$(\ 228.35 \times 7\ )$$

$$21657\ cpm_t = 1.251 \times 10^{-3}\ uMol\ MT$$

$$1\ cpm = 5.78 \times 10^{-8}\ uMol\ MT$$

[2b] Final calculation step for Experiment GO91 and PA91.

$$= cpm_s - cpm_b \times (5.78 \times 10^{-8}\ uMol\ MT/cpm) \times \frac{(\ uL\ TBS)}{kg \times DFs}$$

= uMol MT / kg dwt tissue

To adjust the dry weight to wet weight, divide by (WCV).

uMol MT/ kg wwt = (uMol MT/ kg dwt) / (WCV).

4.2.7. MT Inter-Compartment Comparison

To facilitate inter-compartment comparisons, MT concentrations were normalized by expressing the results in units of uMol MT/cell multiplied by a scaling factor (10^9 for PMN, Ly, and M; 10^{12} for RBCs) to approximate the number of cells in one liter of blood (uMol MT/L).

4.2.8. Statistical Analysis of the MT Results.

A combination of three different statistical tests were applied to the data; the unpaired students t test, the longitudinal paired t test, and One-way ANOVA (level of significance set at 95%) (Rosner, 1986). The unpaired t test was applied to the time course and organ data to test for significant differences in the results between the different exposure groups and the control animals at a set point in time also known as cross-sectional data analysis. The longitudinal paired t test was used to analyze the way individual rats responded to exposure throughout the time course, also known as a follow-up study (Rosner, 1986). In this statistical test, the control time points (T= -20 and 0 h) were averaged and compared to each time point using the paired t test to reflect significant changes from the individual's baseline (initial value) with the passage of time. The One-

way ANOVA was applied to the organ and the inter-compartment comparison data to test for the variance between the different groups and experiments. The data are assumed to follow a normal distribution. The calculated p-values are reported in the figures and tables. The pooled standard deviations were calculated from the summed standard deviations (SD) divided by the total number of data points per time course (n) using the following equation: $\Sigma(SD)/n$ (Rosner, 1986). The pooled SD are indicated in the appropriate figures and the mean $\pm$ SD are reported in the tables. The inter-animal coefficient of variation is equal to the standard deviation divided by the mean times 100 (%CV = $(SD/\bar{x})(100)$). The CV was calculated for each tissue type within a dosage group.

4.3. Experimental Results

4.3.1. MT Isolation, Purification and Characterization

MT protein characteristically has few aromatic amino acids and does not yield normal protein absorption patterns. When fully bound with Cd, the protein absorbs around 250 - 254 nm and exhibits little or no absorbance at 280 nm. The absorbance ratio of 254 / 280 nm provides a characteristic value associated with MT. This high value was observed in the Sephadex G-75 gel filtration separation of the liver supernatant from Cd induced rats (Figure 4.4). Fractions # 34 - 53 contained the highest 254/280 nm ratio were pooled, lyophilized and labelled as crude hepatic rat MT. The crude MT was separated into its isoforms and those fractions containing the MT isoforms (Figure 4.5) were pooled and designated as follows: MT1a (fractions #58 - 61); MT1b (fractions #62 - 65); MT2a (fractions # 95 - 98) and MT2b (fractions # 99 - 103).

SDS-PAGE analysis of the pooled MT fractions showed single bands indicating that the isoforms were sufficiently pure for antibody production and for the RIA procedures (Figure 4.6). The presence of MT protein in the fractions

mentioned above was established using the micro-CSA (Figure 4.7). This figure shows the UV absorbance (254 and 280 nm) as well as the total counts (cpm x 10^{-4}) for the fractions containing the two MT isoforms. The fractions containing the highest 254 nm absorbance also contained the highest concentration of ^{109}Cd.

In the Bio-Rad protein assay, the slope of the standard curve for lysozyme was closer to that of MT and was therefore used as the standard solution in the Bio-Rad assay. This analysis yielded low values for percent protein in the Rat MT1b sample (5.5% by the micro-assay and 8.34% by the mini-assay) compared to 6.47% protein determined for Rabbit MT1 (Sigma) by the micro-assay. The Bio-Rad protein assay was later carried out using equine renal MT (Sigma) as the standard solution. The protein results were considerably higher. A mixture of purified rat MT1 and 2 (see Section 4.2.1, prepared by LMJ Smith) gave a protein estimation of 84 ug or 50% of the actual concentration. However, the polymerized form of the above rat MT 1 and 2 mixture (see Section 4.2.2; Figures 4.6 and 4.8) gave a protein content of 87 ug or 100% of the actual protein concentration.

The molar extinction coefficients (ε) for the rat MT isoforms and the MT isoforms purchased from Sigma (horse and rabbit) are compared to literature values for human and horse kidney MT in Table 4.7. The experimental (ε) values for horse and rabbit were within a reasonable range of the literature (ε) values. The rat MT values were lower than the literature values.

4.3.2. Antibody Production and RIA

4.3.2.1. *Antibody Production*

The polymerized rat MT separation yielded 6.9 mL of product containing 1.16 mg MT / mL (Figure 4.8). The polymerized rat MT was checked for purity by SDS-PAGE which showed a broad band and was therefore considered sufficiently pure for antibody production (Figure 4.6).

The antibody OID profile for sera from rabbit #L-2 shows the control

response in the upper left hand corner (Figure 4.9). The remaining OID tests correspond to the first through the sixth series of MT injections and the maximum response was observed in the fourth through the sixth OID test.

The OID profile for chicken #994 showed the progressive production of anti-rat MT antibodies (Figure 4.10). Yolk preparation #2 showed the control response. Yolk preparations # 15 - 20 showed detectable antibody production.

4.3.2.2. *MT iodination*

In the current study, NSHPP-MT fractions #3 and #4 from the Bolton-Hunter reaction produced the best iodinated rat MT label (^{125}I-Rat MT) although, NSHPP-MT fractions # 6 and #7 had higher 254 nm UV absorbances, their antibody titration curves were lower (data not shown). From the MT iodination elution profile in Figure 4.11a, it is clear that rat ^{125}I-MT was separated from other reaction products. However, in Figure 4.11b it was observed that rabbit ^{125}I-MT eluted with the void volume yielding a poor separation. In Figure 4.11a, the ^{125}I- rat MT peak elutes in the early fractions (#6 - 7) and was small compared to the other reaction products; ^{125}I-NSHPP (fraction # 10), and free ^{125}I (fractions # 14 - 15). In Figure 4.11b, a large peak containing the rabbit ^{125}I-MT label eluted early (fractions # 4 - 6) with smaller peaks eluting later (fractions # 9 and #13-14).

4.3.2.3. *Antibody Binding Capacity*

After each iodination reaction, selected fractions from each peak were tested for antigen - antibody binding capacity using RIA (Table 4.4). Several iodination products from different iodination reactions and several dilutions of 1°Ab (rabbit anti-rat MT and chicken anti-rat MT) were tested for their capacity to bind and precipitate the ^{125}I-MT antigen-antibody complex (Table 4.8). The highest binding was observed when the following conditions were used:

1. ^{125}I- rat MT label iodinated on 10-12-90 using fraction #7 (Figure 4.11a);
2. Rabbit anti-rat MT (1°Ab);

3. Final 1°Ab dilution of 1/1000 in the assay.

Rabbit #L-2 sera were tested for antibody binding capacity and showed a maximum of 30% binding (Table 4.8). The remaining 3 rabbits did not express high enough antibody response for the RIA. Preparation #15 from chicken #994 showed a maximum binding capacity of 6.5% (Table 4.8).

4.3.3. CSA Optimization Study

4.3.3.1. *CSA Interferences and Sample Purity*

The electrophoresis results showed proteins were present in some of the solutions that potentially could interfere with the CSA. In particular, the leukocyte lysates and the RPMI complete cell media had HMW proteins ranging from 31 to 66.5 kD and LMW proteins ranging from 8 to 14 kD. The leukocytes were isolated using RPMI (Figure 4.12). The plasma contained an abundance of HMW proteins ranging from 45 to > 92 kD with some LMW proteins between 8 and 14 kD. RBCs contained more LMW proteins ranging from 8 to 31 kD than HMW proteins ranging from 31 to 66 kD. Urine showed two major bands; one at 14 kD and one at 21 kD (Figure 4.12). Blood products and cells incubated with RPMI were shown by SDS-PAGE to contain other proteins compared to samples without RPMI. Interferences in the CSA were observed in samples incubated with RPMI resulting in high total counts (^{109}Cd) compared to the same samples washed several times with PBS or normal saline prior to analysis. The blood anticoagulant EDTA yielded extremely high total counts in blank solutions and test samples (data not shown). Heparin, however did not interfere with the assay.

4.3.3.2. *Hemoglobin Concentration*

In the case of liver supernatant, almost 98 % of the ^{109}Cd label was precipitated by the 3rd Hb ppt step using a 4 % Hb solution and 85 % was precipitated by a 2 % Hb solution. The 15 mM TBS (blank) CSA analyte from the 3rd Hb ppt step showed 38 % of the ^{109}Cd label had been precipitated in the 2 % Hb and 26 % in the 1 % Hb (Figure 4.13). By comparison, the 30 mM TBS

CSA analyte from the 3rd Hb ppt step showed 99 % of the ^{109}Cd label had been precipitated in the 2 % Hb and 64 % in the 1 % Hb. Proteins or their fragments (25 - 45 kD) were observed with each additional Hb ppt step after step 2 in the 15 mM TBS blanks for both 1 and 2% Hb solutions (Figure 4.14). However, the 30 mM TBS blank did not show increased protein concentration until the 5th Hb ppt step from the 2% Hb solution (Figure 4.14). The protein fragments were more concentrated in the CSA analyte when 15 mM TBS was employed as compared to the 30 mM TBS. The order of increasing protein fragmentation observed by SDS-PAGE was: zero in the 30 mM TBS (1% Hb) < 30 mM TBS (2% Hb) << 15 mM TBS (1% Hb) << 15 mM TBS (2% Hb).

4.3.3.3. *Incubation Temperature*

In the 30 mM TBS blanks, approximately 99 % of the total cpm precipitated from solution by the 3rd Hb step at 100°C, and 90% at 80°C. The reaction temperature did not appear to be critical in the 50 mM TBS blanks where 98 % of total cpm precipitated at 100°C, and 96 % at 80°C (data not shown).

4.3.3.4. *Buffer Concentration*

The supernatant from the 5 and 15 mM TBS solutions showed higher levels of the chromophores as the number of Hb ppt steps increased, compared to the 30 - 100 mM TBS solution supernatants which were clear. By the 3rd Hb ppt step: 49 % of the ^{109}Cd label had been precipitated in the 5 mM; 38 % in the 15 mM; 99 % in the 30 mM; 90 % in 50 mM; and 92 % in the 100 mM TBS blank (for 15, 30, and 100 mM samples see Figure 4.13). SDS-PAGE of the 15 mM TBS blank showed increasing amounts of HMW protein (25 to 45 kD) as the number of Hb ppt steps increased. Slightly elevated amounts of HMW proteins (31 kD) were observed at the 6th Hb ppt step for the 30 mM TBS blank (Figure 4.14). The molarity of the analyte solution dropped considerably with each Hb ppt step because of dilution. At the 3rd Hb step the molarity of the 15 mM TBS blank decreased to 3.7 mM; 30 mM TBS decreased to 7.4 mM; 50

mM TBS decreased to 12.3 mM; and the 100 mM TBS decreased to 24.7 mM. It was shown by SDS-PAGE that Hb fragments in solution and this results in a decrease in the ^{109}Cd precipitation as determined by CSA with buffer concentrations of 15 mM TBS and lower (Figures 4.13 - 4.14). The analyte from the corrected buffer concentration test samples were analyzed by SDS-PAGE and were clean after the 6 Hb ppt step.

4.3.3.5. *Concentration of Cold $CdCl_2$*

The results show that lower ^{109}Cd binding occurred for the 9 and 900 ug $CdCl_2$ radiolabel solutions while higher binding was observed in the 90 ug $CdCl_2$ solution. The optimum for tissues was 90 ug cold $CdCl_2$ in 90 uL radiolabel solution per test sample using the mini-CSA (Figure 4.15).

4.3.3.6. *Column Chromatography*

CSA analyte from 50 mM TBS blank, purified liver supernatant, rat MT1 and MT2, and rabbit MT1 and MT2 revealed the majority of total ^{109}Cd counts were found in fractions #5 - 7 with an approximate MW of 30 - 6 kD (Table 4.9 and Figures 4.16 - 4.19). The maximum total counts (cpm) were detected in fraction #6 (approximate MW = 12.4 kD) for all test samples and corresponds to the descending peak of MT1. These same fractions showed very low 254 and 280 nm absorbance readings (Figures 4.16 - 4.19 and Table 4.9). SDS-PAGE of the CSA analytes showed one band around 10 - 14 kD, and were therefore considered pure and free of interferences (Figure 4.20).

4.3.3.7. *Optimized Conditions for the CSA*

In the systematic study of all the steps in the CSA, the following conditions were found to be optimum for the routine analysis of MT: 1. 2% hemoglobin solution; 2. 100°C heating step (water bath); 3. 50 mM buffer concentration held constant in each of the hemoglobin precipitation steps; 4. 30 ug cold Cd in the micro-CSA ^{109}Cd labelling solution. In addition to the above, blood cell purification steps were inserted in the protocol to remove assay interferences by RPMI. Sample preparation steps were also modified to

incorporate heating, ice bath treatments and centrifugation for all samples prior to the assay (Table 4.6).

4.3.4. MT Determinations by CSA in the *In Vivo* Rat Time Course Experiments

The MT concentration in the cell lysates was close to the detection limit of the CSA (0.8 ug/g), while the MT levels in the tissues were well above the detection limit of 10 ug/g (Eaton and Toal, 1982; Nolan and Shaikh, 1986). Sample interferences or Hb pellet contamination were observed in some samples even after assay optimization (Eaton, 1985; Nolan and Shaikh, 1986; Shaikh and Nolan, 1987).

4.3.4.1. *MT Concentration in Tissues*

This section deals with the comparison of tissue data by the two-sample unpaired t test followed by the ANOVA approach to determine if MT levels in the control and Cd-treated rats were significantly different ($p \leq 0.05$). In the Short Course Experiment PA91, liver MT concentrations for the medium dose (1.25 mg/kg) Cd exposed rats were significantly elevated (99 ± 21 uMol MT/ kg dwt, $p<0.05$), while baseline levels (21 ± 7 uMol MT/kg) were observed in the control (0.0 mg/kg) and low dose (0.25 mg/kg) rats (Figure 4.21 and Table 4.10). Kidney and spleen MT concentrations from Cd-induced rats ranged from 7 to 26 uMol MT/kg and were not significantly different from control values(10 ± 3.5 and 12 ± 5 uMol MT/kg, respectively) shown in Figures 4.22 - 4.23, and Table 4.10. However, lung MT concentrations in the low exposure group (22 ± 2.6 uMol MT/kg) were significantly higher ($p \leq 0.05$) compared to controls (13 ± 2.6 uMol MT/kg, Figure 4.24). Although, lung MT levels were not significantly different from kidney or spleen.

Similar trends to those in the short course rats were seen in the subchronic rats (Figure 4.28 - 4.31 and Table 4.11). Interestingly, rats that died as the experiment progressed gave elevated MT values and hence large standard deviations, especially in kidney and spleen. In these samples, the time

of organ collection varied from 1 to 12 h, depending on the time of death. The time of death was difficult to establish and as a result, tissue necrosis may have occurred. In addition, it is possible that sample contamination occurred during collection and lyophilization.

In Experiment AF90, liver MT concentrations were determined in quadruplicate (Table 4.10). As observed before, the high (2.5 mg/kg) Cd exposed rats showed significantly elevated liver MT levels (140 $\pm$ 9 in the short course and 232 uMol MT/kg dwt in the subchronic experiments), compared to baseline MT levels (21 $\pm$ 7 and 22 $\pm$ 11 uMol MT/kg dwt, respectively) ($p \leq 0.0005$). Maximum MT concentrations were observed in the subchronic rat and were significantly higher ($p \leq 0.0005$) than in the controls (Figures 4.25 - 4.27).

In addition to the two-sample unpaired t test, the organ data were also analyzed by the One-way ANOVA (95% significance level). The ANOVA was used to compare MT levels from a specific organ for all exposure groups in both the short course and subchronic experiments. Liver MT concentration in the controls (0.0 mg/kg) and low (0.25 mg/kg) Cd exposure groups were not significantly different in any of the experiments. However, the medium (1.25 mg/kg) and high (2.5 mg/kg) Cd exposure groups were significantly higher than the controls and low exposure groups in both the subchronic and short course experiments ($p \leq 0.05$). Similarly, the liver MT levels in the high exposure group were significantly elevated compared to the medium group in both experimental protocols. In the ANOVA comparisons of the short course versus the subchronic experiment, the liver MT levels were significantly higher for the subchronic exposure, compared to the short course for both the medium and high Cd dose groups ($p \leq 0.05$); the control and low dose groups were not different. However, the short-course high-dose rats were not significantly different from the subchronic medium dose rats.

In contrast to the liver results, the kidney MT levels for the short course experiment were not significantly different and only the MT levels in the high

dose subchronic rat was significantly elevated above the other exposure groups ($p \leq 0.05$). Similar to the trend observed in liver, ANOVA comparisons revealed the kidney MT concentrations were significantly elevated ($p \leq 0.05$) in the subchronic compared to the short course experiments within the medium and high exposure groups, while the low exposure and controls were not different. However, all of the short course Cd exposed and control rats had significantly lower kidney MT levels than the subchronic medium and high exposure rats ($p \leq 0.05$).

The ANOVA comparison of spleen MT concentrations revealed a trend that was in between the liver and kidney MT results, although the MT concentration in the kidney and spleen were not significantly different. The spleen MT levels in the short course experiments were not significantly different in the controls compared to the low and medium exposure groups. However, the high dose rats were significantly higher than the controls ($p \leq 0.05$). MT levels in the low and medium dose rats were not different, while the high dose was significantly elevated above the low and medium dose groups ($p \leq 0.05$) in the short course experiments. In the subchronic experiment, the control and low dose were not different; both the control and low dose were significantly lower ($p \leq 0.05$) than the medium dose. In contrast to the other organs, the spleen of the subchronic high Cd exposure group was completely decomposed at the time of autopsy making sampling impossible (see Chapter 5).

Similar ANOVA results for the comparison between short course and subchronic experiments at specific exposure levels were observed in the spleen relative to both liver and kidney. Within a dose level, no significant differences were observed in spleen MT concentrations between the short course and subchronic experiments for the controls and low dose groups, while the medium group was significantly lower in the short course compared to the subchronic group ($p \leq 0.05$). In addition, the short-course medium-dose was not different from the subchronic low dose ($p \leq 0.05$). The short-course high-dose was

significantly higher than the subchronic control and low dose. However, the short-course high-dose and the subchronic medium dose were not significantly different.

In lung, MT concentrations did not follow the same trends observed in the other organs studied. The ANOVA results for lung MT levels in the short course experiments revealed the controls were significantly lower than the low and medium exposure rats ($p \leq 0.05$); the low and medium dose rats were not different. The exposure dose comparisons in the subchronic experiments revealed similar trends to the short course, where the controls were significantly lower than the low dose ($p \leq 0.05$) while the control and medium dose were not. In contrast to the short course lung results, the medium dose was significantly lower than the low dose in the subchronic experiments ($p \leq 0.05$). When comparing the subchronic and short course experiments within an exposure group, the trend was again different from the other organs analyzed. The concentrations in the short course controls, as well as in the low dose groups, were significantly lower than their respective doses in the subchronic experiments. However, the medium dose groups showed no difference. In addition, the short course medium dose had significantly lower MT than the subchronic low dose.

In overall organ comparisons using the ANOVA approach, liver MT concentrations in the medium (1.25 mg Cd) and high (2.5 mg Cd) exposure groups were significantly higher ($p \leq 0.05$) than all other tissue types for the short course experiments. Maximum MT levels were observed in the organs of the subchronic rats. A Cd dose-response relationship was observed for MT protein induction in the three experiments; AF90, PA91 and GO91 (Figures 4.21 - 4.31 and Tables 4.10 - 4.11). ANOVA comparisons of the basal MT levels in the organs from the short course control rats revealed the following order: * liver > kidney = spleen = lung, where the liver MT levels were significantly higher than in all other organs, while kidney, spleen and lung MT levels were not

significantly different from each other (* $p \leq 0.05$). ANOVA comparisons of the basal MT levels in the organs from the subchronic control rats revealed the following order: kidney > liver ≥ lung = spleen, where no significant differences were observed, due in part to high standard deviations observed in the kidney. The MT concentrations in the organs from the short-course low Cd-exposure rats were as follows: *liver > *lung > kidney = spleen, where liver MT levels were significantly higher than in all other organs, while lung MT levels were significantly higher than kidney and spleen (* $p \leq 0.05$). The MT concentrations in the organs from the short-course medium and high Cd-exposure rats were as follows: *liver > lung ≥ kidney ≥ spleen, where liver MT levels were significantly higher than in all other organs (* $p \leq 0.05$). The MT concentration in the organs from the subchronic Cd-exposure rats were as follows for the low dose group: *liver > lung ≥ kidney ≥ spleen, where the liver MT levels were significantly higher than kidney and spleen, yet not lung (* $p \leq 0.05$). The MT concentration in the organs from the subchronic medium and high Cd-exposure rats were as follows: *liver >> kidney > lung = spleen, where the liver MT concentrations were significantly higher than in all other organs; those in the kidney, spleen and lung were not significantly different (* $p \leq 0.05$). Spleen and lung samples were not available for the high dose group (Tables 4.10 and 4.11).

4.3.4.2. *MT Concentration in Plasma*

Baseline plasma MT levels (between 0.12 to 0.14 ± 0.05 uMol MT/L) were observed at T = -20 and 0 h for all rats in Experiment PA91. The plasma MT concentrations in the low and medium Cd exposed rats were significantly higher ($p \leq 0.05$, unpaired t test) than the control group from T = 24 to 72 h. Plasma MT levels for the high dose group showed no significant differences between Cd exposed and control groups (Figure 4.32). MT determination for rats E and D were not performed due to insufficient sample volume (Table 4.14). The maximum MT concentration was 0.35 ± 0.05 uMol MT/L for the low dose and 0.55 ± 0.11 uMol MT/L in the medium dose group observed at T = 48

h. Thereafter, the levels continued to decrease for the remainder of the time course. A Cd dose response was observed by T = 24 h ($p \leq 0.05$, paired and unpaired t tests). The control rats showed slight increases in baseline MT levels as the experiment progressed ranging from 0.12 to 0.22 $\pm$ 0.10 uMol MT/L (Figure 4.32 and Tables 4.12 and 4.14). In each exposure group, the individual time points were compared to their corresponding control values using the longitudinal paired t test. The plasma MT levels did not increase significantly in the control rats. However, both the low and medium Cd exposure groups exhibited significant increases ($p \leq 0.05$, paired t test) in MT levels from T = 24 h onwards compared to their own baseline determined at T= -20 and 0 h (Figure 4.32).

By contrast, the subchronic plasma MT concentrations in the GO91 Cd exposed rats were significantly higher than the control rats throughout the time course ($p \leq 0.05$ to 0.0005, unpaired t test, Figure 4.33). Maximum MT levels of 0.59 $\pm$ 0.05 in the low dose rats and 1.0 $\pm$ 0.18 uMol MT/L in the medium dose group were observed at T = 12 h, and decreased thereafter. MT determination for the subchronic high dose rat was not performed due to insufficient sample volume (Table 4.14). The control rats showed baseline MT levels throughout the experiment, with slight increases seen at the later time points (Figure 4.33 and Table 4.13). The longitudinal results of the subchronic plasma MT levels were significantly higher ($p \leq 0.05$, paired t test) at T = 6 and 12 h for the medium (1.25 mg/kg) rats; at T= 6 and 24 h for the control rats (Figure 4.33).

The peak MT levels observed in the subchronic experiment were significantly higher than their respective short course exposure groups. Further comparisons revealed that the relative accumulation levels of Cd and CdMT in the short-course medium-dose results were similar to the low dose subchronic plasma MT levels.

4.3.4.3. *MT Concentration in Erythrocytes*

Baseline RBC MT levels in the range of 0.01 uMol MT/L were found for

all PA91 short course rats for the recovery period (T = -20 and 0 h). Small non-significant fluctuations were observed from T = 6 to 24 h, followed by increased levels of Cd-induced MT which peaked at the later time points (Figure 4.34 and Table 4.15). Control background MT levels of 0.01 uMol MT/L or lower were observed. The low exposure group was not significantly different. However, the medium (1.25 mg/kg) Cd exposure group was significantly higher than the controls ($p \leq 0.05$) from T = 48 h onwards (unpaired t test) and significantly higher than individual initial values from T = 72 h onwards (longitudinal paired t test). The maximum MT concentration for RBCs was 0.03 $\pm$ 0.012 uMol MT/L observed at T = 72 h in the medium Cd exposure group. RBC MT levels were not determined in the earlier high dose group as this parameter was added to the experimental design at a later date.

By contrast, the medium dose subchronic group exhibited elevated RBC MT levels ranging from 0.025 to 0.045 $\pm$ 0.02 uMol MT/L and were significantly higher ($p \leq 0.05$, unpaired t test) than the controls (below 0.007 uMol MT/L) throughout the time course (Figure 4.35 and Table 4.16). However, the control and low dose groups were not significantly different, except at T = 6 h ($p \leq 0.05$, unpaired t test) and represents background MT levels (Figure 4.35 and Table 4.16) comparable to the short course RBC MT results (Figure 4.34 and Table 4.15). There were no subchronic individual differences in RBC MT levels detected by the longitudinal paired t test in contrast to the short course exposure study.

4.3.4.4. *MT Concentration in Polymorphonuclear Leukocytes*

The PMN MT levels in the short course experiment showed no significant differences between the exposed and non-exposed groups studied (Figure 4.36). No trends were observed in the different dose groups of the short course experiments. The PMN data ranged from 0.0 to 0.0065 $\pm$ 0.002 uMol MT/L and was at or below the baseline MT levels detected in RBCs (0.005 to 0.008 uMol MT/L) (Figure 4.36 and Table 4.17). One exception was noted at T= 24 h in the

medium (1.25 mg/kg) Cd group where MT levels were significantly lower ($p \leq 0.05$, unpaired t test) than the controls.

Similarly, the PMN MT levels observed in the GO91 subchronic animals exhibited slightly higher MT concentrations (0.0 to 0.013 $\pm$ 0.008 uMol MT/L) with high standard deviations and significant differences were not observed between any of the treatment groups (Figure 4.37 and Table 4.18). PMN MT levels were not determined in the earlier high dose groups of either protocol as they were not originally part of the experimental design.

4.3.4.5. *MT Concentration in Lymphocytes*

In order to alleviate confusion regarding cell numbers, all WBC cell preparations were counted manually before and after separation of lymphocytes and monocytes (see section 2.3, Figure 2.4). In the short course experiment, baseline lymphocyte MT concentrations (below 0.004 uMol MT/L) were observed for all groups until T = 6 h (Figure 4.38). MT levels were significantly elevated ($p \leq 0.05$, unpaired t test) at T = 24 h in the low and medium Cd exposed groups with the maximum (0.017 $\pm$ 0.005 uMol MT/L) observed in the medium (1.25 mg/kg) group. Maximum levels (0.022 $\pm$ 0.028 uMol MT/L) were observed in the high dose group, although no significant differences were observed due to a high standard deviation (Figure 4.38 and Table 4.21). All lymphocyte MT levels in Cd exposed rats returned to baseline by T = 48 h. The controls showed background MT levels below 0.004 uMol MT/L throughout the time course. A Cd-induced MT response was detected at T = 24 h between Cd and control animals ($p \leq 0.05$ to 0.005) and longitudinally, within the 1.25 mg Cd/kg dosage group ($p \leq 0.05$, Figure 4.38 and Table 4.19).

Baseline lymphocyte MT levels below 0.004 uMol MT/L were detected in all subchronic rats until after the T = 6 h (Figure 4.39). Elevated MT levels were observed at T = 12 and 24 h in the Cd exposed rats, then returned to baseline at T = 48 h. Throughout the experiment, control animals maintained background MT levels below 0.004 uMol MT/L (Figure 4.39 and Table 4.20). Compared to

the short course, the subchronic results followed similar trends however, lower total MT levels (maximum 0.0068 ± 0.0013 uMol MT/L) and higher standard deviations were observed in the subchronic rats. The lymphocyte MT levels in the medium Cd exposure groups were significantly higher compared to the controls ($p \leq 0.05$, unpaired t test) and significantly higher than their initial values at T = 12 h ($p \leq 0.05$, paired t test). Although, the MT levels in the medium group were higher than the low dose, they were not significantly different.

4.3.4.6. *MT Concentration in Monocytes*

Monocyte MT concentrations in all dosage groups except the high dose for the short course experiment, showed baseline levels (0.005 ± 0.002 uMol MT/L) during the recovery period then increased with time until a maximum of 0.024 ± 0.007 uMol MT/L were observed at T = 24 h, returning to baseline at T = 48 h (Figure 4.40). The medium dose group was significantly higher than the controls at T = 6 h ($p \leq 0.05$, unpaired t test). Although a Cd-induced MT response was visible, no significant differences were determined at T = 24 h between Cd exposed and control groups due in part to high standard deviations (Figure 4.40 and Tables 4.22 and 4.24). However, a longitudinal response was observed for the medium exposure group from T = 12 to 48, and 96 h ($p \leq 0.05$, paired t test).

Similar trends for monocyte MT levels were observed in the subchronic animals (Figure 4.41 and Tables 4.23 and 4.24). By contrast however, significant differences were obtained at T = 24 and 96 h ($p \leq 0.05$, unpaired t test) for the low exposure group but not for the higher exposure rats. Interestingly, the monocytes from the low dose subchronic group showed the highest MT concentrations of 0.04 ± 0.01 uMol MT/L with moderate standard deviations. In contrast to the short course experiments, the monocytes from subchronic Cd exposed rats did not show a significant longitudinal MT induction response (Figure 4.41).

In general, the monocyte MT concentrations were higher than those detected in lymphocytes for all of the *in vivo* Cd exposure time course experiments presented in this thesis (Figures 4.38 to 4.41).

4.3.4.7. *MT Inter-Compartment Comparison*

The blood cell inter-compartment comparisons were analyzed by the ANOVA (significance level set at 95%) for specific time points. In the short-course control rats, the basal MT inter-compartment comparison order at T = 0 h was: RBC ≥ M > L > PMN, where no significant differences in MT levels were detected; the PMN MT levels were not detectable. By T = 12 h, the blood cell order was unchanged. Although the RBC MT levels were significantly higher than in the lymphocytes and PMNs, they were lower than monocyte values (* $p \leq 0.05$); the PMN MT levels were not detectable. By contrast, at T= 24 h the monocytes surpassed all other cell types for individual MT concentrations: *M ≥ *PMN > L > RBC, where monocytes and PMN MT levels were significantly higher than in RBCs and lymphocytes, although not significantly different from each other (* $p \leq 0.05$). These results show that basal (control) MT levels differ between the different blood cell compartments (Figure 4.42).

ANOVA comparisons of the MT levels in the blood cell types from the short-course low-dose rats at T = 12 h were: M ≥ RBC ≥ PMN > L, with no significant differences in MT levels; lymphocyte MT levels were not detectable (Figure 4.43). The MT inter-compartment order at T = 24 h in the low dose short-course rats was: *M ≥ RBC > PMN ≥ L, with monocyte MT levels significantly higher than in lymphocytes and PMNs (* $p \leq 0.05$), but not significantly higher than the RBC MT levels. By T = 96 h, the MT compartment order changed to: *RBC > M = PMN ≥ L, with the RBC MT levels significantly higher than in PMNs and lymphocytes (* $p \leq 0.05$), but not different from the monocyte MT levels; lymphocyte MT levels were not detectable (Figure 4.43).

The MT concentrations in the blood compartments from the short-course medium-dose rats at T = 12 h was: *M ≥ *L > RBC > PMN, with monocyte and

lymphocyte MT levels significantly elevated (* $p \leq 0.05$) above those in the RBCs and PMNs, but not significantly different from each other (Figure 4.44). By T = 48 h, the order changed to: *RBC >> L ≥ PMN ≥ M, with the RBC MT levels significantly higher than in all other cells. By T = 72 h, the order was: *RBC >> M ≥ PMN ≥ L, with the RBC MT levels significantly higher than in all other cell MT levels (* $p \leq 0.05$, Figure 4.44). ANOVA comparisons of the MT levels in the lymphocytes and monocytes of the high Cd-exposure rats showed no significant differences between the two compartments at either T = 12 or 24 h.

A Cd-dose response for MT was observed, with a maximum in monocyte MT concentration observed at T = 24 h in the high dose group. Lymphocyte MT concentrations increased with time in a dose response pattern, with the maximum response observed between T = 12 - 24 h, and decreased thereafter. MT levels in PMNs exhibited fluctuations throughout the time course. For the later time points (T = 48 onwards), the RBCs in the Cd exposure groups surpassed the other blood cell types for individual MT concentrations (Figures 4.42 - 4.44). The different blood cell types responded to Cd exposure at different times. Lymphocytes responded early at 12 to 24 h after exposure, the monocytes expressed peak MT levels at 24 h, while the RBCs express increased MT levels at 48 to 72 h after Cd exposure. The pattern observed for the PMNs showed minor fluctuations at 6 and 24 h after exposure. The plasma contribution was significantly higher than all of the blood cell types (Figure 4.32). The general order of MT concentration per cell type was: M > RBC > PMN > Ly. The order was dose dependent and slight changes were observed at higher doses (RBC > M > Ly > PMN).

The general order for basal MT concentrations in the blood cells from subchronic control rats at T = -20 h was: RBC ≥ M > PMN > Ly, where lymphocyte MT levels were not detectable and no significant differences in MT levels were determined between any of the cells (Figure 4.45). The order

changed at T = 12 h to: *PMN > RBC ≥ Ly > M, with the PMN MT levels significantly higher than in all other cells (* $p \leq 0.05$). The RBC and lymphocyte MT levels were below the detection limit and the monocyte MT levels were not detectable. By T = 24 h, the order for basal MT in the subchronic control blood cells changed to: * PMN ≥ RBC > M > Ly, with the PMN MT levels significantly higher than in monocytes and lymphocytes (* $p \leq 0.05$), but not different from the RBC MT levels. Thereafter the order returned to the initial order determined at T = -20 h (Figure 4.45).

The order for MT concentration in the blood cells from subchronic low-dose rats at T = 0 h was: PMN ≥ RBC > M ≥ Ly, where the MT levels were not significantly different from each other. By contrast, the compartment order changed at T = 24 h to: *M > PMN = RBC ≥ Ly, where monocyte MT levels were significantly elevated than in all other cells (* $p \leq 0.05$). By 48 h, no significant differences in MT levels between the cells were observed (Figure 4.46). By contrast, the MT compartment order in the blood cells from the subchronic medium-dose group at T = -20 h was: *RBC >> M = PMN > Ly, where the RBC MT levels were significantly higher than all cells (* $p \leq 0.05$). This trend continued throughout the time course with minor (non-significant) changes in the leukocyte compartment MT levels (Figure 4.47). ANOVA comparisons of the MT levels in the lymphocyte and monocytes of the high Cd exposure rats showed no significant differences between the two compartment at either T = 12 or 24 h.

The blood MT compartmentalization patterns observed in the subchronic experiments were similar to the short course study for the lymphocytes and monocytes. However, the RBCs for the 1.25 mg Cd exposure group were significantly higher than all other blood cell types throughout the time course ($p \leq 0.05$). By contrast to the short course PMN results, the PMN MT levels in the subchronic Cd exposed rats did not exhibit fluctuations, instead a maximum response was observed between T = 12 - 24 h which returned to baseline

values thereafter (Figure 4.45 - 4.47). The plasma contribution to the MT blood compartmentalization was significantly higher than in all other blood cells for all dose groups throughout the experimental time course (Figure 4.33). The general order for MT concentrations at T = 24 h in the controls was: *PMN ≥ RBC > M > Ly (* $p \leq 0.05$). The order changed in the low dose to: *M > PMN ≥ RBC ≥ Ly. By contrast, the order in the medium Cd exposure group was: *RBC >> M > PMN > Ly (* $p \leq 0.05$).

Inter-compartment comparisons, based on observed cellular concentrations of MT, total measured cell counts and typical peripheral cell distribution numbers representative of circulating whole blood result in a concentration order different from that reported above as a 'per cell basis'. The general differential white blood cell count used and attributed to normal rats are 73% lymphocytes, 2.3% monocytes, 22% PMN, 0.5% basophils, and 2.2% eosinophils (Baker et al., 1979). Based on this normal WBC distribution, the order of MT concentration in the control animals was: RBC > Ly > PMN > M. Because of time- and dose-dependent changes observed in WBC populations in Cd-exposed animals, similar calculations were not pursued (see Section 5.4.2 and 5.5.2.2; see also Figures 5.3 - 5.4). However, the general trend was RBC > (Ly ≥ or ≤ PMN) > M, where the ratio between lymphocytes and PMNs changed with dose and time (Figures 5.3 - 5.4).

4.4. Discussion

The discussion of the MT isolation, RIA, and CSA optimization studies are presented in Sections 4.4.1 to 4.4.3, while the salient features of the MT concentrations found in the different tissues and blood cells are detailed in Section 4.4.4 and is followed by an interpretation section (4.4.5). Since MT induction and Cd exposure are closely interrelated, the Cd and MT dynamics model are discussed in Chapter 6.

4.4.1. MT Isolation, Purification and Characterization

G-75 Sephadex gel filtration followed by DEAE ion-exchange separation permitted the isolation of the two isoforms MT1 and MT2. This agrees with earlier work (Margoshes and Vallee, 1957; Vander Mallie and Garvey 1978; Kagi and Kojima, 1987). As expected, the absorbance ratio 254/280 nm yielded elution profiles characteristic of MT and corresponded to those where the ^{109}Cd content was measured.

The observed molar extinction coefficient (ε) values were lower for the isolated rat MT isoforms than for Sigma MT and literature values. There is no ready explanation for this discrepancy of about a factor of ten. The Bio-Rad protein assay results with equine MT as reference material suggested that the protein content of the isolated material was low, presumably due to the presence of buffer. However, this accounts for only about 20% of the discrepancy. Perhaps the Cd content of the purified MT was below saturation, thereby reducing the observed absorbance. Interestingly, the fractions of isolated MT with the highest Cd content (as ^{109}Cd) also exhibited the highest 254/280 absorbance ratio.

The protein assay results for MT purchased from Sigma (rabbit and equine) using the Bio-Rad protein procedure provide evidence of low sensitivity. The Bio-Rad assay, like other methods of protein determination, displays significant protein to protein variation (Bradford, 1976; Van Kley and Hale, 1977; Boyer, 1986; Robyt and White, 1990; Bio-Rad Protein Assay Instruction Manual). A number of reasons are known to account for such unusual responses, including: (i) incomplete denaturation due to the presence of a high number of disulfide linkages; (ii) low positive surface charges due to a low number of arginine residues (lysine residues appear ineffective in dye binding; Reisner et al., 1975); (iii) high concentration of negatively charged amino acids (Reisner et al., 1975); and (iv) low hydrophobicity of the protein. These factors usually reduce the amount of Coomassie Blue dye that adsorbs onto the protein, since the dye is negatively charged and lipophilic (Bradford, 1976;

Sedmak and Grossberg, 1977; Robyt and White, 1990; Aldrich, 1992). Under the acidic conditions of the Bio-Rad assay, CdMT should denature and displacement of the metal ions (Cd^{2+}) would occur resulting in an excess of SH functional groups. Due to the high cysteine content (30%) and possible disulfide formation, incomplete denaturing of the MT is probable. The low arginine content of MT and absence of aromatic amino acids also favor low adsorption by the hydrophobic Coomassie Blue dye (Reisner al., 1975).

The SDS-PAGE separation data and gel filtration results consistently assigned a molecular mass to MT of 10 - 30 kD, considerably higher than the expected value of 6 - 7 kD (Margoshes and Vallee, 1957; Cherian, 1974; Winge et al., 1975; Onosaka and Cherian, 1981; Pleasance et al., 1990; see Section 4.1.1). This suggests that polymerization had taken place as has been reported previously (Kagi and Kojima, 1987). However, overestimation of molecular mass can also occur because low hydrophobicity reduces the binding of the detergent SDS to the protein (Salemme and Zubay, 1983; Hames, 1990; Robyt and White, 1990). As already indicated for Coomassie Blue, this may be relevant for MT (Kagi and Kojima, 1987). Further, incomplete denaturation of MT might also have contributed to low SDS binding thereby reducing the electrophoretic mobility (Hames, 1990).

4.4.2. RIA Determination of MT

Initially, the intent was to investigate the feasibility of using RIA as an independent verification of the CSA for MT determination in tissues and body fluids. However, the RIA exhibited poor sensitivity and reproducibility and there are several possible explanations.

(i) The rat MT isoforms used for the early and more successful antibody titration and RIA feasibility tests (samples dated 10-12-90 to 11-7-90, Table 4.8) were the ion-exchange fractions corresponding to the descending peak of MT1 (labelled as MT1b) and the ascending peak of MT 2 (MT2a). However, these same MT fractions (Figure 4.5) were in high demand for antibody production in

rabbit and chicken, as they previously had produced the strongest antigenic response (Vander Mallie and Garvey, 1978). The rat MT available for most of the RIA and antibody titration testing (samples dated 11-16-90 and onward) were from the less antigenic fractions namely, the ascending peak of MT1 (MT1a) and the descending peak of MT 2 (MT2b), (Vander Mallie and Garvey, 1978).

(ii) The Bolton-Hunter reaction appeared temperamental, and incomplete NSHPP/MT adduct coupling was suspected (Bolton and Hunter, 1973; Vander Mallie and Garvey, 1978; Kagi and Kojima, 1987). It is not clear whether the NSHPP/MT reaction went to completion. It is also possible that the concentration of MT was too low (see Section 4.4.1).

(iii) The 1^{o}Ab (rabbit anti-rat MT serum and chicken anti-rat MT yolk preparations) were not purified after collection and this may be responsible for the high non-specific binding seen in many of the assays. As a result, the % bound for the antibody titration curves and test samples were low (Table 4.8).

(iv) The peak period for antigenic response in the rabbit may well have been missed, judging by the results shown in Figure 4.9.

(v) A review of RIA protocols in the literature which used the double antibody competitive binding complex revealed that a third incubation step is often inserted (of 10 - 24 h duration) after the 2^{o}Ab addition and before the final dilution with PEG (Vander Mallie and Garvey, 1979; Chang et al., 1980; Garvey et al., 1982; Mehra and Bremner, 1983). However, the extra incubation step was not employed in the RIA protocol developed previously in this laboratory (Nieboer et al., 1988a; 1988b). Apparently, the third incubation step facilitates the double antibody complex formation and precipitation. This step might well have improved the antibody titration results observed (Table 4.8).

In view of these difficulties, paired with time and budget constraints, the decision was made to use the CSA alone to determine MT in tissues and body fluids for the *in vivo* rat time course experiments.

4.4.3. CSA Determination of MT

The potential for interferences with the CSA by reagents and sample preparation procedures was reviewed in Section 4.1.2., and stimulated the systematic study of the CSA protocol conditions reported here. Blood cell isolation protocols use many reagents including anticoagulants and RPMI. RPMI complete cell media contains many additives, one of which (albumin) is known to bind many substrates including Cd. The present research showed RPMI blanks and samples containing RPMI had higher ^{109}Cd total counts compared to PBS blanks and samples washed with PBS or normal saline prior to analysis. The anticoagulant, EDTA, often used in the preparation of blood products and blood cells, also was responsible for high total counts in blank solutions and test samples. Heparin however, did not interfere with the assay. Therefore, blood cell samples incubated with RPMI should be isolated and washed thoroughly prior to cell lysis and use in the CSA. Heparin should be used as the anticoagulant in samples marked for MT determination rather than EDTA.

In the TBS blank solution studies, the only possible source of protein contamination during the CSA was from heat-stable Hb fragments (Eaton, 1985) and this was shown to be another source of error. Hb fragmentation may result from low ($<100^{o}C$) incubation temperature and incorrect buffer concentration in the CSA analyte.

The results obtained from the study of cold $CdCl_2$ concentration in the ^{109}Cd radiolabelling solution (see Sections 4.2.5.6 and 4.3.3.5) showed that high levels (300 ug) of cold Cd in the label caused precipitation (salting out) of the radiolabel, while both high (300 ug) and low (3 ug) cold Cd inhibited the complete precipitation of Hb, which resulted in a low estimation of MT concentration during CSA analysis. The results were in agreement with a metal inhibition study previously reported (Eaton, 1985) which indicated that Cd concentrations that were too high (35 uM Cd) inhibited complete precipitation of

Hb. The sensitivity of the assay would also decrease with increasing unlabelled (cold) Cd in solution (Eaton and Toal, 1982; Eaton, 1985).

Examination of the gel filtration mini-column separation of the CSA analyte depicted in Figures 4.16 - 4.19 (see also Section 4.3.3.6) clearly showed that the majority of total ^{109}Cd counts were found in fractions #5 - 7 (approximate MW 30 - 6 kD). This indicates that MT or polymers of MT in the test samples accounted for the majority of total ^{109}Cd counts observed in the CSA (see Section 4.4.1. for discussions on MW and MT polymers). Although the mini-columns are relatively quick for a limited number of samples, their routine use would not be suitable for large numbers, supporting earlier conclusions (Shaikh and Nolan, 1987).

In conclusion, the CSA was considered the more feasible MT assay for the current project.

4.4.4. Salient Features of the Observed MT Concentrations

The salient features of the MT results are described below, followed by an interpretation section. In this and the subsequent section, Figures 6.2, 6.4, and 6.6 have been consulted. In these figures, the data depicted in Chapter 4 are combined. Since MT induction and Cd exposure are closely interrelated, comparative interpretations of the MT results are deferred to Chapter 6. A model describing Cd and MT dynamics is also presented in Chapter 6.

4.4.4.1. *MT Concentration in Tissues*

• In the short course experiments, significantly elevated MT levels were observed at the indicated doses in the liver (medium and high doses), kidney and spleen (high dose only), and lung (low dose), but not at the other doses (Figures 4.21 - 4.27).

• The MT levels for the subchronic experiment depicted in Figures 4.25 - 4.31, showed significant increases in MT levels at the indicated doses in the liver and kidney (high dose), liver (medium dose) and lung (low dose); but not in spleen (high dose not available, see Chapter 5).

• In all tissues except lung, the low (0.25 mg $CdCl_2$ /kg) dose was not sufficient to significantly induce MT production.

• Basel MT levels in control animals were slightly higher in liver (21 uMol MT/kg dwt), compared to 11 uMol MT/kg dwt in the other organs. Subchronic basel MT levels in controls were similar to those in the short course experiment for liver and spleen, but were elevated in kidney (26 uMol MT/kg dwt) and lung (22 uMol MT/kg dwt).

• In the overall organ comparisons by ANOVA, MT levels were higher in the subchronic Cd-exposed rats than the short course.

4.4.4.2. *MT Concentration in Plasma*

• Significant dose- and time-dependent increases in MT levels were observed in the short course animals starting at T = 24 h and returning toward baseline values by T = 96 h (Figure 4.32).

• The subchronic experiment showed dose-dependent 'steady state' MT levels, which were significantly elevated above control levels throughout the 96 h observation period. Plasma MT levels showed transient peaking at T = 6 - 12 h for the low and medium doses (Figure 4.33).

• The plasma MT response to subchronic Cd exposure was rapid, while that for the single exposure was quite slow.

4.4.4.3. *MT Concentration in RBCs*

• Time- and dose-dependent increases in RBC MT levels were observed in the short course (medium dose) animals at T > 24 h, and in the low dose at T = 96 h (Figure 4.34).

• Subchronic RBC MT levels for the medium Cd exposure group showed a dose-dependent 'steady state' which was significantly higher than those for the control and low dose animals throughout the entire 96 h time course (Figure 4.35).

• The maximum MT levels observed in the later time points of the single exposure study approached the initial 'steady state' levels observed in the

subchronic RBCs.

4.4.4.4. *MT Concentration in PMNs*

• Due to the high detection limits, low MT levels, and large standard deviations, it was difficult to identify systematic or transient increases in MT in the PMNs that were significant. (Figures 4.36 - 4.37).

4.4.4.5. *MT Concentration in Lymphocytes*

• The lymphocyte MT response showed transient peaking between 12 and 24 h for all doses, reaching significance in the short course (low and medium dose) and subchronic (medium dose) animals. However, high detection limits, low MT levels, and large standard deviations, made it difficult to assign significance in the short course (high dose) and subchronic (low dose) animals (Figures 4.38 - 4.39).

4.4.4.6. *MT Concentration in Monocytes*

• Due to the high detection limits, low MT levels, and large standard deviations, it is difficult to identify systematic or transient increases in MT that were significant in the monocytes isolated from the short course (all doses) or subchronic (all except in the low dose) animals (Figures 4.40 - 4.41).

• Monocyte MT levels were lower in the subchronic medium-dose group, a trend not observed in the short course animals.

4.4.4.7. *MT Inter-Compartment Comparison*

• The inter-compartment comparisons on a 'per cell basis' showed the induction levels were the highest in the RBCs for both single and multiple Cd-exposures for the medium dose (1.25 mg/kg) while not at the lower doses (Figures 4.42 - 4.47).

