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**Abstract**

The time course of change in tissue Cd, Cu and Zn contents, their distribution in cellular protein fractions as well as the profile of MT gene expression in mouse liver was described over a 7 days period following a single intraperitoneal injection of 2 mg/kg of $CdCl_2$. The result showed that Cd accumulated rapidly in mouse liver. Between 1 hr and 7 days after administration, over 18% of the total Cd administered were found in the liver. Cd administration was also associated with the over-expression of the MT-mRNA. However, the time course of induction was not parallel to the change in tissue Cd content. When separated on a Sephadex G-75 column, majority of Cd was found to bind to the fractions known to contain the metal-binding protein, metallothionein (MT). From day 2 after Cd administration, a small amount was also found associated with the high molecular weight (HMW) proteins. In addition to Cd, tissue Zn content was affected most during the entire study. There was a significant decrease in tissue Zn content during the initial 8 hr but tissue Zn content increased significantly throughout the following 6 days. At 1-7 days, majority of Zn was associated with the HMW protein fraction. Although there was no significant change in total tissue Cu content, distribution of Cu in different protein fractions was detected. While in control aniamls, Cu was mainly associated with the HMW proteins, some was found in the MT fraction on the second day. On the $7^{th}$ day, Cu distribution had deteriorated. Together with changes seen in Cd, the results might suggest that injury had occurred in the tissue at this time. The results of the present study showed that Cd caused a change in subcellular distribution of tissue endogenous metals, which might reflect alteration of cellular functional activities.

## INTRODUCTION

Cd is one of the most toxic metals in the environment. At sublethal doses, the metal had been shown to induce the expression of a number of stress genes (1-5). One of the most well studied gene products closely associated with Cd is metallothionein (MT), a low molecular weight, heat-stable, cysteine-rich cytosolic protein (1). Time course studies demonstrated that cells or tissues respond to Cd by a coordinate sequence of events that would eventually lead to MT induction (3, 5). MT induction could be mediated either directly through Cd or indirectly through specific Zn binding protein (25). The main function of MT is to chelate the toxic metal thus protecting tissues and cells against Cd induced toxicity (6).

Metals are important cellular constituents. They participate in a variety of cellular activities serving as structural and functional components. Majority of the metals is bound to proteins or cytoplasmic organelles. Thus, the distribution of metals in different cytosolic fractions would reflect alteration of functional or structural activities of the cells. Extensive studies had shown that Cd might interfere with the metabolism of essential trace metals (7-10). The correlation between different metals and the genes that are involved in a particular stage of injury had not been defined. In the present study, the effect of a single intraperitoneal injection of a sublethal dose of $CdCl_2$ (2 mg/kg) on mouse liver was studied over a 7-day period. The levels of tissue Cd, Cu, and Zn were measured. Furthermore, the distribution of Cd, Zn and Cu in different cytosolic protein fractions, as well as the expression of MT gene over the same period was analyzed. The purpose was to identify the effect of Cd on endogenous Zn and Cu homeostasis, and the relation of these metals to MT induction.

## MATERIALS AND METHOD

*Animal preparation*

Adult male mice, of the ICR strain with body weight ranging from 31-35 g, were purchased from the Chinese University Animal Unit. They were housed, 10 to each cage, in a temperature and humidity controlled animal unit with 12/12 light-dark cycle. They were allowed free access to purified water (National PJ-24 water purifier) and food (Laboratory Rodent Diet #5001, PMT Feed, Inc., St. Louis, U.S.A.).

Mice were separated into 3 groups. A group of 4 control animals were sacrificed without any treatment. The second group of 28 animals was given a single intraperitoneal injection of 2 mg/kg $CdCl_2$ each. The volume of injection was limited to 0.3 ml. Four animals were sacrificed at 1, 4, 8, 24, 48, 96 and 168 hr after Cd administration. The third group of 28 animals was given a single intraperitoneal injection of the same volume (0.3 ml) of 0.9% NaCl each, and sacrificed at the same times as those given Cd. During the entire study, animals were carefully observed and cared for.

