Comparison of toxicity and disposition of cadmium chloride and cadmium-metallothionein in rats

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Cover: "Cd-salt versus Cd-liver", balans gedragen door Minerva beeldje,
1e helft 19e eeuw, collectie Universiteitsmuseum Utrecht.

Stellingen

1. De selectieve nierstapeling van cadmium na orale belasting is dosis-afhankelijk. Voor een juiste risico inschatting van de orale opname van anorganisch dan wel matrix-gebonden cadmium is het derhalve een "conditio sine qua non" dat dierproeven ook bij lage, niet-toxische cadmium doseringen worden uitgevoerd.
   - *Lehman L.D. and Klaassen C.D. (1986). Toxicol. Appl. Pharmocol., 84, 159-167.*
   - *Dit proefschrift*

2. De selectieve dispositie en toxiciteit van Cd-metallothioneïne in de nieren na orale blootstelling is geringer dan na intraveneuze blootstelling.
   - *Dit proefschrift*

3. Wanneer we het gezondheidsrisico van de inname van cadmium op een juiste wijze willen schatten, dient de ijzer opname mede in de beschouwing te worden betrokken.
   - *Dit proefschrift*

4. Normaanpassing van cadmium in dierlijke produkten is op dit moment niet noodzakelijk.
   - *Maitani T., Waalkes M.P. and Klaassen C.D. (1984). Toxicol. Appl. Pharmacol., 74, 237-243.*
   - *Dit proefschrift*

5. Gezien de structuur van de gember-smaakstoffen shogaol en dehydroparadol is er nog voldoende interessant werk voor toxicologen bij het ophelderen van de rol van kruiden bij de carcinogenese en anti-carcinogenese.
   - *Surh Y.J. and Lu S.S. (1992). Biochem. Int. 27, 179-198.*

6. De recente hypothese van Arch et al. (1992) dat darmtumor-cellen, door expressie van het glycoproteine CD44 op de celmembraan, in staat zijn om het het gedrag van lymfocyten na te bootsen, kan nieuwe mogelijkheden bieden voor drugtherapie waarbij met dit eiwit als target getracht kan worden om de "wolf in schaapskleren" te ontmaskeren.
   - *Arch R., Wirth K., Hofmann M, Ponta H., Matzku S., Herrlich P. and Zöller M. (1992). Science, 257, 682-685.*
   - *Kahn P., Science (1992). Science, 257, 614.*

7. De reversibele binding aan glutathion is een tot nu toe onderschat mechanisme voor het transport van kortlevende reactieve intermediairen.
   - *Vroomen L.H.M., Berghmans M.C.J., Groten J.P., Koeman J.H. and van Bladeren P.J. (1988). Toxicol. Appl. Pharmacol., 95, 53-60.*
   - *Baillie T.A. and Slatter J.G. (1991). Acc. Chem. Res., 24, 264-270.*

8. De progressie van de wetenschap stagneert wanneer er te weinig aandacht wordt besteed aan goed gecoördineerd programmatisch onderzoek en aan evaluerende discussies over centrale hypothesen.

9. Verhoogde celdelingsactiviteit geeft aanleiding tot meer (ge)kanker.
   - *Ames B.N. and Gold L.S. (1990). Science, 249, 970-971.*
   - *"Staatssecretaris Kosto: celdeling geen oplossing voor cellentekort", NRC Handelsblad, 7 juli 1992.*

10. Zo lang de mythe bestaat dat er geen goede part-time wetenschappers zijn, zal het aantal vrouwelijke wetenschappers dat in de wetenschap *blijft* werken, laag blijven.

11. De opvatting dat concurrentie bij subsidiering van projectvoorstellen gezond zou zijn, is niet zonder meer juist. In de moordende concurrentie om financiering van onderzoek, worden summiere resultaten opgepoetst en buitengewoon extravagante beloften gedaan om geldschieters te kietelen.
   - *van Calmhout R., Volkskrant-wetenschap, 23 mei 1992.*
   - *Bell R., Impure Science, John Wiley & Sons, 1992.*

12. Combinatie-toxicologie kan alleen met succes worden bedreven door een team van hoogvliegers, zwaargewichten en diepgravers met lange adem, brede rug, gevoel voor humor en filosofische inslag.

13. Gezien de steeds terugkerende berichten over bodemvervuiling, zou elke nederlander met kinderwens zich eens moeten bezinnen omtrent de aanpassingstrategie van pissebedden. Deze produceren als reactie op de bodemvervuiling vroeg nageslacht, immers uitstel zou wel eens kunnen leiden tot afstel.
   - *Proefschrift M. Donker. Vrije Universiteit Amsterdam.*

Stellingen behorend bij het proefschrift "Comparison of toxicity and disposition of cadmium-chloride and cadmium-metallothioneine in rats". John Groten, Wageningen, 1 december 1992.

La Science se fait non seulement avec
l'esprit mais aussi avec le coeur.

L. Pasteur

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The studies described in this thesis were conducted at the TNO Toxicology and Nutrition Institute, Department of Biological Toxicology, Zeist in collaboration with the Department of Animal Nutrition and Physiology, Wageningen and the Department of Toxicology of the Agricultural University, Wageningen. The research was supported by a grant from the Ministry of Agriculture, Nature Management and Fisheries, The Hague.

ABBREVIATIONS

AAS	Atomic absorption spectrometry
ALAT	Alanine aminotransferase
ANOVA	Analysis of covariance
ALP	Alkaline phosphatase
ASAT	Aspartate aminotransferase
Ca	Calcium
Cd	Cadmium
$CdCl_2$	Cadmium chloride
CdMt	Cadmium-metallothionein
Cu	Copper
DMt	Dietary metallothionein
FAAS	Flame atomic absorption spectrometry
Fe	Iron
GFAAS	Graphite furnace atomic absorption spectrometry
GGT	γ-Glutamyl transpeptidase
Hb	Haemoglobin
HMW	High molecular weight
IMt	Intestinal metallothionein
iv	Intravenously
kg	Kilogram
KMt	Renal metallothionein
LMt	Hepatic metallothionein
Mg	Magnesium
Mn	Manganese
mg	Milligram
ml	Milliliter
μg	Microgram
μl	Microliter
Mt	Metallothionein
P	Phosphorus
PCV	Packed-cell volume
ppm	Parts per million
LDH	Lactate dehydrogenase
MCH	Mean corpuscular haemoglobin
MCV	Mean corpuscular volume
NAG	N-acetyl-ß-D-glucosaminidase
PTWI	Provisional tolerable weekly intake
RBC	Red blood cell
RP-HPLC	Reversed phase-high performance liquid chromatography
SD	Standard deviation
SEM	Standard error of mean
UV	Ultra-violet
Ve	Elution volume
Vo	Void volume
WBC	White blood cell
WHO	World health organization
Zn	Zinc

Chapter 1

GENERAL INTRODUCTION

Food Science and Technology, in part submitted

GENERAL INTRODUCTION

Cadmium (Cd) is an important occupational and environmental pollutant. Cd is widely distributed due to its environmental persistence. It has a long biological half-time which accounts for its bioaccumulation in organisms. Outside the industrial environment, the main route of human Cd exposure is through the food. Although most oral toxicity studies have been performed with inorganic salts of Cd, this is not the chemical form in which Cd occurs in the diet. In food of animal origin one of the main Cd binding ligands has been identified as the protein metallothionein. In plants cadmium is bound, at least partly, to proteins resembling metallothionein. The purpose of the present studies was to establish the toxicity and disposition of Cd-metallothionein in rats. For an assessment of human health risks it is of paramount importance to obtain more information about the bioavailability and toxicity of different Cd-complexes as found in the diet.

1. ENVIRONMENTAL CADMIUM EXPOSURE

1.1 USE AND OCCURRENCE OF CADMIUM

Cd naturally occurs in small quantities throughout the lithosphere. However, the widespread industrial use of Cd has caused a sharp increase in emissions to the environment and consequently to relatively high levels of environmental contamination in many locations. Cadmium occurs in nature in conjunction with zinc and is found wherever zinc is found. Zinc is, in contrast to Cd, an essential metal for most forms of life. Zinc is ubiquitous and due to the close relationship with Cd, it is unlikely that any naturally occurring material will be completely free of Cd. (Elinder, 1985).

Cd is mainly used in the electroplating industry (anti-corrosion agent) and alloy manufacturing, but also in alkaline batteries, pigments and plastic stabilizers. Cd is also a by-product of zinc and lead refineries.

It should be stressed that most uses of Cd are completely dissipative and only a few percent of all Cd currently used, is recycled (Nriagu, 1980; Page et al., 1986)

Cd in soil may occur naturally, but in most cases the pollution is a result of industrial emissions, mining operations, waste incineration and combustion of oil and coal. Sludge-based fertilizers and phosphate fertilizers are important sources of Cd contamination in agricultural soils. Crop grown on contaminated soils take up Cd very efficiently. Due to the bioaccumulation in soil, plants and animals and due to its environmental persistence, Cd can be considered as a food chain contaminant.

1.2 CADMIUM EXPOSURE IN HUMANS

Humans are exposed to Cd via the food, water, air and dust. Outside of the industrial environment, the main source of environmental Cd exposure for the general population is the intake via food. This is illustrated in table 1 derived from Hallenbeck (1985) who has compiled data on the daily intake of cadmium by adults, together with sources contributing to the total. Cd levels in drinking water are generally low and water consumption does not contribute much to the total daily intake of Cd in adults. Smoking is another source which contributes substantially to the daily intake of Cd. Smoking of 20 cigarettes per day will cause an uptake of circa 4 μg per day. In comparison to the intake via the food this does not seem to be that much, but one has to be aware of the fact that the oral absorption of Cd is much lower (4-8%) than inhalatory absorption (15-40%). Outside of smoking, absorption via the lungs can only contribute substantially to the daily intake when individuals are living closely near sources of Cd emission.

Cd is present in virtually all foodstuffs. The highest concentrations can be found in internal

organs (kidney and liver) from cattle, in seafood (oysters, crab, mussels) and in some mushroom species. The Cd concentration in these products can be in the order of mg's per kilogram, even in nonpolluted areas (Elinder, 1985; Robards and Worsfold, 1991; Galal-Gorchev, 1991). In rice and wheat the Cd concentration varies between 10 and 150 μg/kg. Meat, fish and fruit usually contain Cd levels in the order of 1 to 50 μg/kg. The concentrations in dairy products are generally low and in the order of a few micrograms per kilogram. Examples of exceptionally high Cd intake levels, exceeding the PTWI, have been reported due to the above average consumption of mussels, wild mushrooms or internal organs of cattle (Archibald and Kosatsky, 1991; Andersen, 1981; Mc Kinzie et al., 1982). These cases show that the actual Cd intake of some human populations in unpolluted areas is consistently above average due to an atypical diet (dietary habit).

Table 1. Calculated hypothetical total daily intake of cadmium and contributing sources

Individual	Source of cadmium	Intake in μg
non-smoker living in rural area	air	0,0005
	food	4
	water	2
	total	6
smoker living near cadmium source and eating contaminated food	air	25
	food	84
	water	2
	tobacco	4
	total	115

It should be stressed that in some Cd polluted areas the Cd concentration of cereals and meat is much higher than mentioned above. One of the most severe cases in which a population was exposed to highly Cd contaminated food has been reported from Japan and is known as the Itai itai disease (review of Kjellström, 1986). The Japanese rice incident demonstrated that chronic Cd poisoning constitutes a health hazard to the general public through environmental exposure and it showed that the hazard of Cd was not restricted to industrial workers (see also section 4.2). Increased levels of Cd in food as a result of Cd pollution have been reported from many other countries including the Netherlands (Lauwerijs et al., 1980, Sherlock et al., 1983, Elinder, 1985, Copius Peereboom Steegeman and Copius Peereboom, 1989). For instance, in the Netherlands one of the largest polluted areas is the area of the Kempen. The Cd contamination of the soil is due to the Cd emissions from zinc factories; vegetables grown in that region contain fairly high Cd levels (up to 1.2 ppm; Haskoning, 1985). As a consequence the cattle grazing there have elevated Cd levels in liver and kidneys (with maximum values up to 7,66 mg/kg; Spierenburg et al., 1988). Inhabitants eating vegetables of their own gardens, show a higher daily intake than the provisionally tolerated weekly uptake of Cd (Copius Peereboom-Stegeman and Copius Peereboom, 1989).
The calculated intake of Hallenbeck (1985) for an individual living in an unpolluted area is well below the provisional tolerable daily intake of 60-70μg Cd recommended by the WHO (1972). Actual dietary intake levels in Europe and the U.S in the period 1980-1990 are in the order of 15 to 50 μg Cd/day with large individual variations (Gartrell et al., 1986a,b; Louekari et al., 1986; van Dokkum et al., 1989; Robards and Worsfold, 1991; Galal-Gorchev, 1991). Due to the small safety margin between the actual dietary intake and the tolerable level of Cd, dietary exposure to Cd is considered a health problem of major concern in humans.

1.3 Cd-SPECIATION IN FOOD

There is a clear lack of data describing the form, or chemical species, in which Cd occurs in different foodstuffs. In animal tissues of vertebrates and many invertebrates (mollusca, crustacea, insecta) most of the Cd occurs as an inducible, about 7000 dalton (10.000 D apparent molecular weight) cysteine rich protein, called Cd, Zn-thionein, or more generally, metallothionein (Kägi and Nordberg, 1978; Stone and Overnell, 1986; Hamer, 1986; Webb, 1986; Kägi and Kojima, 1987). Vegetable food is even a more important source of dietary Cd intake than animal food. Yet we know little about the form in which Cd occurs in plants.
In plants Cd is at least partially complexed by several metal binding complexes, such as organic acids, metallothioneins and phytoche-

latins. There is increasing evidence that the phytochelatins are the major metal binding proteins in higher plants (Grill et al., 1987, Verkleij et al., 1990). These peptides consist of repetitive γ-glutamylcysteine units with a carboxyl-terminal glycine (poly(γ-EC)G's. Although phytochelatins have certain properties in common with metallothioneins (Cd-cysteine complex, heat stability, inducibility, synthesis from glutathione or its precursor γ-glutamyl cysteine), they belong to a structurally different class of metal binding proteins (Wagner, 1984, Grill et al., 1987, Verkleij et al., 1990). Still, phytochelatins are functionally analogous to metallothioneins (Grill et al., 1987).

It is not yet known whether or not Cd in certain food stuffs is less readily absorbed from the gastrointestinal tract when compared to Cd from other sources. A difference in Cd-availibility of different foodstuffs will certainly have a great impact on the risk evaluation of dietary Cd and on the estimation of tolerable Cd levels in different types of food products.

2 ABSORPTION AND METABOLISM

2.1 GASTRO-INTESTINAL ABSORPTION OF Cd

A large proportion of the ingested cadmium passes the gastrointestinal tract without being taken up in the small intestine. Most toxicokinetics studies performed with inorganic Cd-salts have shown that the Cd retention in rats varies between 0.5 and 2% (Kello and Kostial, 1977; Moore et al., 1973), in mice between 0.5 and 3% (Engstrom and Nordberg, 1979, Andersen et al., 1988), and in apes probably between 2-6% (Nordberg et al., et al., 1971; Suzuki and Taguchi, 1980). These absorption data from experimental animals are highly dependent on the relative dose and the dietary composition (see chapter 3). In a group of 14 human volunteers, the average net intestinal uptake of cadmium was found to be c. 4.5% (McLellan et al., 1978). In the study of Rahola et al (1972) the average Cd absorption in 5 human volunteers was at least 6%. Lastly, in another study the net intestinal uptake of Cd was estimated to be 2.6% in males and 7.5 % in females (Flanagan et al., 1978). It should be emphasized that most studies of gastrointestinal absorption have merely compared the dose given with the amount retained in the body shortly after dosing. This might indicate that the retention in long term experiments is slightly different. Nevertheless, it seems very likely that the gastrointestinal absorption for man and monkeys is somewhat higher than for rodents.

Mechanism of intestinal Cd uptake

Essential metals are generally taken up actively by a carrier system. However, most non-essential metals, including Cd seem to be taken up in non-specific way. There are no data in the literature that argue for an active carrier-mediated uptake of Cd in the mucosal cell layer. In general, the Cd uptake can be divided in two steps:

step 1 **step 2**
luminal Cd ----> *mucosal Cd* ----> *systemic Cd*

Step 1 represents the uptake from lumen into the intestinal mucosa. Foulkes and co-workers (1980; 1986) showed that step 1 may further be separated into step 1a, representing the electrostatic binding of Cd to the outside of the brush border, followed by step 1b, representing internalization by a passive, temperature-dependent process. Step 1b is probably related to membrane fluidity and does not occur via pinocytose. This assumption was based on the finding (Foulkes, 1988, Bevan and Foulkes, 1989) that Cd is internalized into brush border vesicles and erythrocytes in vitro; neither of these have appreciable pinocytic activity. Furthermore, it was shown that step 1 follow first order kinetics at luminal Cd concentrations up to 200 μM (Foulkes et al., 1980) and at a oral dose of 10 μg/kg body weight (Goon and Klaassen 1989).

Step 2 represents passage across the basolateral membrane into the serosal fluid and blood stream and appeared to be rather constant at luminal Cd concentrations between 2 and 200 μM. It is proposed that the rate of step 2 is probably much lower than the rate of step 1, (Kello et al., 1978; Foulkes 1988) and thus step 1 may have a regulatory role in the absorption of Cd. The difference between the rates of step 1 and step 2 of the intestinal Cd uptake will result in the accumulation of Cd in the intestinal tissue. However, the actual Cd accumulation in the

intestine is probably much lower due to the continuous renewal of the intestinal epithelium and desquamation of the old mucosa (Valberg et al., 1976).

Role of intestinal metallothionein in the control of Cd absorption

It has been shown that the protein metallothionein in the intestine is inducible by both zinc (Zn) and Cd (Richards and Cousins, 1975; Ouelette et al., 1982; Clarkson et al., 1985; Min et al., 1991). It was originally proposed by Richards and Cousins that the intestinal metallothionein could help retain the excess of zinc in the mucosa and, in this manner, controls the zinc absorption. Consequently it was suggested that the intestinal Mt is also a major determinant of the intestinal Cd absorption (Squib et al., 1976; Engström and Nordberg, 1979). However, the findings on the possible role of Mt in the control of Cd absorption are contradictory. For example, Kello (et al., 1978) found no effect of the intestinal Mt induction on the ratio between step 1 and step 2 of the Cd uptake. In contrast the work of Foulkes and McMullen (1986) showed that induction of intestinal Mt decreases step 2 of Cd uptake. Andersen (1989) postulated that in the study of Foulkes the binding capacity of intestinal Mt was exhausted in uninduced animals, which were exposed to relatively high Cd levels. Consequently induction of intestinal Mt by zinc will lead to a reduction of step 2. In the study of Kello the relatively lower Cd doses could not saturate the intestinal Mt in unexposed animals. This hypothesis is not fully conclusive since a recent study of Goon and Klaassen (1989) using a Cd dose (100 μg Cd/kg recirculating perfusion) even higher than in the study of Foulkes and McMullen (5 -30 μg/kg body weight without recirculated perfusion) did not show any effect on Cd absorption by Zn-preinduction of intestinal Mt.

The analysis of Cd-transport and the role of intestinal Mt in isolated intestinal segments can provide information of only limited quantitative significance to evaluate the absorption in the intact animal. The difficulty arises from the fact that the absorption *in vitro* can not be influenced by dietary factors, by the changes in rate of intestinal transit and by the gastric and intestinal secretions. It should therefore be emphasized that it has been shown that *in vivo* metallothionein does play a significant role in the intestinal uptake of Cd at a dose level of 5 mg/kg (Min et al., 1991). The study shows that intestinal Mt is involved in the selective renal disposition of Cd. We will discuss this further in section 2.2.

2.2 Cd-DISTRIBUTION

After uptake (step 2 of gastrointestinal absorption), Cd is mainly transported via the blood plasma, although the main part (90-95%) of blood Cd is bound to the red blood cells. In plasma, the main part of Cd is present as Cd-albumin and Cd-metallothionein. There are several studies reviewing the Cd distribution in animals and man and it is beyond the scope of this introduction to discuss this matter in detail. In short, two main sites of storage of Cd are the liver and kidneys. About 40-80 % of the body burden is found in these two organs and in the case of low level exposure, about 30-50% is stored in the kidneys alone (Bremner, 1979; Nordberg et al., 1985, Bernard and Lauwerijs, 1986; Foulkes et al.,1990). Within the kidneys, the highest concentration is found in the cortex. Particularly high concentrations are found in the proximal tubular cells.

In humans, it has been shown that in kidneys a maximum Cd level is reached at an age of 40-50 and in liver the accumulation decreases after 30 years of age. In most human studies the renal cortex level is about 10 to 30 times higher than that in liver at an age of about 50 (Nordberg et al., 1985; Elinder, 1985b).

Role of intestinal metallothionein in distribution of cadmium

It has been shown in mice (Engström et al., 1979), rats (Lehman and Klaassen 1986) and in quails (Scheuhammer, 1988) that after absorption of very low amounts of ionic Cd through the intestine Cd is mainly deposited in the kidneys, whereas high doses of Cd predominantly result in hepatic Cd deposition. It was proposed that a low amount of Cd will mainly be bound to endogenous metallothionein of the intestine, which will be released in the systemic circulation and then be deposited in the kidneys.

This theory is based on the findings that after intravenous injection of low molecular weight

complexes such as Cd-cysteine or Cd-metallothionein, Cd in the blood stream remains bound to these low molecular weight proteins. In this form Cd will be efficiently taken up in the kidneys by glomerular filtration and subsequent reabsorption (Nordberg et al., 1975; Tanaka et al., 1975; Squibb et al., 1984). However, after a higher dose (100 μg/kg or higher) the available endogenous circulating Mt pool is overloaded (see also 2.1) and Cd will bind to high molecular plasma proteins and then be deposited in the liver (Lehman and Klaassen, 1986; Ohta and Cherian, 1991). This is supported by the fact that intravenous injection of Cd-proteins with a low Cd-affinity or intravenous injection of Cd-ions, will result in a redistribution of Cd to high molecular weight plasma proteins such as albumin. These Cd-albumin complexes are indeed deposited in the liver (Garvey and Chang, 1981; Suzuki, 1984). Thus, the binding form of Cd in blood is of crucial importance for the final distribution to the main storage organs.
Just recently it has been shown that pre-induction of intestinal Mt indeed causes a selective renal Cd accumulation (Min et al., 1991). Moreover, the immunological identification of intestinal metallothionein in the blood plasma supports the theory that intestinal Mt is indeed released into the systemic circulation (Elsenhans et al., 1991).

Role of hepatic metallothionein in the distribution of cadmium

The distribution between renal and hepatic Cd is not only dose- and species- but also time-dependent: with increasing time after exposure, the kidney levels will increase due to redistribution from the liver (Gunn and Gould, 1957; Lucis and Lucis, 1969; Webb, 1979). The redistribution of Cd with time might be supported by the hypothesis, originally proposed by Piscator (1964), that Cd, Zn-metallothionein is transported from the liver to the kidneys. In the first few hours after uptake in the liver most of the cadmium in the liver is bound to high molecular weight proteins in the cytosol and then within 24 hours is reassociated to freshly synthesized metallothionein to form a Cd, Zn-Mt. There is some evidence to believe that hepatic Cd, Zn-Mt is indeed present extracellularly in liver sinusoids (Banerjee et al., 1982) which might be released from the liver due to normal turnover of hepatocytes or due to increased cell death (Dudley et al., 1985; Webb et al., 1986). It has been shown that hepatic metallothionein containing Cd and Zn released into plasma, contains merely Cd and copper (Cu). Due to it's high affinity to Cu it seems likely that zinc might be replaced by copper and the plasma metallothionein serves under these conditions as a carrier protein for copper. (Webb, 1979; Suzuki, 1984).
Thus, Cd-metallothionein derived from the intestine (after low oral doses of Cd) or liver (after high oral doses of Cd), is likely to be reabsorbed into the renal proximal cells after glomerular filtration. The metallothionein protein will be broken down by the lysosomes and nonmetallothionein-bound Cd will be released in the kidney cells. This will stimulate the (de novo) synthesis of renal, endogenous metallothionein. This Mt contains in contrast to the hepatic Cd, Zn- Mt both Cd, Zn and Cu (Suzuki, 1984). There is a constant renewal and turnover of endogenous and exogenous metallothionein in the renal cortex.

2.3 METALLOTHIONEIN: INDUCTION, BIOLOGICAL ROLE AND OCCURRENCE

In animals, the protein Mt is most abundant in the cytoplasm of liver, kidneys, intestine and pancreas. The biologically half-life of Mt's generally ranges from 1 to 4 days, depending on the type of metal bound. The primary structure of Mt is characterized by a high content of cysteine, serine and glycine and its lack of aromatic amino acids and histidine. Most characteristics of Mt are shown in table 2. According to Fowler (et al., 1987) and Kägi and Schäffer (1988) mammalian and most metallothioneins of mollusc and crustacea belong to class 1 metallothioneins. Class 2 comprises Mt's from sea urchin, wheat and yeast, whereas class 3 contains the atypical polypeptides such as poly-γ-ECg's derived from plants (see section 1.3). Class 1 mammalian Mt's usually contain two major fractions called Mt-1, Mt-2, differing at neutral pH by a single negative charge.
Due to its high affinity for and inducibility by non-essential metals such as Hg, Cd and Pt the protein serves as a detoxification agent for toxic metals. This hypothesis was originally proposed by Piscator (1964). Due to the short biological half-life there is a constant degradation and

renewal of Cd-Mt in the liver and kidneys explaining the long biologic halflife of Cd in these organs. As shown in section 2.2, Mt probably plays a key role in the transport and distribution of Cd throughout the body. There are numerous articles available reviewing both biochemistry and biological function of the protein (Webb, 1979; Webb, 1986; Kägi and Kojima, 1987; Richards, 1989; Waalkes and Goering, 1990).

The physiological function of Mt is probably related to the Zn and Cu homeostasis, and it has indeed been shown that high Mt levels are present during periods of high Zn demand. It is thought that Mt supplies Zn within rapidly growing tissues which might declare the high Mt levels at birth (Wong and Klaassen, 1979). The role of Mt as an Zn and Cu donor might be supported by the finding that apoenzymes which require Zn and/or Cu have been activated in vitro after incubation with Zn,Cu-thioneine (Udom and Brady, 1980; Geller and Winge, 1982; Goering and Fowler 1987). However, to the best of our knowledge at present no such evidence has been shown in vivo.
Except Mt's role in metal metabolism, there are several other chemical and physical treatments

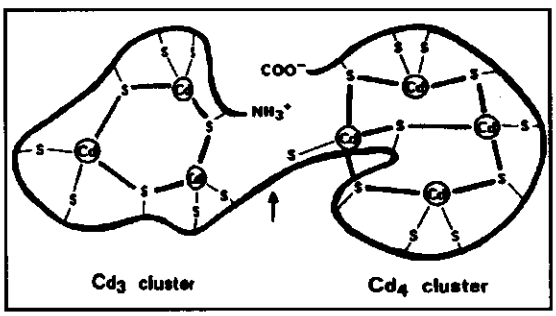

Fig.1. Schematic representation of the structure of Cd,Mt from rat liver (From Winge and Miklossy, 1982)

which are known to induce Mt synthesis (see review of Kägi and Schäffer, 1988). In the case of exposure to electrophilic attack (O2, free radicals, alkylating agents) it has been suggested that Mt detoxifies reactive intermediates due to the presence of nucleophilic thiol groups which have a neutralizing activity. Mt has been shown to be an effective scavenger of oxygen radicals after radiation-induced oxidative stress (Thornalley and Vasak, 1985; Matsubura et al., 1987).
It should be stressed that, despite all the research, the exact function(s) of Mt is not yet known. Further research in the field of molecular biology, biochemistry and physiology is certainly required.
The role of Mt in the transport and distribution of cadmium has been discussed in section 2.2 The consequence of the presence of Mt in the diet will be discussed in 3.2. Lastly, the role of Mt in the nephrotoxicity of Cd will be discussed in section 4.1.

Table 2

Properties of metallothionein
molecular weight 6000-7000
metal clusters with thiolate ligands
high cysteine and metal content
deficient in aromatic acids and histidine
inducible by metals and other treatments
heat stability
optical properties characteristic of metal-thiolate bonds

3 FACTORS INFLUENCING CADMIUM ABSORPTION AND DISTRIBUTION

3.1 DOSE

There is a surplus of evidence to suggest that the dose level and the body retention in laboratory animals are positively correlated. In the study of Engström and Nordberg (1979) mice exposed between 1 and 37000 μg Cd/kg show an intestinal absorption in the range of 0.4 and 3.2 %. Lehman and Klaassen (1986) found a similar effect in rats since the percentage of the dosage retained 7 days after administration increased from 0.4% at the 1 μg/kg dosage to 1.65 % at the 100 μg/kg and higher dosages. Similar observations were made in quails (Scheuhammer, 1988) and mice (Andersen 1988). The studies of Scheuhammer (1988) and Goon and Klaassen (1989) have shown that the increase in absorption due to increased dosages does not appeared to occur gradually. It seems that at dosages lower than 10-100 μg/kg the total absorption is quite constant, ranging from 0.4-

0.8 % whereas at dosages of 100 μg/kg and higher the intestinal absorption is at least 10 times higher.
It is believed that in step 1 of the intestinal uptake the endogenous Mt pool is not overloaded (see 2.1) and the uptake follows first order kinetics. However, at high dosages the intestinal Mt pool is saturated with Cd and the Cd uptake will be facilitated. Moreover, the same authors suggest that high doses of Cd are directly toxic to the mucosa which will causes the abrogation of the integrity of the intestinal wall, which might explain the enhanced Cd absorption. Histopathological changes as reported in other studies (Valberg et al., 1976; Richardson and Fox, 1975) indeed do occur at dose levels comparable to the high dosages used in the disposition studies mentioned above.
In summary, the Cd concentration in the intestinal lumen determines the final Cd absorption in the body. In addition, as shown in section 2.2, the oral Cd dose is also a major determinant of the differential Cd distribution between liver and kidneys.

3.2 SPECIATION

Although most Cd disposition studies have used inorganic salts of Cd, this is clearly not the chemical form in which Cd occurs in the diet (see section 1.3). Several studies therefore dealt with the Cd-distribution after oral exposure to Cd salts compared to biologically incorporated Cd. For example, internal organs of Cd-exposed pigs, oysters, scallops and canned crab (table 3) have been used as source material for exposure to biologically incorporated Cd. It seems very likely that in this source material part of the Cd will be complexed with class 1 metallothionein. However, Cd-binding ligands were not characterized prior to use and the results of these studies are difficult to interpret.
In several other reports the oral Cd absorption was studied after administration of purified CdMt (Cherian, 1979; Maitani et al., 1984; Ohta and Cherian, 1991). From these studies it appears that at Cd absorption from CdMt is lower than from inorganic Cd salts, at least at the dose levels chosen. Moreover, these studies show that the liver to kidney ratio of the Cd concentration is lower after exposure to CdMt compared to inorganic Cd salts. This effect was mainly due to the selective renal Cd disposition after oral CdMt exposure. Since it is well known that CdMt after iv administration preferentially accumulates in the kidneys, it has been suggested that oral CdMt or CdMt fragments can pass the intestinal mucosa and consequently show the same renal disposition as parenteral CdMt. In this regard some evidence suggest that the sequence of events for intestinal uptake of CdMt is different from that for $CdCl_2$: It has been shown (Klein et al., 1986; Crews et al., 1989) that indeed at least part of the CdMt was not affected by *in vitro* treatment with gastro-intestinal proteolytic enzymes. Furthermore Cherian (1979) and Ohta and Cherian (1991) found after oral exposure to exogenous CdMt that a major portion of ingested Cd still was associated to a protein with a moleculair weight of c. 10,000 dalton, whereas after exposure to ionic Cd the majority of the Cd was bound to a protein with high molecular weight. This suggests that exogenous CdMt (fragments) does in fact reach the intestine, which indicates that there is a difference in metabolic pathway between CdMt and $CdCl_2$. However, rats exposed to low doses of $CdCl_2$ show, similar to dietary CdMt, selective renal Cd accumulation and it is not clear whether the selective renal accumulation after CdMt exposure is also dose dependent. If the uptake of CdMt is dose-dependent than it seems possible that the difference in renal Cd-accumulation between CdMt and $CdCl_2$ is not due to a difference in metabolic routes, but simply due to the difference in intestinal Cd absorption.

3.3 DIETARY FACTORS

The effect of protein, fat and fibre.

Rats fed semisynthetic diets, high in fat, low in fibre, show a much higher body retention of Cd than rats fed standard rat diets (Andersen, 1989). Furthermore, rodents fed semi-synthetic diets with milk (Engström and Nordberg, 1979; Kello and Kostial, 1977b), or casein (Revis, 1981; Uthe and Chou, 1979) retained a significant larger fraction of orally administered cadmium than animals fed a cereal based diet. Finally, Rabar and Kostial (1981) found a larger Cd retention in animals fed human diet components such as milk, meat and bread than in rats fed normal diets. It should be noted that these semi-synthetic diets are much closer to the

Table 3. Oral studies on the disposition of cadmium from inorganic and organic Cd sources

Source material	Type of study	Dose, animal	Absorption and distribution	References
$CdCl_2$ versus CdMt	open-ended duodenal perfusion, 60 min.	100 μM Cd, rat	Body retention CdMt twice as low.	Valberg et al., 1977
Cd-sulfate versus Cd-lettuce leaves	semi-synthetic diet once for 5 hours in 12 days experiment	40-80 μg/2.5 g diet in rat	Slightly increased uptake of Cd from lettuce as compared to Cd-salt	Welch et al., 1978
$CdCl_2$ versus Cd Glutathione versus CdMt	oral gavage, once for 4 hours	60 μg Cd per mouse	Intact isolation of ingested CdMt from intestinal mucosa? CdMt shows lower hepatic and higher renal Cd-accumulation	Cherian, 1979
Cd-sulfate versus Cd-scallops. (Chemical form of Cd unknown)	semi-synthetic diet, 28 days	5 mg/kg diet, in rat	No significant difference in retention between inorganic & biologically incorporated Cd	Lagally et al., 1980
$CdCl_2$ versus Cd-porcine kidney/liver versus Cd-lobster (Cd-form not identified)	semi-synthetic diet, 90 days	21 mg Cd/kg diet in rat	Cd uptake from Cd-lobster digestive gland and from Cd-porcine liver/kidney is lower than from inorganic Cd. No selective renal accumulation of biologically incorporated Cd.	Uthe and Chou, 1980
$CdCl_2$ versus CdMt	oral gavage, five times in 5 weeks	20 μg Cd/kg mouse	Total CdMt retention in body is lower. Renal Cd accumulation for CdMt is higher	Cherian, 1983
$CdCl_2$ versus Cd-oyster. Cd is bound to 1000 D peptide	semi-synthetic diet, 28 days	1.8 mg Cd/kg diet, in mouse	Oyster Cd was retained at a lower rate in all tissues. Relative renal Cd accumulation for Cd-oyster higher.	Siewicki et al., 1983
$CdCl_2$ versus CdMt	oral gavage, once for 3 days	0.5 mg Cd/kg mouse	CdMt shows higher kidney/liver Cd ratio than $CdCl_2$ due to the low Cd-accumulation in liver	Maitani et al., 1984
$CdCl_2$ versus Cd-oyster (Chemical form of Cd in oyster unidentified)	semi-synthetic diet, 14 days	0.4 mg Cd/kg diet in mouse	No significant difference in Cd retention. Relative renal Cd accumulation for Cd-oyster is somewhat higher.	Sullivan et al., 1984
$CdCl_2$ versus Cd_3phytate	Gavage, once for 10 days	1 mg/kg rat	At normal phytate concentration in diet no difference in body retention.	Jacki et al., 1985
$CdCl_2$ versus Cd-crab (chemical form of Cd in crab unidentified)	semisynthetic diet, 6, 12, 24 weeks	4 mg Cd/kg diet	Accumulation in liver and kidneys is twice as low for Cd incorporated in crab meat.	Maage and Julshamn, 1987
$CdCl_2$ versus Cd-spinach/Cd mussels	semisynthetic diet, 4 weeks	30 mg Cd/kg diet in rat	Hepatic Cd accumulation from Cd-mussels is lower than from Cd-spinach or $CdCl_2$	Sinkeldam et al., 1989
$CdCl_2$ versus CdMt	cannulated jejunum segments, 60 min.	50 μg/0.5 ml per rat	Intestinal and hepatic uptake of CdMt is lower than for $CdCl_2$. Renal accumulation is similar for both Cd-forms	Ohta and Cherian, 1991

human "western type of diet" than standard rodent diet and therefore, the apparently higher intestinal uptake in humans as compared to rodents is not necessarily due to a difference in uptake mechanism, but might be attributed to the differences in diet composition. Animals fed Cd in combination with sea food or meat products show a lower retention of cadmium than rats fed Cd combined with casein (Lagally et al., 1980; Uthe and Chou, 1979; Siewicki et al., 1983). This indicates a lower retention of cadmium bound to animal protein. It should be emphasized that in studies, comparing the Cd disposition of inorganic Cd-salt with organic Cd from seafood and meat, the animals exposed to inorganic Cd have to be fed a similar amount of uncontaminated sea food or meat. This in order to avoid the effect of the animal protein on the Cd-disposition.

Interaction with minerals

There is some evidence that several trace elements can inhibit step 1 of the Cd absorption in the intestine in vitro (Sahagian et al., 1967) and in situ (Foulkes, 1985). It is suggested that during step 1 of the absorption Cd is electrostatically bound to the surface membrane and since this is a non-specific effect, many polyvalent cations can inhibit the Cd uptake by neutralizing the membrane charge (Foulkes, 1985; 1988). Over the past twenty years several elements like Zn, Cu, Fe, Ca, P, Mn, Mg and Se have been shown to interfere with the Cd-metabolism in animals and in humans (Task-group on Metal Interactions, 1978; Fox et al., 1983; Chmielnicka and Cherian, 1986). It has been suggested that Cd competes with Zn, Ca and Fe for the specific carriers, but non of these specific interactions has been proven.

Several studies have shown that diets with a low Calcium (Ca) content can increase the body retention of Cd (Larson and Piscator, 1971; Hamilton and Smith, 1978). Moreover it is shown after in situ perfusion of the intestine, that a high Ca dose can inhibit Cd absorption (Foulkes, 1980). The reversed reaction i.e. the inhibition of the Ca absorption by Cd has also been reported (Ando et al., 1977). This interaction is of special importance because of the suggested role of Cd in the osteomalacia of the Itai-itai disease.

Interactions between the uptake of Cd and Fe in animals have also been reported frequently. For Fe-deficient animals it has been shown that both the uptake of Cd in the intestinal mucosa (step 1) and its transport into the body (step 2) is increased (Hamilton and Valberg, 1974; Flanagan et al., 1978; Fox et al., 1979). For man it has been shown that persons with low iron stores (mostly females) as indicated by low serum ferritin levels absorbed more Cd than persons with high iron stores (Flanagan et al., 1978, Shaikh and Smith, 1980; Bunker et al., 1984). On the other hand, it has been shown in labo-ratory animals that dietary supplements of iron protect against Cd accumulation (Fox et al, 1971; Fox, 1980) and against Cd-intoxication (Pond and Walker 1972). The mechanism behind the competition of Fe and Cd is still not understood.