• The monocytes exhibited the highest MT induction response compared to all other leukocytes for all of the Cd exposures studied; the monocyte MT levels were also higher than the RBC levels at the low dose (0.25 mg/kg) in both experimental protocols (Figures 4.42 - 4.47).

• Perusal all of the data related to leukocytes (Figures 4.36 - 4.41) and

RBCs (Figures 4.34 - 4.35) demonstrated transient responses in the leukocytes while the RBCs showed a gradual increase toward a systemic 'steady state' response.

• The major contributors to overall MT concentration in the blood were the RBCs and lymphocytes, followed by the PMNs, and to a lesser extent, the monocytes. The individual contributions depends on the total number of each cell type in peripheral whole blood.

4.4.5. Interpretation of the Observed MT Levels

4.4.5.1. *MT Concentration in Tissues*

The basal MT concentrations observed in the organs of control animals were similar to earlier reported values of 10 to 16 uMol MT/kg dwt for liver and 6 to 50 uMol MT/kg dwt for kidney (Onosaka and Cherian, 1981; Eaton and Toal, 1982; Nolan and Shaikh, 1986). Both the liver and kidney MT concentrations in the present study clearly illustrated a dose-dependent Cd-induced synthesis of MT. The liver clearly responds more strongly to Cd exposure than the kidney. It appears that the kidney became somewhat more important after multiple Cd exposures. The ineffectiveness of the low dose (0.25 mg Cd/kg) may represent the level of no-response for MT induction in organs other than lung.

The relative proportions of induced MT in the organs studied were similar to that reported previously, although the absolute values were lower likely due to differences in dose and route of Cd administration (Onosaka and Cherian, 1981; Eaton and Toal, 1982; Onosaka et al., 1984; Shaikh and Tohyama, 1984; Waalkes et al., 1985; EHC,1992a). The strong response observed in liver concurs with the hypothesis that after uptake, Cd is accumulated mainly by the liver and results in induction of MT synthesis, which appears to occur to a lesser degree in the other organs and blood cells (Friberg et al., 1986a; Goyer, 1991). The extensive necrosis of the spleen observed during autopsy in the high-dose subchronic animal suggests that the MT levels were insufficient to detoxify the higher Cd concentrations. Further discussions regarding cellular changes upon

histological examination are deferred to Chapter 5

4.4.5.2. *MT Concentration in Plasma*

The delay in appearance of MT in plasma observed in the short course experiment is consistent with the time required for internal distribution after uptake, synthesis of MT in the liver and subsequent release into the circulatory system (Bremner and Morrison, 1986; Bremner et al., 1987). Presumably, the rapid rise (0 to 12 h) in plasma MT concentrations for the subchronic animals reflects a system poised for MT synthesis (ie., permanently induced). The rather rapid clearance of plasma MT levels in the same animals concurs with its excretion in urine (Cherian and Shaikh, 1975; Shaikh and Tohyama, 1984; Jin et al., 1987c) and presumably also uptake into tissues (eg. kidney).

4.4.5.3. *MT Concentration in Erythrocytes*

The MT data suggest that the low dose administered just exceeded the no-response level, while the medium dose was clearly responsible for significant RBC MT induction. Further, the MT induction levels obtained in the short course experiment were close to the induction capacity observed in the subchronic groups. The time frame for induction of RBC MT in the short course experiment (1 - 4 days) is in agreement with earlier rat (Garty et al., 1981; Garty et al., 1986) and mice studies (Nordberg et al., 1971b; Tanaka et al., 1985; Tanaka et al., 1986; Tanaka et al., 1987). Since MT is synthesized only in RBC progenitors, this time frame reflects the time required for cell maturation. The typical life span for RBCs is 120 days in humans (Hall and Malia, 1984) and 84 days in the rat (Ringler and Dabich, 1979). It is generally accepted that induction occurs primarily in the erythroblasts located in the erythropoietic tissues, (spleen and bone marrow) and thus MT induced-reticulocytes enter circulation gradually (Garty et al., 1981; Tanaka et al., 1985; Tanaka et al., 1987). In the subchronic animals, the dose administered at T = 0 h had only a gradual positive impact on the already elevated MT levels in RBCs (data not available for the high dose). The near 'steady state' enhancement observed in

subchronic experiments presumably reflects a sustained induction of the precursor cells in erythropoietic tissues (Tanaka et al., 1985; Tanaka et al., 1987).

4.4.5.4. *MT Concentration in WBCs*

Even though the scatter in the MT concentrations found in WBCs does not permit detailed interpretation, a few general comments are warranted. Clearly, the PMNs exhibit the lowest MT levels, which is consistent with *in vitro* studies that indicate a low level of MT induction (Enger et al., 1983; Peavy and Fairchild, 1987; Harley et al., 1989). The highest MT concentrations were found in monocytes of the short course animals, which suggest a transient response to current exposure. For the lymphocytes, the data suggests some enhancement of MT in the subchronic animals. In addition in both experimental protocols, there is a suggestion of a transient presence of MT 12 to 24 h after the administered dose (Enger et al., 1983; Peavy and Fairchild, 1987; Harley et al., 1989). Lymphocytes and monocytes showed lower MT levels in the medium and high subchronic groups compared to the short course experiment, which may reflect cell specific Cd cytotoxicity (see Chapter 3, Sections 3.1.1, 3.4.6 - 3.4.7.).

It is obvious that the CSA protocol as employed in the present study lacks the sensitivity and reproducibility required to detect with confidence the low levels and small increases of MT in WBCs. The small quantities of cells available was an obvious limitation.

4.4.5.5. *MT Inter-Compartment Comparison*

The inability to establish clearly defined patterns of MT induction in WBCs prevents a detailed comparison of MT in the various blood cell compartments. There are two obvious factors that explain the greater variability in MT determination in WBCs. First, compared to WBCs, the amount of MT present in one liter of blood for RBCs is higher by about a factor of 10. Second, cell losses during the separation procedures are expected to have been more

significant for WBCs than for RBCs because of lower cell numbers. The increased MT concentrations in RBCs in part reflects the increased cell numbers and not necessarily a higher induction capacity of these cells, in agreement with earlier conclusions (Hildebrand and Cram, 1979; Garty et al., 1981).

4.5. Conclusions

The *in vivo* rat studies have illustrated a clear Cd-induced, dose-response for the synthesis of MT in major tissues (liver and kidney), plasma and RBCs. The plasma MT response was gradual in the single exposure study, while that for the subchronic Cd-exposure was relatively rapid and superimposed on an enhanced 'steady state' background. A similar elevated 'steady state' background was evident for MT in RBCs in the subchronic animals. The pre-induced animal data suggest that the liver can respond quickly to a demand for MT synthesis, which then becomes available for immediate redistribution into the plasma compartment, as supported in earlier studies (Bremner and Morrison, 1986; Bremner et al., 1987). The RBC and plasma results indicate that MT levels may serve as reliable indicators of both short- and long-term Cd exposures. Time of sampling post-exposure is critical and may help to distinguish between current and long-term (body burden) contributions.

Although there is some suggestion that MT induction in monocytes responds to current Cd exposure, and that in lymphocytes to both current and previous Cd exposures, improvements in sensitivity over the CSA protocol are required in the determination of MT in these cellular compartments.

Table 4.1 Principal Methods of Metallothionein Determination.

Method	Principle	References
Cadmium Saturation Assay (CSA)	MT protein concentration calculated from ^{109}Cd binding ratio of 7 mol Cd per mol MT.	Chen and Ganther, (1975); Eaton and Toal, (1982); Nolan and Shaikh, (1986).
Mercury Saturation Assay	MT concentration is determined from mercury binding to the protein.	Piotrowski et al,(1973); Kotsonis and Klaassen,(1977).
Polarography	MT is calculated from measured sulphydryl groups.	Olafson and Sim, (1979); Onosaka and Cherian, (1982).
Radioimmunoassay (RIA)	MT is assessed employing ^{125}I labelled MT and a double antibody precipitation.	Vander Mallie and Garvey, (1979); Garvey et al., (1982).
Enzyme-Linked Immunosorbent Assay (ELISA)	MT measured directly from fluorescence of precipitated protein by MT specific double antibody complex.	Thomas et al., (1986); Garvey et al., (1987).

Table 4.2. Reagents Employed in MT Isolation and Purification.

Reagent	Preparation and Comments
AFB	0.1 M ammonium formate buffer, pH 7.4 (3.85 mL formic acid in 600 mL DDW, pH with 8.5 mL conc. NH_4OH, adjust with dilute NH_4OH, make up to 1 L, store 4^{o}C).
BBS	0.125 M borate buffered saline, pH 8.6 (47.6 g $Na_2B_4O_7$: 10 H_2O in 980 mL DDW pH with HCl, store at room temperature).
PBS	0.15 M phosphate buffered saline, pH 7.3 (0.2 g KCl, 0.2 g KH_2PO_4, 8.0 g NaCl and 1.15 g Na_2HPO_4 in 950 mL DDW, pH with HCl, make up to 1L).
0.5 M Tris-HCl	60.5 g Tris (MW 121) in 950 mL DDW, pH with 2 M HCl and make up to 1L.
TBS	50 mM Tris buffered saline, pH 8.5 (6.05 g Tris (MW 121) in 950 mL DDW, adjust pH with HCL and make up to 1 L).
5 mM Tris-HCl	Mix 0.605 g Tris in 950 mL DDW, adjust pH 8.5 with 2 M HCL and make up to 1L or prepared from 500 mM Tris stock (20uL 0.5 M Tris diluted to 1 L).

Table 4.3. Reagents Employed in the RIA for MT Quantification.

Reagent	Comments
TGB	0.05 M Tris gelatin buffer (0.05 M Tris, 0.1 % gelatin, 0.02 % NaN_3, pH 8.0) stored at 4^{o}C.
Tris (EDTA/NRS)	50 mM EDTA and 2.5% normal rabbit serum (NRS, supplied by Dr. Hatton's Laboratory) made up in TGB.
GARGG	1/20 dilution of goat anti-rabbit IgG (GARGG) with TGB.
RACGG	1/20 dilution of rabbit anti-chicken IgG (RACGG) with TGB.
1^{o}Ab	Rabbit anti-rat MT or chicken anti-rat MT (produced by LMJ Smith). 1^{o}Ab initial dilution with Tris (EDTA/NRS), several initial dilutions were tested from 1/100 to 1/1000 to yield final assay dilutions of 1/1000 to 1/10000. Maximum binding observed with the final dilution of 1/1000 in the RIA reaction tubes.
4% PEG	Polyethyleneglycol (PEG) made up in TGB.
^{125}I-MT	Radiolabel diluted with TGB to yield ~ 50,000 cpm / 50 uL.
MT standard	Rabbit MT1 from Sigma (No. M-5267). Prepare a stock solution 0.5 mg/mL in TGB, from which serial dilutions were made with TGB to yield $5x10^{-1}$, $5x10^{-2}$, $5x10^{-3}$, $5x10^{-4}$; and $2x10^{-2}$ ug/mL.

Table 4.4. Radioimmunoassay (RIA) Protocol [a].

	T = 0 h	T = 24 h		T = 30 h		
	Volume in microliters					
	Sample	Tris	1°Ab[b]	^{125}I-MT	2°Ab[c]	PEG
Total Counts	—	—	—	50	—	—
NSB Control[d]	—	450	—	50	100	1000
MT Standard	400	—	50	50	100	1000
Test Sample	300	100	50	50	100	1000

a. Additional details are provided in Section 4.2.3. Calculated % Bound = 100 X (Test-NSB/Total-NSB).

b. 1°Ab depicts the primary antibody, rabbit or chicken anti-rat MT.

c. 2°Ab depicts the secondary antibody, goat anti-rabbit IgG (GARGG) or rabbit anti-chicken IgG (RACGG).

d. Control for non-specific binding (NSB).

Table 4.5 Reagents Employed in the Micro-CSA.

Reagent	Preparation and Comments
Stock A ^{109}Cd solution:	100 uL pure ^{109}Cd, carrier free (1.3 uCi /uL) diluted with 100 uL DDW to yield 200 uL Stock A (0.65 uCi/uL).
^{109}Cd used in the assay:	
For Tissues -	Mix together 20 uL Stock A (0.65 uCi/uL), 100 uL $CdCl_2$ (10 ug $CdCl_2$/uL) and 880 uL 0.87% NaCl to yield 1000uL of assay label (13 uCi ^{109}Cd/ 1000 ug $CdCl_2$/1000 uL).
For Blood Cells -	Mix together 20 uL Stock A (0.65 uCi/uL), 10 uL $CdCl_2$ (10 ug $CdCl_2$/uL) and 970 uL 0.87% NaCl to yield 1000uL of assay label (13 uCi ^{109}Cd/ 100 ug $CdCl_2$/ 1000 uL).
$CdCl_2$	Mix 100 mg $CdCl_2$ in 10 mL 0.87% NaCl.
2 % hemoglobin	2% Hb (w/v) in 0.87% NaCl (normal saline, pH. 7.4) make fresh or freeze at - 20°C.
500 mM Tris-HCl	Mix 60.5 g Tris in 950 mL DDW, adjust to pH 8.5 with 2 M HCl and make up to 1L.
50 mM Tris-HCl	Mix 6.05 g Tris in 950 mL DDW, adjust pH 8.5 with 2 M HCL and make up to 1L or prepared from 500 mM Tris.
0.87% NaCl	Physiological saline, mix 8.7 g NaCl in 1L DDW.

Table 4.6 Optimized Mini-CSA Conditions[a].

1. Partial purification of samples prior to the CSA was carried out:
   For Tissues: homogenization, heat / ice precipitation, and centrifugation prior to the assay.
   For Blood Cells: heparin was used as anti-coagulant; cells in contact with RPMI were washed twice with PBS or TBS prior to cell lysis; all cells received at least two freeze/ thaw sonication steps; heat/ice precipitation and centrifugation prior to assay.
2. When a contaminate was suspected, SDS-PAGE or gel filtration mini-column chromatography was recommended.
3. Excess ^{109}Cd label was precipitated with 2% Hemoglobin solution.
4. Incubation temperature: 100°C (water bath).
5. Minimum buffer concentration: 50 mM TBS.
6. Concentration of $CdCl_2$ in 90 uL of ^{109}Cd labelling solution (LS).
   For tissues: 90 ug $CdCl_2$, LS = 13 uCi ^{109}Cd/1000 ug $CdCl_2$/1000 uL NaCl.
   For cell lysates: 9 ug $CdCl_2$, LS = 13 uCi ^{109}Cd/100 ug $CdCl_2$/1000 uL NaCl.
7. To optimize precision, assay starting times were staggered when running more than 8 test samples.
8. Careful separation of the Hb pellet is crucial.

a. See Figure 4.3 for CSA Flowchart.

Table 4.7 Experimental and Reported Molar Extinction Coefficients (ε) values for Purified MT [a].

Reagent	Source	(ε) values	Reference
Experimental ε Values at 250 nm:			
Rat MT1	Smith	1.03×10^3 $M^{-1}cm^{-1}$	
Rat MT2 a	Smith	2.02×10^3 $M^{-1}cm^{-1}$	
Rat MT2 b	Smith	8.88×10^2 $M^{-1}cm^{-1}$	
Horse MT	Sigma	4.00×10^4 $M^{-1}cm^{-1}$	
Rabbit MT	Sigma	6.00×10^4 $M^{-1}cm^{-1}$	
Reported ε Values at 250 nm:			
Human MT		1.72×10^4 $M^{-1}cm^{-1}$	Pulido et al., (1966)
Horse MT		1.46×10^4 $M^{-1}cm^{-1}$	Kagi and Vallie, (1961)
Rat MT		7.90×10^4 $M^{-1}cm^{-1}$	Winge et al., (1975)

a. Calculated from the measured absorbance using Beer's Law.

Table 4.8. Results of Antibody Binding to Metallothionein [a].

	^{125}I-Rabbit MT			^{125}I-Rat MT	
1°Ab Dilution	% Bound	Iodination Date	1°Ab Dilution	% Bound	Iodination Date
1°Ab Rabbit anti-rat MT:					
1/5000	3.4	11-14-90	1/5000	21.2	10-12-90
1/5000	9.0	11-21-90	1/5000	3.1	11-07-90
1/1000	2.0	11-14-90	1/5000	4.0	11-16-90
1/1000	5.4	11-21-90	1/1000	22.9	10-12-90
			1/1000	14.5	10-17-90
			1/1000	9.3	10-17-90
1°Ab Chicken anti-rat MT:					
Chicken # 994 (prep 15)			1/1000	3.3	10-12-90
Chicken # 995 (prep 15)			1/1000	6.5	10-12-90
Chicken # 991 (prep 12)			1/1000	4.8	10-12-90

a. The RIA protocol is outlined in Section 4.2.3 and Table 4.4. The % bound values represent separate iodinations and are calculated as % bound = 100 x (Max - NSB/Total -NSB). Where NSB denotes nonspecific binding.

Table 4.9. Optimization of CSA: Sephadex G-50 Gel Filtration of CSA Analyte; 50 mM TBS, Liver Supernatant and Purified MT solutions (a).

Sample	Fraction no.	254nm	280nm	Cpm x10e-4	Sample	254nm	280nm	Cpm x10e-4
b.					d.			
Liver	1	0.009	0.007	0.000	Rabbit	0.005	0.005	-0.001
	2	0.009	0.008	-0.002	MT1 &	0.003	0.003	0.000
	3	0.005	0.004	-0.001	MT2	0.004	0.004	-0.001
	4	0.928	0.792	0.147		0.037	0.019	0.205
	5	0.937	0.803	0.366		0.168	0.054	1.071
	6	0.179	0.151	1.495		0.277	0.051	1.945
	7	0.065	0.052	1.566		0.210	0.038	1.505
	8	0.050	0.037	0.644		0.079	0.022	0.469
	9	0.085	0.053	0.138		0.033	0.019	0.120
	10	0.145	0.084	0.030		0.031	0.026	0.016
	11	0.164	0.083	0.007		0.030	0.028	0.005
	12	0.121	0.049	0.002		0.019	0.018	0.000
c.					e.			
Rat MT1	1	0.016	0.014	-0.001	Blank	0.005	0.005	0.000
& MT2	2	0.006	0.005	0.002		0.005	0.005	-0.001
	3	0.005	0.005	0.001		0.005	0.005	0.002
	4	0.010	0.007	0.056		0.012	0.013	0.001
	5	0.045	0.035	0.632		0.022	0.025	0.003
	6	0.025	0.030	1.748		0.017	0.021	0.008
	7	0.022	0.017	1.424		0.017	0.019	0.006
	8	0.019	0.017	0.495		0.020	0 019	0.004
	9	0.030	0.026	0.128		0.031	0.027	0.002
	10	0.035	0.032	0.047		0.032	0.029	0.001
	11	0.028	0.026	0.026		0.028	0.026	0.001
	12	0.016	0.015	0.017		0.019	0.017	-0.002

a. CSA parameters: 2% Hb,100 C water bath, 90 ug Cold Cd, 50 mM TBS buffer (concentration held constant). The data shown above are depicted in Figures 4.16 - 4.19.
b. Liver supernatant was isolated from Cd induced rats.
c. Rat MT1 and MT2 was isolated from Cd induced rats.
d. Rabbit MT1 and MT2 was purchased from Sigma Chemical Co.
e. Blank solution was 50 mM Tris - HCl buffer.

Table 4.10. MT Concentration in Tissues, Short Course Experiments.

Tissue	Administered Dose (mg/kg)	Individual Concentrations[a,b] (uMol MT/kg dwt)					Mean ± SD (minimum n=3)		CV[c] (%)
Liver	0.00 mg/kg	29	27	17	13	20	21	6.8	32
	0.25 mg/kg	26	27	35	–	–	29	4.9	17
	1.25 mg/kg	75	115	107	–	–	99	21.2	21
	2.50 mg/kg	149	141	131	–	–	140	9.0	6
Kidney	0.00 mg/kg	9.0	7.3	16	7.3	11	10	3.5	35
	0.25 mg/kg	13	13	8.4	–	–	11	2.7	23
	1.25 mg/kg	15	14	17	–	–	15	1.9	12
	2.50 mg/kg	26	20	14	–	–	20	5.8	29
Spleen	0.00 mg/kg	16	5.0	12	15	12	12	5.0	42
	0.25 mg/kg	14	14	15	–	–	14	0.5	3
	1.25 mg/kg	9.4	14	14	–	–	12	2.7	21
	2.50 mg/kg	22	24	26	–	–	24	2.4	10
Lung	0.00 mg/kg	14	11	16	–	–	13	2.6	20
	0.25 mg/kg	19	24	21	–	–	22	2.6	12
	1.25 mg/kg	16	21	21	–	–	19	3.0	16

a. Values for 2.5 mg/kg samples are reported as averages of quadruplicate determinations for liver and in triplicate for spleen and kidney, remaining values are single determinations.
b. For rat codes see Table 2.1: 0.0 mg/kg = T, U, AA, B, E, from left to right respectively; 0.25 mg/kg= V, W, X; 1.25 mg/kg = P, Q, R; 2.5 mg/kg = A, D, F.
c. Inter-animal coefficient of variation (CV %).

Table 4.11. MT Concentration in Tissues, Subchronic Experiments.

Tissue	Administered Dose (mg/kg)	Individual Concentration (uMol MT / kg dwt) [a,b]			Mean ± SD n = 3		CV (%) [c]
Liver	0.0 mg/kg	14	19	34	22	11	48
	0.25 mg/kg	35	53	42	43	9.0	21
	1.25 mg/kg	139	153	190	161	27	17
	2.5 mg/kg	232	--	--	—	—	—
Kidney	0.0 mg/kg	13	18	48	26	19	73
	0.25 mg/kg	11	12	31	18	11	62
	1.25 mg/kg	31	41	35	36	4.9	14
	2.5 mg/kg	107	--	--	—	—	—
Spleen	0.0 mg/kg	13	15	14	14	0.6	4
	0.25 mg/kg	16	12	12	13	2.7	20
	1.25 mg/kg	15	29	24	23	7.0	31
Lung	0.0 mg/kg	24	23	20	22	2.0	9
	0.25 mg/kg	30	27	--	29	1.7	6
	1.25 mg/kg	19	22	25	22	2.6	12

a. Each rat received three IP injections of the indicated CdCl2 dose over a 1.5-2 month period. The third injection was administered at T = 0 h. For rat codes see Table 2.2.

b. Values for 2.5 mg/kg tissue samples are reported as averages of quadruplicate determinations for liver and in triplicate for spleen and kidney, remaining values are single determinations.

c. Inter-animal coefficient of variation (% CV).

Table 4.12. MT Concentration in Plasma, Short Course Experiment PA91.

Time (h)	MT Concentration (uMol MT/L)[a]			Mean ± SD[b]		CV (%)[c]
Administered Dose: 0.00 mg/kg				minimum (n=3)		
	T	U	AA			
-20	0.08	0.11	0.18	0.12	0.05	40
0	0.11	0.20	0.11	0.14	0.05	38
6	0.11	0.21	0.14	0.16	0.05	32
12	0.18	0.17	0.18	0.18	0.004	2.5
24	0.14	0.22	0.18	0.18	0.04	22
48	0.24	0.17	0.25	0.22	0.04	20
72	0.16	0.14	0.23	0.18	0.05	28
96	0.31	0.23	0.12	0.22	0.10	45
Administered Dose: 0.25 mg/kg						
	V	W	X			
-20	0.13	0.13	0.14	0.13	0.01	4.8
0	0.08	0.11	0.09	0.09	0.02	21
6	0.07	0.13	0.11	0.10	0.03	31
12	0.11	0.17	0.17	0.15	0.04	24
24	0.29	0.24	0.23	0.25	0.03	14
48	0.38	0.30	0.38	0.35	0.05	13
72	0.27	0.29	0.20	0.25	0.04	17
96	0.14	0.11	0.19	0.15	0.04	30
Administered Dose: 1.25 mg/kg						
	P	Q	R			
-20	0.09	0.15	0.05	0.10	0.05	48
0	0.10	0.20	0.09	0.13	0.06	45
6	0.15	0.22	0.30	0.23	0.08	33
12	0.18	0.24	0.35	0.26	0.09	33
24	0.32	0.42	0.39	0.38	0.05	13
48	0.54	0.44	0.66	0.55	0.11	20
72	0.44	0.40	0.52	0.45	0.06	13
96	0.34	0.33	0.39	0.35	0.03	8.3

a. Capital letters denote individual rats (see Table 2.1).
b. Mean ± standard deviation (minimum n = 3).
c. Inter-animal coefficient of variation (% CV).

Table 4.13. MT Concentration in Plasma, Subchronic Experiment GO91.

Time (h)	MT Concentration (uMol MT/L) [a]			Mean ± SD [b]		CV (%) [c]
Administered Dose: 0.00 mg/kg				(minimum n=2)		
	G	H	I			
-20	0.10	0.12	0.05	0.09	0.04	42
0	0.14	0.11	0.10	0.12	0.02	19
6	0.15	0.13	0.10	0.13	0.02	17
12	0.18	0.13	0.14	0.15	0.02	16
24	0.19	0.18	0.13	0.17	0.03	19
48	0.21	0.13	0.14	0.16	0.05	29
72	0.13	0.19	-	0.16	0.04	25
96	0.10	0.15	-	0.12	0.03	23
Administered Dose: 0.25 mg/kg						
	J	K	L			
-20	0.27	0.24	0.23	0.24	0.02	9.4
0	0.36	0.26	0.22	0.28	0.07	26
6	0.43	0.54	0.51	0.49	0.06	12
12	0.56	-	0.62	0.59	0.05	7.8
24	0.34	-	0.39	0.37	0.04	10
48	0.38	-	0.35	0.36	0.02	5.4
72	0.40	-	-	-	-	-
96	0.26	-	-	-	-	-
Administered Dose: 1.25 mg/kg						
	M	N	O			
-20	0.45	0.36	0.47	0.42	0.06	14
0	0.32	0.38	0.40	0.37	0.04	11
6	0.69	0.82	0.76	0.76	0.07	8.7
12	0.81	1.15	1.04	1.00	0.18	18
24	0.52	0.73	0.53	0.59	0.12	20
48	0.46	0.51	0.40	0.46	0.05	11
72	0.59	0.62	0.48	0.57	0.07	13
96	0.51	-	0.34	0.42	0.12	28

a. Capital letters denote individual rats (see Table 2.2).
b. Mean ± standard deviation (minimum n = 2).
c. Inter-animal coefficient of variation (% CV).

Table 4.14. MT Concentration in Plasma, Short Course AF90 and Subchronic CD90 Experiments.

Time (h)	MT Concentration (uMol MT/L) [a]			Mean ± SD [b]		CV (%) [c]
Short Course:						
Administered Dose: 0.00 mg/kg				minimum (n=2)		
	B	E				
-20	3.74E-02	-		-	-	-
0	4.75E-02	-		-	-	-
6	5.06E-02	-		-	-	-
12	8.56E-03	-		-	-	-
24	3.04E-02	-		-	-	-
34	-	-		-	-	-
48	-	-		-	-	-
96	-	-		-	-	-
Administered Dose: 2.50 mg/kg						
	A	F	D			
-20	4.12E-02	-	-	-	-	-
0	4.98E-02	5.72E-02	-	5.35E-02	5.21E-03	10
6	4.59E-02	7.35E-02	-	5.97E-02	1.95E-02	33
12	6.30E-02	4.21E-01	-	2.42E-01	2.53E-01	105
24	2.20E-01	1.71E-01	-	1.95E-01	3.51E-02	18
34	-	2.73E-02	-	-	-	-
96	-	-	-	-	-	-
Subchronic:						
Administered Dose: 2.50 mg/kg						
	C					
-20	-			-	-	-
0	-			-	-	-
12	-			-	-	-

a. Capital letters denote individual rats (see Tables 2.1 & 2.2). There was insufficient sample for E, D, and C plasma MT determinations.
b. Mean ± standard deviation (minimum n = 2).
c. Inter-animal coefficient of variation (% CV).

Table 4.15. MT Concentration in Erythrocytes, Short Course Experiment PA91.

Time (h)	MT Concentration (uMol MT/L) [a]			Mean ± SD [b]		CV (%) [c]
Administered Dose: 0.00 mg/kg				minimum (n=3)		
	T	U	AA			
-20	1.44E-02	0.00E+00	0.00E+00	4.79E-03	8.30E-03	173
0	1.07E-02	0.00E+00	0.00E+00	3.56E-03	6.17E-03	173
6	1.02E-02	0.00E+00	0.00E+00	3.40E-03	5.89E-03	173
12	6.60E-03	6.55E-03	1.35E-02	8.88E-03	4.00E-03	45
24	0.00E+00	0.00E+00	0.00E+00	0.00E+00	0.00E+00	-
48	3.74E-03	0.00E+00	0.00E+00	1.25E-03	2.16E-03	173
72	0.00E+00	0.00E+00	3.85E-03	1.28E-03	2.23E-03	173
96	0.00E+00	0.00E+00	2.68E-03	8.93E-04	1.55E-03	173
Administered Dose: 0.25 mg/kg						
	V	W	X			
-20	1.77E-02	0.00E+00	3.62E-03	7.10E-03	9.34E-03	132
0	0.00E+00	7.23E-03	0.00E+00	2.41E-03	4.17E-03	173
6	0.00E+00	1.25E-02	1.68E-02	9.77E-03	8.74E-03	89
12	5.98E-03	0.00E+00	9.47E-03	5.15E-03	4.79E-03	93
24	4.60E-03	0.00E+00	1.73E-02	7.29E-03	8.95E-03	123
48	2.87E-02	6.35E-03	0.00E+00	1.17E-02	1.51E-02	129
72	1.09E-02	0.00E+00	1.21E-02	7.66E-03	6.66E-03	87
96	5.08E-03	1.20E-02	3.63E-02	1.78E-02	1.64E-02	92
Administered Dose: 1.25 mg/kg						
	P	Q	R			
-20	4.92E-03	9.94E-03	8.37E-03	7.75E-03	2.57E-03	33
0	2.05E-03	1.12E-02	7.28E-03	6.84E-03	4.59E-03	67
6	4.77E-03	2.18E-02	1.07E-02	1.24E-02	8.66E-03	70
12	1.25E-02	6.37E-03	4.54E-03	7.81E-03	4.18E-03	54
24	0.00E+00	0.00E+00	9.41E-03	3.14E-03	5.43E-03	173
48	1.82E-02	1.52E-02	2.19E-02	1.84E-02	3.35E-03	18
72	2.07E-02	4.32E-02	2.33E-02	2.91E-02	1.23E-02	42
96	1.74E-02	1.89E-02	2.07E-02	1.90E-02	1.63E-03	9

a. Capital letters denote individual rats (see Table 2.1).
b. Mean ± standard deviation (minimum n = 3).
c. Inter-animal coefficient of variation (% CV).

Table 4.16. MT Concentration in Erythrocytes, Subchronic Experiment G091.

Time (h)	MT Concentration (uMol MT/L) [a]			Mean ± SD [b]		CV (%) [c]
Administered Dose: 0.00 mg/kg				(minimum n=2)		
	G	H	I			
-20	8.52E-03	8.70E-03	0.00E+00	5.74E-03	4.97E-03	87
0	1.42E-03	0.00E+00	3.56E-03	1.66E-03	1.79E-03	108
6	0.00E+00	0.00E+00	3.64E-03	1.21E-03	2.10E-03	173
12	0.00E+00	0.00E+00	7.33E-04	2.44E-04	4.23E-04	173
24	0.00E+00	9.33E-03	7.84E-03	5.72E-03	5.01E-03	88
48	0.00E+00	0.00E+00	4.72E-03	1.57E-03	2.73E-03	173
72	6.36E-03	5.92E-03	-	6.14E-03	3.14E-04	5.1
96	8.71E-03	0.00E+00	-	4.35E-03	6.16E-03	141
Administered Dose: 0.25 mg/kg						
	J	K	L			
-20	1.63E-02	1.16E-02	8.59E-03	1.21E-02	3.87E-03	32
0	7.00E-03	4.72E-03	1.57E-02	9.14E-03	5.80E-03	63
6	9.84E-03	7.55E-03	9.09E-03	8.83E-03	1.16E-03	13
12	0.00E+00	-	3.06E-03	1.53E-03	2.16E-03	141
24	6.08E-03	-	8.56E-03	7.32E-03	1.75E-03	24
48	6.67E-03	-	0.00E+00	3.33E-03	4.72E-03	141
72	1.68E-03	-	-	-	-	-
96	0.00E+00	-	-	-	-	-
Administered Dose: 1.25 mg/kg						
	M	N	O			
-20	4.29E-02	1.49E-02	1.45E-02	2.41E-02	1.63E-02	67
0	4.87E-02	4.09E-02	2.05E-02	3.67E-02	1.45E-02	40
6	2.62E-02	1.91E-02	3.79E-02	2.77E-02	9.47E-03	34
12	6.60E-02	8.28E-03	4.27E-02	3.90E-02	2.90E-02	74
24	3.06E-02	3.25E-02	3.11E-02	3.14E-02	1.00E-03	3.2
48	3.61E-02	3.00E-02	3.82E-02	3.48E-02	4.26E-03	12
72	2.54E-02	3.97E-02	6.91E-02	4.47E-02	2.23E-02	50
96	3.41E-02	-	4.71E-02	4.06E-02	9.15E-03	23

a. Capital letters denote individual rats (see Table 2.2).
b. Mean ± standard deviation (minimum n = 2).
c. Inter-animal coefficient of variation (% CV).

Table 4.17. MT Concentration in Polymorphonuclear Leukocytes, Short Course Experiment PA91.

Time (h)	MT Concentration (uMol MT/L) [a]			Mean ± SD [b]		CV (%) [c]
Administered Dose: 0.00 mg/kg				minimum (n=3)		
	T	U	A A			
-20	0.00E+00	0.00E+00	9.30E-03	3.10E-03	5.37E-03	173
0	0.00E+00	0.00E+00	0.00E+00	0.00E+00	0.00E+00	-
6	3.57E-03	7.47E-03	3.09E-03	4.71E-03	2.40E-03	51
12	0.00E+00	0.00E+00	0.00E+00	0.00E+00	0.00E+00	-
24	5.79E-03	5.02E-03	8.80E-03	6.54E-03	2.00E-03	31
48	1.91E-03	0.00E+00	9.60E-03	3.84E-03	5.08E-03	132
72	1.03E-02	1.96E-03	0.00E+00	4.10E-03	5.50E-03	134
96	1.58E-03	1.66E-04	5.41E-03	2.39E-03	2.71E-03	114
Administered Dose: 0.25 mg/kg						
	V	W	X			
-20	8.46E-03	2.16E-03	3.31E-03	4.64E-03	3.35E-03	72
0	5.19E-03	0.00E+00	3.64E-03	2.94E-03	2.66E-03	91
6	3.69E-03	5.57E-03	0.00E+00	3.09E-03	2.83E-03	92
12	3.70E-03	1.80E-03	2.96E-03	2.82E-03	9.57E-04	34
24	4.11E-03	2.04E-03	7.51E-03	4.55E-03	2.76E-03	61
48	1.03E-03	1.85E-03	0.00E+00	9.61E-04	9.27E-04	96
72	0.00E+00	1.51E-03	8.78E-03	3.43E-03	4.69E-03	137
96	4.88E-04	2.06E-03	0.00E+00	8.50E-04	1.08E-03	127
Administered Dose: 1.25 mg/kg						
	P	Q	R			
-20	0.00E+00	0.00E+00	0.00E+00	0.00E+00	0.00E+00	-
0	0.00E+00	0.00E+00	0.00E+00	0.00E+00	0.00E+00	-
6	0.00E+00	1.62E-03	3.72E-03	1.78E-03	1.87E-03	105
12	3.28E-03	7.37E-03	1.43E-03	4.03E-03	3.04E-03	75
24	0.00E+00	0.00E+00	7.95E-04	2.65E-04	4.59E-04	173
48	3.78E-03	3.03E-04	0.00E+00	1.36E-03	2.10E-03	154
72	1.78E-04	7.97E-04	8.51E-03	3.16E-03	4.64E-03	147
96	0.00E+00	0.00E+00	0.00E+00	0.00E+00	0.00E+00	-

a. Capital letters denote individual rats (see Table 2.1).
b. Mean ± standard deviation (minimum n = 3).
c. Inter-animal coefficient of variation (% CV).

Table 4.18. MT Concentration in Polymorphonuclear Leukocytes, Subchronic Experiment G091.

Time (h)	MT Concentration (uMol MT/L) [a]			Mean ± SD [b]		CV (%) [c]
Administered Dose: 0.00 mg/kg				(minimum n=2)		
	G	H	I			
-20	0.00E+00	6.47E-03	3.12E-03	3.20E-03	3.24E-03	101
0	1.32E-03	3.76E-03	6.63E-04	1.91E-03	1.63E-03	85
6	8.41E-03	7.57E-04	1.58E-03	3.58E-03	4.20E-03	117
12	1.55E-03	8.77E-03	7.88E-03	6.07E-03	3.94E-03	65
24	1.35E-02	1.28E-02	6.12E-03	1.08E-02	4.08E-03	38
48	2.41E-03	3.08E-03	1.94E-03	2.48E-03	5.75E-04	23
72	2.13E-03	5.67E-03	-	3.90E-03	2.50E-03	64
96	0.00E+00	0.00E+00	-	-	-	-
Administered Dose: 0.25 mg/kg						
	J	K	L			
-20	1.70E-03	1.47E-03	4.29E-03	2.49E-03	1.57E-03	63
0	0.00E+00	0.00E+00	2.26E-02	7.52E-03	1.30E-02	173
6	2.64E-03	1.35E-02	9.83E-03	8.66E-03	5.52E-03	64
12	4.59E-03	-	1.41E-02	9.33E-03	6.70E-03	72
24	4.30E-03	-	1.19E-02	8.12E-03	5.41E-03	67
48	3.54E-03	-	1.40E-02	8.79E-03	7.43E-03	85
72	0.00E+00	-	-	-	-	-
96	0.00E+00	-	-	-	-	-
Administered Dose: 1.25 mg/kg						
	M	N	O			
-20	3.86E-03	0.00E+00	8.88E-03	4.25E-03	4.45E-03	105
0	0.00E+00	7.72E-04	0.00E+00	2.57E-04	4.46E-04	173
6	1.32E-02	5.22E-03	4.97E-03	7.81E-03	4.71E-03	60
12	2.21E-02	6.70E-03	1.06E-02	1.31E-02	8.00E-03	61
24	1.57E-02	0.00E+00	6.58E-03	7.44E-03	7.90E-03	106
48	4.58E-03	8.66E-04	0.00E+00	1.82E-03	2.44E-03	134
72	3.07E-03	2.11E-03	3.11E-03	2.76E-03	5.65E-04	20
96	1.54E-02	-	5.48E-03	1.04E-02	7.03E-03	67

a. Capital letters denote individual rats (see Table 2.2).
b. Mean ± standard deviation (minimum n = 2).
c. Inter-animal coefficient of variation (% CV).

Table 4.19. MT Concentration in Lymphocytes, Short Course Experiment PA91.

Time (h)	MT Concentration (uMol MT/L) [a]			Mean ± SD [b]		CV (%) [c]
Administered Dose: 0.00 mg/kg				minimum (n=3)		
	T	U	AA			
-20	0.00E+00	0.00E+00	0.00E+00	0.00E+00	0.00E+00	-
0	0.00E+00	4.98E-04	0.00E+00	1.66E-04	2.87E-04	173
6	2.19E-03	0.00E+00	0.00E+00	7.30E-04	1.26E-03	173
12	0.00E+00	0.00E+00	9.13E-04	3.04E-04	5.27E-04	173
24	8.42E-04	0.00E+00	0.00E+00	2.81E-04	4.86E-04	173
48	0.00E+00	4.30E-03	4.64E-04	1.59E-03	2.36E-03	149
72	4.98E-04	0.00E+00	0.00E+00	1.66E-04	2.87E-04	173
96	0.00E+00	0.00E+00	0.00E+00	0.00E+00	0.00E+00	-
Administered Dose: 0.25 mg/kg						
	V	W	X			
-20	0.00E+00	1.32E-03	0.00E+00	4.42E-04	7.65E-04	173
0	0.00E+00	1.83E-03	0.00E+00	6.08E-04	1.05E-03	173
6	0.00E+00	0.00E+00	0.00E+00	0.00E+00	0.00E+00	-
12	0.00E+00	0.00E+00	0.00E+00	0.00E+00	0.00E+00	-
24	1.56E-03	5.48E-03	3.65E-03	3.56E-03	1.96E-03	55
48	3.32E-03	0.00E+00	2.58E-03	1.97E-03	1.74E-03	89
72	0.00E+00	0.00E+00	0.00E+00	0.00E+00	0.00E+00	-
96	0.00E+00	0.00E+00	0.00E+00	0.00E+00	0.00E+00	-
Administered Dose: 1.25 mg/kg						
	P	Q	R			
-20	0.00E+00	1.71E-03	0.00E+00	5.70E-04	9.88E-04	173
0	2.43E-03	2.74E-03	6.08E-03	3.75E-03	2.03E-03	54
6	7.61E-04	3.42E-03	0.00E+00	1.39E-03	1.80E-03	129
12	2.43E-02	0.00E+00	0.00E+00	8.11E-03	1.41E-02	173
24	2.23E-02	1.17E-02	1.83E-02	1.74E-02	5.34E-03	31
48	1.14E-03	3.89E-03	1.56E-03	2.20E-03	1.48E-03	67
72	1.96E-03	7.47E-04	2.09E-03	1.60E-03	7.40E-04	46
96	4.30E-03	0.00E+00	1.33E-03	1.87E-03	2.20E-03	117

a. Capital letters denote individual rats (see Table 2.1).
b. Mean ± standard deviation (minimum n = 3).
c. Inter-animal coefficient of variation (% CV).

Table 4.20. MT Concentration in Lympbocytes, Subchronic Experiment G091.

Time (h)	MT Concentration (uMol MT/L) [a]			Mean ± SD [b]		CV (%) [c]
Administered Dose: 0.00 mg/kg				(minimum n=2)		
	G	H	I			
-20	0.00E+00	0.00E+00	0.00E+00	0.00E+00	0.00E+00	-
0	3.83E-03	5.08E-03	1.10E-03	3.34E-03	2.04E-03	61
6	1.10E-03	9.13E-04	3.18E-03	1.73E-03	1.26E-03	73
12	0.00E+00	0.00E+00	1.37E-03	4.56E-04	7.90E-04	173
24	0.00E+00	1.99E-03	2.74E-03	1.58E-03	1.42E-03	90
48	2.81E-03	9.52E-04	0.00E+00	1.25E-03	1.43E-03	114
72	1.83E-03	3.33E-03	-	2.58E-03	1.07E-03	41
96	1.24E-03	0.00E+00	-	6.22E-04	8.80E-04	141
Administered Dose: 0.25 mg/kg						
	J	K	L			
-20	2.74E-03	7.91E-03	0.00E+00	3.55E-03	4.02E-03	113
0	1.22E-03	0.00E+00	6.39E-03	2.54E-03	3.39E-03	134
6	6.57E-03	4.56E-03	0.00E+00	3.71E-03	3.37E-03	91
12	2.74E-03	-	7.30E-03	5.02E-03	3.23E-03	64
24	1.64E-03	-	7.82E-03	4.73E-03	4.37E-03	92
48	1.29E-03	-	0.00E+00	6.44E-04	9.11E-04	141
72	0.00E+00	-	-	-	-	-
96	1.64E-03	-	-	-	-	-
Administered Dose: 1.25 mg/kg						
	M	N	O			
-20	1.15E-03	0.00E+00	4.69E-03	1.95E-03	2.45E-03	126
0	0.00E+00	0.00E+00	1.04E-03	3.48E-04	6.02E-04	173
6	3.76E-03	0.00E+00	0.00E+00	1.25E-03	2.17E-03	173
12	5.90E-03	5.13E-03	6.92E-03	5.98E-03	8.95E-04	15
24	8.08E-03	7.00E-03	5.48E-03	6.85E-03	1.31E-03	19
48	0.00E+00	2.54E-03	1.96E-03	1.50E-03	1.33E-03	89
72	7.82E-04	0.00E+00	3.55E-03	1.44E-03	1.87E-03	129
96	0.00E+00	-	1.62E-03	8.11E-04	1.15E-03	141

a. Capital letters denote individual rats (see Table 2.2).
b. Mean ± standard deviation (minimum n = 2).
c. Inter-animal coefficient of variation (% CV).

Table 4.21. MT Concentration in Lymphocytes, Short Course AF90 and Subchronic Experiments CD90.

Time (h)	MT Concentration (uMol MT/L) [a]			Mean ± SD [b]		CV (%) [c]
Short Course:						
Administered Dose: 0.00 mg/kg				minimum (n=2)		
	B	E				
-20	0.00E+00	0.00E+00		0.00E+00	0.00E+00	-
0	2.60E-04	1.41E-03		8.34E-04	8.12E-04	97
6	1.17E-03	0.00E+00		5.87E-04	8.31E-04	141
12	3.27E-03	4.02E-03		3.64E-03	5.32E-04	15
24	1.36E-03	0.00E+00		6.82E-04	9.65E-04	141
34	-	1.20E-03		-	-	-
96	1.06E-02	-		-	-	-
Administered Dose: 2.50 mg/kg						
	A	F	D			
-20	2.08E-03	4.73E-04	1.01E-04	8.84E-04	1.05E-03	119
0	2.30E-03	5.17E-03	2.45E-03	3.31E-03	1.62E-03	49
6	7.54E-04	0.00E+00	-	3.77E-04	5.33E-04	141
12	6.72E-04	4.10E-02	-	2.08E-02	2.85E-02	137
24	8.67E-04	2.02E-03	1.77E-03	1.55E-03	6.08E-04	39
34	-	6.34E-03	-	-	-	-
96	2.11E-03	-	-	-	-	-
Subchronic:						
Administered Dose: 2.50 mg/kg						
	C					
-20	4.41E-03			-	-	-
0	0.00E+00			-	-	-
12	8.07E-04			-	-	-

a. Capital letters denote individual rats (see Tables 2.1 and 2.2).
b. Mean ± standard deviation (minimum n = 2).
c. Inter-animal coefficient of variation (% CV).

Table 4.22. MT Concentration in Monocytes, Short Course Experiment PA91.

Time (h)	MT Concentration (uMol MT/L) [a]			Mean ± SD [b]		CV (%) [c]
Administered Dose: 0.00 mg/kg				minimum (n=3)		
	T	U	AA			
-20	6.22E-03	5.03E-07	1.23E-04	2.11E-03	3.56E-03	168
0	2.43E-03	5.76E-03	0.00E+00	2.73E-03	2.89E-03	106
6	0.00E+00	7.47E-04	5.69E-03	2.14E-03	3.09E-03	144
12	0.00E+00	7.91E-03	2.15E-03	3.35E-03	4.09E-03	122
24	1.83E-02	7.94E-03	8.53E-03	1.16E-02	5.84E-03	50
48	2.63E-03	1.25E-02	0.00E+00	5.06E-03	6.61E-03	131
72	9.88E-03	7.31E-03	0.00E+00	5.73E-03	5.13E-03	89
96	0.00E+00	0.00E+00	0.00E+00	-	-	-
Administered Dose: 0.25 mg/kg						
	V	W	X			
-20	5.53E-03	0.00E+00	4.24E-03	3.26E-03	2.89E-03	89
0	0.00E+00	4.85E-04	7.21E-03	2.57E-03	4.03E-03	157
6	1.04E-02	4.27E-03	6.92E-03	7.21E-03	3.10E-03	43
12	0.00E+00	3.43E-02	4.97E-03	1.31E-02	1.85E-02	142
24	1.61E-02	2.53E-02	1.38E-02	1.84E-02	6.04E-03	33
48	0.00E+00	1.51E-02	0.00E+00	5.04E-03	8.74E-03	173
72	2.54E-03	1.72E-02	6.62E-03	8.78E-03	7.56E-03	86
96	6.43E-04	0.00E+00	5.81E-03	2.15E-03	3.18E-03	148
Administered Dose: 1.25 mg/kg						
	P	Q	R			
-20	7.21E-03	2.80E-03	5.72E-03	5.24E-03	2.24E-03	43
0	1.12E-03	3.91E-03	1.53E-03	2.19E-03	1.51E-03	69
6	1.50E-02	9.30E-03	8.66E-03	1.10E-02	3.51E-03	32
12	2.06E-03	0.00E+00	0.00E+00	6.86E-04	1.19E-03	173
24	3.02E-02	1.51E-02	2.55E-02	2.36E-02	7.75E-03	33
48	1.61E-03	3.09E-04	0.00E+00	6.39E-04	8.54E-04	134
72	1.18E-02	4.12E-03	8.44E-03	8.12E-03	3.86E-03	47
96	1.14E-03	9.42E-05	0.00E+00	4.12E-04	6.34E-04	154

a. Capital letters denote individual rats (see Table 2.1).
b. Mean ± standard deviation (minimum n = 3).
c. Inter-animal coefficient of variation (% CV).

Table 4.23. MT Concentration in Monocytes, Subchronic Experiment G091.