*Preparation of tissue samples and tissue metal analysis*

Mice were sacrificed painlessly and rapidly by cervical dislocation and the liver was removed and rinsed with ice-cold saline (0.9% NaCl). Approximately 1 g of liver was blotted dry and digested in 5 ml $HNO_3$ (70%), 2 ml double-distilled water (Millipore) and 1 ml Pb (as internal standard) in a microwave oven (CME Microwave Technology, USE). The digestion program was set according to the manufacturer's protocol for bovine liver in closed vessels (Appl. Note: B1-5). After digestion, 5 ml of the digestate was diluted to 10 ml in an acid treated volumetric flask with double-distilled water, and stored, tightly capped, in a plastic tube for metal analysis. In order

to reduce metal contamination, plasticware was used whenever possible. All glassware and plasticware were pre-soaked in weak acid (5% $HNO_3$) followed by thorough rinsing with ultra pure water. All reagents were made with double-distilled water. Tissue Cd, Zn and Cu content were measured simultaneously in an inductively coupled plasma atomic emission spectrometer (ICPAES, Perkin Elmer Optima 3000XL). Correction was made on the recovery of Pb, which serves as internal standard. The reliability of metal analysis was monitored by parallel analysis of a reference standard (bovine liver, US-NIST Standard Reference Material 1577b).

*Preparation of cDNA probes*

Metallothionein (MT) cDNA probes were generated by PCR of total RNA derived from mouse liver. The primers were designed on the basis of the published cDNA sequence of MT (5`-CCCAACTGCTCCTGCTCCAC-3` and 5`-GTCACTTCAGG-CACAGCACG-3') (11). The PCR was run for 35 cycles with 56°C annealing cycle (1 min), 72°C extension cycle (1 min 30 s), and a 95°C denaturing cycle (50 s), plus final incubation at 72°C for 3 min. The 186 bp PCR product for MT was purified, subcloned into pUC 18 and subjected to dideoxy sequencing for verification.

*Northern blot analysis*

A portion of liver from both groups of animals were homogenized in TRIZOL Reagent (Gibco BRL) using a glass-Teflon homogenizer according to the manufacturer's instructions. Briefly, after a 5 min incubation at room temperature (24°C), chloroform was added to the homogenate. The upper aqueous phase was collected and the RNA was precipitated by mixing with isopropyl alcohol. The RNA pellet was washed once with 75% ethanol, air-dried and redissolved in RNase-free water. $A_{260}/A_{280}$ ratios were between 1.6-1.8. Northern blots were generated by separating 20 μg of the total RNA on 1% agarose gels containing formaldehyde. The separated RNAs were transferred to nylon membranes (Hybond-N, Amersham

Pharmacia Biotech). Hybridization was carried out with the PCR-derived mouse MT-cDNA labeled with [α-$^{32}$P]dCTP (3000 Ci/mmol, Amersham Pharmacia Biotech) using a Ready-To-Go DNA labeling kit (Amersham Pharmacia Biotech). Membrane signals were detected using Kodak scientific imaging film (BioMax MS). The adequacy of RNA loading was assessed by the presence of 18S rRNA. Results were digitized in a transilluminator (Ultra-Violet Products, England). Liver sample from each animal was processed separately.

*Quantitation of tissue MT*

The total cytosolic protein was heated at 90°C for 5 min and rapidly cooled in ice. The precipitated protein was removed by centrifugation. MT in the supernatant was measured by a modified Cd-binding assay (12). Instead of using radioactive tracer for measuring the MT-bound Cd, the amount of Cd bound to MT was measured by graphite furnace atomic absorption spectrometry at wavelength of 228.8 nm (22, 23).

*Binding of Cd, Zn and Cu in different cytosolic protein fractions*

A separate set of animals was used. At 1, 2, 4 and 7 days after Cd injection, liver was dissected and rinsed in 0.9% NaCl to remove as much blood as possible. The tissue was blotted dry, weighed accurately and homogenized in 1:5 w/v pre-chilled 0.01 M Tris-HCl, pH 7.4 containing 2 mM PMSF. The homogenate was centrifuged at 100,000 x g (Hitachi HIMAC ultracentrifuge) for 60 min at 4°C. The supernatant, which contained the total cytosolic proteins, was collected. Five-ml of the proteins (calibrated to the same amount of proteins in each sample) was applied to a Sephadex G-75 column (2.6 x 70 cm) for separation by size. Prior to addition of the sample, the void volume of the column was calibrated with 0.1% blue dextran. The proteins were then eluted with 0.01 M Tris-HCl, pH 7.4 containing 0.02 % sodium azide at a flow rate of 30 ml/hr at room temperature. Seven-ml fractions were collected. The protein

in each fraction was determined by UV absorbance at 280nm (Varian 634), and the Cd, Zn and Cu contents in each fraction were determined by flame atomic absorption spectrometry at the specified wavelength for the different metals.