Similar to Ca, Zinc (Zn) can inhibit the intestinal transport of Cd after in situ perfusion, although the concentration of Zn required for this effect is very high (Foulkes, 1980; 1985). It appears that animals fed Cd with low Zn levels show increased Cd absorption (Jacobs et al. 1978b). Except for the site of absorption Cd and Zn interact at many other sites in the body, as might be predicted from their similar chemical properties. An important site for interaction can be found in the protein metallothionein (see section 2.3). It has been shown that the increase of Zn in the renal cortex of horses is related to the production of metallothionein which contains Cd as well as Zn (Nordberg et al., 1979). Human autopsy studies have shown that the Zn concentration increases in kidney cortex with increasing Cd concen-tration (Piscator and Lind, 1972) probably due to formation of metallo-thionein, binding both Cd and Zn.

In conclusion, mineral-deficient diets can increase the gastro-intestinal absorption of Cd. However, information on the protective effect of mineral supplements in the diet, is rather restricted. Furthermore, most researchers have studied single metal-cadmium interactions and there are only few studies that have examined the effect of dietary combinations of minerals (Banis et al., 1969; Jacobs et al., 1978a; 1978b, 1983). The impact of dietary minerals on the absorption of biologically incorporated Cd has not yet been investigated.

4 CHRONIC ORAL TOXICITY

4.1 ANIMAL STUDIES

The effects of repeated oral administration of inorganic cadmium, mainly as the chloride salt, have been extensively studied in laboratory animals and these studies have often been reviewed (Fielder and Dale, 1983; Friberg et al., 1986; Foulkes, 1986; Ros and Slooff, 1988). Target organs are the kidneys, the haematopoietic system, the bones and the liver.
The carcinogenic potential of Cd is still under debate: the heavy metal is a potent carcinogen for the testis and prostate and at the sites of injection (see review of Waalkes and Oberdörster, 1990), but there is no evidence of carcinogenicity in rats and mice after oral administration (Collins et al., 1992).
This section will be restricted to some comments on non-neoplastic lesions observed in the kidneys, the haematopoietic system and the liver.

Renal effects

The most characteristic toxic effect after oral exposure to cadmium is nephrotoxicity, the effects usually being limited to the proximal tubules. At high doses the glomeruli and the renal blood vessels may also be affected. Table 4 summarizes some oral Cd studies in rats and monkeys showing the relationship between renal cortical Cd-concentration and the renal toxicity. Most animal data indicate that at a renal cortex Cd level of 100-200 mg Cd/kg tissue renal tubular damage will occur. For example Sugawara et al. (1974) found slight tubular damage at a renal Cd level of 91 mg/kg and severe renal effects were noted at 224 mg/kg renal tissue in Wistar rats. Primates appear to have similar sensitivity to renal effects in rats. However, the critical concentration of Cd in the renal cortex was higher in primates ($\pm$600 mg/kg) than in the rat (Fielder and Dale, 1983).
Atrophy and degeneration of tubular cells are the most striking histopathological lesions. Cd-induced proteinuria is the most characteristic sign of renal tubular damage. This proteinuria is mainly due to impairment of renal tubular reabsorption, which affects the reabsorption of all proteins, but especially low molecular weight proteins such as microglobulines, lysozyme and retinol binding protein (Kjellström, 1986; Bernard et al., 1991). Another approach for early detection of nephropathy is the detection of enzymuria due to increased urinary excretions of kidney-derived enzymes. Increased enzyme activity in urine of enzymes such as LDH, NAG indicates cell damage, whereas the increase enzyme activity of ALP and GGT indicates desquamation of the brush border of proximal tubular cells (Nomiyama et al., 1975; Dubach et al., 1988; Gatta et al., 1989; Stonard, 1990).

Role of CdMt in renal toxicity

The main part of the Cd which reaches the kidneys is bound to metallothionein, which is either derived from the liver after degradation of Cd-albumin, or derived from the intestinal tract after transfer across the intestinal wall. The role of CdMt in the kidneys is somewhat paradoxeal since on the one hand it seems that synthesis of renal metallothionein protects against Cd toxicity, but on the other hand parenteral administration of CdMt leads to more pronounced nephrotoxicity than parenteral administration of inorganic Cd salts (Nordberg et al., 1975; Squib et al., 1979). This is due at least in part to a higher accumulation of Cd in the kidneys from CdMt exposure (Tanaka et al., 1975). CdMt is reabsorbed into the proximal renal cells and after intracellular degradation (see section 2.2) free Cd is released within the cell, which probably interferes with zinc-dependent enzymes and causes damage to the metabolism of the cell (Kjellström, 1986; Foulkes, 1990). Cd-toxicity is believed to depend on the amount of free ionic Cd present in the cell which is not sequestered by available endogenous metallothionein or glutathione (Jin et al., 1987; Suzuki et al., 1989). Alternatively, it has been suggested that CdMt will damage the brush border during reabsorption of the CdMt complex (Cherian, 1982) and indeed, a higher sensitivity of the kidneys to Cd in the form of CdMt has also been observed (Sendelbach and Klaassen, 1988). The Mt protein will be degraded by lysosomes and non Mt-bound Cd will be released in the cell, which will stimulate the synthesis of renal, endogenous Mt. As shown in section 2.2, there is a constant renewal and turnover of endogenous and exogenous metallothionein in the renal cortex. The critical renal Cd concentration is the particular Cd concentration after long term renal Cd

Table 4. Renal effects after long term oral exposure in relation to renal cadmium levels

Species	Administration	Cd Dose, Duration before effect	Reported findings	Renal cortex concentr.	Reference
Rat	$CdCl_2$ in water	50-200 mg/l water, 8.5 months	Renal histological changes at all doses levels	44-180 mg/kg	Kawai et al., 1976
Female wistar rat	$CdCl_2$ in diet	10 and 50 mg/kg diet, 41 weeks	High mortality at 22 weeks. After 41 weeks: severe tubule lesions at 50 mg/kg, slight effects at 10 mg/kg	91 and 224 mg/kg	Sugawara & Sugawara 1974
Female wistar rat	$CdCl_2$ in water	200 mg/l 8 months	Proteinuria at 8 month . Histology at 11 months revealed no lesions	200 mg/kg	Bernard et al., 1981
Male Sprague-Dawley rat	unspecified Cd-salt in water, high and low protein diets	50 mg/l, 90 days	Necrotic tubular cells	15 mg/kg normal diet 30 mg/kg high protein	Revis, 1981
Male wistar rat	unspecified Cd salt in water	50 mg/l, 24 weeks	Tubular necrosis, glomerular fibrosis, cell hypertrophy	60 mg/kg	Aughey et al., 1984
Male rabbit	$CdCl_2$ in diet	300 mg/kg diet, 4 and 10 months	Aminoaciduria and enzymuria at after 4 months. Proteinuria and glucosuria at 10 months	200 and 300 mg/kg	Nomiyama et al., 1975
Male Rhesus monkey	$CdCl_2$ in diet	3-100 mg/kg in diet for 30 months	β_2-microglobulinaria and aminoaciduria in week 40, glycosuria from week 60	560-635 mg/kg in week 10-60	Nomiyama et al., 1982

accumulation at which the amount of free (non Mt-bound) cadmium is high enough to cause cytotoxicity (Nordberg et al., 1985; Foulkes et al., 1990).

It has to be emphasized that acute nephrotoxicity due to parenteral administered bolus amounts of CdMt has only little relevance to the progressive renal accumulation of Cd in chronically exposed animals where the plasma CdMt levels are much lower. Whether the difference in renal toxicity between inorganic Cd salt and CdMt also applies to the oral route has not yet been investigated.

It seems possible that part of the CdMt in food (i.e. exogenous CdMt) might also reach the kidneys intact (see section 3.2). This might be relevant for assessing the health risk of biologically incorporated Cd.

Effects on the liver and the haematopoietic system

Several studies have shown that signs of anaemia after long-term oral exposure to cadmium develop in male rats (Wilson et al. 1941, Pond and Walker, 1972; Prigge et al., 1977). Although nephrotoxicity is regarded as the characteristic effect, decreased haemoglobin concentration and decreased packed cell volume are among the early signs of chronic, peroral toxicity of Cd (Elinder et al., 1986).

As discussed in section 2, after oral exposure to high Cd levels Cd-transport across the intestinal wall will be saturated and Cd bound to albumin in the blood will accumulate in the liver. Consequently, hepatic metallothionein will be induced, which then acts as a Cd-scavenger and decreases Cd toxicity. Therefore, overt signs of acute toxicity in the liver are most likely related to non-metallothionein bound Cd.

Studies of Dudley (et al., 1985) have shown that Cd is an effective hepatotoxin after chronic parenteral exposure. Moreover, other studies (Tanaka et al., 1981; Cain and Griffiths, 1980) have demonstrated that chemically induced hepatotoxicity in animals previously exposed to Cd, causes leakage of CdMt from the liver, which will be taken up in the kidneys (see section 2.2).
Dudley (et al., 1985) emphasized that Cd-induced hepatotoxicity may play a role in the nephrotoxicity seen in rats after long term exposure to Cd. However, the regulatory role of the liver is restricted when small amounts of Cd are ingested via the food, because the majority of the metal bypasses the liver and accumulates directly in the kidneys (section 2.2). It seems therefore not surprising that in mammals the level of Cd in the renal cortex is much higher than in liver (Elinder, 1985b).

4.2 OBSERVATIONS IN MAN

There is an extensive data base of case-reports from Cd alloy factories, in the U.K., USA and Japan and nickel cadmium factories or cadmium production plants in the UK, Sweden, Japan and Belgium (Lauwerijs et al., 1980; Fielder and Dale, 1983; Kjellström, 1986; Foulkes, 1986, Ros and Slooff, 1988). In workers exposed to Cd for prolonged period the principal organs affected are the kidneys and lung. It is important to point out that, similar to the results with animals, in most long-term studies in humans, renal effects preceded or occurred simultaneously with other effects and seem to develop at lower doses than those needed to produce prostatic cancer or lung cancer (Kjellström, 1986; Foulkes, 1990). Moreover, the relationship between Cd exposure and lung or prostatic cancer remains arbitrary (Sullivan and Waterman, 1988).
The most extreme cases of human toxicity after environmental Cd exposure have been those with renal dysfunction and bone symptoms, often diagnosed as "Itai itai disease". This disease occurred in epidemic proportions among the inhabitants of the Fuchu area in Japan, who ingested chronically Cd contaminated rice. In several of these cases the Cd intake through rice ranged from 500-2000 μg/day. The etiology of the disease does not only point in the direction of Cd-exposure, but the poor nutrition of the patients (low protein, Ca, Fe and Vitamin D) also was a necessary factor for the development of the high incidence of bone effects in that particular area. Since the improvement of the nutrional status of the local population and the decrease of the Cd contamination of food the Itai itai disease has disappeared (Kjellström, 1986b).

Similar to what has been found in animal studies, proteinuria in humans is characterized by an increased excretion of low molecular weight proteins such as β_2-microglobuline retinol binding protein and/or metallothionein (Bernard and Lauwerijs, 1991). Moreover, urinary excretion of N-acetyl-ß-glucosaminidase (NAG) as indicator of renal dysfunction is also suggested to be a reliable indicator of Cd exposure in humans (Nogawa et al., 1983; Mutti, 1989; Kawada et al., 1989).

Dose-response relationship

The critical renal concentration used in the risk assessment of Cd is 200 mg Cd/kg tissue and is mainly based on epidemiological studies with young, healthy workers in industry and on laboratory experiments with animals. In both cases the data concern exposure to inorganic Cd. It is difficult to extrapolate critical renal Cd levels to a critical daily intake level since Cd risk estimates are seriously hampered by uncertainties in both the assumptions (percentage absorption, intake levels, dietary form, etc.) as well the data base used for the calculations. For example, based on pharmacodynamic models (Kjellström and Nordberg, 1978; Kjellström, 1986c) it is estimated that an oral intake of 300-400 μg/day will cause renal dysfunction after lifetime exposure. On the other hand epidemiologic studies from Japan (Nogawa et al., 1989) suggest that renal damage will occur at a daily Cd intake of 100-200 μg/day.
Thus, safety-limits should be used very cautiously (Kjellström, 1986; Ros and Slooff, 1988; Archibald and Kosatsky, 1991).
The provisionable tolerable weekly intake (PTWI) recommended by the World Health Organization (WHO) is 0.4-0.5 mg based on a tolerable intake of 1 μg/kg/day (60-70 μg Cd/day; WHO, 1972). An oral daily intake of 1 μg Cd/kg in non-smokers for 50 years will lead to a mean cortex concentration of circa 50 mg/kg.

An average diet provides at least 100-250 μg/week and smoking of 20 cigarettes per day provides between 2-5μg of absorbed cadmium, which corresponds to 50-300 μg/week via oral intake. Smokers are therefore close to the WHO limit for tolerable Cd intake (Kjellström, 1986c). Inhabitants of the Dutch Kempen eating vegetables of their own gardens, show a higher daily intake than the PTWI of Cd of 400-500 μg/week (Copius Peereboom-Stegeman and Copius Peereboom, 1989). Inhabitants of the Kempen therefore may incur a higher health risk than the general Dutch population. This might be supported by the finding that renal function tests have indicated an increased excretion of RBP and NAG in the inhabitants of the Kempen (Kreis et al., 1987). Recent findings in Belgium have shown that almost 10% of the general population showed a Cd excretion higher than 2 μg/24 hours corresponding to an renal cortex level of 50 mg/kg at the age of 50 (Buchet et al., 1990). These Cd excretion levels are at least in 10% of the cases correlated to slight renal dysfunction (tested a.o. by the renal excretion of NAG and $ß_2$-microglobuline). This is inconsistent with the fact that a renal Cd concentration of 200 mg Cd/kg is believed to cause a higher incidence of renal dysfunction. The authors suggest that the difference might be due to the healthy worker effect leading to underestimates of the health risk for the general population (Buchet et al., 1990). The results have not been confirmed yet by other industrialized countries.

5 ANALYTICAL ASPECTS: ISOLATION AND QUANTIFICATION OF METALLOTHIONEIN

The protein metallothionein was first isolated from equine renal cortex (Margoshes and Vallee, 1957) and further characterized by Kagi and Vallee (1960, 1961). Since then, the most common techniques used in isolation of the protein are gel-filtration and ion-exchange chromatography.
To quantitate Cd binding proteins in mammalian tissue most studies make use of metal saturation techniques, gel-filtration techniques and immuno assays (Webb, 1986; Richards, 1989). The metal saturation assays appeared to be similar in precision and recovery of Mt to assays that directly measure the protein moiety, such as radioimmunoassays (Dieter et al., 1985). Molecular biology techniques have been developed to quantitate Mt mRNA levels (Hamer, 1986; Peterson and Mercer, 1988).
In the last years clear analytical improvements have been made to study Mt heterogeneity. For instance, the use of reversed phase high performance liquid chromatography systems has been applied to characterize different Mt isoforms expressed by different organs and animal species (Klauser et al., 1983; Richards and Steele, 1987; van Beek and Baars, 1988).
Although RP-HPLC does not achieve a detection sensitivity comparable with RIA techniques, its advantage over RIA and the other Mt-assay techniques is that it can, in one step, provide information concerning concentration of isometallothioneins in the tissue. Moreover the application of RP-HPLC to the isolation and characterization of Mt isoforms has demonstrated its superior capability in resolving additional isoforms not previously detected using conventional techniques. Lastly, the difference in chromatographic behaviour of different isoforms on HPLC offers the unique possibility to study simultaneously the metabolic fate of administered Mt and endogenous Mt. Figure 2 shows chromatograms of a reversed-phase HPLC system, demonstrating the UV-spectra of Mt-isoforms purified from liver of various species. The spectral analysis shows that different isoforms have similar UV spectra.

6 OBJECTIVES AND APPROACH

The main source of human Cd exposure is the diet and in the diet Cd is to a large extent associated with the protein metallothionein. However, health risk assessment of Cd is mainly based on oral studies with inorganic Cd salts. Therefore information is required on the difference in bioavailability and toxicity between inorganic Cd salts and CdMt. The main objectives of the studies described in the present thesis were:

- To compare toxicity of inorganic Cd and CdMt in rats and in cell cultures of target organs,
- To compare the dose-dependent kinetics of Cd-uptake from CdMt and inorganic Cd
- To establish differences in metabolic pathways between CdMt and $CdCl_2$ to predict disposition and toxicity at environmentally relevant doses.

The following approach was used to accomplish

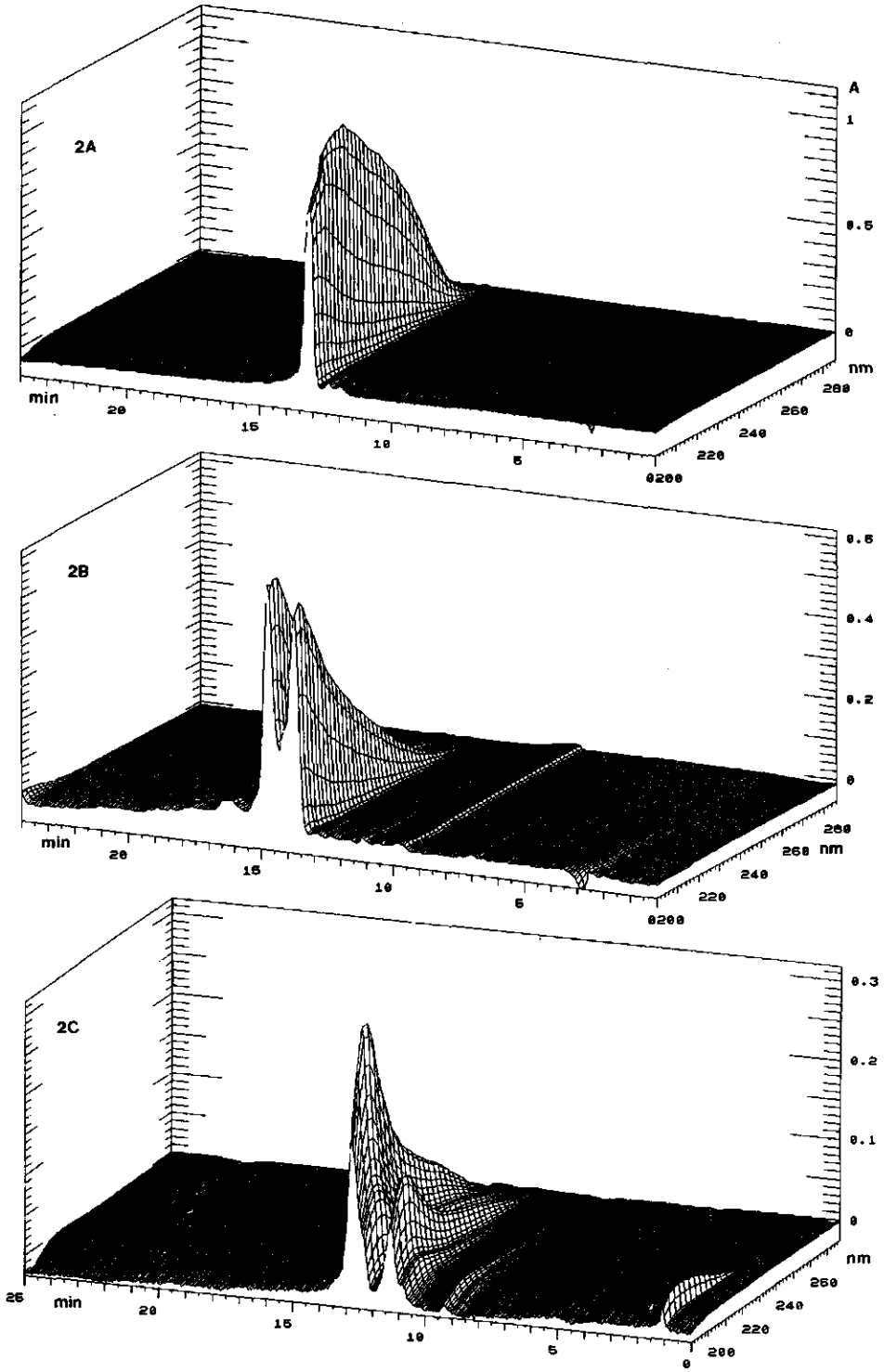

Fig. 2. Reversed-phase HPLC elution profile on a Hypersil ODS (250x6mm) column of purified hepatic CdMt isoforms. The separation was performed using 10 mM-sodium phosphate (solvent 1) and 10 mM phosphate buffer in acetonitrile (40:60, v/v solvent 2) as eluting solvents. Mt-samples were eluted with a linear gradient of 0-5% solvent 2 in 5 min and from 5 to 20% solvent 2 in 15 min with a flow rate of 1 ml/min. UV-spectrum (200-300nm) was determined with a diode-array spectrofotometer. X-axis=retention time, Y-axis=Absorbance, Z-Axis=UV-spectrum. A: Rat CdMt isoform-2, B= Pig CdMt isoform-2, C= Horse CdMt

these objectives. In **Chapter 2** a 4-week feeding study in rats is described to detect differences in early signs of toxicity between $CdCl_2$ and CdMt. The purpose of the studies described in **Chapter 3** was to establish the kinetics of the Cd uptake and to investigate the dose-dependent renal disposition of both $CdCl_2$ and CdMt. This study provides more insight into the observed differences in toxicity between CdMt and $CdCl_2$. **Chapter 4** deals with the Cd-accumulation and toxicity of CdMt and $CdCl_2$ in cell culture of the target organs. It shows differences in sensitivity between both Cd-forms at a cellular level. **Chapter 5** describes studies on interaction of minerals with the accumulation and toxicity of $CdCl_2$. The study identifies those minerals which are most effective inhibitors of Cd uptake. The aim of **Chapter 6** was to investigate the interaction of minerals with the absorption of CdMt. The results of Chapter 5 and 6 together provide information on the metabolic uptake routes of CdMt and $CdCl_2$. **Chapter 7** deals with the metabolic fate of CdMt in the intestine and kidneys after oral and intravenous administration. The study puts the findings on differences in toxicity, disposition and mineral interaction between CdMt and $CdCl_2$ into perspective.
The long-term diet study of **Chapter 8** was one of the end-points of the thesis to determine the nephrotoxicity after the chronic feeding of inorganic Cd and CdMt.

The outcome of these studies is discussed in the "Concluding remarks" within the frame work of the risk assessment for the human consumer.

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CHAPTER 2

COMPARISON OF THE TOXICITY OF INORGANIC AND LIVER-INCORPORATED CADMIUM: A 4-WK FEEDING STUDY IN RATS

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COMPARISON OF THE TOXICITY OF INORGANIC AND LIVER-INCORPORATED CADMIUM: A 4-WK FEEDING STUDY IN RATS

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Abstract—The toxicity of cadmium was examined in rats fed diets containing either tissue-incorporated cadmium or cadmium salt for 4 wk. The test diets contained 30 mg cadmium/kg either as cadmium chloride, or as cadmium incorporated in pigs' livers; the control group was fed a diet containing liver from a pig not treated with cadmium. Over 90% of the cadmium present in the pigs' livers was bound to metallothionein. Analysis of the diet and determination of the food consumption revealed that both cadmium-fed groups were exposed to similar dietary cadmium levels. There was no adverse effect on general health or survival. Feeding cadmium resulted in growth retardation and slightly decreased water intake. Moreover, both cadmium-treated groups showed clear signs of anaemia and increased plasma aspartate and alanine aminotransferase activities. For the group fed cadmium chloride, all of these effects were more pronounced than for the group fed cadmium incorporated in liver. Microscopic examination of the liver and kidneys, however, did not reveal any lesion that could be attributed to the cadmium treatment. After exposure to cadmium the spleen showed decreased extramedullary haematopoiesis, an effect that was also more pronounced after feeding of the cadmium chloride than after feeding liver-incorporated cadmium. The differences in the extent of the toxic effects between the inorganic and the tissue-incorporated cadmium were accompanied by differences in the cadmium concentrations in liver and kidneys: the feeding of cadmium incorporated in pigs' livers resulted in about half the accumulation of cadmium in the rats' livers that took place after intake of a diet containing cadmium chloride. In contrast a much less marked difference in cadmium accumulation was observed in the kidneys. Since humans are usually exposed to tissue-incorporated cadmium these findings deserve further investigation, with special attention to the observed difference in tissue accumulation.

INTRODUCTION

The major source of environmental exposure to cadmium for the general population is the intake in food (Foulkes, 1986). Although previous studies have evaluated cadmium toxicity after exposure to inorganic cadmium, this is not the main chemical form in which cadmium is present in food. In vertebrates, some shellfish and many other animals one of the main cadmium-binding ligands has been identified as the protein metallothionein (Webb, 1986). In plants cadmium is bound, at least partly, to proteins with similarities to metallothioneins (Kägi and Kojima, 1987; Wagner, 1984). The effects of repeated oral administration of inorganic cadmium, mainly as the chloride salt, have been extensively studied in laboratory animals. Although nephrotoxicity is regarded as the characteristic toxic effect, decreased haemoglobin concentration and decreased packed cell volume are among the early signs after chronic, peroral exposure to cadmium (Elinder, 1986).

It is not known whether cadmium bound to metallothionein is as toxic as cadmium chloride. Several studies have examined the distribution of inorganic cadmium and purified cadmium-metallothionein (Cd-Mt) after single oral doses of the metal compounds (Cherian, 1979; Maitani *et al.*, 1984). However none of these reports has compared the early signs of toxicity after continuous exposure to cadmium through the diet. Moreover, whether the absorption of a single dose of purified Cd-Mt is comparable with the absorption of Cd-Mt, as present in meat products or plants given chronically in low doses, has never been clarified. Studies of the toxicity of cadmium, in the different forms in which it is present in human foods, are therefore needed.

The present study was intended to compare the toxicity, in rats, of cadmium as present in pigs' liver with that of cadmium chloride. To achieve a more or less continuous oral exposure the cadmium compound was mixed with the diet. For a meaningful interpretation of the results the cadmium-binding ligand in pigs' liver was identified as a mixture of two metallothionein isoforms. In preliminary experiments the sensitivity of the strain of rats used was examined and a dietary level of 30 mg cadmium/kg food was established as suitable for use in subsequent, comparative studies (E. J. Sinkeldam *et al.*, unpublished data, 1986).

Abbreviations: Cd-Mt = cadmium metallothionein; HPLC = high-performance liquid chromatography.

MATERIALS AND METHODS

Test substance. Cadmium chloride with a purity, as specified by the supplier, of at least 99% was obtained from E. Merck, AG, Darmstadt, FRG.

Preparation of cadmium incorporated in pigs' livers. Three young male pigs, initially weighing 50 ± 4 kg, were housed separately in metabolism cages. The animals were injected intramuscularly with cadmium chloride dissolved in saline according to the following schedule: on days 0, 9, 12, 15 and 18 with 3 mg cadmium/kg body weight and on days 3 and 6 with 6 mg cadmium/kg body weight. Four weeks after the last injection the animals were killed and the livers were removed. The livers were pooled, homogenized, lyophilized and stored at −20°C. Livers obtained from two untreated pigs were handled in the same way and served as control material. Cadmium analysis of freeze-dried liver samples (see below) revealed that the cadmium and zinc contents were 950 and 700 mg/kg tissue, respectively.

Characterization of the cadmium-binding proteins in pig liver. Lyophilized liver tissue was homogenized using a teflon pestle in two volumes of 10 mM-Tris-HCl (pH 7.4, 4°C). The homogenate was centrifuged at 10,000 *g* for 15 min, decanted and again centrifuged at 100,000 *g* for 70 min. Cytosol (150 μl) was then applied to a column of Superose 12 (30 × 1 cm, Pharmacia, Uppsala, Sweden) which was equilibrated with a 0.05 mM-Tris-KCl buffer (pH 7.4). The column was calibrated with standard proteins of known molecular mass (varying from 67 to 12.4 kDa). Fractions of 0.35-ml eluent were analysed for cadmium content. Pigs' liver Cd-Mt was further analysed using a LKB liquid chromatography system. Pigs' liver cytosol was heat-treated (4 min, 90°C) and centrifuged for 10 min at 10,000 *g*. The supernatant (50 μl) was separated on a Hypersil ODS (20 × 0.3 cm) column (Chrompack, The Netherlands) using a 10 mM-sodium phosphate buffer (pH 7.2; solvent-1) and 10 mM-sodium phosphate buffer in acetonitrile (60:40; solvent-2) as eluting solvents. A gradient from 0 to 5% solvent-2 in 5 min and from 5 to 20% solvent-2 in 15 min was used with a flow-rate of 0.4 ml/min. The UV spectrum (190–280 nm) of the cadmium-binding molecule was determined with a diode array spectrophotometer (LKB 2140) coupled to the high-performance liquid chromatography system. Purified rat metallothionein isoforms 1 and 2 (Mt-1 and Mt-2) were used as standards on HPLC as described for purified chicken metallothionein by Richards and Steele (1987). Rat metallothionein was purified from the livers of rats that had previously been injected with $CdCl_2$ dissolved in saline according to the following schedule: on day 0 and 12 with 3 mg/kg sc and on day 4, 6, 8 and 10 with 1.2 mg/kg body weight. Two days after the last injection the livers were perfused with 0.9% NaCl, excised and homogenized (1:2, w/v) in 10 mM-Tris–144 mM-KCl buffer (pH 7.4) using a teflon pestle. The homogenate was centrifuged as described above for pigs' liver. The cytosol was filtered through a 0.22-μm filter (Millipore, Molsheim, France) and chromatographed on a Sephadex G-75 column (5 × 60 cm, Pharmacia) with 10 mM-Tris-HCl (pH 7.4, 4°C). The metallothionein-containing fractions were determined by the relative elution volume Ve/Vo = ±2) and were applied to a Sephadex DEAE-A25 column (2.5 × 20 cm) and eluted with a linear gradient of 10–300 mM-Tris-HCl (pH 8.5, 4°C). The fractions that correspond to Mt-1 and Mt-2 (as determined by the cadmium content of the eluate as described below) were pooled, dialysed against water (Amicon YM membrane), and lyophilized.

Table 1. Percentage composition of the diets

Ingredient	Diet code		
	Control	$CdCl_2$	Cd-liver
Casein	20.17	20.17	20.49
Pigs' liver, untreated	3.14	3.14	—
Pigs' liver, cadmium treated	—	—	3.14
Wheat white flour	54.12	54.12	53.80
Mineral mixture*	4.24	4.24	4.24
Vitamin ADEK preparation†	0.36	0.36	0.36
Vitamin B mixture‡	0.24	0.24	0.24
Lard	17.73	17.73	17.73
Cadmium chloride	—	0.0049	—
Zinc chloride	0.0035	0.0035	—

*The mineral mixture contained (per gram): 399 mg KH_2PO_4, 389 mg $CaCO_3$, 142 mg NaCl, 58 mg $MgSO_4$, 5.7 mg $FeSO_4.7H_2O$, 0.9 mg $ZnCl_2$, 0.8 mg $CuSO_4.5H_2O$, 4.6 mg $MnSO_4.2H_2O$, 0.02 mg $CoCl_2.6H_2O$, 0.007 mg KI, 0.08 mg $KCr(SO_4)_2.12H_2O$.

†The ADEK vitamin preparation contained (per gram): 939 mg vitamin A concentrate (2250 IU/g), 30 mg vitamin D concentrate (750 IU/g), 1.0 mg vitamin E preparation (50%), 1.0 mg menadione-Na-bisulphite (K_3), 30 mg wheat starch.

‡The vitamin B mixture contained (per gram): 3 mg thiamin.HCl, 2.25 mg riboflavin, 4.5 mg pyridoxin.HCl, 15 mg niacin, 6 mg Ca-pantothenate, 0.075 mg biotin, 0.75 mg folic acid, 37.50 mg vitamin B_{12} (0.1%), 931 mg choline chloride.

Diets. A semi-synthetic powdered basal diet was composed in such a way that it mimics a Western type of human diet (high in fat, low in fibre, without excessive amounts of minerals and vitamins). One diet consisted of the basal diet supplemented with the high-cadmium pig-liver homogenate described above, providing 30 mg cadmium/kg diet. A second diet contained the basal diet supplemented with cadmium chloride to a dietary level of 30 mg cadmium/kg and untreated liver from the control pigs, in an amount equal to the amount of liver eaten by the first group. The third diet contained the basal diet and the untreated liver sample without additional cadmium and was used as the control diet. The percentage compositions of the test diets are given in Table 1. The diets were analysed for moisture, crude protein, methionine, cystine, crude fat, calcium, phosphorus, zinc, copper, iron, magnesium and cadmium (Table 2).

Animals and maintenance. Male, weanling, Wistar-derived SPF-bred rats (Bor; WISW) were obtained from F. Winkelmann (Institute for the Breeding of Laboratory Animals GmbH & Co. KG, Borchen, FRG). At the beginning of the study the rats were about 4 wk old. We used young, growing animals to

Table 2. Chemical analyses of the diets

Constituent	Diet code		
	Control	$CdCl_2$	Cd-liver
Moisture (%)	10.2	10.5	9.3
Crude protein (%)	27.0	26.8	27.1
Methionine (%)	0.72	0.71	0.71
Cystine (%)	0.28	0.26	0.27
Crude fat (%)	18.9	18.9	18.9
Calcium (%)	0.66	0.66	0.66
Phosphorus (%)	0.63	0.61	0.63
Zinc (mg/kg)	48	48	51
Copper (mg/kg)	12	11	11
Iron (mg/kg)	56	63	59
Magnesium (mg/kg)	510	510	518
Cadmium (mg/kg)	0.1	29.2	30.9

study the effects of cadmium on body-weight gain. The rats were housed under conventional conditions, in suspended stainless-steel cages fitted with a wire-mesh floor and front. The room temperature was maintained at 22 ± 2°C and the relative humidity at 40–70%. A 12-hr light/dark cycle was maintained and the number of air changes was about ten/hr. Before the experiment all rats were fed the basal diet without any further additions. Drinking-water was supplied in glass bottles which were cleaned once each week. Food and water were provided *ad lib.*

Experimental design and treatment. The rats were acclimatized to the animal facilities for 8 days and were then allocated to three groups of ten animals using a computer-generated random-number table. A few rats were re-allocated in order to equalize the initial mean body weights of the various groups. Each treatment group (housed in groups of five) received one of the three test diets. Analysis of the test diets revealed that under the experimental conditions the levels of cadmium were between 98 and 103% of the intended level of 30 mg/kg diet.

Observations. The rats were weighed at weekly intervals and were observed daily for condition and behaviour. Food and water intake were measured over weekly periods throughout the study.

Haematology and clinical chemistry. Blood samples were collected from the tips of the tails of all animals on day 22 and were examined for haemoglobin concentration, packed cell volume, erythrocyte and total and differential leucocyte counts. At autopsy heparinized blood samples collected from the abdominal aorta of all rats were centrifuged at 1250 *g* for 15 min, using Sure-sep II dispensers (General Diagnostics). The plasma was then analysed for alkaline phosphatase, aspartate aminotransferase, alanine aminotransferase and urea.

Urinalysis. On day 24 rats were deprived of water for 24 hr, and of food for 16 hr. Urine was collected from the individual animals during the last 16 hr of the deprivation period and its volume (calibrated tubes), density (Bellingham and Stanly refractometer) and calcium content were determined.

Pathology. On day 28 the animals were killed by exsanguination from the abdominal aorta whilst under light ether anaesthesia, and autopsied. Immediately after evisceration the pancreas, testes, kidneys, spleen, liver, lungs and small intestine were weighed and the organ:body-weight ratio was calculated. Samples of the weighed organs of all animals were fixed in 4% neutral phosphate buffered formaldehyde solution. The tissue samples were processed and embedded in paraffin wax, sectioned at 5 μm, stained with haematoxylin and eosin, and then examined microscopically.

Metal analysis. Zinc, calcium and copper were determined by flame atomic absorption spectrometry. The samples were dry-ashed at 500°C and dissolved in hydrochloric acid. Cadmium was determined by flame atomic absorption spectrometry or graphite furnace atomic absorption spectrometry after a wet digestion of the sample by sulphuric acid.

Statistical analysis. Data on body weights were evaluated by a one-way analysis of covariance, followed by Dunnett's multiple-comparison tests. The laboratory determinations and organ weights were evaluated by a one-way analysis of variance, followed by Dunnett's multiple-comparison tests. Differential white blood cell counts were analysed by the Mann–Whitney U-test.

RESULTS

Gel filtration of the liver cytosol from cadmium-treated pigs revealed that more than 90% of the cadmium eluted with a relative elution coefficient (Ve/Vo) corresponding to a protein molecular mass of 10 kDa (Fig. 1). A reversed-phase HPLC system was used to characterize further the Cd-Mt from the porcine cytosol. The HPLC chromatogram (Fig. 2b)

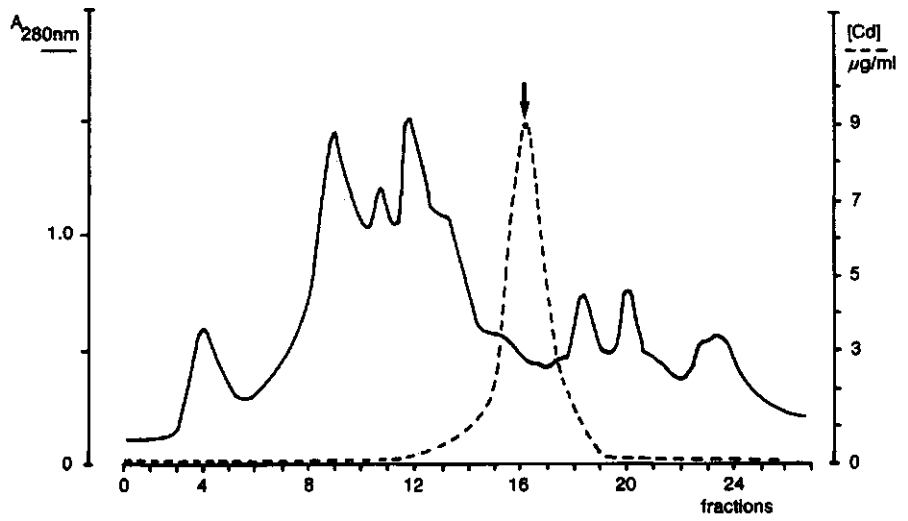

Fig. 1. Elution profile on a Superose 12 column (30 × 1 cm, Pharmacia) of 200 μl liver cytosol from pigs treated intramuscularly with $CdCl_2$ for 3 wk. Flow 0.3 ml/min. The cadmium content of fractions (0.35 ml) was determined by atomic absorption spectrometry. The arrow indicates a molecular mass of 10 kDa and corresponds to the cadmium-containing fractions. The molecular mass was estimated using four molecular-mass markers ranging from 78 to 12.4 kDa.