Time (h)	MT Concentration (uMol MT/L) [a]			Mean ± SD [b]		CV (%) [c]
Administered Dose: 0.00 mg/kg				(minimum n=2)		
	G	H	I			
-20	7.16E-03	4.02E-03	7.88E-03	6.35E-03	2.05E-03	32
0	3.30E-03	0.00E+00	0.00E+00	1.10E-03	1.91E-03	173
6	9.41E-03	2.84E-03	1.47E-03	4.58E-03	4.25E-03	93
12	0.00E+00	0.00E+00	0.00E+00	0.00E+00	0.00E+00	-
24	5.89E-03	0.00E+00	0.00E+00	1.96E-03	3.40E-03	173
48	0.00E+00	0.00E+00	6.19E-03	2.06E-03	3.57E-03	173
72	1.28E-02	0.00E+00	-	6.38E-03	9.02E-03	141
96	4.13E-04	0.00E+00	-	2.07E-04	2.92E-04	141
Administered Dose: 0.25 mg/kg						
	J	K	L			
-20	1.73E-02	1.22E-02	1.78E-04	9.90E-03	8.80E-03	89
0	5.33E-03	0.00E+00	3.84E-03	3.06E-03	2.75E-03	90
6	6.61E-03	0.00E+00	0.00E+00	2.20E-03	3.82E-03	173
12	0.00E+00	-	5.44E-03	2.72E-03	3.84E-03	141
24	4.93E-02	-	3.17E-02	4.05E-02	1.24E-02	31
48	0.00E+00	-	9.99E-03	5.00E-03	7.07E-03	141
72	0.00E+00	-	-	-	-	-
96	5.41E-03	-	-	-	-	-
Administered Dose: 1.25 mg/kg						
	M	N	O			
-20	9.36E-03	0.00E+00	0.00E+00	3.12E-03	5.41E-03	173
0	1.07E-02	1.03E-02	3.00E-03	8.01E-03	4.35E-03	54
6	1.20E-02	1.62E-03	0.00E+00	4.53E-03	6.49E-03	143
12	0.00E+00	0.00E+00	9.88E-03	3.29E-03	5.70E-03	173
24	8.65E-03	2.35E-02	4.75E-03	1.23E-02	9.91E-03	81
48	0.00E+00	2.20E-03	0.00E+00	7.34E-04	1.27E-03	173
72	7.69E-03	0.00E+00	1.15E-02	6.40E-03	5.87E-03	92
96	1.37E-03	-	2.04E-03	1.70E-03	4.72E-04	28

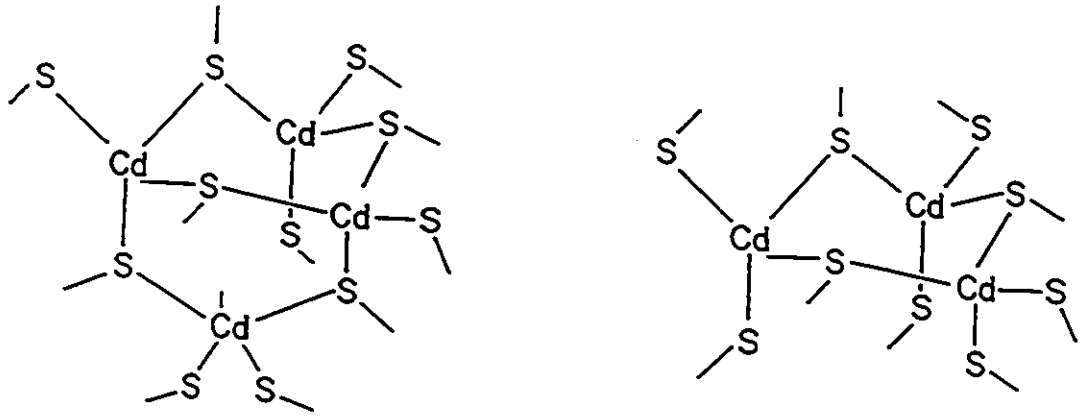
a. Capital letters denote individual rats (see Table 2.2).
b. Mean ± standard deviation (minimum n = 2).
c. Inter-animal coefficient of variation (% CV).

Table 4.24. MT Concentration in Monocytes, Short Course AF90 and Subchronic Experiments CD90.

Time (h)	MT Concentration (uMol MT/L) [a]			Mean ± SD [b]		CV (%) [c]
Short Course:						
Administered Dose: 0.00 mg/kg				minimum (n=2)		
	B	E				
-20	4.92E-02	3.43E-02		4.18E-02	1.06E-02	-
0	0.00E+00	3.71E-02		1.85E-02	2.62E-02	141
6	8.49E-02	3.23E-03		4.41E-02	5.78E-02	131
12	1.70E-02	4.35E-02		3.03E-02	1.88E-02	62
24	0.00E+00	1.78E-02		8.92E-03	1.26E-02	141
34	-	1.03E-01		-	-	-
96	0.00E+00	-		-	-	-
Administered Dose: 2.50 mg/kg						
	A	F	D			
-20	2.00E-02	0.00E+00	2.14E-02	1.38E-02	1.20E-02	87
0	0.00E+00	3.16E-02	0.00E+00	1.05E-02	1.82E-02	173
6	1.94E-02	1.12E-02	-	1.53E-02	5.80E-03	38
12	2.69E-02	0.00E+00	-	1.35E-02	1.91E-02	141
24	9.53E-04	1.41E-02	5.34E-02	2.28E-02	2.73E-02	120
34	-	0.00E+00	-	-	-	-
96	7.51E-04	-	-	-	-	-
Subchronic:						
Administered Dose: 2.50 mg/kg						
	C					
-20	1.49E-02			-	-	-
0	1.24E-02			-	-	-
12	2.66E-03			-	-	-

a. Capital letters denote individual rats (see Tables 2.1 and 2.2).
b. Mean ± standard deviation (n = 2).
c. Inter-animal coefficient of variation (% CV).

Figure 4.1. Proposed Structure of Clusters A and B of Mammalian Metallothionein



Cluster A: 4 metal atoms **Cluster B: 3 metal atoms**

Adapted from Hunt et al., 1984

The structure was based on ^{113}Cd-NMR data

Figure 4.2. Tissue Isolation and Purification Methods for CSA and MT Protein Preparations.

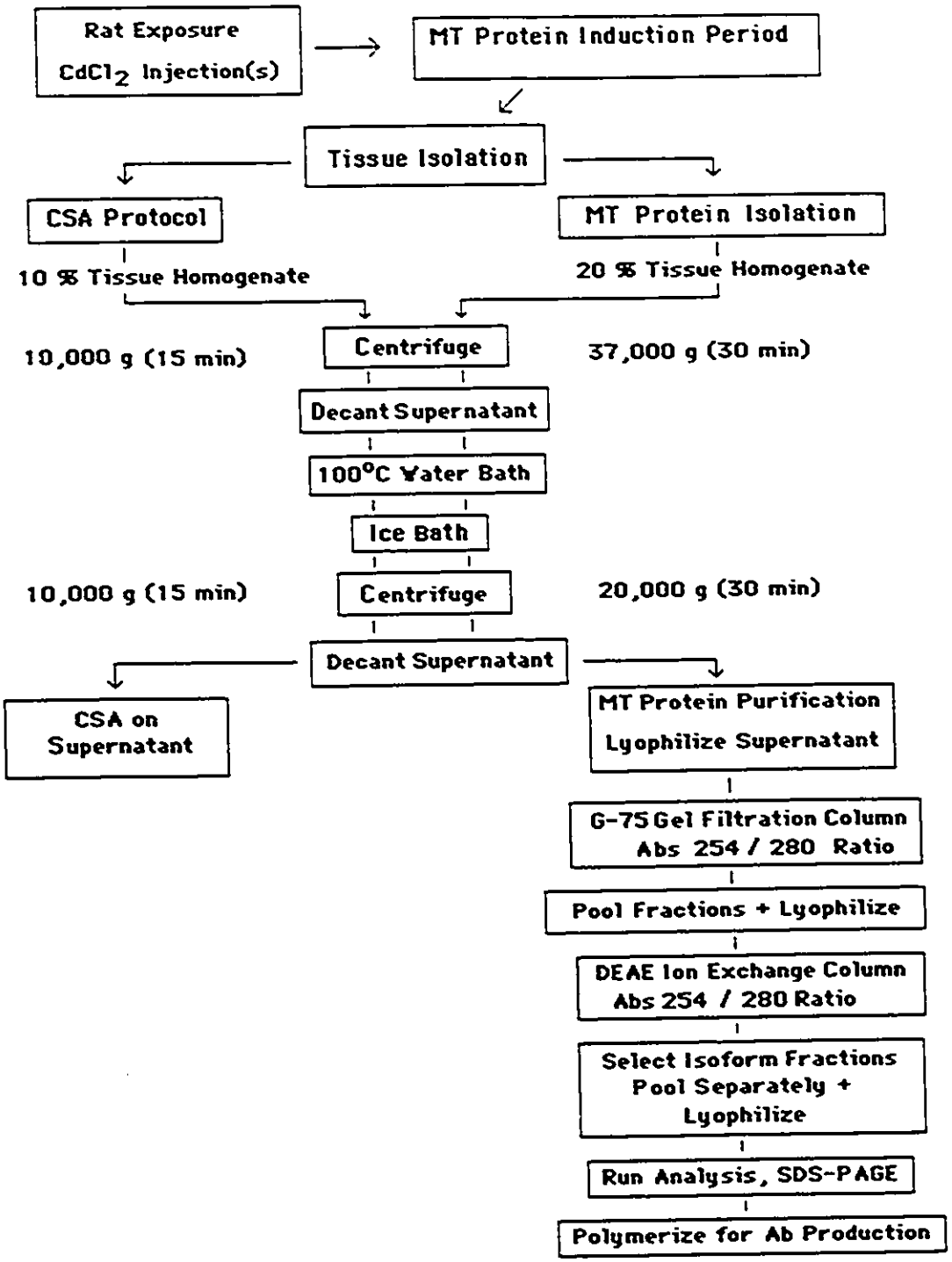


Figure 4.3. Micro - CSA Procedure Flowchart

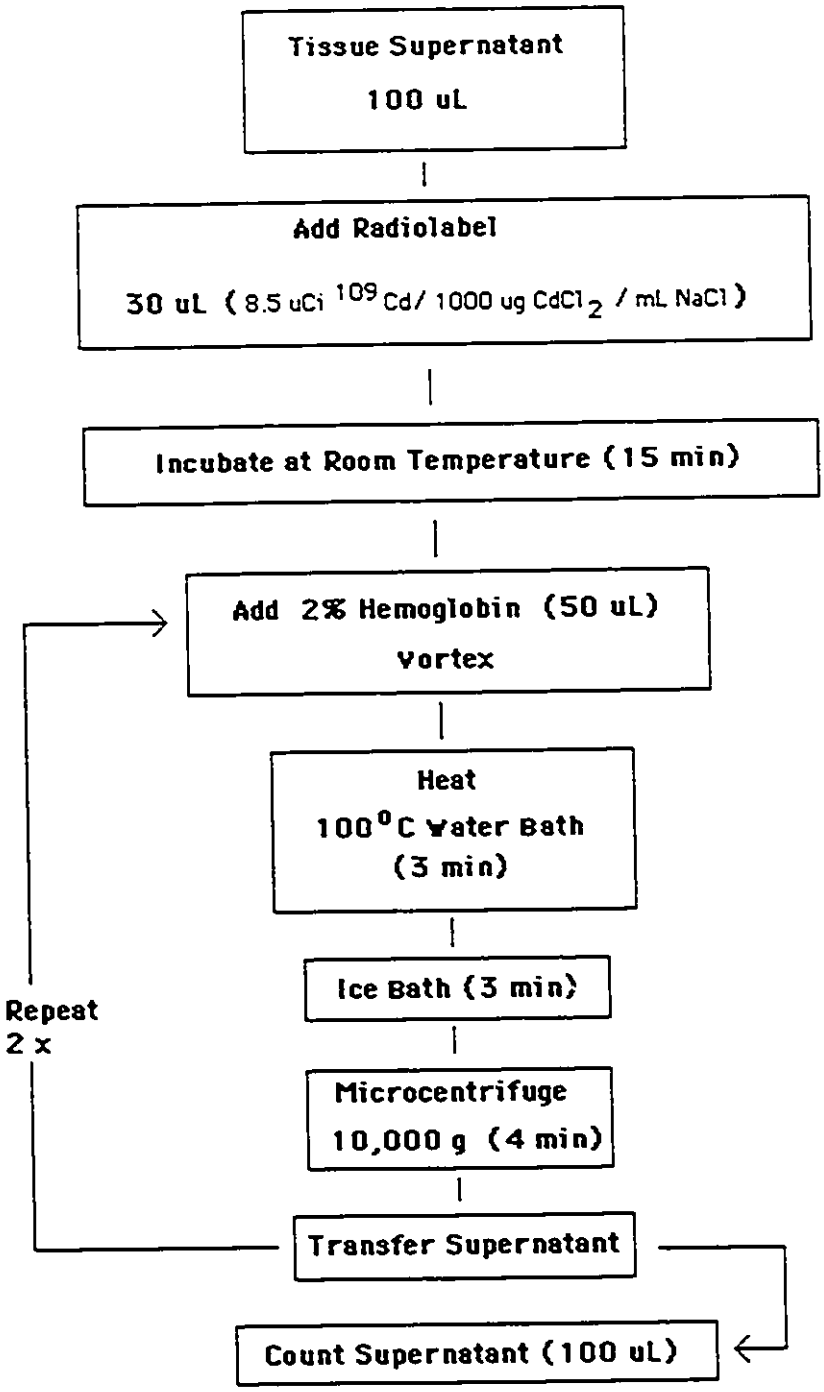
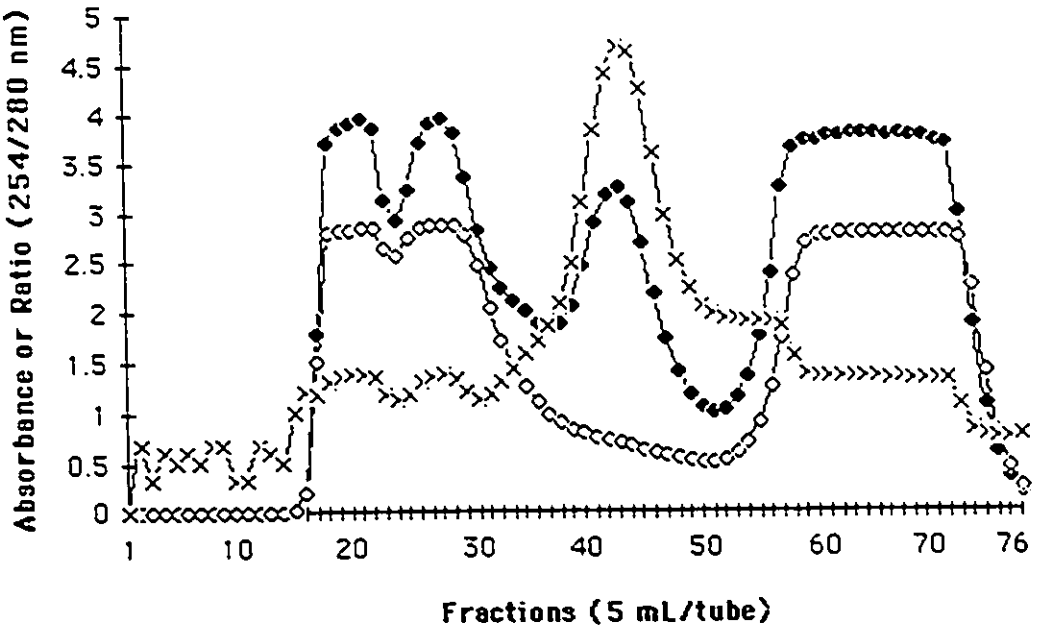


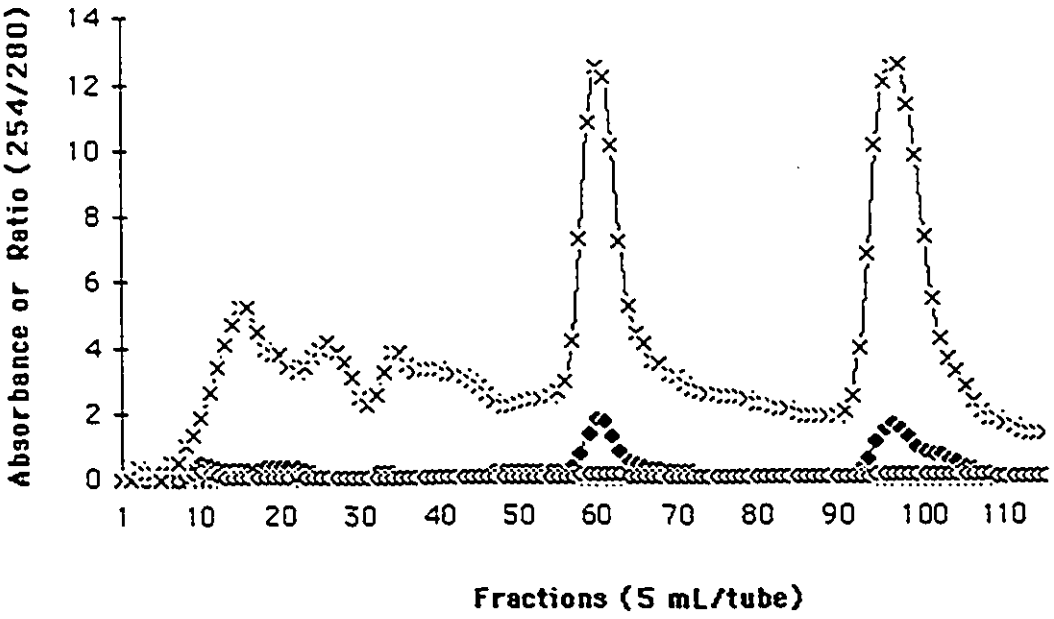
Figure 4.4. Separation of Hepatic Rat MT by Sephadex G-75 Gel Filtration Column Chromatography.



Legend to figure:

Livers from $CdCl_2$ induced rats were homogenized (20% w/v) with 0.1 M ammonium formate buffer, centrifuged, received alternate heat and ice treatments, recentrifuged, lyophilized and the rehydrated product was applied to a Sephadex G-75 gel filtration column (2.6 x 70 cm, flow rate 1 ml/min) and eluted with 0.01 M ammonium formate buffer. The fractions (5 mL/tube) were monitored at 254nm (◆), 280 nm (◇), and the absorbance ratio (254/280) were calculated (⋊). The fractions with the highest 254/280 absorbance ratio, (Fractions = 34 - 53) were pooled, lyophilized overnight and stored at -20°C. The resulting lyophilized material (crude hepatic rat MT) was further purified and separated into the two MT isoforms (Figure 4.5).

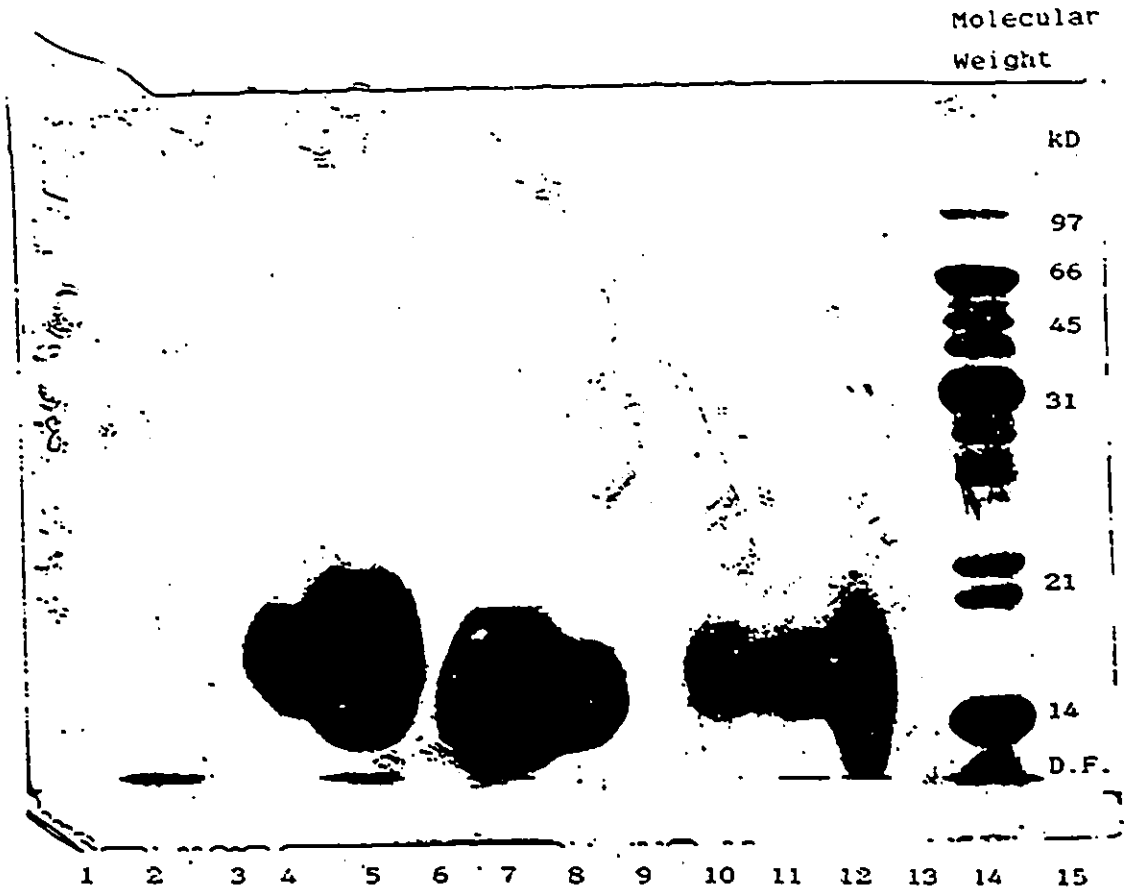
Figure 4.5. Rat MT Isoform Purification on DEAE A25 Ion - Exchange Column Chromatography.



Legend to figure:

The lyophilized G-75 Sephadex gel filtration product from crude hepatic rat MT homogenate was applied to the DEAE A25 ion-exchange column (2.6 x 30 cm) and eluted with a 0.005 - 0.5 M Tris - HCl gradient, pH 8.6. Fractions were monitored at 254 nm (◆), and 280 nm (◇), and the absorbance ratio 254 / 280 were calculated (-x-). The fractions with the highest ratio's for the two peaks were pooled, lyophilized and labelled as the following: F# 58 - 61 (MT 1a); F# 62 - 65 (MT 1b); F#95 - 98 (MT 2a); and F# 99 - 103 (MT 2b). The MT isoforms were dialysed against borate buffered saline, lyophilized and stored desiccated at -20°C.

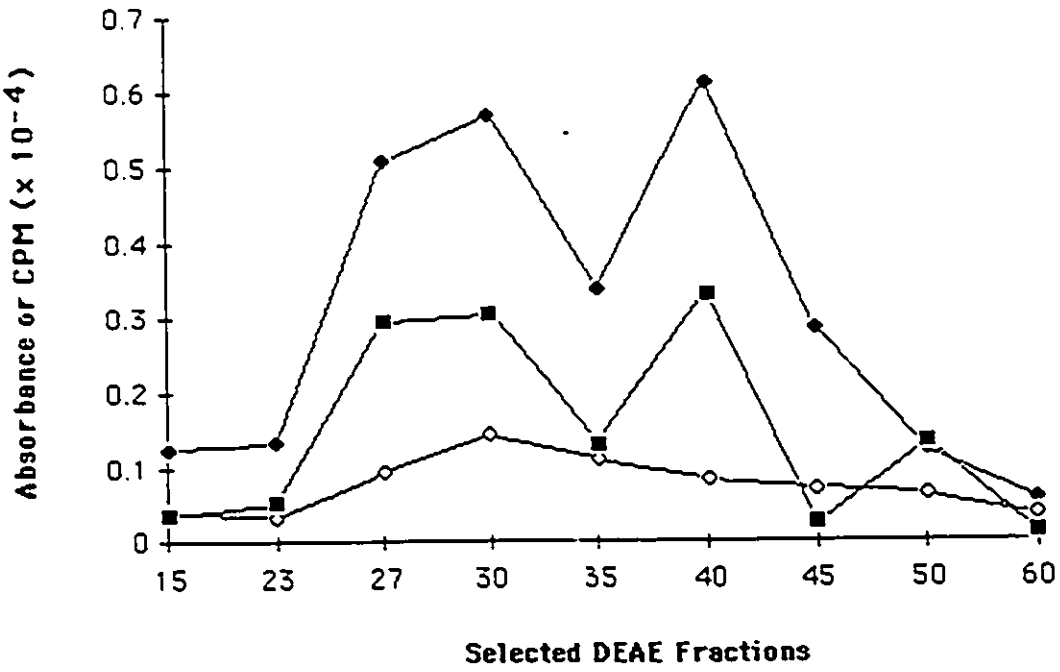
Figure 4.6. SDS-PAGE of Purified Rat MT1, MT2 Isoforms and Polymerized Rat MT1 & MT2.



Legend to figure:

Several purified Rat MT isoforms and polymerized Rat MT1 & MT2 mixture were separated by SDS-PAGE (4% stacking and 12% separating gels) slab gels using the method of Laemmli (1960) and the Bio-Rad procedure. In lane 2, LMW standards (Sigma MW SDS-17, range 2.5-17 kD); lane 4, MT1a; lane 5, MT1b; lane 7, MT2a; lane 8, MT2b, lanes 10-11, MT1a from an earlier isolation; lane 12, Polymerized Rat MT1 & MT2 mixture used for antibody production; and lane 14, HMW standards (Bio-Rad #161-0304, range 14.4-98 kD). The gels were stained with Bio-Rad Silver Stain (#161-0443). The bands centered at ~ 16 kD suggest polymerization.

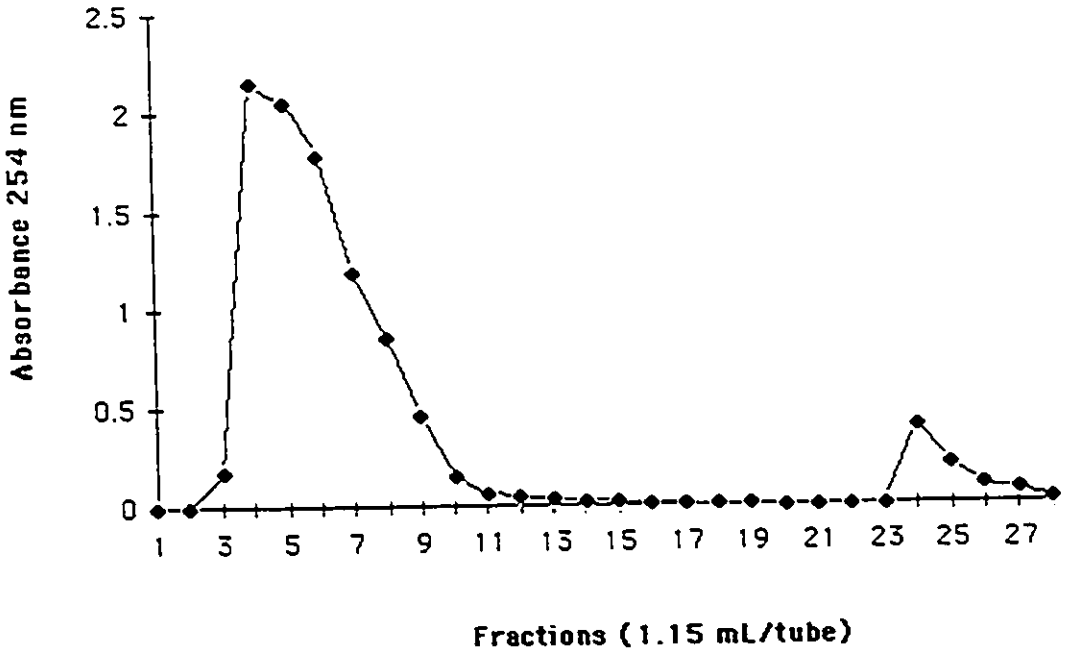
Figure 4.7. MT Quantification of the Rat MT Isoforms Separated by the DEAE Ion-Exchange Column.



Legend to figure:

Selected fractions from a DEAE A25 ion-exchange column separation of Rat MT into the two MT isoforms were tested by the CSA protocol for MT quantification. The early column fractions were not plotted in order to save space. The fractions were monitored at 254 nm ◆, 280 nm ◇, and by CSA reported as total cpm (x 10^{-4}) ■.

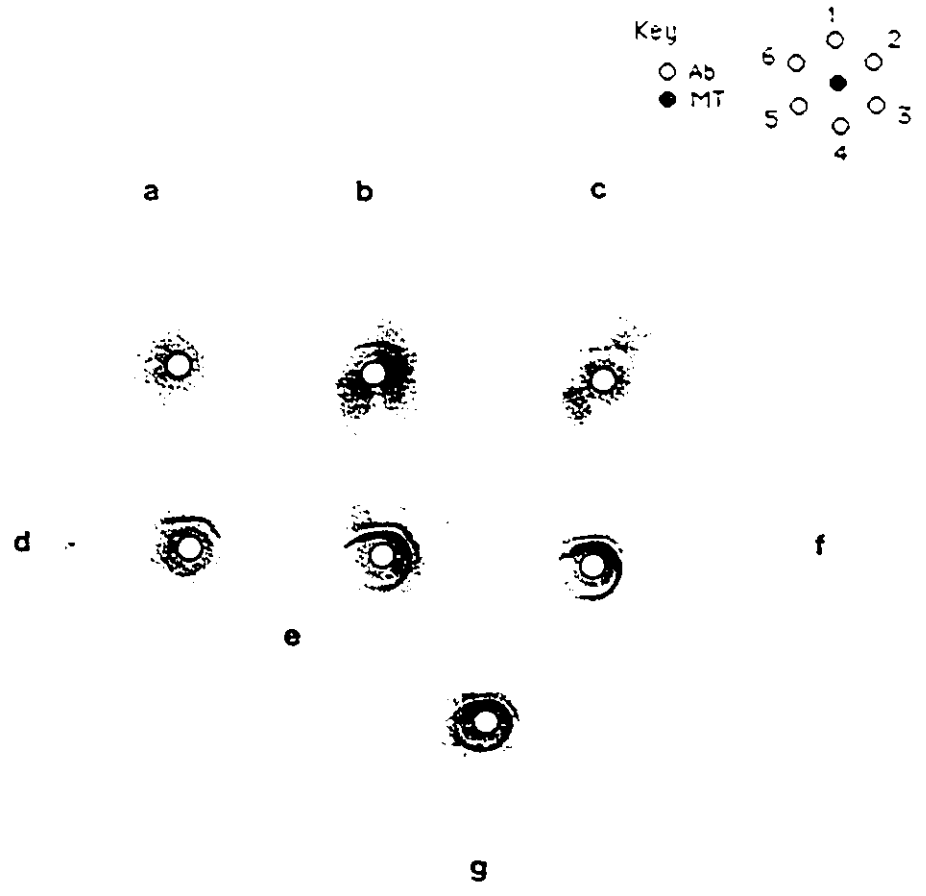
Figure 4.8. Rat MT1 and MT2 Polymerization Product Separated on Sephadex G-75 for Antibody Production.



Legend to figure:

A concentrated stock solution of purified Rat MT 1 and MT 2 isoforms (20 mg/ml) were mixed together and stored at -20 C. Aliquots of approximately 400 uL containing 8 mg MT 1 & 2 were diluted with borate buffered saline to 1 mL. The polymerization reaction began with the addition of 5 uL of 25% Gluteraldehyde. The glut-MT reaction continued for 2 hours at room temperature with stirring. The reaction was quenched with lysine (final concentration of 0.1M) and allowed to react for 45 minutes. The polymerized MT was separated from free lysine and glutaraldehyde over a Sephadex G-75 column (1.5 x 50 cm) and the fractions monitored for 254 nm absorbance. Fractions #4 - 9 were pooled, total volume 6.9 mL containing 1.16 mg MT/mL. The polymerized MT was stored at -20°C and aliquots were used for antibody production.

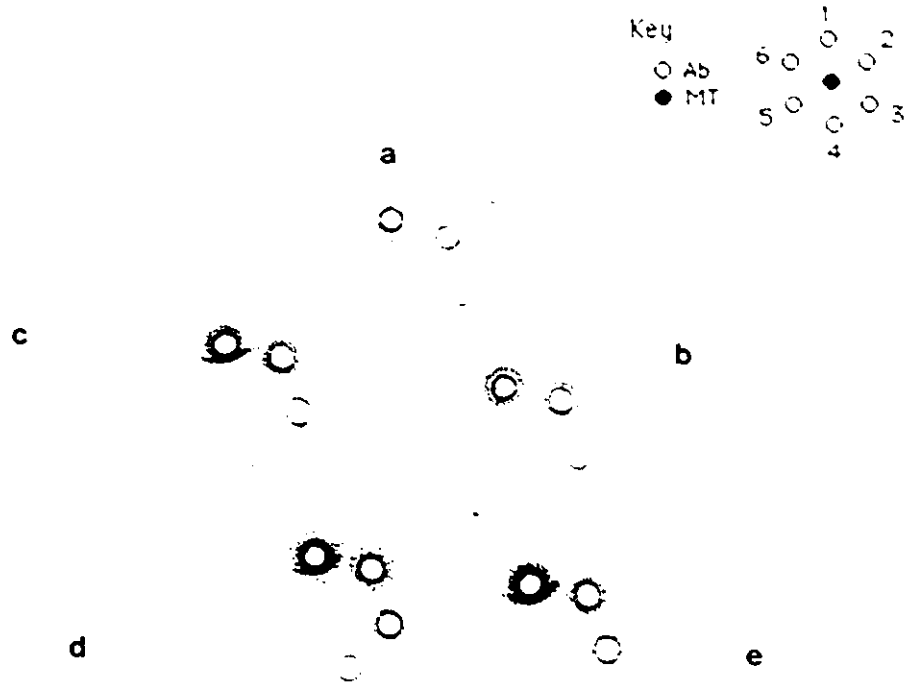
Figure 4.9. The Ouchterlony Immunodiffusion Profile for Rabbit Anti-Rat MT Antibody Production.



Legend to figure:

The sera from rabbit L-2 was routinely tested for antibody production using Ouchterlony immunodiffusion (OID) plates. The OID profile for rabbit L-2 shows the control response (a) in the upper left hand corner. The remaining six OID tests correspond to the antibody response from rat MT1 &2 injections: (b) primary Ab response, (10-13-89), (c) secondary (12-7-89), (d) third (1-9-90), (e) fourth (1-16-90), (f) fifth (4-9-90), and (g) sixth (6-14-90). In each OID test, the center well contained 5 uL of Rat MT (2 mg MT/ml). The six surrounding wells contained 10 uL of rabbit anti-rat MT sera diluted as: position #1 (undiluted serum), #2 (50% dilution), #3 (75%) #4 (87%), #5 (94%), and #6 (97%) dilution in PBS. The antibody production observed for rabbit L-2 was representative of the four rabbits immunized. See Section 4.2.2 and 4.3.2.

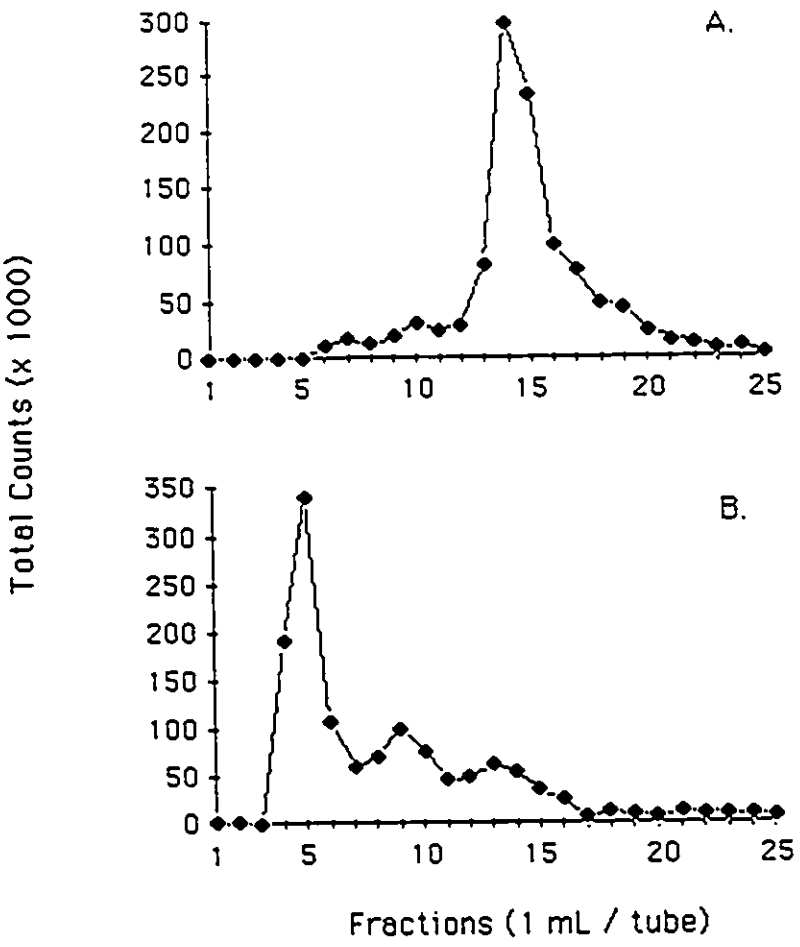
Figure 4.10. The Ouchterlony Immunodiffusion Profile for Chicken Anti-Rat MT Antibody Production.



Legend to figure:

The chicken yolk IgG preparations were routinely tested for antibody production using Ouchterlony immunodiffusion (OID) plates. The OID profile for chicken #994 shows the control response (a) in the upper left hand corner. The remaining four OID tests correspond to antibody response from rat MT 1&2 injections. (b) Ab preparation #8, (c) #15, (d) #17 and (e) #20. In each OID test, the center well contained 5 uL of Rat MT (2 mg MT/ml). The six outside wells contained 10 uL of chicken anti-rat MT diluted as: position #1 undiluted yolk antibody, #2 (50% dilution), #3 (75%), #4 (87%), #5 (94%) and #6 (97%) yolk antibody dilution in PBS. The antibody production observed for chicken #994 was representative of the three birds immunized. See Section 4.2.2 and 4.3.2.

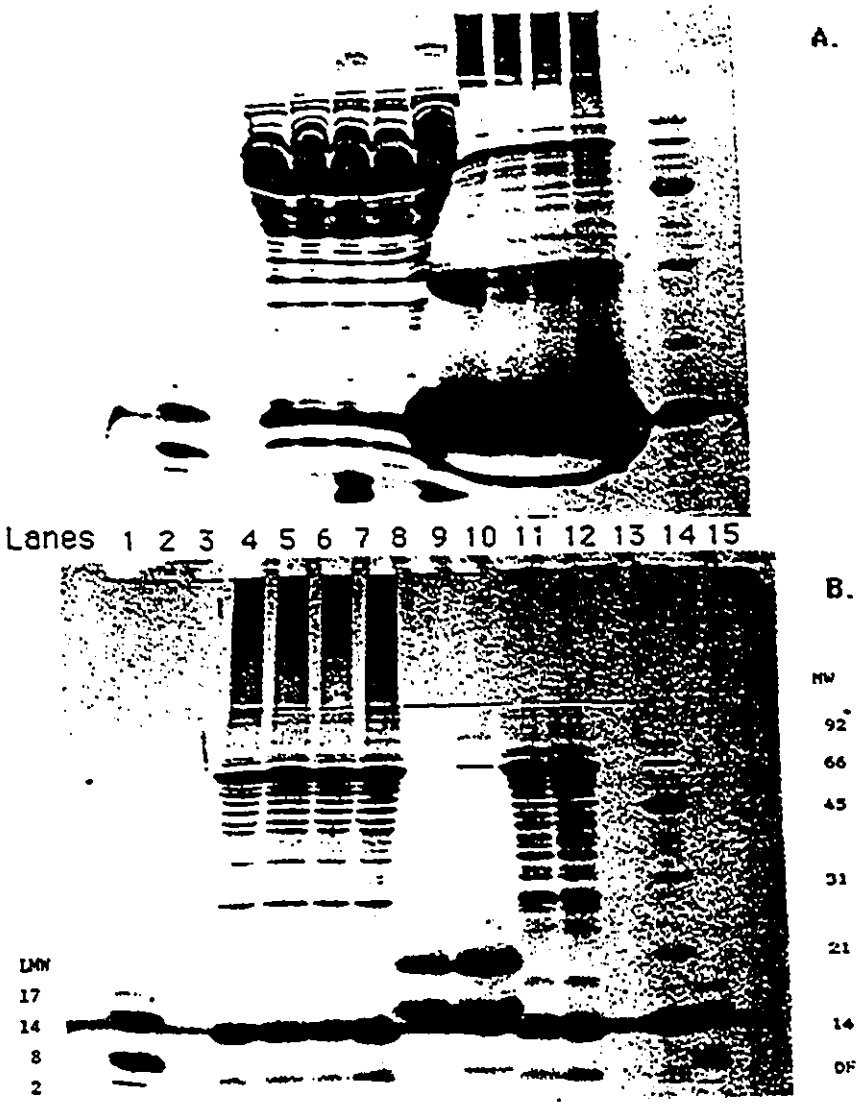
Figure 4.11. MT Iodination Products by G-50 and G-25 Sephadex Gel Filtration.



Legend to figure:

Figure A. Represents ^{125}I-Rat MT label iodination reaction of 10-12-90, eluted with TGB over a G-50 Sephadex column. Figure B. Represents ^{125}I-Rabbit MT label iodination reaction of 11-21-90, eluted with TGB over a G-25 Sephadex column. Antibody binding results are tabulated in Table 4.8.

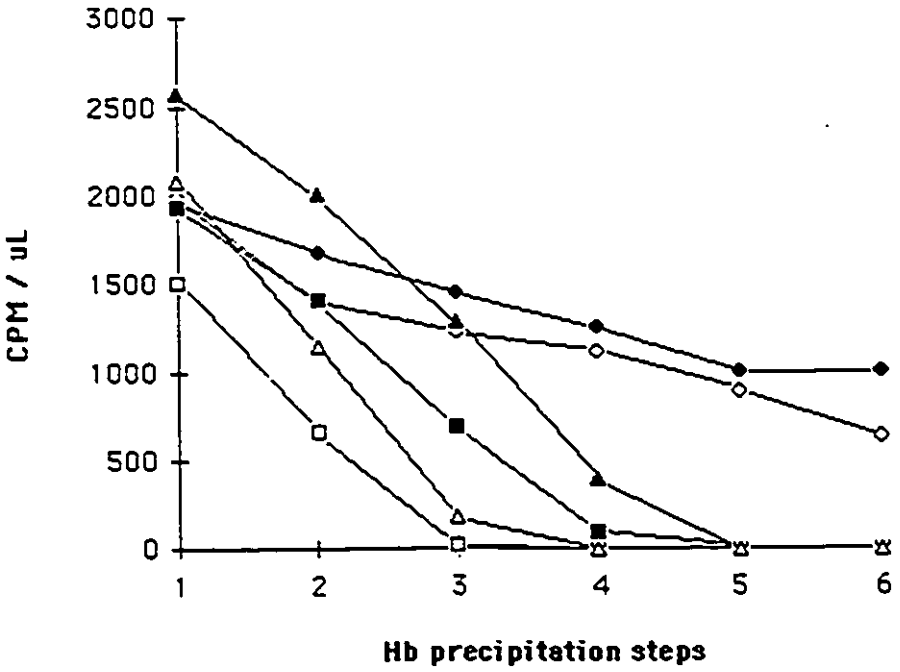
Figure 4.12. SDS-PAGE of Sample and Reagent Purity.



Legend to figure:

Several sample types and RPMIc Media were separated by SDS-PAGE (4% stacking and 12% separating gels) slab gels using Laemmli buffer system, Bio-Rad procedure. Gel A: Lane 2, LMW standards (Sigma MW SDS-17, range 2.5-17 kD); lanes 4-8, plasma from a $CdCl_2$ induced rat; lanes 9-12, RBC and PMN from a $CdCl_2$ induced rat; lane 14, HMW standards (Bio-Rad #161-0304, range 14.4-98 kD). Gel B: Lane 2, LMW standards; lanes 4-7, leukocyte isolation in RPMIc; lanes 8-9, urine; lanes 10-11, RPMIc cell media; lane 13, HMW; lane 14, LMW standards. The gels were stained with Bio-Rad Silver Stain (#161-0443). Other than the urine samples, all other samples contained an array of proteins.

Figure 4.13. Effect of Buffer and Hemoglobin Concentrations on the CSA.



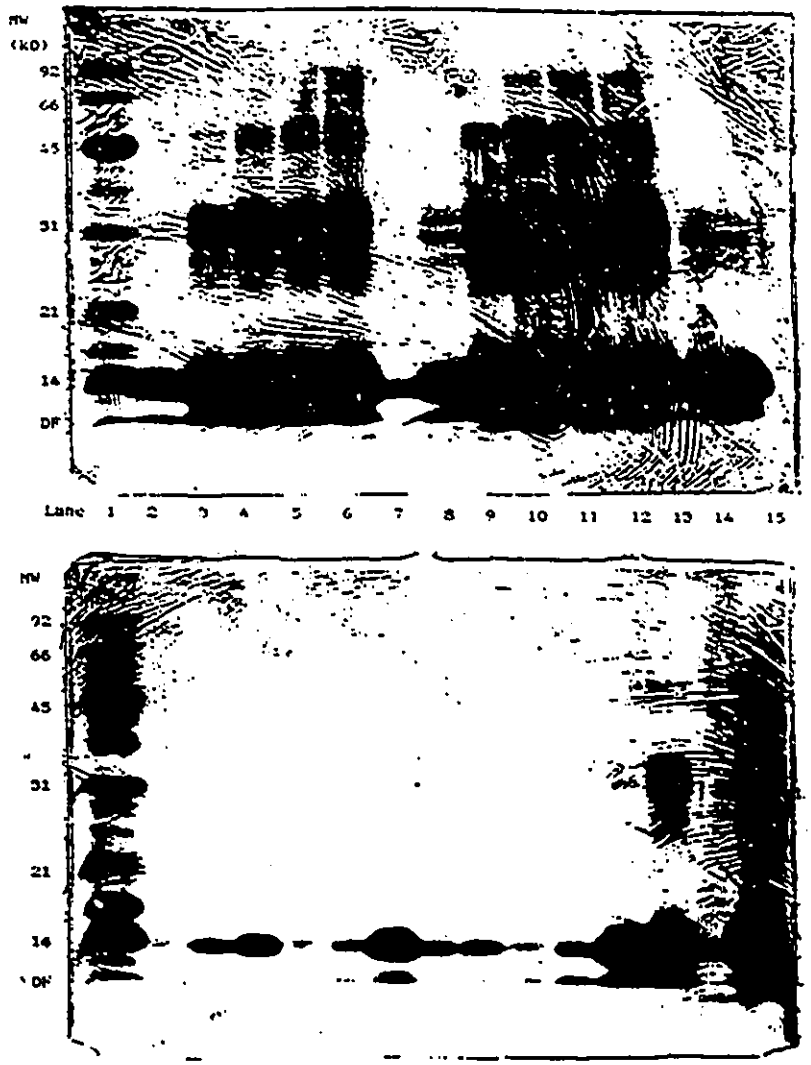
Legend to figure:

Several buffer concentrations (15, 30, and 100 mM Tris buffered saline) and hemoglobin concentrations (1 and 2 % Hb) were examined by the CSA.

·◆- 15(1)	·■- 30(1)	·▲- 100(1)
·◇- 15(2)	·□- 30(2)	·△- 100(2)

The assay involved six Hb precipitation steps and aliquots from each step were counted for the ^{109}Cd remaining in solution (cpm/uL sample).

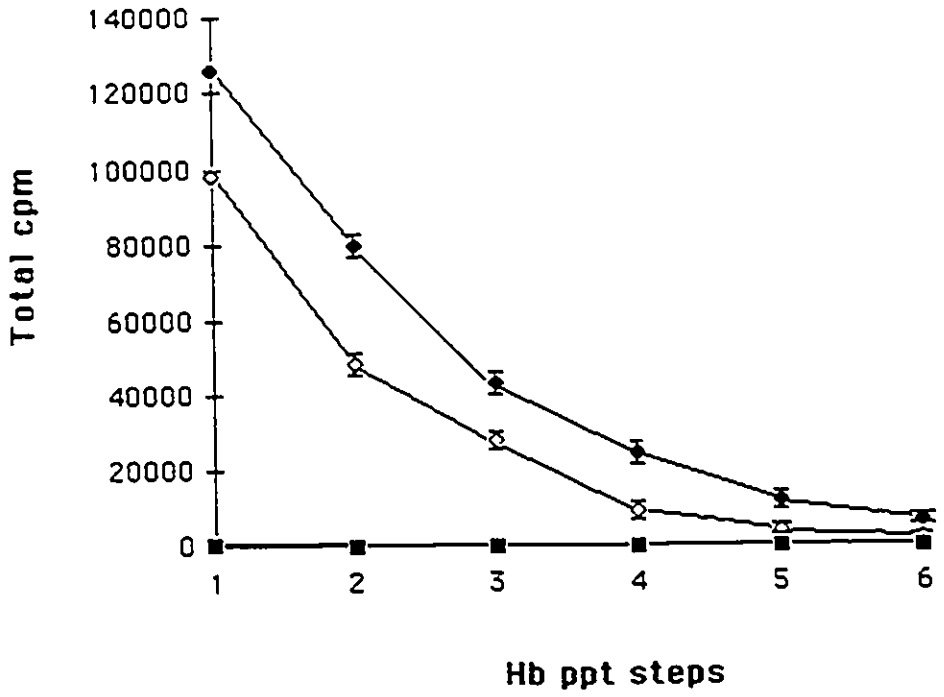
Figure 4.14. Effects of Buffer Concentration and Impurities on MT Determination by CSA.