## RESULTS AND DISCUSSION:

*Control values of mouse liver metal content*

Table 1 shows the liver content of Cd, Zn and Cu in control untreated mice and those after given a single intraperitoneal injection of 0.9% NaCl. The results demonstrated that the values of the three metals were within the same order of magnitude but there were variations at different times after injection. In subsequent studies, changes in liver metal content were compared with the NaCl treated animals at the corresponding time to accommodate the effect of injection stress.

*Time course of change in metal contents in mouse liver*

Figure 1 shows the time course of change in tissue Cd as well as Cu and Zn in mouse liver after a single intraperitoneal injection of Cd. At 1 hr after injection, mean Cd level had increased to 9 mg/kg and remained high over the entire study. Considering that the liver/body ratio of mice to be 4% (13), for a 30 g mouse, the weight of the liver would be 1.2 g. When the total amount of Cd administered was 60 μg, it would mean that 18% of the total Cd administered was located in the liver at 1 hr. The percentage was increased to 32% (16 μg/g wet wt) on the $7^{th}$ day. Although the absolute amount of Cd was increased, the difference in tissue metal content between 1 and 168 hr were not statictically significant when analyzed by ANOVA. The results is consistent with literature finding that showed a rapid (within 2 hr) distribution of Cd

in liver and the metal remained in liver for a prolonged period (> 3 days) of time (14). The change in liver Cd profile serves as a reference for subsequent discussion.

In addition to the change in tissue Cd content, there was a decrease in tissue Zn content during the initial 4 hr, but the content increased significantly at 24 hr and remained elevated until the 7$^{th}$ day (168 hr). The results of this study showed that Cd caused significant changes in tissue Zn content both acutely during the initial 24 hr and subacutely during the following 6 days. The results is consistent with previous study which showed a significant increase in rat liver Zn content 72 hr after a single intraperitoneal injection of 1 mg/kg $CdCl_2$ solution (9). Cu content was increased significantly only briefly at 24 and 168 hr after injection.

*Time course of change in MT-mRNA in mouse liver*

Cd is an efficient inducer of MT. Figure 2 is a representative profile that shows the change in MT-mRNA content during the entire study period. MT gene expression increased gradually reaching maximum at the 8$^{th}$ hr, reduced and remained low at 24 hr until the 7$^{th}$ day. The results are consistent with that described in the literature (15). Results of the present study also showed that MT induction lags behind the rapid increase in Cd, and neither the change in tissue endogenous Zn nor Cu correlates with MT induction.

*Distribution of Cd in different cytosolic proteins fractions*

To further examine changes in Cd, Cu and Zn within the liver tissue, and their relation with MT, study was undertaken to examine the distribution of metals in different cytosolic protein fractions. Figure 3 shows the Sephadex G-75 column separated cellular proteins into two different fractions when examined by UV absorption at 280 nm. The fractions were divided into the high molecular weight (HMW) proteins that

were eluted between fractions 11-13, immediately after the void volumn (fraction 10-11); and the low molecular weight (LMW) molecules at fractions 30-35. These peaks were found located at the same fractions in all samples studied (data not shown) and were used as reference points for metal profile. At 1 and 4 days after Cd administration, a Cd-binding peak was seen in fractions 21-25, a peak intermediate to the HMW and the LMW fractions. The fractions did not show significant absorbance at 280 nm, which is consistent with the amino acid content of MT, i.e. no appreciable aromatic amino acid within the sequence (16). The elution volume is ~2 folds of the void volume, a position where MT should be eluted (17-18).

Figure 4 shows a time dependent change in Cd distribution in the different cytosolic fractions. At 1 to 7 days after Cd injection, the Cd-bound MT fraction was clearly evident. Furthermore, tissue MT content was elevated (Figure 5). These results were not consistent with that seen in Figure 2 for MT-mRNA. Similar descrepency was also noted in other experiments exposing rodents to a single sublethal dose of Cd (15, 28). However, the data would be consistent if considering that at 1 day after Cd injection, cell had synthesized enough MT and there would only be a small amount of free Cd to initate further MT induction. Starting from the second day, some Cd was detected in the HMW protein fractions. At the 7$^{th}$ day, significant amount of Cd was bound to the HMW. A binding of Cd to the HMW proteins was reported in studies using HPLC-AAS after repeated subcutaneous injection of Cd to rats (20). The present results could indicated that loading of MT had reached maximum at this time (day 7) and the excess Cd would be available to stimulate MT induction as reflected by a marked increase in MT-mRNA seen at 168 h (Figure 2).