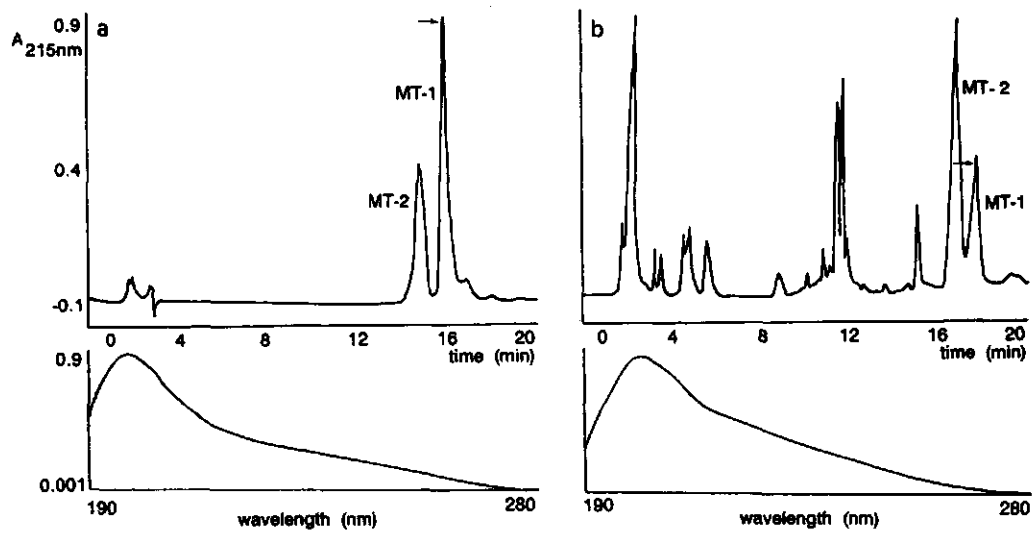

Fig. 2. Reversed-phase HPLC elution profile on a Hypersil ODS (200 × 3 mm i.d.) column. The separation was performed using 10 mM-sodium phosphate (solvent-1) and 10 mM-sodium phosphate buffer in acetonitrile (60:40, v/v; solvent-2) as eluting solvents. The injected sample was eluted with a linear gradient of 0–5% solvent-2 in 5 min and from 5 to 20% solvent-2 in 15 min with a flow-rate of 0.4 ml/min. The injection volume was 50 μl. a = purified rat liver metallothionein, b = heat-treated pig liver cytosol. The arrow indicates the domain of MT-1 used for the UV spectrum (λ 190–280 nm) given in the figure below the chromatogram.

exhibited two major isoforms. For comparison, a chromatogram depicting the separation of the metallothionein isoforms from rat liver is shown in Fig. 2a. The spectral analysis of porcine Mt-1 showed that the cadmium-binding protein in the pig liver had a UV spectrum similar to that of purified rat metallothionein isoform 1 (Fig. 2: λ_{max} at 199 nm, broad shoulder at 250 nm, λ_{min} at 280 nm). Based on molecular weight, cadmium-binding properties, heat-stability and spectral analysis it was concluded that more than 90% of the cadmium bound to pig liver was associated with metallothionein.

All rats appeared to be healthy throughout the 4-wk study. However, growth and food intake were significantly decreased in both groups fed cadmium. Rats fed tissue-incorporated cadmium gained more weight than those fed $CdCl_2$ especially during the last 2 wk of the study (Table 3). Both cadmium-treated groups showed a lower water consumption than the control group but not significantly so (Table 3).

Haemoglobin concentration and packed cell volume were considerably decreased in both cadmium-treated groups (Table 4). These effects were most pronounced in the group fed $CdCl_2$. The lowered haemoglobin concentration in the group treated with $CdCl_2$ was accompanied by a significant decrease in the mean corpuscular volume, the mean corpuscular haemoglobin and the mean corpuscular haemoglobin concentration. These effects occurred to a lesser extent in the rats fed cadmium bound to metallothionein (Table 4). The white blood cell variables were not affected by cadmium treatment (data not shown).

An increase in the plasma aspartate aminotransferase and alanine aminotransferase activities was observed in both cadmium treated groups. This effect was most pronounced in the animals fed $CdCl_2$ (Table 5). There were no other treatment-related differences in clinical chemistry parameters between the groups. The volume, density and calcium content of the urine produced during 24 hr (see Materials and Methods) did not show treatment-related differences between the groups (data not shown).

The group fed the diet supplemented with $CdCl_2$ showed a significant increase of the relative weight of

Table 3. Mean values of body weight, food consumption and water consumption in rats fed 30 ppm cadmium in the diet

Duration of exposure (days)	Body weight (g)†			Food consumption (g/rat/week)‡			Water consumption (ml/rat/week)‡		
	Control	$CdCl_2$	Cd-liver§	Control	$CdCl_2$	Cd-liver§	Control	$CdCl_2$	Cd-liver§
0	67.6 ± 1.8	67.7 ± 1.6	67.6 ± 1.5						
7	113.9 ± 2.4	108.0 ± 2.0**	107.4 ± 2.0**	74.1	68.5	68.8	113.7	102.2	105.2
14	163.8 ± 3.5	151.3 ± 2.6**	153.0 ± 2.7**	98.0	87.2	90.2	163.0	136.4	144.2
21	208.0 ± 4.1	193.7 ± 3.3**	199.9 ± 4.3	109.4	100.3	105.4	165.3	152.7	158.3
28	252.7 ± 4.3	230.1 ± 3.4**	239.6 ± 5.3*	110.8	100.1	107.2	155.3	135.6	148.9
Overall means	161.4	150.1	153.6	98	89	92.9	149.4	131.7	139.1

†Body-weight values are means ± SEM for groups of ten animals. The values of the mean body weights marked with asterisks differ significantly (COVAR + Dunnett's test) from the control diet (*$P < 0.05$; **$P < 0.01$).

‡The mean values for food/water consumption were recorded weekly for two cages each of five animals. The values for food/water consumption by the test animals did not differ significantly from the values for animals on the control diet (ANOVA + least square significant difference test).

§Cadmium incorporated in liver was obtained by treating pigs with $CdCl_2$ and processing the livers as described in Materials and Methods.

Table 4. Mean haematological findings in male rats fed 30 ppm cadmium in the diet for 22 days

Parameter	Diet code		
	Control	$CdCl_2$	Cd-liver
Hb (mmol/l)	8.6 ± 0.1	6.2 ± 0.1**	7.9 ± 0.1**
RBC (10^9/ml)	5.8 ± 0.1	5.7 ± 0.1	5.7 ± 0.1
PCV (l/l)	0.402 ± 0.004	0.319 ± 0.006**	0.382 ± 0.007*
MCV (fl)	69.8 ± 1.1	56.0 ± 0.8**	66.7 ± 1.6
MCH (fmol)	1.49 ± 0.02	1.09 ± 0.02**	1.39 ± 0.02**
MCHC (mmol/l)	21.3 ± 0.4	19.5 ± 0.4**	20.8 ± 0.2

Hb = haemoglobin concentration RBC = red blood cell count
PCV = packed-cell volume MCV = mean corpuscular volume
MCH = mean corpuscular haemoglobin
MCHC = mean corpuscular haemoglobin concentration
Values are means ± SEM for groups of ten rats. Those values marked with asterisks differ significantly (ANOVA + Dunnett's test) from the corresponding control value (*$P < 0.05$; **$P < 0.01$; two-sided).

the small intestine and a significant decrease of the relative weight of the liver (Table 6). The microscopic examination of the liver, kidneys, spleen, testes, pancreas, lungs and small intestine revealed substance-related changes in the spleen only. The extramedullary haematopoiesis that was observed in the spleen of the controls was decreased in incidence and degree in the cadmium-treated groups. The decrease was most pronounced in the group fed $CdCl_2$ where extramedullary haematopoiesis was absent.

Feeding 30 mg $CdCl_2$/kg for 28 days resulted in a mean cadmium concentration in the liver of 6.71 mg/kg tissue, which is more than twice the amount found in animals fed a similar amount of liver-incorporated cadmium (Table 6). The copper level in the liver was slightly increased in the animals fed $CdCl_2$ whereas the zinc concentration of the liver was increased in both cadmium-treated groups.

In contrast to the liver, the kidneys show a much less marked difference in cadmium accumulation between the cadmium-treated groups; feeding cadmium incorporated in liver resulted in only a slightly lower cadmium concentration in the kidney than feeding $CdCl_2$. In contrast to the liver the copper level in the kidneys was decreased in both cadmium-treated groups whereas the zinc concentration was significantly increased only in the group fed cadmium incorporated in liver (Table 6).

DISCUSSION

In the present study several well known signs of cadmium toxicity occurred after repeated oral exposure to $CdCl_2$ or cadmium incorporated in pigs' liver. The cadmium compound in the pigs' liver was characterized to help understand the difference in cadmium uptake from a diet containing $CdCl_2$ or cadmium incorporated in tissue. Reversed-phase HPLC resolved two isoforms of metallothionein from porcine liver cytosol using purified rat metallothionein as a Cd-Mt standard. These two isoforms together accounted for over 90% of the total cadmium present in the pigs' liver preparation. The existence of porcine metallothionein isoforms has been reported previously by other investigators (Mehra and Bremner, 1984; Richards and Steele, 1987).

The daily intake of cadmium in the present study was 0.29–0.45 mg/rat or 2–3 mg cadmium/kg body weight. Although the exposure level of the rats was similar for both cadmium compounds, the severity of the toxic effects observed was different: signs of anaemia, decreased weight gain, decreased extramedullary haematopoiesis and increased ALAT and ASAT activity were all more pronounced in the rats fed $CdCl_2$. Moreover, the increase of ALAT/ASAT activity in the blood plasma of rats fed with $CdCl_2$ was accompanied by a significant decrease in relative liver weight. However, both cadmium-treated groups showed no treatment-related histopathological changes in the liver. This result is somewhat surprising since, for example, Andersen *et al.* (1988) found

Table 5. Clinical chemistry of the plasma of rats fed 30 ppm cadmium in the diet for 28 days

Parameters	Diet code		
	Control	$CdCl_2$	Cd-liver
ALAT (units/l)	56.9 ± 1.0	75.2 ± 2.2**	64.7 ± 1.5**
ASAT (units/l)	22.5 ± 0.8	50.0 ± 2.7**	32.5 ± 1.6**
ALP (units/l)	410.9 ± 23.8	410.0 ± 20.4	380.0 ± 22.0
Urea (mmol/l)	7.39 ± 0.47	6.7 ± 0.34	7.39 ± 0.18

ALAT = alanine aminotransferase
ASAT = aspartate aminotransferase ALP = alkaline phosphatase
Values are means for groups of ten rats. Those marked with asterisks differ significantly (ANOVA + Dunnett's test) from the corresponding control value (*$P < 0.05$; **$P < 0.01$).

Table 6. Relative organ weights and metal concentrations in liver and kidneys of rats fed 30 ppm cadmium in the diet for 28 days

Parameters	Diet code		
	Control	$CdCl_2$	Cd-liver
Relative organ weight (g/kg body weight)			
Pancreas	1.9 ± 0.09	2.26 ± 0.13	1.97 ± 0.08
Testes	10.92 ± 0.24	11.86 ± 0.32	11.45 ± 0.39
Spleen	2.48 ± 0.12	2.54 ± 0.13	2.48 ± 0.13
Lung	6.27 ± 0.08	6.49 ± 0.14	6.44 ± 0.09
Small intestine	26.70 ± 1.1	33.0 ± 0.8**	29.30 ± 0.70
Liver	47.7 ± 0.6	45.1 ± 0.4**	46.2 ± 0.70
Kidneys	7.99 ± 0.1	7.84 ± 0.13	7.95 ± 0.12
Metal concn in liver (mg/kg)			
Cadmium	0.08 ± 0.02	6.71 ± 0.2**	3.09 ± 0.18**
Zinc	30.5 ± 0.4	32.5 ± 0.3*	32.5 ± 0.7*
Copper	4.4 ± 0.1	5.0 ± 0.1**	4.1 ± 0.1
Metal concn in kidneys (mg/kg)			
Cadmium	0.07 ± 0.02	8.47 ± 0.4**	6.52 ± 0.35**
Zinc	27.9 ± 0.4	28.4 ± 0.9	32.1 ± 0.8**
Copper	16.3 ± 1.1	10.5 ± 0.7**	7.3 ± 0.3**

Values are means ± SEM for ten rats; those marked with asterisks differ significantly (ANOVA + Dunnett's test) from the corresponding control value (*$P < 0.05$; **$P < 0.01$).

histopathological changes in the livers of mice 10 days after oral exposure to a single dose of $CdCl_2$ (30 mg/kg body weight), and Elinder (1986) reported, that for detecting the long-term effects of cadmium on the liver, liver morphology is a more sensitive parameter than the liver-enzyme activities in the blood. In contrast, Dudley *et al.* (1985) showed significantly increased plasma activities of aspartate aminotransferase and alanine aminotransferase 2 wk after sc exposure to $CdCl_2$ whereas morphological signs of hepatic injury did not occur until 4 wk after exposure.

The differences in the extent of signs of adverse effects between $CdCl_2$ and incorporated cadmium are accompanied by differences in the cadmium concentrations in the liver and kidneys. The ratio between the cadmium concentration of the liver and the kidneys was 0.79 in the group fed $CdCl_2$ and 0.47 after feeding of liver-incorporated cadmium. Similar findings have been reported by Maitani *et al.* (1984) in mice after a single oral administration of $CdCl_2$ or liver-incorporated cadmium. The observed difference is almost solely due to the difference in accumulation in the liver and not to any marked difference in the uptake by the kidneys.

Crews *et al.* (1989) have found that the majority of cadmium in pig-kidney metallothionein-like protein survives *in vitro* gastro-intestinal digestion, which may indicate that at least part of the cadmium is able to recombine with the metallothionein-like protein at higher pH after passage through the stomach. It has been suggested that cadmium-metallothionein can pass through the intestinal mucosa partially intact because of the constantly high kidney/liver concentration after oral exposure to Cd-Mt (Cherian 1979; Maitani *et al.*, 1984). If this theory is correct, Cd-Mt after entering the bloodstream would then accumulate preferentially in the kidneys and not in the liver. The suggestion that Cd-Mt can be taken up intact is further supported by the work of Cherian (1979) who found that a major portion of the ingested Cd-Mt was isolated intact from the intestinal mucosa 4 hr after oral exposure to Cd-Mt. However, no hard evidence exists that exogenous Cd-Mt can pass the intestinal barrier.

There are two possible explanations of the difference in the extent of effects of $CdCl_2$ and tissue-incorporated cadmium. First, there is a difference in the accumulation in the target organs, and secondly there may be a difference in the sensitivity of the target organs to the two compounds.

There are no data available on the liver toxicity of Cd-Mt *in vivo*. However, from recent *in vitro* studies (Beattie *et al.*, 1987; Bracken *et al.*, 1989) it appears that $CdCl_2$ is more toxic to primary hepatocyte cultures than Cd-Mt. The difference in the signs of liver toxicity between the group fed a diet containing $CdCl_2$ and the diet with Cd-Mt in the present study is therefore probably not only caused by the difference in cadmium accumulation but also by a difference in the sensitivity of the liver cells to $CdCl_2$ and Cd-Mt.

It is not surprising that no renal toxicity was observed in the present study, since the cadmium concentrations were too low for acute toxicity and the exposure time too short for chronic toxicity (Friberg *et al.*, 1974; Kjellström, 1986). After iv injection of Cd-Mt or $CdCl_2$ several research groups concluded that Cd-Mt was more nephrotoxic than the $CdCl_2$ (Cherian *et al.*, 1976; Nordberg *et al.*, 1975; Squibb *et al.*, 1979). This can at least partly be ascribed to the higher accumulation of Cd-Mt in the kidneys observed after parenteral exposure (Tanaka *et al.*, 1975). However, from the experiment of Sendelbach and Klaassen (1988) it appears that Cd-Mt is more nephrotoxic than $CdCl_2$ after iv administration despite a similar degree of accumulation in the kidneys, which points to a difference in the mechanisms of action of the two compounds. If such a difference also applies to the oral route of exposure, then our results may be valuable for assessing the risks to humans from cadmium consumed in animal products.

Exposure to Cd-Mt seems to lead to a lower hepatotoxicity than exposure to corresponding amounts of $CdCl_2$. Since both compounds accumulate to a similar extent in the kidneys, nephrotoxicity may at least be equal in severity for both forms of cadmium.

Anaemia is one of the well known signs of toxicity after peroral exposure to cadmium and is thought to be related to the effects of cadmium on the metabolism of iron or zinc (Elinder, 1986). For the time being we have no sound explanation for the difference in the degrees of anaemia caused by the two dietary forms of cadmium. It is surprising that the erythropoiesis in the spleen is reduced by the cadmium treatment. However, the incidence of extramedullary haematopoiesis in rats of this strain and age is quite variable. Therefore no significance is attached to the disappearance of extramedullary haematopoiesis in the $CdCl_2$-treated group. More research is being undertaken to determine the kinetics of the Cd-Mt uptake after oral exposure, and the role of the small intestine in the uptake and distribution of tissue-incorporated cadmium.

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Chapter 3

CADMIUM ACCUMULATION AND METALLOTHIONEIN CONCENTRATIONS AFTER 4-WEEK DIETARY EXPOSURE TO CADMIUM CHLORIDE OR CADMIUM-METALLOTHIONEIN IN RATS

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Cadmium Accumulation and Metallothionein Concentrations after 4-Week Dietary Exposure to Cadmium Chloride or Cadmium–Metallothionein in Rats

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Cadmium Accumulation and Metallothionein Concentrations after 4-Week Dietary Exposure to Cadmium Chloride or Cadmium–Metallothionein in Rats. GROTEN, J. P., SINKELDAM, E. J., LUTEN, J. B., AND VAN BLADEREN, P. J. (1991). *Toxicol. Appl. Pharmacol.* **111**, 504–513. The distribution of cadmium was examined in rats fed diets containing either cadmium–metallothionein (CdMt) or cadmium chloride ($CdCl_2$) for 4 weeks. The test diets contained 3, 10, or 30 mg Cd/kg diet (3, 10, or 30 ppm) as CdMt or 30 mg Cd/kg diet (30 ppm) as $CdCl_2$. A second study was performed to establish the Cd content in liver and kidneys after exposure to low doses of both CdMt and $CdCl_2$ (1.5 and 8 ppm Cd). The feeding of CdMt resulted in a dose- and time-dependent increase of the Cd concentration in liver, kidneys, and intestinal mucosa. Rats fed 30 ppm CdMt consistently showed less Cd accumulation in liver and intestinal mucosa than did rats fed 30 ppm $CdCl_2$. However, renal accumulation in rats fed 30 ppm was similar until Day 28 regardless of Cd form. At lower dietary Cd levels (1.5 and 8 ppm), relatively more Cd is deposited in the kidneys, although even at these doses the kidney/liver ratio of Cd is still higher with CdMt than with $CdCl_2$. Tissue metallothionein (Mt) levels in the intestinal mucosa were relatively constant but always higher after $CdCl_2$ exposure than after CdMt exposure. Mt levels in both liver and kidney increased after $CdCl_2$ or CdMt exposure during the course of study. Although Mt levels in liver were higher after $CdCl_2$ intake (30 ppm) than after CdMt intake (30 ppm), renal Mt concentrations were the same for both groups. In fact on Day 7, CdMt administration resulted in slightly higher Mt levels than $CdCl_2$ administration, suggesting a direct accumulation of exogenous CdMt in the kidneys. In conclusion, after oral exposure to CdMt in the diet there is a relatively higher Cd accumulation in the kidneys. However, the indirect renal accumulation via redistribution of Cd from the liver might be lower than after $CdCl_2$ exposure. Which of these two phenomena is decisive in the eventual level of renal toxicity of Cd after long-term oral intake could determine the toxicological risk of the chronic intake of biologically incorporated Cd.

The main source of environmental exposure to Cadmium (Cd) for nonsmokers is food (Foulkes 1986). Due to the small safety margin between the actual level of dietary intake and the toxic level of Cd (Buchet *et al.*, 1990), human dietary exposure to Cd has become a major concern. In this regard, although most toxicity studies have used inorganic salts of Cd, this is clearly not the chemical form in which Cd occurs in the diet. In animals one of the main Cd-binding ligands is the protein metallothionein (Webb, 1986). A similar protein, phytochelatin, is the major Cd-binding protein in plants (Wagner, 1984; Kägi and Kojima, 1987).

Previous studies have shown that parenteral administration of cadmium–metallothionein (CdMt) leads to a more pronounced nephrotoxicity than parenteral administration of inorganic Cd salts (Nordberg *et al.*, 1975; Cherian *et al.*, 1976; Squibb *et al.*, 1979). This is due at least in part to a higher accumulation

of Cd in the kidneys from CdMt exposure (Tanaka *et al.*, 1975). It is thought that CdMt is reabsorbed into the renal proximal cells after glomerular filtration. The exogenous CdMt is degraded in lysosomes and free Cd, released within the cell, will cause damage (Squibb *et al.*, 1979; Kjellström, 1986). Cd-cytotoxicity is believed to be dependent on the amount of free ionic Cd present in the cell which is not sequestered by available endogenous metallothionein or glutathione (Jin *et al.*, 1987; Suzuki and Cherian, 1989). Alternatively, it is suggested that CdMt will damage the brush border during reabsorption of the CdMt complex (Cherian, 1982) and, indeed, a higher sensitivity of the kidneys to Cd in the form of CdMt has also been observed (Sendelbach and Klaassen, 1988).

Several studies have examined the distribution of Cd salts and CdMt after oral exposure. A few such studies used biologically incorporated Cd. For example, internal organs of Cd-exposed pigs (Uthe and Chou, 1974) or whole oysters (Sullivan *et al.*, 1984) have been used as source material for exposure to biologically incorporated Cd. The results of these studies are difficult to interpret since Cd-binding ligands were not characterized prior to use. Other researchers have studied the oral absorption of Cd after gavage of CdMt. For example, Cherian (1979, 1983) used purified rat CdMt, while Maitani *et al.* (1984) used CdMt added to rat liver homogenate. The gavage studies using CdMt as the only Cd-binding ligand have demonstrated that the liver to kidney ratio of Cd concentrations was much lower after exposure to CdMt compared to $CdCl_2$. This was mainly due to a selective deposition in the kidneys after CdMt exposure (Cherian 1979, 1983; Maitani *et al.*, 1984). However, in these studies, the animals were exposed by gavage and no kinetics of the Cd disposition were determined. Moreover, since rats exposed to very low doses of $CdCl_2$ show selective renal Cd accumulation (Lehman and Klaassen, 1986), the question remains whether the selective renal accumulation after CdMt exposure is dose-dependent.

In a previous study we attempted to simulate normal dietary exposure of humans to CdMt through the food chain by incorporating CdMt into the diet instead of using gavage (Groten *et al.*, 1990). In this way, possible effects of dietary factors were taken into account. It is known that dietary factors like minerals, vitamins, and proteins affect the intestinal Cd uptake or the Cd toxicity (Fox, 1979; Taskgroup on Metal Interactions, 1978; Groten *et al.*, 1991). It was shown that there is a clear difference in toxicity in rats fed either inorganic ($CdCl_2$) or pig liver-incorporated Cd in which Cd was mainly bound to porcine CdMt. Signs of Cd toxicity, such as decreased weight gain, anemia, and liver damage, were all more pronounced after exposure to $CdCl_2$ than after feeding of CdMt (Groten *et al.*, 1990).

The present study was intended to establish the kinetics of the Cd uptake and the CdMt concentration in the kidneys, liver, and intestinal mucosa during 4-week oral exposure to CdMt via the food and to study the dose-dependent Cd disposition. Such a comparative study might provide more insight into the observed differences in toxicity between inorganic and Mt-bound Cd.

MATERIALS AND METHODS

Test Substance

Cadmium chloride, with a purity of at least 99% as specified by the supplier was obtained from E. Merck (AG Darmstadt, FRG).

Preparation of Cd Incorporated into Pig Liver

Three male pigs, initially weighing 50 ± 4 kg, were housed separately in metabolism cages. The animals were injected in the thigh intramuscularly with $CdCl_2$ dissolved in saline according to the following schedule: on Days 0, 9, 12, 15, and 18 with 3 mg Cd/kg and on Days 3 and 6 with 6 mg Cd/kg.

Four weeks after the last injection the animals were killed and the livers were removed. The livers were pooled, homogenized, lyophilized, and stored at −30°C. Livers obtained from two untreated pigs were handled in the same way. Cd analysis of freeze-dried liver samples (see below)

revealed that the Cd and Zn content was 950 and 700 mg/kg tissue, respectively. In a previous study (Groten *et al.*, 1990) the Cd-binding ligand in the pig liver that accounted for almost 90% of the total Cd present was characterized as CdMt.

Diets

A semisynthetic powdered basal diet was composed in such a way that it mimics a Western-type human diet: high in fat, low in fiber, without excessive amounts of minerals and vitamins (Sinkeldam *et al.*, 1990). Four diets consisted of the basal diet supplemented with the pig liver homogenates described above, providing dietary levels of 0, 3, 10, or 30 ppm Cd. A fifth diet contained the basal diet supplemented with $CdCl_2$ to a dietary level of 30 ppm Cd (for chemical composition of the diets see Groten *et al.*, 1990). Mineral analysis of the test diets revealed that the Zn content was 46.5 ± 1.5 ppm, the Fe content was 77 ± 12 ppm, and the protein concentration was 27.1 ± 1.1%.

Animals and Maintenance

Male, weanling, Wistar-derived SPF-bred rats (Bor; WISW) were obtained from F. Winkelmann (Institute for the Breeding of Laboratory Animals GmbH & Co. KG, Borchen, FRG). At the beginning of the study the rats were 4 to 5 weeks old. They were housed under conventional conditions in suspended stainless-steel cages fitted with a wire-mesh floor and front. The room temperature was maintained at 22 ± 2°C and the relative humidity at 40–70%. A 12-hr light/dark cycle was maintained and the number of air changes was about 10/hr. Prior to the experiment all rats were fed the basal diet with no further additions. Drinking water was supplied in glass bottles which were cleaned weekly. Food and tap water were provided *ad libitum*. Diets were provided as a meal mash in stainless steel cans. The food was covered by a perforated stainless steel plate which effectively prevented spillage. Food intake was measured by weighing the feeders. On Days 4 and 7 and once weekly thereafter, the leftovers in the feeders were weighed and discarded prior to filling the feeders with fresh portions.

Experimental Design and Treatment

The rats were acclimatized to the animal facilities for 8 days and were then allocated into five groups of 18 animals using a computer-generated random number table. A few rats were reallocated in order to equalize the initial mean body weight in the various groups. Each treatment group (housed in groups of five) received one of the five test diets. Analysis of the test diets revealed that under the experimental conditions the actual levels of Cd were between 93 and 97% of the intended level.

Observations and Analyses

The rats were weighed at weekly intervals and were observed daily for condition and behavior. Food and water intakes were measured over weekly periods throughout the study.

Tissue preparation. On Days 2, 4, 7, 14, 21, and 28 three animals of each group were killed by exsanguination from the abdominal aorta while under light ether anesthesia and necropsied. Immediately after evisceration the kidneys, liver, and small intestine were weighted and the medulla of the kidneys was removed using a pair of tweezers. After weighing, both the kidney cortex and the liver were immediately frozen using liquid nitrogen and finally kept in an −80°C freezer prior to use. To remove all intestinal contents, a cannula which was connected to a reservoir with a 10-ml dispenser was inserted into the duodenum. Every intestine was rinsed thoroughly with 60 ml of phosphate-buffered saline (six strokes of a 10-ml dispenser). Finally the small intestine was sliced lengthwise at one side and was placed on an ice-cold petri dish (20 cm diameter). The intestinal mucosa was scraped with an object glass at an angle of 40° to the surface. Mucosal scrapings were immediately frozen in liquid nitrogen and kept in a −80°C freezer.

The tissues were thawed just prior to analysis and homogenized using a teflon pestle in a 10 mM Tris–HCl buffer (pH 7.4, 4°C). An aliquot of the homogenized tissue was used for the metal analysis. The rest of the homogenate was centrifuged at 9,000*g* for 20 min and the supernatant (S9 mixture) was used for CdMt analysis.

Cd and CdMt analysis. Zn was determined by flame atomic absorption spectrometry (FAAS). The samples were dry-ashed at 500°C and dissolved in hydrochloric acid. Cd was determined by FAAS or graphite furnace atomic absorption spectrometry (GFAAS) after a wet digestion of the sample by sulfuric acid. The tissue level of CdMt was determined by the Cd–hemoglobin assay as developed by Onosaka and Cherian (1978) and modified by Eaton and Toal (1982). A 200-μl aliquot of heat-treated S9 homogenate of liver, intestine, or kidneys was saturated by incubation with 200 μl of 10 mM Tris-buffered $CdCl_2$ (0.26 mM Cd). After 10 min the incubation mixture was processed through two sequential treatments with 2% bovine hemoglobin (Hb; Sigma, St. Louis) and heating (2 min, 95°C). In both steps the denatured Hb protein was spun down (8,000*g*, 10 min). After the second step, the supernatant was decanted and the Cd content was determined by atomic absorption spectrometry.

From the resulting mean values (duplicate/animal), the concentration of Mt in the original solution was calculated using a molar apo-Mt:Cd ratio of 1:7 (Winge and Miklossy, 1982). The Mt concentration was determined in the con-

trols and at the Cd dose levels of 30 ppm Cd on Days 2, 7, 14, and 28.

Second Experiment

After the main experiment it was decided to perform a second experiment to establish the kidney/liver Cd ratio after 28 days of exposure to low levels of both CdMt and $CdCl_2$. For that purpose five groups of rats (five animals/group) were fed with a diet that contained 1.5, 8, or 30 ppm Cd as $CdCl_2$ and 1.5 and 8 ppm Cd as CdMt. On Day 28 all animals were killed. Experimental design, tissue preparation, and AAS analysis were performed as in the main study.

Statistical Analysis

Data on body weights were evaluated by a one-way analysis of covariance, followed by Dunnett's multiple comparison tests. The laboratory determinations and organ weights were evaluated by a one-way analysis of variance, followed by Dunnett's multiple comparison tests. The data on the ratio between the Cd content of kidney and liver were evaluated by a two-way analysis of variance for the overall effect and by a paired *t* test for the effect on the separate days.

RESULTS

General condition and behavior of the rats appeared to be normal throughout the 4-week study. Body weight gain and food consumption were similar in all groups (Table 1). The daily intake of Cd in rats fed 3 ppm CdMt varies from 125 to 260 μg/kg body wt. For rats fed 30 ppm Cd as $CdCl_2$ or CdMt the daily intake varied from 1 to 2.5 mg/kg body wt. The variation in dose is due to the body weight gain. There were no consistent differences in organ weights or food consumption among the test groups.

Feeding of pig CdMt produced a dose- and time-dependent increase of the Cd concentration in all organs (Fig. 1). However, at the dose level of 3 ppm Cd in the diet the time-dependent increase was not statistically significant in the liver and kidneys. In contrast to the kidneys and the liver the intestinal mucosa already contained fairly high Cd levels after 2

TABLE 1

MEAN VALUES OF BODY WEIGHT AND FOOD CONSUMPTION IN RATS FED WITH 3–30 ppm CADMIUM IN THE DIET

Days of exposure	Body weight (g)[a]					Food consumption (g/rat/day)[b]				
	Control	CdMt[c] (3 ppm Cd)	CdMt (10 ppm Cd)	CdMt (30 ppm Cd)	$CdCl_2$ (30 ppm Cd)	Control	CdMt (3 ppm Cd)	CdMt (10 ppm Cd)	CdMt (30 ppm Cd)	$CdCl_2$ (30 ppm Cd)
0	62.5 ± 1.0	62.3 ± 1.1	62.4 ± 1.1	62.4 ± 1.1	62.5 ± 1.0	—	—	—	—	—
4	79.6 ± 1.5	84.4 ± 1.3	8.7 ± 1.6	82.5 ± 1.6	81.5 ± 1.5	8	8	7.9	7.5	7.6
7	97.7 ± 1.9	103.4 ± 1.6	101.5 ± 2.7	102 ± 1.9	99.4 ± 2.2	9.2	9	9.2	8.7	8.5
14	137.9 ± 3.2	147.3 ± 2.0	145.5 ± 4.2	144.8 ± 2.5	138 ± 3.7	11.3	11.5	11.6	11.1	10.3
22	183.2 ± 4.3	202.2 ± 2.8	191.8 ± 8.4	203.4 ± 7	186 ± 10.6	13.2	13.5	9.8	13.5	10.3
28	219.2 ± 11.8	238.6 ± 2.5	235.6 ± 16.5	222.3 ± 7	220.7 ± 14.1	10.3	10.7	10.9	10.3	9.6

[a] Body weight values are means ± SEM for groups of 18 animals on Day 0 to three animals on Day 28. The values of the mean body weights did not differ significantly from the control value (COVAR + Dunnet's test).

[b] The mean values for food consumption are recorded weekly in two cages of five animals each. The values for food consumption did not differ significantly from the control diet (ANOVA + least square significant difference test).

[c] CdMt was obtained by treating pigs with $CdCl_2$ and processing the livers as described under Materials and Methods.

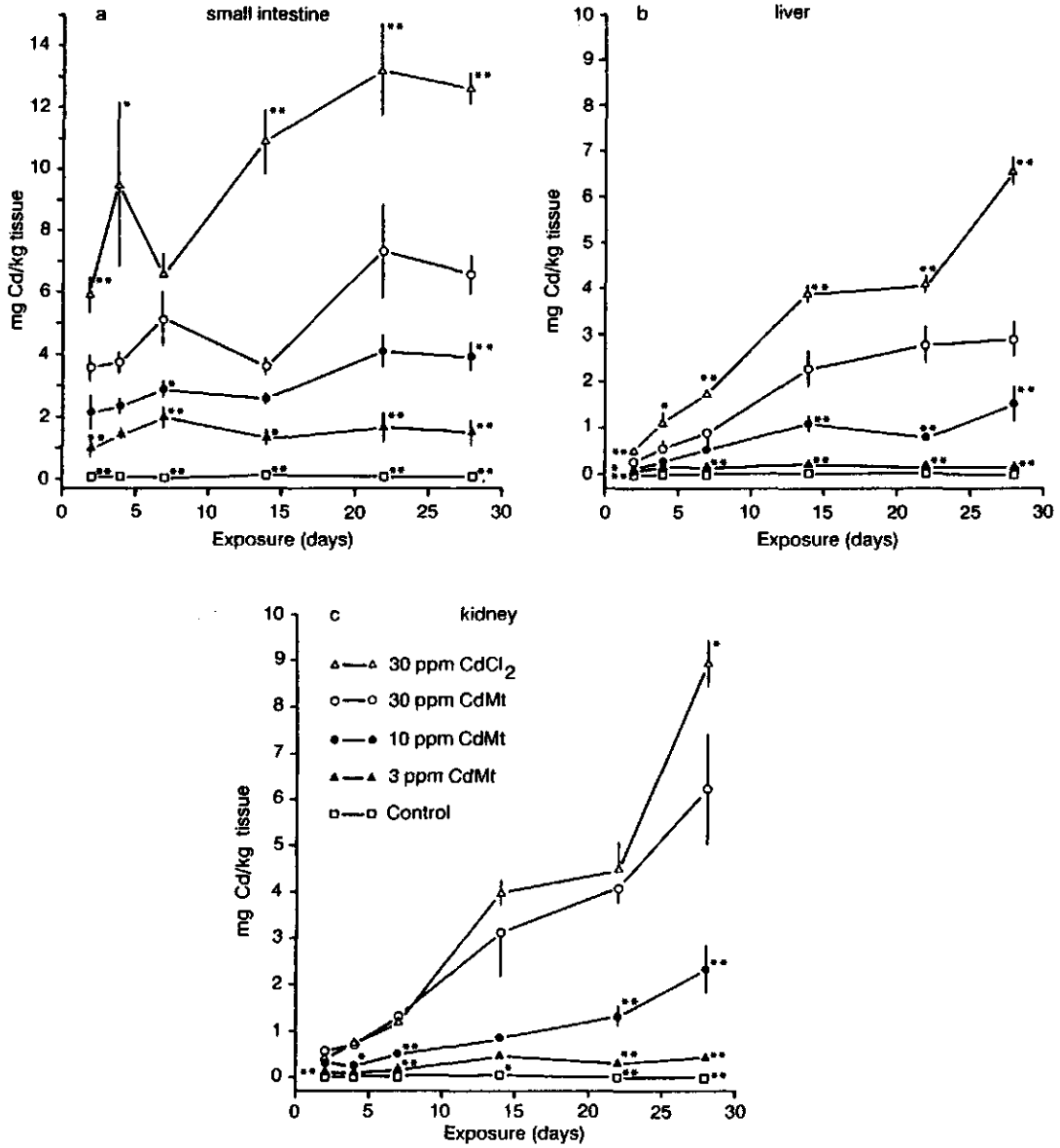

FIG. 1. Cd concentrations in small intestine, liver, and kidneys after oral exposure to $CdCl_2$ (30 mg Cd/kg diet) or pig's CdMt (3, 10, or 30 mg Cd/kg diet) for 28 days. Every value is the mean ± SEM of three rats. All values marked with an asterisk differ significantly from the group fed 30 mg Cd/kg as CdMt (**$p < 0.01$ and *$p < 0.005$). Note the y-axis of the intestine is plotted from 0 to 15 mg/kg and the y-axis of the liver and kidneys is plotted from 0 to 10 mg/kg.

days of exposure to either inorganic or biologically incorporated Cd.

After 28 days the Cd concentration in the intestinal mucosa is more than twice as high after the feeding of $CdCl_2$ as after the feeding of CdMt (Fig. 1a). A similar pattern was found for the Cd accumulation in the liver; from Day 2 onward the feeding of $CdCl_2$ resulted in almost twice the amount of Cd in the liver compared with the feeding of CdMt (Fig. 1b).

In sharp contrast no difference in the Cd concentration between $CdCl_2$ and CdMt ex-

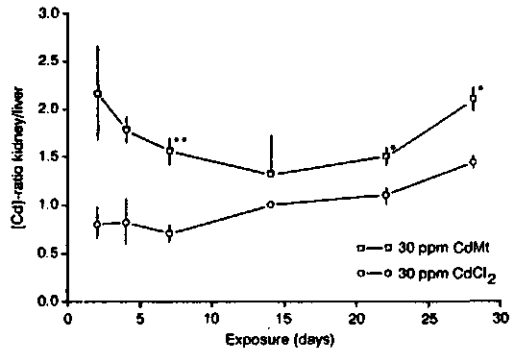

FIG. 2. The ratio between kidney and liver Cd concentrations of rats fed 30 mg Cd/kg diet. Each value is the mean ± SEM of three rats. Values marked with an asterisk in the group fed $CdCl_2$ differ significantly from the group fed 30 mg Cd/kg as pig's CdMt (**$p < 0.01$ and *$p < 0.005$).

posure in the kidneys was observed during the first 22 days of the study (Fig. 1c). Only on Day 28 did the animals fed $CdCl_2$ show a significantly higher Cd accumulation in the kidneys than the animals fed CdMt, but the difference was much smaller than that observed for liver and small intestine.

A plot of the kidney/liver ratio of Cd concentration versus time of exposure (Fig. 2) shows that oral exposure to CdMt leads to a significantly higher ratio than the feeding of inorganic Cd on Days 4, 22, and 28 and when regarding the whole experimental period. Initially, the Cd distribution from CdMt is very different from the distribution of Cd originating from $CdCl_2$. However, the difference becomes less pronounced with time and from Day 15 onward the increase in the relative contribution of the Cd content of the kidneys is similar for both Cd forms, although CdMt gives a consistently higher Cd ratio between kidneys and liver.