Legend to figure:

The 15 and 30 mM TBS blank analyte (without MT) from the Hb precipitation steps 1 - 6 were separated by SDS-PAGE (4% stacking and 12% separating gels) slab gels using Laemmli buffer system, Bio-Rad procedure. Gel A : CSA analyte from 15 mM TBS blank; lane 1, HMW standards (Bio-Rad #161-0304); lanes 2-6, Hb ppt steps 2 - 6 using 1% Hb; lanes 7-12, Hb ppt steps 1 - 6 using 2% Hb; lane 14, 1% Hb; lane 13 & 15 were empty. Gel B: CSA analyte from 30 mM TBS blank; lane 1, HMW standards; lanes 2-7, Hb ppt steps 1-6 using 1% Hb; lanes 8-13, Hb ppt steps 1-6 using 2% Hb; lane 14, Hb ppt step 1 using 1% Hb and 15 mM TBS; and lane 15, 2% Hb solution. The gels were radioactive and stained with Bio-Rad Silver Stain (#161-0443). In gel A the amount of protein fragments increased with the number of Hb ppt steps. Increasing buffer concentration to 30 mM minimized protein fragmentation.

Figure 4.15. Optimization of CSA: Frequency of Hemoglobin Precipitation Steps (Liver Supernatant) Mean ± SD, n=3.



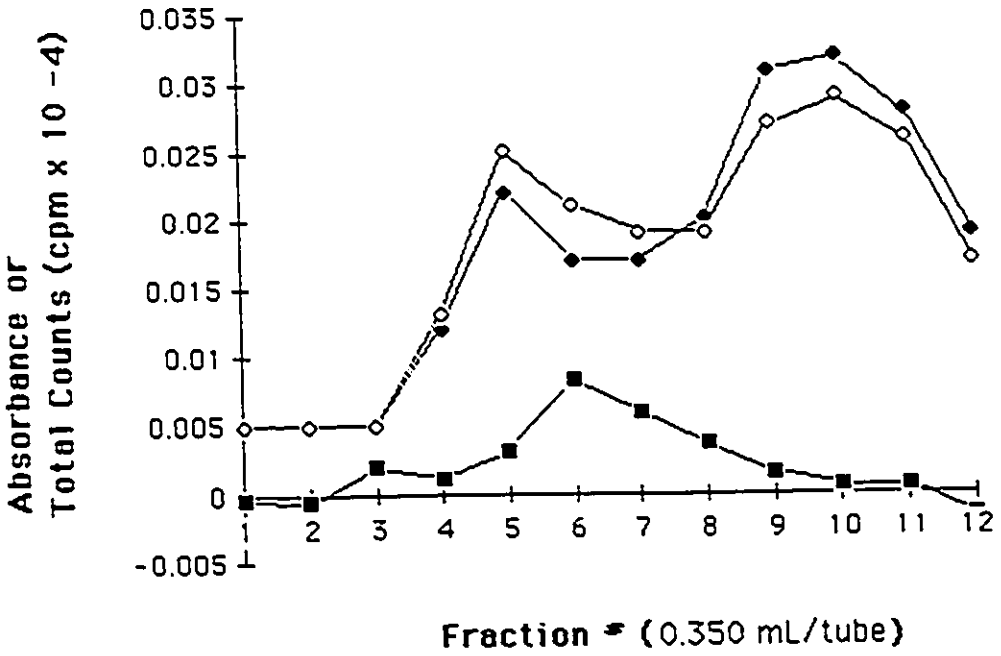
Legend to figure:

Experimental parameters: 2 % Hb ppt solution ; 100 ^{0}C water bath ; 90 ug cold $CdCl_2$ in test label; buffer concentration held constant at 50 mM TBS.

- ·◆· liver supernatant from Cd exposed rats.
- ·○· liver supernatant from saline exposed rats.
- ·■· Blank (50mM TBS).

Each group was significantly different ($p < 0.004$) from each other ; statistical analysis was by a two - tailed, paired t test with 2 degrees freedom.

Figure 4.16. Optimization of CSA: Gel Filtration of CSA Analyte (50 mM TBS Blank) Mean, n=3.

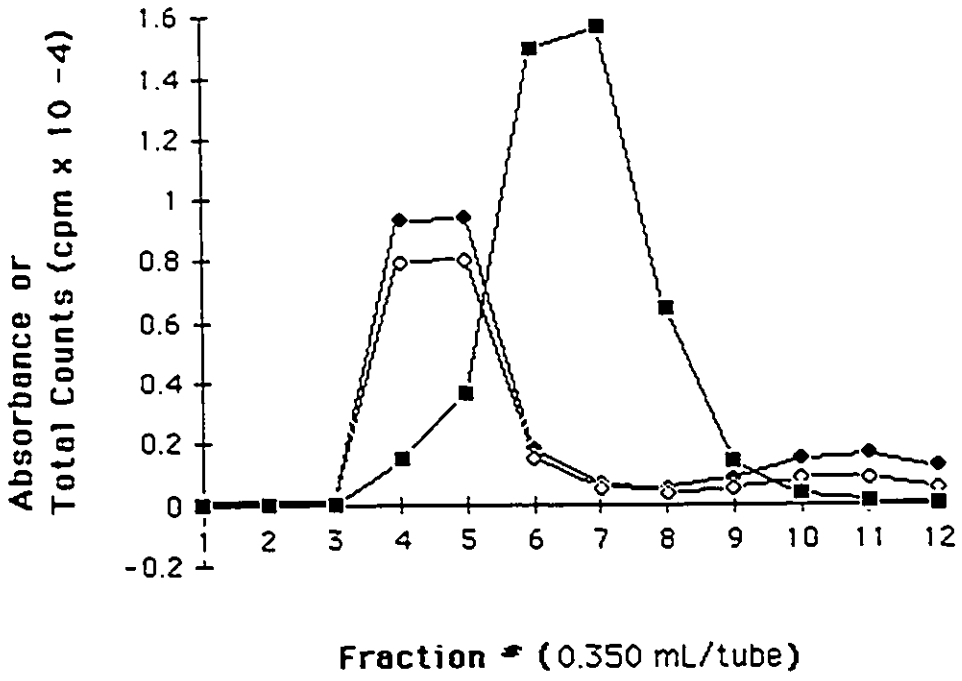


Legend to figure:

The 50 mM TBS blank analyte from the third Hb precipitation step was passed over a calibrated G-50 fine Sephadex mini-column (10 x 1 cm). The fractions were measured for UV absorbances at 254 and 280 nm and total ^{109}Cd counts (cpm x 10^{-4}). The following molecular mass standards were employed: dextran blue (Vo) and albumin (66 kD) eluted in fraction #4; Cytochrome C (12.4 kD) eluted in fraction #6 (see Table 4.9.).

-◆- 254 nm UV Abs; -◇- 280 nm UV Abs; -■- cpm (total counts x 10^{-4}).

Figure 4.17. Optimization of CSA: Gel Filtration of CSA Analyte (Liver Supernatant) Mean, n=3.

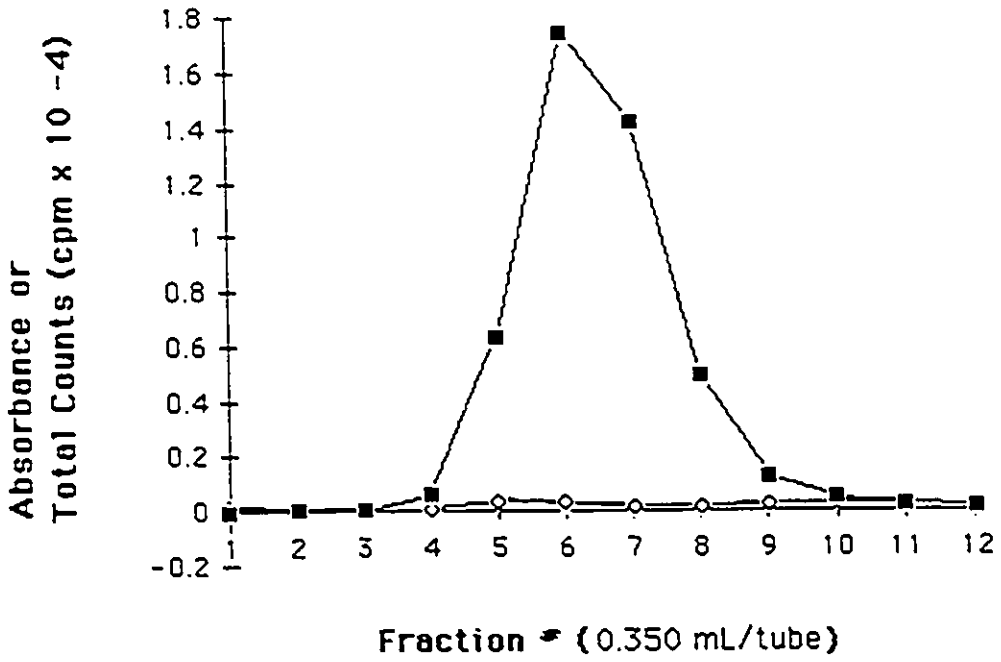


Legend to figure:

Liver analyte from the third Hb precipitation step was passed over a calibrated G-50 fine Sephadex mini-column (10 x 1 cm). The fractions were measured for UV absorbances at 254 and 280 nm and total ^{109}Cd counts (cpm x 10^{-4}). The following molecular mass standards were employed: dextran blue (Vo) and albumin (66 kD) eluted in fraction #4; Cytochrome C (12.4 kD) eluted in fraction #6 (see Table 4.9.).

-◆- 254 nm UV Abs; -◇- 280 nm UV Abs; -■- cpm (total counts x 10^{-4}).

Figure 4.18. Optimization of CSA: Gel Filtration of CSA Analyte (Purified Rat MT 1 and MT2). Mean, n=3.

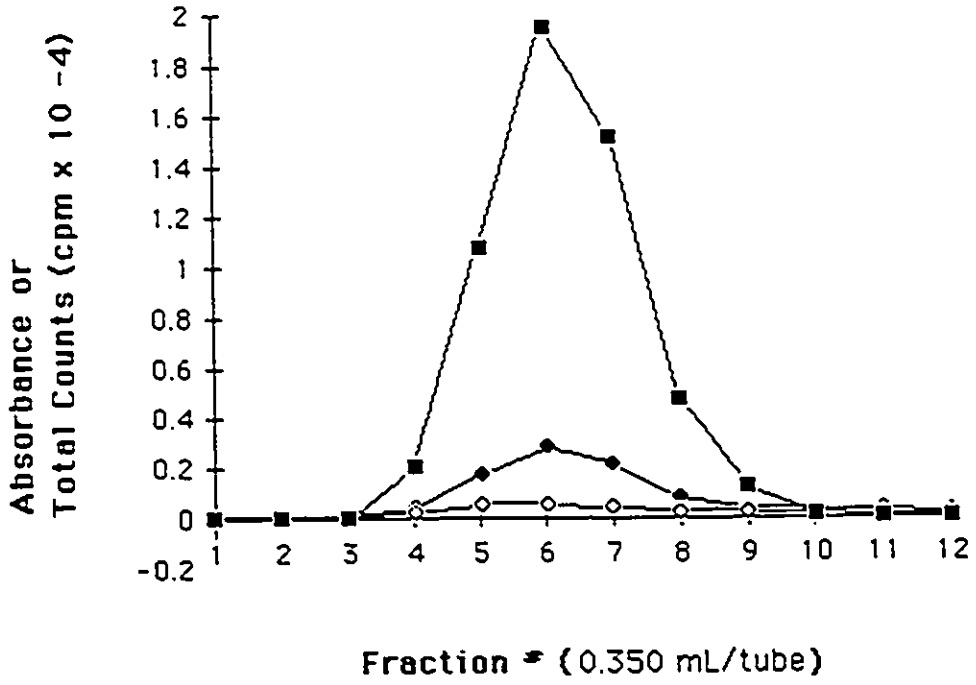


Legend to figure:

Rat MT1 and MT2 analyte from the third Hb precipitation step was passed over a calibrated G-50 fine Sephadex mini-column (10 x 1 cm). The fractions were measured for UV absorbances at 254 and 280 nm and total ^{109}Cd counts (cpm $\times 10^{-4}$). The following molecular mass standards were employed: dextran blue (Vo) and albumin (66 kD) eluted in fraction #4; Cytochrome C (12.4 kD) eluted in fraction #6 (see Table 4.9.).

-◆- 254 nm UV Abs; -◇- 280 nm UV Abs; -■- cpm (total counts x 10^{-4}).

Figure 4.19. Optimization of CSA: Gel Filtration of CSA Analyte (Sigma Rabbit MT1 and MT2) Mean, n=3.

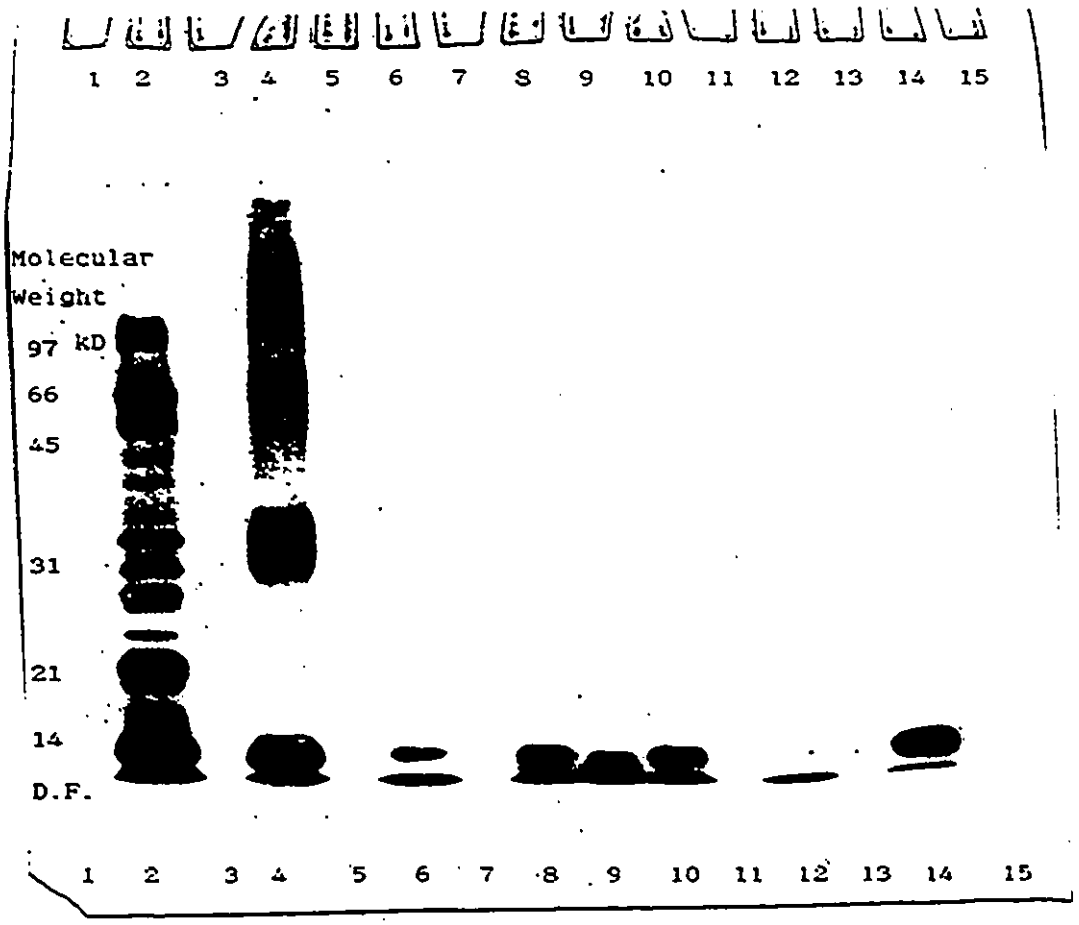


Legend to figure:

Rabbit MT1 and MT2 analyte from the third Hb precipitation step was passed over a calibrated G-50 fine Sephadex mini-column (10 x 1 cm). The fractions were measured for UV absorbances at 254 and 280 nm and total ^{109}Cd counts (cpm x 10^{-4}). The following molecular mass standards were employed: dextran blue (Vo) and albumin (66 kD) eluted in fraction #4; Cytochrome C (12.4 kD) eluted in fraction #6 (see Table 4.9.).

-◆- 254 nm UV Abs; -○- 280 nm UV Abs; -■- cpm (total counts x 10^{-4}).

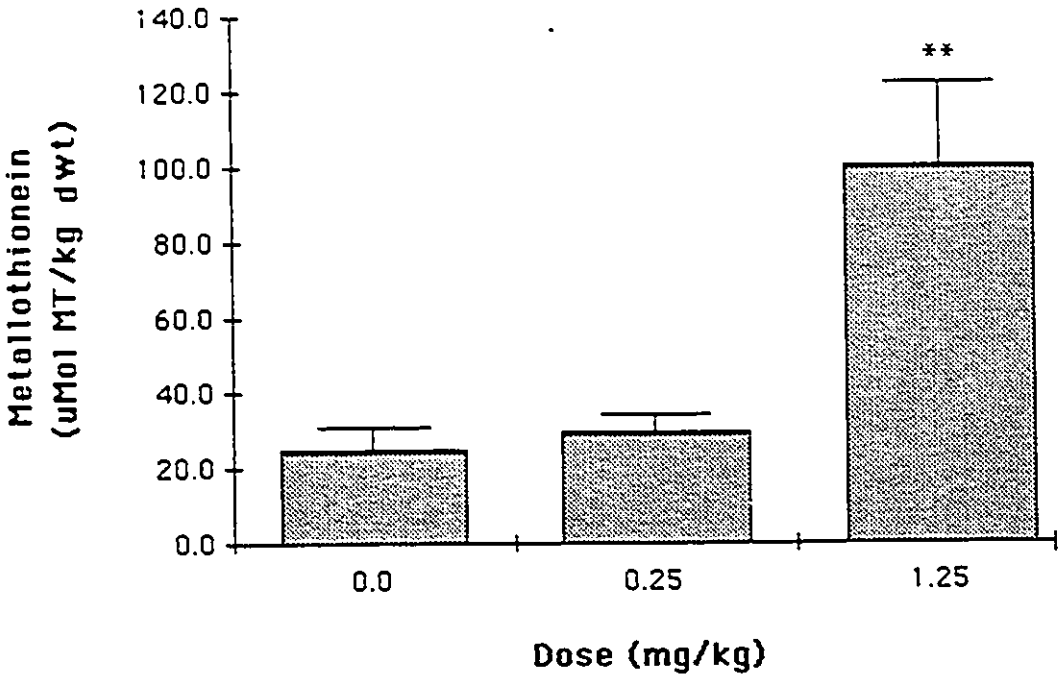
Figure 4.20. Optimization of CSA: SDS-PAGE of CSA Analyte Purity.



Legend to figure:

The CSA analyte from the third Hb precipitation step were separated by SDS-PAGE (4% stacking and 12% separating gels) slab gels using Laemmli buffer system, Bio-Rad procedure. Lanes 1,3,5,7,11,13, and 15 were empty. In lane 2, the HMW standards (Bio-Rad #161-0304, low range 14.4 - 97.4 kD); lane 4, liver supernatant from a $CdCl_2$ induced rat; lane 6, purified Rat MT1 & MT2 (LMJ Smith); lane 8, rabbit MT1 & MT2 (Sigma); lane 9, rabbit MT1 (Sigma); lane 10, rabbit MT2 (Sigma); lane 12, 50 mM Tris blank; and lane 14, purified rat MT1 & MT2 stock (not CSA analyte). Solutions resolved in lanes 4 - 12 were radioactive. The gel was stained using Coomassie brilliant blue R-250. All samples contained a clearly separated MT band (c. 10 - 14 kD). Only the unpurified liver supernatant (lane 4) showed the presence of HMW constituents.

Figure 4.21. Metallothionein Concentration in Liver, Short Course Experiment PA91.

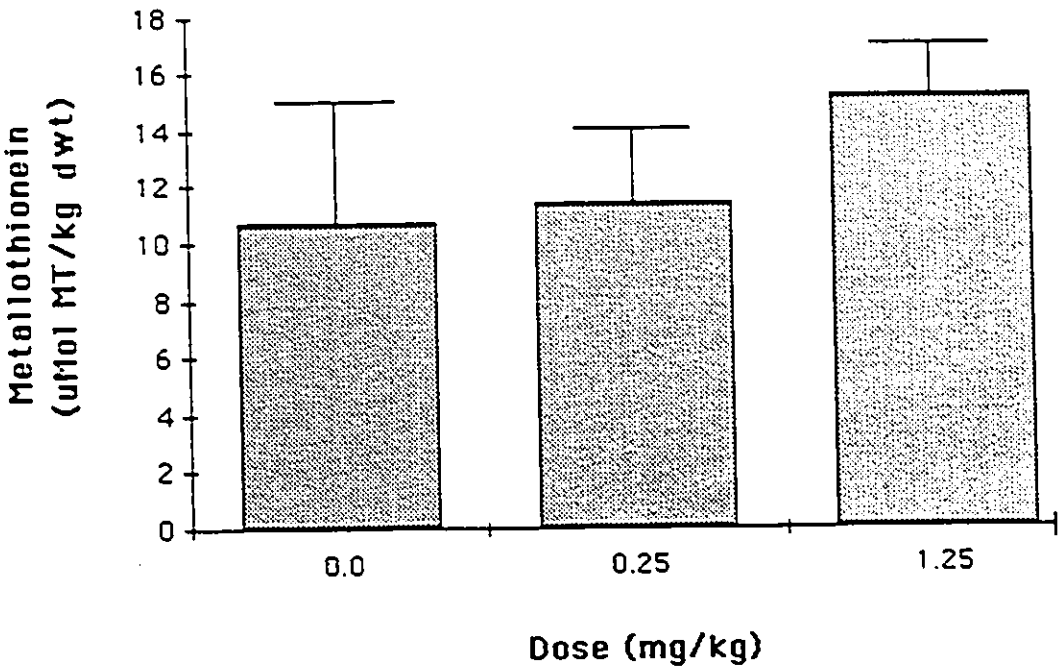


Legend to figure (administered dose):

0.0 mg/kg (T,U,AA); 0.25 mg/kg (V,W,X); 1.25 mg/kg (P,Q,R);

Each rat received a single IP injection at T = 0 h. The bar graph represents the mean ± standard deviation (n=3), ** $p<0.005$. See Table 4.10 for individual MT concentrations.

Figure 4.22. Metallothionein Concentration in Kidney, Short Course Experiment PA91.

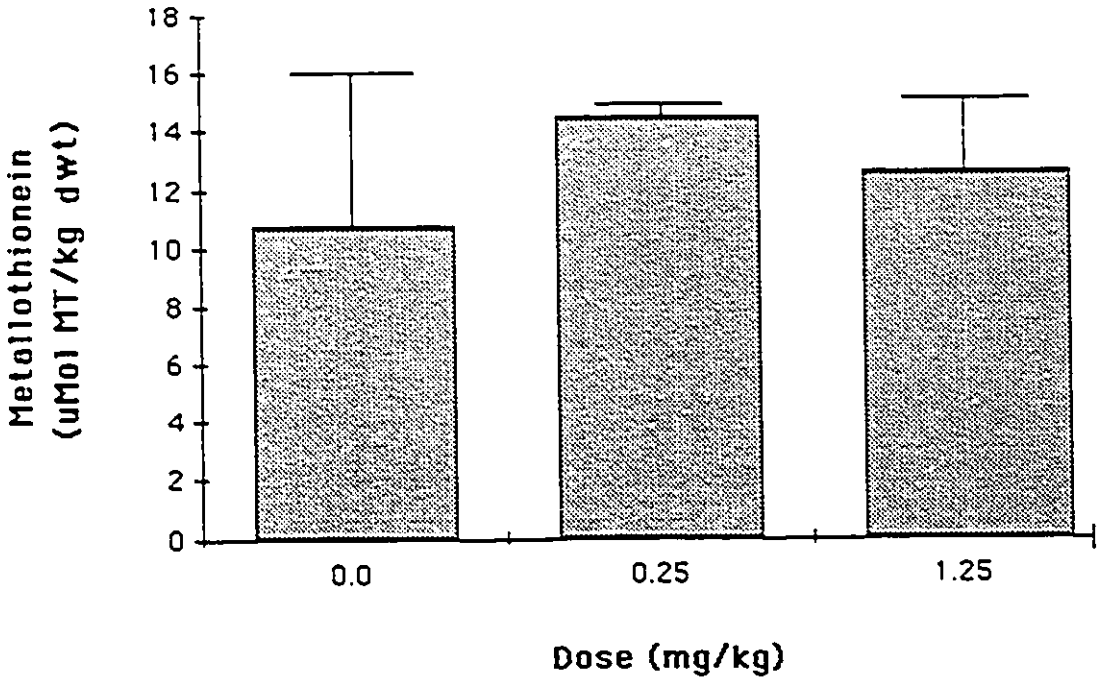


Legend to figure (administered dose):

0.0 mg/kg (T,U,AA); 0.25 mg/kg (V,W,X); 1.25 mg/kg (P,Q,R);

Each rat received a single IP injection at T = 0 h. The bar graph represents the mean ± standard deviation (n=3). See Table 4.10. for individual MT concentrations.

Figure 4.23. Metallothionein Concentration in Spleen, Short Course Experiment PA91.

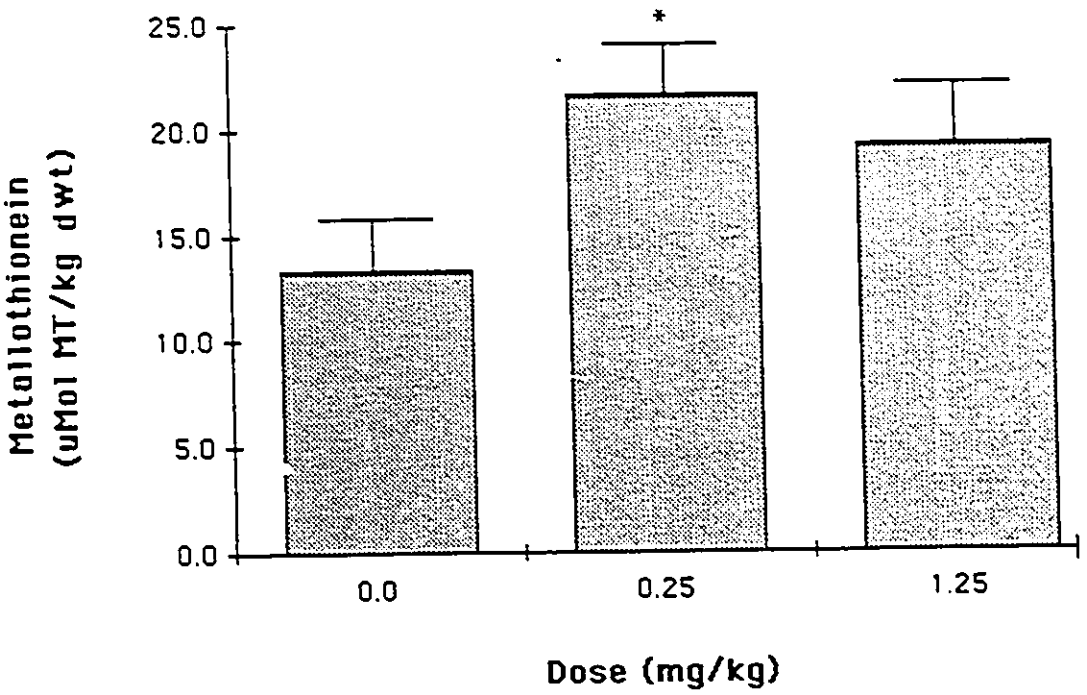


Legend to figure (administered dose):

0.0 mg/kg (T,U,AA); 0.25 mg/kg (V,W,X); 1.25 mg/kg (P,Q,R);

Each rat received a single IP injection at T = 0 h. The bar graph represents the mean ± standard deviation (n=3). See Table 4.10. for individual MT concentrations.

Figure 4.24. Metallothionein Concentration in Lung, Short Course Experiment PA91.

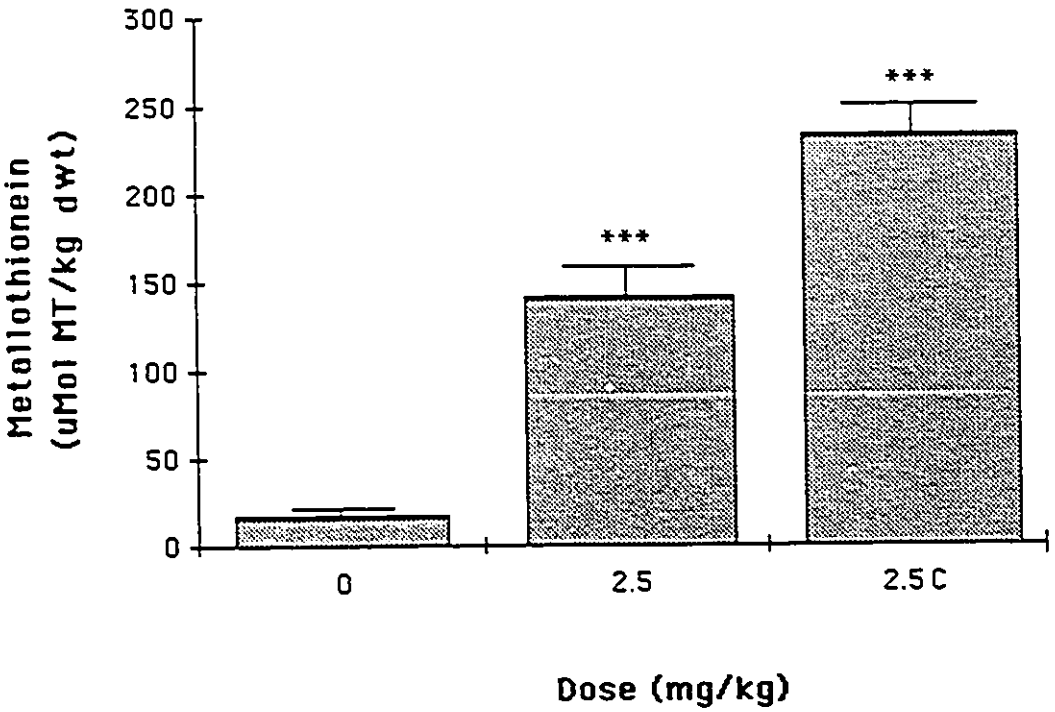


Legend to figure (administered dose):

0.0 mg/kg (T,U,AA); 0.25 mg/kg (V,W,X); 1.25 mg/kg (P,Q,R);

Each rat received a single IP injection at T = 0 h. The bar graph represents the mean ± standard deviation (n=3), * $p<0.05$. See Table 4.10. for individual MT concentrations.

Figure 4.25. Metallothionein Concentration in Liver, (Short Course AF90 and Subchronic CD90 Experiments).



Legend to figure (administered dose):

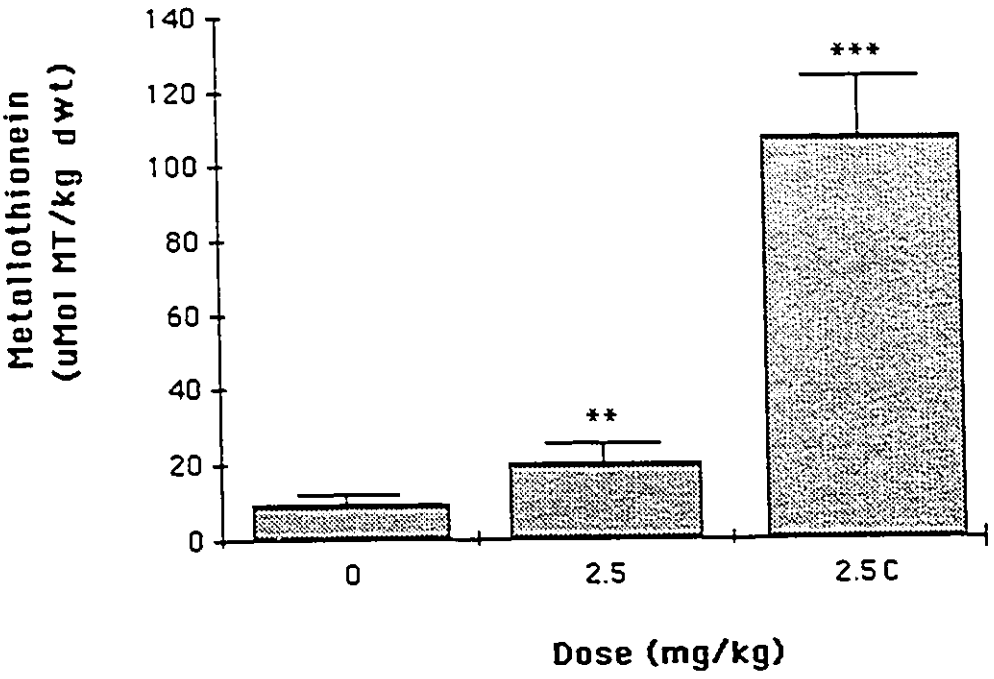
Short Course Dose: 0.0 mg/kg (B,E); 2.5 mg/kg (A,F,D). Each rat received a single IP injection at T = 0 h.

Subchronic Dose: 0.0 mg/kg (D); 2.5C mg/kg (C); Each rat received three IP injections in total of the indicated doses over 2 month period The third injection (2.5 mg/kg) was administered to both rats at T = 0 h,

The bar graph represents the mean ± standard deviation, n = 4.

*** $p<0.0005$. See Tables 4.10. and 4.11.

Figure 4.26. Metallothionein Concentration in Kidney, (Short Course AF90 and Subchronic CD90 Experiments).



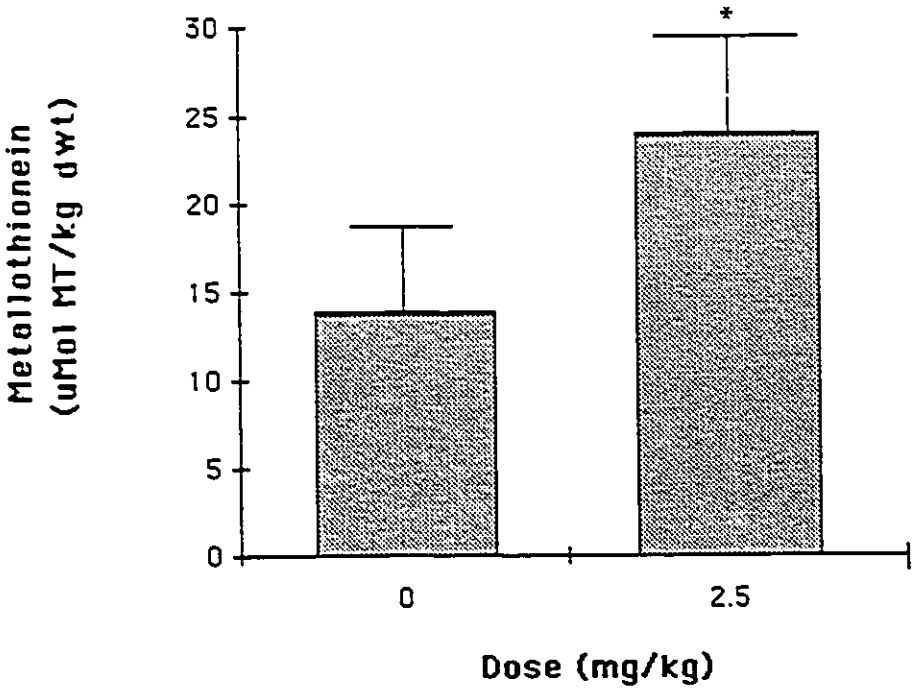
Legend to figure (administered dose):

Short Course Dose: 0.0 mg/kg (B,E); 2.5 mg/kg (A,F,D). Each rat received a single IP injection at T = 0 h.

Subchronic Dose: 0.0 mg/kg (D); 2.5C mg/kg (C); Each rat received three IP injections in total of the indicated doses over 2 month period The third injection (2.5 mg/kg) was administered to both rats at T = 0 h,

The bar graph represents the mean ± standard deviation, n = 4.
** $p<0.005$, *** $p<0.0005$. See Tables 4.10. and 4.11.

Figure 4.27. Metallothionein Concentration in Spleen, (Short Course AF90 and Subchronic CD90 Experiments).



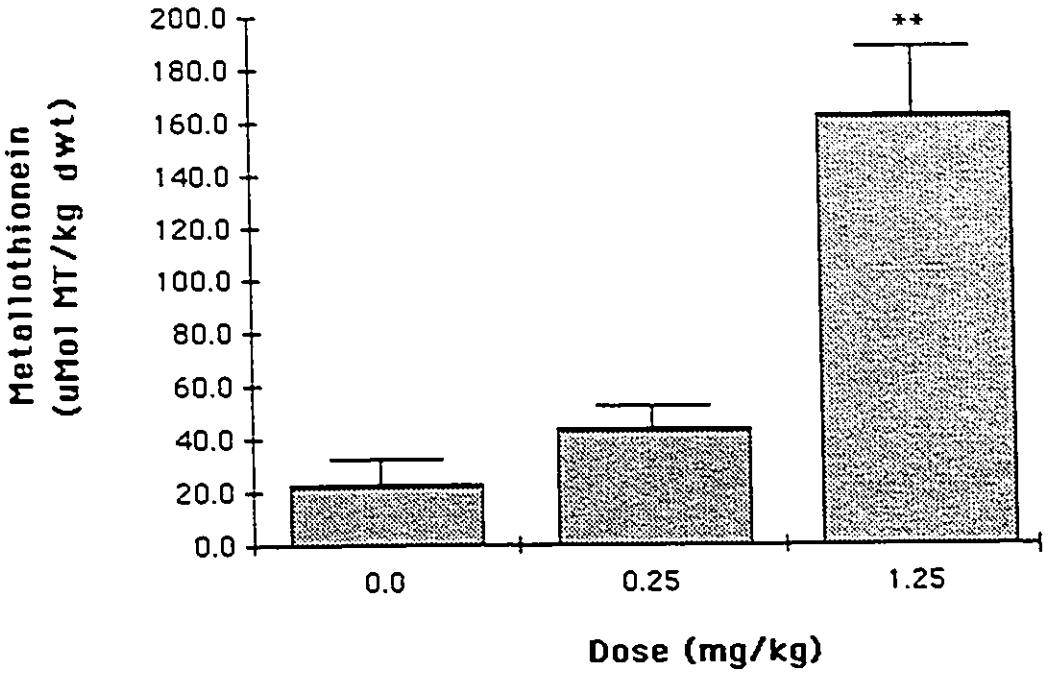
Legend to figure (administered dose):

Short Course Dose: 0.0 mg/kg (B,E); 2.5 mg/kg (A,F,D). Each rat received a single IP injection at T = 0 h.

Subchronic Dose: 0.0 mg/kg (D); 2.5C mg/kg (C); Each rat received three IP injections in total of the indicated doses over 2 month period The third injection (2.5 mg/kg) was administered to both rats at T = 0 h,

The bar graph represents the mean ± standard deviation, n = 3. * $p<0.05$. See Tables 4.10. and 4.11. Spleen sample was not available for rat C, see text for details.

Figure 4. 28. Metallothionein Concentration in Liver, Subchronic Experiment G091.

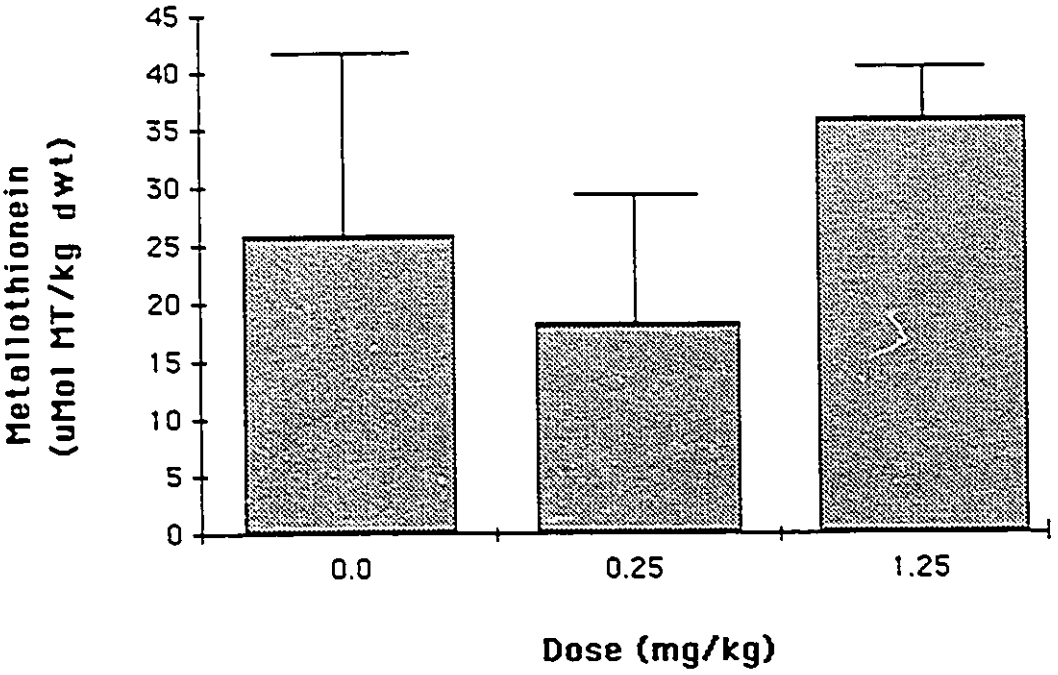


Legend to figure (administered dose):

0.0 mg/kg (G,H,I); 0.25 mg/kg (J,K,L); 1.25 mg/kg (M,N,O);

Each rat received a total of three IP injections of the indicated doses over a 1.5 - 2.0 month period, (except M had 0.5 months). The third injection was administered at T = 0 h. The following rats died as the experiment progressed: K after 6 h, I and L after 48 h, and N after 72 h. The bar graph represents the mean ± standard deviation (n=3). ** $p<0.005$, see Table 4.11.

Figure 4.29. Metallothionein Concentration in Kidney, Subchronic Experiment G091.

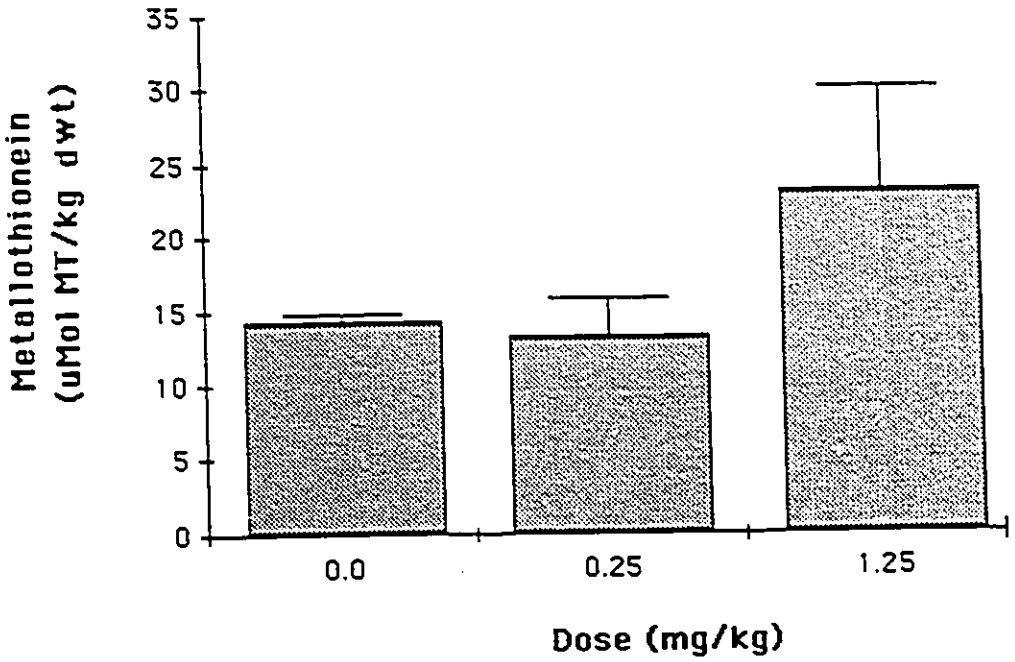


Legend to figure (administered dose):

0.0 mg/kg (G,H,I); 0.25 mg/kg (J,K,L); 1.25 mg/kg (M,N,O);

Each rat received a total of three IP injections of the indicated doses over a 1.5 - 2.0 month period, (except M had 0.5 months). The third injection was administered at T = 0 h. The following rats died as the experiment progressed: K after 6 h, I and L after 48 h, and N after 72 h. The bar graph represents the mean ± standard deviation (n=3), see Table 4.11.

Figure 4.30. Metallothionein Concentration in Spleen, Subchronic Experiment G091.

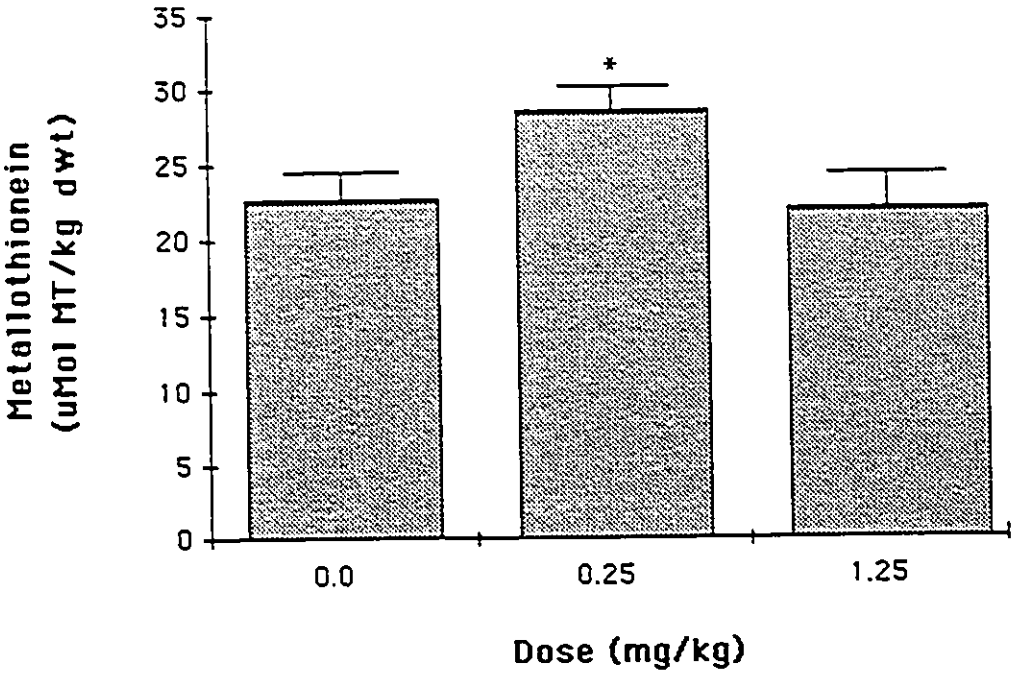


Legend to figure (administered dose):

0.0 mg/kg (G,H,I); 0.25 mg/kg (J,K,L); 1.25 mg/kg (M,N,O);

Each rat received a total of three IP injections of the indicated doses over a 1.5 - 2.0 month period, (except M had 0.5 months). The third injection was administered at T = 0 h. The following rats died as the experiment progressed: K after 6 h, I and L after 48 h, and N after 72 h. The bar graph represents the mean ± standard deviation (n=3), see Table 4.11.

Figure 4.31. Metallothionein Concentration in Lung, Subchronic Experiment 6091.

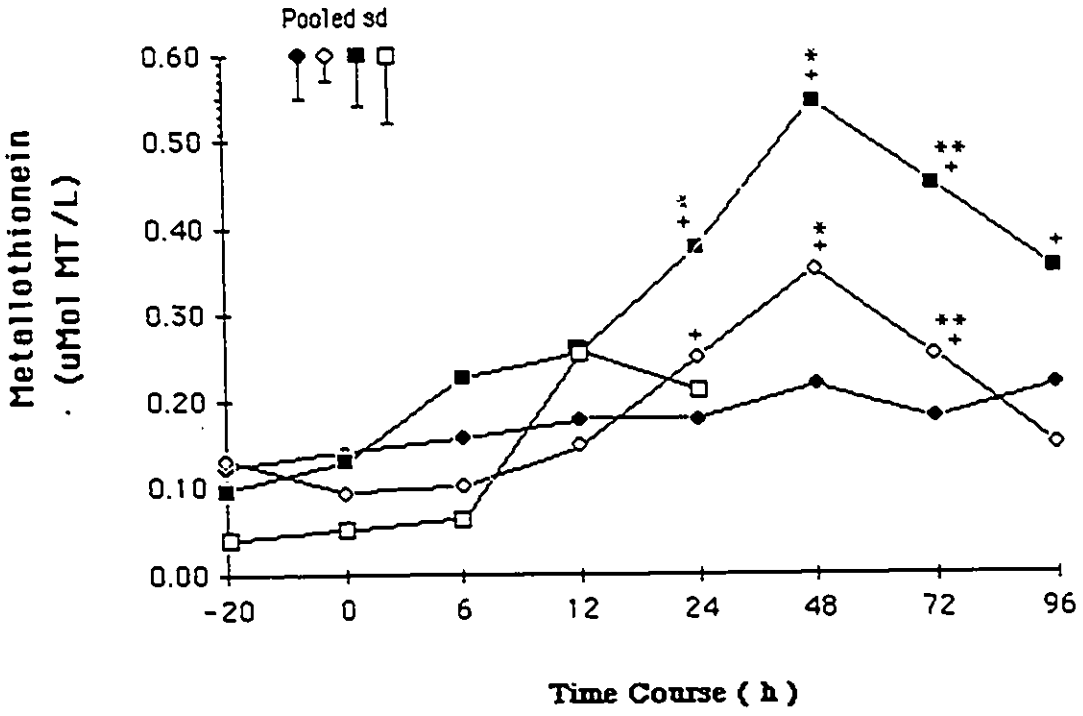


Legend to figure (administered dose):

0.0 mg/kg (G,H,I); 0.25 mg/kg (J,K,L); 1.25 mg/kg (M,N,O);

Each rat received a total of three IP injections of the indicated doses over a 1.5 - 2.0 month period, (except M had 0.5 months). The third injection was administered at T = 0 h. The following rats died as the experiment progressed: K after 6 h, I and L after 48 h, and N after 72 h. The bar graph represents the mean ± standard deviation (n=3). * $p<0.05$, see Table 4.11.