*Distribution of Zn and Cu in different cytosolic protein fractions*

One of the interesting findings is the elevated tissue Zn content at days 1 to 7 after Cd administration. Zn is an important trace metal and its level in tissue is carefully

regulated. It is thus believed that an elevated liver Zn content would reflect significant changes within the tissue. Figure 6 shows the distribution of Zn during the study period when Zn level was elevated. Throughout the entire study, Zn was not found associated with the MT fraction. This is consistent with the relative affinity of Zn and Cd to MT (21). Majority of Zn was bound to the HMW protein fractions. The increased amount of Zn associated with the HMW fractions would suggest either a change in the levels of the high molecular weight Zn-binding proteins or a binding of Zn to proteins that normally do not contain Zn. Recently, Sheline et al. (24) showed that in neuronal cells, Zn could bind and inhibit the activity of glycolytic enzymes, leading to a progressive loss of ATP and resulting in neuronal cell death. Correlation between Zn and cellular energy metabolism is being studied in liver cells in our laboratory.

Although the total tissue Cu content was not changed (Figure 1), the distribution of Cu in different cytosolic protein fractions was not the same. Figure 7 shows that Cu in the HMW protein pool reduced over time. Beginning on the second day, an increasing amount of Cu was seen in the MT fraction. In addition, there was a small fraction of protein appearing at fractions 16-18, which also contains Cd. It could be hypothesized that at this time, events occurred in cells that led to a release of Cu from its normal binding sites on the HMW proteins. The free Cu could bind to MT. On the $7^{th}$ day, the distribution of Cu deteriorated. Together with the distribution profile of Cd, it might suggest that significant injury had occurred in the liver.

Reports in the literature had described changes in endogenous Cu distribution. In marine organisms, Cd could change Cu and Zn concentrations in various subcellular fractions including the mitochondria and microsomes in the hepatopancreas of *Nassarius reticulatus* (26). A shift of Cu to the MT-fraction was demonstrated in *Perna viridis* upon exposure to Cd using similar chromatographic technique (19). Kinetically, Cu has higher affinity to MT than Cd (21). Should there be a release of

Cu due to a drastic change in intracellular protein profile, it is possible that the released Cu could displace Cd from the Cd-MT complex resulting in an increase in Cu in the MT fraction as observed on the 7$^{th}$ day in Figure 7. A recent study had indicated that when MT was saturated with Cu it could become a prooxidant and may cause oxidative damage in liver (27). Research should be conducted to correlate the change in Cu distribution to free radicals generation.

The current study provided a description of change in tissue metals as well as MT gene expression in mouse liver. Consistent with the current hypothesis which states that Cd initiate a sequence of events that eventually lead to the observed toxicity (5), the current results demonstrated that besides altering gene expression, changes could also be observed in the distribution of endogneous trace metals, which would reflect an alteration of functional activities of the cells.

## ACKNOWLEDGEMENT

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Table 1. Tissue metal content in control mice and mice after handling stress

| | Tissue metal content (μg/g wet wt ±SEM) [1] | | |
|---|---|---|---|
| | Cd | Cu | Zn |
| Control | 0.05±0.02 | 4.66±0.42 | 24.88±0.35 |
| hr after NaCl-injection | | | |
| 1 | 0.04±0.01 | 4.91±0.20 | 27.21±1.23 |
| 4 | 0.07±0.03 | 4.86±0.40 | 27.45±1.31 |
| 8 | 0.04±0.004 | 4.76±0.53 | 27.66±1.78 |
| 24 | 0.03±0.03 | 4.93±0.22 | 27.98±2.40 |
| 48 | 0.01±0.003 | 4.59±0.25 | 27.82±0.78 |
| 96 | 0.09±0.06 | 4.58±0.21 | 26.72±0.99 |
| 168 | 0.043±0.01 | 5.03±0.33 | 29.21±0.68 |

[1] each value represents the mean and SEM from 4 animals.

FIGURE LEGENDS:

Figure 1. Liver Cd, Zn and Cu content over 7 days in stress controls and in animals after intraperitoneal injection of a single dose of $CdCl_2$ (2 mg/kg). Each point represents the mean and SEM of 4 animals. The hollow symbols are stress controls and the solid symbols are Cd treated animals. * indicates that the value is significantly different from the corresponding control.