A comparison of the ratio between renal and hepatic Cd concentrations at low doses of $CdCl_2$ and CdMt was performed in a separate study. Cd accumulation after feeding 30 ppm $CdCl_2$ was determined in both studies: the kidney/liver Cd ratios were 1.4 and 1.3, respectively, for the main and the second study. The ratio of kidney/liver Cd concentration is dose dependent as feeding of a low dose of CdMt or $CdCl_2$ (1.5 ppm Cd) resulted in a relatively higher Cd disposition in the kidneys than the feeding of a high dose (30 ppm Cd). However, at all dose levels tested the kidney/liver Cd ratio is still higher after feeding of CdMt than after feeding of $CdCl_2$ (Table 2).

From the organ weights it was calculated that the total Cd intake in liver and kidneys at the doses of 2, 8, and 30 ppm Cd was 3.5, 39.2, and 105 μg Cd, respectively, after feeding $CdCl_2$ and 2.4, 9.6, and 39.3 μg, respectively,

TABLE 2

MEAN VALUES OF LIVER AND KIDNEY CONCENTRATIONS IN RATS FED $CdCl_2$ OR CdMt FOR 28 DAYS AT THREE DIETARY Cd LEVELS

Diets	Cd concentration in mg/kg tissue		Cd ratio kidney/liver
	Liver	Kidney cortex	
1.5 ppm $CdCl_2$	0.20 ± 0.05 (2.6)	0.43 ± 0.2 (0.87)	2.41 ± 0.62
8 ppm $CdCl_2$	2.34 ± 0.61 (32.45)	3.10 ± 0.64 (6.78)	1.34 ± 0.16
30 ppm $CdCl_2$	7.64 ± 1.47 (84.6)	9.98 ± 2.0 (19.66)	1.31 ± 0.19
1.5 ppm CdMt	0.14 ± 0.02 (1.72)	0.37 ± 0.04 (0.76)	2.75 ± 0.50
8 ppm CdMt	0.54 ± 0.38 (6.37)	1.15 ± 0.5 (2.23)	2.50 ± 0.70
30 ppm CdMt[a]	2.93 ± 0.39 (28.0)	6.26 ± 1.2 (11.26)	2.10 ± 0.20

Note. Cd concentrations are means ± SD for groups of five animals. The value between brackets gives the mean total Cd intake in liver and kidneys, respectively, in μg Cd. For details of the experiment see Materials and Methods.

[a] Values of the 30 ppm CdMt dose are derived from the first experiment and are means ± SD of three animals.

after feeding CdMt. Thus, the total Cd intake in liver and kidneys for 30 ppm CdMt and 8 ppm $CdCl_2$ is approximately equal, although Table 2 shows that the renal Cd content is more than twice as high after feeding 8 ppm Cd as CdMt than after feeding 30 ppm Cd as $CdCl_2$.

The CdMt content of the various organs was also measured: After feeding of 30 ppm Cd as $CdCl_2$ or as CdMt the Mt levels in liver and kidneys were significantly increased compared to the control (Figs. 3b and 3c). In contrast to the kidneys and the liver, the Mt concentration in the intestinal mucosa is already significantly raised after 2 days of exposure to either of the Cd forms, but the Mt concentration does not increase during the course of the study (Fig. 3a). From Day 14 onward, a significant difference in the Mt content in liver and intestine based on the treatment form of Cd occurred (Figs. 3a and 3b), as $CdCl_2$ leads to higher Mt levels than CdMt. However, renal Mt levels for both Cd forms were equal. In fact on Day 7 the feeding of CdMt resulted in significantly higher Mt levels in the kidneys than did the feeding of $CdCl_2$ (Fig. 3c).

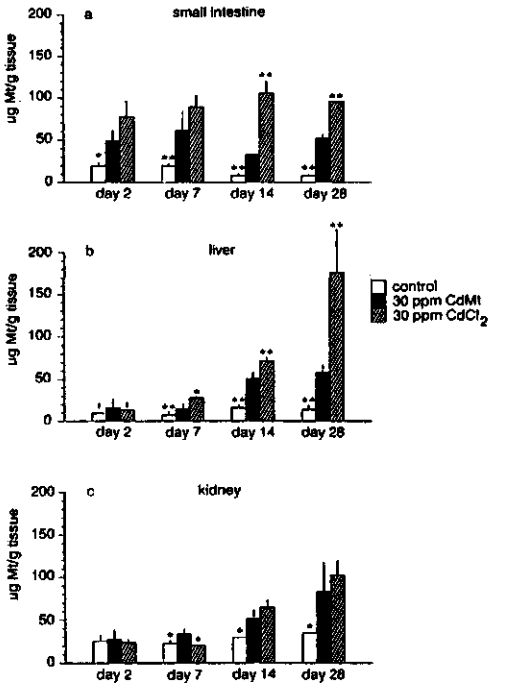

FIG. 3. Mt concentrations in small intestine, liver, and kidneys after oral exposure to $CdCl_2$ or pig's CdMt (30 mg Cd/mg) for 28 days. Every value is the mean ± SD of three rats. Values marked with an asterisk in the control group and in the group fed $CdCl_2$ differ significantly from the group fed CdMt (**$p < 0.01$ and *$p < 0.005$).

DISCUSSION

In the present investigation the disposition of biologically incorporated Cd, specifically Cd associated with Mt, was studied. In this regard, some evidence suggests that the sequence of events for intestinal uptake of CdMt is different from that for $CdCl_2$. The first potential difference between $CdCl_2$ and CdMt can be found in the gastrointestinal stability of CdMt. Cherian (1979) and Ohta and Cherian (1991) found that a major portion of ingested CdMt could be isolated intact from the intestinal mucosa after oral exposure to CdMt. Crews *et al.* (1989) have shown that indeed at least part of a pig kidney or equine CdMt-like protein survives *in vitro* treatment with gastrointestinal proteolytic enzymes. Thus CdMt does in fact appear to reach the intestine. It is well known that CdMt after iv administration preferentially accumulates in the kidneys, unlike inorganic salts of Cd that accumulate in the liver (Nordberg *et al.*, 1975; Tanaka *et al.*, 1975; Squibb *et al.*, 1979; Sendelbach and Klaassen, 1988; Maitani *et al.*, 1988). Furthermore, it has been suggested that CdMt can pass the intestinal mucosa at least partially intact because of the high kidney/liver ratio found after single bolus oral exposure to CdMt (Cherian, 1979; Maitani *et al.*, 1984; Ohta and Cherian, 1991). Thus the results of the present study showing a high kidney/liver ratio of Cd concentration also indicate that CdMt incorporated into the diet is at least partially absorbed intact.

The general theory for $CdCl_2$ disposition is that after absorption of very low amounts of ionic Cd through the intestine the Cd ions are initially bound to available endogenous Mt and transported preferentially to the kidneys

(Lehman and Klaassen, 1986). However, after a higher dose (100 μg Cd/kg body wt or higher), the available endogenous circulating Mt pool is overloaded and Cd will bind to higher molecular weight plasma proteins such as albumin and then be deposited in the liver (Lehman and Klaassen, 1986). To what extent $CdCl_2$ or other inorganic salts are accumulated by the kidneys after oral administration thus depends on the dose. This hypothesis is strengthened by the present results which show that the selective renal disposition of Cd from $CdCl_2$ is dose-dependent after subchronic administration. A level of 1.5 ppm Cd as $CdCl_2$ in the diet resulted in a higher proportion of the total dose depositing in the kidneys after 4 weeks of exposure than did a dose level of 30 ppm Cd as $CdCl_2$. Moreover at the 30 ppm level the Cd accumulation in the kidneys after feeding of $CdCl_2$ increases with time. Initially the ratio of the kidney/liver Cd concentration is low and comparable to the ratio observed after a single similar dose in the study of Lehman and Klaassen (1986), but after 28 days of exposure the Cd concentration in the kidneys is higher than in the liver. However, after exposure to dietary CdMt the kidney/liver ratio of Cd is always higher than after $CdCl_2$ intake, irrespective of dose. In the first week of exposure to 30 ppm Cd in the diet the Mt concentration in the kidneys was even higher after administration of CdMt than after exposure to $CdCl_2$. This, in conjunction with the fact that similar renal Cd concentrations were observed until Day 7, might support the theory (Cherian, 1979; Maitani *et al.*, 1984; Ohta and Cherian, 1991) that exogenous CdMt indeed passes the intestinal barrier and reaches the kidneys intact. The differential disposition of $CdCl_2$ and CdMt between liver and kidney is less pronounced at lower doses of Cd, but still large enough to indicate metabolic differences.

In contrast to the liver and kidneys the Mt and Cd levels of the intestine are rather constant, which might be explained by the continuous renewal of the intestinal epithelium and desquamation of the "old" mucosa. Cd, which is retained in the intestinal wall, is thus released back into the lumen due to this epithelial sloughing and is available for reabsorption by lower parts of the intestine (Valberg *et al.*, 1976; Foulkes, 1986). An equilibrium between uptake and desquamation would result in constant Mt and Cd levels of the intestine in chronically exposed animals. The fact that the Cd content in fact slightly increased might indicate that relatively more Cd is bound to the same amount of CdMt in the course of the study as proposed by Goyer *et al.* (1990) for kidney and liver Mt after parenteral administration of $CdCl_2$. On the other hand, it might also indicate that other Cd-binding ligands are involved in the intestinal retention of Cd or CdMt rather than intestinal Mt.

The ratio of kidney to liver concentrations increased during the third and fourth weeks of exposure after both $CdCl_2$ and CdMt administration. This could be attributed to the frequent observation that after chronic Cd exposure kidney Cd levels increase due to a gradual redistribution from the liver (Nordberg *et al.*, 1985). At all doses tested, $CdCl_2$ leads to a higher Cd content of the liver than CdMt. After toxic insult or as a consequence of the age-related degeneration of the liver (Dudley *et al.*, 1985; Nordberg *et al.*, 1985), more Cd is thus available for redistribution from the liver to the kidneys after exposure to an inorganic Cd salt than after exposure to CdMt. This raises the question of what the impact of the Cd redistribution from the liver to the kidneys will be during long-term oral intake in the final level of renal intoxication.

In conclusion, after oral exposure to CdMt in the diet there is a relatively higher accumulation of Cd in the kidneys. However, the indirect renal accumulation via redistribution of Cd from the liver might be lower than after $CdCl_2$ exposure. Which of these two phenomena is decisive in the eventual level of renal toxicity of Cd after long-term oral intake could determine the toxicological risk of the chronic intake of biologically incorporated Cd.

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Chapter 4

COMPARATIVE TOXICITY AND ACCUMULATION OF CADMIUM CHLORIDE AND CADMIUM-METALLOTHIONEIN IN PRIMARY CELLLS AND CELL LINES OF RAT INTESTINE, LIVER AND KIDNEY

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COMPARATIVE TOXICITY AND ACCUMULATION OF CADMIUM CHLORIDE AND CADMIUM-METALLOTHIONEIN IN PRIMARY CELLS AND CELL LINES OF RAT INTESTINE, LIVER AND KIDNEY

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Abstract—The protective role of metallothionein (Mt) in the toxicity of Cadmium (Cd) is controversial, since Cd bound to Mt is more nephrotoxic than ionic Cd after parenteral exposure and less hepatotoxic than ionic Cd after oral exposure. This study compared the uptake and toxicity *in vitro* of $CdCl_2$ and two isoforms of rat cadmium metallothionein (CdMt-1 and CdMt-2) using primary rat kidney cortex cells primary rat hepatocytes, liver hepatoma cell line H-35, kidney epithelial cell line NRK52-E and intestinal epithelial cell line IEC-18. The molar ratio of Cd was 2.1 and 1.4 mol Cd/mol Mt for CdMt-1 and CdMt-2, respectively. Monolayer cultures were incubated for 22 hr with $CdCl_2$, CdMt-1 or CdMt-2 and Cd-accumulation was examined at Cd levels of 0.25–10 μM-Cd. Cells exposed to $CdCl_2$ accumulated more Cd in 22 hr than cells exposed to an equimolar amount of CdMt. For $CdCl_2$ the Cd accumulation is directly related to the Cd concentration in the medium; however, for CdMt an increase in Cd concentration in the medium above 2 μM had no effect on the Cd accumulation in the cells. At Cd concentrations above 2 μM, therefore, the difference in Cd accumulation between $CdCl_2$ and CdMt was greater (5–6 times) than at concentrations below 2 μM (1–2 times). Cytotoxicity was examined in the Cd-concentration range from 0.25 to 100 μM by determining the lactate dehydrogenase (LDH) release in the medium and the neutral red uptake in the cells. Under these culture conditions $CdCl_2$ was at least 100 times more toxic than CdMt-1 or CdMt-2 in all cell types tested. Primary hepatocyte cultures were ten times more sensitive (50% LDH release at 1–2 μM) to $CdCl_2$ intoxication than primary cultures of renal cortical cells or the intestinal cell line (50% LDH release at 10–20 μM). Hepatic and renal cell lines were less sensitive (50% LDH release at 20–35 μM) than the corresponding primary cultures. No difference in sensitivity towards CdMt-1 or CdMt-2 was found for the various cell types tested. To investigate the influence of the molar Cd ratio of CdMt on cytotoxicity, the Cd content of CdMt-1 (2.1 mol Cd/mol Mt) was artificially raised *in vitro* to 5 mol/mol Mt. Compared with native CdMt, CdMt with a high molar Cd ratio in primary renal cultures showed a 15% increase in LDH release at a Cd concentration of 1500 μM in the medium. In conclusion, exogenous CdMt is far less toxic than $CdCl_2$ to cell cultures in a serum-free medium. Whereas $CdCl_2$ in all cases showed dose-dependent Cd accumulation, Cd accumulation due to CdMt exposure in all cell types tested reached a plateau at medium Cd concentrations of 2 μM. The low cellular Cd uptake of CdMt and the corresponding low cytotoxicity supports previously reported results *in vivo*, showing that the difference in toxicity between CdMt and $CdCl_2$ is associated with a difference in Cd distribution.

INTRODUCTION

Occasionally humans are exposed to inorganic cadmium (Cd), but the general population is mainly exposed to cadmium through food (Foulkes, 1986; Robards and Worsfold, 1991). In food a major portion of the Cd is bound to metal-binding proteins such as phytochelatins and metallothioneins. Metallothionein (Mt) is known to have a key role in the detoxification of heavy metals in animals (Waalkes, 1990; Webb, 1986). There is some evidence that rats exposed to cadmium-metallothionein (CdMt) by way of the food show Cd-accumulation preferentially in the kidneys (Groten *et al.*, 1991; Maitani *et al.*, 1984; Ohta and Cherian, 1991). On the other hand, after oral exposure to inorganic Cd, Cd accumulates also to a considerable extent in the liver. It is thought that Cd ions, once absorbed in the intestine, are bound to high molecular weight plasma proteins such as albumin and to Mt. The Cd–albumin complex is

Abbreviations: AAS = atomic absorption spectrometry; Cd = cadmium; CdMt = cadmium-metallothionein; EDTA = ethylenediaminetetraacetic acid; FAAS = flame atomic absorption spectrometry; GFAAS = graphite furnace atomic absorption spectrometry; H-35 = hepatoma cell line; IEC-18 = intestinal epithelial cell line; LDH = lactate dehydrogenase; Mt = metallothionein; NRK-52E = normal rat kidney (epithelial like) cell line; PBS = phosphate buffered saline.

taken up in the liver, where after degradation of the Cd–albumin, Cd is bound again to hepatic Mt. Cd bound to intestinal or hepatic Mt is taken up in the kidneys, and after degradation of this Cd-complex, Cd is bound to renal Mt (Lehman and Klaassen, 1986; Ohta and Cherian, 1991; Webb, 1986). The binding to proteins such as albumin and Mt thus serves as a protective mechanism to scavenge Cd from the blood. However, the protective role of Mt is rather controversial since it has been shown that CdMt after iv injection is even more nephrotoxic than ionic Cd. This can largely be ascribed to the higher renal accumulation of CdMt after parenteral exposure (Cherian *et al.*, 1976; Nordberg *et al.*, 1975; Squibb *et al.*, 1979; Tanaka *et al.*, 1975). In contrast to the acute nephrotoxicity of parenteral CdMt, it has been shown that signs of subacute toxicity such as anaemia and hepatotoxicity are less pronounced after dietary exposure to CdMt than after exposure to $CdCl_2$ (Groten *et al.*, 1990). The lower toxicity of CdMt in this case correlates well with the fact that the intestinal and hepatic uptake of Cd after CdMt exposure is lower than after exposure to $CdCl_2$ (Groten *et al.*, 1991; Maitani *et al.*, 1984). Indeed, studies *in vitro* using primary hepatocytes have confirmed that exogenous CdMt is less toxic to primary hepatocytes than $CdCl_2$ and this is due, at least in part, to a difference in Cd-uptake (Beattie *et al.*, 1987; Sendelbach *et al.*, 1989). However, isolated renal epithelial cells are apparently more sensitive to CdMt *in vitro*, in spite of a low Cd uptake (Cherian 1982, 1985). In addition, renal accumulation of Cd *in vivo* was almost similar for both CdMt or $CdCl_2$ when given orally (Groten *et al.*, 1991).

A reliable comparison of the data from different cytotoxicity studies using cells of different tissue origin has been difficult to date, because for example, the method of preparation of CdMt (e.g. species, molar Cd ratio) and the culture conditions (e.g. use of serum) have not always been identical. For this reason the study reported here was undertaken to compare the accumulation and toxicity of CdMt and $CdCl_2$ in primary cultures and cell lines grown under the same conditions and using one identical batch of native CdMt. This comparative study provides more insight into the difference in toxicity between exogenous CdMt and $CdCl_2$, and the difference in sensitivity between hepatic, renal and intestinal cells *in vitro*. The results may eventually shed light on similar differences *in vivo*.

MATERIALS AND METHODS

Cell culture

Primary cells. Rat hepatocytes were isolated using the two-step collagenase perfusion technique described by Seglen (1976) and modified by Paine *et al.* (1979).

The cells were plated in 10-cm tissue culture dishes (Costar, Cambridge, MA, USA) or 6-well tissue culture plates (Costar) at a density of 8×10^6 cells/dish in 10 ml medium or 1×10^6 cells/well, respectively. Cell viability, as determined by trypan blue exclusion, ranged from 80 to 95%. The culture medium was Williams medium E, supplemented with 3% (v/v) newborn calf serum, 1 μM-insulin, 10 μM-hydrocortisone and 50 mg gentamicin/litre. The Cd exposure took place 24 hr after isolation of the hepatocytes.

Rat primary kidney cells were isolated according to the method of Jones *et al.* (1979) as modified by Bruggeman *et al.* (1989). The cells were plated on 6-cm tissue culture dishes in 5 ml medium or 6-well tissue culture plates in 2 ml medium at a density of $2–3 \times 10^6$ cells/dish or 0.4×10^6 cells/well, respectively. Cell viability, as determined by trypan blue exclusion, ranged from 70 to 90%. The cells became confluent between 3 and 5 days after plating. The culture medium was Williams E, supplemented with 10% foetal calf serum (FCS), penicillin–streptomycin (100 U, mg/litre). The Cd exposure took place 24 hr after confluency.

Cell lines. NRK-52E epithelial kidney cells (passage 17–20) and IEC-18 intestinal epithelial cells (passage 19–25) were cultured in HEPES-buffered Dulbecco's modified Eagle's medium. H-35 liver hepatoma cells were cultured in Ham's F12. All cell lines were obtained from ATCC (Rockville, MD, USA). All media were supplemented with 2 mM-glutamine, 5% FCS and 5000 units penicillin–streptomycin/l. Cells were seeded on 10-cm dishes in 10 ml culture medium or 6-well tissue culture plates in 2.5 ml culture medium at a density of 2×10^6 cells/dish or 0.3×10^6 cells/well, respectively, and became confluent between 2 and 3 days after plating. Cd exposure took place 24 hr after confluency.

Preparation of CdMt

Rat Mt was purified from livers of male Wistar [Crl:WI(WU)Br] rats that previously had been injected sc with $CdCl_2$ dissolved in saline according to the following schedule: on day 0 and 12 with 3 mg/kg and on day 4, 6, 8 and 10 with 1.2 mg/kg body weight. Two days after the last injection the livers were perfused with 0.9% NaCl, excised and homogenized (1:2, w/v) in 10 mM-Tris/144 mM-KCl buffer (pH 7.4) using a teflon pestle. The homogenate was centrifuged at 10,000 *g* for 15 min, decanted and again centrifuged at 100,000 *g* for 70 min. The cytosol was filtered through a 0.22-μM filter (Millipore GS, Molsheim, France) and chromatographed on a Sephadex G-75 column (5 × 60 cm, Pharmacia, Uppsala, Sweden) with 10 mM-Tris–HCl. The Mt containing fractions were determined by the relative elution volume ($V_e/V_o = \pm 2$) and were applied to a Sephadex DEAE-A25 column (2.5 × 20 cm) and eluted with a linear gradient of 10–300 mM-Tris–HCl

(pH 8.5, 4°C). The fractions that corresponded to CdMt-1 and CdMt-2 (as determined by the cadmium content of the eluate) were pooled, dialysed against water (Amicon YM membrane, Danvers, Ireland) and lyophilized. HPLC analysis on hypersil ODS HPLC steel columns (UV absorption wavelengths ranging from 190 to 280 nm, as described by Groten *et al.*, 1990) revealed one single peak for every isoform with less than 8% impurities in the preparation. Finally, the lyophilized material of several isolations was pooled to obtain 29 mg lyophilized Mt-1 and 38 mg Mt-2 from 80 ml liver cytosol. Both isoforms contained Zn and Cu in addition to Cd. It should be emphasized that, *in vivo*, Cd-induced Mt is almost always found in a mixed-metal form consisting of Cd, Zn and small amounts of Cu (Holt *et al.*, 1985; Otvos *et al.*, 1987; Webb, 1986). The molar ratios of Cd/Zn/Cu in Mt-1 and Mt-2 were 1:1.2:0.04 and 1:1.4:0.06, respectively. Based on a purity of 90% (as determined by the Cd–haemoglobin assay of Onosaka *et al.*, 1978 and modified by Eaton and Toal, 1982) Mt-1 and Mt-2 contained 2.1 mol Cd/mol Mt and 1:4 mol Cd/mol Mt, respectively.

Preparation of Cd-containing media

A filter-sterilized stock solution of $CdCl_2$ in demineralized water was pipetted into the serum-free culture medium. CdMt was weighed, dissolved in a small volume of serum-free culture medium and sterilized through a 0.22-μM filter (Millipore GV, Low Protein Binding). All Cd-containing media were prepared freshly before each experiment. The Cd concentration of the medium was checked by atomic absorption spectrometry (AAS) as described below and was always between 80 and 110% of the intended Cd level.

Cd accumulation

To examine the Cd accumulation, primary cultures and cell lines in monolayer culture were incubated for 22 hr in serum-free culture medium with a final Cd concentration of 0.25, 0.5, 1, 2, 5 and 10 μM, as $CdCl_2$, CdMt-1 or CdMt-2. The Cd-accumulation studies were performed in triplicate in 10-cm tissue culture dishes. After treatment, the Cd-containing medium was removed and the dishes were rinsed twice with 0.02% ethylenediaminetetraacetic acid (EDTA) in phosphate buffered saline (PBS) to remove adherent Cd, followed by rinsing the dishes twice with PBS to remove EDTA. Cells were detached with a teflon cell scraper (Costar, Cambridge, MA, USA), collected in 3 ml PBS and cooled to 4°C. The cells (except primary kidney cells) were spun down gently at 50 *g* for 10 min, the supernatant was removed and the cells were resuspended in 0.8 ml Tris–HCl (10 mM, pH 7.4); this was done to concentrate the cell suspension after being detached in PBS buffer. In this way, more than 90% of the accumulated Cd was recovered in the pellet consisting of intact cells and less than 10% was lost in the supernatant derived from damaged cells. For kidney cells the Cd recovery in the cell pellet was much lower, owing to the fact that many cells were damaged while being detached; primary kidney cells were therefore collected directly after being detached in 1 ml PBS followed by sonification. The cell yield after the primary kidney cells were detached was therefore 8–15% lower than for other cell types. All cell suspensions were sonicated on ice for 20 sec with a sonifier (Soniprep 150, MSE, UK). The cell homogenate was then centrifuged at 9000 *g* for 20 min (4°C). Finally, the supernatant (S-9-mixture) was decanted and stored at −20°C before analysis.

The samples were dry-ashed at 500°C and dissolved in hydrochloric acid. Cd in the cells was determined by flame AAS (FAAS) or graphite furnace AAS (GFAAS) after wet digestion of the sample by sulphuric acid. The Cu and Zn concentration of the CdMt was analysed by GFAAS and FAAS respectively. The total protein content in the S-9 mixture was determined according to the method of Lowry *et al.* (1951) using a centrifugal analyser (Cobas-Bio). Cell accumulation is always expressed as μg Cd/mg cellular protein.

Cd toxicity

Toxicity was examined at Cd concentrations ranging from 0.5 to 100 μM. Two toxicity tests were used: (1) LDH release (membrane permeability); (2) NR uptake (pinocytic uptake). For all cell types, morphology and cell attachment were examined using phase-contrast light microscopy. For determination of LDH release, cells were seeded on 6-well plates as described previously. After 22 hr exposure to one of the Cd compounds in a serum-free medium, this medium was removed and kept at 4°C. Cells were detached as described previously and sonicated in PBS in a volume equivalent to the medium. LDH enzyme activity was measured in the medium and in the cells. The increase in the percentage LDH release was determined as the LDH activity in the medium divided by the total LDH activity present in cells and medium. The 50% LDH release was determined by extrapolation as the concentration at which 50% of the LDH activity was present in the medium. For the NR uptake the cells were seeded on a 96-well tissue culture plate (Costar) and exposed for 22 hr to the Cd-containing media. NR uptake was determined using a slight modification of the method of Babich and Borenfreund (1990). The 50% inhibition of NR uptake was the concentration at which the NR uptake was reduced by 50% compared with untreated cells.

To compare the cytotoxicity of native CdMt and CdMt with a high molar ratio of Cd, CdMt-1 *in vitro* was saturated with Cd. For that purpose, 6.5 mg CdMt-1 was dissolved in 1 ml Tris–HCl buffer

(10 mM, pH 7.4) containing 5.5 M-Cd as $CdCl_2$ and incubated for 15 min at room temperature. To separate Cd bound to CdMt from free ionic Cd, the Mt sample was applied to a Sephadex G-75 column (2.5 × 70 cm, Pharmacia, Uppsala, Sweden) and chromatographed with a volatile 25 mM-ammonium acetate buffer (pH 7). Fractions of 2 ml were collected and the Cd content was determined. The gel filtration revealed two Cd-containing peaks: the first peak with a relative elution volume (V_e/V_o) of ±2 contained the Mt fractions and the second peak contained free ionic Cd not bound to Mt, which elutes later on the Sephadex column (V_e/V_o = 2.9–3.4). As determined by AAS analysis, the saturated CdMt-1 contained 5 mol Cd/mol Mt.

The toxicity of the two metallothioneins with different molar Cd ratios (2 mol Cd/mol Mt and 5 mol Cd/mol Mt, respectively) was tested using proximal renal cultures only. Owing to the limited amount of material the experiment was performed in a 24-well plate (Costar) with 200 µl medium/well. Toxicity was determined at actual Cd concentrations ranging from 10 to 1500 µM, depending on the cell type used, and LDH release into the medium was measured 22 hr after exposure.

Statistical analysis

The data relating to Cd content in the individual cell types were evaluated by a one-way analysis of variance, followed by pairwise *t*-tests. A Bonferroni correction was applied to the significance level to adjust the false-positive error rate for multiple comparisons (Dixon, 1988).

RESULTS

Cd accumulation

At non-toxic $CdCl_2$ levels (0.25–2 µM) the cellular Cd concentration was directly related to the concentration of $CdCl_2$ in the medium. At higher $CdCl_2$ concentration (5–10 µM-Cd) the accumulation data are unreliable because some cell types (such as hepatocytes, IEC-18) showed decreased cell viability at

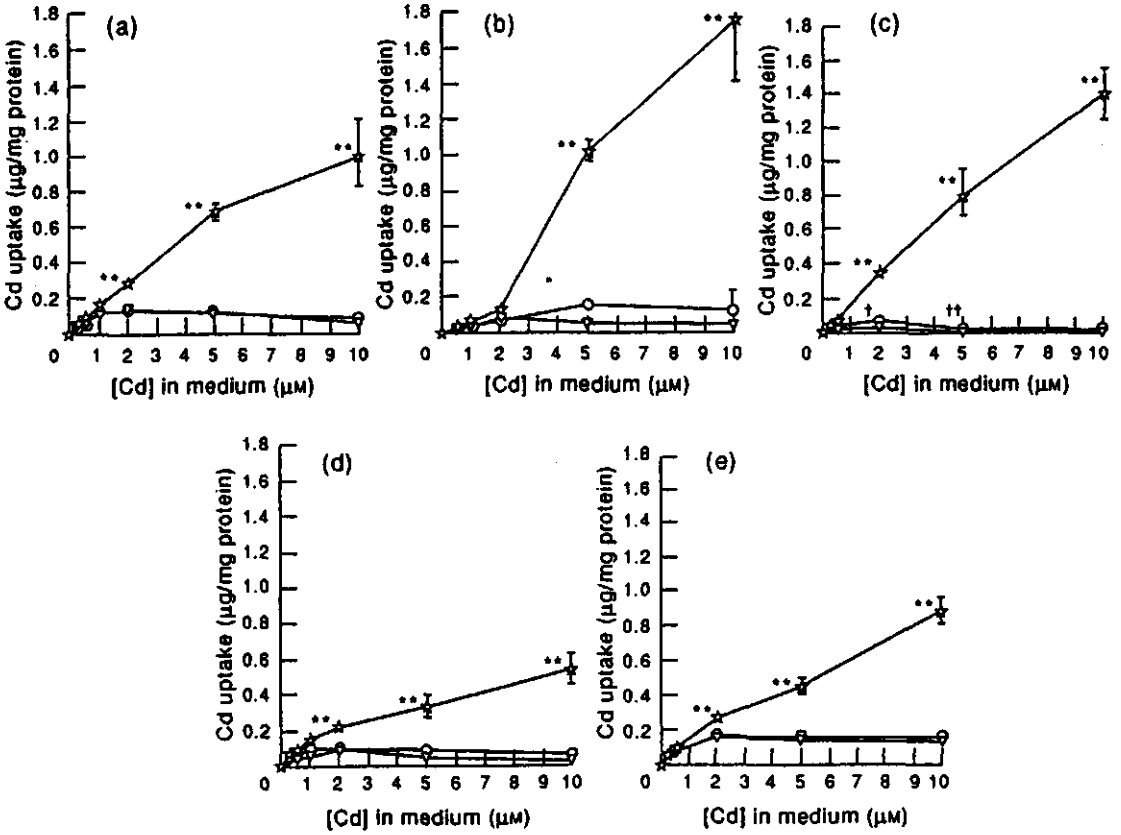

Fig. 1. Intracellular cadmium (Cd) concentrations in relation to Cd exposure-concentration in five types of cells: (a) IEC-18 intestinal cell line; (b) primary hepatocytes; (c) H-35 hepatoma cell line; (d) primary renal cortical cells; (e) NRK-52E kidney cell line. Cells were exposed for 22 hr to $CdCl_2$ (✡), cadmium-metallothionein (CdMt)-1 —○—, or CdMt-2 (—▽—). Every value is the mean of results from three tissue culture dishes. Vertical bars indicate standard deviation (SD). When no bars are present, the SD was smaller than the size of the plotted data point. Values marked with an asterisk in the group treated with $CdCl_2$ differ significantly from the groups exposed to CdMt-1 and -2 (**$P < 0.05$; *$P < 0.1$). Values marked with a dagger in the group treated with CdMt-1 differ significantly from the groups exposed to CdMt-2 (††$P < 0.05$, †$P < 0.1$).

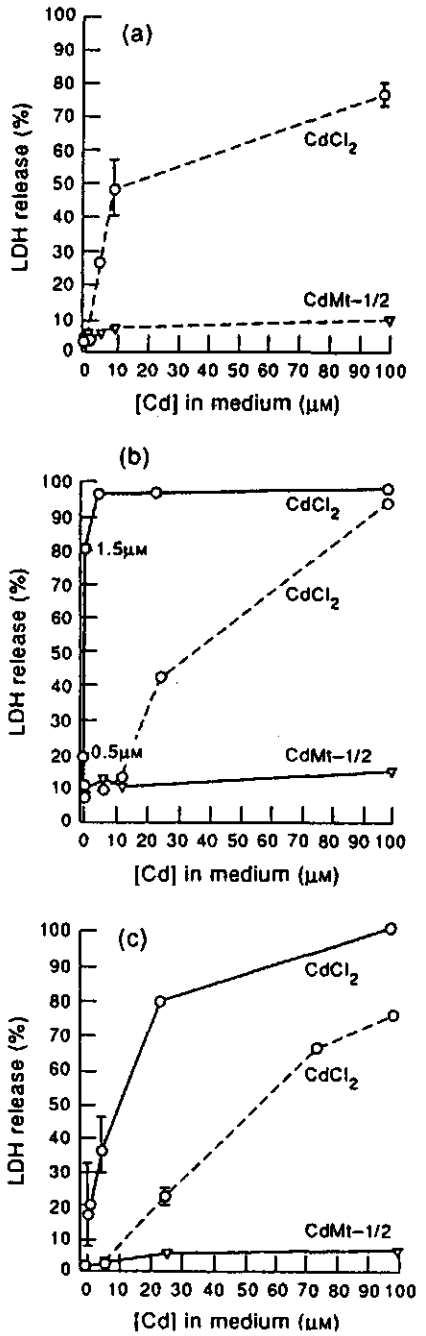

Fig. 2. Concentration-dependent toxicity of Cd as measured by lactate dehydrogenase (LDH) release into the medium. Cells were treated with $CdCl_2$ (upper line(s)) (○), CdMt-1 or CdMt-2 for 22 hr. CdMt-1 and CdMt-2 are shown together as one (bottom) line (▽). Vertical bars indicate standard deviations. The LDH release of H-35 and NRK-52E cells after CdMt treatment is not depicted as LDH release gives the same results as with primary hepatocytes and primary renal cortical cells. Data points are means ± SD from three replicate tissue culture dishes. When no bars are present, the SD was smaller than the size of the plotted data point. (a) IEC-18 (---); (b) hepatocytes (——) and H-35 cell line (---); (c) renal cortical cells (——) and NRK-52E kidney cell line (---).

Table 1. Cellular toxicity of $CdCl_2$ in five types of cell culture

Cells	LDH_{50}* (μM)	NR_{50}† (μM)
Primary hepatocytes	1–2	1–2
Primary renal cortical cells	10–20	10–20
H-35 hepatoma cell line	20–25	15–20
NRK-52E kidney cell line	25–35	25–35
IEC-18 intestinal cell line	10–15	5–10

*LDH_{50} = the concentration at which 50% of the lactate dehydrogenase (LDH) activity is present in the medium.

†NR_{50} = the concentration at which the neutral red (NR) uptake is reduced by 50% compared with untreated cells. Every parameter was measured at least in two separate sets of experiments. Every experiment consisted of three replicate dishes (LDH assay) or eight replicate wells (NR assay). For details of incubation see Methods.

these levels (see Figs 2a,b). The intracellular Cd concentration at a dose of 1 μM-Cd in the medium (which is not toxic for most cell types) varied between 0.08 and 0.2 μg/mg protein. In general, the cellular Cd concentration after $CdCl_2$ exposure in primary cultures and cell lines of liver was higher than in the renal cultures (Fig. 1b–d). Cells exposed to CdMt-1 or CdMt-2 showed a dose-related cellular Cd concentration up to 2 μM-Cd in the medium. However, above 2 μM the Cd accumulation of both CdMt isoforms did not increase further with increasing Cd concentrations in the medium. Therefore, at high Cd concentration in the medium all cell types exposed to CdMt accumulated much less Cd than cells exposed to the same concentration of $CdCl_2$. After exposure to CdMt-1, Cd accumulation was slightly, but consistently, higher than after exposure to CdMt-2. The difference in accumulation between CdMt-1 and CdMt-2 became statistically significant at several Cd concentrations in the liver cell line (Fig. 1c).

Cd toxicity

The toxicity of $CdCl_2$, CdMt-1 or CdMt-2 to primary cells of liver and kidney, as measured by LDH release into the medium, is depicted in Fig. 2(b,c). For comparison the toxicity of $CdCl_2$ to cell lines of liver and kidney is shown as a dotted line in the same figures. The NR uptake is not depicted since the results were very similar to the LDH release data. It appears that the differences in accumulation between $CdCl_2$ and CdMt (Fig. 1) are accompanied by a marked difference in cytotoxicity (Fig. 2). LDH release into the medium of cells without any Cd treatment varied according to cell type, but was always between 3 and 8% for the cell lines. LDH release in control incubations from primary cultures was higher. In untreated hepatocytes and proximal renal cells, respectively, 10 and 16% of the LDH activity was present in the medium after 22 hr of culture. After 22 hr treatment with $CdCl_2$, primary hepatocytes are the most sensitive to $CdCl_2$ intoxication, since increased LDH release was seen at Cd levels of 0.5 μM and higher (Fig. 2b). It seems that both primary hepatocytes and the hepatoma cell line were more sensitive to $CdCl_2$ than were the kidney primary cultures and cell line.

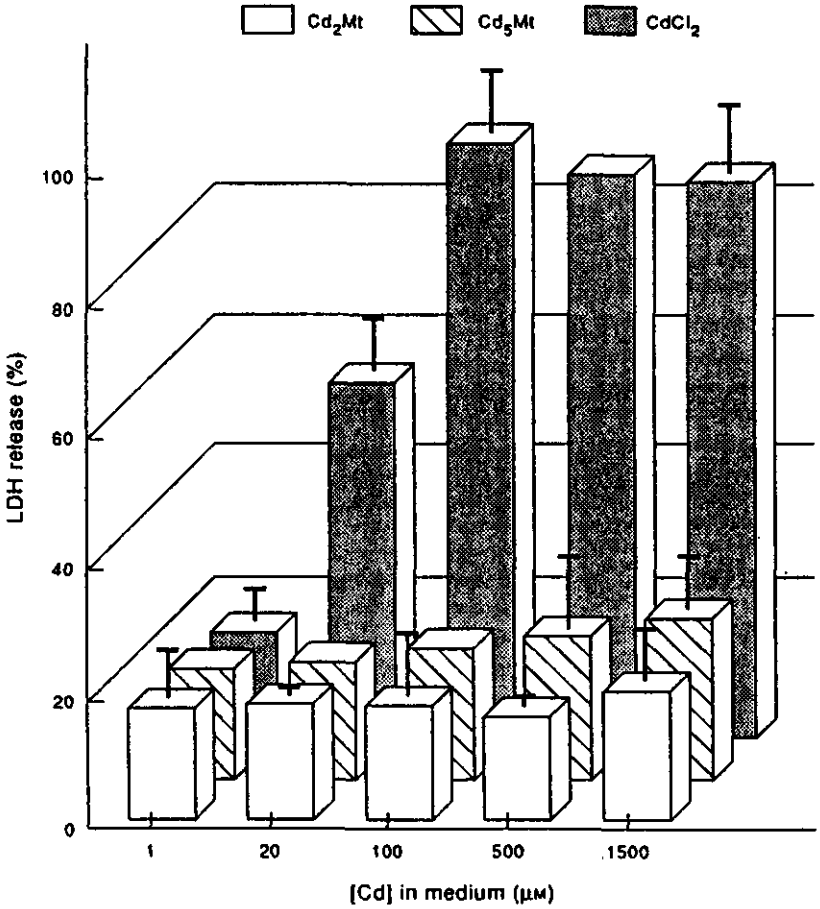

Fig. 3. Concentration-dependent toxicity of Cd as measured by LDH release into the medium. Cells were treated with $CdCl_2$ (▨), CdMt-1 with 2 mol Cd/mol Mt (Cd2Mt, ■) or CdMt-1 with 5 mol Cd/mol Mt (Cd5Mt, ▧) for 22 hr. Data points are means ± SD from three replicate tissue culture wells on a 24-well tissue culture plate. When no bars are present, the SD was smaller than the size of the plotted data point.