Figure 4.32. Metallothionein Concentration in Plasma, Short Course Experiments.

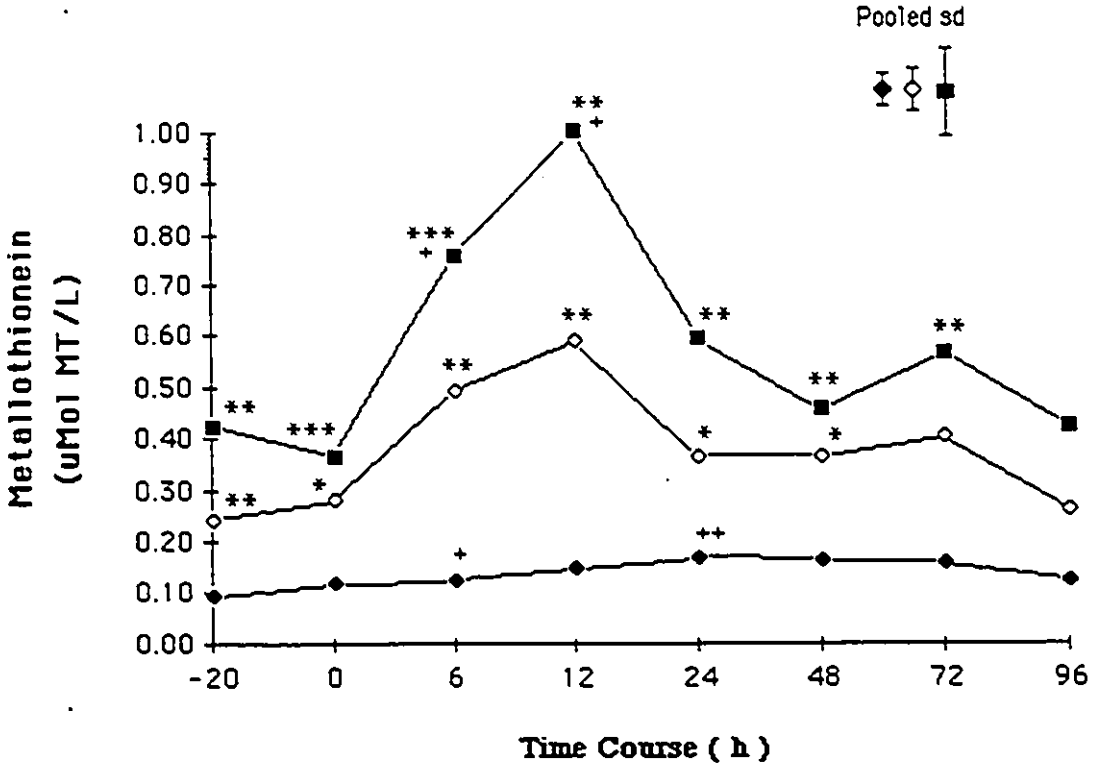


Legend to figure (administered dose):

◆ 0.0 mg/kg ; ◇ 0.25 mg/kg ; ■ 1.25 mg/kg ; □ 2.5 mg/kg;

Each rat received a single IP injection at T = 0 h. The data points represent the mean (n=3). The pooled standard deviations are; ◆ (.05), ◇ (.03), ■ (.06), and □ (.08). *p<0.05, **p<0.005 (unpaired t-Test); +p<0.05 (longitudinal paired t-Test). See Tables 4.12 and 4.14. for individual MT concentrations.

Figure 4.33. Metallothionein Concentration in Plasma, Subchronic Experiments.

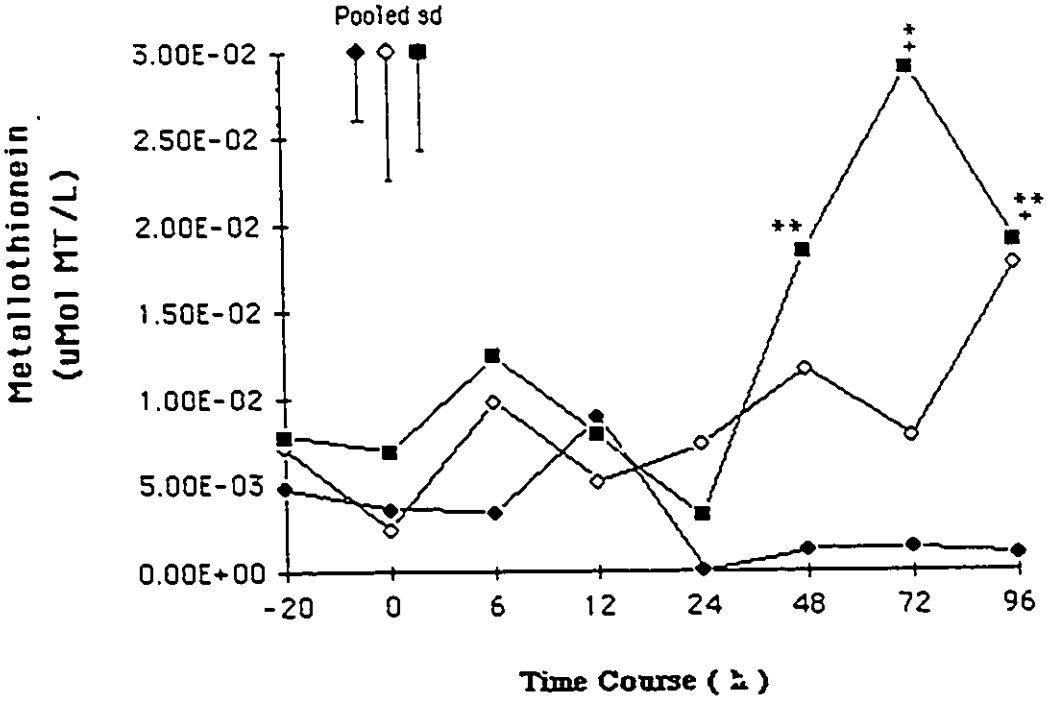


Legend to figure (administered dose):

·◆· 0.0 mg/kg (G,H,I); ·◇· 0.25 mg/kg (J,K,L); ·■· 1.25 mg/kg (M,N,O);

Each rat received a total of three IP injections of the indicated doses over a 1.5 - 2.0 month period, (except M had 0.5 months). The third injection was administered at T = 0 h. The following rats died as the experiment progressed: K after 6 h, I and L after 48 h, and N after 72 h. The data points represent the mean (n=3), pooled standard deviations are; ·◆·(.03), ·◇·(.04), and ·■·(.09). * $p<0.05$, ** $p<0.005$, *** $p<0.0005$ (unpaired t-Test). + $p<0.05$, ++ $p<0.005$ (longitudinal paired t-Test). See Table 4.13.

Figure 4.34. Metallothionein Concentration in Erythrocytes, Short Course Experiments.

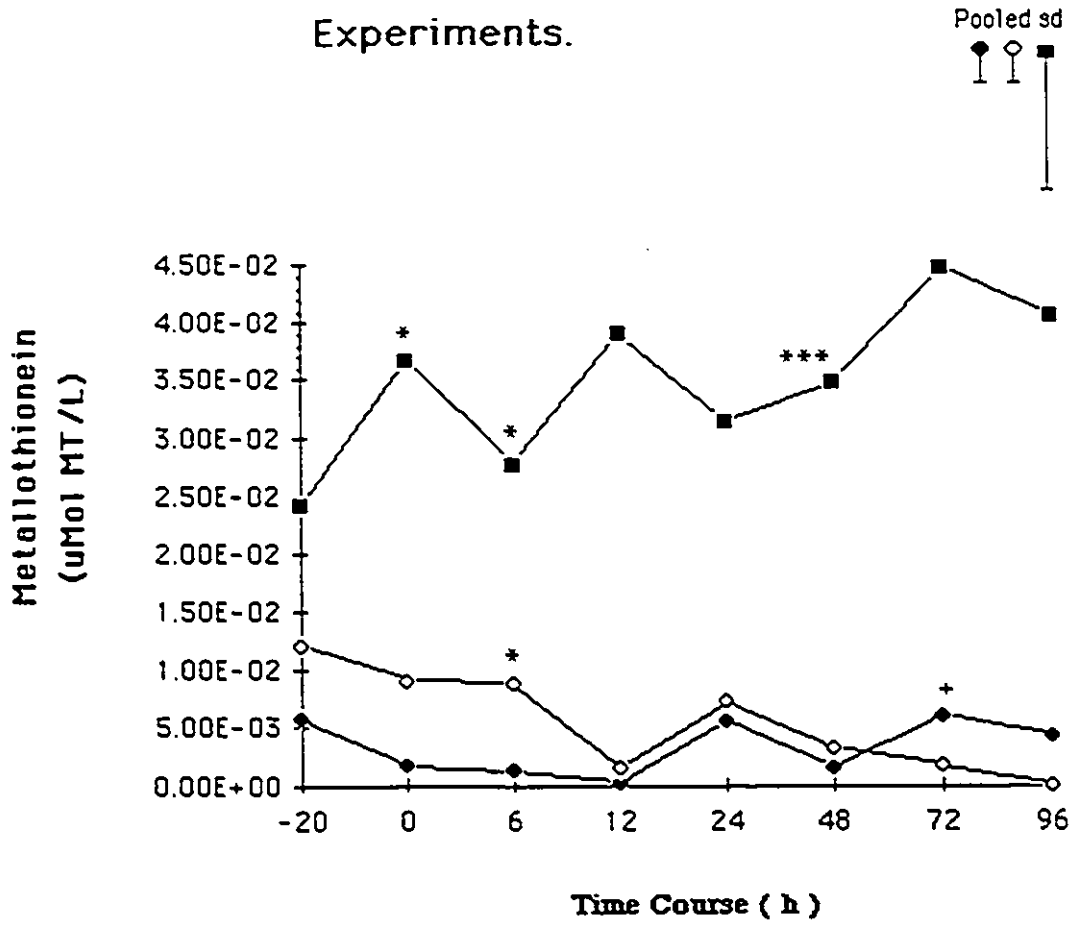


Legend to figure (administered dose):

·◆- 0.0 mg/kg (T,U,AA); ·◇- 0.25 mg/kg (V,W,X); ·■- 1.25 mg/kg (P,Q,R);

Each rat received a single IP injection at T = 0 h. The data points represent the mean (n = 3). The pooled standard deviations are; ·◆- (0.004), ·◇- (0.008), and ·■- (0.006). *p<0.05, **p<0.005 (unpaired t-Test); +p<0.05 (longitudinal paired t-Test). See Table 4.15. for individual MT concentrations.

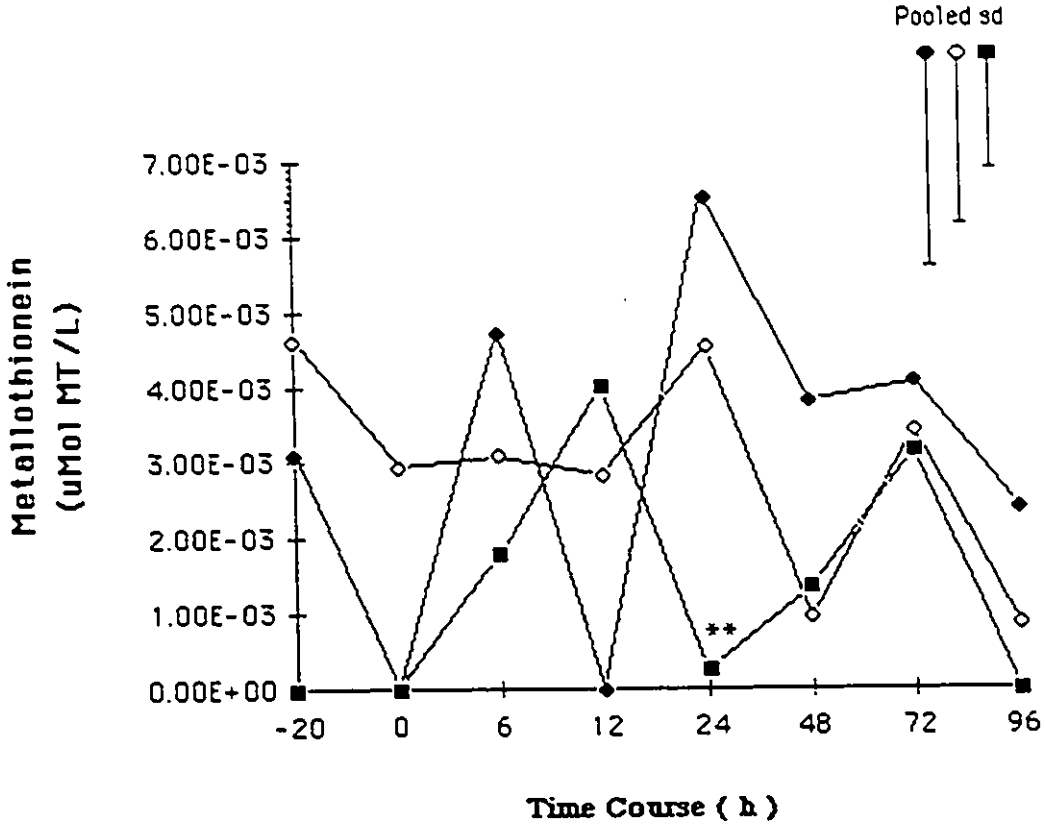
Figure 4.35. Metallothionein Concentration in Erythrocytes, Subchronic Experiments.



Legend to figure (administered dose):

-◆- 0.0 mg/kg (G,H,I); -◇- 0.25 mg/kg (J,K,L); -■- 1.25 mg/kg (M,N,O);
Each rat received a total of three IP injections of the indicated doses over a 1.5 - 2.0 month period, (except M had 0.5 months). The third injection was administered at T = 0 h. The following rats died as the experiment progressed: K after 6 h, I and L after 48 h, and N after 72 h. The data points represent the mean (n=3), pooled standard deviations are; -◆- (.003), -◇- (.003), and -■- (.013). * $p<0.05$, *** $p<0.0005$ (unpaired t-Test). + $p<0.05$ (longitudinal paired t-Test). See Table 4.16.

Figure 4.36. Metallothionein Concentration in Polymorphonuclear Leukocytes, Short Course Experiments.

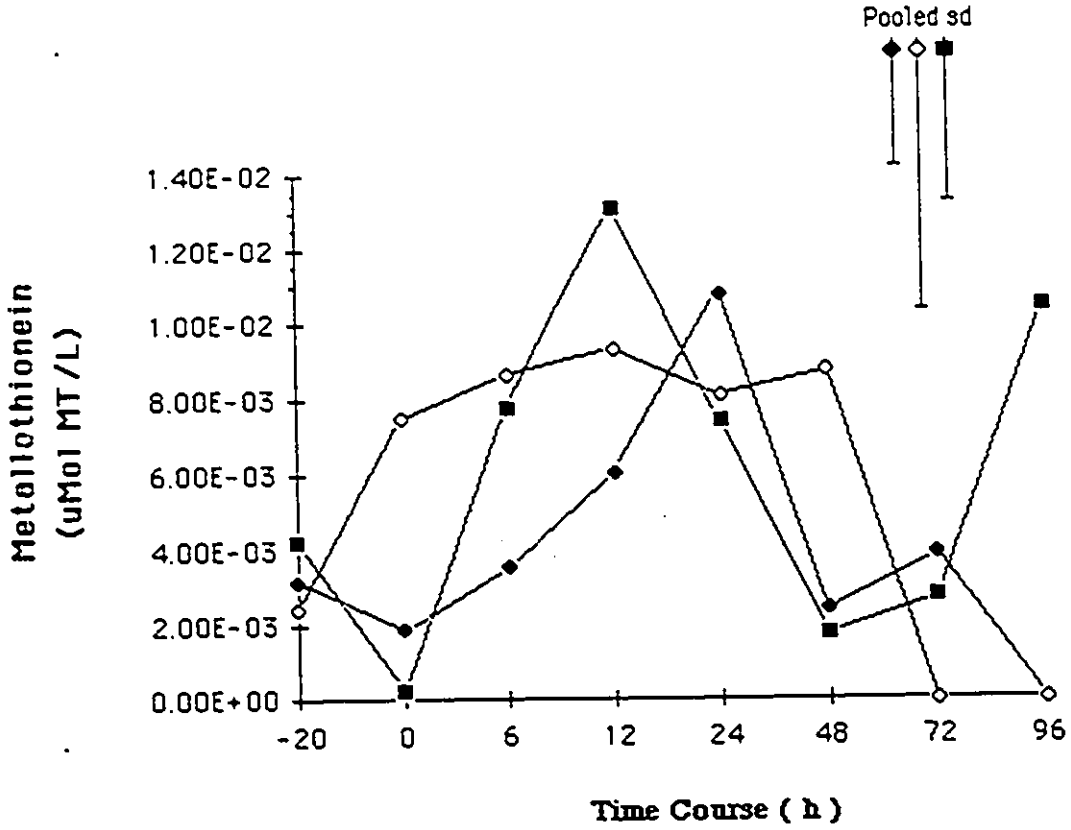


Legend to figure (administered dose):

-◆- 0.0 mg/kg (T,U,AA); -◇- 0.25 mg/kg (V,W,X); -■- 1.25 mg/kg (P,Q,R);

Each rat received a single IP injection at T = 0 h. The data points represent the mean (n = 3). The pooled standard deviations are; -◆-(.0028), -◇-(.0024), and -■- (.0015). **p<0.005 (unpaired t-Test). See Table 4.17. for individual MT concentrations.

Figure 4.37. Metallothionein Concentration in Polymorphonuclear Leukocytes, Subchronic Experiments.

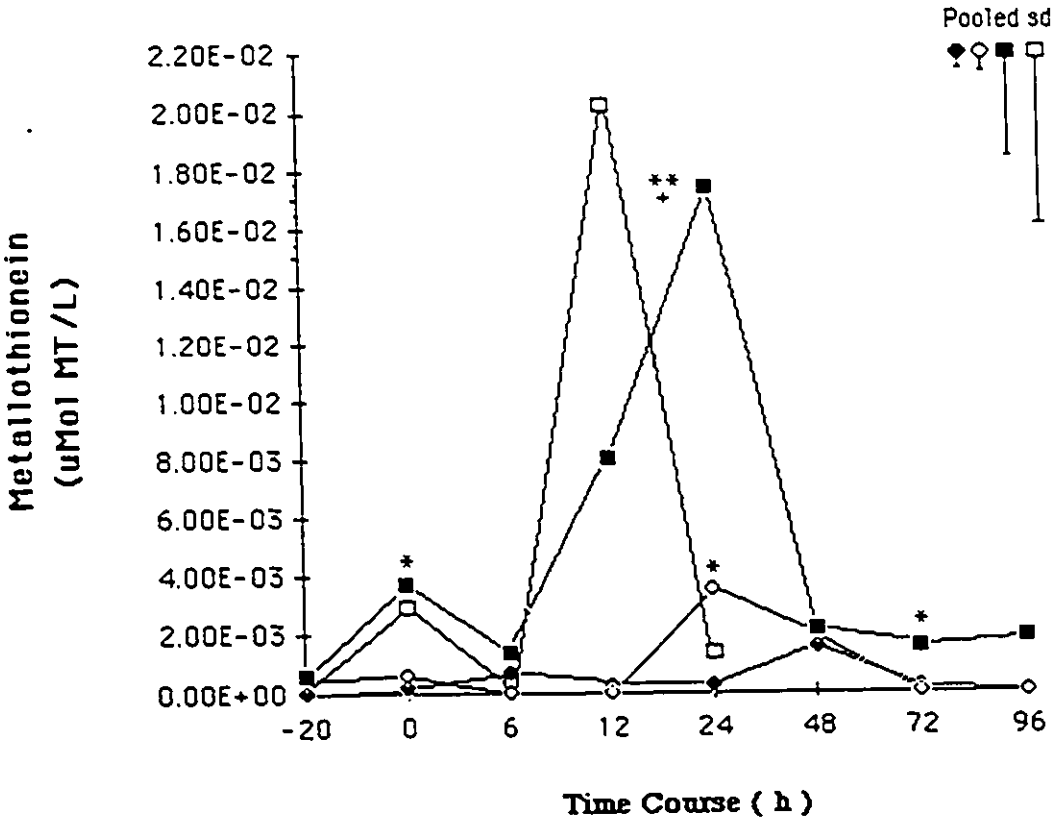


Legend to figure (administered dose):

◆ 0.0 mg/kg (G,H,I); ◇ 0.25 mg/kg (J,K,L); ■ 1.25 mg/kg (M,N,O);

Each rat received a total of three IP injections of the indicated doses over a 1.5 - 2.0 month period, (except M had 0.5 months). The third injection was administered at T = 0 h. The following rats died as the experiment progressed: K after 6 h, I and L after 48 h, and N after 72 h. The data points represent the mean (n=3), pooled standard deviations are; ◆ (.003), ◇ (.007), and ■ (.004) See Table 4.18.

Figure 4.38. Metallothionein Concentration in Lymphocytes, Short Course Experiments.

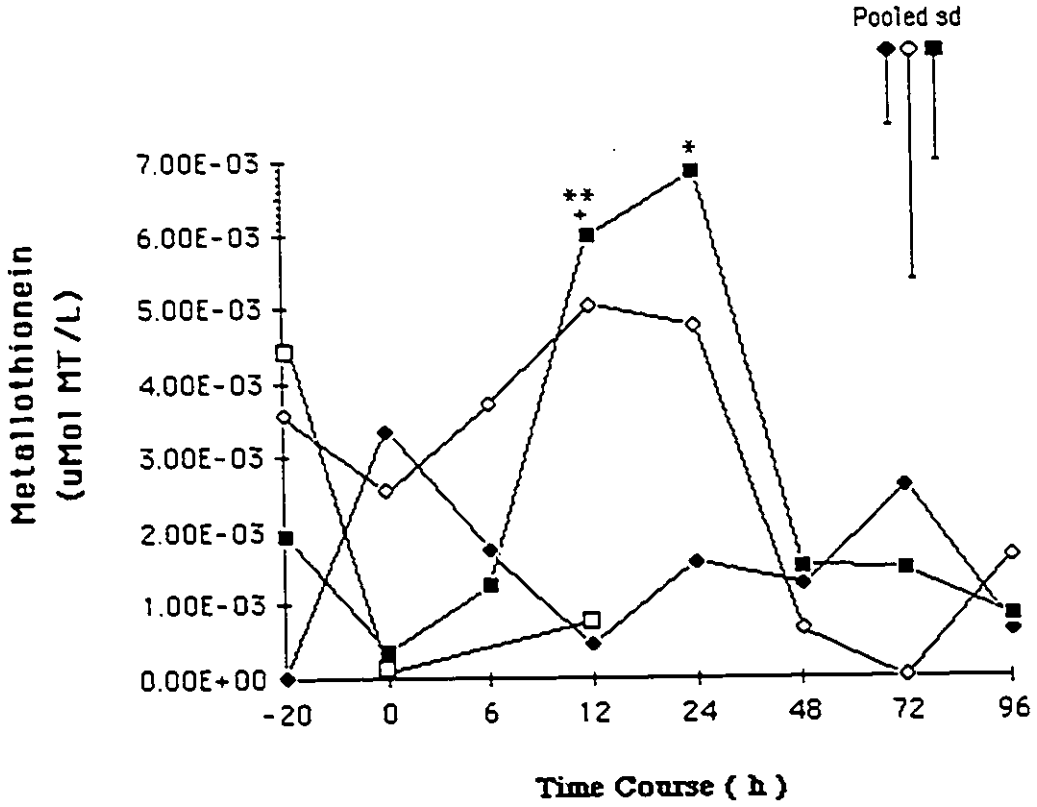


Legend to figure (administered dose):

-◆- 0.0 mg/kg ; -○- 0.25 mg/kg ; -■- 1.25 mg/kg ; -□- 2.5 mg/kg;

Each rat received a single IP injection at T = 0 h. The data points represent the mean (n = 3). The pooled standard deviations are; -◆-(.0006), -○-(.0007) -■- (.0036) and -□-(.0065). *p<0.05, **p<0.005 (unpaired t-Test); +p<0.05 (longitudinal paired t-Test). See Tables 4.19 and 4.21. for individual MT concentrations.

Figure 4.39. Metallothionein Concentration in Lymphocytes, Subchronic Experiments.

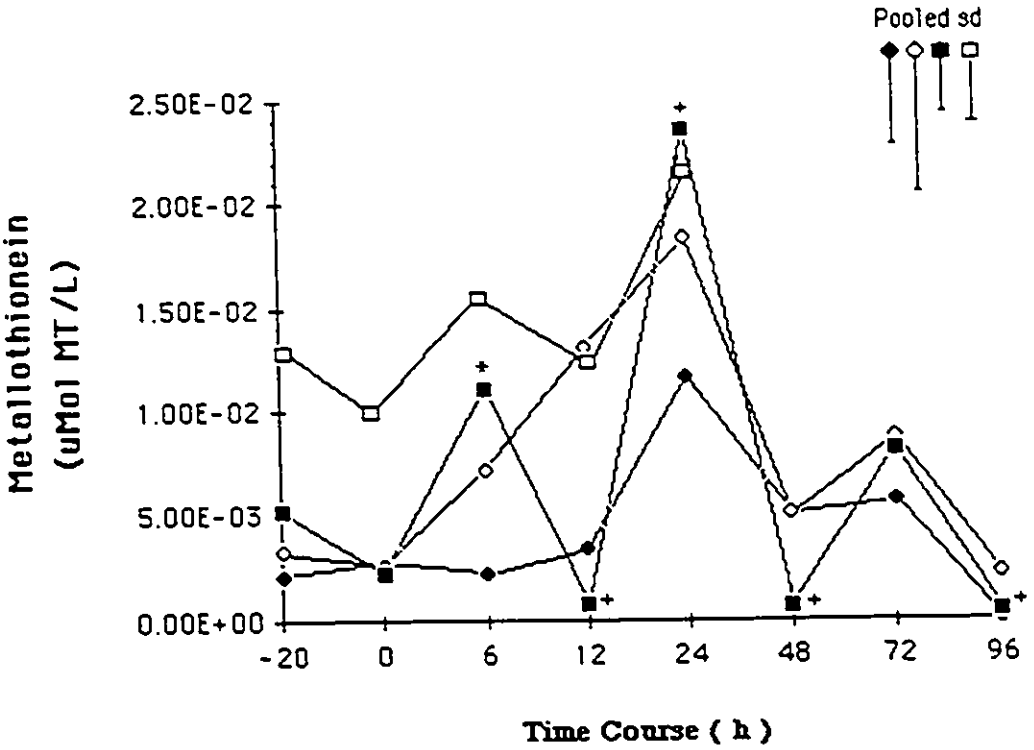


Legend to figure (administered dose):

◆ 0.0 mg/kg ; ○ 0.25 mg/kg ; ■ 1.25 mg/kg; ▭ 2.5 mg/kg;

Each rat received a total of three IP injections of the indicated doses over a 1.5 - 2.0 month period, (except M had 0.5 months). The third injection was administered at T = 0 h. The following rats died as the experiment progressed: K after 6 h, I and L after 48 h, and N after 72 h. The data points represent the mean (n=3), pooled standard deviations are; ◆ (.001), ○ (.003), and ■ (.0015). * $p<0.05$, ** $p<0.005$ (unpaired t-Test). + $p<0.05$ (longitudinal paired t-Test). See Tables 4.20. and 4.21.

Figure 4.40. Metallothionein Concentration in Monocytes, Short Course Experiments.

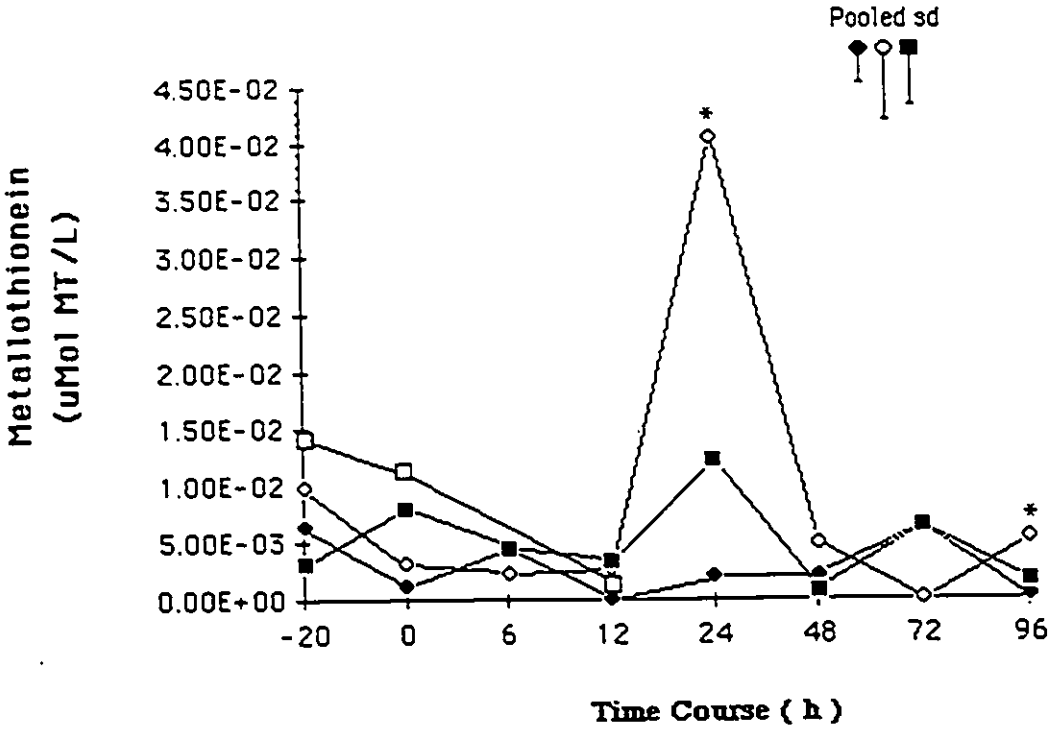


Legend to figure (administered dose):

◆- 0.0 mg/kg ; ◇- 0.25 mg/kg ; ■- 1.25 mg/kg ; □ 2.5 mg/kg;

Each rat received a single IP injection at T = 0 h. The data points represent the mean (n=3). The pooled standard deviations are; ◆- (.0045), ◇- (.0068), ■- (.0027) and □ (.003). *p<0.05 (unpaired t-Test); +p<0.05 (longitudinal paired t-Test) See Tables 4.22. and 2.24. for individual MT concentrations.

Figure 4.41. Metallothionein Concentration in Monocytes, Subchronic Experiments.

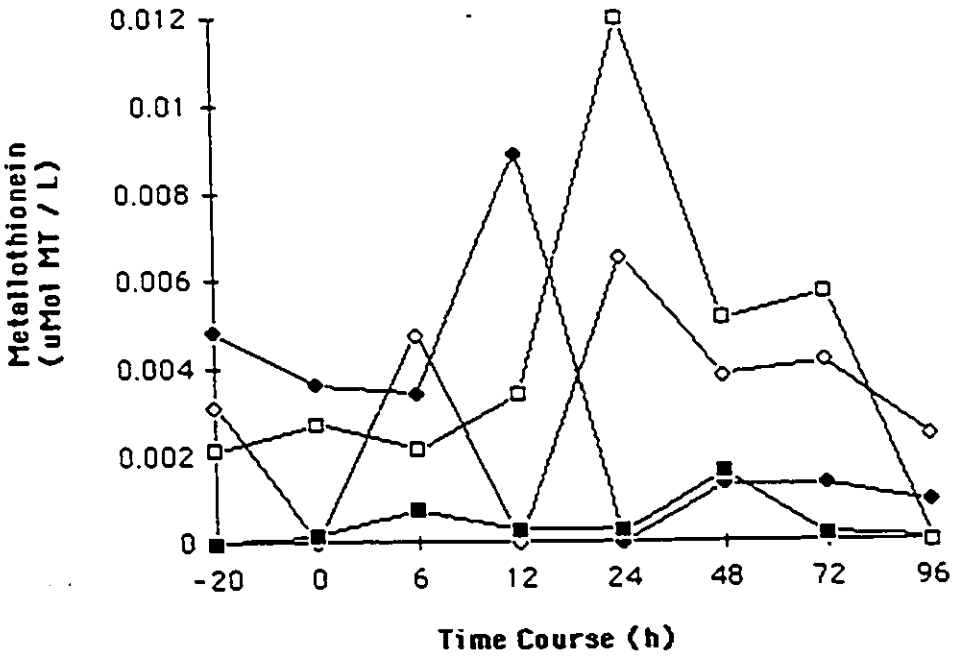


Legend to figure (administered dose):

-◆- 0.0 mg/kg ; -○- 0.25 mg/kg ; -■- 1.25 mg/kg; -□- 2.5 mg/kg;

Each rat received a total of three IP injections of the indicated doses over a 1.5 - 2.0 month period, (except M had 0.5 months). The third injection was administered at T = 0 h. The following rats died as the experiment progressed: K after 6 h, I and L after 48 h, and N after 72 h. The data points represent the mean (n=3), pooled standard deviations are; -◆-(.003), -○-(.006), and -■-(.005). * $p<0.05$ (unpaired t-Test). See Tables 4.23 and 4.24.

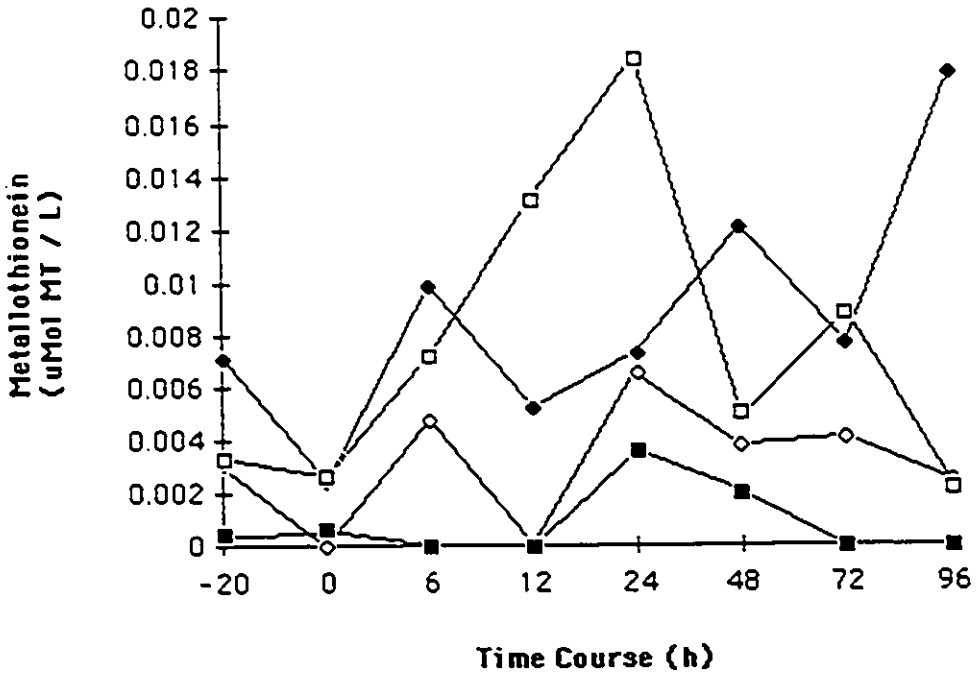
Figure 4.42. Comparison of Individual Control Blood Cell Compartments for MT Concentration, Short Course Experiment PA91.



Legend to figure:

Each rat received a single IP injection at T = 0 h. The data points represent the mean (n=3). The individual blood cell compartments are: (-◆-) RBC, (-○-) PMN, (-■-) Lymphocytes, and (-□-) Monocytes for the control group.

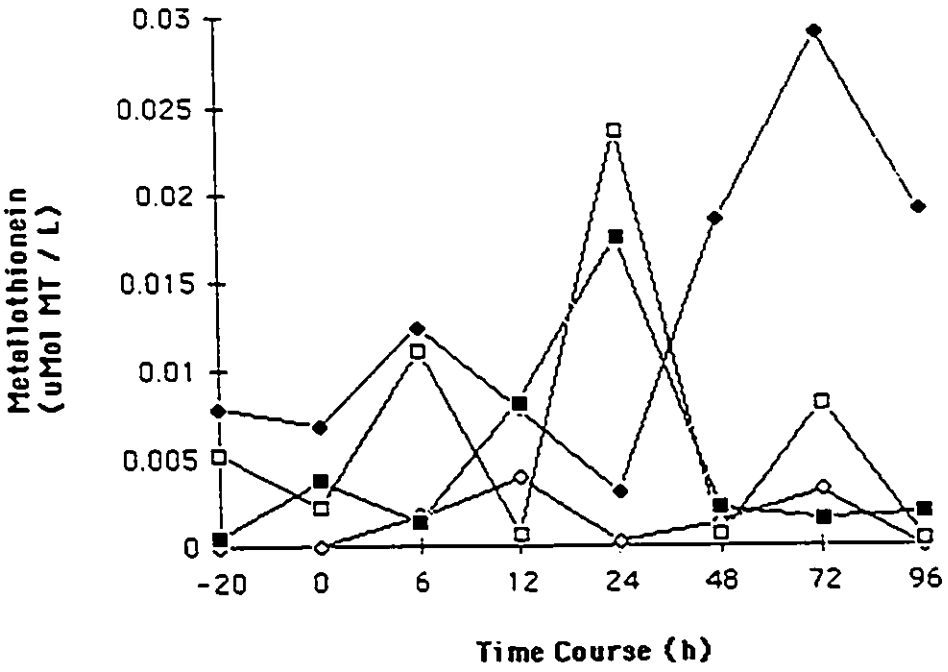
Figure 4.43. Comparison of Individual Low Dose Blood Cell Compartments for MT Concentration, Short Course Experiment PA91.



Legend to figure:

Each rat received a single IP injection at T = 0 h. The data points represent the mean (n=3). The individual blood cell compartments are: (·◆-) RBC, (·○-) PMN, (·■-) Lymphocytes, and (·□-) Monocytes for the low dose (0.25 mg Cd/kg) group.

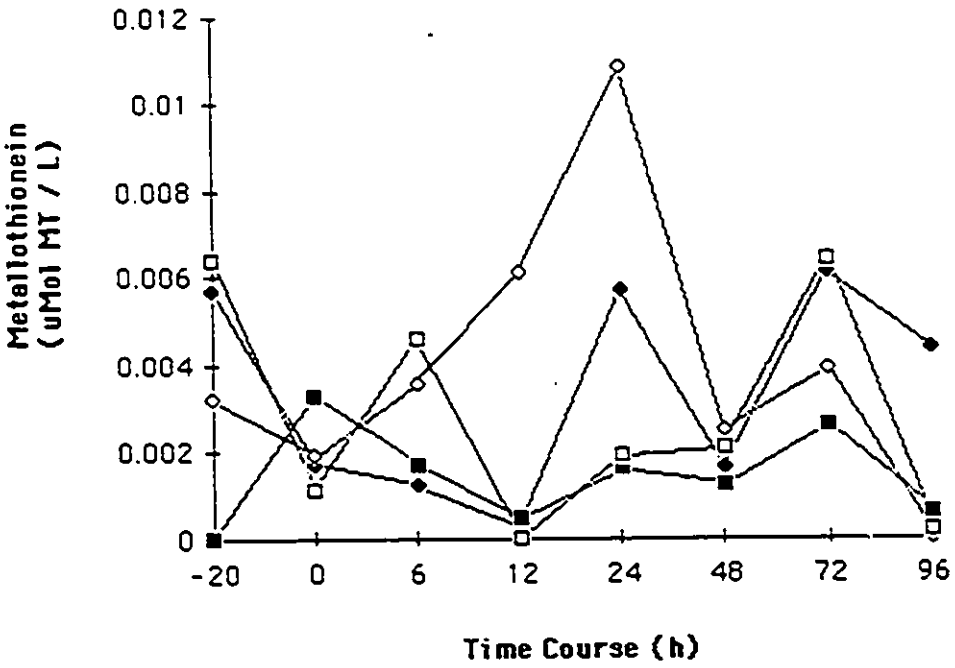
Figure 4.44. Comparison of Individual Medium Dose Blood Cell Compartments for MT Concentration, Short Course Experiment PA91.



Legend to figure:

Each rat received a single IP injection at T = 0 h. The data points represent the mean (n=3). The individual blood cell compartments are: (-◆-) RBC, (-○-) PMN, (-■-) Lymphocytes, and (-□-) Monocytes for the medium dose (1.25 mg Cd/kg) group.

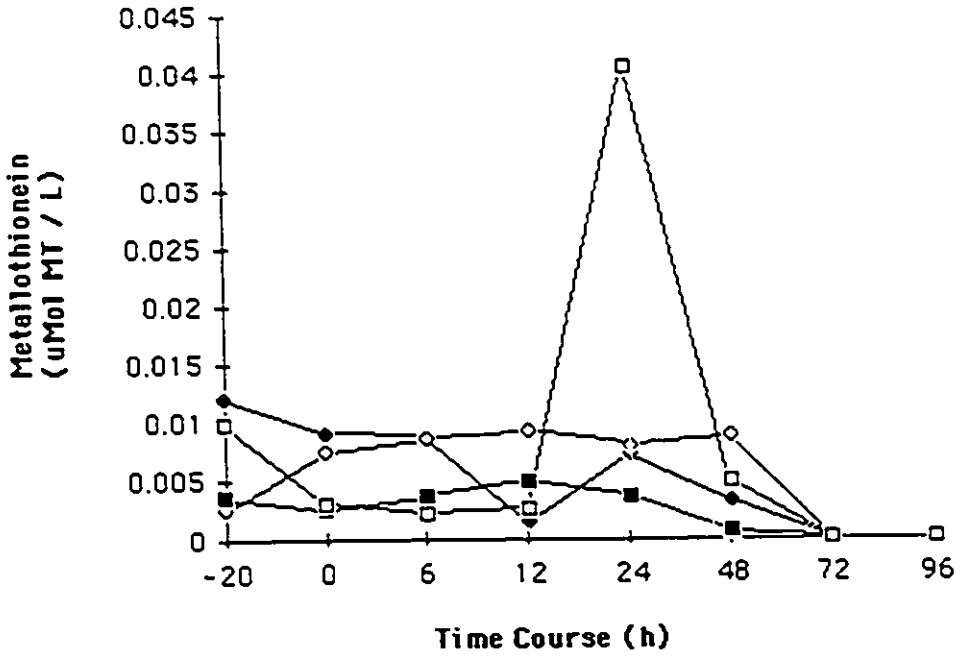
Figure 4.45. Comparison of Individual Control Blood Cell Compartments for MT Concentration, Subchronic Experiment G091.



Legend to figure:

Each rat received a total of three IP injections of the indicated doses over a 1.5 - 2.0 month period. The third injection was administered at T = 0 h. The data points represent the mean (n=3). The individual blood cell compartments are: (-◆-) RBC, (-◇-) PMN, (-■-) Lymphocytes, and (-□-) Monocytes for the control group.

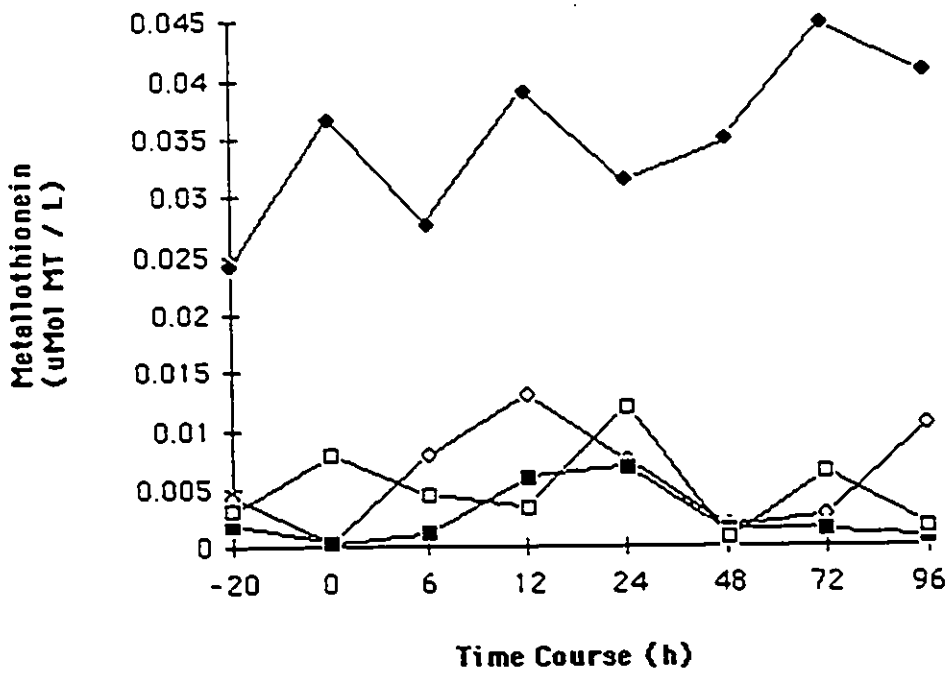
Figure 4.46. Comparison of Individual Low Dose Blood Cell Compartments for MT Concentration, Subchronic Experiment G091.



Legend to figure:

Each rat received a total of three IP injections of the indicated doses over a 1.5 - 2.0 month period. The third injection was administered at T = 0 h. The data points represent the mean (n=3). The individual blood cell compartments are: (-◆-) RBC, (-◇-) PMN, (-■-) Lymphocytes, and (-□-) Monocytes for the low dose (0.25 mg Cd / kg) group.

Figure 4.47. Comparison of Individual Medium Dose Blood Cell Compartments for MT Concentration, Subchronic Experiment G091.



Legend to figure:

Each rat received a total of three IP injections of the indicated doses over a 1.5 - 2.0 month period. The third injection was administered at T = 0 h. The data points represent the mean (n=3). The individual blood cell compartments are: (-◆-) RBC, (-◇-) PMN, (-■-) Lymphocytes, and (-□-) Monocytes for the medium dose (1.25 mg Cd /kg)group.

CHAPTER 5:

5. CADMIUM-INDUCED PHYSIOLOGICAL CHANGES AND HEALTH ASSESSMENT.

5.1. Introduction

An integral part of the Cd time-course experiments involved the comparison of cellular changes and compartmental levels of Cd and MT observed in rats. A recent study in peripheral blood leukocytes from healthy, non-smoking human donors demonstrated evidence for inter-individual differences in their ability to induce MT and respond to Cd exposure (Harley et al., 1989). These results revealed that *in vitro* monocytes and lymphocytes synthesized MT in response to Cd exposure, whereas polymorphonuclear leukocytes (PMN) did not, supporting earlier studies (Enger et al., 1983; Peavy and Fairchild, 1987). A recent rodent study showed that Cd induced toxicity in lymphocytes and monocytes (Koizumi et al., 1987), while the resistance to Cd induced toxicity was considerably higher in PMNs (Enger et al., 1983).

As illustrated in Table 5.1, the WBC differential cell counts for rats are different from those in humans (McDonald et al., 1978; Baker et al., 1979); by contrast cell function (chemical defense, antibody production, protein synthesis, and phagocytosis) appears similar (Baker et al., 1979; Carter and Bazin, 1979; Ringler and Dabich, 1979; Hall and Malia, 1984).

Lymphocytes, unlike other white blood cells, have long survival times (estimated between 5 - 7 days in rats) and are extensively recirculated in the lymphatic system, blood, and lymphoid (spleen, lymph nodes, and alimentary canal) tissues (Carter and Bazin, 1979; Hall and Malia, 1984). The recirculation time for T-cells is 14 - 18 h, and 24 - 28 h for B-cells (Carter and Bazin, 1979).

Monocytes are capable of microbial killing in a similar manner to PMNs which involves phagocytosis of damaged cells and foreign agents (for example, particulates, carbon, silica, and coal-dust), processing of antigens for presentation to lymphocytes, chemotaxis, and diapedesis to injury sites (Hall and Malia, 1984). The monocyte constitutes an intermediate cell stage in the development of tissue macrophages. Monocytes leave the circulatory system to reside in the tissues, where they differentiate into macrophages (Hall and Malia, 1984). The life span of rat monocytes in the circulatory system ranges from 19 to 108 h, with an average of 88 h (Carter and Bazin, 1979).

Since PMNs were shown not to synthesize MT to any significant degree in response to Cd exposure (Enger et al., 1983; Peavy and Fairchild, 1987; Harley et al., 1989; also see Chapter 4), another response to Cd was examined. PMNs produce enzyme-containing granules which are released upon stimulation by foreign agents. Leukocyte alkaline phosphatase activity (LAPA) is one measure of PMN granular activation due to toxicant stimulation. Monocytes and lymphocytes, generally do not show alkaline phosphatase activity.