Figure 2. A profile of the change in liver MT-mRNA over the 7 days study period following a single intraperitoneal injection of $CdCl_2$. No signal was detected in stress control animals. The values in the graph were mean and SEM of 2 animals after the images were digitized.

Figure 3. Sephadex G-75 chromatography profile for UV-280 and Cd of the cytosolic protein fractions in mouse liver 1 and 4 days after given a single intraperitoneal administration of 2 mg/kg $CdCl_2$.

Figure 4. The Sephadex G-75 chromatographic profile of Cd in mouse liver at 1, 2, 4 and 7 days after administration of $CdCl_2$ (2 mg/kg). The line on the x-z plane shows the location of the fractions in which MT would be detected.

Figure 5. Liver MT content over the 7-day study period. Each point represents the mean and SEM of 3 animals.

Figure 6. The Sephadex G-75 chromatographic profile of Zn in mouse liver at 1, 2, 4 and 7 days after administration of $CdCl_2$ (2 mg/kg).

Figure 7. The Sephadex G-75 chromatographic profile of Cu in mouse liver at 1, 2, 4 and 7 days after administration of $CdCl_2$ (2 mg/kg).

Figure 1

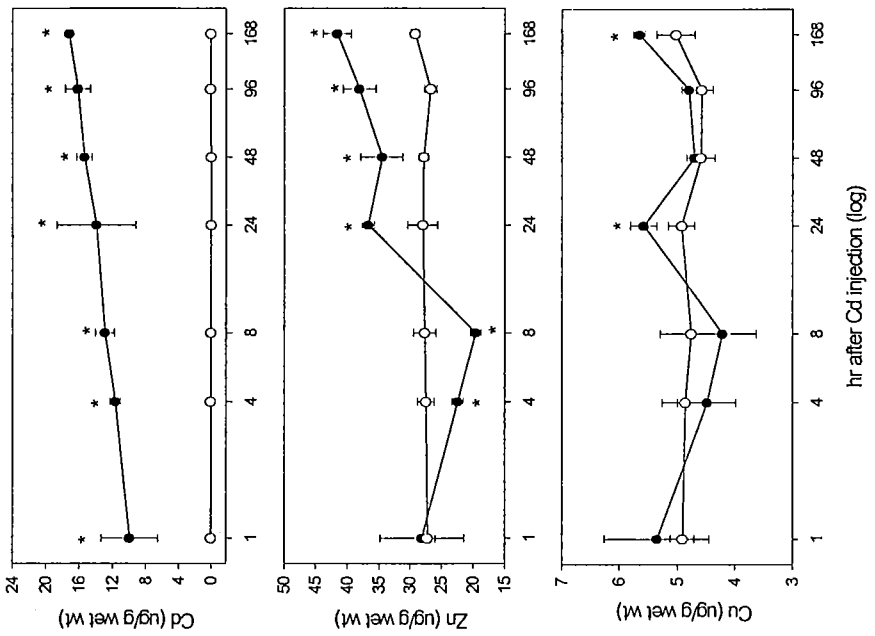

Cd (ug/g wet wt)
Zn (ug/g wet wt)
Cu (ug/g wet wt)
hr after Cd injection (log)