Table 1 shows the calculated Cd concentration for $CdCl_2$ at which 50% of the LDH is released into the medium or the Cd concentration at which NR uptake in the cell was reduced by 50%. In primary hepatocyte cultures the 50% LDH release or 50% reduced NR uptake is reached at a value 10 times lower (1–2 μM-Cd) than in primary renal cortical cultures (10–20 μM). The intestinal epithelial cell culture IEC-18 seems to be more sensitive to $CdCl_2$ than the other cell lines (Fig. 2a) and the 50% LDH release or NR uptake was reached at a Cd concentration of ±10 μM (Table 1). As shown in Table 1, the Cd level at which 50% LDH release was reached, was almost similar to the Cd concentration at which 50% reduction of the NR uptake was obtained. The two indicators of cytotoxicity used in this investigation correspond well to the morphological differences in cell attachment observed.

$CdCl_2$ is consistently much more toxic to primary cultures and cell lines than CdMt-1 or CdMt-2. No increase in LDH release, no reduced NR uptake and no morphological changes were observed in cells exposed to equimolar Cd concentrations in the form of CdMt.

To investigate whether CdMt becomes toxic at higher Cd concentrations than those tested in the main experiment (0–100 μM-Cd), a separate cytotoxicity study was performed in primary renal cells with Cd concentrations up to 1.5 mM-Cd (Fig. 3). Although toxicity starts for $CdCl_2$ at 10 μM-Cd, no signs of toxicity could be observed for native CdMt-1 (2.1 mol Cd/mol Mt) up to 1.5 mM-Cd. For CdMt-1 in which the Cd content had been raised artificially to 5 mol Cd/mol Mt, LDH release was slightly (although not statistically significantly) increased.

DISCUSSION

CdMt is one of the main forms in which Cd is present in the diet (Chmielnicka and Cherian, 1986; Foulkes, 1986). Compared with $CdCl_2$, both the intestinal and hepatic uptake of CdMt *in vivo* are low (Groten *et al.*, 1991; Maitani *et al.*). In agreement with this, the present results *in vitro* also indicate a

low cellular uptake of CdMt by the intestinal and hepatic cells. Moreover, both *in vivo* (Groten *et al.*, 1990) and *in vitro*, the difference in Cd accumulation between inorganic Cd and CdMt is accompanied by a considerable difference in hepatotoxicity. However, the difference in Cd accumulation between $CdCl_2$ and CdMt cannot be put forward as the only reason for the difference in hepatotoxicity: for instance, primary hepatocyte cultures show signs of toxicity due to $CdCl_2$ treatment between 0.5 and 2 μM-Cd, but not by an equimolar Cd concentration in the form of CdMt, although at these exposure levels there are no distinct differences in cellular Cd concentrations between hepatocytes exposed to $CdCl_2$ or CdMt.

The exact mechanism by which cadmium induces cytotoxicity still remains to be elucidated. In general, Cd cytotoxicity is believed to be dependent on the amount of free ionic Cd present in the cell that has not been sequestered by available endogenous Mt or other thiols (Glennas and Rugstad, 1984; Jin *et al.*, 1987; Sendelbach *et al.*, 1989; Stacey, 1986). The cytotoxicity of CdMt has been investigated in the kidneys in particular, but the mechanism involved is still not understood. CdMt is thought to be taken up by endocytosis (Cherian, 1979) and cytotoxicity may be a consequence of intracellular Cd release after degradation of the protein (Cain and Holt, 1983; Maitani *et al.*, 1988; Squibb *et al.*, 1979). It has been suggested that hepatic or intestinal uptake of CdMt will also take place by way of endocytosis (Beattie *et al.*, 1987; Ohta and Cherian, 1990). However, the fact that in this study both CdMt-1 and CdMt-2 are less hepatotoxic than $CdCl_2$, in spite of a similar Cd concentration in the hepatocytes, seems to argue against an acute release of Cd from exogenous CdMt after this metalloprotein has been taken up by the cell.

Alternatively, it has been suggested that CdMt damages the brush-border or cell membrane during reabsorption of the CdMt, because toxicity of CdMt to renal cells has been observed (Cherian, 1982) in spite of a lower cellular uptake for CdMt than for ionic Cd. In contrast to this, it has been shown *in vivo* that CdMt, both after parenteral and oral exposure, is taken up more easily in the kidneys than ionic Cd (Cherian *et al.*, 1976; Groten *et al.*, 1991; Kjellström, 1986; Maitani *et al.*, 1984 and 1988; Tanaka *et al.*, 1975). This selective renal uptake of CdMt *in vivo* is probably due to direct and efficient glomerular filtration and reabsorption of CdMt in the proximal cells; thus, the difference in accumulation between CdMt and $CdCl_2$ in proximal cells *in vivo* is attributable to a difference in glomerular filtration, which determines the availability of the Cd-compound for the proximal tubule cells in the pro-urine. This cannot be simulated *in vitro* when proximal tubule cells are exposed directly, without the interference of glomeruli, to equimolar-Cd amounts of both Cd forms. On the other hand, the study *in vitro* reported here does show that proximal renal cells, like hepatic and intestinal cells, are not very sensitive to CdMt under these culture conditions. Thus, previous results indicating that renal epithelial cells are specifically sensitive to CdMt *in vitro* (Cherian, 1982) could not be confirmed. It should be emphasized that the difference in sensitivity might be due to the difference in experimental conditions, with regard to cell isolation, Mt purification and/or culture conditions. Most of the cells in the primary renal culture of the present experiment have previously been identified as proximal tubular cells, since the histochemical detection of γ-glutamyltranspeptidase showed bright red areas demonstrating the presence of the proximal tubular brush-border enzyme (Bruggeman *et al.*, 1989). In our study, all cells were cultivated under what were, according to the literature, optimal conditions including FCS; only during exposure were the cells cultivated overnight in a serum-free medium. This was done to compare this study with the work of Beattie *et al.* (1987) and Sendelbach *et al.* (1989), using only small amounts of serum or even serum-free medium during the exposure period. Furthermore, it is known that serum proteins can markedly decrease the availability of Cd as $CdCl_2$ in the medium and the transport into the cell (Corrigan and Huang, 1983; Klug *et al.*, 1988). Possibly for that reason, the cytotoxicity of $CdCl_2$ in the present study was 20 times higher (50% LDH release at 10–20 μM-Cd) than in the study of Cherian (1982 and 1985), who observed cytotoxicity on $CdCl_2$ treatment at a concentration of 200–400 μM-Cd.

Electron microscopy in the present study (data not presented) revealed that the intestinal cells and the renal proximal cells possessed numerous, thin microvilli covering the surface membrane, which would greatly enhance the efficiency of the cell in its absorptive function. It has been suggested that the absorption of Cd ions takes place by endocytosis, but to date there is no evidence to prove this assumption. Furthermore, it has been shown for ionic Cd that internalization of the surface-bound Cd fraction occurs in erythrocytes and in membrane vesicles prepared from the intestinal brush border, ruling out the role of ATP-dependent pinocytosis (Bevan and Foulkes, 1989; Foulkes, 1988). Nevertheless, Cd accumulation in intestinal brush borders from $CdCl_2$ is higher than from CdMt (Sugawara *et al.*, 1988).

The uptake of CdMt was suggested to take place by way of receptor-mediated endocytosis (Cherian, 1982; Foulkes, 1986). This might be supported by the finding that the rat intestinal epithelial cell line (IEC) and primary cells of intestine and kidneys of the rat are, indeed, capable of protein endocytosis from the culture medium (Adams, 1984; Heyman *et al.*, 1989; Maunsbach, 1966; Wall and Maack, 1985). If such an uptake route exists, it could certainly account for the slow rate of accumulation of CdMt in the present study. In this experiment, however, we did not study the form in which Cd crossed the cell membrane.

The study reported here has shown that the cellular Cd concentration as a result of Cd uptake from CdMt reaches a plateau at a concentration of about 2 μM. CdMt-1, which has a higher degree of Cd saturation than CdMt-2, leads to slightly, but consistently, higher Cd accumulation in several cell types tested, indicating that there is a transport mechanism involved in the uptake of the protein, irrespective of the Cd content. In agreement with this, it has been shown *in vivo* by Suzuki *et al.* (1979) and Holt *et al.* (1985) that the uptake of Cd in the kidneys after exposure to CdMt is higher when the Cd:Zn ratio in the injected Mt is raised. However, it has also been shown that an increase in the Cd content of the Mt molecule does not dramatically affect the toxicity in the kidneys (Suzuki *et al.*, 1979). This was also seen in the present study *in vitro*, when primary renal cell cultures exposed to CdMt with an artificially raised Cd content showed a slight toxic response only at very high Cd concentrations. Severe cell damage after CdMt exposure in the range 40 to 80 μM, as shown by others (Cherian, 1982 and 1985), cannot be explained by assuming a higher molar Cd ratio within CdMt.

In summary, the cytotoxicity of CdMt is lower than the toxicity of inorganic Cd for all cell types tested, even when the Cd content of CdMt is artificially raised. In general, the difference in cytotoxicity between CdMt and $CdCl_2$ corresponds well to a difference in cellular uptake of Cd, and no cell type tested was specifically sensitive to CdMt, at least not under the culture conditions, described. This is in agreement with results *in vivo* (Groten *et al.*, 1990 and 1991) showing a difference in toxicity between CdMt and $CdCl_2$ attributable to a difference in organ distribution of Cd.

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Chapter 5

INTERACTION OF DIETARY Ca, P, Mg, Mn, Cu, Fe, Zn, AND Se WITH THE ACCUMULATION AND ORAL TOXICITY OF CADMIUM IN RATS

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INTERACTION OF DIETARY Ca, P, Mg, Mn, Cu, Fe, Zn AND Se WITH THE ACCUMULATION AND ORAL TOXICITY OF CADMIUM IN RATS

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Abstract—The toxicity of Cd was examined in rats fed diets containing 30 mg Cd/kg as $CdCl_2$ for 8 wk. The Cd-containing diets were supplemented with various combinations of the minerals Ca, P, Mg, Mn, Cu, Fe, Zn and Se in order to investigate the protective effect of these mineral combinations on Cd accumulation and toxicity. The mineral combinations were chosen such that the effect of the individual components could be analysed. At the end of the 8-wk feeding period, the Cd concentrations in the liver and renal cortex were 13.9 and 19.5 mg/kg body weight, respectively. The feeding of 30 mg Cd/kg diet alone resulted in well known Cd effects, such as growth retardation, slight anaemia, increased plasma transaminase activities and alteration of Fe accumulation. Only supplements that contained extra Fe resulted in a significant protection against Cd accumulation and toxicity. The most pronounced effect was obtained using a supplement of Ca/P, Fe and Zn, which resulted in a 70–80% reduction in Cd accumulation in the liver and kidneys, as well as a reduction in Cd toxicity. The protective effect of the mineral combinations was mainly due to the presence of Fe^{2+}, but in combinations with Ca/P and Zn the effect of Fe was most pronounced. Compared with Fe^{2+} the protective effect of Fe^{3+} was significantly lower. Addition of ascorbic acid to Fe in both forms improved the Fe uptake, but consequently did not decrease Cd accumulation. Thus, the mineral status of the diet may have a considerable impact on the accumulation and toxicity of Cd, fed as $CdCl_2$ in laboratory animals. For the risk assessment of Cd intake, special consideration should be given to an adequate intake of Fe.

INTRODUCTION

Outside of the industrial environment, most of the body burden of Cd is derived from the diet. Several studies over the past 15 years have demonstrated that dietary nutrients like Zn, Fe, Mn, Cu, Se, Ca, ascorbic acid and vitamin D alter the intestinal uptake and/or the oral toxicity of Cd in animals and humans (Chmielnicka and Cherian, 1986; Fox, 1979; Nordberg *et al.*, 1985; Task Group on Metal Interactions, 1978).

There is some evidence that several metals can inhibit Cd absorption in the intestine *in vitro* (Sahagian *et al.*, 1967) and *in vivo* (Foulkes, 1985). However, the mechanisms of both Cd absorption and the way in which trace elements interfere with this process are still not clear (Foulkes, 1986). Most investigations have focused on a single metal–metal interaction, and few attempts have been made to compare the actual mineral status of other trace elements in the same study. Because of the complexity of trace-element interactions more extensive studies are required in which the effect of several minerals can be considered simultaneously. The authors are aware of only a few studies that have examined the effect of a dietary supplement of a combination of minerals (Banis *et al.*, 1969; Jacobs *et al.*, 1977, 1978b and 1983). Therefore, in order to gather more information on the protective effect of nutrient supplements in excess of nutritional requirements, a more extensive study was undertaken in the present paper. Eight minerals were studied, which, according to the literature, are thought to be effective in lowering Cd accumulation in the body (Task Group on Metal Interactions, 1978). All other trace elements were kept constant, at a level meeting the nutrient requirements of the laboratory rat (National Research Council, 1978).

In preliminary experiments, the sensitivity of the strain of rats used was examined and a dietary level of 30 mg Cd/kg diet was established as suitable for use in subsequent studies (Groten *et al.*, 1990).

The study comprised two experiments. The first was designed to compare the effect on Cd toxicity of diets supplemented with seven combinations of minerals, such that the effect of a single component could be analysed. In the second experiment, further studies were undertaken on those individual trace elements that were found, in experiment 1, to be the most effective in reducing Cd toxicity.

MATERIALS AND METHODS

Chemicals. $CdCl_2$ with a purity of at least 99%, was obtained from E. Merck AG (Darmstadt, Germany). The compounds used in the mineral supplements were $MgCl_2 \cdot 6H_2O$, $CaHPO_4 \cdot 2H_2O$, KH_2PO_4, $FeSO_4 \cdot 7H_2O$, $CuSO_4 \cdot 5H_2O$, $ZnCl_2$, $Na_2SeO_3 \cdot 5H_2O$ and $MnSO_4 \cdot H_2O$. All were obtained from E. Merck (analytical grade).

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Abbreviations: AAS = atomic absorption spectrometry.

Table 1. Percentage composition of basal diet of rats fed diets containing 30 mg Cd/kg and supplements of various combinations of minerals

Ingredient	% Composition
Casein	22.53
White wheat-flour	54.72
Mineral mixture*	4.24
Vitamin ADEK preparation†	0.36
Vitamin B mixture‡	0.24
Lard	17.91

*In 1 g mineral mixture: 399 mg KH_2PO_4, 389 mg $CaCO_3$, 142 mg NaCl, 58 mg $MgSO_4$, 5.7 mg $FeSO_4 \cdot 7H_2O$, 0.9 mg $ZnCl_2$, 0.8 mg $CuSO_4 \cdot 5H_2O$, 4.6 mg $MnSO_4 \cdot H_2O$, 0.02 mg $CoCl_2 \cdot 6H_2O$, 0.007 mg KI and 0.08 mg $KCr(SO_4)_2 \cdot 12H_2O$.

†In 1 g vitamin mixture: 2113 IU vitamin A concentrate, 704 IU vitamin D concentrate; 30 mg vitamin E preparation (50%), 1 mg menadione-Na-bisulphite (K_3) and 30 mg wheat-starch.

‡In 1 g vitamin B mixture: 3 mg thiamine·HCl, 2.25 mg riboflavin, 4.5 mg pyridoxine·HCl, 15 mg niacin, 6 mg Ca-pantothenate, 0.075 mg biotin, 0.75 mg folic acid, 37.50 mg vitamin B_{12} (0.1%) and 931 mg choline chloride.

Animals and maintenance. Weanling, Wistar-derived specific-pathogen-free rats (Bor; WISW) were obtained from F. Winkelmann (Institute for the Breeding of Laboratory Animals GmbH & Co. KG, Borchen, Germany). At the beginning of the study the rats were about 5 wk old. They were housed under conventional conditions, in suspended stainless-steel cages fitted with a wire-mesh floor and front. The room temperature was kept at 22 ± 2°C, and the relative humidity at 40–70%. A 12-hr light/dark cycle was maintained, and the number of air changes was about 10/hr. Prior to the experiment, all rats were fed the basal diet without any further additions. Drinking-water was supplied in glass bottles, which were cleaned once a week. Food and water were provided *ad lib.*

Experiment 1

Diets. A semi-synthetic, powdered basal diet (Table 1) was composed to resemble a western type of human diet (high in fat, low in fibre, without excessive amounts of minerals and vitamins). The mineral content of the basal diet was based on the nutrient requirements of the rat according to the National Research Council (1978). The experiment consisted of 11 test groups (diet codes A–K). Ca and P were always added as one combination, because they interactively affect the other's bioavailability (Spencer, 1984; Zemel and Linkswiler, 1981). Therefore, the ratio between Ca and P in the diet should be kept constant upon addition. To obtain 30 mg Cd/kg diet, 0.049% $CdCl_2$ (w/w) was added to the basal diet. Mineral analysis of the 11 diets (see Table 2) revealed that, under the experimental conditions, the actual levels of Cd were between 87 and 105% of the intended level of 30 mg/kg. Control groups A and C contained 170 and 190 μg Cd/kg diet, respectively. All minerals were distributed homogeneously throughout the diet, considering the low variation between individual sample data (data not shown). One batch of each of the diets was stored in a freezer at *c.* −20°C until use. Twice a week the diets in the feeders were refreshed with a portion of the test diets (thawed immediately before use).

Experimental design and treatment. The rats were acclimatized to the animal facilities for 1 wk, and were then allocated to 11 groups of 10 animals using a computer-generated random number table. A few rats were reallocated in order to equalize the initial mean body weight in the various groups. Each treatment group (housed in groups of 5) received one of the test diets. On day 28, 3 rats were autopsied and on day 56 autopsy was conducted on 7 rats.

Observations and analyses. The rats were weighed at weekly intervals and were observed daily for condition and behaviour. Food and water intake were measured over weekly periods throughout the study.

Haematology and clinical chemistry. Blood samples were collected from the tip of the tail of all animals on days 24 and 51, and were examined for haemoglobin concentration, packed cell volume, erythrocyte and total leucocyte counts. At autopsy (days 28 and 56), heparinized blood samples collected from the abdominal aorta of all rats were centrifuged at 1250 *g* for 15 min, using Sure-sep II serum-plasma separators (Organon Teknika, Durham, NC, USA). The plasma was then analysed for aspartate aminotransferase and alanine aminotransferase.

Mineral content of liver and kidneys. At autopsy, on days 28 and 56, the Cu, Zn and Cd levels were determined in both liver and kidney cortex (see Analysis of minerals).

Experiment 2

Diets. The purpose of experiment 2 was to determine whether the effect of the mixture Ca/P, Zn and Fe could be attributed to the presence of Ca/P, Zn or Fe ($^{2+}$ or $^{3+}$) alone. The experiment consisted of two sets of separate bioassays. In both sets, two control groups were taken into account: one control group was fed the Cd-supplemented diet without any mineral supplement. A second control group was fed the Cd diet supplemented with a total mineral mixture of Ca/P, Zn and Fe^{2+}. The test groups in the first set consisted of diets with a supplement of the single compounds Ca/P, Zn or Fe^{2+} in order to check the contribution of each single trace element that, according to experiment 1, was effective in reducing Cd toxicity. Two other test groups in the first set consisted of a Cd diet supplemented with Fe^{3+} or $Fe^{3+} + 0.25\%$ ascorbic acid. This allowed the comparison of possible effects of Fe^{2+} and Fe^{3+}. In the second set, the control groups were similar to those in the first set. The test groups in the second set consisted of Cd diets supplemented with Fe^{2+}, $Fe^{2+} + 0.25\%$ ascorbic acid or 0.25% ascorbic acid alone, to check the effect of ascorbate on the effect of Fe^{2+}. Twice a week the diets in the feeders were refreshed with a portion of the tests diets (thawed immediately before use). This procedure proved to be essential for the outcome of the study. In an initial experiment where the diet was not refreshed and was kept at room temperature, the protective effect of Fe was much less pronounced.

Experimental design. The rats were acclimatized to the animal facilities for 1 wk and were then reallocated to 7 groups in the first set and to 5 groups in the second set. Each group consisted of 5 animals, obtained using a computer-generated random number

Table 2. Mineral analyses of diets containing 30 mg Cd/kg and supplements of various combinations of minerals, which were fed to rats

Ingredients	Minerals (% or mg/kg diet)								
	Ca (%)	P (%)	Zn (mg/kg)	Cu (mg/kg)	Fe (mg/kg)	Mg (%)	Mn (mg/kg)	Se (mg/kg)	Cd (mg/kg)
Experiment 1									
A: Basal diet	0.67	0.61	28	11	46	0.046	56	0.11	0.17
B: + Cd	0.66	0.63	27	10	39	0.046	54	0.10	30.5
C: + TMM	1.28	1.32	140	51	195	0.25	235	0.85	0.19
D: + Cd + TMM	1.28	1.34	140	51	185	0.25	235	0.78	28
E: + Cd + Ca/P, Mg, Cu	1.3	1.33	29	46	35	0.24	45	0.11	29.5
F: + Cd + Ca/P, Fe^{2+}, Zn	1.29	1.35	125	7	215	0.046	48	0.09	28
G: + Cd + Ca/P, Se, Mn	1.28	1.3	29	11	42	0.046	250	0.88	26
H: + Cd + Mg, Fe^{2+}, Se	0.64	0.59	28	10	245	0.26	60	0.84	30
I: + Cd + Mg, Zn, Mn	0.66	0.6	125	8	46	0.26	270	0.1	29.5
J: + Cd + Fe^{2+}, Cu, Se	0.66	0.6	29	51	215	0.047	260	0.09	31.5
K: + Cd + Cu, Zn, Se	0.66	0.6	140	70	40	0.046	54	0.62	30
Experiment 2									
Set 1									
A: Basal diet + Cd	0.67	0.60	27	—	70	—	—	—	30
B: + Cd + Ca/P, Zn, Fe^{2+}	1.3	1.27	140	—	270	—	—	—	27
C: + Cd + Fe^{2+}	0.67	0.61	27	—	290	—	—	—	32
D: + Cd + Fe^{3+}	0.67	0.60	29	—	270	—	—	—	29
E: + Cd + Fe^{3+} + VC	0.67	0.61	27	—	250	—	—	—	29
F: + Cd + Ca/P	1.2	1.31	28	—	65	—	—	—	30
G: + Cd + Zn	0.66	0.59	140	—	65	—	—	—	28
Set 2									
A: Basal diet + Cd	0.68	0.61	28	—	80	—	—	—	28
B: + Cd + Ca/P, Zn, Fe^{2+}	1.3	1.29	135	—	265	—	—	—	26
C: + Cd + Fe^{2+}	0.68	0.60	28	—	285	—	—	—	28
D: + Cd + Fe^{2+} + VC	0.68	0.61	28	—	290	—	—	—	27
E: + Cd + VC	0.68	0.61	28	—	80	—	—	—	29

TMM = total mineral mixture of Ca, P, Mg, Mn, Cu, Fe^{2+}, Zn and Se VC = vitamin C

table (see experiment 1). Each treatment group (housed in groups of 5) received one of the test diets for 28 days.

Observations and analyses. The rats were weighed at weekly intervals and were observed daily for condition and behaviour. Food and water intakes were measured over weekly periods throughout the study.

Mineral content of liver and kidneys. At autopsy, on day 28, the liver and kidneys were removed and weighed. Part of the liver and kidney cortex were prepared for atomic absorption spectrometry (AAS) analysis (see Analysis of minerals). The Fe, Zn and Cd content was determined in both the liver and kidney cortex.

Analysis of minerals

Samples taken from each of the test diets were analysed to check the content of Cd and minerals in the diet. For the determination of Ca, Mg, Mn, Zn and Fe, samples of 5 g of each diet were dry-ashed at 500°C. The residues were dissolved in hydrochloric acid. The mineral content was determined by flame AAS. Se was determined by fluorimetry. Samples of 1 g were digested with perchloric acid/nitric acid. After reduction with hydrochloric acid Se was coupled to 2,3-diaminonaphthalene and extracted into cyclohexane. The samples for P determination were ashed and dissolved in nitric acid. The P content was determined gravimetrically after precipitation with a sulphate–molybdate solution. For the determination of Zn and Fe in the liver and kidneys, samples (1–2 g) were wet-digested with sulphuric acid and nitric acid, and analysis was performed by flame AAS or gas–flame AAS. The measurement of Cd in diets and organs was determined by flame AAS or gas–flame AAS after wet-digestion of the samples (1–2 g) with sulphuric acid and nitric acid (Muys, 1984).

Statistical analysis

Experiment 1. Data on body weights, laboratory determinations and organ weights were evaluated using a one-way analysis of covariance, followed by Dunnett's multiple comparison tests. Differential white blood cell counts were analysed using the Mann–Whitney U-test. The data for animals killed on day 28 were analysed separately from those killed on day 56. The measurements in experiment 1, as shown in Tables 3 and 4 and Figs 2, 3 and 4, were subjected to the aforementioned individual statistical analysis. However, there are no two groups in experiment 1 that differ in the addition of just one mineral. Therefore, all data from experiment 1 were also subjected to multiple analysis of variance; one analysis for experimental groups A–D and one for groups D–K. This method of analysis, as shown in Fig. 6, uses all eight experimental groups, and the comparisons drawn will be more accurate than comparisons with two groups. For groups A–D a two-way ANOVA was used to assess the effects of Cd addition, addition of a total mineral mixture, and the possible enhancement or inhibition of the effect of Cd by the total mineral mixture. In the analysis of groups D–K, the influence of seven minerals was studied on only eight experimental groups. A complete design with all possible combinations of minerals would take 2^7 experimental groups, so the present experiment was one-sixteenth of a 2^7 design. This design is exemplified by Box *et al.* (1978).

Experiment 2. Both bioassays in experiment 2 were analysed using a one-way ANOVA, followed by Student's *t*-test to evaluate differences of interest. In

Table 3. Mean haematological findings on days 24 and 51 (experiment 1) from rats fed diets containing 30 mg Cd/kg and supplements of various combinations of minerals

	RBC (10^9/ml)	Hb (mmol/litre)	PCV (litre/litre)	MCV (fl)	MCH (fmol)	MCHC (mmol/litre)	WBC
A: Basal Diet	6.1 ± 0.1	8.2 ± 0.1	0.406 ± 0.05	66.6 ± 1.35	1.35 ± 0.03	20.3 ± 0.4	13.4 ± 1.2
	7.3 ± 0.11	9.2 ± 0.2	0.46 ± 0.007	62.7 ± 1.4	1.26 ± 0.03	20.1 ± 0.2	14.8 ± 1.3
B: + Cd	5.9 ± 0.1	5.7 ± 0.3**	0.297 ± 0.01**	50.3 ± 1.5**	0.97 ± 0.03**	19.2 ± 0.4	18.2 ± 1.0*
	7.9 ± 0.2	5.9 ± 0.2**	0.34 ± 0.01**	42.6 ± 0.8**	0.75 ± 0.02**	17.7 ± 0.2**	20 ± 0.7**
C: + TMM	5.8 ± 0.2	8.4 ± 0.1	0.42 ± 0.004	71.7 ± 1.6	1.44 ± 0.04	20.2 ± 0.2	18.1 ± 1.6**
	7.0 ± 0.2	9.3 ± 0.2	0.464 ± 0.01	66.0 ± 1.8	1.32 ± 0.03	20 ± 0.1	13.2 ± 1.5
D: + Cd + TMM	5.8 ± 0.2	8.1 ± 0.1	0.4 ± 0.003	69.0 ± 1.5	1.4 ± 0.02	20.3 ± 0.3	16.4 ± 1.0
	7.2 ± 0.2	9.1 ± 0.1	0.45 ± 0.004	64.9 ± 1.6	1.31 ± 0.03	20.1 ± 0.3	16.0 ± 0.7
E: + Cd + Ca/P, Mg, Cu	5.6 ± 0.1	5.3 ± 0.1**	0.29 ± 0.01**	51.1 ± 1.1**	0.94 ± 0.02**	18.5 ± 0.6**	20.1 ± 1.8**
	7.9 ± 0.2	5.5 ± 0.2**	0.32 ± 0.012	40.7 ± 1.0**	0.7 ± 0.02**	17.2 ± 0.1**	19.8 ± 0.7**
F: + Cd + Ca/P, Fe^{2+}, Zn	5.9 ± 0.1	8.2 ± 0.2	0.39 ± 0.007	67.7 ± 0.9	1.4 ± 0.02	20.6 ± 0.3	14.7 ± 1.4
	7.1 ± 0.1	9.2 ± 0.2	0.45 ± 0.005	64.1 ± 0.5	1.3 ± 0.02	20.3 ± 0.3	15.9 ± 0.9
G: + Cd + Ca/P, Se, Mn	6.1 ± 0.4	5.8 ± 0.2**	0.29 ± 0.01**	48.5 ± 2.9**	0.96 ± 0.06**	19.8 ± 0.3	16.6 ± 0.7
	7.9 ± 0.3	6.4 ± 0.3**	0.35 ± 0.02**	44.6 ± 1.7**	0.81 ± 0.2**	18.1 ± 0.2**	17.5 ± 1.1**
H: + Cd + Mg, Fe^{2+}, Se	6.4 ± 0.1	8.4 ± 0.1	0.415 ± 0.01	64.9 ± 2.1	1.31 ± 0.03	20.2 ± 0.3	16.1 ± 0.6
	7.2 ± 0.1	9.3 ± 0.1	0.47 ± 0.01	65.2 ± 0.4	1.29 ± 0.01	19.8 ± 0.1	15.9 ± 0.86
I: + Cd + Mg, Zn, Mn	6.2 ± 0.2	6.3 ± 0.3**	0.326 ± 0.02**	52.6 ± 2.7**	1.02 ± 0.05**	19.4 ± 0.5	17.2 ± 1.4
	8.4 ± 0.2**	7.6 ± 0.2	0.406 ± 0.01**	48.5 ± 2.1**	0.91 ± 0.04**	18.7 ± 0.1**	16.4 ± 0.9
J: + Cd + Fe^{2+}, Cu, Se	6.1 ± 0.1	8.3 ± 0.1	0.4 ± 0.003	65.6 ± 1.2	1.36 ± 0.02	20.7 ± 0.3	16.8 ± 0.6
	7.3 ± 0.1	9.4 ± 0.1	0.476 ± 0.01	65.4 ± 1.3	1.29 ± 0.02	19.7 ± 0.2	16.5 ± 1.00
K: + Cd + Cu, Zn, Se	5.9 ± 0.1	5.9 ± 0.2**	0.31 ± 0.01**	52.2 ± 1.5	1.0 ± 0.03**	19.2 ± 0.2	17.5 ± 0.6
	8.0 ± 0.2**	6.9 ± 0.5**	0.38 ± 0.02**	47.1 ± 2.5**	0.85 ± 0.05**	18.1 ± 0.3**	17.5 ± 1.0

Hb = haemoglobin concentration MCH = mean corpuscular haemoglobin MCHC = mean corpuscular haemoglobin concentration
MCV = mean corpuscular volume PCV = packed cell volume RBC = red blood cell count
TMM = total mineral mixture of Ca, P, Mg, Mn, Cu, Fe^{2+}, Zn and Se WBC = white blood cell count
The top value is the mean ± SEM of groups of 5 rats on day 24. The bottom value is the mean ± SEM of groups of 7 rats on day 51. Those marked with asterisks differ significantly (ANOVA + Dunnett's test) from the corresponding control value (diet A) (*$P < 0.05$; **$P < 0.01$; two-sided).

the first bioassay, groups with Fe^{2+}, Fe^{3+}, Ca/P and Zn, and the group containing Ca/P, Fe^{2+} and Zn, were compared with the group to which no extra mineral was added. In this way the individual effects of each mineral could be assessed. A separate comparison was carried out, in both bioassays, of the groups containing Fe^{3+} and Fe^{3+} + ascorbic acid, and the groups containing Fe^{2+} and Fe^{2+} + ascorbic acid, to determine the effect of ascorbic acid.

RESULTS

All rats in the experiments appeared to be healthy throughout the 4- and 8-wk studies and none of the rats died.

Experiment 1

During the study, body weight increased steadily in all groups (Fig. 1). From day 7 to day 56 the mean gain in body weight was significantly decreased, compared with control group A, in rats that were fed diets without mineral supplements (group B) or diets with mineral supplements that did not contain Fe^{2+} (groups E, G, I and K). In these groups, the food intake was reduced by about 10% in the first month (data not shown). Body weights of rats that were all fed Cd-enriched diets with a mineral supplement containing Fe (groups D, F, H and J) were generally comparable with the controls (groups A and C), and there were no noticeable and consistent differences in food intake.

On days 24 and 51, animals fed the Cd-enriched diet without mineral supplements showed a decrease in the haemoglobin concentration and packed cell volume, and subsequently in the mean corpuscular volume, and the mean corpuscular haemoglobin concentration. The white blood cell counts were significantly increased (Table 3). Supplementation of the

Table 4. Mean clinical findings in the plasma on days 28 and 56 (experiment 1) of rats fed diets containing 30 mg Cd/kg and supplements of various combinations of minerals

	ALAT (units/litre)		ASAT (units/litre)	
Diet	Day 28	Day 56	Day 28	Day 56
A: Basal diet	30.5 ± 2.6	28.2 ± 4.1	57.8 ± 2.5	61.7 ± 4.2
B: + Cd	66.1 ± 7.5**	63.1 ± 4.7**	85 ± 6.5**	89.1 ± 4.4**
C: + TMM	27.8 ± 1.5	27.7 ± 2.1	57.0 ± 1.6	52.4 ± 2.4
D: + Cd + TMM	45.2 ± 7.6	36.4 ± 1.4	64.9 ± 3.7	52.4 ± 2.4
E: + Cd + Ca/P, Mg, Cu	60.2 ± 7.6**	67.7 ± 3.5**	82.8 ± 6.9*	91.5 ± 3.3**
F: + Cd + Ca/P, Fe^{2+}, Zn	35.7 ± 0.8	35.2 ± 2.3	59.1 ± 5.9	54.8 ± 2.2
G: + Cd + Ca/P, Se, Mn	63.2 ± 4.3**	65.9 ± 5.3**	84.3 ± 6.3**	94.6 ± 5.9**
H: + Cd + Mg, Fe^{2+}, Se	41.9 ± 3.4	37.4 ± 2.4	65.4 ± 4.5	57.7 ± 2.2
I: + Cd + Mg, Zn, Mn	60.5 ± 7.6**	54.5 ± 3.4**	84.8 ± 1.6**	68.7 ± 3.1
J: + Cd + Fe^{2+}, Cu, Mn	44.6 ± 5.5	34.6 ± 1.7	65.9 ± 7.4	57.9 ± 2.0
K: + Cd + Cu, Zn, Se	65.0 ± 5.3**	58.3 ± 3.0	84.6 ± 5.6**	74.4 ± 4.4

ALAT = alanine aminotransferase ASAT = aspartate aminotransferase
TMM = total mineral mixture of Ca, P, Mg, Mn, Cu, Fe^{2+}, Zn and Se
Values are means ± SEM for groups of 3 rats on day 28 and groups of 7 rats on day 56. Those marked with asterisks differ significantly (ANOVA + Dunnett's test) from the corresponding control value (diet A) (*$P < 0.05$; **$P < 0.01$).

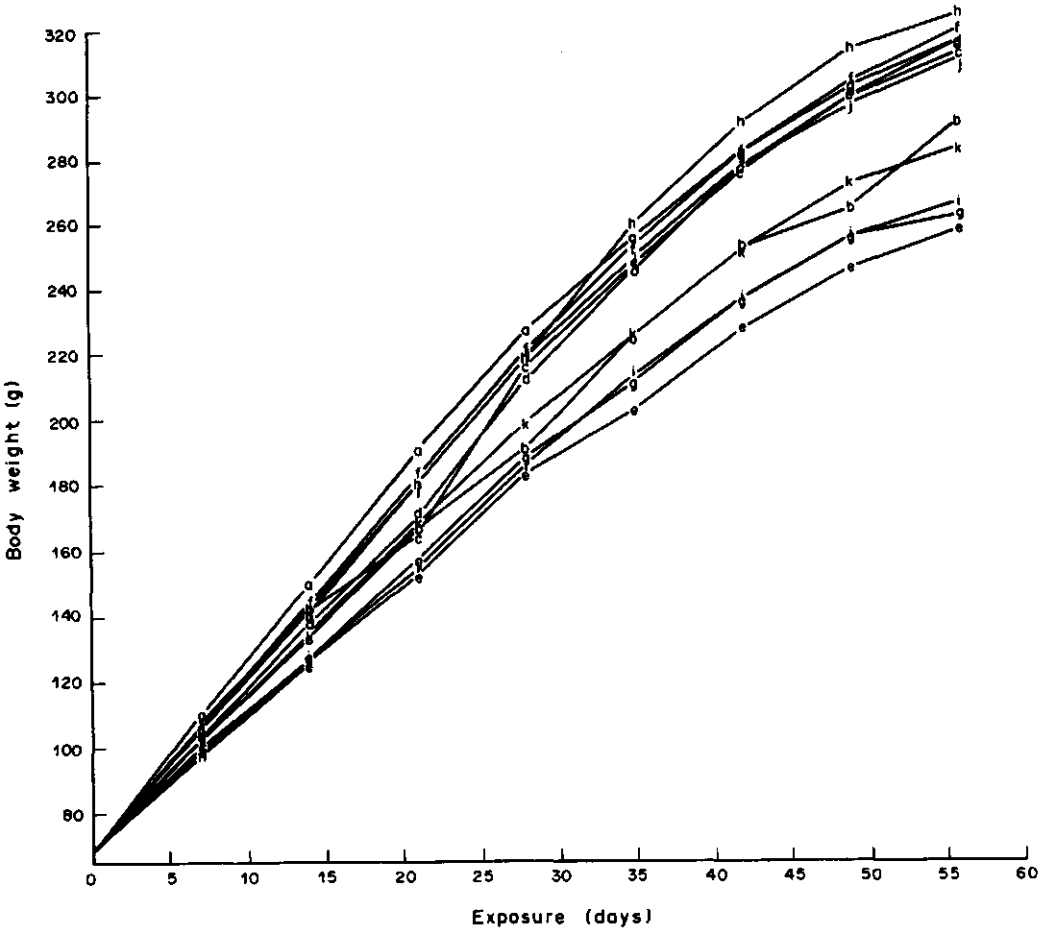

Fig. 1. Mean body weights (g) of rats in experiment 1 fed diets containing 30 mg Cd/kg and supplements of various combinations of minerals, for groups of 10 animals. The values of the mean body weight in animals fed with diets b, e, i, g and k differ significantly from the control diet a from day 14 to day 56 (analysis of covariance + Dunnett's test). Codes a–k stand for diet codes A–K. The mineral supplement of all diet codes (A–K) is given in Table 2.