The mature erythrocyte (RBC) exposed to Cd *in vitro* showed no significant uptake of Cd and no synthesis of MT (Hildebrand and Cram, 1979; Enger et al., 1983), while *in vivo* animal studies showed significant increases in RBC Cd and MT levels (Nordberg et al., 1971b; Garty et al., 1981; Tanaka et al., 1985; Tanaka et al., 1987; also Chapters 3 and 4). Clearly erythroblasts (immature RBCs) or their precursors which contain nuclei must be responsible for the induction of MT (Garty et al., 1981; Tanaka et al., 1985; Tanaka et al., 1987).

Polychromatophilia (color variations) of the RBC observed in differential blood cell slides constitutes an index to increased erythropoiesis and occurs to a greater degree in diseased animals (Ringler and Dabich, 1979). The packed cell volume (PCV) serves as a measurement of the concentration of cells in a known volume of blood. Reported mean PCV values range from 40 to 54% and

are considered normal for rats (Ringler and Dabich, 1979).

This chapter summarizes the impact of *in vivo* Cd exposure on both peripheral blood cells and on the general health of the rats employed in the project described in this thesis.

5.2. Experimental Procedures

The experimental details, cell isolation, tissue histology, and health assessment are described in detail in Chapter 2. In all experiments, whole blood slides were prepared at each time point to assess white blood cell (WBC) differential counts. These slides were stained with Wrights stain and 100 consecutive WBCs were counted using 100x objective and oil immersion. The data were expressed as percent of 100 cells.

Total white (WBC) and red blood counts (RBC) were determined by a Coulter Counter using normal operating procedures. Two serial dilutions of the whole blood samples were necessary; first a 1:500 for WBC and then a second dilution of 1:100 for the RBC to yield a total dilution factor of 1:50,000. Thus 20 uL of whole blood was diluted in 9.98 mL of Isoton II for WBC counting, followed by diluting 100 uL of this solution with 9.90 mL of Isoton II for RBCs, which were counted immediately. Zap-isoton (3 drops) were added to each WBC vial in order to lyse all RBCs and the samples were counted within thirty minutes. The normal correction factor was applied for RBCs using the Coulter Counter Coincidence Correction Chart.

Prior to organ collection, all rats were examined for gross pathological abnormalities and determination of their health status. All rats were anesthetized with pentobarbital to allow organ assessment and tissue collection (see Chapter 2 for details).

5.3. Health Assessment

5.3.1. Body Weight

Body weights were recorded at each time point. The short course exposed groups showed minor fluctuations in body weight throughout the time course (Table 5.2). There were no significant differences between the short-course saline and Cd exposed rats using the unpaired t test. The elevated initial body weight of rat D reflects age and not toxicity. The high dose group (2.5 mg/kg) showed a sustained decrease in body weight throughout the course of the experiment, and is interpreted as toxic effects of the treatment.

Except for rat C, the animals in the subchronic experiment showed minor fluctuations in body weight throughout the time course, with no consistent significant differences observed between saline and low Cd-exposed rats using the unpaired t test (Table 5.3). A significant reduction was only observed in the medium dose group at T = 48 h ($p \leq 0.05$, unpaired t test). It is important to note that rat M was a replacement rat and its relatively low weight reflects age and not necessarily Cd toxicity, while the reduced body weight of rat C ($p \leq 0.05$, unpaired t test) may be considered the result of Cd toxicity.

5.3.2. Organ Weight

The major organ weights (wwt) from the short course animals did not show significant differences between the control and Cd exposed rats as determined by the unpaired t test (Table 5.4). The organ weights from the subchronic animals showed a slight dose-dependent increases in liver weights ($p \leq 0.16$), while the kidney, spleen and lung were not significantly different (Table 5.5).

5.3.3. Packed Cell Volume (PCV)

Analysis of the PCV data indicated that some fluctuation occurred apparently due to repetitive blood sampling (Table 5.6 and 5.7). Decreases were observed when multiple samples were collected in a 12-hour period. The rats appeared to be within or close to the normal range of 40 to 54% PCV (Ringler and Dabich, 1979).

5.4. Physiological and Cellular Changes

5.4.1. Leukocyte Alkaline Phosphatase Activity (LAPA)

The Cd exposed groups showed a dose-dependent increase in LAPA scores beyond the normal range (20 to 180, Sigma Procedure #86) compared to the control groups which stayed close to normal values (Figures 5.1 - 5.2, and Tables 5.8 - 5.9). The LAPA scores for all short course Cd exposed groups were significantly higher than the control group during the later time points, T = 24 to 48 h onwards ($p \leq 0.05$, unpaired t test). By contrast, only the subchronic medium dose group exhibited LAPA values significantly higher than the controls ($p \leq 0.05$, unpaired t test). Longitudinal analysis of the short course LAPA scores showed significantly higher levels ($p \leq 0.05$) in the Cd exposed groups compared to their initial values (Figure 5.1). No significant differences were observed in the longitudinal analysis of the LAPA scores for the subchronic rats (Figure 5.2).

5.4.2. Differential White Blood Cell (WBC) Counts

Examination of the WBC differential cell counts revealed that the lymphocyte to PMN ratio changed in a dose-response manner (Figures 5.3 - 5.4). The monocyte, eosinophil, and basophil numbers stayed within the normal levels (Table 5.1). Significant changes in the cellular populations were not observed in the control groups. The PMN levels increased gradually over the time course and returned to normal levels by T = 72 h for the control group in both protocols and the short course low-dose group (Figures 5.3 and 5.4). The PMN levels for the subchronic low-dose group were significantly higher than the corresponding control PMN levels at T = 6 and 12 h ($p \leq 0.05$, unpaired t test), thereafter returning to normal values (Figures 5.4). By contrast, the medium dose group showed increases in the PMN numbers which remained higher than normal for the duration of the subchronic experiment ($p \leq 0.05$, unpaired t test, Figure 5.4). The PMN numbers from the short course animals were significantly

higher than control levels at T = 12 h for the medium dose group and at T = 12 and 24 h for the high dose group ($p \leq 0.05$, unpaired t test, Figure 5.3).

The lymphocyte numbers followed a reversal of the PMN trends described above, where the numbers of lymphocytes decreased in a dose and time-dependent manner. The short course low-dose group showed significantly lower lymphocyte numbers compared to the control group at T = 12 h ($p \leq 0.05$, unpaired t test, Figure 5.3), thereafter returning to normal levels. The short course medium and high dose groups showed significantly lower number of lymphocytes at T = 12 and 24 h ($p \leq 0.05$, unpaired t test, Figure 5.3), returning to normal values thereafter. Lymphocyte levels from the subchronic low and medium dose groups were significantly lower than the control group at T = 6 - 48 h (low dose) and at all time points for the medium dose group ($p \leq 0.05$, unpaired t test, Figure 5.4).

The monocyte numbers in the short-course animals generally stayed within normal limits except at T = 12 h, at which low and medium dose groups showed significantly higher numbers compared to the control group ($p \leq 0.05$, unpaired t test, Figure 5.3 and Table 5.10). Only the monocyte levels in the subchronic low-dose group showed significantly higher numbers compared to the control group at T = 24 and 96 h ($p \leq 0.05$, unpaired t test, Figure 5.4 and Table 5.11).

5.4.3. Total WBC Counts

The total WBC counts showed slight fluctuations with time, although no significant differences were observed between the control and Cd exposed groups for either protocol (Figure 5.5 and Tables 5.12 - 5.13). Total WBC counts were not available for the high dose group in experiment AF90, as this was not included in the original experimental design.

5.4.4. Erythrocytes (RBC)

The total RBC counts from the short course and subchronic rats showed no significant differences between the Cd exposed and control groups (Figure

5.6 and Tables 5.14 - 5.15). Total RBC counts were not available for the earlier high dose group (AF90) as already explained.

During the analysis of the WBC differential slides, the RBCs were monitored for viability. Increased polychromatophilia was observed in a time and dose-dependent manner in all treatment groups. Polychromatophilia was observed with increasing frequency in the short course animals at T = 48 h onwards, while the subchronic animals showed varying degrees throughout the time course. Other abnormalities (roulex and burrs) were also observed in the high dose group for both protocols.

5.4.5. Tissues (Liver, Kidney, Spleen, and Lung)

5.4.5.1. *Gross Pathology*

Gross (visible) pathological changes were observed in liver and spleen of the short-course and subchronic Cd-exposure animals at the medium (1.25 mg/kg) and high (2.5 mg/kg) doses. The most striking changes were observed in the subchronic high-dose (2.5 mg/kg) rat C, which died early (T = 12 h). The spleen was totally necrotic. The liver was abnormally shaped, atrophied, and covered by fibrotic membranes. Also visible were multiple adhesions to intestines, kidneys, stomach, pancreas, fatty tissue and liver. The pathological changes became less severe in the remaining subchronic and short course animals in a dose-response manner. In the short course animals, capsular fibrosis was evident, and the lungs showed slight discoloration. There were no visible abnormalities in the organs from the short course low-dose (0.25 mg/kg) rats. All organs examined appeared normal and healthy for the control (0.0 mg/kg) rats. The pathological changes visible in the lungs were less pronounced than the other organs. Subchronic rats K (0.25 mg/kg) and N (1.25 mg/kg), showed focal white circular spots in the lung. These changes were less severe in rat K than in rat N (see Section 5.4.5.2. and Figure 5.16).

5.4.5.2. *Histopathology*

Histologically, the selected organs of a control rat and one short course

low-dose rat appeared normal as defined by previous authors (Reith and Ross, 1977; Copenhaver et al., 1978; Bivin et al., 1979). The tissue from the medium-dose subchronic rat showed some capsular changes in most organs examined (Figures 5.7, 5.12, 5.14). In the liver, evidence of fatty infiltration and hyperplasia of the tunica-serosa and connective tissue capsule (capsule of Glisson) were observed, indicating fibrosis (Figure 5.7). The structural changes in cells were considered minor in the rats exposed to subchronic Cd (1.25 mg/kg) compared to the control (Figure 5.8) and lower dosage groups. Tissues for the high dose animals were freeze-dried and not suitable for histological examination. Therefore, from here on, only the results for the subchronic medium-dose and the control animals will be discussed. Individual hepatocyte necroses and degenerate cells were observed in the subchronic Cd animal (Figure 5.9). The affected hepatocytes stained darker and were more angular in shape compared to normal hepatocytes. Regions of vacuolated hepatocytes and dense cytoplasmic inclusions were also observed (Figure 5.10). In addition, erythropoiesis and vacuolated hepatocytes were seen in the Cd exposed rat, but not in the control (Figure 5.11). The concentration of iron in the liver of the Cd treated animals did not appear to increase with dose and were not considered different from the control using Perls Prussian Blue stain (PPB). There was no evidence of increased hemolysis in the liver. Overall, the hepatocyte changes were considered minor.

In the kidney, evidence of slight hyperplasia of the connective tissue capsule was seen (Figure 5.12). The structural changes observed in the Cd-exposed kidneys were minor. Slight thickening of the glomerulus was observed compared to the control. Kidneys from the subchronic rat stained with periodic acid Schiff (PAS) showed that the proximal tubular structure was distorted (Figure 5.13). The changes were slight and focal. A portion of the tubules showed degeneration of the luminal aspect of the cells, with degeneration of the brush border and nuclear pyknosis in the subchronic rat while other areas

appeared unaffected by Cd exposure (Figure 5.13). The glomerulus of the control rat was delicate, while that of the Cd exposed rat had a coarse appearance.

In the spleen, fibrosis of the capsule was evident (Figure 5.14). A noticeable reduction in the size of the malpighian corpuscles of the white pulp and increased cellularity of the red pulp was observed compared to the control (Figure 5.15). In addition, a mild increase in hemopoietic cells, pigmented cells and hemosiderin deposits was observed. The same animal showed a slight increase in iron concentration in the spleen.

In the lung, slight pleural thickening and opacification was seen. There were no major changes in the histology of the lungs in the Cd exposed groups, although some small fibrotic nodules were observed using H&E and Trichrome stains (Figure 5.16). In addition, regional emboli were observed within a few vessels from several animals, regardless of treatment.

Examination of the histological slides was performed by Dr. D. deSa, Professor of Pathology at McMaster University Medical Center.

5.5. Discussion

5.5.1. Health Assessment

The observed reduction in the body weight of the subchronic high-dose (2.5 mg/kg) rat C and to a lesser extent, the subchronic medium dosed animal (1.25 mg/kg) may be viewed as an indicator of acute Cd toxicity. There was no significant evidence of any consistent body-weight reduction in the other rats, although minor weight-loss trends were observed in the low and medium Cd-exposure groups. Neither were there significant alterations in the weights of liver and kidney. Significant alterations in the organ weights were not evident using the unpaired t test.

The PCV data for the time course animals appeared to be within or close to the normal range of 40 to 54% (Ringler and Dabich, 1979). The overall

assessment of the general health of the experimental animals indicated that all of the rats were within the normal range for the parameters tested (body and organ weights, PCV and general appearance) except subchronic rat C (high dose) and to a lesser degree, the subchronic medium-dose group.

5.5.2. Physiological and Cellular Changes

5.5.2.1. *Leukocyte Alkaline Phosphatase Activity*

The LAPA results (Figures 5.1 - 5.2, and Table 5.8 and 5.9) clearly showed evidence of PMN granular activation in response to Cd exposure. The increases in LAPA was both time- and dose-dependent (longitudinal or unpaired t test, $p \leq 0.05$) for the short course animals. By contrast, the overall LAPA levels in the subchronic animals were close to or within the normal range of 20 - 180, with the highest response occurring for the intermediate dose of 1.25 mg/kg (unpaired t test, $p \leq 0.05$). These results suggest that the LAPA response is most sensitive in short-term exposures.

5.5.2.2. *Differential and Total WBC Counts*

The observed changes in PMN and lymphocyte cell ratios occurred in a time and dose-dependent manner in response to the IP Cd exposure. These observations support the role of lymphocytes in cellular defense, where lymphocytes are known to leave the peripheral circulation for the lymphatic system and are able to recirculate (Carter and Bazin, 1979; Hall and Malia, 1984). Rat lymphocytes are known to have a blood recirculation time of 14 - 18 h for T-cells and 24 - 28 h for B-cells (Carter and Bazin, 1979). In normal rat blood, most of the lymphocytes are the small cell type, with 60 - 70% of the small lymphocytes constituting T-cells and 30 - 40% B-cells (Carter and Bazin, 1979). The significant decreases observed in lymphocyte cell numbers might indicate that either the removal of lymphocytes from peripheral circulation into the tissues and the lymphatic system (Carter and Bazin, 1979) or lymphocyte sub-populations exhibited sensitivity to Cd resulting in lethal cellular toxicity (Koizumi et al., 1987; also see Section 3.4.6). While the lymphocyte numbers

decreased in response to IP Cd exposure, the PMNs increased. The combined effects of movement and activation of these two cell types may well have contributed to some of the variability observed in the Cd and MT levels measured in these cells (see Chapters 3 and 4).

The overall total WBC counts did not change significantly in response to Cd exposure suggesting that the animals in the medium and low exposure doses were not significantly compromised during the time course experiment. The total WBC counts were not available for the high dose animals.

5.5.2.3. *Erythrocytes*

The overall total RBC counts were not significantly different after Cd exposure from the control group and attest to the overall well being of the animals tested. The increased polychromatophilia observed with time may reflect erythropoiesis in response to repetitive blood sampling and Cd exposure, which is consistent with the expectation of MT induction in the erythroblast or earlier progenitors (Nordberg et al., 1971b; Hildebrand and Cram, 1979; Garty et al., 1981; Tanaka et al., 1985; Tanaka et al., 1987; see also Chapters 3 and 4) but not in the mature RBCs (Hildebrand and Cram, 1979).

5.5.2.4. *Gross Pathology*

The gross pathological changes of the selected organs (liver, kidneys, spleen and lung) occurring in response to IP $CdCl_2$ injections were both time- and dose-dependent, with the most striking changes occurring in the high and medium dose groups (see Section 5.4.5.1). The organs from the control and short course low-dose groups appeared normal and healthy (Bivin et al., 1979). Most of the external changes of the liver, spleen and to a lesser extent, the kidneys maybe related to the route of exposure (IP). However, the changes observed in the lung may have been due to either old particulate emboli or the Cd exposure (personal communication with Dr. D. deSa). The subchronic Cd exposure demonstrated Cd-induced toxicity rather than protection (see also Chapters 3 and 4).

5.5.2.5. *Histopathology*

Changes in the visceral capsules were observed in both exposure protocols indicating early Cd toxicity, while evidence of cellular necrosis was only seen in the subchronic medium-dose animals. The hepatocyte necrosis initiated by $CdCl_2$ were similar to lesions reported earlier (Rehm and Waalkes, 1990). The splenic capsular fibrosis is a nonspecific change that may have been the result of cell degeneration in response to Cd exposure (personal communication with Dr. D. deSa). The renal findings support earlier studies which showed that Cd-induced nephropathic changes occurred after long-term exposure, generally after MT induction and when renal cortex Cd concentrations approached 200 ug/g wwt (Cherian et al., 1976; Goyer et al., 1984; Suzuki and Cherian, 1987; Hamada et al., 1991). The renal ultrastructural changes observed have been reported previously (Goyer et al., 1989) for exposure periods comparable to that in the subchronic time course (2 - 8 weeks). By contrast to our findings, morphological changes were not observed in the liver (Goyer et al., 1989). Increased hemopoiesis was observed in the subchronic rat (1.25 mg/kg) supporting previous observations of increased polychromatophilia (Section 5.4.4.) as well as increased Cd and MT concentrations in RBCs with time (Chapters 3 and 4). The regional emboli in the lung vessels of several animals were possibly related to the introduction of foreign agents or bacteria during the cannulation procedure or specimen collection and are not necessarily related to Cd exposure (personal communication with Dr. D. deSa). However, regions of increased fibrosis in the lung were observed in the Cd-exposed animals.

5.6. Conclusions

The body weight, total WBC, and RBC counts suggest that, generally speaking, the test animals were in good health. However, changes in the differential WBC counts and LAPA indicated a time- and dose-dependent

activation and movement of leukocytes due to Cd-exposure. In addition, the pathological changes observed indicate that some Cd toxicity occurred in both the short course and subchronic experiments (medium and high doses). Only the subchronic medium dose (high dose not available) showed evidence of histological changes to the organs examined.

Table 5.1. Comparison of Rat and Human WBC Differential Counts.

Rat WBC Differential Counts

Type	Number %	Function	Cd	MT
PMN	22 (9-34)%	CD, P	-	-
Lym.	73 (65-84)	AB,PS, P,CD	+	+
Mono.	2.3 (0-5)	P,AP,PS,CD	+	+
Eosin.	2.2 (0-6)	CD, P	?	?
Baso.	0.5 (0-1.5)	CD, P	?	?

Human WBC Differential Counts

Type	Number %	Function	Cd	MT
PMN	60 (40-75)	CD, P	-	-
Lym.	30 (20-45)	AB,PS, P,CD	+	+
Mono.	6 (2-10)	P,AP,PS,CD	+	+
Eosin.	3 (1-6)	CD, P	?	?
Baso.	0.5 (0-1)	CD, P	?	?

Key:

AB - antibody production, AP - antigen presentation, CD - chemical defense, P - phagocytosis, PS - protein synthesis. The number % denotes the mean with the range reported in parenthesis. The (+) or (-) represents the presence or absence of Cd / MT in the leukocytes. The (?) indicates these cell types were not examined in this study.

Table 5.2. Body Weights for the Short Course Rats.

Dosage mg/kg	ID#	Body Weight (kg) -20	0	6	12	24	48	72	96 h
0.00	AA	0.337	0.335	0.328	0.325	0.332	0.339	0.340	0.340
	B	0.272	0.264	0.258	0.254	0.264	0.257	0.260	0.266
	E	0.320	0.300	0.292	0.288	0.294	0.292	-	-
	T	0.298	0.291	0.287	0.282	0.286	0.294	0.293	0.285
	U	0.327	0.324	0.313	0.313	0.311	0.322	0.333	0.336
	Mean	0.311	0.303	0.296	0.292	0.297	0.301	0.307	0.307
	± SD	0.026	0.028	0.027	0.028	0.026	0.031	0.037	0.037
0.25	V	0.327	0.331	0.327	0.321	0.321	0.323	0.336	0.339
	W	0.330	0.333	0.321	0.314	0.321	0.330	0.330	0.338
	X	0.319	0.332	0.323	0.320	0.324	0.323	0.323	0.333
	Mean	0.325	0.332	0.324	0.318	0.322	0.325	0.330	0.337
	± SD	0.006	0.001	0.003	0.004	0.002	0.004	0.007	0.003
1.25	P	0.320	0.317	0.307	0.303	0.296	0.295	0.308	0.310
	Q	0.321	0.318	0.306	0.298	0.291	0.305	0.310	0.315
	R	0.325	0.320	0.310	0.315	0.318	0.317	0.325	0.333
	Mean	0.322	0.318	0.308	0.305	0.302	0.306	0.314	0.319
	± SD	0.003	0.002	0.002	0.009	0.014	0.011	0.009	0.012
2.5	A	0.260	0.260	0.260	0.254	0.249	0.245	0.235	0.254
	F	0.322	0.290	0.284	0.280	0.273	0.271	-	-
	D^	0.483	0.469	-	0.457	0.450	-	-	-
	Mean	0.291	0.275	0.272	0.267	0.261	0.258	0.235	0.254
	± SD	0.044	0.021	0.017	0.018	0.017	0.018	-	-

^ The elevated body weight for rat D reflects age not toxicity within the group and the data was not included in the statistical comparison.

Table 5.3. Body Weights for the Subchronic Time Course Rats.

Dosage mg/kg	ID #	Body Weight (kg) -20	0	6	12	24	48	72	95 h
0.00	G	0.553	0.550	0.546	0.542	0.545	0.546	0.535	0.532
	H	0.591	0.588	0.578	0.567	0.573	0.565	0.562	0.559
	I	0.577	0.585	0.576	0.571	0.561	0.550	-	-
	Mean	0.574	0.574	0.567	0.560	0.560	0.554	0.549	0.546
	± SD	0.019	0.021	0.018	0.016	0.014	0.010	0.019	0.019
0.25	J	0.486	0.492	0.488	0.476	0.478	0.467	0.467	0.465
	K	0.542	0.546	0.538	-	-	-	-	-
	L	0.618	0.625	0.614	0.601	0.602	0.605	-	-
	Mean	0.549	0.554	0.547	0.539	0.540	0.536	0.467	0.465
	± SD	0.066	0.067	0.063	0.088	0.088	0.098	-	-
1.25	M^	0.364	0.357	0.351	0.345	0.341	0.345	0.351	0.351
	N	0.534	0.525	0.507	0.500	0.495	0.488	0.493	-
	O	0.566	0.564	0.542	0.530	0.533	0.521	0.519	0.534
	Mean	0.550	0.545	0.525	0.515	0.514	*0.505	0.509	0.534
	± SD	0.023	0.028	0.025	0.021	0.027	0.023	0.015	-
2.50	C	*0.307	*0.293	*0.270	*0.268	*0.265	-	-	-

^ Rat M was a replacement rat and its relatively low body weight indicates age not toxicity, and the data was not included in the statistical comparison.

* $p < 0.05$, unpaired t test.

Table 5.4. Organ Weights for the Short Course Rats.

Dosage mg/kg	Tissue	Organ Weight (g wet wt)			Mean	± SD
		AA	T	U		
0.00	Liver	14.75	10.55	12.71	12.67	2.10
	Kidney - L	1.49	1.14	1.25	1.29	0.18
	- R	1.53	1.20	1.18	1.30	0.20
	Spleen	0.92	0.74	1.04	0.90	0.15
	Lung	1.20	0.79	1.28	1.09	0.26
		V	W	X		
0.25	Liver	14.29	13.67	13.05	13.67	0.62
	Kidney - L	1.15	1.14	1.14	1.14	0.01
	- R	1.16	1.19	1.13	1.16	0.03
	Spleen	0.93	0.85	1.05	0.94	0.10
	Lung	1.19	1.45	1.40	1.35	0.14
		P	Q	R		
1.25	Liver	11.62	12.46	13.15	12.41	0.77
	Kidney - L	1.11	1.20	1.28	1.20	0.09
	- R	1.13	1.24	1.25	1.21	0.07
	Spleen	1.06	1.11	0.90	1.02	0.11
	Lung	1.21	1.16	1.25	1.21	0.05

Table 5.5. Organ Weights for the Subchronic Time Course Rats.

Dosage mg/kg	Tissue	Organ Weight (g wet wt)			Mean	± SD
		G	H	I		
0.00	Liver	17.61	18.24	-	17.93	0.45
	Kidney - L	1.53	1.65	-	1.59	0.08
	- R	1.45	1.59	-	1.52	0.10
	Spleen	1.05	1.33	-	1.19	0.20
	Lung	1.67	1.67	-	1.67	0.00
		J	K	L		
0.25	Liver	15.43	24.44	-	19.94	6.37
	Kidney - L	1.61	2.20	-	1.91	0.42
	- R	1.62	2.09	-	1.86	0.33
	Spleen	1.28	0.85	-	1.07	0.30
	Lung	1.52	1.61	-	1.57	0.06
		M *	N	O		
1.25	Liver	13.18	24.69	20.04	22.37	3.29
	Kidney - L	1.15	2.16	1.61	1.64	0.51
	- R	1.16	2.15	1.68	1.66	0.50
	Spleen	1.02	1.19	1.78	1.33	0.40
	Lung	1.20	1.39	2.02	1.54	0.43

* Rat M was a replacement rat and the organ weights indicates age not toxicity.

Table 5.6. Packed Cell Volumes for the Short Course Rats.

Time (h)	Packed Cell Volume (PCV)[a]			Mean[b]	± SD
Administered Dose: 0.0 mg					
	T	U	AA		
-20	43	44	38	42	3.2
0	41	44	43	43	1.5
6	37	38	39	38	1.0
12	40	32	41	38	4.9
24	35	34	37	35	1.5
48	38	33	39	37	3.2
72	38	42	41	40	2.1
96	32	38	43	38	5.5
Administered Dose: 0.25 mg					
	V	W	X		
-20	36	42	44	41	4.2
0	44	42	38	41	3.1
6	42	44	42	43	1.2
12	42	44	43	43	1.0
24	30	38	42	37	6.1
48	38	39	41	39	1.5
72	42	41	39	41	1.5
96	41	44	43	43	1.5
Administered Dose: 1.25 mg					
	P	Q	R		
-20	44	40	39	41	2.6
0	43	41	38	41	2.5
6	35	39	44	39	4.5
12	30	44	44	39	8.1
24	30	43	32	35	7.0
48	32	32	34	33	1.2
72	36	40	35	37	2.6
96	36	33	37	35	2.1

a. Capital letters denote individual rats (see Table 2.1).
b. Mean ± standard deviation (n=3).

Table 5.7. Packed Cell Volumes for the Subchronic Time Course Rats.

Time (h)	Packed Cell Volume (PCV) [a]			Mean	± SD [b]
Administered Dose: 0.0 mg					
	G	H	I		
-20	42	45	44	44	1.5
0	47	44	43	45	2.1
6	47	43	48	46	2.6
12	44	48	47	46	2.1
24	41	38	40	40	1.5
48	38	38	35	37	1.7
72	38	39	-	39	0.7
96	39	41	-	40	1.4
Administered Dose: 0.25 mg					
	J	K	L		
-20	46	47	48	47	1.0
0	43	43	44	43	0.6
6	47	46	48	47	1.0
12	43	-	46	45	2.1
24	42	-	43	43	0.7
48	43	-	43	43	0.0
72	40	-	-	40	-
96	39	-	-	39	-
Administered Dose: 1.25 mg					
	M	N	O		
-20	41	42	43	42	1.0
0	42	43	40	42	1.5
6	38	44	43	42	3.2
12	38	45	44	42	3.8
24	40	40	42	41	1.2
48	37	38	32	36	3.2
72	38	40	33	37	3.6
96	38	-	35	37	2.1

a. Capital letters denote individual rats (see Table 2.2).
b. Mean ± standard deviation (n=3).

Table 5.8. Leukocyte Alkaline Phosphatase Activity (LAPA) Scores for the Short Course Rats.

ID #	Time (h) -20	0	6	12	24	48	72	96
				[a]				
Dose: 0.0 mg		LAPA Scores						
T	90	99	95	96	59	116	94	103
U	81	90	67	57	72	113	73	133
AA	88	87	86	110	129	106	148	138
Mean	86	92	83	88	87	112	105	125
± SD	5	6	14	27	37	5	39	19
Dose: 0.25 mg								
V	78	70	136	112	132	139	163	190
W	101	91	120	144	170	133	198	168
X	71	74	96	123	115	128	165	165
Mean	83	78	117	126	139	133	175	174
± SD	16	11	20	16	28	6	20	14
Dose: 1.25 mg								
P	80	75	125	163	77	162	205	153
Q	82	98	134	97	82	164	145	198
R	73	95	122	114	77	167	179	214
Mean	78	89	127	125	79	164	176	188
± SD	5	13	6	34	3	3	30	32
Dose: 2.50 mg								
A	80	107	87	193	218	-	-	346
D	99	111	-	-	193	-	-	-
F	95	79	109	121	166	-	-	-
Mean	91	99	98	157	192	-	-	346
± SD	10	17	16	51	26	-	-	-

a. Normal LAPA scores range from 24 - 180 using FBB stain.

Table 5.9. Leukocyte Alkaline Phosphatase Activity (LAPA) Scores for the Subchronic Time Course Rats.

ID #	Time (h) -20	0	6	12	24	48	72	96
				[a]				
Dose: 0.0 mg	LAPA Scores							
G	88	116	109	150	100	107	108	116
H	103	92	98	99	130	80	118	72
I	111	96	116	99	110	94	-	-
Mean	101	101	108	116	113	94	113	94
± SD	12	13	9	29	15	14	7	31
Dose: 0.25 mg								
J	92	90	104	109	113	100	143	97
K	93	129	111	-	-	-	-	-
L	130	115	92	108	117	110	-	-
Mean	105	111	102	109	115	105	143	97
± SD	22	20	10	1	3	7	-	-
Dose: 1.25 mg								
M	171	173	144	177	149	140	149	127
N	134	141	135	221	139	134	160	-
O	156	128	169	157	123	165	149	192
Mean	154	147	149	185	137	146	153	160
± SD	19	23	18	33	13	16	6	46
Dose: 2.5 mg								
C	103	136	-	143	-	-	-	-

a. Normal LAPA scores range from 24 - 180 using FBB stain.

Table 5.10. WBC Differential Counts, Short Course Experiment.

Time	WBC Differential Counts (Mean ± SD, n=3)									
Dose: 0.0 mg										
	PMN		**Ly**		**Mo**		**Eos**		**Baso**	
-20	14.5	8.2	77.5	10.0	6.5	0.9	1.0	1.7	0.3	0.4
0	34.8	15.2	58.7	14.0	5.6	1.5	0.8	0.4	0.0	0.0
6	32.4	7.0	62.6	7.2	4.4	2.3	0.2	0.3	0.4	0.7
12	27.1	10.3	70.0	10.6	2.6	1.6	0.0	0.0	0.3	0.4
24	26.4	9.3	69.4	8.6	3.4	1.1	0.8	0.8	0.0	0.0
48	24.1	2.8	69.8	2.9	4.4	1.1	1.3	1.0	0.3	0.5
72	17.7	2.9	77.0	4.4	5.0	1.0	0.3	0.6	0.0	0.0
96	19.8	6.1	75.4	6.3	4.3	1.0	0.4	0.5	0.3	0.5
Dose: 0.25 mg										
	PMN		**Ly**		**Mo**		**Eos**		**Baso**	
-20	15.3	4.5	76.7	5.5	7.3	1.2	0.7	0.6	0.0	0.0
0	11.3	7.1	81.3	10.8	6.0	3.0	1.0	1.7	0.3	0.6
6	28.0	6.1	65.7	2.5	5.3	3.1	1.0	1.0	0.0	0.0
12	26.7	5.1	66.3	4.9	6.3	2.1	0.3	0.6	0.0	0.0
24	33.3	6.4	62.7	7.0	3.7	1.5	0.3	0.6	0.0	0.0
48	28.3	3.8	65.0	3.6	6.0	1.0	0.7	0.6	0.0	0.0
72	20.3	3.1	72.7	3.1	5.0	1.7	2.0	1.7	0.0	0.0
96	16.7	10.0	78.7	12.3	3.3	2.1	1.3	1.5	0.0	0.0
Dose: 1.25 mg										
	PMN		**Ly**		**Mo**		**Eos**		**Baso**	
-20	12.7	1.5	78.0	2.6	8.0	2.0	0.3	0.6	1.0	0.0
0	23.7	5.9	67.7	5.5	7.0	2.6	1.0	1.0	0.3	0.6
6	48.0	10.6	48.7	9.5	3.0	1.7	0.3	0.6	0.0	0.0
12	39.7	7.6	54.0	7.5	5.3	0.6	0.7	0.6	0.0	0.0
24	37.0	4.4	58.7	3.1	2.0	1.7	2.3	3.2	0.0	0.0
48	31.7	10.5	62.7	10.5	4.7	0.6	0.7	1.2	0.3	0.6
72	26.0	8.5	69.0	7.5	4.0	1.0	1.0	0.0	0.0	0.0
96	27.7	9.3	64.0	12.1	6.7	1.5	1.3	1.5	0.3	0.6
Dose: 2.5 mg										
	PMN		**Ly**		**Mo**		**Eos**		**Baso**	
-20	21.3	8.4	71.2	9.2	6.2	1.3	1.0	1.3	0.2	0.3
0	51.0	18.4	46.0	17.3	2.7	1.3	0.2	0.3	0.2	0.3
6	68.5	14.8	25.5	9.9	3.3	2.5	0.5	0.7	1.0	1.4
12	80.0	4.2	16.3	1.8	3.0	1.4	0.0	0.0	0.8	1.1
24	65.5	2.0	31.0	4.4	2.3	1.0	0.5	0.5	0.8	1.0
48	-	-	-	-	-	-	-	-	-	-
72	-	-	-	-	-	-	-	-	-	-
96	35.5	-	53.0	-	9.0	-	0.0	-	2.5	-

Table 5.11. WBC Differential Counts, Subchronic Experiment.

Time	WBC		Differential Counts (Mean ± SD, n=3)							
Dose: 0.0 mg										
	PMN		**Ly**		**Mo**		**Eos**		**Baso**	
-20	12.0	4.0	80.7	8.1	6.0	2.0	1.3	2.3	0.0	0.0
0	13.3	7.6	80.7	7.5	5.3	3.2	0.7	1.2	0.0	0.0
6	33.0	5.0	60.3	5.7	6.0	2.6	0.3	0.6	0.3	0.6
12	30.3	6.4	63.7	4.6	5.3	1.5	0.7	0.6	0.0	0.0
24	20.3	6.4	74.7	5.5	4.7	0.6	0.0	0.0	0.3	0.6
48	21.0	2.6	70.7	3.1	6.3	2.1	2.0	2.6	0.0	0.0
72	12.0	2.8	78.5	3.5	7.5	0.7	1.0	0.0	1.0	0.0
96	18.5	3.5	71.5	2.1	7.0	0.0	2.5	0.7	0.5	0.7
Dose: 0.25 mg										
	PMN		**Ly**		**Mo**		**Eos**		**Baso**	
-20	11.3	3.1	83.3	3.5	4.3	0.6	1.0	1.0	0.0	0.0
0	18.3	10.2	72.7	8.1	7.3	3.1	1.7	0.6	0.0	0.0
6	53.7	3.1	39.3	1.5	5.7	2.5	1.3	1.2	0.0	0.0
12	53.5	3.5	39.5	2.1	3.0	0.0	4.0	1.4	0.0	0.0
24	36.0	11.3	53.0	9.9	7.5	0.7	3.0	0.0	0.5	0.7
48	37.5	12.0	52.0	8.5	2.0	1.4	8.0	1.4	0.5	0.7
72	12.0	-	79.0	-	5.0	-	3.0	-	1.0	-
96	19.0	-	68.0	-	9.0	-	4.0	-	0.0	-
Dose: 1.25 mg										
	PMN		**Ly**		**Mo**		**Eos**		**Baso**	
-20	32.3	4.0	61.3	1.2	5.0	2.6	1.3	1.2	0.0	0.0
0	29.7	6.7	61.0	4.4	7.7	2.1	1.7	0.6	0.0	0.0
6	63.7	9.0	29.0	7.8	5.7	0.6	1.3	0.6	0.0	0.0
12	54.0	10.5	37.7	6.1	7.0	2.6	0.7	1.2	0.3	0.6
24	37.0	5.3	53.3	3.5	8.7	2.5	0.7	0.6	0.3	0.6
48	36.3	8.6	51.0	7.2	8.2	1.8	4.3	1.5	0.0	0.0
72	37.3	6.7	52.3	4.5	6.7	2.5	3.7	3.1	0.0	0.0
96	49.5	0.7	43.0	2.8	4.0	1.4	3.5	2.1	0.0	0.0
Dose: 2.5 mg										
	PMN		**Ly**		**Mo**		**Eos**		**Baso**	
-20	40.0	-	51.0	-	8.0	-	1.0	-	0.0	-
0	69.5	-	21.5	-	9.0	-	0.0	-	0.0	-
6	-	-	-	-	-	-	-	-	-	-
12	64.0	-	25.0	-	10.5	-	0.5	-	0.0	-
24	-	-	-	-	-	-	-	-	-	-
48	-	-	-	-	-	-	-	-	-	-
72	-	-	-	-	-	-	-	-	-	-
96	-	-	-	-	-	-	-	-	-	-

Table 5.12. Total White Blood Cell Counts, Short Course Experiment.

Time (h)	Total WBC (cells/L)[a]			Mean[b]	± SD
Administered Dose: 0.0 mg/kg					
	T	U	AA		
-20	6.79E+09	1.16E+10	6.74E+09	8.36E+09	2.77E+09
0	8.28E+09	1.14E+10	8.62E+09	9.43E+09	1.71E+09
6	6.54E+09	6.11E+09	9.41E+09	7.35E+09	1.80E+09
12	6.62E+09	6.29E+09	7.90E+09	6.93E+09	8.53E+08
24	7.28E+09	7.13E+09	8.76E+09	7.72E+09	9.03E+08
48	1.64E+10	8.49E+09	8.69E+09	1.12E+10	4.51E+09
72	8.33E+09	1.32E+10	1.12E+10	1.09E+10	2.45E+09
96	1.24E+10	1.37E+10	1.18E+10	1.26E+10	9.43E+08
Administered Dose: 0.25 mg/kg					
	V	W	X		
-20	7.38E+09	6.87E+09	4.97E+09	6.41E+09	1.27E+09
0	7.98E+09	7.09E+09	6.37E+09	7.14E+09	8.08E+08
6	9.95E+09	8.17E+09	5.69E+09	7.94E+09	2.14E+09
12	8.97E+09	8.77E+09	6.01E+09	7.92E+09	1.65E+09
24	7.84E+09	5.82E+09	6.58E+09	6.75E+09	1.02E+09
48	1.25E+10	6.85E+09	5.77E+09	8.38E+09	3.63E+09
72	1.28E+10	7.03E+09	5.66E+09	8.51E+09	3.81E+09
96	1.49E+10	7.62E+09	7.65E+09	1.01E+10	4.20E+09
Administered Dose: 1.25 mg/kg					
	P	Q	R		
-20	1.06E+10	1.05E+10	4.99E+09	8.71E+09	3.22E+09
0	8.85E+09	1.15E+10	7.99E+09	9.45E+09	1.84E+09
6	9.94E+09	5.78E+09	9.47E+09	8.40E+09	2.28E+09
12	6.57E+09	1.78E+10	9.87E+09	1.14E+10	5.78E+09
24	5.90E+09	1.09E+10	4.86E+09	7.21E+09	3.22E+09
48	6.38E+09	1.53E+10	7.27E+09	9.66E+09	4.93E+09
72	8.74E+09	1.10E+10	6.51E+09	8.74E+09	2.24E+09
96	9.86E+09	1.01E+10	9.09E+09	9.69E+09	5.41E+08

a. Total WBC counts were determined using a Coulter Counter (Model 3805).
b. Mean ± standard deviation (n = 3).

Table 5.13. Total White Blood Cell Counts, Subchronic Experiment.

Time (h)	Total WBC (cells/L)[a]			Mean[b]	± SD
Administered Dose: 0.0 mg/kg					
	G	H	I		
-20	7.89E+09	1.05E+10	1.01E+10	9.50E+09	1.41E+09
0	1.06E+10	1.12E+10	1.17E+10	1.12E+10	5.74E+08
6	1.10E+10	1.31E+10	1.63E+10	1.35E+10	2.66E+09
12	9.18E+09	1.15E+10	1.26E+10	1.11E+10	1.75E+09
24	1.15E+10	9.68E+09	1.17E+10	1.09E+10	1.10E+09
48	1.14E+10	1.08E+10	1.23E+10	1.15E+10	7.45E+08
72	8.34E+09	9.46E+09	-	8.90E+09	7.88E+08
96	1.31E+10	1.48E+10	-	1.40E+10	1.19E+09
Administered Dose: 0.25 mg/kg					
	J	K	L		
-20	1.19E+10	7.99E+09	9.29E+09	9.74E+09	2.01E+09
0	9.46E+09	6.59E+09	7.01E+09	7.69E+09	1.55E+09
6	1.20E+10	9.65E+09	1.16E+10	1.11E+10	1.28E+09
12	1.05E+10	-	1.13E+10	1.09E+10	5.47E+08
24	8.39E+09	-	7.14E+09	7.77E+09	8.85E+08
48	1.06E+10	-	6.61E+09	8.61E+09	2.83E+09
72	1.27E+10	-	-	1.27E+10	-
96	1.38E+10	-	-	1.38E+10	-
Administered Dose: 1.25 mg/kg					
	M	N	O		
-20	6.50E+09	8.74E+09	1.11E+10	8.77E+09	2.28E+09
0	5.33E+09	9.23E+09	1.08E+10	8.46E+09	2.82E+09
6	1.09E+10	1.30E+10	1.65E+10	1.35E+10	2.83E+09
12	6.85E+09	1.09E+10	1.51E+10	1.09E+10	4.12E+09
24	9.25E+09	9.99E+09	8.76E+09	9.33E+09	6.21E+08
48	1.09E+10	1.39E+10	1.17E+10	1.22E+10	1.53E+09
72	9.15E+09	1.58E+10	1.48E+10	1.32E+10	3.58E+09
96	1.02E+10	-	1.74E+10	1.38E+10	5.08E+09

a. Total WBC counts were determined using a Coulter Counter (Model 3805).
b. Mean ± standard deviation (minimum n = 2).

Table 5.14. Total Red Blood Cell Counts, Short Course Experiment

Time (h)	Total RBC (cells/L)[a]			Mean	± SD[b]
Administered Dose: 0.0 mg/kg					
	T	U	AA		
-20	6.09E+12	6.53E+12	7.96E+12	6.86E+12	9.75E+11
0	5.83E+12	5.05E+12	7.42E+12	6.10E+12	1.21E+12
6	5.16E+12	5.27E+12	7.12E+12	5.85E+12	1.10E+12
12	6.94E+12	6.25E+12	6.22E+12	6.47E+12	4.07E+11
24	6.71E+12	4.76E+12	5.60E+12	5.69E+12	9.78E+11
48	7.64E+12	5.52E+12	6.01E+12	6.39E+12	1.11E+12
72	5.34E+12	5.90E+12	5.81E+12	5.68E+12	3.01E+11
96	5.18E+12	5.75E+12	6.41E+12	5.78E+12	6.16E+11
Administered Dose: 0.25 mg/kg					
	V	W	X		
-20	5.95E+12	6.28E+12	6.33E+12	6.19E+12	2.05E+11
0	5.96E+12	7.05E+12	6.01E+12	6.34E+12	6.13E+11
6	6.30E+12	7.25E+12	7.14E+12	6.90E+12	5.18E+11
12	5.68E+12	7.04E+12	6.50E+12	6.41E+12	6.85E+11
24	6.42E+12	7.72E+12	6.88E+12	7.01E+12	6.61E+11
48	5.04E+12	6.31E+12	5.82E+12	5.72E+12	6.40E+11
72	5.25E+12	5.77E+12	7.02E+12	6.01E+12	9.10E+11
96	5.45E+12	7.82E+12	6.40E+12	6.56E+12	1.19E+12
Administered Dose: 1.25 mg/kg					
	P	Q	R		
-20	6.60E+12	5.63E+12	5.29E+12	5.84E+12	6.80E+11
0	5.47E+12	5.56E+12	5.44E+12	5.49E+12	6.24E+10
6	4.80E+12	5.24E+12	7.48E+12	5.84E+12	1.44E+12
12	3.46E+12	6.95E+12	6.10E+12	5.50E+12	1.82E+12
24	5.42E+12	5.60E+12	4.30E+12	5.11E+12	7.04E+11
48	5.03E+12	5.65E+12	5.23E+12	5.30E+12	3.16E+11
72	4.46E+12	3.79E+12	4.22E+12	4.16E+12	3.39E+11
96	5.26E+12	4.34E+12	5.54E+12	5.05E+12	6.28E+11

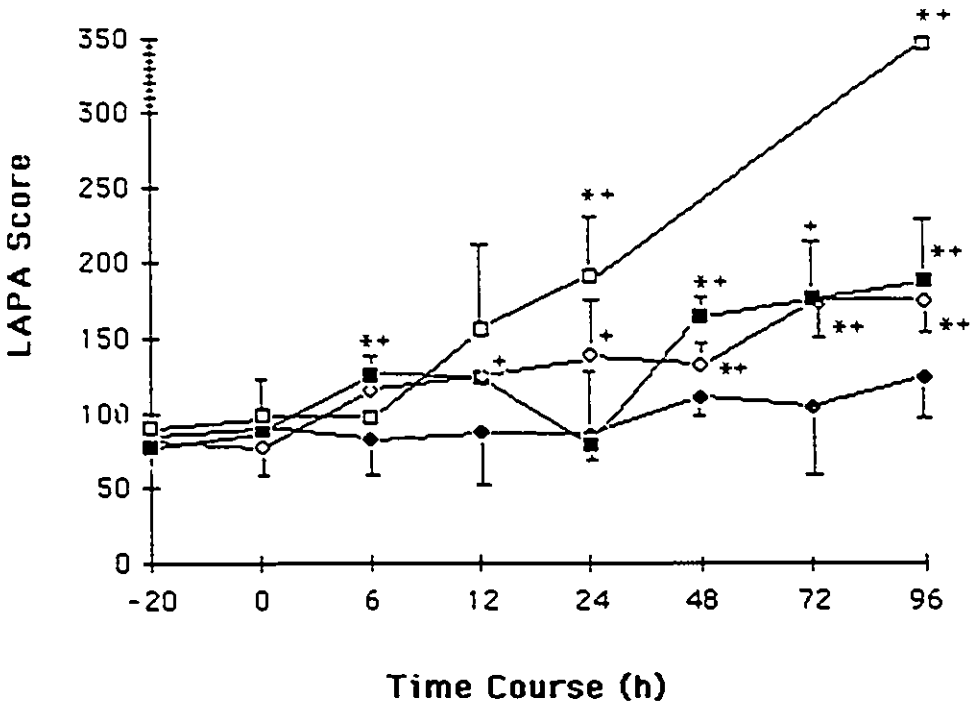
a. Total RBC counts were determined using a Coulter Counter (Model 3805).
b. Mean ± standard deviation (n = 3).

Table 5.15. Total Red Blood Cell Counts, Subchronic Experiment.

Time (h)	Total RBC (cells/L)[a]			Mean[b]	± SD
Administered Dose: 0.0 mg/kg					
	G	H	I		
-20	6.50E+12	7.00E+12	6.84E+12	6.78E+12	2.53E+11
0	7.88E+12	7.07E+12	7.86E+12	7.60E+12	4.59E+11
6	7.60E+12	6.35E+12	7.68E+12	7.21E+12	7.46E+11
12	7.67E+12	8.05E+12	7.55E+12	7.76E+12	2.58E+11
24	6.44E+12	6.53E+12	7.22E+12	6.73E+12	4.28E+11
48	6.32E+12	6.26E+12	5.99E+12	6.19E+12	1.75E+11
72	6.16E+12	6.55E+12	-	6.35E+12	2.76E+11
96	5.85E+12	6.03E+12	-	5.94E+12	1.27E+11
Administered Dose: 0.25 mg/kg					
	J	K	L		
-20	7.49E+12	7.66E+12	7.65E+12	7.60E+12	9.67E+10
0	7.20E+12	7.11E+12	7.40E+12	7.23E+12	1.48E+11
6	6.83E+12	7.41E+12	8.01E+12	7.42E+12	5.90E+11
12	7.54E+12	-	7.24E+12	7.39E+12	2.09E+11
24	4.60E+12	-	7.19E+12	5.89E+12	1.83E+12
48	6.79E+12	-	6.81E+12	6.80E+12	1.41E+10
72	6.58E+12	-	-	6.58E+12	-
96	5.49E+12	-	-	5.49E+12	-
Administered Dose: 1.25 mg/kg					
	M	N	O		
-20	5.61E+12	5.93E+12	5.78E+12	5.77E+12	1.63E+11
0	6.04E+12	6.09E+12	6.06E+12	6.06E+12	2.75E+10
6	6.47E+12	7.04E+12	6.95E+12	6.82E+12	3.06E+11
12	6.36E+12	7.44E+12	6.95E+12	6.92E+12	5.41E+11
24	6.47E+12	6.96E+12	6.67E+12	6.70E+12	2.44E+11
48	5.80E+12	5.85E+12	5.78E+12	5.81E+12	3.33E+10
72	6.01E+12	6.42E+12	5.16E+12	5.86E+12	6.42E+11
96	6.30E+12	-	5.53E+12	5.92E+12	5.44E+11

a. Total RBC counts were determined using a Coulter Counter (Model 3805).
b. Mean ± standard deviation (minimum n = 2).