Figure 2

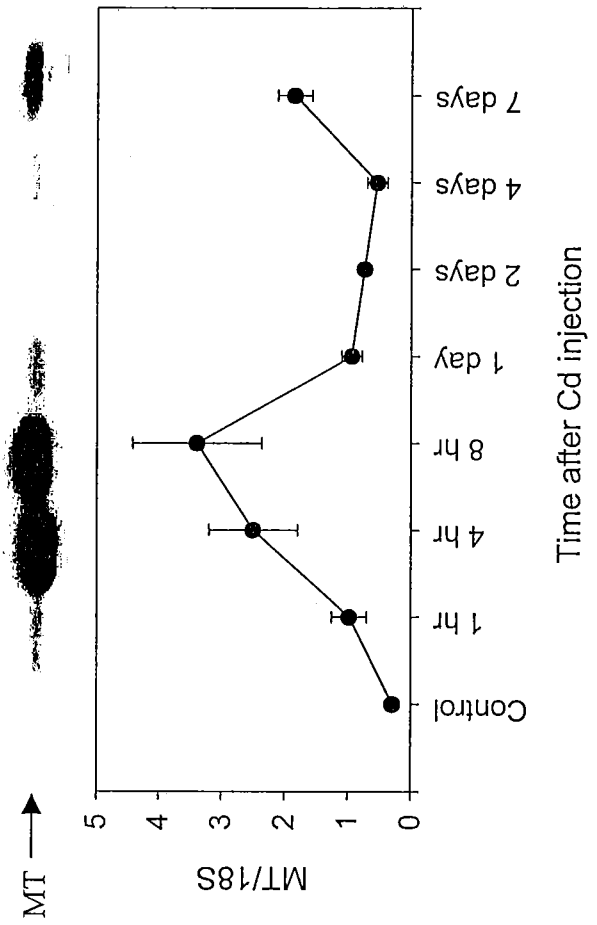

MT
MT/18S
0
1
2
3
4
5
Control
1 hr
4 hr
8 hr
1 day
2 days
4 days
7 days
Time after Cd injection

Figure 3

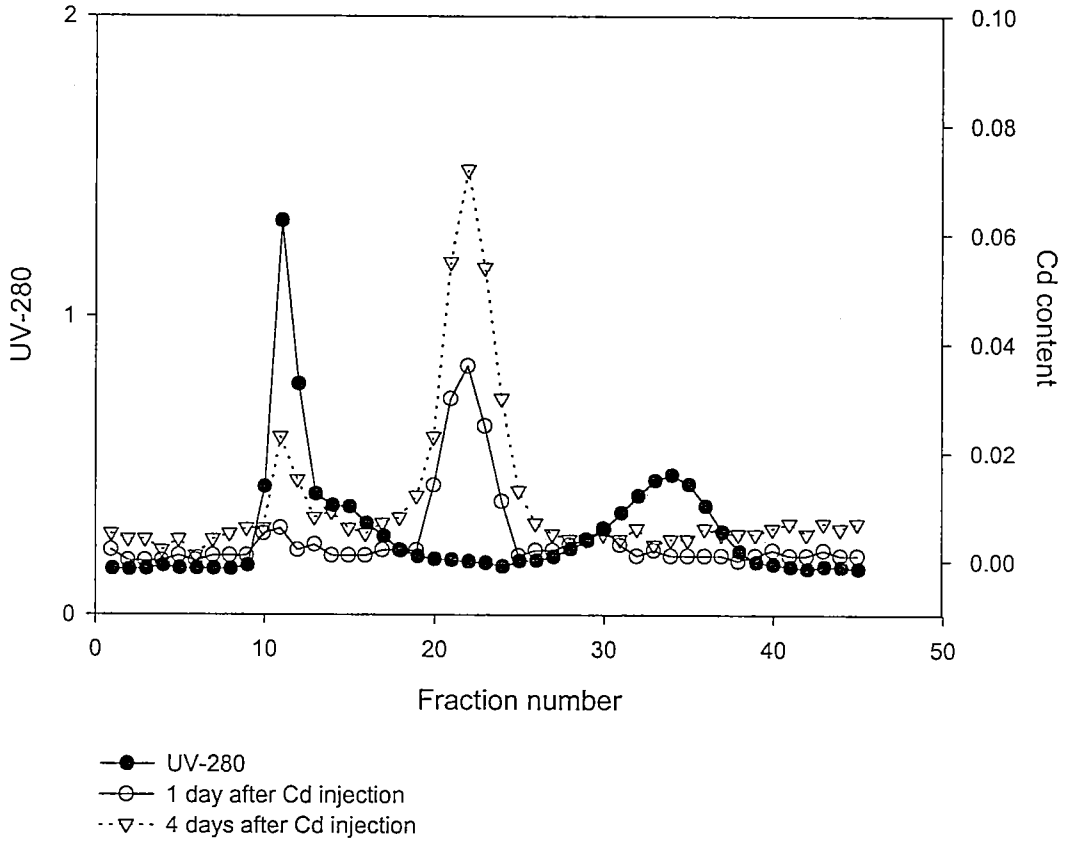

2
1
0
UV-280
0.10
0.08
0.06
0.04
0.02
0.00
Cd content
0
10
20
30
40
50
Fraction number
UV-280
1 day after Cd injection
4 days after Cd injection