Cd diet with mineral mixtures that did not contain Fe showed similar haematological findings. However, animals fed the Cd diet supplemented with a mineral mixture that contained Fe (groups D, F, H and J) showed no changes in the haematological parameters (Table 3 and Fig. 6). The data on white blood cell counts in Table 3 are not fully conclusive since group C, which is a control group, also showed an increase in the white blood cell count. However, the statistical analysis in Fig. 6 shows that rats receiving mineral supplements with Fe have significantly less white blood cells compared with supplements without Fe. An increase in the plasma aspartate aminotransferase and alanine aminotransferase activities was observed in all rats treated with 30 mg Cd/kg diet that did not receive supplements. Rats fed the Cd diet supplemented with mineral mixtures that contained Fe (groups D, F, H and J) did not show a significant increase in the aminotransferase activities (Table 4 and Fig. 6).

All Cd-treated groups showed Cd accumulation in the liver and kidneys (Fig. 2). No Cd accumulation was noticeable in either control group. There was an almost linear increase in the Cd concentration from day 28 (Fig. 2a) to day 56 (Fig. 2b) in the rats fed Cd without additional Fe in the supplement (groups B, E, G, I and K). However, in the animals fed Cd with the total mineral supplement (group D) or the animals fed Cd with a mineral supplement that contained Fe^{2+} (groups F, H and J) the Cd accumulation in the liver and kidneys was much lower compared with animals fed Cd without a Fe supplement. The protection against Cd accumulation was highest in the groups fed Cd with a mineral mixture that contained Ca/P, Zn and Fe (Fig. 2). In these groups there was hardly any increase in the Cd content of the liver from day 28 to day 56.

Figure 6 shows the overall difference in the Cd accumulation of all groups combined fed Cd with Fe, compared with all groups combined fed Cd without Fe. Groups fed Cd supplemented with Fe showed, overall, significantly less Cd accumulation than the groups fed Cd without Fe (Fig. 6). On day 56, all Cd-treated groups showed a slightly lower Zn content in the liver. This effect was most pronounced in animals with a high Cd accumulation (Zn data not shown).

Cd treatment resulted in a significant decrease in the Fe content of the liver (Figs 3a and b). After

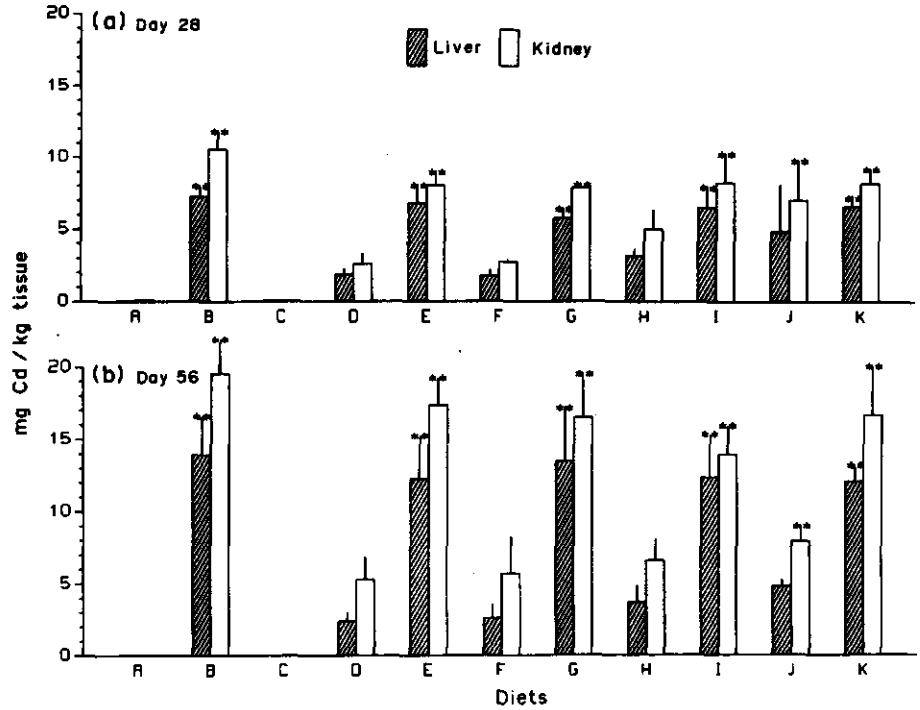

Fig. 2. Cd concentration in the liver and kidneys of rats in experiment 1 fed diets containing 30 mg Cd/kg and supplements of various combinations of minerals. All values are means ± SD of 3 rats on day 28 (Fig. 2a) and of 7 rats on day 56 (Fig. 2b). Those marked with asterisks differ significantly (ANOVA + Dunnett's test) from diet D ($P < 0.05$; $^{**}P < 0.01$). The mineral supplement of all diet codes (A–K) is given in Table 2.

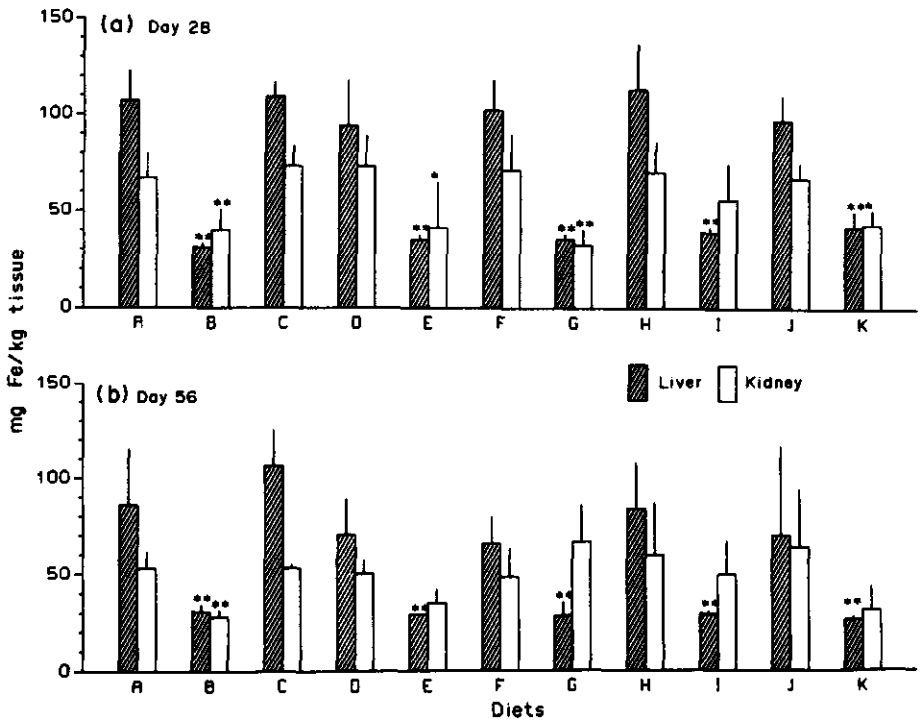

Fig. 3. Fe concentration in the liver and kidneys of rats in experiment 1 fed diets containing 30 mg Cd/kg and supplements of various combinations of minerals. All values are means ± SD of 3 rats on day 28 (Fig. 3a) and of 7 rats on day 56 (Fig. 3b). Those marked with asterisks differ significantly (ANOVA + Dunnett's test) from diet D ($^{*}P < 0.05$; $^{**}P < 0.01$). See Table 2 for the mineral supplement of each diet code.

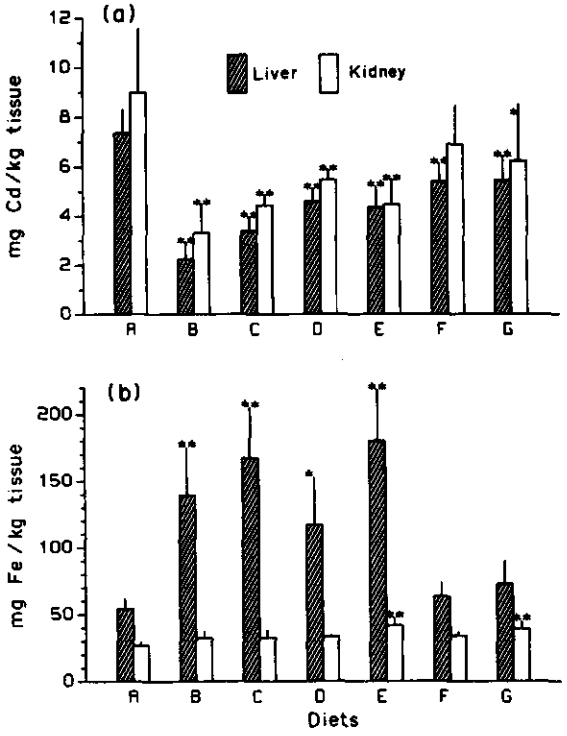

Fig. 4. Cd (Fig. 4a) and Fe (Fig. 4b) concentrations in the liver and kidneys of rats in the first set of experiment 2 fed diets containing 30 mg Cd/kg and supplements of various combinations of minerals, after 28 days of exposure. All values are means ± SD of 5 animals. Those marked with asterisks differ significantly (ANOVA + Dunnett's test) from diet A (*$P < 0.05$; **$P < 0.01$). See Table 2 for mineral supplements of each diet code.

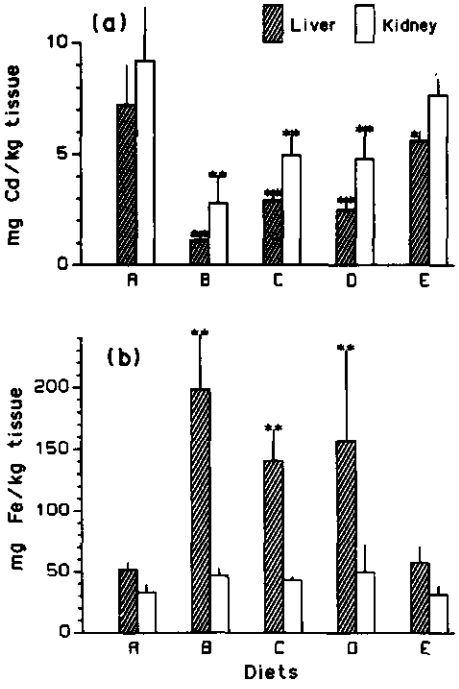

Fig. 5. Cd (Fig. 5a) and Fe (Fig. 5b) concentrations in the liver and kidneys of rats in the second set of experiment 2 fed diets containing 30 mg Cd/kg and supplements of various combinations of minerals, after 28 days of exposure. All values are means ± SD of 5 animals. Those marked with asterisks differ significantly (ANOVA + Dunnett's test) from diet A (*$P < 0.05$; **$P < 0.01$). See Table 2 for mineral supplements of each diet code.

supplementation of the Cd-enriched diet with extra Fe in the mineral mixture, the Fe level of the liver was normal (compared with control group A). The Fe content in the kidneys showed very high variations between animals. There was still a significant decrease in the Fe content of animals fed a diet without any additional Fe in the mineral supplement (for the overall effect, see Fig. 6; the individual effects of the groups B, E, G and K are shown in Fig. 3).

There were no noticeable differences in the relative liver and kidney weights among the groups on any of the dissection days (data not shown).

Experiment 2

The mineral mixture Ca/P, Zn and Fe showed a similar protection against Cd accumulation as in experiment 1: the Cd accumulation in both liver and renal cortex was reduced by up to 80%. When animals were fed a Cd diet supplemented with either Fe^{2+}, Zn or Ca/P, only the Fe group showed a significant decrease in the Cd content in the liver and kidneys (Fig. 4). However, the Cd decrease after the Fe^{2+} supplement was significantly less than with the mineral mixture that also contained Ca/P and Zn. The protection against Cd accumulation after addition of Fe^{3+} was significant, although less pronounced than after addition of Fe^{2+}. Addition of ascorbic acid to the Fe^{3+} supplement did not significantly improve the Cd reduction. However, addition of ascorbic acid to a Cd diet slightly reduced Cd accumulation in the liver and kidneys without addition of extra Fe (Fig. 5).

DISCUSSION

To study the competition between orally administered Cd and other minerals during subchronic exposure, eight different minerals were added in different combinations to the diet of rats. Seven diets supplemented with three-to-four minerals were used in order to obtain a restricted number of diets, which still could provide clear information on the effect of the individual trace elements on Cd toxicity. After consuming a diet containing 30 mg Cd/kg for 8 wk, male rats showed a number of well known Cd effects, such as growth retardation, anaemia, decreased Ca concentration of the femur, increased plasma transaminase activities and alteration of Fe metabolism. Based on the food intake, it was calculated that the daily intake of Cd was 0.17–0.36 mg Cd/rat, which is equal to 1.5–2.5 mg Cd/kg body weight.

The exposure of the rats to Cd was similar in all Cd diets. However, the addition of certain mineral mixtures diminished the toxic effects of Cd. The differences in toxicity between unsupplemented Cd diets and some of the Cd diets supplemented with minerals were accompanied by differences in the Cd content of the liver and kidneys. The results clearly demonstrate that the protective effect of a total mineral mixture against Cd toxicity was due to the

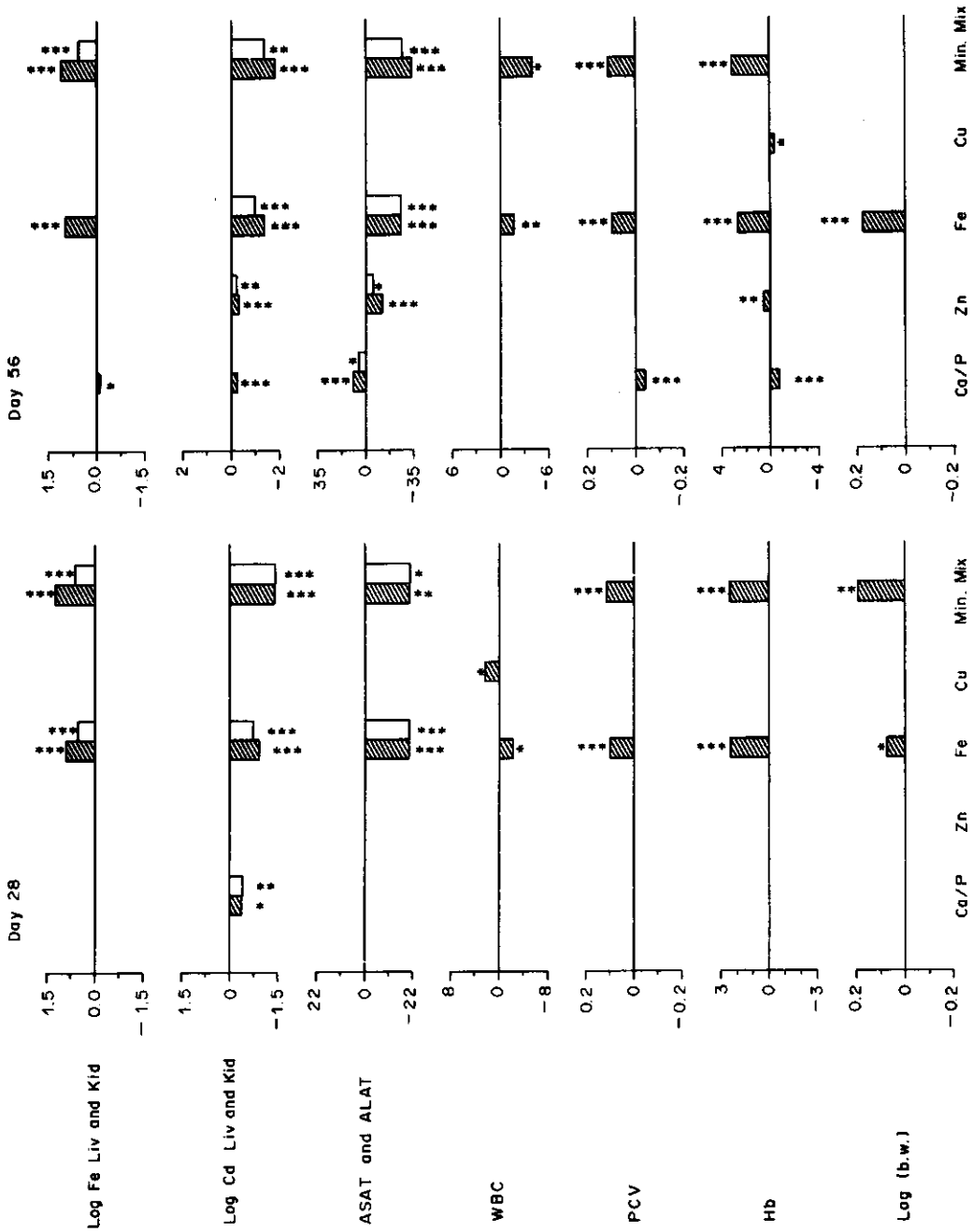

Fig. 6. Effects of Ca/P, Zn, Fe, Cu and total mineral mixture in the presence of Cd on selected variables in rats fed diets containing 30 mg Cd/kg and supplements of various combinations of minerals. Only effects significantly differing from 0 are shown ($0.01 < *P < 0.05$; $0.001 < **P < 0.01$; $***P < 0.001$). Effects are means of the groups with the relevant mineral minus the means of the groups without the mineral. Results of total mineral mixture relate to groups A–D, results of Ca/P, Zn, Cu and Fe are from groups D–K. LogCd/Fe Liv/Kid = logarithm of Cd/Fe content (mg/kg) in the liver and kidneys; log (b.w.) = logarithm of body weight (g). All other abbreviations are explained in Tables 3 and 4. Whenever two parameters are shown in one Fig. (log Cd/Fe LIV and KID or ASAT and ALAT), a dashed bar relates to the first parameter in the list (log Cd/Fe LIV or ASAT) and an open bar relates to the second parameter (log Cd/Fe KID or ALAT).

presence of extra Ca/P, Zn and Fe. The protective effect of Fe was more pronounced than the effect of Ca/P and Zn. On the other hand, a combined supplement of these three minerals was more effective in preventing Cd accumulation than a single supplement of one of the minerals.

Previous studies have indicated a correlation between the Fe and Cd content in the diet and the Cd and Fe absorption in animals. For Fe-deficient animals it was thought that the uptake of Cd in the intestinal mucosa and its transport from the intestine to other compartments of the body is

increased (Flanagan *et al.*, 1978; Fox, 1979; Hamilton and Valberg, 1974; Valberg *et al.*, 1976). In Fe-supplemented diets in which Fe^{2+} was added to the diet, the uptake and transport of Cd seemed to be lowered in quails (Fox *et al.*, 1971 and 1980). Moreover, the addition of Fe^{2+} was effective in preventing signs of Cd toxicity in rats and pigs (Pond and Walker, 1972 and 1973). From the studies of Fox *et al.* (1971 and 1980) and from the present results it appears that the protective effect of Fe in birds and rodents is probably due to decreased Cd uptake through the gastro-intestinal tract.

Since we showed that Fe was the most effective mineral in rats for preventing Cd accumulation, the next question to answer was whether Fe^{2+} and Fe^{3+} were equally effective. Fox *et al.* (1971) showed that feeding of Fe^{2+} is more effective in preventing anaemia in birds caused by Cd than the feeding of Fe^{3+}. The present study shows similar findings for rats, since the protective effect of feeding Fe^{2+} is more pronounced than with Fe^{3+}.

The enhancement of Fe absorption by ascorbic acid has been repeatedly reported for man (Bothwell *et al.*, 1982; Bowering *et al.*, 1976; Hallberg and Rossander, 1982). Several authors have shown reduced Cd toxicity by combined administration of ascorbic acid and Fe, owing to the improved Fe absorption (Fox *et al.*, 1980; Maji and Yoshida, 1974). In the present study, the dose level of ascorbic acid chosen does not significantly affect the Cd accumulation of the Fe^{3+}-fortified diet. However, when ascorbic acid is given alone, the Fe absorption is not improved, whereas the Cd accumulation is significantly lowered. Therefore, the improvement in Fe absorption seems to be not the only effect of ascorbic acid on the gastro-intestinal uptake of Cd.

The present investigation has shown that Cd accumulation in rats is mainly influenced by Fe and not by other minerals. This finding raises the question whether the intestinal transport of Cd in rats is specifically mediated by the Fe transfer system, since interaction between Cd and the Fe transport system has already been shown previously (Hamilton and Valberg, 1974; Leon and Johnson, 1985; Schäfer and Forth, 1984). However, the mechanism behind the competition of Fe and Cd is still not understood. Schafer and Forth (1984) postulated that Cd competes with the Fe transfer system mainly by binding to mucosal transferrin. Mucosal transferrin appears to be an important determinant in Fe uptake in the intestine (Huebers *et al.*, 1983). A second explanation for the interaction of Fe with Cd accumulation is based on the work of Petro and Hill (1987), who showed that ip injection of Fe greatly increased hepatic metallothionein production in rats. The studies of Cousins *et al.* (1977), Valberg *et al.* (1976) and Eaton and Toal (1982) have shown that Cd is associated with metallothionein-like proteins in the intestine. Changes in the metallothionein level in the intestine by Fe may result in an alteration of Cd retention. However, it is unknown whether Fe can change metallothionein production in the gastro-intestinal tract. A third protein that might contribute to the competition between Cd and Fe is ferritin. Although the level of Cd binding to ferritin is low compared with the binding of Fe, the possibility exists that the uptake of small amounts of Cd may involve binding to intestinal ferritin (Fox *et al.*, 1980; Joshi and Zimmerman, 1988).

In quails, Jacobs *et al.* (1978b) found that doubling of the Zn content (60 ppm) in the diet reduced the Cd uptake of the liver. The beneficial effect of a combined supplement of Zn together with Cu and Mn has been reported by Jacobs *et al.* (1977 and 1978a,b). They showed, by studying the individual trace elements, that only Zn caused the protective effect against Cd accumulation (Jacobs *et al.*, 1983). This observation seems to be in agreement with our findings. Moreover, Banis *et al.* (1969) found that a combined administration of Zn and Fe had a protective effect against Cd accumulation in rats. From our study it appeared that a supplement of Fe together with Zn and Ca/P (groups F and D in experiment 1) was the most effective diet against the retention of Cd. Thus, the results of Banis and those from our studies indicate a protective effect of dietary Zn, especially when combined with extra Fe.

The role of Ca is not clear. Several studies have shown that diets with a low Ca content can increase body retention of Cd (Hamilton and Smith, 1978; Norberg *et al.*, 1985). Moreover, Foulkes (1980) has shown that, after *in situ* perfusion of the intestine, a high Ca dose can inhibit Cd absorption. From the present study it appeared that a Ca/P supplement slightly reduced Cd accumulation and improved the protective effect of Fe in both experiments. However, the Ca and P supplement introduced signs of toxicity such as elevated levels of liver enzymes in the plasma and decreased packed cell volume and haemoglobin concentration in the blood as indicated in Fig. 6. Thus, although Ca/P influences Cd accumulation, it appears that Ca/P protects against Cd toxicity only in the presence of Fe in the mineral supplement.

Considering the other minerals, from the results obtained in this comparative study it appears that high levels of the elements Mg, Mn, Se or Cu did not affect the uptake of Cd and did not protect against Cd toxicity in rats.

In conclusion, in a direct comparison of eight potentially effective minerals in the diet, the amount of which was accurately defined, only combinations with Fe were found to protect significantly against the toxicity of $CdCl_2$; the protective effect appeared to be based on lowering Cd accumulation. For man, the present results indicate that special consideration should be given to an adequate Fe intake when assessing the health risks of Cd intake.

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Chapter 6

DIETARY IRON LOWERS THE INTESTINAL UPTAKE OF CADMIUM-METALLOTHIONEIN IN RATS

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Dietary iron lowers the intestinal uptake of cadmium-metallothionein in rats

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It has been shown that addition of extra calcium/phosphorus (Ca/P), zinc (Zn) and iron (Fe^{2+}) to the diet results in a significant protection against cadmium (Cd) accumulation and toxicity in rats fed inorganic Cd salt. However, it is not clear whether the presence of these mineral supplements in the diet also protects against the Cd uptake from cadmium-metallothionein. The present study examines the influence of Ca/P, Zn and Fe^{2+} on the Cd disposition in rats fed diets containing either 1.5 and 8 mg Cd/kg diet as cadmium-metallothionein (CdMt) or as cadmium chloride ($CdCl_2$) for 4 weeks. The feeding of Cd resulted in a dose-dependent increase of Cd in intestine, liver and kidneys. The total Cd uptake in liver and kidneys after exposure to CdMt was lower than after exposure to $CdCl_2$. At the low dietary Cd level and after addition of the mineral supplement, the kidney/liver concentration ratio increased. However, this ratio was always higher with CdMt than with $CdCl_2$, suggesting a selective renal disposition of dietary CdMt. The uptake of Cd from $CdCl_2$ as well as from CdMt was significantly decreased by the presence of a combined mineral supplement of Ca/P, Zn and Fe^{2+}. The protection which could be achieved was 72 and 75% for CdMt and 85 and 92% for $CdCl_2$ after doses of 1.5 mg/kg and 8 mg/kg respectively. In a following experiment it was shown that the protective effect of the mineral mixture against CdMt was mainly due to the presence of Fe^{2+}. It seems clear that Cd speciation and the mineral status of the diet have a considerable impact on the extent of Cd uptake in rats.

Cd^{2+}; Cadmium chloride; Cadmium-metallothionein; Accumulation; (Rat)

1. Introduction

Outside of the industrial environment, most of the body's burden of cadmium orginates from the diet (Foulkes, 1986; Robards, 1991). Due to the small safety margin between the actual level of the dietary intake and the toxic level of Cd (Buchet et al., 1990), human dietary exposure to Cd is at present a major concern.

In food of animal and vegetable origin, Cd is mainly associated with the protein metallothionein (Wagner, 1984; Webb, 1986; Kági, 1987). In spite of numerous studies performed with $CdCl_2$, a clear lack of information on the toxicological risk of CdMt exists. Recently, it has been shown that signs of toxicity (e.g., anemia, hepatotoxicity) in subacute studies are less pronounced in rats exposed to CdMt than in rats exposed to inorganic Cd-salt (Groten et al., 1990). This correlates well with the fact that the intestinal and hepatic uptake of Cd after CdMt exposure is lower than after exposure to $CdCl_2$ (Maitani et al., 1984; Groten et al., 1991b). However, in spite of the lower total Cd uptake in experiments with CdMt, the renal Cd accumulation from CdMt is relatively higher than from $CdCl_2$ (Maitani et al., 1984; Groten et al., 1991b; Ohta and Cherian, 1991). The most acceptable hypothesis explaining this finding is that CdMt is, at least partially, absorbed intact and is then immediately deposited in the kidneys (Groten et al., 1991b; Ohta and Cherian, 1991).

Several dietary factors including vitamins, proteins, and minerals are known to affect the intestinal Cd uptake of inorganic Cd-salts (Fox, 1979; Nordberg, 1985; Chmielnicka and Cherian, 1986). Recently it has been shown that a combined mineral supplement of Ca/P, Zn and Fe was very effective in preventing Cd toxicity and resulted in an 80% decrease of the Cd accumulation after feeding of inorganic Cd (Groten et al., 1991a). However, the impact of these minerals on the absorption of biologically incorporated Cd has not been investigated.

The present study describes the influence of mineral supplements on the uptake of Cd after exposure to CdMt in two successive 4-week feeding studies in rats.

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2. Materials and methods

2.1. Chemicals

Cadmium chloride with a purity, as specified by the supplier, of at least 99% was obtained from E. Merck, Darmstadt, Germany. Compounds used in the mineral mixtures were $CaHPO_4 \cdot 2H_2O$, KH_2PO_4, $Fe(2^+)SO_4 \cdot 7H_2O$, $ZnCl_2$, all obtained from E. Merck, Darmstadt, Germany (analytical grade).

2.2. Animals and experimental design

Albino male rats, Wistar outbred (Hsd/Cpb:WU) were obtained from a colony maintained under SPF conditions at Harlan/CPB, Austerlitz, Netherlands. At the beginning of the study the rats were 5–6 weeks old. They were housed under conventional conditions, in suspended stainless steel cages fitted with a wire-mesh floor and front. The room temperature was maintained at 22 ± 2°C and the relative humidity at 40–70%. A 12 h light/dark cycle was maintained and the number of air changes was about ten/h. Prior to the experiment all rats were fed a basal diet without any further additions. Drinking water was supplied in glass bottles which were cleaned once weekly. Food and water were provided ad libitum.

The rats were acclimatized to the animal facilities for 1 week and were then allocated to test groups of five animals using a computer generated random number table. A few rats were reallocated in order to equalize the initial mean body weight in the various groups. Each treatment group (housed in groups of five) received one of the test diets. On day 28 all rats were autopsied.

2.3. Preparation of cadmium incorporated in pig liver

Seven young male pigs, initially weighing 57 ± 5 kg, were housed separately in metabolism cages. The animals were injected intravenously (vena jugularis) with cadmium chloride dissolved in saline according to the following schedule: on days 0, 2, 5 and 7 with 0.6 mg Cd/kg body weight, on days 9 and 12 with 0.9 mg Cd/kg body weight and on days 14, 16, 19, 21 and 23 with 1.15 mg Cd/kg body weight. 3 days after the last injection the animals were killed and the livers were removed. The livers were pooled, homogenized, lyophilized and stored at −20°C. Livers obtained from two untreated pigs were handled in the same way and served as control material. Analysis of freeze-dried liver samples revealed that the Cd, Zn and Fe content was 950, 530 and 460 mg/kg tissue, respectively. In a previous study (Groten et al., 1990) the Cd-binding ligand in the pig liver that accounted for almost 90% of the total Cd present was characterized as metallothionein.

2.4. Diets

The study comprised two experiments; the first one was designed to compare the effect on Cd accumulation of diets supplemented with one mineral mixture known to protect efficiently against $CdCl_2$. A second

TABLE 1

Mineral analyses of the diets.

Diets	Minerals						
	Calcium (%)	Phosphorus (mg/kg)	Zinc (mg/kg)	Copper (mg/kg)	Iron (mg/kg)	Cadmium (mg/kg)	Nitrogen (%)
Experiment 1							
8 ppm $CdCl_2$ – Min [a]	0.65	0.63	49	2.2	79	7.5	4.38
2 ppm $CdCl_2$ – Min	0.64	0.63	47	2.6	77	1.6	4.37
8 ppm $CdCl_2$ + Min	1.42	1.38	138	2.2	273	7.5	4.19
2 ppm $CdCl_2$ + Min	1.42	1.39	135	2.0	295	1.4	4.24
8 ppm CdMt – Min	0.65	0.63	49	2.9	79	8.5	4.52
2 ppm CdMt – Min	0.64	0.63	47	2.6	71	1.6	4.45
8 ppm CdMt + Min	1.44	1.22	139	3.2	288	8.5	4.37
2 ppm CdMt + Min	1.44	1.23	138	2.4	283	1.6	4.32
Experiment 2							
10 ppm $CdCl_2$	0.67	61	35	–	70	9.7	4.38
10 ppm $CdCl_2$ + Min	1.44	1.5	145	–	280	8.4	4.17
10 ppm $CdCl_2 + Fe^{2+}$	0.66	0.64	34	–	260	9.8	4.3
10 ppm CdMt	0.68	0.61	35	–	65	9.3	4.4
10 ppm CdMt + Min	1.43	1.4	140	–	270	8.4	4.1
10 ppm CdMt + Ca/P	1.46	1.5	34	–	75	8.4	4.26
10 ppm CdMt + Zn	0.67	0.63	145	–	65	9.9	4.38
10 ppm CdMt + Fe^{2+}	0.66	0.62	34	–	260	9.6	4.33

[a] Min = Total mineral mixture of Ca/P, Zn, Fe^{2+}.

experiment was undertaken to determine the effect of the individual elements used in the mixture of the first experiment.

2.4.1. Experiment 1

A semi-synthetic powdered basal diet was composed to resemble a Western type of human diet (see Groten et al., 1990). The mineral content of the basal diet was based on the nutrient requirements of the rat according to the National Research Council (1978). Four diets consisted of the basal diet supplemented with lyophilized pig liver from Cd-treated pigs providing a dietary level of 1.5 and 8 mg Cd/kg diet. Two of these diets were supplemented with a mineral mixture of Ca/P, Zn and Fe^{2+} which according to previous observations (Groten et al., 1991a) was the most effective against Cd accumulation. Four other diets consisted of the basal diet supplemented with $CdCl_2$ and lyophilized liver homogenate of control pigs which were not treated with Cd, provided dietary levels of 1.5 and 8 mg Cd/kg diet. The mineral analysis of the eight diets (see table 1) revealed that, under the experimental conditions, the actual levels of Cd were 94% and 106% of the intended level for $CdCl_2$ and CdMt respectively.

One batch of each of the diets was stored in a freezer at −20°C until use. Twice a week the diets in the feeders were refreshed with a portion of the test diets (thawed immediately before use).

2.4.2. Experiment 2

The purpose of experiment 2 was to determine whether the effect of the mixture Ca/P, Zn and Fe^{2+} could be attributed to the presence of Ca/P, Zn or Fe^{2+} alone. The control group was fed a Cd diet (10 mg/kg diet) either as $CdCl_2$ or CdMt supplemented with a combined mineral mixture consisting of Ca/P, Zn and Fe^{2+}. A third diet contained $CdCl_2$ supplemented with Fe^{2+} alone. Three diets contained CdMt (10 mg/kg diet) together with a supplement of the single compounds Ca/P, Zn or Fe^{2+}. The mineral analysis of the diets (see table 1) revealed that, under the experimental conditions, the actual levels of Cd were between 84 and 99% of the intended level for both $CdCl_2$ and CdMt.

2.5. Observation and analyses

The rats were weighed at weekly intervals and were observed daily for condition and behaviour. Food and water intake were measured over weekly periods throughout the study. At autopsy on day 28 liver and kidneys were removed and weighed. In experiment 1 the small intestinal mucosa was also removed and treated as described elsewhere (Groten et al., 1991b). Part of the liver, intestine or the kidney cortex was prepared for AAS analysis of the Fe, Zn and Cd content (Muys et al., 1984; Groten et al., 1990).

2.6. Statistical analysis

Data on body weights were evaluated by a one-way analysis of covariance, followed by Dunnett's multiple comparison tests. The laboratory determinations and organ weights were evaluated by a one-way analysis of variance, followed by Dunnett's multiple comparison tests.

3. Results

The general condition and behavior of the rats fed $CdCl_2$ or CdMt appeared to be normal throughout the 4-week study. Body weight gain and food consumption were similar in all groups and there were no consistent differences in organ weights between the control animals and the animals fed Cd (data not shown).

3.1. Experiment 1

Feeding of CdMt and $CdCl_2$ produced a dose-dependent Cd accumulation in liver, kidneys, as well as intestine (fig. 1). The Cd concentration achieved after 28 days of dietary Cd exposure was highest in the intestinal mucosa and lowest in the liver. At a dose of 8 mg/kg the Cd concentration in all three tissues was significantly higher after feeding of $CdCl_2$ than after

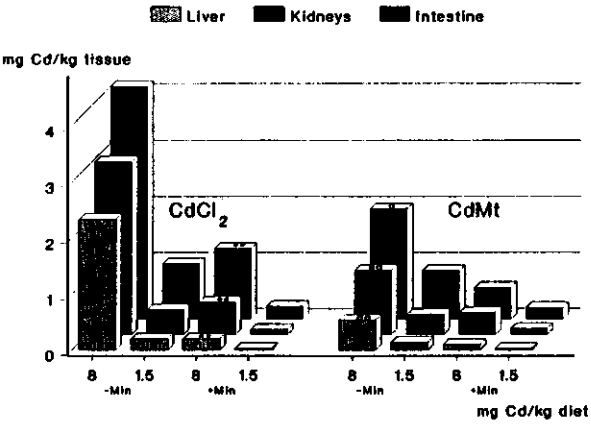

Fig. 1. Cadmium concentration in liver, kidneys and small intestine in experiment 1. Rats were fed for 28 days a diet containing $CdCl_2$ or CdMt with and without a mineral mixture of Ca/P, Zn and Fe^{2+} (+Min/−Min). All values are means of five rats. Those CdMt groups marked with an 'o' differ significantly (ANOVA + Dunnett's test) from the $CdCl_2$ group of a corresponding dose (^{o} $P < 0.05$, ^{oo} $P < 0.01$). Those +Min groups marked with an asterisk differ significantly from the corresponding −Min group (ANOVA + Dunnett's test: ** $P < 0.01$).

CdMt. From the organ weights it was calculated that the total Cd uptake in liver and kidneys together at the dose levels of 1.5 and 8 mg/kg was 3.5 and 39.2 μg respectively after feeding of $CdCl_2$, and 2.5 and 8.6 μg respectively after feeding of CdMt (fig. 2). The difference in tissue Cd concentrations between CdMt and $CdCl_2$ became less pronounced at a dose of 1.5 mg/kg (fig. 1). However as the calculation in fig. 2 shows, the total Cd amount in liver and kidneys together at the low $CdCl_2$ dose was still higher than for CdMt. Supplementation of the Cd diet with the mineral mixture lowered the Cd accumulation considerably: the protection which could be achieved was 72 and 75% when CdMt was given and 85 and 92% when $CdCl_2$ was given at 1.5 and 8 mg/kg respectively. A plot of the ratio between Cd concentration in kidneys and liver (fig. 2) versus dose shows that oral exposure to 8 mg/kg CdMt led to a higher Cd concentration ratio than the feeding of 8 mg/kg $CdCl_2$. The difference became less pronounced at a dose of 1.5 mg/kg. Addition of the mineral mixture resulted in an increase in the kidney/liver ratio for both Cd forms. The increase in the Cd ratio after addition of extra minerals was more pronounced with CdMt than with $CdCl_2$: the kidney/liver [Cd] ratio in animals fed 1.5 mg Cd/kg without extra minerals for $CdCl_2$ and CdMt was 2.4 and 2.8 respectively, whereas after addition of the mineral mixture the ratio increased to 3.1 and 4.2 respectively (fig. 2).

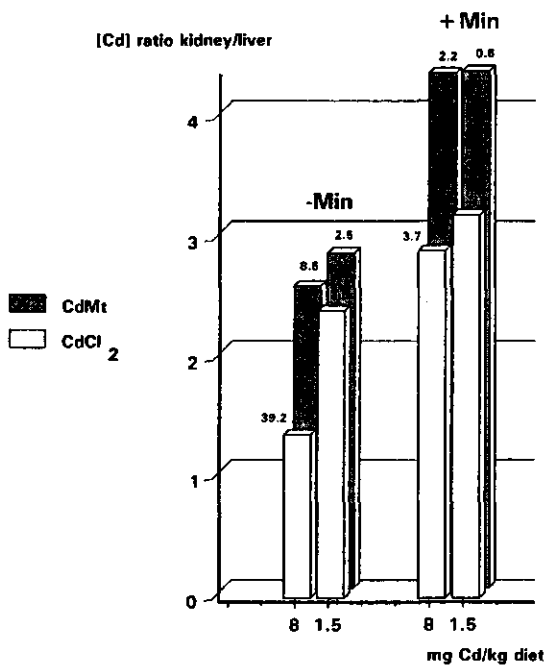

Fig. 2. Ratio between kidney and liver Cd concentration of rats fed 1.5 and 8 mg/kg Cd as $CdCl_2$ or CdMt. Each value is the mean of five individual calculated kidney/liver ratios of five rats. Values marked with an asterisk in the group fed 8 mg/kg $CdCl_2$ + Min differ significantly from the group fed 8 mg/kg CdMt + Min (* $P < 0.005$). The value above/next to each bar gives the mean total Cd uptake in liver and kidneys together (in μg Cd). For details of the experiment see Materials and methods section. Min = Mineral mixture of Ca/P, Zn and Fe^{2+}.