Figure 5.1. Leukocyte Alkaline Phosphatase Activity, Short Course Experiment.

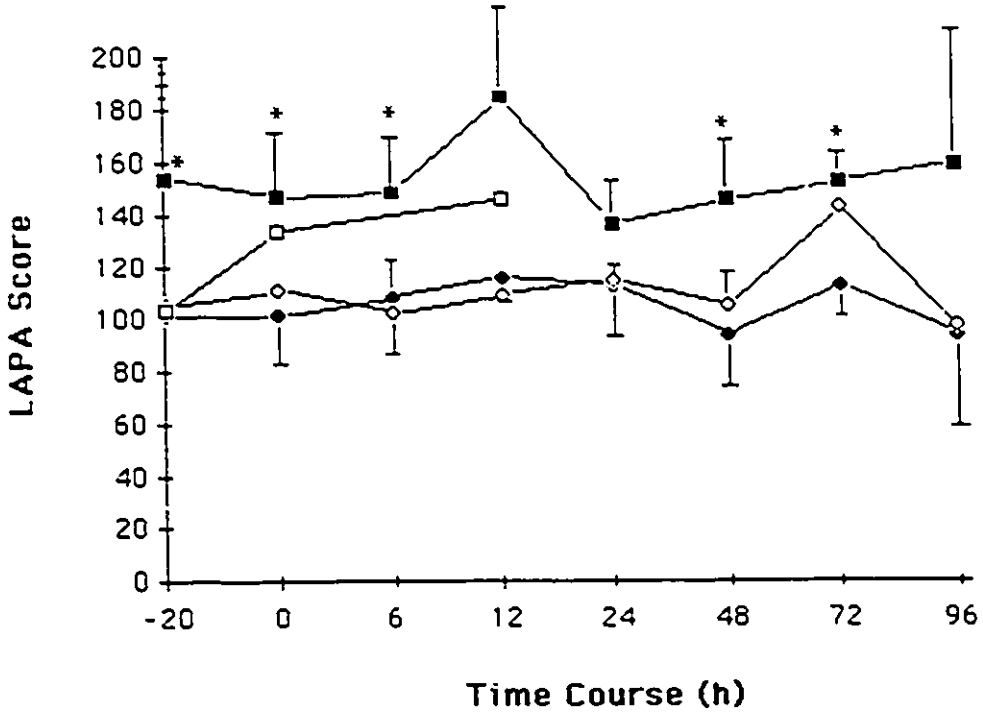


Legend to figure:

◆- 0.0 mg/kg ; ◇- 0.25 mg/kg ; ■- 1.25 mg/kg ; □- 2.5 mg/kg;

Each rat received a single IP injection of the indicated doses administered at T = 0 h. The data points represent the mean ± standard deviation, (n=3). * $p<0.05$ (unpaired t test), + $p<0.05$ (longitudinal t test). Normal LAPA scores using the fast blue base (FBB) stain range from 20 - 180 with a mean of 91. See Table 5.8.

Figure 5.2. Leukocyte Alkaline Phosphatase Activity, Subchronic Experiment.

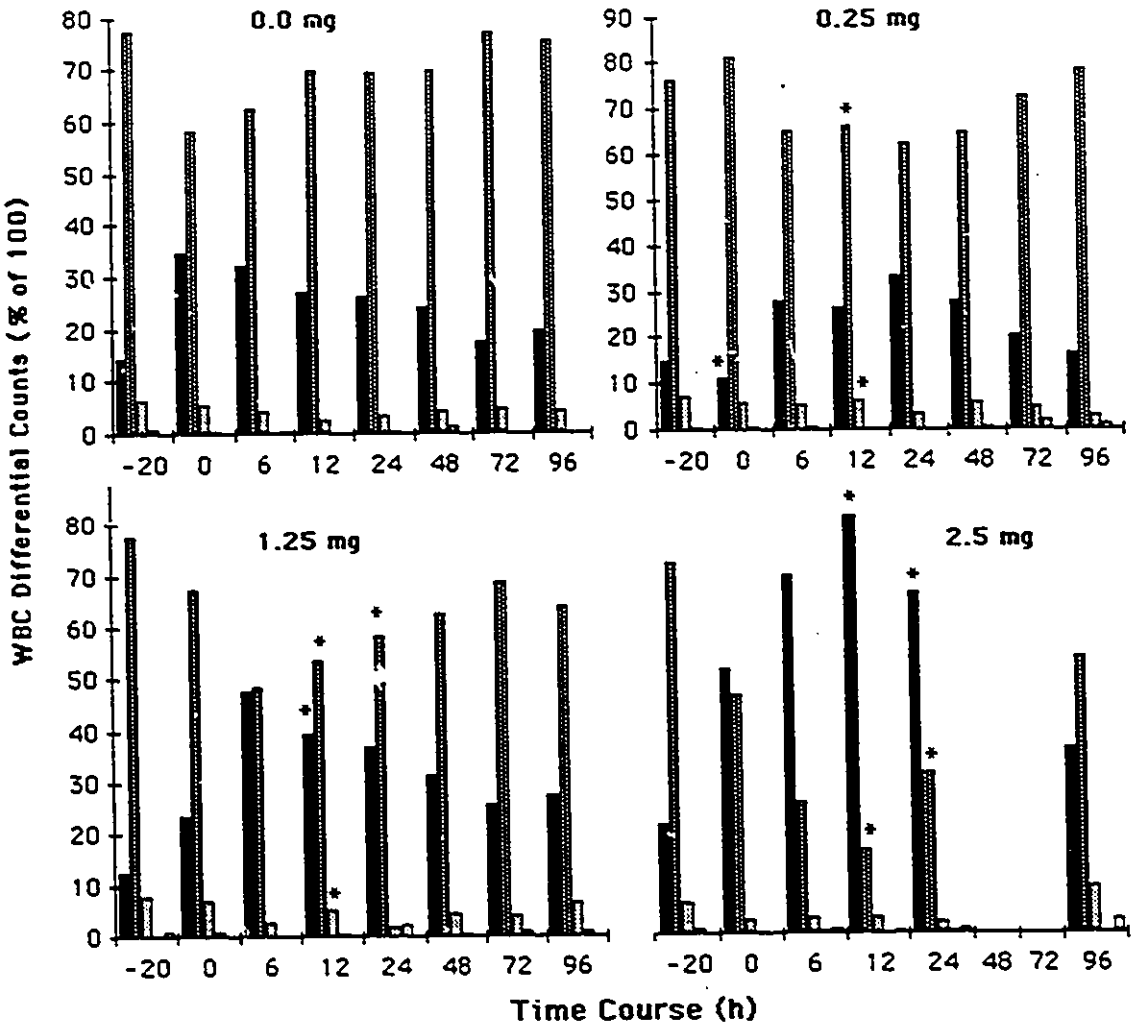


Legend to figure:

-◆- 0.0 mg/kg ; -○- 0.25 mg/kg ; -■- 1.25 mg/kg ; -□- 2.5 mg/kg;

Each rat received a total of three IP injections of the indicated doses over a 1.5 - 2.0 month period. The third injection was administered at T = 0 h The data points represent the mean ± standard deviation, (n=3). * $p<0.05$ (unpaired t test). Normal LAPA scores using the fast blue base (FBB) stain range from 20 - 180 with a mean of 91. See Table 5.9.

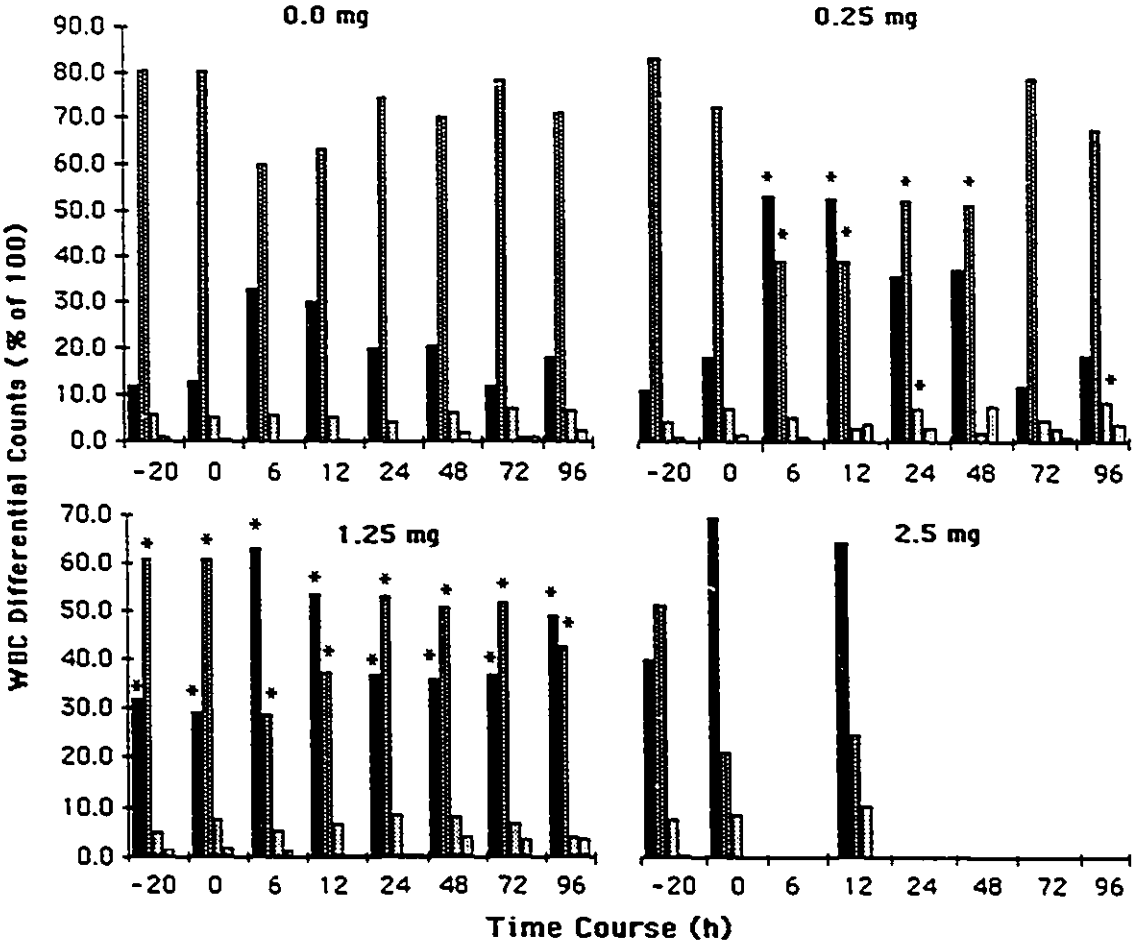
Figure 5.3. White Blood Cell Differential Counts, Short Course Experiment.



Legend to Figure:

Each treatment group received a single IP injection of the indicated dosage at T = 0h. The bar graphs represent the mean (n=3), the standard deviations are reported in Table 5.10. * $p < 0.05$ (Unpaired t test), relative to the corresponding control values.

Figure 5.4. White Blood Cell Differential Counts, Subchronic Experiment.

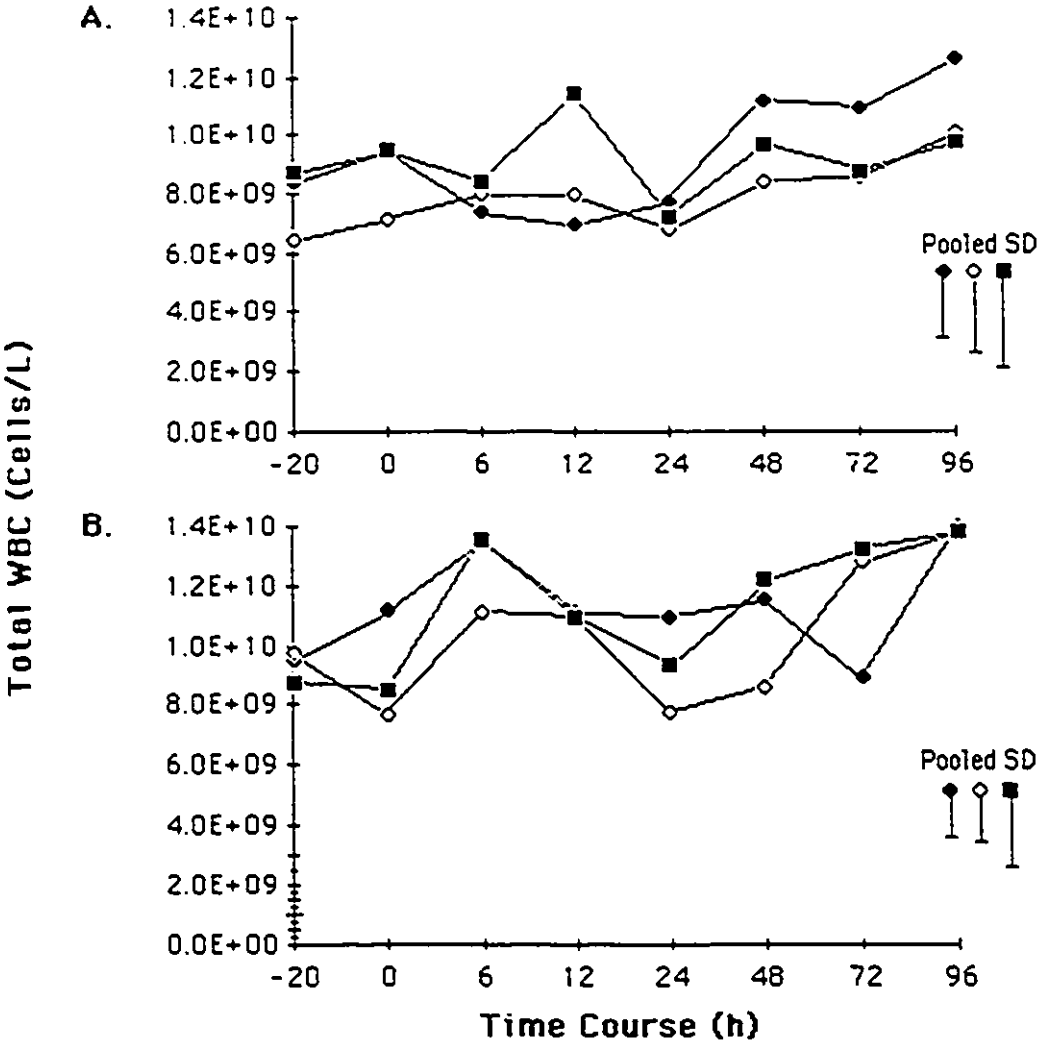


Legend to Figure:

PMN Ly Mo Eos Baso

Each treatment group received three IP injections of the indicated dosage over a 1.5 - 2 month period. The third injection was administered at T = 0h. The bar graphs represent the mean (n=3), the standard deviations are reported in Table 5.11. * $p<0.05$ (Unpaired t test), relative to the corresponding control values.

Figure 5.5. Total White Blood Cell Counts, Short Course and Subchronic Experiments.

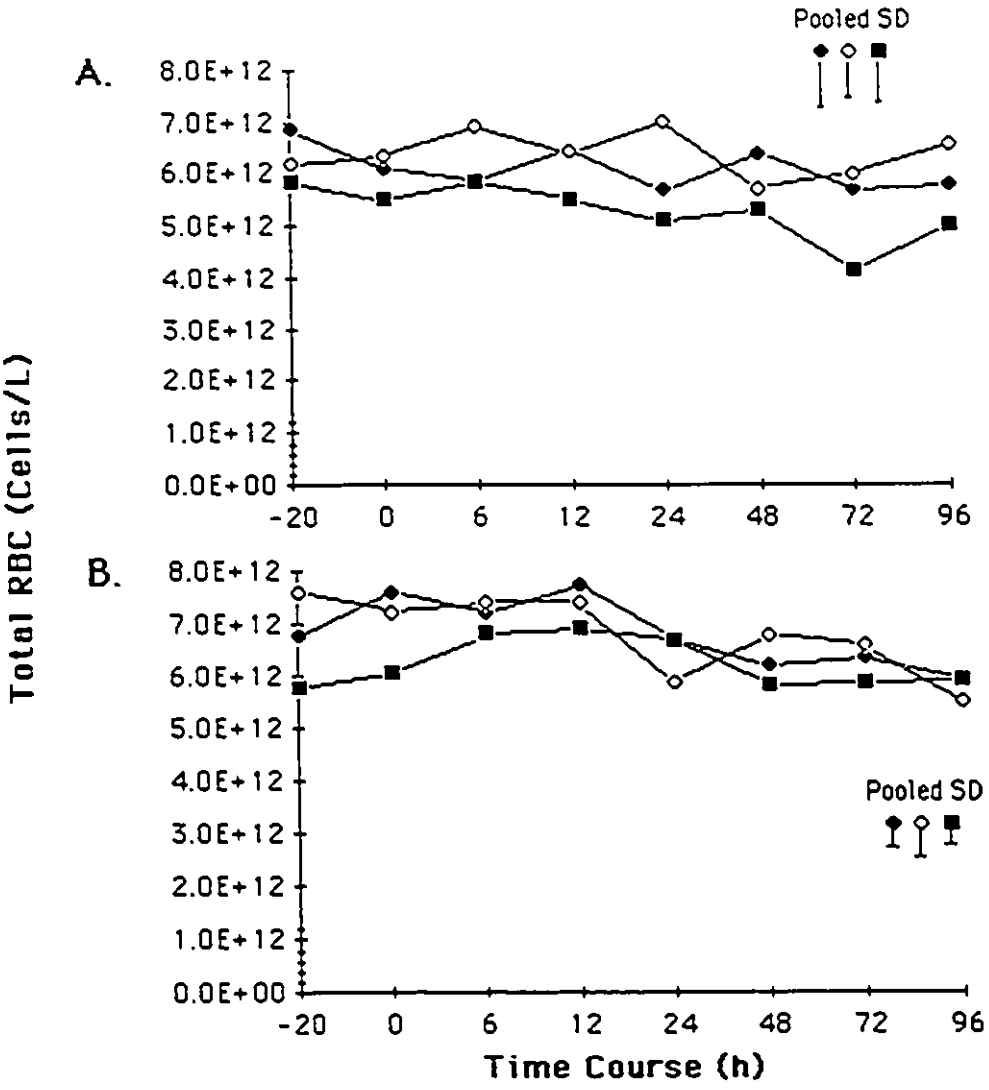


Legend to figure:

-◆- 0.0 mg/kg ; -◇- 0.25 mg/kg ; -■- 1.25 mg/kg ;

Figure A., represents the short course protocol where each rat received a single IP injection at T = 0h of the indicated doses. Figure B., represents the subchronic protocol where each rat received a total of three IP injections of the indicated doses over a 1.5-2 month period. The third injection was administered at T = 0 h. The data points represent the mean (n=3), * $p<0.05$ (unpaired t test). See Tables 5.12 and 5.13.

Figure 5.6. Total Red Blood Cell Counts, Short Course and Subchronic Experiments.

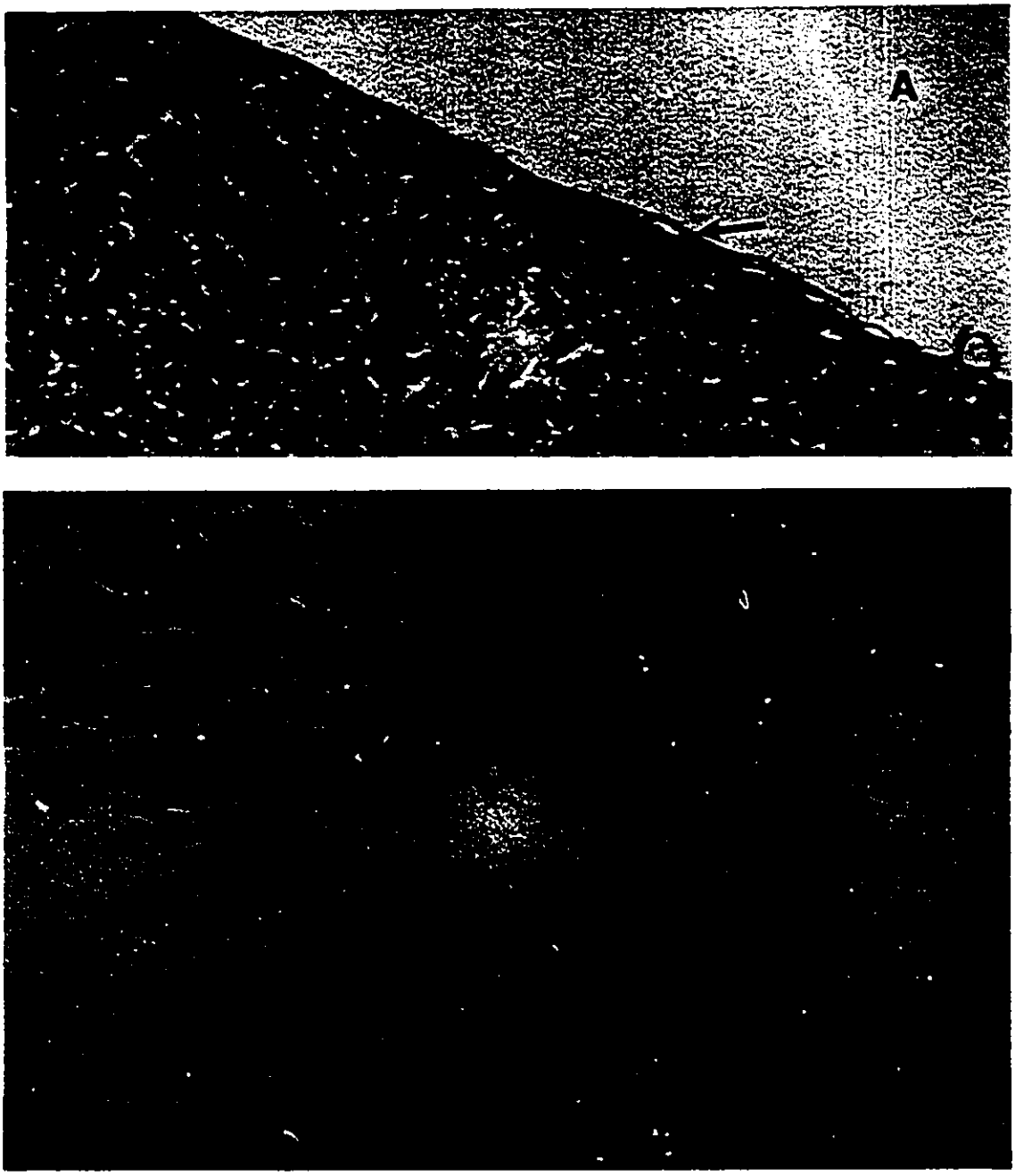


Legend to figure:

·◆- 0.0 mg/kg ; ·◇- 0.25 mg/kg ; ·■- 1.25 mg/kg ;

Figure A., represents the short course protocol where each rat received a single IP injection at T = 0h of the indicated doses. Figure B., represents the subchronic protocol where each rat received a total of three IP injections of the indicated doses over a 1.5-2 month period. The third injection was administered at T = 0 h. The data points represent the mean (n=3), * $p<0.05$ (unpaired t test). See Tables 5.14 and 5.15.

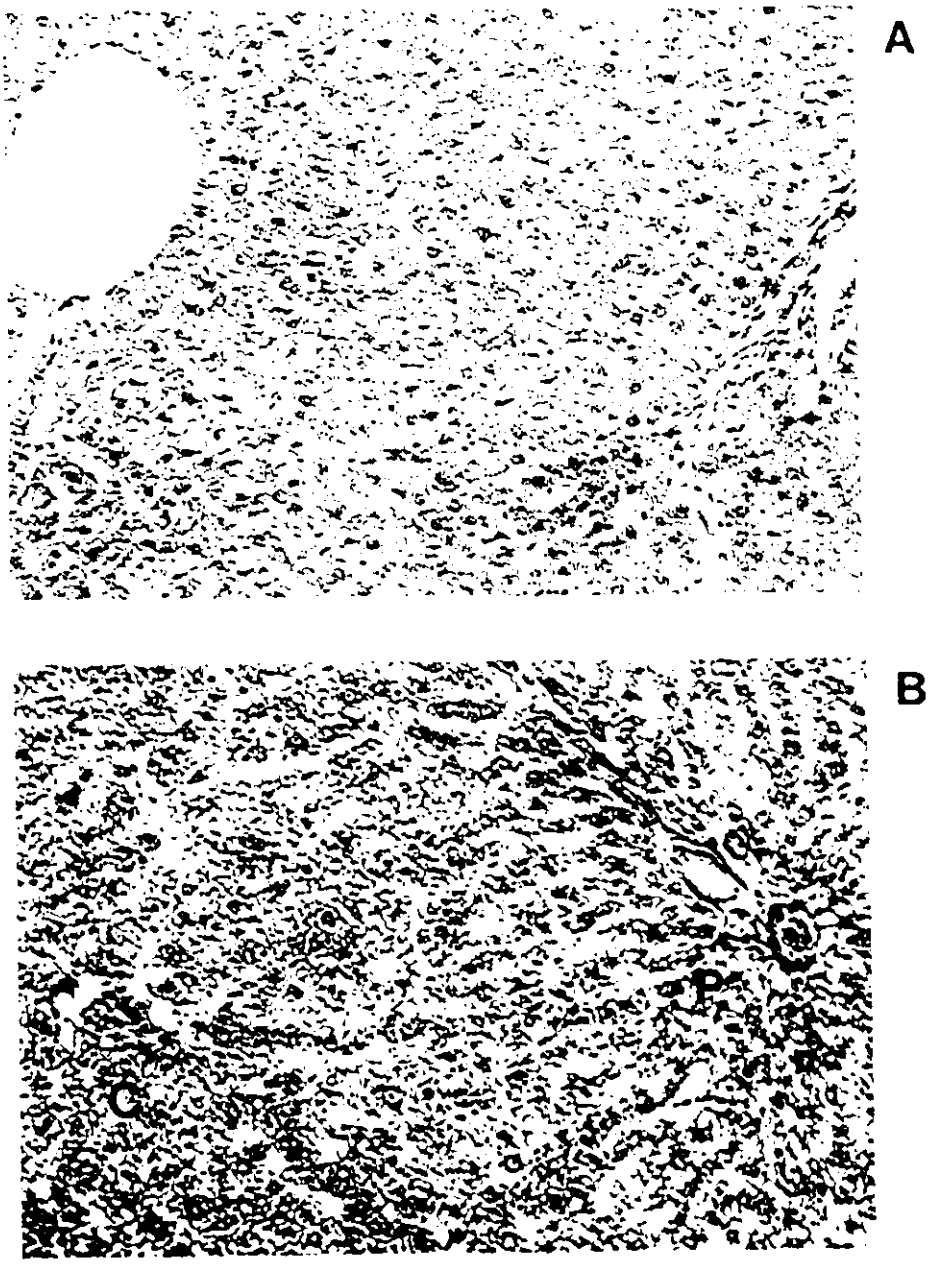
Figure 5.7. Light Micrographs of Cd-Induced Liver Capsular Fibrosis.



Legend to Figure:

Light microscopy of liver showing normal capsular membranes (plate A) from control rat and a region of capsular fibrosis (plate B) in the subchronic rat (1.25 mg $CdCl_2$/kg, IP). H&E, 40x magnification.

Figure 5.8. Light Micrographs of Control Rat Liver.



Legend to Figure:

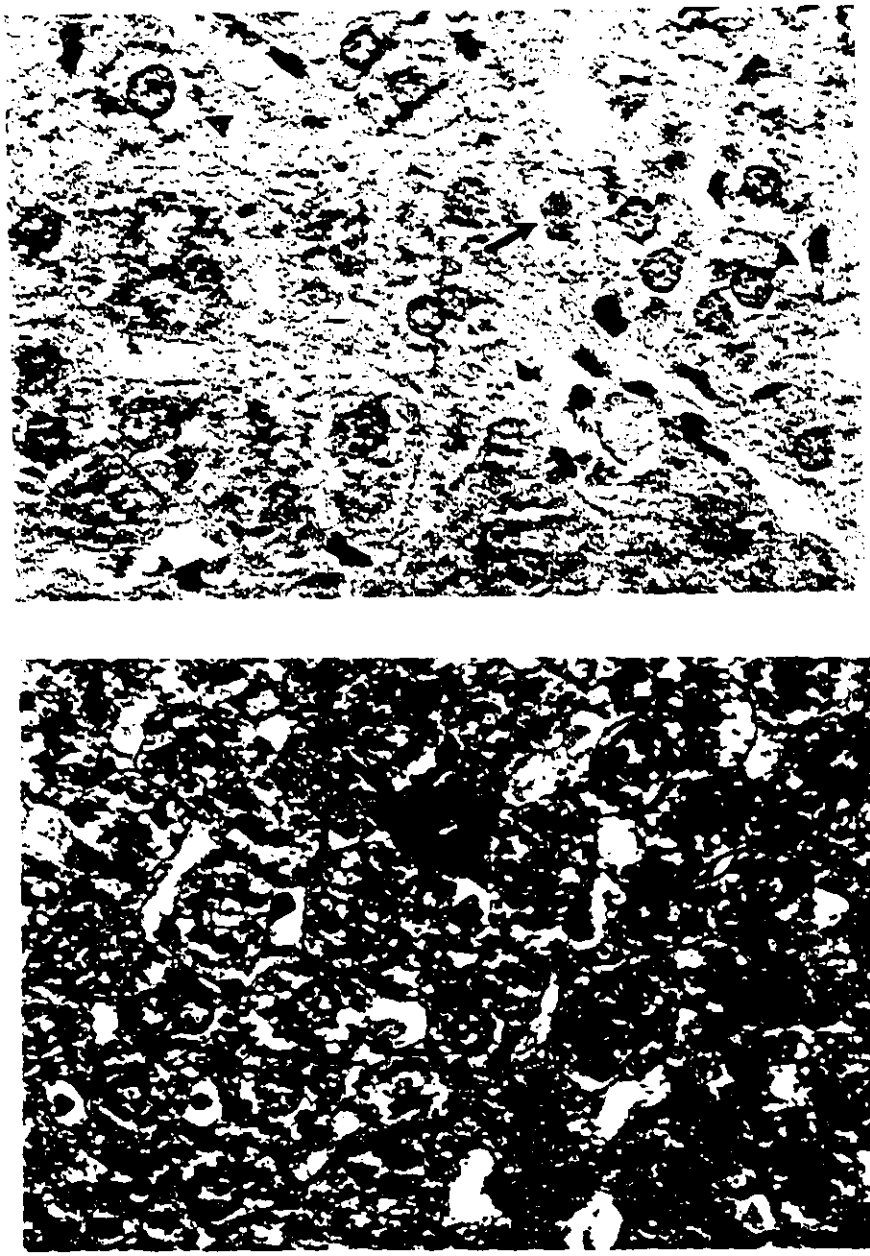
Light microscopy of normal rat liver architecture. Compared to plate A (H&E), plate B (CAB) features a portal triad (P) and central vein (C). Magnification 175 x.

Figure 5.9. Light Micrographs of Degenerated Hepatocytes from a Subchronic Rat.

Legend to Figure:

Light microscopy showing small clusters of degenerated hepatocytes (arrows) near the central vein from the subchronic rat (1.25 mg $CdCl_2$/kg, IP). H&E (plate A) and CAB (plate B) stains; magnification 550 x.

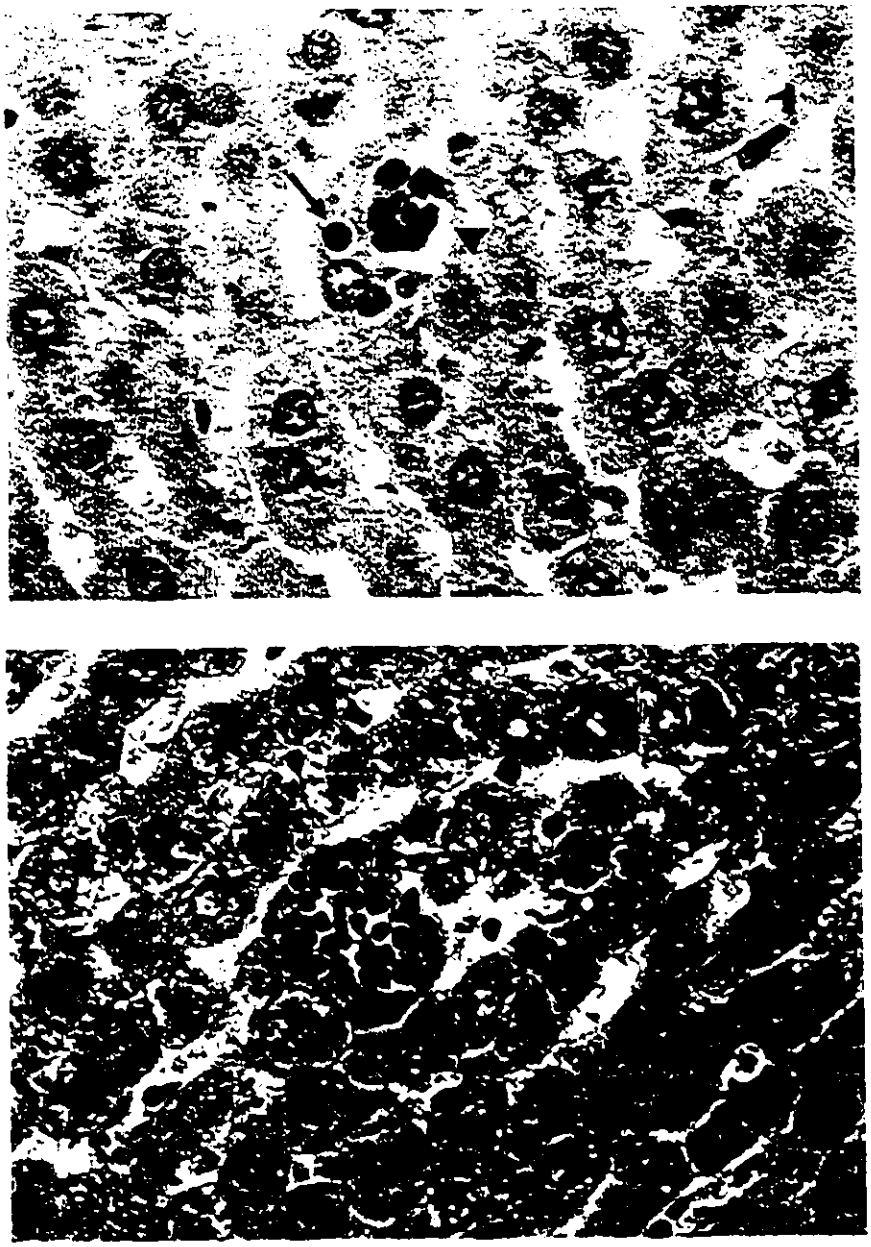
Figure 5.10. Light Micrographs of Vacuolated Hepatocytes from a Subchronic Rat.



Legend to Figure:

Plate A shows regions of vacuolated hepatocytes (arrow head) and dense cytoplasmic inclusions (arrow) in the subchronic rat (1.25 mg $CdCl_2$/kg, IP); H&E, magnification 630 x. Plate B (from the same rat) also shows vacuolated cells and a degenerate hepatocyte (arrow); CAB, 630 x

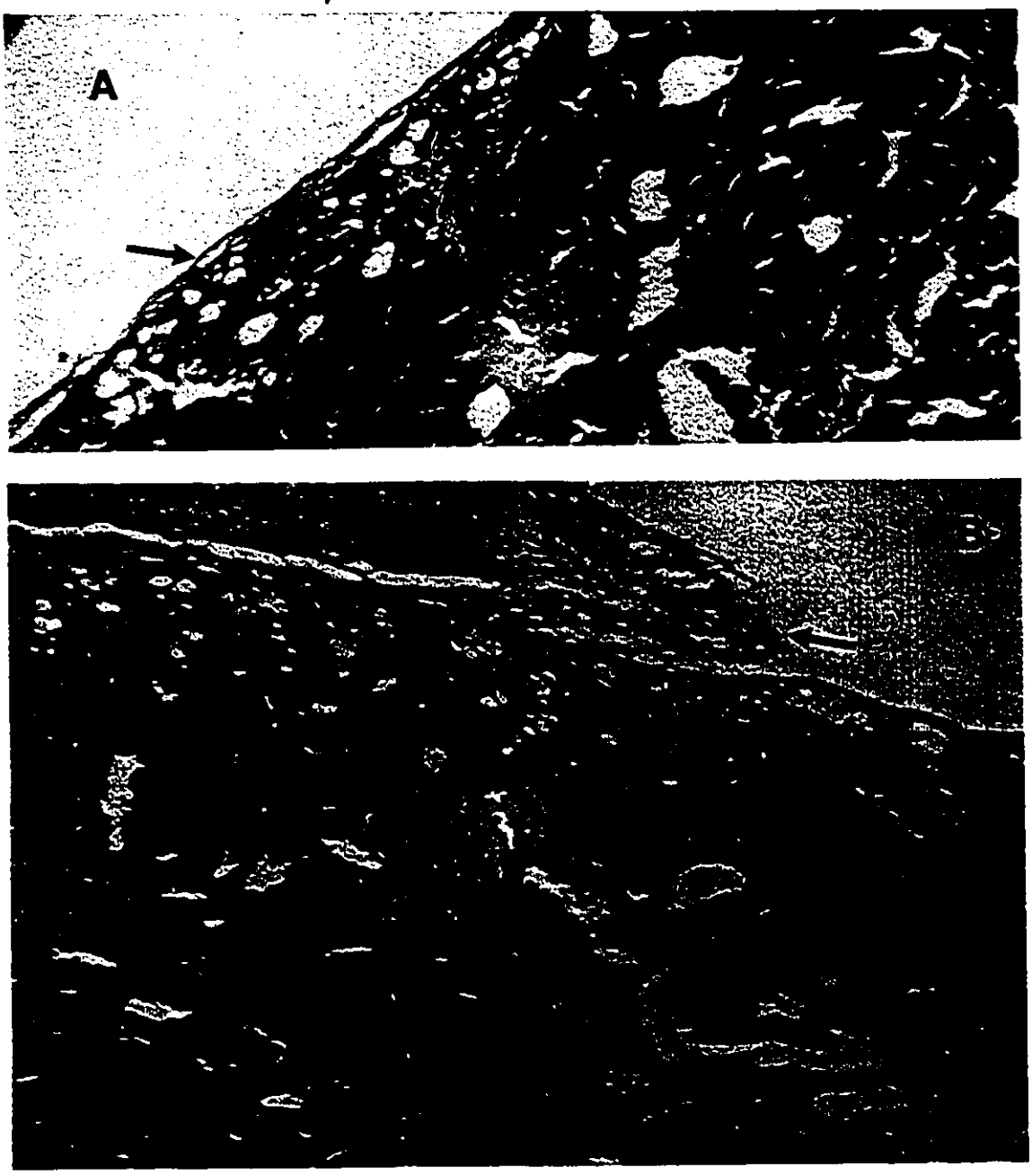
Figure 5.11. Light Micrographs of Cd-Induced Hemopoiesis in the Liver from a Subchronic Rat.



Legend to Figure:

Hemopoiesis (arrow head) and dense cytoplasmic inclusions (arrow) were seen in the liver from the subchronic rat (1.25 mg $CdCl_2$/kg, IP); H&E, magnification 630 x (plate A). Plate B shows an island of erythropoiesis (arrow head) amid vacuolated hepatocytes from the same rat; CAB, magnification 550 x.

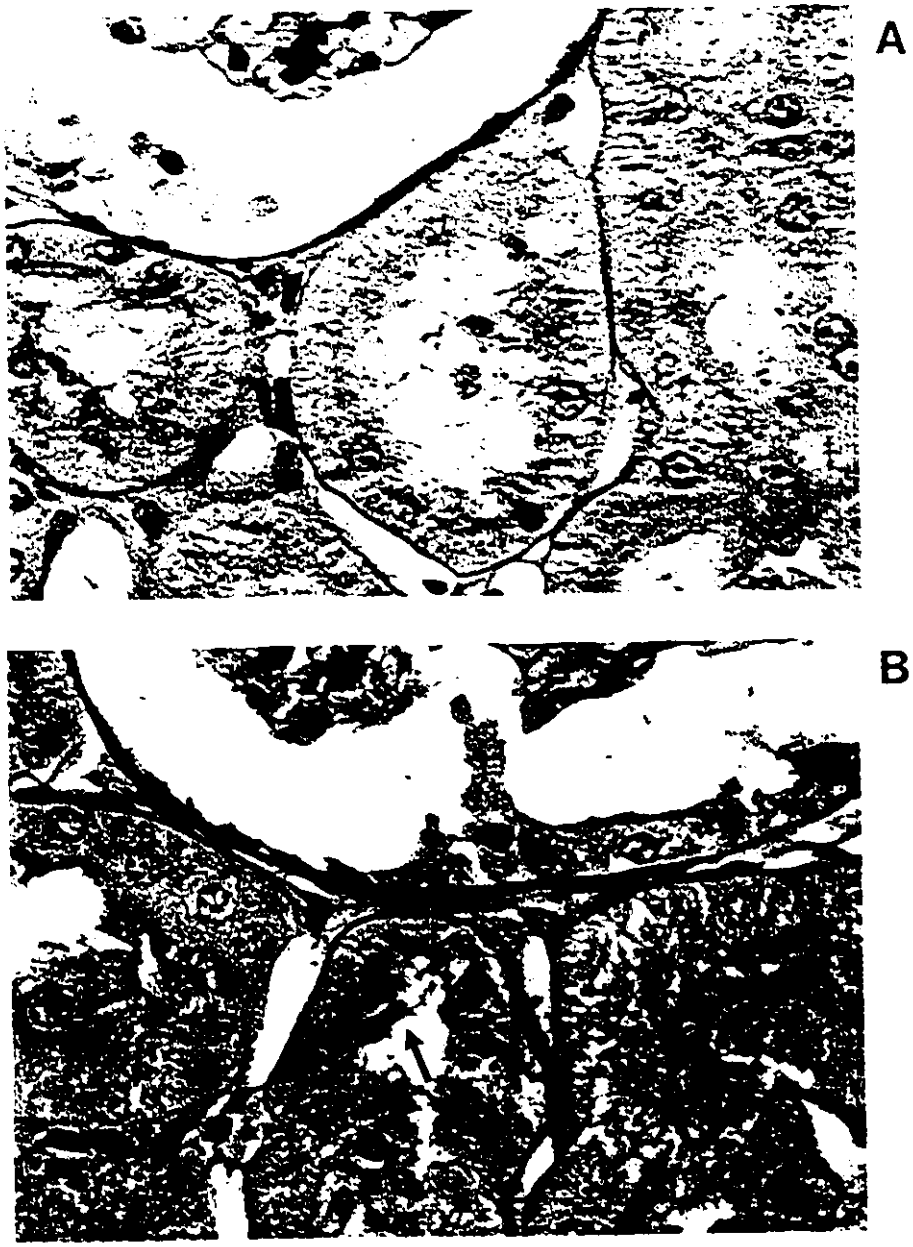
Figure 5.12. Light Micrographs of Cd-Induced Renal Capsular Fibrosis.



Legend to Figure:

Light microscopy of kidney showing normal capsular membranes (plate A) from control rat. Plate B shows a region of capsular fibrosis in the subchronic rat (1.25 mg $CdCl_2$/kg, IP); H&E, 40x magnification.

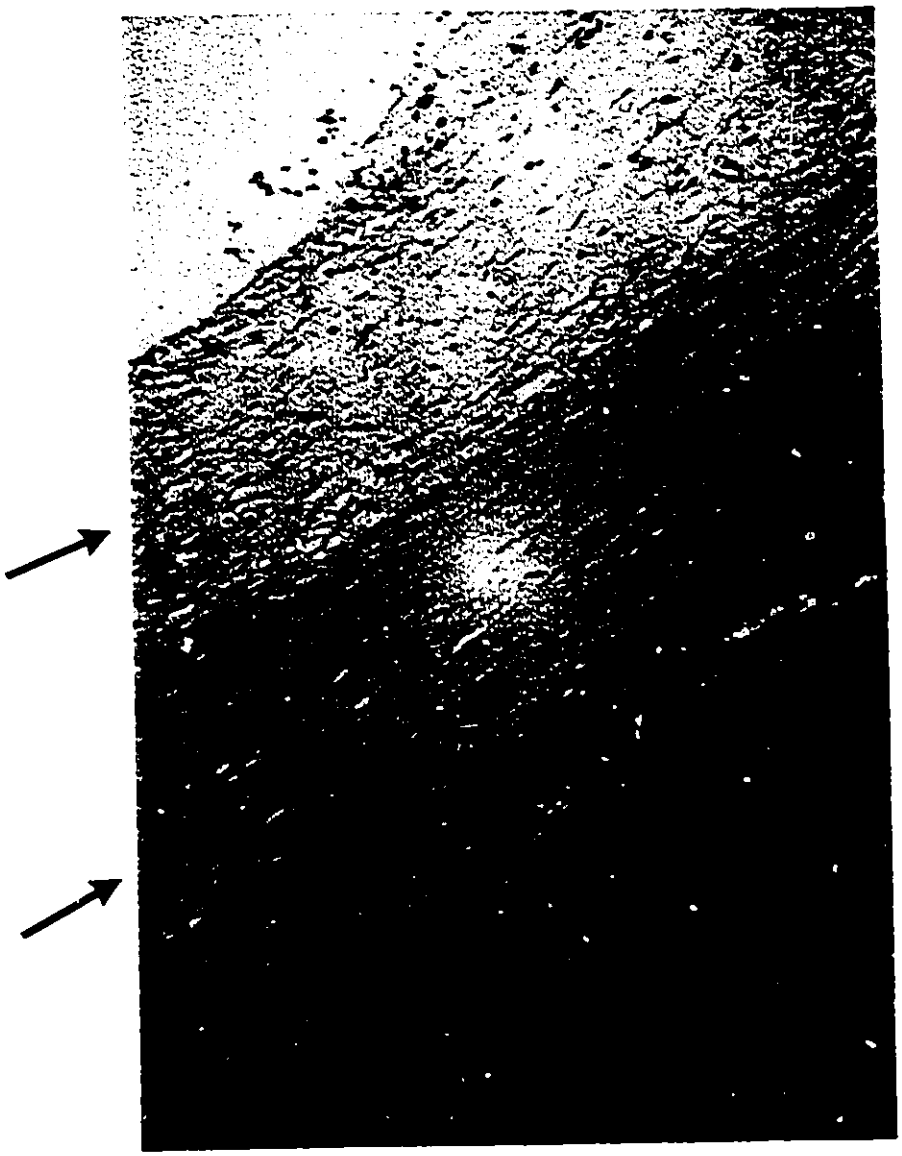
Figure 5.13. Light Micrographs of Renal Tubules from Control and Subchronic Cd-Exposed Rats.



Legend to Figure:

Light microscopy of normal proximal tubules from control rat kidney (plate A). Plate B shows degeneration of the brush border and nuclear pyknosis (arrow) from the subchronic rat (1.25 mg $CdCl_2$/kg, IP). PAS stain, magnification 630 x.

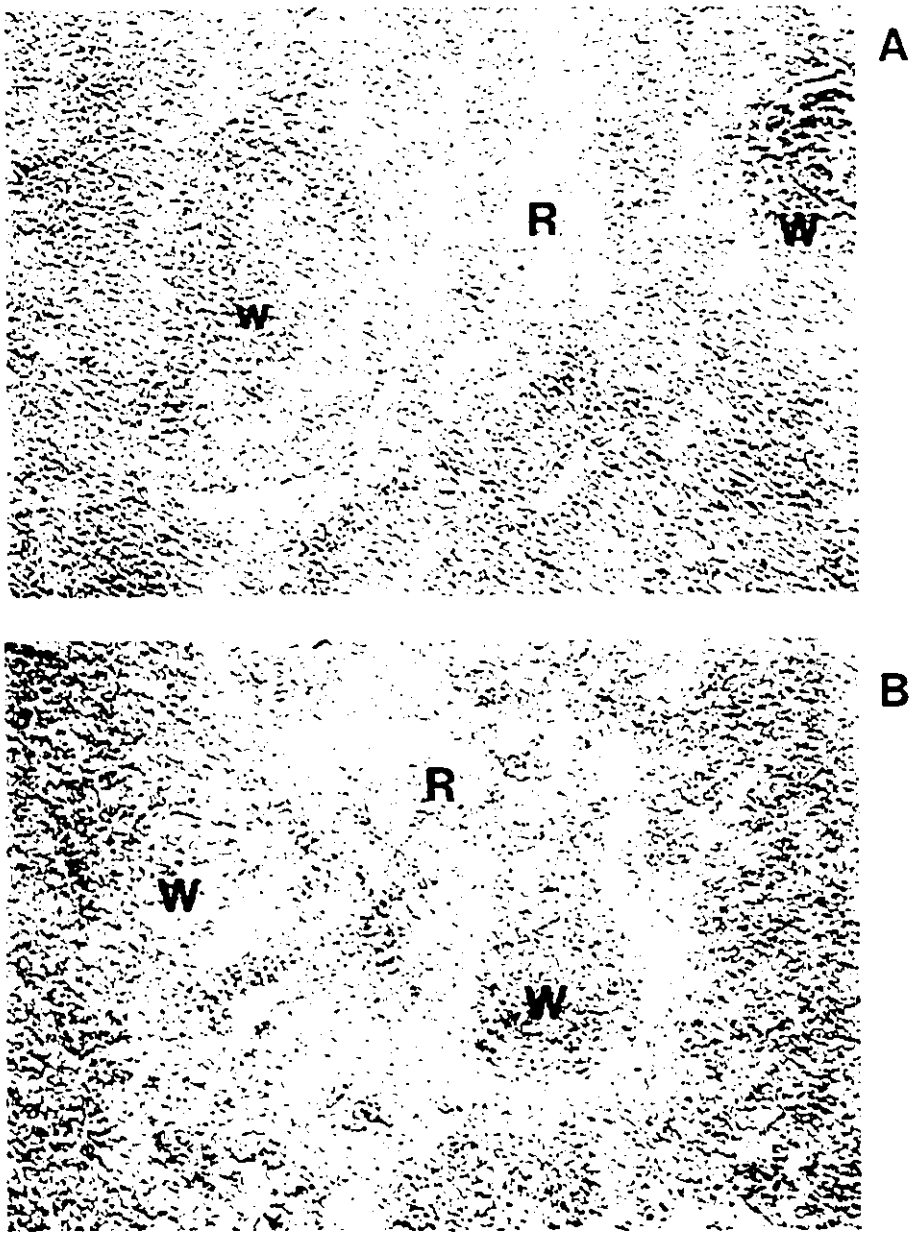
Figure 5.14. Cd-Induced Capsular Fibrosis of the Spleen in Rat.