Figure 4

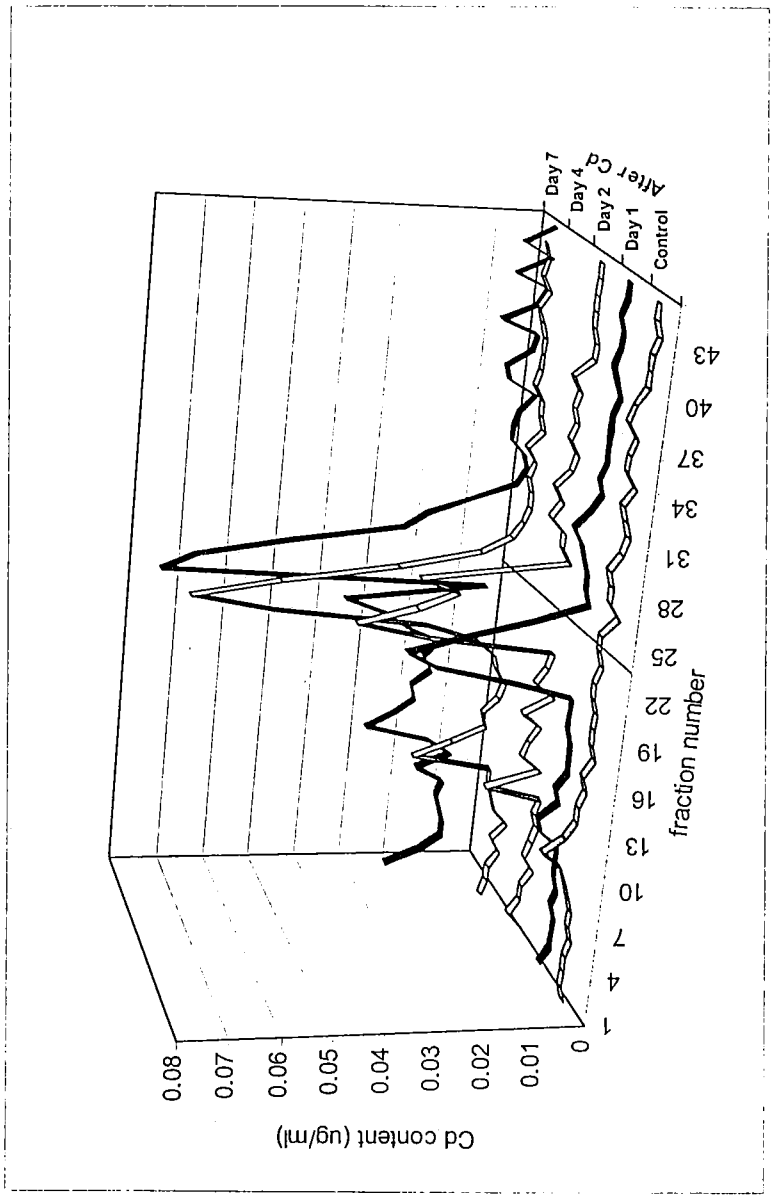

Cd content (ug/ml)
0.08
0.07
0.06
0.05
0.04
0.03
0.02
0.01
0
1
4
7
10
13
16
19
22
25
28
31
34
37
40
43
fraction number
After Cd
Day 7
Day 4
Day 2
Day 1
Control