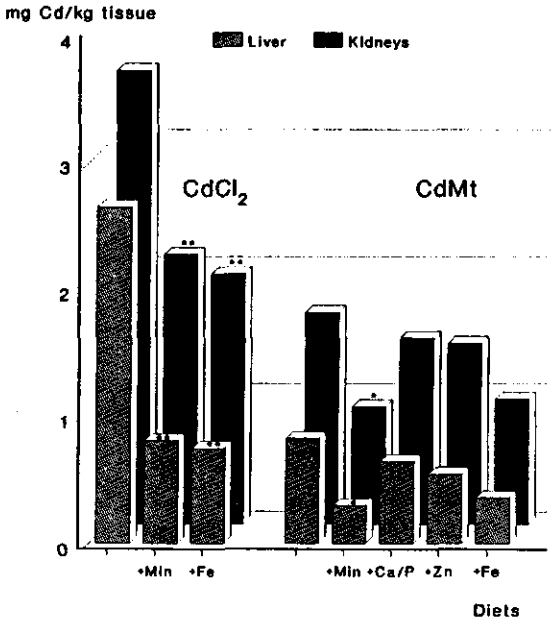

Fig. 3. Cadmium concentration in liver and kidneys in rats of experiment 2 after 28 days of exposure to 10 mg/kg Cd. All values are means of five animals. Those groups marked with an asterisk differ significantly from the corresponding group fed Cd without additional minerals (ANOVA + Dunnett's test): * $P < 0.05$, ** $P < 0.01$. + Min = diet supplemented with total mineral mixture of Ca/P, Zn and Fe^{2+}, + Fe = Fe supplement, + Ca/P = Ca/P supplement, + Zn = Zn supplement.

3.2. *Experiment 2*

In the second experiment the effect on CdMt uptake of the individual elements used in the combined mineral supplement of experiment 1 was studied. The mineral mixture Ca/P, Zn and Fe^{2+} showed a slightly lower protection in experiment 2 than at the 8 mg/kg Cd level in experiment 1: the Cd accumulation in the second experiment was reduced up to 59% and 71% for CdMt and $CdCl_2$ respectively. When animals were fed a CdMt diet supplemented with either Fe^{2+}, Zn or Ca/P, only the Fe^{2+} group showed a significant decrease in the Cd content in the liver and kidneys (fig. 3). For both CdMt and $CdCl_2$ the decrease in Cd uptake after the Fe^{2+} supplement was almost similar to the decrease resulting from the mineral mixture that also contained Ca/P and Zn.

4. Discussion

After addition of a mineral supplement consisting of Ca/P, Zn and Fe^{2+} to a diet with CdMt there are two striking differences compared with the effect of these minerals on $CdCl_2$. Firstly, the total protection of the mineral mixture against Cd uptake from CdMt is lower than for inorganic Cd, and secondly relatively more Cd is deposited in the kidneys with CdMt than with $CdCl_2$. The fact that the mineral supplement also protects against CdMt – albeit less pronounced than against $CdCl_2$ – might be explained by the partial degradation of CdMt in the gastrointestinal tract (Klein et al., 1986). Once degraded, Cd from CdMt will show the same metabolic behavior as inorganic Cd-salt and the uptake in liver and kidneys will be decreased by mineral suppletion. On the other hand, rats fed CdMt supplemented with extra minerals showed a strong increase in the ratio of the kidney/liver Cd concentration. An explanation for the high kidney/liver Cd ratio after feeding of CdMt has been given previously (Maitani et al., 1984; Groten et al., 1991b) and orginates from the fact that a portion of ingested CdMt is not degraded (Klein et al., 1986, Crews et al., 1989), but does in fact appear to reach the intestine intact (Cherian, 1979; Ohta and Cherian, 1991). Since the selective renal disposition of CdMt is higher with extra minerals in the diet, it seems that the mineral supplement does not affect the absorption of the intact exogenous CdMt, which will then reach the kidneys unhampered.

In previous studies we have shown that a combined mineral supplement of Ca/P, Zn and Fe^{2+} resulted in a 70–80% reduction of Cd accumulation in liver and kidney as well as a reduction of Cd toxicity in rats fed 30 ppm Cd as $CdCl_2$. The present study shows that the protection against Cd uptake is even 10–20% higher at a $CdCl_2$ intake of 1.5 mg/kg. However, at low dietary Cd levels or after addition of a combined mineral supplement, relatively more Cd is accumulated in the kidneys than in the liver. This selective renal disposition might be explained by the theory that low amounts of ionic Cd, once absorbed through the intestine, are initially bound to available endogenous metallothionein, which will be transported preferentially to the kidneys (Lehman and Klaassen, 1986; Scheuhammer, 1988). However, at higher Cd doses the available endogenous circulating metallothionein pool is overloaded and Cd will also be bound to plasma proteins such as albumin and then be deposited in the liver (Lehman and Klaassen, 1986). Since addition of the mineral supplement decreases the Cd absorption, it will prevent to some extent the overload of the intestinal metallothionein pool and as a consequence the kidney/liver ratio will increase.

Ca/P and Zn given alone only slightly reduce the Cd intake of CdMt. A similar effect of these minerals was observed after feeding of $CdCl_2$ (Groten et al., 1991a). The protective effect of the mineral mixture is thus almost solely due to the presence of Fe^{2+} and this finding supports the theory that the intestinal transport of Cd in rats is mediated by the Fe transfer system (Hamilton and Valberg, 1974; Leon and Johnson, 1982; Schäfer and Forth, 1984). The decrease in Cd uptake by Fe^{2+} will certainly result in a clear protection against Cd toxicity since in previous studies it was shown that a decrease of Cd accumulation in rats fed Cd is followed by the disappearance of signs of Cd toxicity (Groten et al., 1990, 1991a).

Thus, Fe^{2+} suppletion of the diet is a very effective way to prevent Cd accumulation and Cd toxicity from feeding of both inorganic Cd and biologically incorporated Cd. The results indicate that Cd speciation and the mineral status of the diet have a considerable impact on the ultimate uptake of Cd in rats.

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Chapter 7

METABOLIC FATE OF EXOGENOUS CADMIUM-METALLOTHIONEIN AFTER ORAL AND INTRAVENOUS ADMINISTRATION IN RATS

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THE METABOLIC FATE OF EXOGENOUS Cd-METALLOTHIONEIN AFTER ORAL AND INTRAVENOUS ADMINISTRATION IN RATS

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Abstract-In the present study an HPLC-based method is used to distinguish between endogenous and exogenous metallothioneins, and to study their metabolic fate simultaneously. For that purpose, Cd-metallothionein isoforms were purified from liver (LMt), kidneys (KMt) and intestine (IMt) of rats. Ion exchange chromatography revealed two isoforms in all rat tissues (Mt-1 and Mt-2) and both isoforms eluted as single discrete peaks on the HPLC system, except for the kidney in which case both isoforms eluted together as one single peak. Purified hepatic Mt isoform-1 (LMt-1) was radiolabeled with ^{109}Cd and administered orally or intravenously at a low, non-toxic dose to rats. ^{109}CdLMt-1 administered i.v. preferentially distributed to the kidneys (approximately 45% of the dose). The Cd concentration in the kidneys increased rapidly between 5 and 30 min after administration and then remained constant for at least 48 hours. In contrast, in the liver only 6-9% of the dose was retained. HPLC analysis showed that 5 min after administration of LMt-1, Cd in the kidneys is no longer bound to intact LMt-1, but to heat-resistant degradation products of Mt of comparable molecular weight. Within 24 hours almost 40% of the Cd in the kidney was bound to KMt.
After oral administration of ^{109}CdLMt the Cd retention in the intestinal mucosa reached its maximum 5 hours after exposure, i.e. 1.5 % of the dose. Within 24 h. most of the administered Cd was again excreted by the faeces. At all time points, the majority of the Cd in the intestine was bound to heat-resistant proteins with a molecular mass similar to Mt. However, HPLC analysis showed that Cd was no longer bound to intact LMt-1; 35% of the Cd was transferred to IMt and the remainder of the Cd was bound to Mt-derived products. These Mt-like products originate from LMt-1, but also from IMt because they can also be found after oral exposure to inorganic Cd. After oral administration of LMt-1, Cd was equally distributed to kidneys and liver, but the retention was very low and isoform identification proved to be impossible. In *in vitro* experiments with renal and intestinal homogenates, the Cd was rapidly exchanged between the exogenous (LMt-1) and endogenous Mt-isoforms (KMt or IMt). The extent of Cd exchange was dependent on the concentration ratio between exogenous and endogenous Mt isoforms. Thus, after oral exposure to low doses of exogenous CdMt, the Cd ions will be bound to endogenous CdMt due to the rapid exchange and due to gradual degradation of exogenous CdMt. The rapid liberation of Cd-ions at low CdMt levels implies that at low oral doses exogenous CdMt will show the same metabolic behaviour as inorganic Cd.

INTRODUCTION

Intestinal absorption of low doses of Cd will mainly lead to renal cadmium (Cd) accumulation, whereas after high doses of Cd, the Cd is predominantly stored in the liver (Engstrom and Nordberg, 1979, Lehman and Klaassen, 1986; Scheuhammer, 1988). It has been proposed that a low amount of ionic Cd will mainly be bound to endogenous metallothionein (Mt) of the intestine, which will be released in the systemic circulation and then be deposited in the kidneys (Min et al., 1991). However, after a higher oral dose, the available endogenous Mt pool is overloaded and Cd will also bind to e.g. plasma proteins and then be deposited in the liver (Suzuki, 1984; Lehman and Klaassen, 1986). In the liver Cd will be associated to hepatic Mt, which is probably released from the liver in the systemic circulation due to normal turnover of hepatocytes or due to increased cell death (Dudley et al., 1985; Webb, 1986) and is only

then transported to the kidneys.

Cd in the diet is in part present as Cd-Mt. This dietary Mt can to some extent survive the treatment with gastro-intestinal proteolytic enzymes (Klein et al., 1986; Crews et al. '89) and can be taken up in the intestinal mucosa (Cherian et al., 1979; Ohta and Cherian, 1991). If released from the intestinal mucosa into the systemic circulation, exogenous CdMt shows, similar to endogenous CdMt, a selective renal disposition (Maitani et al., 1984; Ohta and Cherian 1991; Groten et al., 1991). However the degradation of dietary Mt, absorbed in the intestine, has so far not been studied in detail.

CdMt which has been absorbed in the kidneys is presumably degraded within hours whereas the renal Cd concentration remains almost constant for days (Cain and Holt, 1983; Squib et al., 1984). Studies on Cd-speciation in kidneys and intestine have thus far been performed with bolus amounts of CdMt and employ gel-filtration techniques to estimate the molecular size of the Cd-binding proteins. Under these conditions it remains uncertain whether Cd is exchanged between endogenous Mt in the tissue and exogenous Mt taken up by the tissue. The fate of low doses of exogenous CdMt is not known in detail, although it has been suggested that before degradation of the CdMt in the tissue the Cd^{2+} is already liberated and will displace zinc in the renal endogenous Mt, that is already present (Webb and Etienne, 1977).

In the last few years reversed phase-high performance liquid chromatography (RP-HPLC) has been applied to characterize different Mt isoforms expressed by different organs (Klauser et al., 1983; Richards and Steele, 1987; Waalkes et al., 1988; Groten et al., 1990). The advantage of HPLC over other Mt-detection techniques like RIA, mRNA-assays or metal saturation assays is that it can, in one step, provide information concerning the isoforms present in the tissue. The HPLC technique was applied in the present study to investigate simultaneously the Cd binding to endogenous and exogenous Mt, making use of the fact that different rat Mt-isoforms show different elution profiles on RP-HPLC (Groten et al., 1992). For that purpose purified CdMT was administered orally or intravenously to rats at low Cd-concentrations and the fate of radiolabeled Cd was studied.

MATERIALS AND METHODS

Chemicals. $^{109}CdCl_2$ was obtained from Amersham International (Bucks., U.K.) as stock solutions of 1mCi and 100μCi/ml in 0.1 N HCl (spec. activity 41.1 and 47.7 MBq/μg Cd). Acetonitrile (HPLC grade) was purchased from Westburg (Leusden, NL). All other chemicals (analytical grade) were obtained from E. Merck AG (Darmstadt, FRG).

Animals and Maintenance. Albino male rats, Wistar outbred (Crl:WI(WU)Br) were obtained from a colony maintained under SPF-conditions at Charles River Wiga, Sulzfeld FRG. At the beginning of the study the rats were 10-12 weeks old, weighing 250-300g. During the experimental part they were housed in macrolon cages. The room temperature was maintained at 22 $\pm$ 2 °C. and the relative humidity at 40-70 %. A 12 hr-light/dark cycle was maintained. Drinking water was supplied in glass bottles which were cleaned twice a week. Food (pelletized cereal-based stock diet) and water were provided ad libitum. The rats were acclimatized to the animal facilities for at least 3 days and were then allocated to test groups.

Purification of CdMt. Rat Mt-isoforms were purified from liver, kidney and intestinal mucosa from rats which were treated with $CdCl_2$ as described elsewhere (Groten et al., 1990). Briefly, the tissue homogenates (cytosol) were filtered through a 0.22 μm filter and chromatographed on a Sephadex G-75 column (5 x 60 cm, Pharmacia, Sweden) with 10 mM Tris.HCl (pH 7.4, 4°C). The CdMT-containing fractions (Ve/Vo = 2 $\pm$ 0.2) were applied to a Sephadex DEAE-A25 column (2.5 x 20 cm) and eluted with a linear gradient of 10-300 mM Tris.HCl (pH 8.5, 4°C). The fractions which corresponded to Mt-1 and Mt-2 were dialyzed against water (Amicon YM membrane, the Netherlands) and lyophilized. The lyophilized Mt powder was dissolved in deionized water and further analyzed on RP-HPLC (see below). Rat liver isoform-1 (LMt-1) was pooled from several isolations and used as test material for the *in vivo* and *in vitro* studies. The molar ratio of Cd:Zn:Cu in LMt-1 was 1:1:0.04 respectively. Based on a purity of 90% (as determined by the Cd-Haem assay of Eaton

and Toal, 1982) LMT-1 contained 2.8 mol Cd/mol Mt.

HPLC analysis. Purified Mt and heat treated organ homogenates were applied to an RP-HPLC system using a stainless steel Hypersil 5 ODS (250 x 4.6 mm) column (Chrompack, NL) eluted with a 10 mM sodium phosphate buffer (pH 7.2) (solvent-1) and 10 mM sodium phosphate buffer in acetonitrile (40:60) (solvent-2) as eluting solvents. Samples were eluted with a linear gradient of 0-5% solvent-2 in 5 min and from 5 to 20% solvent-2 in 15 min with a flow rate of 1 ml/min. After UV detection at 220 nm fractions of 0.25 ml eluens were collected and counted for ^{109}Cd in a gamma counter (LKB Rackgamma, Sweden). ^{109}Cd-recovery on HPLC varied between 80-95%.

Renal Cd speciation after parenteral exposure to ^{109}CdLMt-1. LMt-1 (1.5 mg) was incubated with ^{109} $CdCl_2$ (40 μCi) for 15 minutes in 2 ml deionized water. After incubation the ^{109}CdLMT was diluted with a sterile solution of concentrated NaCl to obtain a final solution of 0.9 % NaCl containing 200 μg LMt1/ml (5.1 μCi/ml). Prior to injection purity of radiolabeled LMt-1 was checked on HPLC. The ^{109}CdLMt-1 solution was injected into the tail vein of rats (0.5 ml/300 gr body weight) and on 5 min., 30 min, 4 h and 24 hours after injection two animals/group were sacrificed and liver and kidneys were removed and immediately emerged in liquid nitrogen. All tissues were homogenized in 2 volumes of 10 mM Tris-144 mM KCl buffer (pH 7.4, on ice) and S9-mixture was prepared by centrifugation at 10.000g for 20 min (4°C). The renal homogenates were applied to Sephadex gel-filtration columns (60x1.5 cm, 35 ml/h, 2 ml/fraction) and after heat treatment (1 min, 100°C) to the RP-HPLC system. Gel-filtration profiles were divided in three major areas: High molecular weight (HMW) proteins larger than 10kD, Mt-like proteins between 3 and 10kD and compounds smaller than 3 kD.

Intestinal Cd-speciation after oral exposure to ^{109}CdLMt-1 . LMt-1 (4 mg) was incubated with $^{109}CdCl_2$ (100 μCi) for 15 minutes in 2 ml 10 mM Tris.HCl and finally diluted to a concentration of 500 μg Mt/ml.
The animals, fasted overnight, were orally exposed by gavage to the ^{109}CdLMt-1 (1 ml/200gr body weight) and after 5 min., 4 h and 24 hours two animals/group were sacrificed and intestine, liver and kidneys were removed. The intestinal mucosa was scraped off as described previously (Groten et al.,1991) and the mucosal scrapings as well as the liver and the kidneys were immediately immersed in liquid nitrogen. All tissues were homogenized in 2 volumes of 10 mM Tris-144 mM KCl buffer (pH 7.4) on ice and were prepared in the same way as described for renal homogenates after parenteral exposure.

Renal and intestinal Cd-speciation after in vitro exposure to $^{109}CdCl_2$ or ^{109}CdLMt-1. To obtain a radiolabeled Mt, 500 μg LMt-1 was dissolved in 1ml (10 mM) Tris.HCl buffer (pH 8.0) and incubated for 10 minutes with $^{109}CdCl_2$ (1-5 μCi). After the radiolabeling ^{109}CdLMt-1 was kept on ice prior to use. In preliminary experiments it was established that 98 % of the ^{109}Cd remained bound to LMt-1 after extensive dialysis through an amicon ultramembrane. The purity of the radiolabeled LMt-1 was established by HPLC which revealed one single peak of ^{109}Cd-labeled LMt-isoform 1 (see fig 1.). An equimolar Cd amount as $CdCl_2$ was prepared by dissolving 32μg $CdCl_2$ in 1ml 10 mM TrisHCl buffer together with $^{109}CdCl_2$ (1-5μCi). Aliquots of the ^{109}CdLMT-1 or $^{109}CdCl_2$ solution (20-100μl) were added to 500 μl renal or intestinal homogenates of untreated rats. The total amount of radioactivity added to the homogenates was always between 50.000 and 100.000 dpm/100μl. Mt-concentration in the kidneys and intestine of untreated rats was 65 and 28 μg /g tissue respectively and the renal Mt concentration in kidneys of Cd-treated rats was 250μg/g tissue (determined according to the method of Eaton and Toal, 1982). The amount of ^{109}CdLMt-1 which was added to the renal and intestinal homogenates was chosen in such a way that the molar ratio between endogenous Mt (KMt or IMt) and exogenous Mt (LMt-1) was 5:1, 1:1 and 1:3 respectively. After vigorously mixing, the homogenates were incubated for 15 seconds at 37°C and subsequently heat-treated for 1.5 minutes at 100°C. Lastly, the heat-treated tissue homogenates were spun down at 10,000g for 5 minutes in an eppendorf centrifuge and the supernatant was decanted and kept on ice prior to HPLC analysis. Stability of the CdMt-complexes in renal tissue was tested by incubating LMt-1 for 24 hours in renal homogenates at 4°C and at 37 °C, followed by the work up procedure described above.

Stability of ^{109}CdLMt-1 in blood. Blood of untreated rats was collected in a heparinized culture tube and diluted 1:1 with 10 mM Tris-HCl buffer. Ten μl of the ^{109}CdLMt-1 solution was added to 600 μl blood to obtain a concentration of 9 μg LMt-1/ml, which is comparable to the Mt concentration in the blood in the in vivo experiment after iv administration of CdMt. The blood was heat treated and centrifuged as described above. Finally the supernatant was applied to the RP-HPLC.

RESULTS

In order to fully characterize the HPLC separation of rat Mt, tissue cytosol was fractionated using conventional chromatography techniques. Ion exchange chromatography of pooled gel-filtration samples revealed that in all tissues of the rat, Mt was present in two main isoforms (Fig.1a). In the intestine the majority of the Cd was however bound to isoform 2, whereas in the liver and kidney the Cd was equally divided among both isoforms. Mts which had undergone previous purification (i.e. low-pressure gel-permeation and anion-exchange chromatography) were lyophilized and subjected to RP-HPLC which revealed that purified Mt isoform 1 and 2 from liver and intestine eluted as single discrete peaks with retention times of 12.9 ($\pm$0.3) and 14.8 min ($\pm$ 0.3) respectively. Thus, liver Mt isoforms (LMt) and intestinal Mt isoforms (IMt) showed an identical chromatographic behaviour on HPLC (1b). In contrast, both kidney isoforms eluted together on HPLC

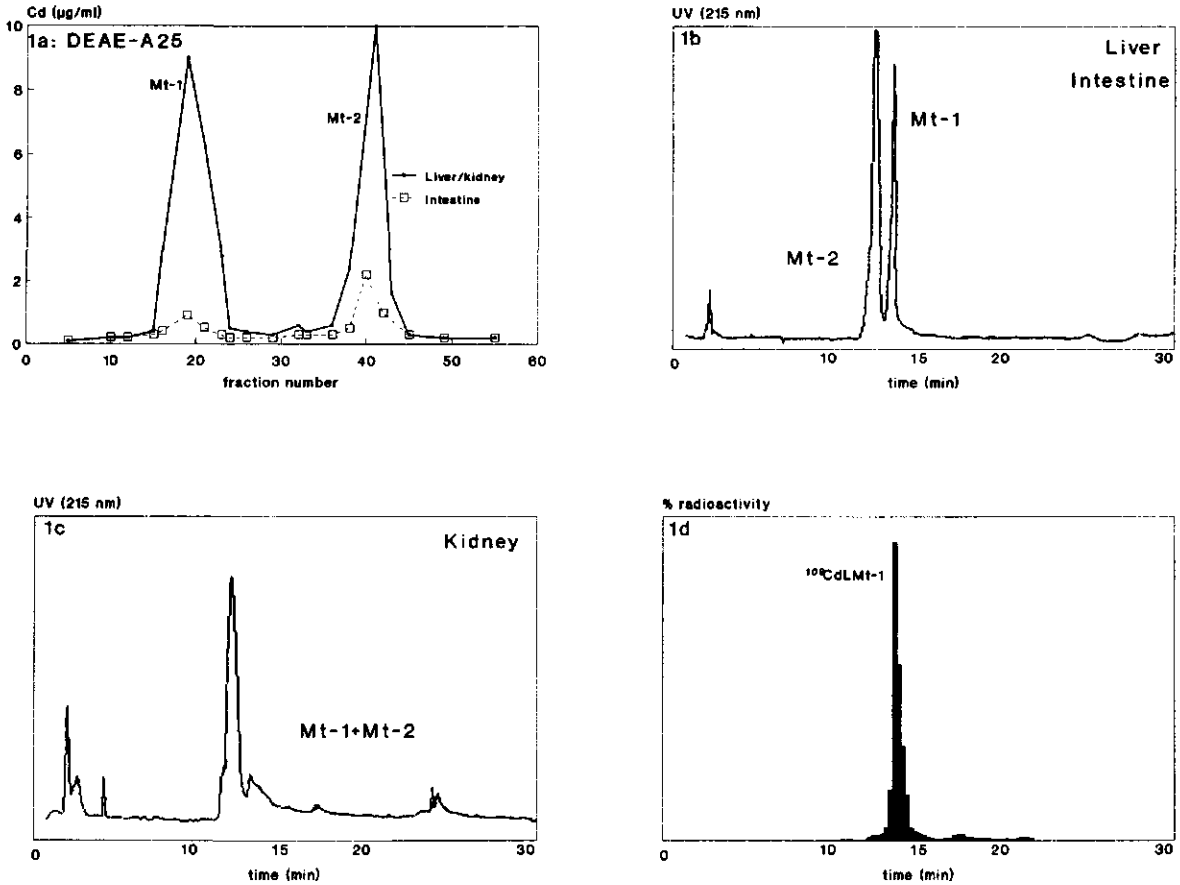


Fig. 1

1a. Anion exchange chromatography (gradient of 10-300 mM Tris.HCl pH8.5) of rat Cd-Mt isolated by preparative gel-filtration from liver/kidney/intestine cytosol. After purification and lyophilization the Cd-Mt isoforms were further analysed on reversed phase HPLC: 1B: HPLC profile of rat Mt-1 and 2 from liver and intestine, 1c: HPLC elution profile of Mt1 and 2 from kidney. Rats were treated with $CdCl_2$ as described in method section. 1d HPLC elution profile of LMt-1 after radiolabeling with ^{109}Cd. Mt1/Mt2 = Metallothionein isoform 1/2.

as a one single peak on 13 min, almost similar to the retention time of isoform-2 of liver and intestine (Fig.1c). Thus, ion-exchange chromatography was superior to RP-HPLC in resolving Mt-1 and -2 from rat kidney.
In all *in vitro* and *in vivo* experiments LMt-1 was labeled with ^{109}Cd. Purity of these $^{109}CdLMt$-1 probes was checked on HPLC. HPLC analysis showed that all radioactivity eluted as one single peak at 12.9 min, which corresponded to the UV signal of the purified LMt-1 (Fig.1d).

Table 1. Cd disposition (as percentage of radioactive dose) in intestine, liver and kidneys after oral and intravenous administration of $^{109}CdLMt$ in rats. Two animals were killed per time interval (every experiment was performed twice).

Oral administration	Cd in Faeces (%)	Cd in intestinal mucosa (%)	Cd in liver (%)	Cd in Kidney (%)
2 h.	-	0.5±0.3	0.04±0.1	0.04
5 h.	-	1.6±0.4	0.05±0.03	0.05±0.01
24 h.	84,2±1	1.0	0.07±0.02	0.06±0.02
Intravenous administration				
5 min	-	-	6±1	26±2
30 min	-	-	7±1	38±2
4 h.	-	-	8±2	45±3
24 h.	-	-	9±2	43±3

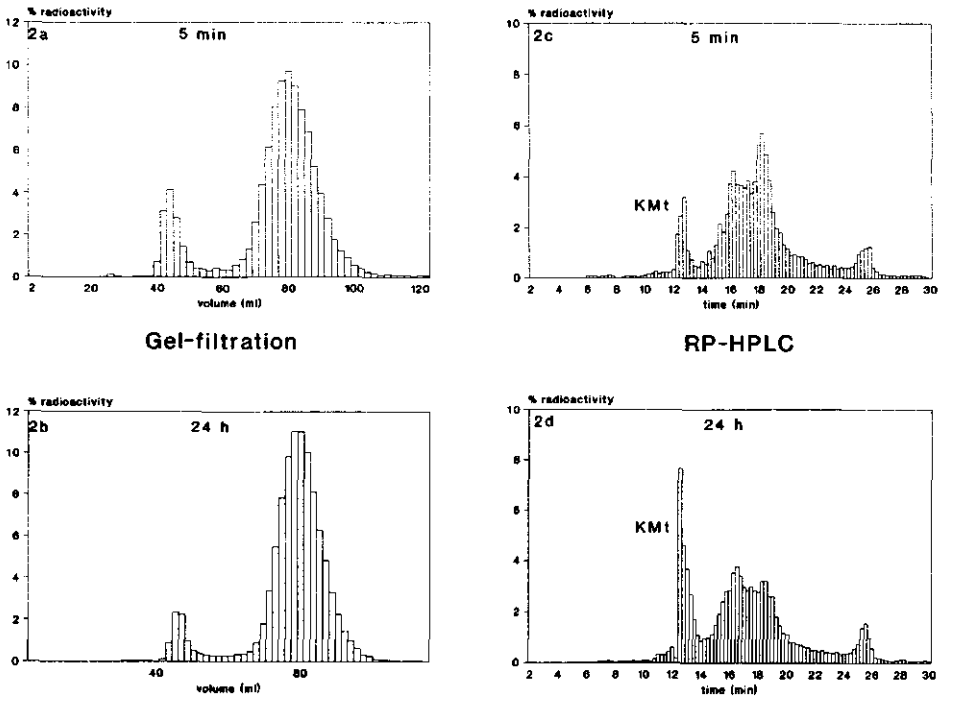


FIG. 2: Left side: Gel-filtration profiles of kidney S9 homogenate from rats which were i.v. dosed with $^{109}CdLMt$-1 2a=5 min, 2b=24 h. Vo=42 ml. Right side: RP-HPLC elution profiles of heat treated renal homogenate. 2c=5min, 2d=24h. See for experimental details Materials and Methods

Table 1 shows the distribution of Cd with time after the i.v. administration of ^{109}CdLMt. Cd administered as CdMt preferentially distributed to the kidneys (approximately 45% of the dose). Cd concentration in the kidneys increased rapidly between 5 and 30 min after administration and then remained constant for at least 24 hours. The Cd-accumulation from CdMt in the kidneys appeared to be 5-8 times as high than in the liver. Fig 2. illustrates the gel-filtration and HPLC elution profiles of the renal cytosol at 5 min and 24 hours after i.v. admininistration of ^{109}CdLMt-1. It appeared that already 5 min after administration of CdLMt-1 Cd is not bound anymore to this LMt. Instead, a small proportion of the Cd eluted at 13 min, a retention time which corresponds with that of purified KMt. The rest of the radioactivity eluted in the area between 15 and 20 min. and at 25 min during the wash program of the column. These Cd ligands have not been further identified, but on the gel-filtration column they behave like Mt-proteins; the majority of the ^{109}Cd in the kidneys was bound to proteins in the area between 3-10 kD (Ve/Vo=2±0.2). One day after iv injection of LMt-1 the amount of Cd bound to KMt was substantially increased; 37% of the radioactivity elutes in the peak at 13 min and the amount of Cd eluting in the area between 15 and 20 min. was reduced. Gel-filtration profiles of the kidney homogenate, (see also Fig.2) showed that a small percentage was bound to high molecular weight proteins (Ve/Vo=1±0.2). The Cd binding to HMW proteins decreased with time; 5 minutes after administration of LMt, 20% of the radioactivity in the kidney was bound to high molecular weight proteins whereas 24 hours after injection this proportion was only 6%. It should be emphasized that gel-filtration profiles show the total radioactivity present in the renal homogenate, whereas HPLC profiles only show

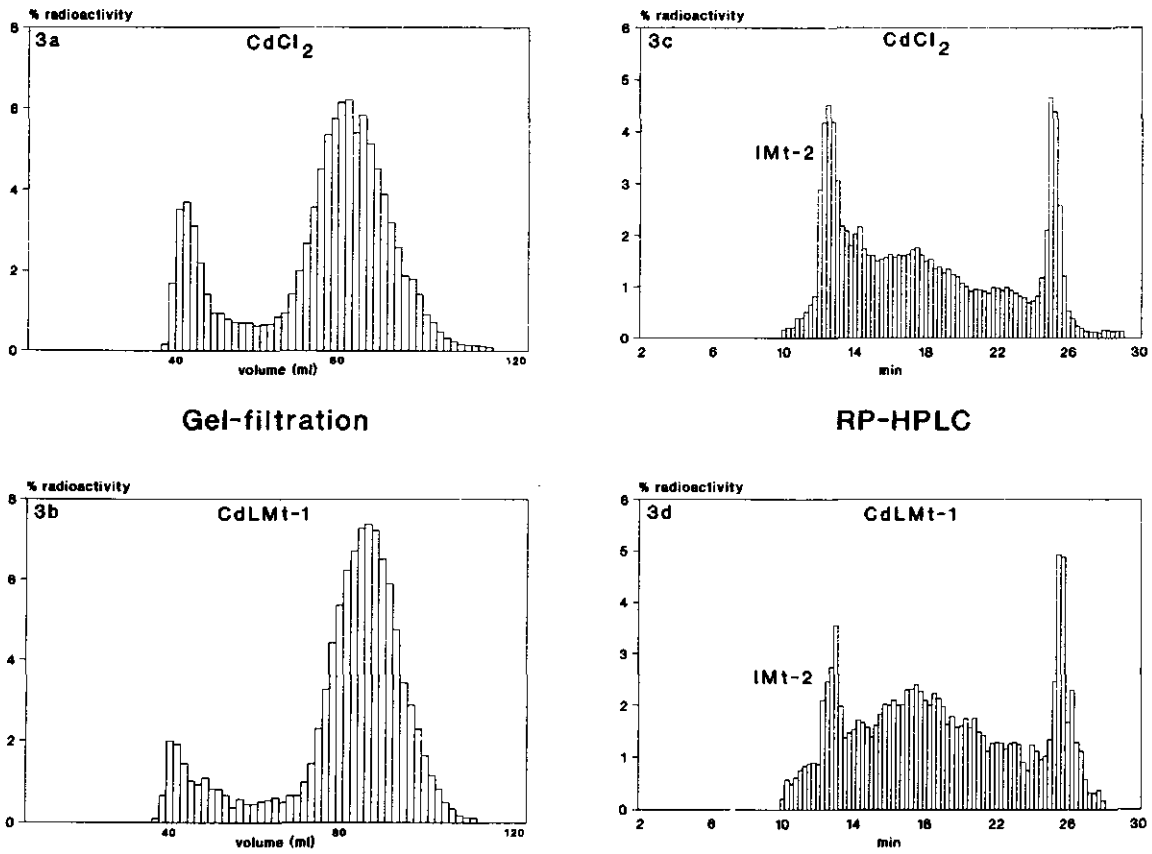


Fig. 3. Left side: Gelfiltration profiles of intestinal mucosal S9-homogenate in rats 5 hours after oral exposure to $CdCl_2$ (3a) or CdLMt-1 (3b). Right side: RP-HPLC elution profile of heat treated mucosal homogenate in same rats. 3c=$CdCl_2$, 3d=CdLMt-1.

heat-resistant Cd-binding ligands. The Cd recovery of heat treated renal homogenate was 75 and 85% after 5 min and 24 hours respectively. The main part of the radioactivity which is lost in the pellet will presumably be bound to high molecular weight proteins, which seems to be in agreement with the gel-filtration profiles.

Table 1 also shows the distribution of Cd with time after the oral administration of CdLMt-1. The Cd retention in the intestinal mucosa after oral exposure to LMt-1 reached its maximum 5 hours after oral gavage. At that time a large proportion of the radioactivity could be retrieved in the coecum (data not shown), but within 24 hours almost 85% of the original dose was excreted by the faeces. HPLC analysis of the intestinal mucosa 5 hours after exposure to LMt-1 showed that Cd was hardly bound to intact LMt-1 (Fig. 3d); only 10% of the Cd eluted at 14 min. 20 % of the Cd is bound to IMt-2 and the rest of the radioactivity is found in a broad area with a retention time between 15 and 22 min. For comparison, rats exposed for 5 hours to $CdCl_2$ (Fig. 3c) showed a similar HPLC pattern, although relatively more Cd is bound to IMt-2 and IMt-1 (45%). The rest of the Cd mainly elutes from the column in the area between 15 and 20 min and during column rinsing at 25 min. Due to the low Cd disposition in the kidneys, no HPLC studies could be performed on the renal homogenates. Gel-filtration profiles of the intestinal mucosa (Fig 3a) show that 5 hours after $CdCl_2$ administration 20% of the Cd is bound to HMW proteins, whereas after exposure to CdLMt-1 (Fig. 3b) only 6% was bound to HMW proteins. The ^{109}Cd recovery in the supernatant of heat treated mucosa tissue was 81% and 94% for respectively $CdCl_2$ and CdLMt. This implies that 19 and 6% of the Cd binding proteins was not heat-resistant, which is in agreement with the percentage of Cd bound to high molecular weight proteins after gel-filtration. Thus, the majority of the Cd in rats exposed to low doses of CdMt or $CdCl_2$ is bound to heat-resistant proteins. These proteins have a molecular weight in the range of Mt, but on HPLC these Cd-ligands do not behave like purified Mt and eluted in a broad area between 15 and 20 min.

After addition of $^{109}CdCl_2$ to renal homogenates *in vitro*, a large fraction of the radioactivity elutes at 13 min, which corresponds with the retention time of purified renal metallothionein (fig 4a). The rest of the radioactivity, which is not bound to KMt elutes from the column at 25 min., which is during the wash program with 100% buffer B. When $^{109}CdCl_2$ is added to renal homogenates of rats, which are pre-treated with $CdCl_2$ the KMt becomes the main binding protein and there is hardly any radioactivity at 25 min. (data not shown). After addition of ^{109}CdLMt-1 to the renal homogenates in a concentration which is five times as high as the total endogenous (KMt) concentration (fig4b), the main part of the radioactivity elutes at 14.8 min, corresponding with the retention time of purified ^{109}CdLMt. This indicates that the Cd remained bound to CdLMt-1. However, when the LMt-1 concentration was lowered, the radioactivity of ^{109}Cd redistributed from LMt towards endogenous renal Mt (fig 4c and 4d). Thus the ratio between KMt/LMt concentration determines the final Cd distribution in the renal tissue.

Figure 5 shows the fate of ^{109}CdLMt-1 in renal homogenates during a 24 hours incubation period at 4 °C and at 37 °C. LMt was added at a concentration equal to the endogenous KMt concentrations. Immediately after incubation of CdLMt-1 at 37°C the ^{109}Cd is distributed over KMt and LMt-1. After 4 hours of incubation the LMt-1 has disappeared while Cd is still bound to KMt. The rest of the radioactivity eluted at 25 min during the wash program. After 24 hours neither LMt or KMt was visible anymore and the ^{109}Cd elutes in a broad area between 15 and 20 min and again during the wash program of the column. In contrast, when CdLMt-1 is incubated with the renal homogenate at 4°C, the redistribution from LMt-1 towards KMt takes place very slowly and 24 hours after incubation, there is still some of the LMt-1 present in the homogenate. Nevertheless, the fact that Cd also redistributes at 4°C, indicates that to obtain a meaningful result, it is of crucial importance that tissue preparation in the *in vivo* experiments will be performed as quickly as possible and under ice-cold storage conditions. The ^{109}Cd recovery in the heat-treated renal homogenate after 15 sec., 4 h., and 24.h of incubation was respectively 80, 50 and 20 % at 37°C and 80, 75 and 60 % at 4°C. This shows that at 37 °C after degradation of LMt-1, Cd became bound to proteins which are not heat-resistant.