Legend to Figure:

Light microscopy of spleen showing regions of capsular fibrosis (see arrows) in the subchronic rat (1.25 mg $CdCl_2$/kg, IP), H&E, 40 x magnification

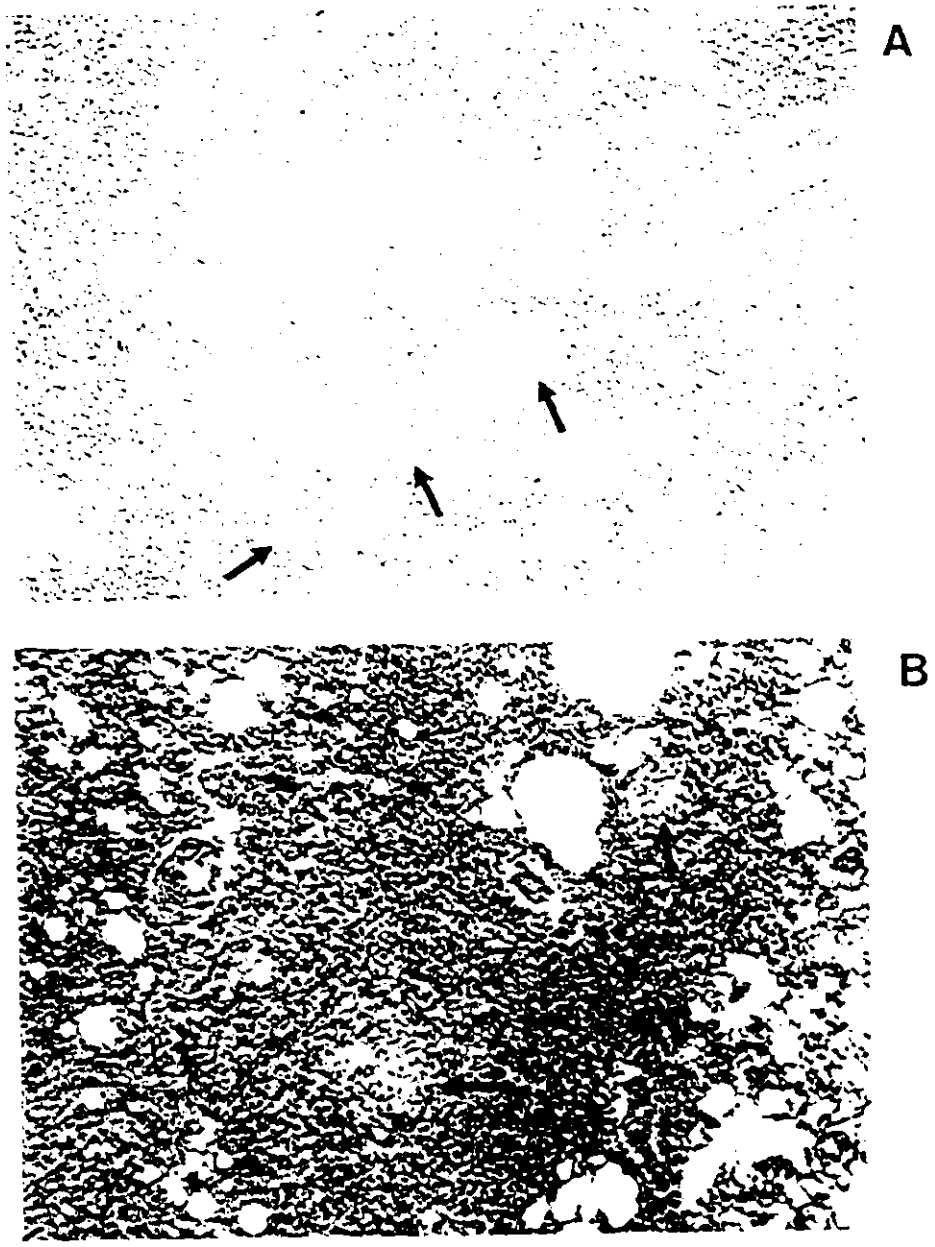
Figure 5.15. Light Micrograph of Spleen from Control and Subchronic Rats.



Legend to Figure:

Normal spleen tissue showing white (W) and red pulp (R) regions in the control rat (plate A). Plate B depicts the reduced size of malpighian corpuscles of white pulp and increased cellularity of red pulp in the spleen from the subchronic rat (1.25 mg $CdCl_2$/kg, IP), H&E, magnification 75 x.

Figure 5.16. Light Micrographs of Lung from a Subchronic Rat.



Legend to Figure:

Small nodules or aggregates (arrow) were observed probably within the blood vessel of the lung from the subchronic rat (1.25 mg $CdCl_2$/kg, IP) (plate A), H&E, magnification 75 x. In the same animal, the two nodules stained green (plate B) indicate fibrosis (arrow) in the lung, trichrome, magnification 100 x

CHAPTER 6:

6. INTEGRATED DISCUSSION

6.1. Introductory Comments

In this final chapter of the thesis, the results presented and discussed in Chapters 3 to 5 are integrated by the use of a dynamics model for the absorbed Cd and induced CdMT in the rat. To facilitate this, the figures presented in Chapters 3 and 4 are combined for easier reference (see Figures 6.1 - 6.7). The dynamics model depicted in Figure 6.8 constitutes a composite of concepts and ideas suggested by others (Kjellstrom and Nordberg, 1978; Lauwerys, 1983a;b; Petering et al., 1984; Kjellstrom and Nordberg, 1985; Elinder, 1985a,b; Petering and Fowler, 1986; Friberg et al., 1986a; Goyer, 1991; Matsuno et al., 1991b), but presented in a manner allowing integrated discussion of the data obtained in the present study.

6.2. Integration and Interpretation of the Results

6.2.1. Absorption

It may be assumed that the bulk of the $CdCl_2$ injected into the peritoneal cavity is absorbed by the highly vascularized peritoneal membranes permitting transport into the circulatory system (Figure 6.8). The plasma Cd data summarized in Figure 6.3 suggest that absorption was very rapid since peak concentration occurred at the first time point (T = 6 h) after exposure.

The pathological examination at necropsy provided some evidence of histological damage to the capsular membranes of the organs located in the peritoneal cavity due to $CdCl_2$ exposure in both the short course (medium and high doses) and subchronic (all doses) experiments (see Section 5.4.5). Contact tissue damage (cytotoxicity) due to Cd salts has been reported by other

workers (Duval and Grubb, 1986; Waalkes et al., 1989).

6.2.2. Distribution (Blood Compartment; see Figure 6.8)

A perusal of the Cd concentration data summarized in Figures 6.3 and 6.5 clearly indicate that the distribution from plasma into cellular compartments depends on the the experimental protocol (short course versus subchronic). As for plasma, Cd appeared rapidly in the WB compartment. Comparison of the RBC Cd time-course (Figure 6.3) with that of MT (Figure 6.4) strongly suggests that the appearance of Cd in RBCs during the first 24 h, is due to direct uptake, while the subsequent increase appears to be due to CdMT. Interestingly, in the subchronic experiments there is no increase in RBC Cd after the last injection suggesting that the newly absorbed Cd is unavailable for cellular uptake. The similarity of the enhanced 'steady state' levels observed for both Cd (Figure 6.3) and CdMT (Figure 6.4) may be interpreted that the measured Cd is bound to MT. Presumably Cd is also present as CdMT in lymphocytes in the subchronic animals, while PMNs showed no evidence of Cd accumulation (Figures 6.5 and 6.6). However, there is some indication of transient elevation of Cd in lymphocytes and monocytes in the short course protocol. It is not immediately evident from the results what form Cd was in for these two cell types (Figures 6.5 and 6.6). Examination of Cd and CdMT levels in plasma provided indirect evidence of the involvement of the liver in Cd distribution and metabolism. The disappearance of plasma Cd and appearance of plasma MT suggests redistribution of CdMT from liver to plasma (Figures 6.3, 6.4, and 6.8). These results are in agreement with earlier studies (Cherian and Shaikh, 1975; Bremner and Morrison, 1986; Bremner et al., 1987; Jin et al., 1987c; Wang et al., 1993).

6.2.3. Distribution (Tissues; see Figure 6.8)

The data clearly show the movement of Cd from the plasma (Figure 6.3) into the liver and kidney (high dose), and to a lesser extent to the other tissues (Figure 6.1). It appears that the plasma clearance into the tissues is slower in

the short course animals, suggesting the basal tissue MT levels were insufficient to bind and remove the administered dose during the first 24 h (Figures 6.3 - 6.4). By contrast, the rapid removal of Cd from the subchronic animals and the immediate peaking of plasma MT presumably indicate that the overall induction levels in the tissues (especially liver) were sufficient for complete Cd clearance from the blood compartment (Figures 6.1 - 6.4). The elevation of Cd (Figure 6.1) and that of CdMT (Figure 6.2) in the tissues are complementary in time and this observation agrees with earlier work (Onosaka and Cherian, 1981; Eaton and Toal, 1982; Onosaka et al., 1984; Shaikh and Tohyama, 1984; Waalkes et al., 1985; EHC,1992a).

The RBC Cd and CdMT data (Figures 6.3 - 6.4), as well as the tissue Cd data (Figure 6.1), suggest that the hemopoietic tissues (bone marrow and spleen) did accumulate sufficient levels of Cd to induce MT in RBC and presumably WBC progenitor cells (see Figure 6.8). Evidence for MT induction in the spleen was not highly significant (Figure 6.2), although there was a mild increase. The origin of MT in this organ, be it from splenocytes, hemopoietic cells, or damaged blood cells, could not be determined in this study. The tissue pathology suggests that there was insufficient MT induction in the spleen to prevent cell necrosis (see Chapter 5), pointing to RBCs as the major Cd source.

6.2.4. Elimination (see Figure 6.8)

Examination of the fecal Cd data for both experimental protocols (Figure 6.7) suggests that the both Cd and presumably CdMT are eliminated via the bile and feces. The relative amount of Cd was greater in the feces of the short course animals than that observed in the subchronic animals, suggesting that with increasing total body MT induction there is a reduction of fecal elimination of Cd. Although the urinary Cd data is very limited, the subchronic levels appeared to be higher by about a factor of approximately 1.5 (Figures 3.26 - 3.27). Together these results, along with the knowledge of tissue Cd and CdMT levels, suggest that Cd and CdMT is eliminated via the feces and urine, but that

after MT induction, a shift to greater urinary excretion and tissue deposition seems to occur, supporting earlier conclusions (Klaassen and Kotsonis, 1977; Lauwerys, 1983a; Lauwerys, 1983b; Klaassen, 1985; Friberg et al., 1986a).

6.3. Conclusions

The jugular cannulation approach developed for sequential blood sampling has both advantages and disadvantages. Body samples can be collected over a 96 h time period from the same animal, thereby reducing inter-animal variation. The dynamics of Cd uptake and distribution and Cd-induced MT synthesis can thus be assessed in a single animal and be linked to the corresponding tissue results. The main disadvantage is that duplicate sampling at a specific time point is limited by the volume of blood that can be drawn. However, this limitation is partially compensated by the ability to collect many samples over an extensive time period permitting longitudinal statistical comparisons with fewer animals. Another limitation is that the sample collection period is short compared to the time involved in chronic exposures.

Although some inter-animal and intra-animal variation in metabolic response to saline or Cd exposure is to be expected, the variation observed no doubt also reflected the limitations in the analytical protocols (CSA, EAAS), specifically related to small sample size and losses during cell isolation procedures. Clearly, the determination of MT in WBCs was limited by the sensitivity of the CSA protocol employed.

The data obtained support the hypothesis that Cd initially is circulated primarily to the liver for incorporation into MT (Kjellstrom and Nordberg, 1978; Kjellstrom and Nordberg, 1985). In both experimental protocols there was no strong evidence that Cd (or CdMT) was preferentially transferred to the kidneys. It is believed that in tissues the CdMT complex undergoes degradation with release of Cd^{2+} to the surrounding tissues resulting in further MT induction or cellular toxicity (Friberg et al., 1986a; Goyer, 1991). The turnover time for CdMT

in the tissues has been documented to be between 1 and 5 days (Kotsonis and Klaassen, 1981; Monia et al., 1986). It is believed that the process of Cd turnover takes place with little consequence until saturation of MT occurs and cellular damage results (Lauwerys 1983a). Tissue damage was especially evident in the kidneys, liver and spleen in the present study.

The evidence presented in this thesis clearly establishes Cd in RBCs and whole blood as reliable short- and long-term indices of exposure and possible body burden. MT levels in plasma and RBCs constitute similar markers. Further, Cd in lymphocytes shows promise as a long-term indicator and in monocytes as a short-term index of exposure (Enger et al., 1983; Koizumi et al., 1987; Harley et al., 1989). There is some evidence of MT induction in monocytes (short-term) and in lymphocytes (short- and long-term) in response to Cd exposure, although improvements in CSA sensitivity are necessary in order to make this a reliable analytical method. It is clear that the time of sampling for Cd and MT is critical and may help to distinguish between current and long-term (body burden) contributions, depending on the blood compartment. The short-term Cd accumulation and MT induction in the WBCs and the relatively long-term response in RBCs correlates with cell physiology, function, and life span.

An assessment of the tissue pathological and histological changes indicated that acute Cd toxicity occurred in the short course medium-dose rats (1.25 mg/kg) with negligible changes for the low dose rats (0.25 mg/kg). Enhanced Cd cytotoxic effects were observed in the subchronic medium-dose rats (1.25 mg/kg) and to a lesser degree in the low dose rats (0.25 mg/kg). In general, the subchronic rats exhibited more pronounced pathological changes than those in the short course experiment. The pathologic and histologic observations suggested that preexposure to Cd at the doses tested did not afford protection against the toxic effects of additional Cd exposure.

Figure 6.1. Cd Concentration in the Short Course and Subchronic Organs.

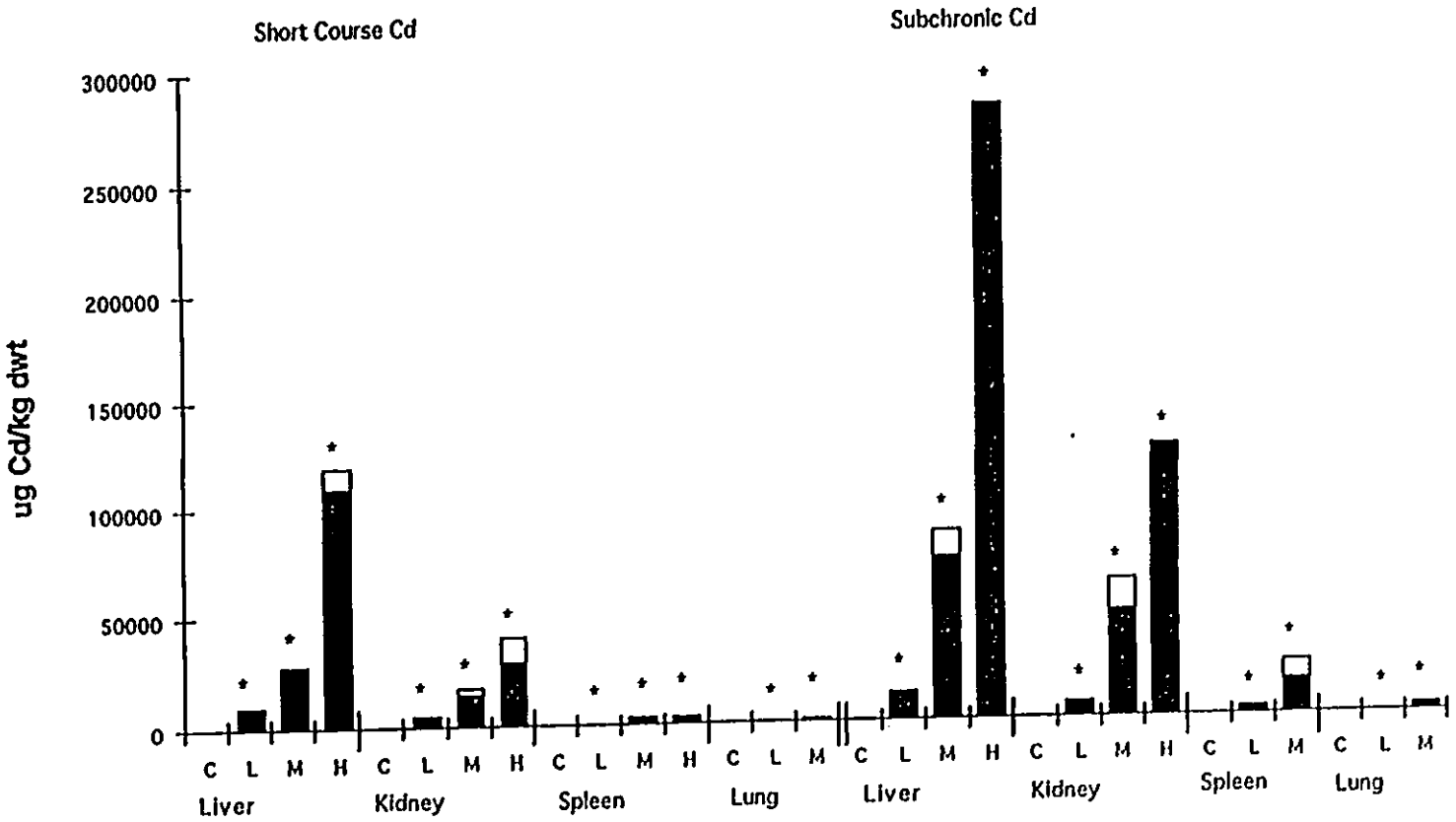


Figure Legend:
C (control), L (low, 0.25 mg Cd/kg), M (medium, 1.25 mg/kg), H (high 2.5 mg/kg).
Each rat received a single IP injection at T=0h. The bar graphs represent the mean ± standard deviation (n=3); * $p \leq 0.05$.

Figure 6.2. MT Concentration in the Organs from Short Course and Subchronic Cd Exposed Rats.

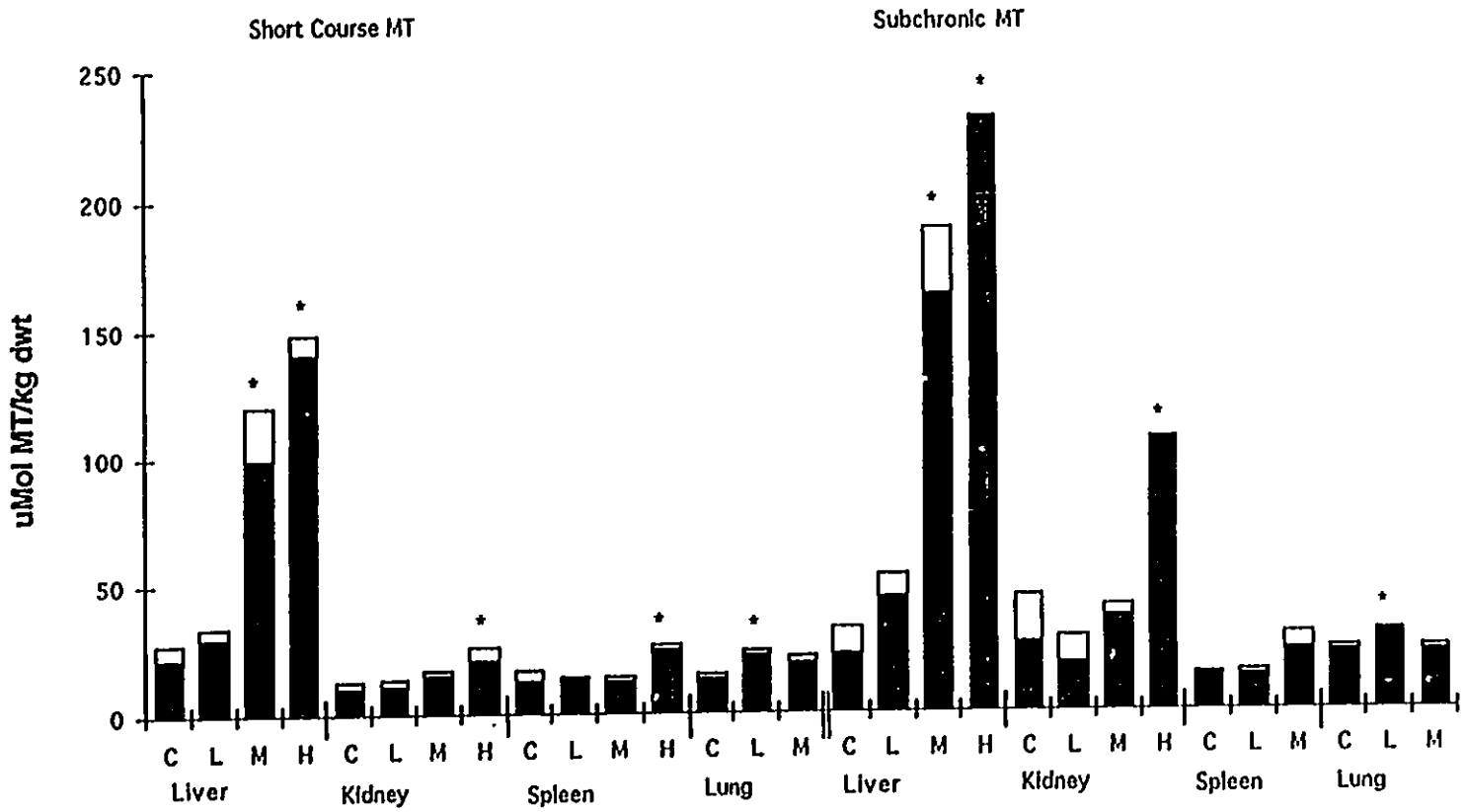


Figure Legend:
C (control), L (low, 0.25 mg Cd/kg), M (medium, 1.25 mg/kg), H (high 2.5 mg/kg).
Each rat received an IP injection at T=0h. The bar graphs represent the mean ± standard deviation (n=3); * $p \leq 0.05$.

Figure 6.3. Cd Concentration in the Blood, Plasma, and RBCs from Short Course and Subchronic Rats.

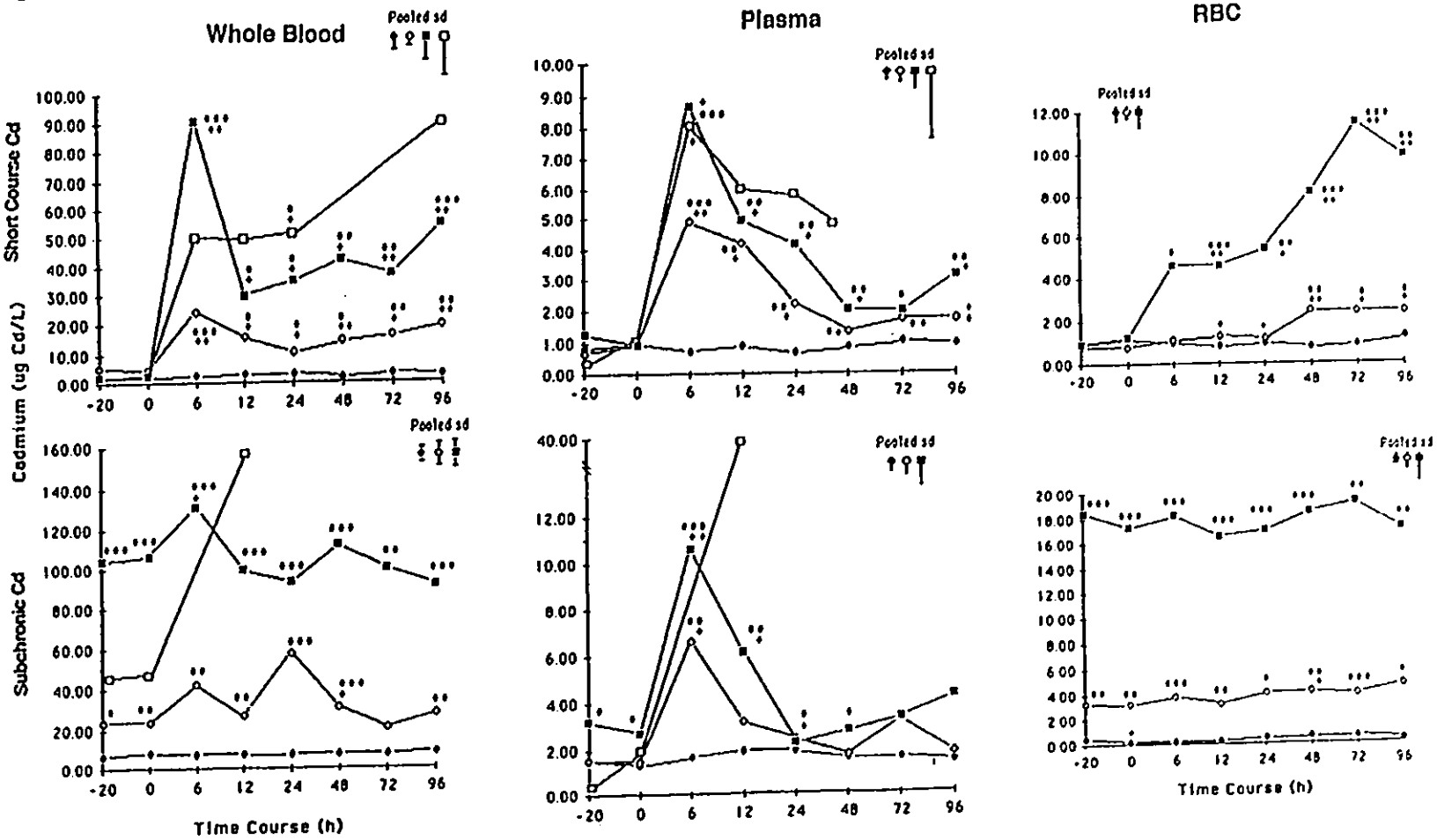


Figure Legend:
◆ (control), ◇ (low, 0.25 mg Cd/kg), ■ (medium, 1.25 mg/kg), □ (high, 2.5 mg/kg).
Each rat received an IP injection at T=0h. The data points represent the mean (n=3) and pooled standard deviations (SD) are shown as bars;
* $p \leq 0.05$ unpaired t test, + $p \leq 0.05$ longitudinal paired t test.

Figure 6.4. MT Concentration in the Plasma and RBCs from Short Course and Subchronic Cd Exposed Rats.

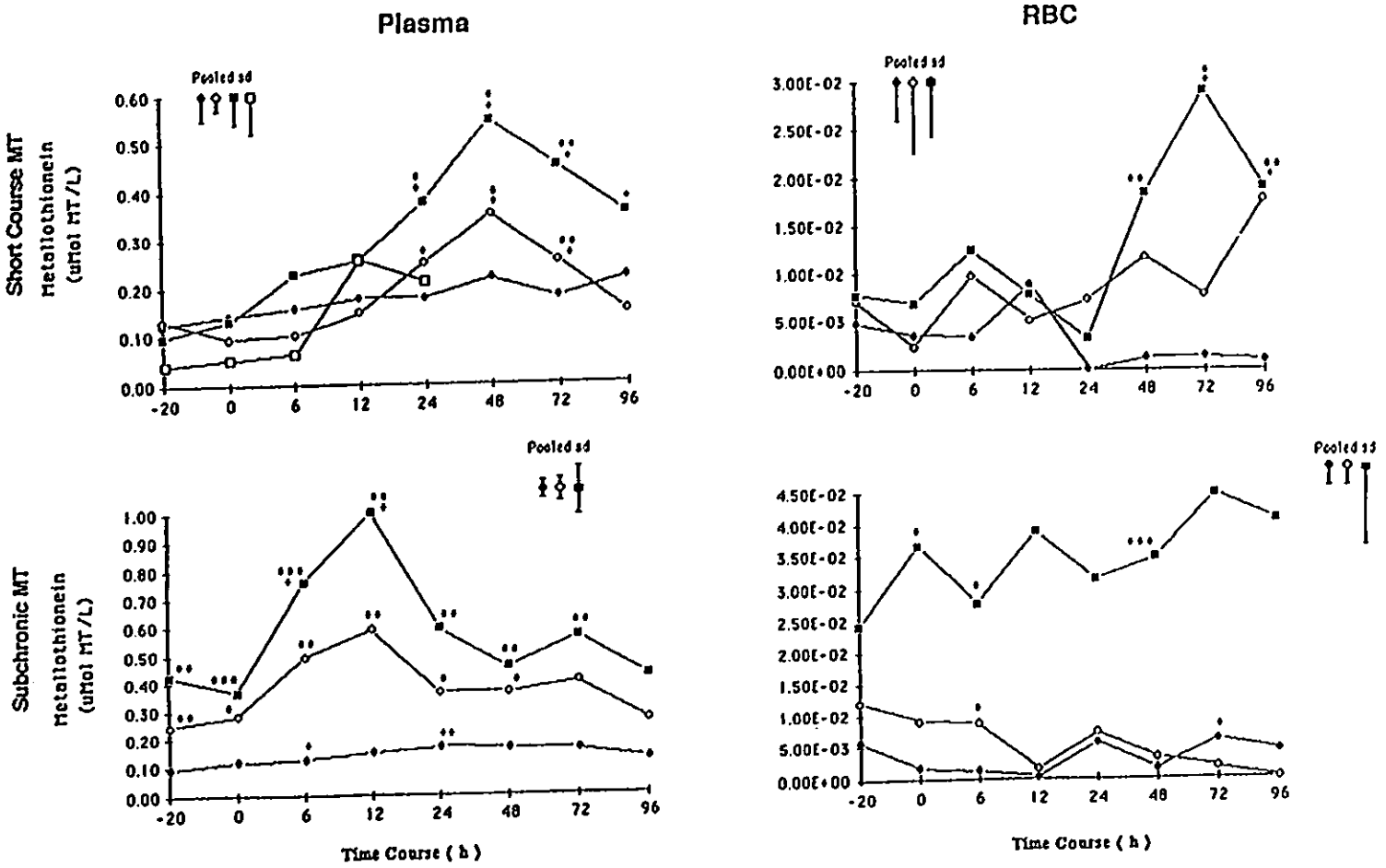


Figure Legend:

(control), (low, 0.25 mg Cd/kg), (medium, 1.25 mg/kg), (high, 2.5 mg/kg). Each rat received an IP injection at T=0h. The data points represent the mean (n=3) and pooled standard deviations (SD) are shown as bars; * $p \leq 0.05$ unpaired t test, + $p \leq 0.05$ longitudinal paired t test.

Figure 6.5. Cd Concentration in the Lymphocytes, Monocytes, and PMNs from Short Course and Subchronic Rats.

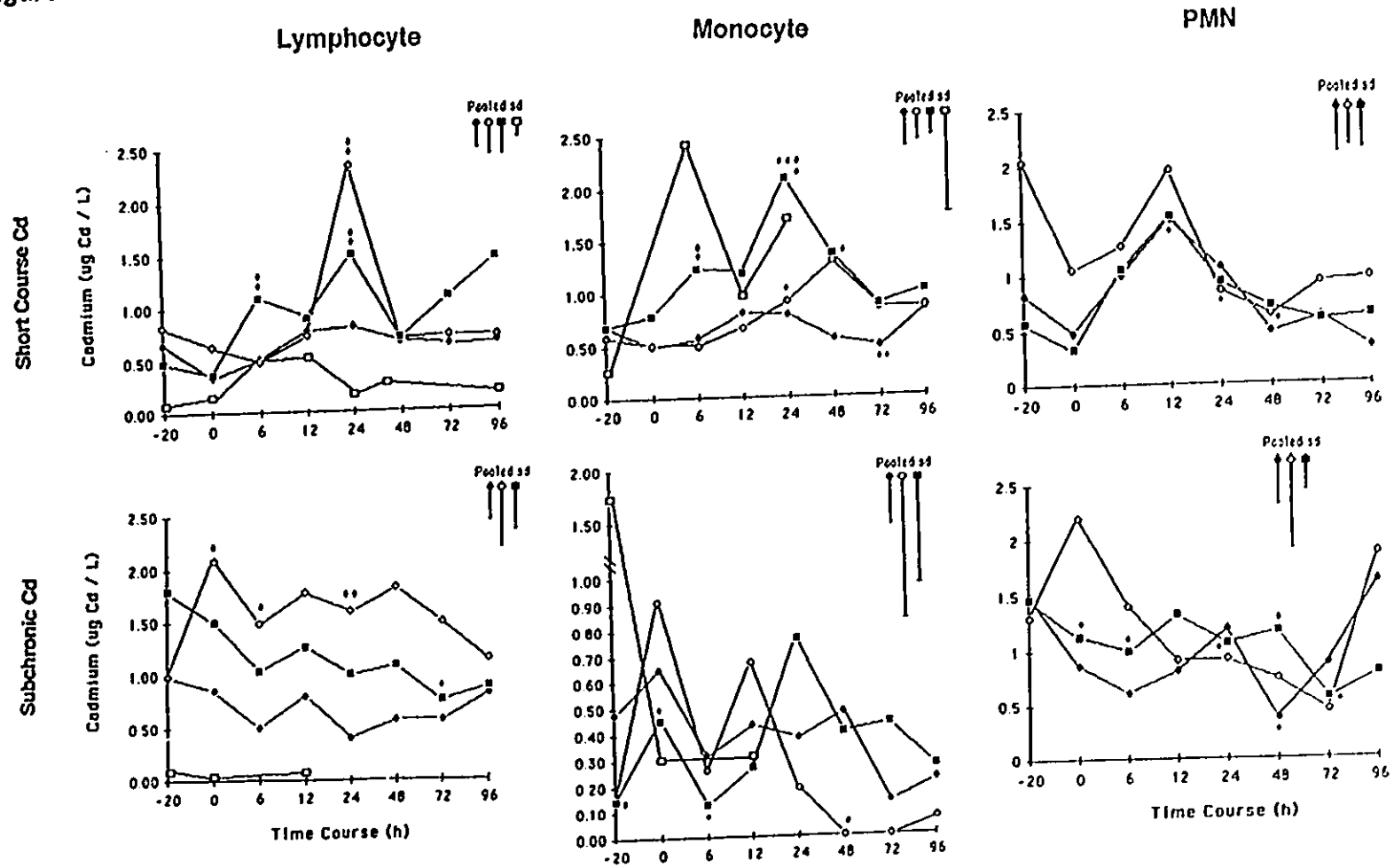


Figure Legend:
♦ (control), ◇ (low, 0.25 mg Cd/kg), ■ (medium, 1.25 mg/kg), □ (high, 2.5 mg/kg).
Each rat received an IP injection at T=0h. The data points represent the mean (n=3) and pooled standard deviations (SD) are shown as bars;
* $p \leq 0.05$ unpaired t test, + $p \leq 0.05$ longitudinal paired t test.

Figure 6.6. MT Concentration in the Lymphocytes, Monocytes, and PMNs from Short Course and Subchronic Cd Exposed Rats.

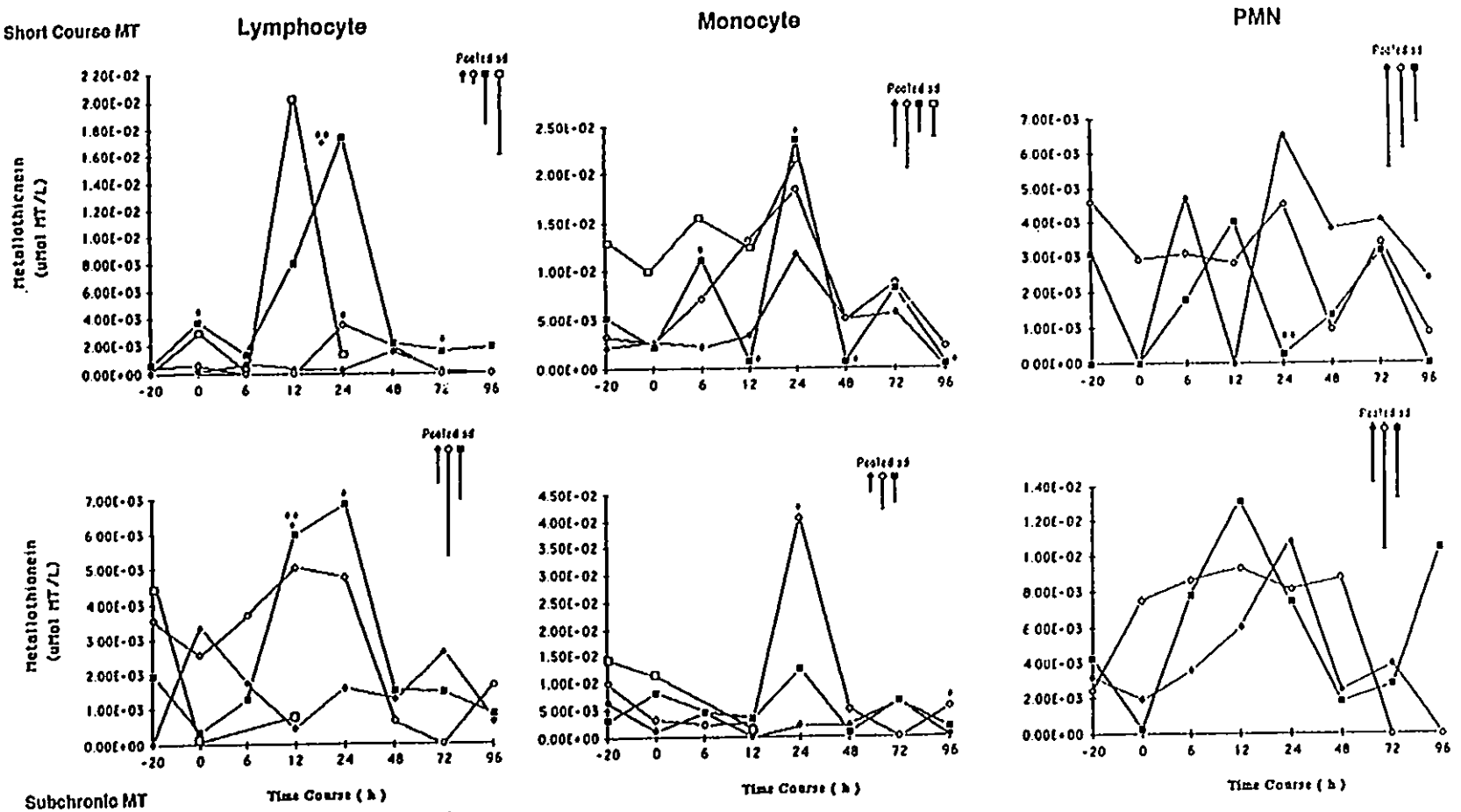


Figure Legend:

-♦- (control), -○- (low, 0.25 mg Cd/kg), -▪- (medium, 1.25 mg/kg), -○- (high, 2.5 mg/kg). Each rat received an IP injection at T=0h. The data points represent the mean (n=3) and pooled standard deviations (SD) are shown as bars; * $p \leq 0.05$ unpaired t test, + $p \leq 0.05$ longitudinal paired t test.

Figure 6.7. Cd Concentration in the Feces from Short Course and Subchronic Cd Exposed Rats.

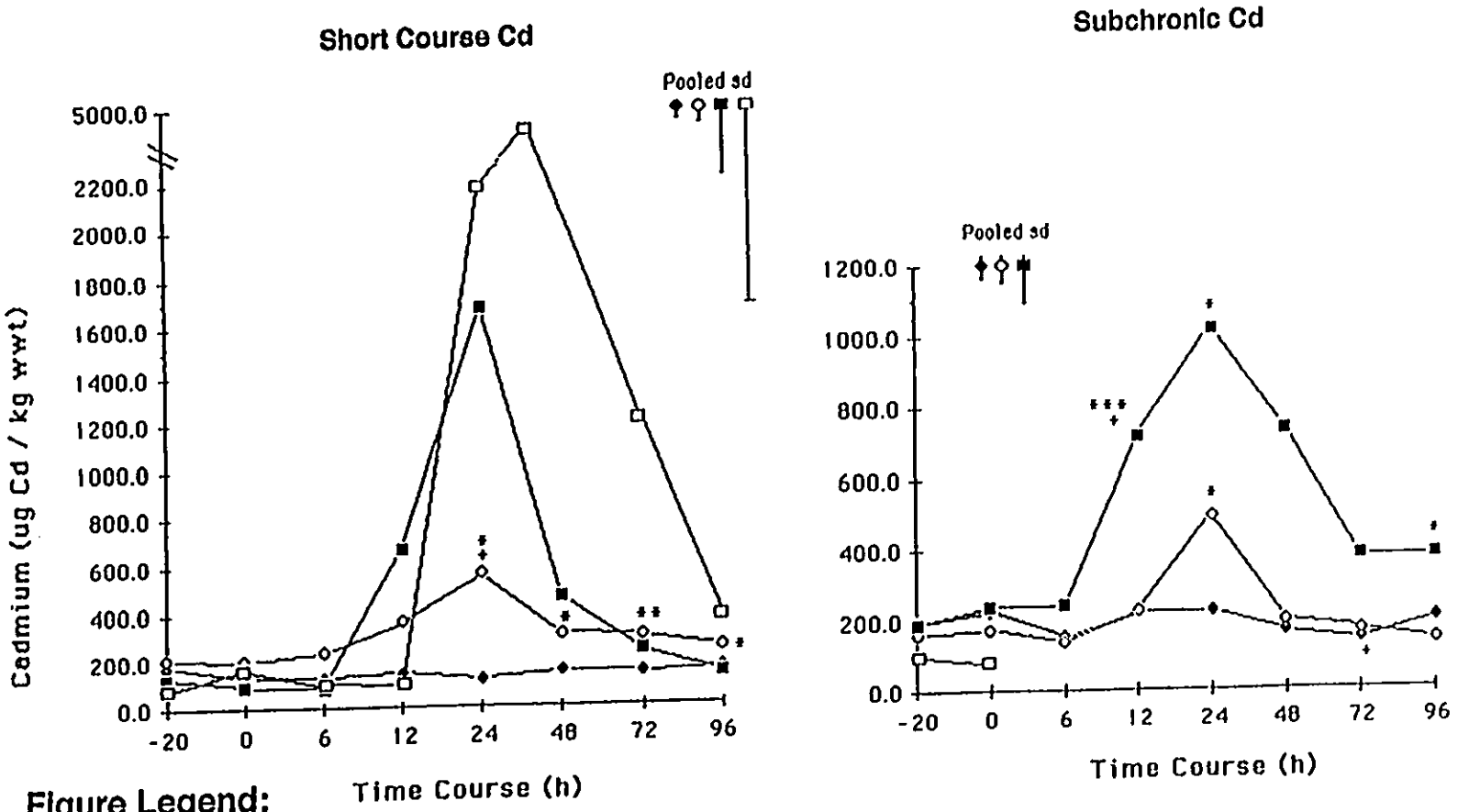
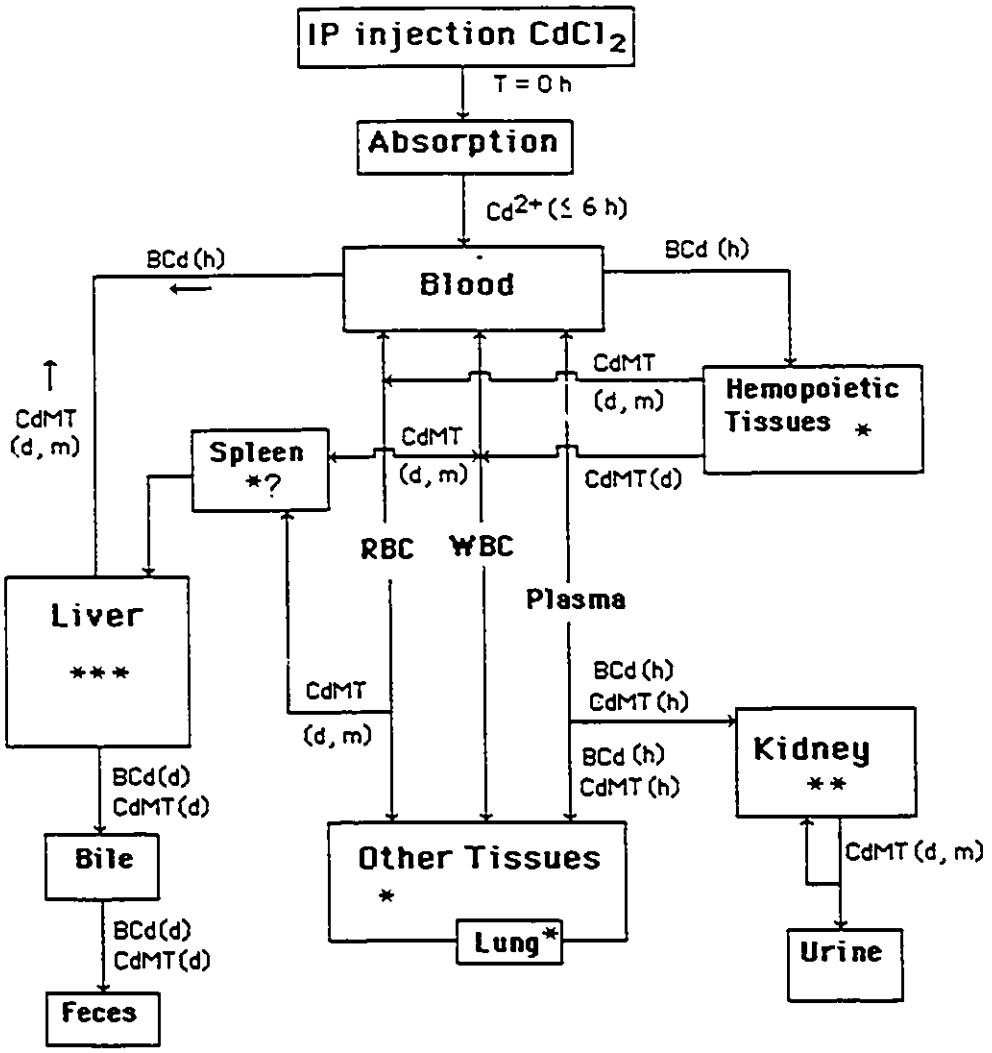


Figure Legend: ♦ (control), ○ (low, 0.25 mg Cd/kg), ■ (medium, 1.25 mg/kg), □ (high, 2.5 mg/kg). Each rat received an IP injection at T=0h. The data points represent the mean (n=3) and pooled standard deviations (SD) are shown as bars; * $p \leq 0.05$ unpaired t test, + $p \leq 0.05$ longitudinal paired t test.

Figure 6.8. In Vivo Dynamics Model for Cd and MT in Rat.



Legend to Figure:

Diagramatical representation of the distribution of Cd and CdMT in the *in vivo* rat model. The (BCd) represents Cd bound to HMW plasma proteins, albumin, amino acids, and transferrin. The (CdMT) represents Cd bound to metallothionein. The letters in parenthesis denote the response time and/or residence time: (h) hours, (d) days, (m) months. The (*) depicts the degree of MT induction. See Section 6.2 for details.

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APPENDIX 1

Several exploratory (pilot) experiments were performed in order to select the analytical protocols for the *in vivo* Cd experiments and to determine the minimum sample volumes necessary to determine Cd and MT concentrations. The pilot studies indicated that a minimum of 50 - 75 uL plasma was necessary to obtain reliable EAAS results for Cd and 75 - 100 uL plasma for MT by CSA. For both the Cd and MT protocols, the higher volume was used to increase the reproducibility of the results. The volume of blood cell lysate needed for EAAS was 200 - 300 uL (depending on the cell number) compared to 100 uL lysate solution for the CSA. The initial lysate volume was higher for EAAS so as to compensate for the extra dilution steps during analysis. Empirically it was shown that 0.4 mL WB yielded a clean 0.2 mL aliquot of plasma and sufficient leukocytes for cell separations and analytical determinations (see Figure 2.4). All sample manipulations were carried out in acid-washed microcentrifuge tubes prior to splitting into aliquots for Cd and MT analyses, in order to minimize Cd contamination of the EAAS samples.

These pilot studies also provided insight into the selection of the dosage regime of $CdCl_2$. It was determined that doses higher than 2.5 mg $CdCl_2$/kg bwt were excessively toxic, causing death in several instances. Consequently, the high dose was selected as 2.5 mg $CdCl_2$/kg bwt. The medium dose (1.25 mg/kg) was 50% of the high dose and the low dose (0.25 mg/kg) was 10% of the high dose.