Figure 5

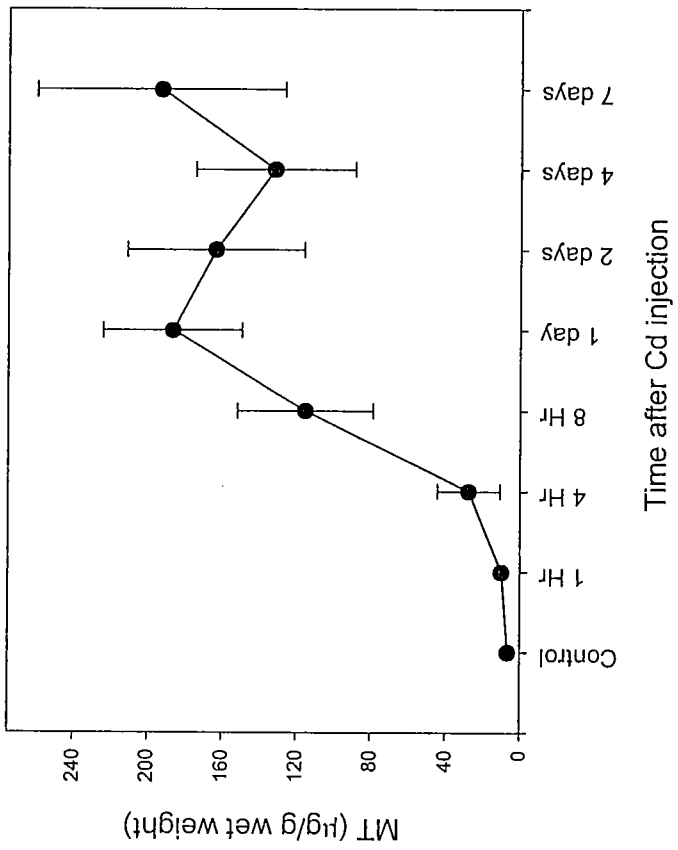
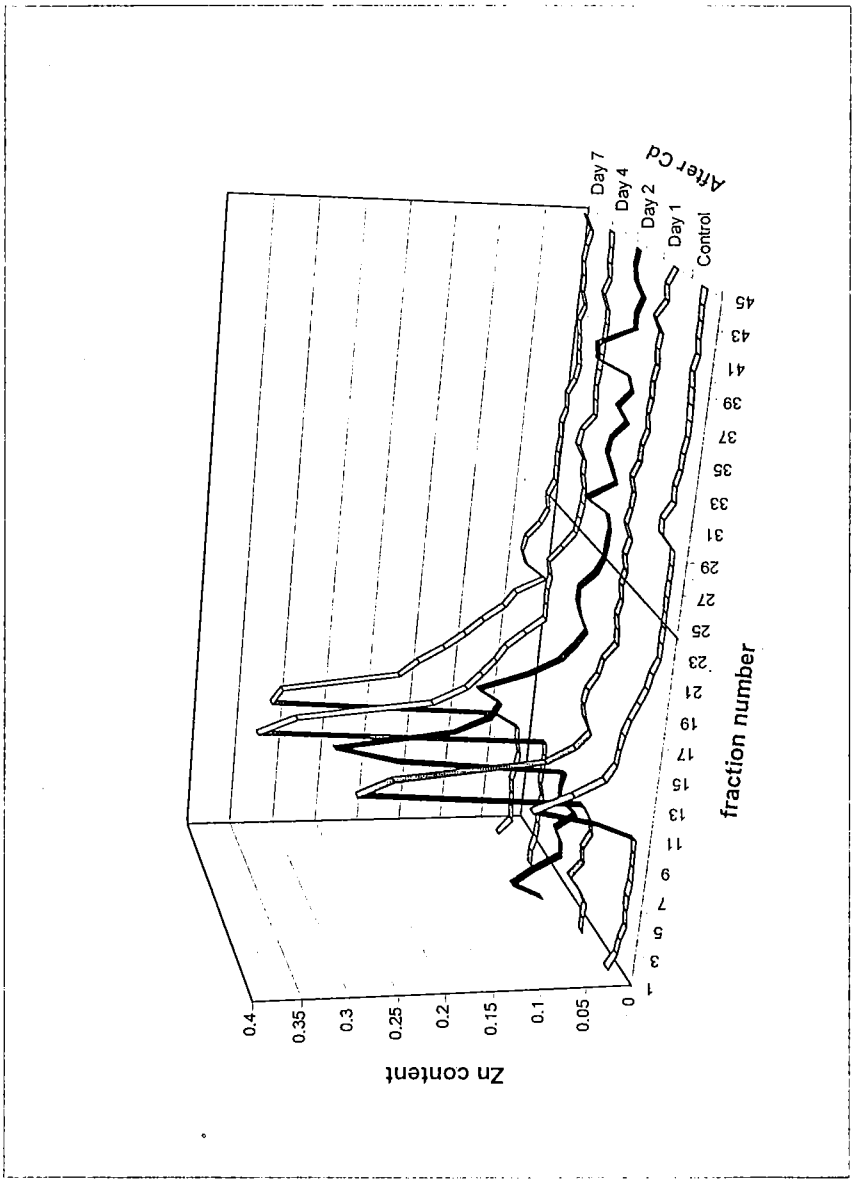

MT (µg/g wet weight)
0
40
80
120
160
200
240
Control
1 Hr
4 Hr
8 Hr
1 day
2 days
4 days
7 days
Time after Cd injection

Figure 6


After Cd
Day 7
Day 4
Day 2
Day 1
Control
fraction number
Zn content
0.4
0.35
0.3
0.25
0.2
0.15
0.1
0.05
0
1
3
5
7
9
11
13
15
17
19
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