After incubation of intestinal homogenates with $^{109}CdCl_2$ *in vitro* the Cd ions are mainly bound to IMt-2 and not to IMt-1 (Fig. 6a). This is in

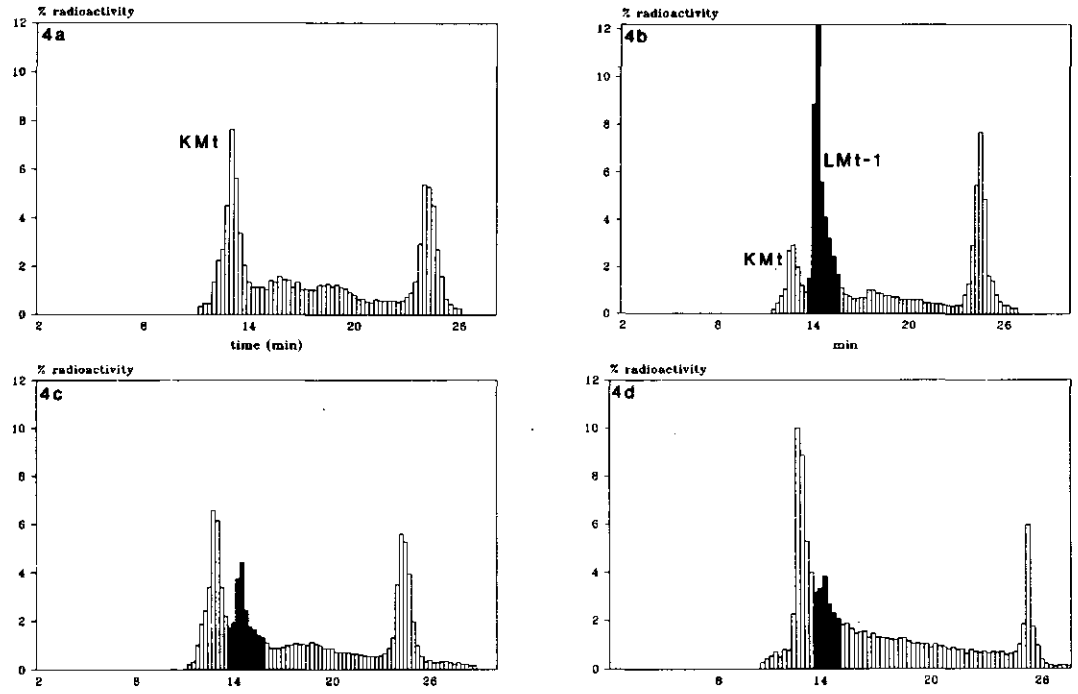


Fig.4. HPLC elution profiles of renal homogenates after 15 sec. incubation with $CdCl_2$ (4A), or LMT-1 in a concentration ratio LMt:KMt of 5:1 (4B), 1:1 (4C) and 1:3 (4D). The area drawn black, co-elutes with LMt-1.

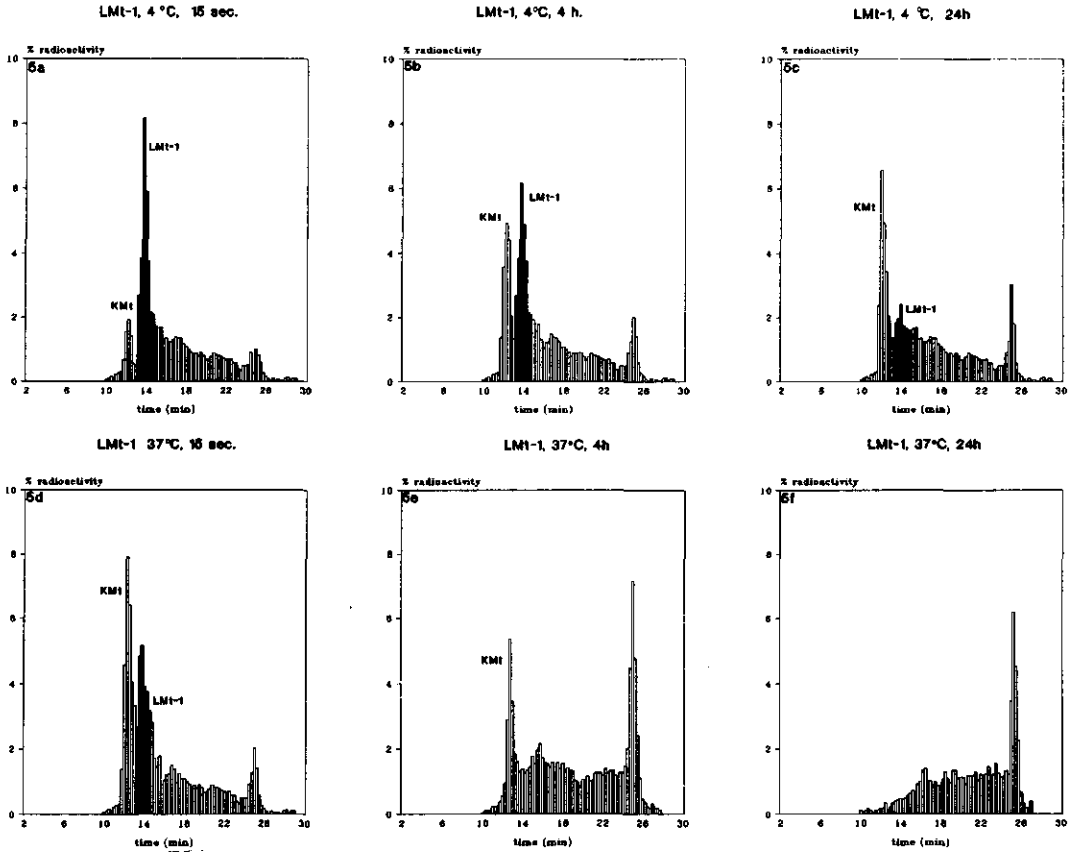


Fig. 5. HPLC elution profiles showing degradation of LMT and KMt in renal homogenates after in vitro incubation with LMt-1 (ratio LMt:KMt=1:1) for 15 sec. (5a,d), 4h. (5b,e) and 24 h. (5c,f) at 4°C and 37°C respectively. The area drawn black, co-elutes with purified LMt-1. For details, see method section.

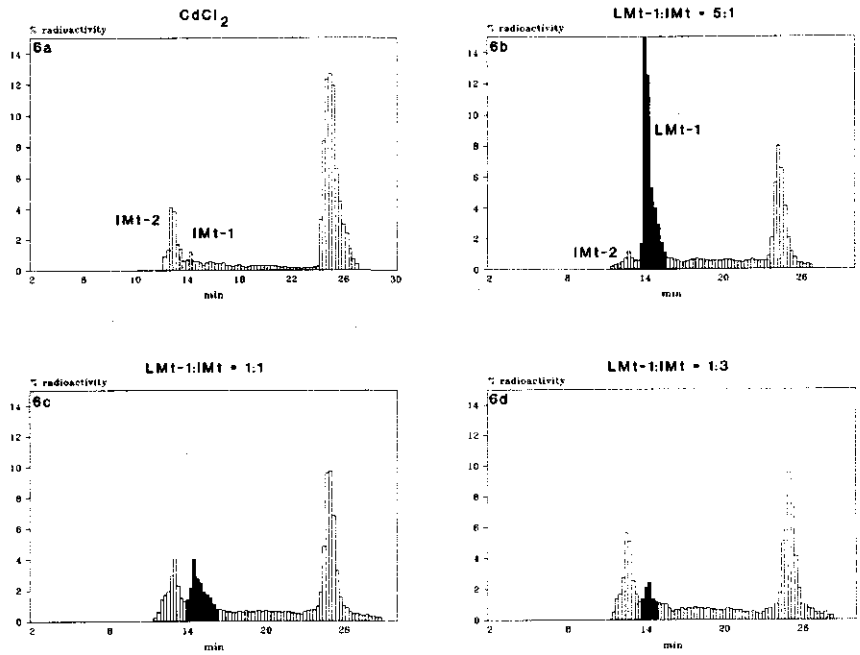
agreement with the fact that IMt-2 is the main metallothionein isoform in untreated rats (Fig 1). However, the total amount of Cd which was bound to endogenous Mt in the intestine (14%) is lower than observed for the kidneys (Fig 4a). The difference can be explained by the fact that the intestinal Mt concentration is twice as as low as the renal Mt concentration (see Materials and Methods). After addition of ^{109}CdLMt to the intestinal homogenate in a LMt/IMt ratio of 5 to 1 (i.e. LMt concentration 5 times as high as the endogenous IMt concentration) the majority of the ^{109}Cd remained bound to LMt (Fig 6b). At a lower Mt concentration ratio between LMt and IMt the ^{109}Cd is distributed over LMt-1 and IMt-2 (fig 6c and d).

Lastly, LMt-1 was incubated with whole blood for 30 min. The LMt concentration was equal to that in the in vivo experiment after i.v. injection. HPLC analysis of the heat-treated blood sample revealed one single peak at 12.9 min, which indicates that Cd is firmly bound to the LMt in the systemic circulation.

DISCUSSION

Although ion-exchange chromatography showed two main Mt-isoforms in both liver and kidneys of rats the renal and hepatic Mt do not behave similarly on RP-HPLC; renal Mt-1 and Mt-2 could not be separated whereas hepatic Mt isoforms were complete distinguishable. However, the fact that renal Mt isoforms elutes on HPLC as one peak at 13 min, allows a distinction from hepatic Mt isoform-1 (with a retention time of 14.8 min). This advantage also applies for the intestine since IMt-2, eluting at 12.9 min is the main Cd-binding ligand in mucosal scrapings and is completely distinguishable from LMt-1. The main research objective of the present study was to investigate, in one step, the metabolic fate of both endogenous and exogenous CdMt in intestinal and renal tissue of rats which were exposed to low oral or iv doses of CdLMt-1. The detection limit of the HPLC system for purified Mt-samples is approximately 0.5 μg Mt based on the UV sig-nal at 215nm. However, in samples of whole tissue homogenates the detection limit is decreased to approximately 3-4 μg/injection. Therefore RP-HPLC in this study was conducted with phosphate buffer at neutral pH to be sure that metal-thiolate clusters of Mt remain intact (Richards and Steele, 1987). Under these conditions the sensitivity of the system was highly increased by studying the ^{109}Cd elution profile in addition to the UV-chromatogram at 215nm. This approach, to study the difference in chromatographic be-haviour by means of the Cd elution profile, has been reported to be succesfull in distin-guishing species-specific (i.e. pig and rat) metallothionein isoforms (Groten et al., 1992). Renal Cd accumulation after oral chronic exposure to cadmium is due to the release of CdMt from intestine and liver into the systemic circulation (Dudley et al., 1985; Min et al., 1991). After i.v. administration of a low, non-toxic dose to rats, CdMt was taken up predominantly by the kidneys and the maximum concentration was reached within 30 min after adminsitration. These results are in agreement with studies reported by Nordberg et al. (1975), Tanaka et al. (1975), and Dorian et al. (1992). After injection of LMt, 80% of the Cd is bound to Mt-like proteins, and this percentage increased with time. Already 5 min after i.v. injection of LMt-1, Cd in the kidneys was no longer bound to intact LMt-1 and, within 24 hours a substantial amount of the Cd in the kidneys, released from LMt-1 was bound to endogenous renal Mt (KMt). A considerable proportion of the Cd was bound to other heat-resistant compounds which had a retention time between 15 min and 20 min on the HPLC system, but on a gel-filtration column behaved like proteins with a molecular size in the Mt-range. It seems likely that these Cd binding ligands are slightly modified or degraded products of endogenous and exogenous metallothioneins. This is in line with the fact that *in vitro* a similar concentration of exogenous CdMt was degraded in renal tissue homogenates within a few hours (Fig 5). The rapid degradation of CdMt *in vitro* seems to be in agreement with the results of Webb and Etienne (1977) and Min et al. (1992). However the Cd exchange between endogenous and exogenous Mt is not solely due to degradation of LMt-1 because Cd was rapidly, i.e. within 15 sec. exchanged between LMt-1 and KMt *in vitro*, without the detection of Mt-degradation products on the HPLC. The Cd exchange was dependent on the ratio between the exogenous Mt (=LMt) and the endogenous Mt (KMt) concentration in the tissue. The Cd release from exogenous metallothionein in the present study appears to be a faster proces than the Cd release from rat CdMt

Fig 6. HPLC elution profile of heat-treated intestinal mucosa after 15 sec. in vitro incubation with $CdCl_2$ (6a) or CdLMt-1 with ratio LMt:KMt of 5:1 (6b), 1:1 (6c), 1:3 (6d).



as used by Cain and Holt (1983) and Squibb et al.(1984). This difference can be explained by the fact that the CdMt concentration used was much higher than in the present study. Thus, a larger proportion of the Cd remains bound, at least initially, to the exogenous Mt and in this situation the Cd release is determined by the gradual degradation of exogenous CdMt (Cain and Holt, 1983; Squibb et al., 1984). It appeared that the in vitro degradation of exogenous LMt-1 in the present study occurred faster (4 hours) than the degradation of the endogenous KMt (24 hours). Similar findings have been shown by Feldman et al. (1978) and Min et al (1986) and it has been suggested that the lysosomal degradation of endogenous and exogenous CdMt mainly takes place via cystein protease (Min et al., 1992).

At a high oral dose, a proportion of the dietary CdMt can pass the intestinal wall and will reach the kidneys intact (Cherian, 1979). Consequently, the Cd concentration ratio between kidney and liver is higher after exposure to CdMt than after exposure to inorganic Cd salts (Maitani et al., 1984; Groten et al., 1991). In the second part of the study the intestinal Cd speciation was studied at low oral doses of CdMt. For comparison, the Cd disposition after a similar Cd dose in the form of organic Cd-salts was also examined. The intestinal and hepatic uptake of $CdCl_2$ was higher than for CdMt, which seems to be in agreement with the results of Ohta and Cherian (1991). However, the total renal Cd disposition after 24 hours was similar for both Cd compounds (data of $CdCl_2$ not shown). HPLC analysis of the intestinal mucosa revealed that a large fraction of the Cd from CdMt and $CdCl_2$ elutes in an broad area between 15 and 20 min. Rats exposed to CdMt showed always more of these compounds than rats exposed to $CdCl_2$. Whether these Mt derived Cd-ligands can reach the bloodstream remains unsolved. These Cd ligands have not been identified, but the in vitro incubation of LMt-1 in intestinal or renal homogenates revealed similar Cd elution profiles. Therefore these compounds are probably modified or degraded products of CdMt with a molecular weight still in the Mt-range. The Cd binding to endogenous intestinal Mt was very constant at all time points and was always slightly higher for $CdCl_2$ than for LMt-1.

The present result that after exposure to $CdCl_2$ or CdMt the majority of the Cd is bound to Mt-like proteins stands in contrast to the results of Ohta and Cherian (1991), who showed that Cd after exposure to inorganic Cd was predominantly bound to high molecular weight proteins. However, the intestinal Cd absorption in the present study was much lower (0.25 μg/kg tissue) and it can be assumed that the intestinal Mt pool is sufficient to retain most of the Cd-ions (Goon and Klaassen, 1989). If we assume that LMt-1 has reached the intestine intact (Crews et al., 1989), it can be calculated that the amount of LMt-1 taken up by the mucosa was approximately 6 μg/gr tissue. This implies that the LMt-1 concentration is much lower than the IMt-1 content. In this regard, the in vitro experiment with intestinal homogenate showed that, similar to the in vitro experiment with the renal homogenates, Cd rapidly exchanges between various isoforms, depending on the Mt concentration ratio. It is thus likely that at least part of the Cd from dietary CdMt is exchanged towards IMt, before degradation of the LMt complex has taken place. Further redistribution of Cd only takes place after liberation of Cd^{2+} due to degradation of the Mt.

Thus, both Mt degradation and the Cd exchange are important determinants of the Cd-speciation in the tissue. For low levels of exogenous CdMt the rapid exchange of Cd^{2+} towards endogenous CdMt is the decisive factor in the disposition, making its fate similar to that of inorganic Cd salts.

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Chapter 8

COMPARISON OF RENAL TOXICITY AFTER LONG-TERM ORAL ADMINISTRATION OF CADMIUM CHLORIDE AND CADMIUM-METALLOTHIONEIN IN RATS

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COMPARISON OF RENAL TOXICITY AFTER LONG-TERM ORAL ADMINISTRATION OF CADMIUM CHLORIDE AND CADMIUM-METALLOTHIONEIN IN RATS

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Abstract-There is a clear lack of information on the toxicological risk of dietary intake of CdMt. The present study was intended to establish dose dependent Cd disposition and to investigate differences in renal toxicity after long term dietary exposure to cadmium-metallothionein (CdMt) or cadmium chloride ($CdCl_2$) in rats. Male Wistar rats were fed diets containing 0.3, 3, 30 and 90 mg Cd/kg diet (0.3, 3, 30, 90 ppm) either as CdMt or as $CdCl_2$ for 10 months. In rats fed 30 and 90 ppm $CdCl_2$ the Cd concentrations in intestine, liver and kidneys were all higher than in rats fed the same doses CdMt and the kidney/liver ratio of Cd concentration was higher with CdMt than with $CdCl_2$. At the lower Cd concentrations (0.3 and 3 ppm) no differences in Cd accumulation between CdMt and $CdCl_2$ fed groups were observed and the kidney/liver Cd ratio was also similar. Rats exposed to 3, 30 and 90 ppm Cd showed a significant increase in Cd concentration in liver and kidneys both after 4 and 10 months of exposure. When based on the amount of CdMt per milligram Cd in the tissue, rats fed CdMt showed a similar relative CdMt concentration in liver and kidney to rats fed $CdCl_2$. First signs of renal injury, indicated by an increase of urinary LDH activity were seen 4 months after exposure to 90 ppm $CdCl_2$. After 8 and 10 months the renal effect from 90 ppm $CdCl_2$ became more pronounced and urinary enzyme activity of LDH, NAG and ALP were all elevated. The only clinical effect of CdMt at the dose level of 90 ppm, was a slight increase in urinary GGT activity at 8 and 10 months. Determination of β_2-microglobuline in urine did not reveal treatment related changes in any of the groups. Histopathological changes (a.o. glomerulo-nephrosis and basophilic tubules) were observed after 10 months of exposure in rats fed 30 and 90 ppm $CdCl_2$. Rats fed 90 ppm CdMt also showed slight histomorphological changes, but the effect was less pronounced than from $CdCl_2$ and was mainly restricted to the tubules. Thus, no difference was observed in renal disposition between CdMt and $CdCl_2$ after long term exposure to low ($\leq$3ppm) dietary doses. Since the absolute concentration in the kidney is lower after high doses of CdMt than after $CdCl_2$, nephrotoxicity seems to be mainly related to total renal Cd concentrations. Therefore the health risk of dietary intake of CdMt at environmentally relevant doses (i.e. < 2 ppm) seems not be different from the intake of $CdCl_2$.

INTRODUCTION

Food is the major source of human Cd exposure (Robards and Steele, 1991). Since metallothionein is one of the main Cd binding ligands in animals (Webb, 1986; Waalkes and Goering, 1991), Cd in food of animal origin will largely be present as CdMt. However, most oral toxicity studies have been performed with inorganic Cd and there is a clear lack of information on the toxicological risk of biologically incorporated Cd, viz. CdMt. It has been shown that signs of toxicity (e.g., anemia and hepatotoxicity) in subacute studies are less pronounced in rats exposed to CdMt than in rats exposed to inorganic Cd-salts (Groten et al., 1990). This correlates well with the fact that the intestinal and hepatic uptake of Cd after CdMt exposure is lower than after exposure to $CdCl_2$ (Groten et al., 1991a). However, in spite of the lower total uptake of CdMt, the renal Cd accumulation from CdMt is relatively higher than from $CdCl_2$ (Maitani et al, 1984; Ohta and Cherian 1991). Because of the high kidney to liver ratio of Cd concentration after oral exposure to CdMt it has been suggested that CdMt can pass the intestinal mucosa and can reach the kidneys at least partially intact (Maitani et al., 1984; Ohta and Cherian, 1991). This might have implications

for human risk from cadmium absorbed from animal products since CdMt is known to be a potential nephrotoxic compound after parenteral administration (Nordberg et al., 1975; Squibb et al., 1979; Sendelbach and Klaassen, 1988). The differential disposition of $CdCl_2$ and CdMt between liver and kidney is less pronounced at lower dietary doses, but still large enough in a sub-acute experiment to indicate metabolic differences between both Cd-forms (Groten et al., 1991a). Although the kidneys are the critical organ after long-term dietary exposure to cadmium, no long term studies have been performed clarifying the potential risk of kidney injury due to the dietary intake of CdMt. Moreover it is unclear whether the differential disposition between kidneys and liver occurs after long term exposure at environmentally relevant Cd levels in the diet. The present study was intended to establish the dose dependent Cd disposition and to investigate differences in renal toxicity after long term dietary exposure to CdMt or $CdCl_2$ in rats.

MATERIALS AND METHODS

Test substance. Cadmium chloride, with a purity of at least 99% as specified by the supplier was obtained from Merck-Schuchardt (Hohenbrunn, FRG).

Preparation of CdMt incorporated in pig's liver. Thirteen male pigs, initially weighing 76 ± 7.5 kg, were housed separately in metabolism cages. The animals were injected intravenously (vena jugularis) with $CdCl_2$, dissolved in saline according to the following schedule: on Days 0, 2, 4, 7, 9, 10, 11 an 12 with 0.6 mg/kg, on Days 14 and 15 with 1 mg Cd/kg, Day 16 with 1.3 mg/kg, Days 17, 18 and 20 with 1.5 mg Cd/kg, and again on Day 20 and Day 22 with 2.5 mg/kg body weight.
Two days after the last injection the animals were killed under pentobarbital anaesthesia and the livers were removed. The livers were pooled, homogenized, lyophilized and stored in plastic sealed bag at -30° C. Livers obtained from untreated pigs (public slaughter house) were handled in the same way. Mineral analysis of the freeze dried liver samples revealed that the Cd, Zn, Fe and Ca content was 2000, 820, 555 and 190 ppm in the lyophilized liver of Cd-treated pigs and 0.45, 205, 600 and 170 ppm in the liver of control pigs.

Gel-permeation and HPLC analysis (see Groten et al., 1990, 1992b) revealed that more than 95% of the Cd was bound to pig's metallothionein.

Diets. A semi-synthetic powdered basal diet was composed as described previously (Groten et al., 1990; 1991a). Four diets consisted of the basal diet supplemented with $CdCl_2$ and control liver and additional zinc, four other diets consisted of the basal diet supplemented with the Cd-enriched pig liver described above. The diets, providing dietary Cd levels of 0, 0.3, 3, 30, 30 mg/kg, were prepared five times during the study and each batch was stored in sealed plastic bags for 2 months in a -20° C freezer. Mineral analysis of the test diets revealed that under the experimental conditions the actual Cd levels were between 85 and 95 % of the intended levels (table 1). The Ca,P, Zn and Fe content of the diet is of crucial importance for the outcome of the study (Groten et al, 1991b, 1992a). The Fe, Zn, Ca, P content of the diets was respectively 70±20ppm, 65±10ppm, 0.5±0.1% and 0.45±0.05%.

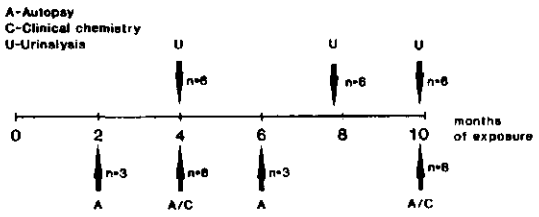
Animals and maintenance. Albino, male rats, Wistar outbred (Hsd/Cpb:WU), were obtained from a colony maintained under SPF-conditions at Harlan/CPB (Austerlitz, NL). At the beginning of the study the rats were 9-10 weeks old. They were housed under conventional conditions in suspended stainless-steel cages fitted with a wire-mesh floor and front. The room temperature was maintained at 22 ± 3 °C and the relative humidity at 40-70% with a 12- hr light/dark cycle. During an acclimatization period of 10 days the rats were fed the basal diet with no further additions. Drinking water was supplied in glass bottles which were cleaned once weekly. Food and water were provided ad libitum. Food/water intake was measured by weighing the feeders/bottles.

Experimental design. The rats were allocated by weight into 9 groups of 20 animals using a computer-generated random number table. Each treatment group (housed in groups of five) received one of the nine diets for 10 months. Autopsy was performed after 2, 4, 6 and 10 month of exposure on respectively 3, 6, 3 and all remainding rats (=max. 8). Clinical chemistry was performed after 4 and 10 months and urine analyses were carried out after 4, 6 and 10 months

General observations. Food and water consumptions were measured 10 times during the

study at weekly intervals in order to establish any differences in Cd dose between CdMt or $CdCl_2$ exposed rats.

Fig. 1. Experimental design of the study. Rats were fed for 10 months Cd diets containing 0, 0.3, 3, 30 and 90 ppm Cd as $CdCl_2$ or Cd-Metallothionein. For further details see Materials and methods



Clinical chemistry. At autopsy on day 112 and day 294 heparinized blood samples collected from the abdominal aorta of all rats were centifuged at 1250 g for 15 min, using Sure-sep II dispensers (General Diagnostics, The plasma was then analysed for alkaline phosphatase (ALP), aspartate (ASAT) and alanine amino transferase (ALAT), γ-glutamyl transpeptidase (GGT), lactate dehydrogenase (LDH) activity and for albumin, urea and creatinin. Analyses were performed on a Cobas-Bio Centrifugal Analyzer using Baker and Boehringer reagent kits.

Urinalysis. On days 106, 218, and 287 six rats/group were acclimatized for two days to wire-mesh metabolic cages. Thereafter, urine was collected in ice-cooled beakers for two 24 hrs. periods. Water and food was provided ad libitum. After collection, urine of both days was pooled, vigorously mixed and the following determinations were made: volume, pH, creatinine, total protein, alkaline phosphatase activity (ALP), lactate dehydrogenase activity (LDH) and γ-glutamyl transferase activity (GGT), N-Acetyl-ß-D-glucosaminidase (NAG) activity and $ß_2$-microglobine. $ß_2$-microglubuline was measured at the Catholic University of Louvain (Belgium) according to the method of Viau (et al., 1986). Except NAG and microglobulin all analyses were performed on a Cobas centrifugal analyzer using Baker and Boehringer reagent kits. NAG was performed colorimetrically using a Boehringer reagent kit. Urine concentration ability was tested 1 week after urinalysis. For that purpose rats were deprived of water for 24 hours and urine was collected during the last 16 hours of the deprivation period to determine volume and density.

Cd analysis and pathology. On day 56 and day 168 animals of each group were killed by exsanguination from the abdominal aorta while under light ether anesthesia and necropsied. Immediately after evisceration the kidneys, small intestine and liver were weighed and the medulla of one kidney was removed for metal analysis. The intestines were rinsed with phosphate buffered saline as described previously (Groten et al., 1991a) and part of the duodenum and ileum were removed for metal analysis. Cd analysis was performed as described previously (Groten et al., 1991b).
In addition to this procedure, on days 112 and 294 one liver lobe and cross sectional parts of one kidney were fixed in 4% neutral phosphate buffered formaldehyde solution. The tissue samples were processed and embedded in paraplast, sectioned 4 μm, stained with haematoxylin and eosin, and then microscopically examined. The rest of the tissue samples were frozen in liquid nitrogen and finally stored at -80 °C for metallothionein analysis.

Metallothionein analysis. Samples of liver and kidney were thawed and after homogenization with a teflon pestle in a 10 mM Tris-HCl buffer (pH 7.4, 4°C), the homogenates were centrifuged (9,000*g* for 20 min) and the supernatant used for CdMt analysis. The tissue level of CdMt was determined by the Cd-hemoglobin assay as modified by Eaton and Toal (1982). From the resulting mean values (duplicate per animal), the concentration of Mt was calculated using a molar apo-Mt:Cd ratio of 1:7 (Winge and Miklossy, 1982). Mt concentration was determined after 4 and 10 months of exposure.

Statistical Analysis. Data on body weights were evaluated by a one way analysis of covariance (covariate: body weight at the start of the treatment) followed by Dunnet's multiple comparisons tests. The laboratory determinations and organ weights were evaluated by a one-way analysis of variance, followed by Dunnet's multiple comparison tests. Histopathological changes were evaluated by Fisher's exact probability test.

RESULTS

General condition and behaviour of the rats was normal during the study. Body weight gain and food consumption were similar in all groups (data not shown). Figure 2 shows that feeding of $CdCl_2$ or CdMt produced a dose- and time-dependent increase of the Cd concentration in all organs. At the dietary Cd level of 0.3 ppm the time dependent increase of the Cd content in the organs was less obvious than at the higher dose levels and the difference in Cd concentration with the control animals was not statistically significant. At dietary Cd levels of 0.3 and 3 ppm there were no consistent differences in Cd disposition between $CdCl_2$ and CdMt. In contrast, at dietary Cd concentrations of 30 ppm and 90 ppm the Cd disposition in the organs was much higher after feeding of $CdCl_2$ than after feeding of CdMt (Fig.2). This effect was most pronounced in the liver. Figure 3 illustrates the difference in the distribution of Cd to kidneys and liver after feeding of $CdCl_2$ and CdMt. After 10 months a higher Cd concentration was achieved in kidneys than liver at all dietary Cd levels. However, the ratio of kidney/liver Cd concentration was inversely proportional with dose. Thus, as the oral dosage increased, more Cd was found in the liver and as a result the kidney/liver ratio of Cd concentration decreased. Moreover, at the dietary concentration of 30 and 90 ppm the Cd ratio was higher in rats fed CdMt than in rats fed $CdCl_2$. At the lower dietary doses of 3 and 0.3 ppm there was no significant difference between both Cd forms in differential Cd disposition between liver and kidneys. Table 1 shows that the endogenous metallothionein concentration of control kidneys was almost three times as high as in liver. Tissue Mt levels appeared to be rather constant between the 4th and 10th month of the experiment. Rats exposed to 30 and 90 ppm Cd showed a significant

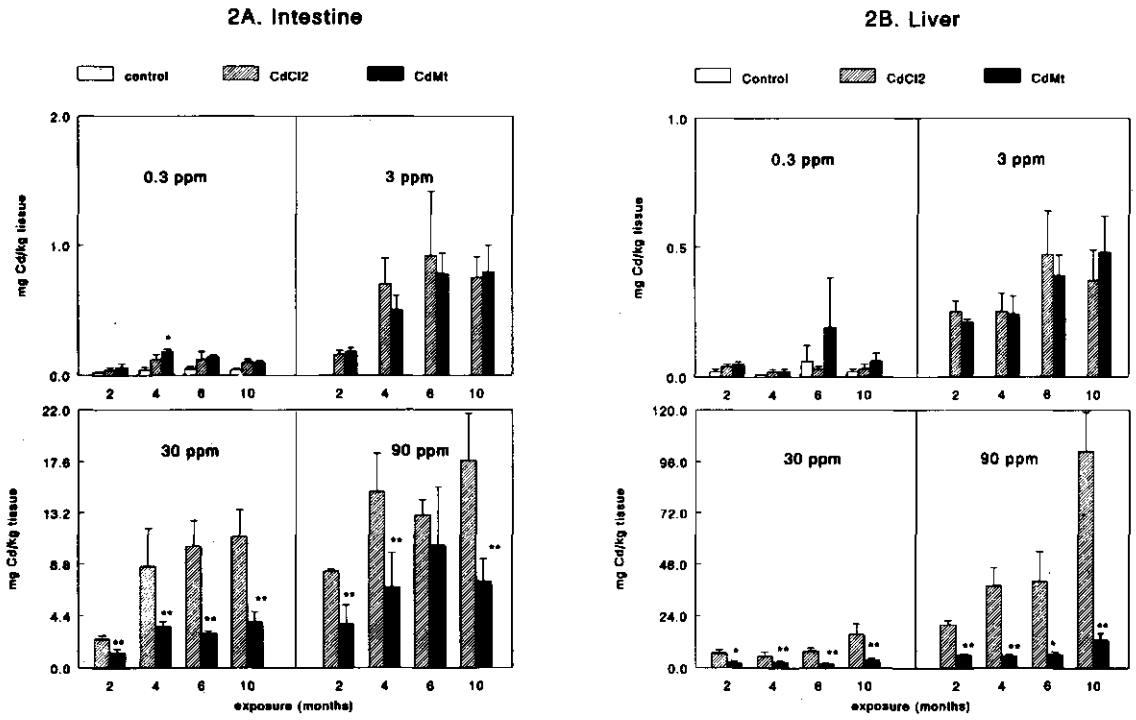
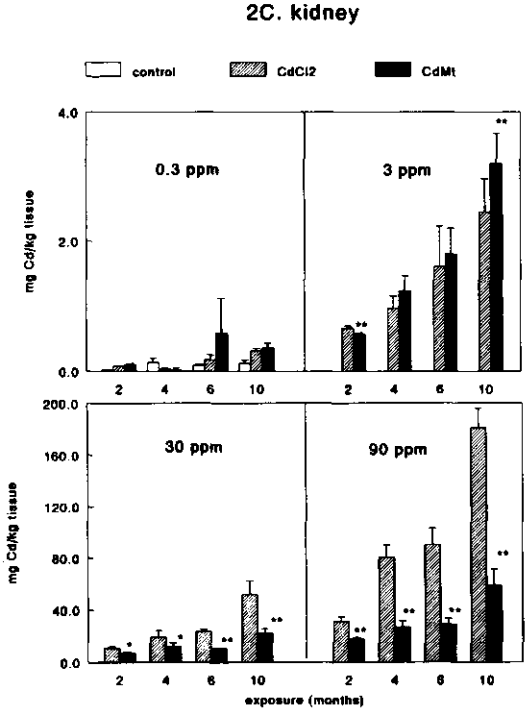


FIG. 2. Time dependent Cd concentrations in small intestine, liver and kidneys after oral exposure to 0.3, 3, 30 and 90 ppm $CdCl_2$ or CdMt. Values represent the mean ± S.D. (n=3, 6, 3 and 8 for respectively month 2, 4, 6, and 10). All values marked with an "" in the groups fed CdMt differ significantly from the groups fed $CdCl_2$ of a corresponding dose. (*o $p<0.05$, **oo $p<0.01$). Fig.1A=Intestine, 2B=Liver, 2C=Kidneys.*

increase in Mt concentration both after 4 and 10 month of exposure. Mt induction was dose- and time-dependent, however the Cd concentration in the organs at the dose level of 0.3 ppm was not sufficiently high to cause Mt induction.



Mt induction in liver was higher than in the kidneys. This is also illustrated in Fig.4 which shows the relationship between the Cd and Mt concentration in liver and kidneys after 10 month of exposure to CdMt or $CdCl_2$. When based on the amount of Mt per milligram Cd in the tissue rats fed CdMt showed a similar relative Mt concentration to rats fed $CdCl_2$.

The first renal changes in the study were seen after 4 months of exposure. At that time, rats fed 90 ppm $CdCl_2$ showed an increase in the LDH enzyme activity in the urine (Fig 5). Figure 5 shows the total mean enzyme excretion per rat per 24 hours, but similar results were seen when enzyme excretion was expressed per amount of creatinine or total protein. The mean renal cortical Cd concentration in these rats was 80 mg/kg (cf. Fig.2). After 8 months of exposure the effect in the 90 ppm $CdCl_2$ became more pronounced and both LDH and NAG as well as the brush border enzyme ALP were elevated in urine samples of the rats. At none of the other dose levels any significant increase in these urinary enzyme levels was observed. It must be emphasized that the blood plasma enzyme levels of ALP, LDH, GGT did not show any differences between control and Cd-treated animals, indicating that the enzymatic changes in urine are kidney-related. In rats fed 90 ppm Cd as CdMt the urinary ALP excretion seems to be elevated at all time points, although the effect was never statistically significant due to the high interindividual variation. The only significant clinical effect in urine samples of CdMt treated rats was a small (but significant and consistent) increase in the GGT activity, an effect which was not observed after $CdCl_2$ treatment. In spite of the observed enzymuria in the rats fed 90 ppm $CdCl_2$ none of the rats showed changes in their ability to concentrate urine (data not shown). $ß_2$-microglobulin was measured as a potential early indicator of low molecular weight proteinuria (Bernard et al, 1991). However, the interindividual variation in $ß_2$-microglobulin levels observed in male rats were too large for this measurement to reveal treatment-related changes (data not shown).

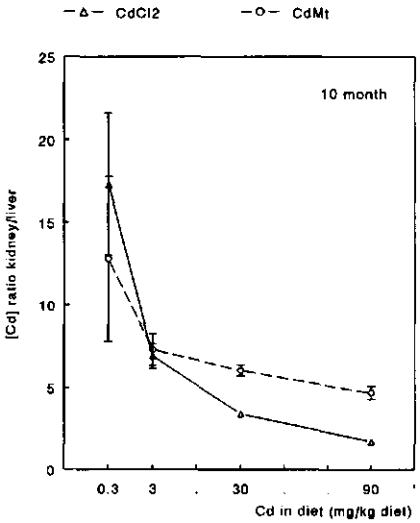
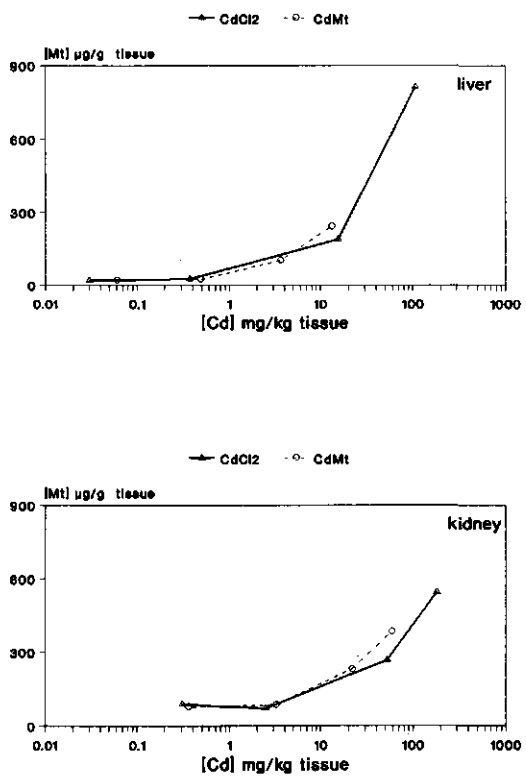


Fig. 3. The ratio between kidney and liver Cd concentrations of rats fed $CdCl_2$ or CdMt for 10 month. Values represent the mean ± SE of 8 individually calculated Cd ratio's.

Table 1. Metallothionein concentrations in liver and kidneys of rats after 4 and 10 month of exposure to $CdCl_2$ or CdMt. Every value is the mean of 6 rats ± S.D. All values marked with an "" differ significantly from the controls. Values marked with an "o" in the groups fed CdMt differ significantly from the groups fed $CdCl_2$ of a corresponding dose ($^{o}p<0.05$, $^{**o}p<0.1$).*

	MONTH 4 Liver	Kidney	MONTH 10 Liver	kidney
Control	16.47±2.71	47.14±4.35	16.67±3.08	63.3±16.49
0.3 ppm CdCl	16.88±2.46	48.49±3.51	20.27±3.3	87.7±9.65
3 ppm CdCl	21.76±5.97	55±13.39	26.8±10.26	70.81±18.41
30 ppm CdCl	84.17±18.95**	158±47.74**	190±22.62**	267.34±4.22**
90 ppm CdCl	306.0±78.69**	283.93±64.35**	814±96.56**	545.6±273.7**
0.3 ppm CdMt	17.15±1.63	55.45±39.12	19.57±3.13	77.17±8.0
3 ppm CdMt	26.58±9.97	62.4±14.34	25.19±5.43	85.21±10.0
30 ppm CdMt	58.53±14.74	177.64±43.48**	101.95±49.7**oo	229.59±14.3**
90 ppm CdMt	111.21±9.84**oo	219.31±28.23**o	243±61.3**oo	383.27±47.3**o

FIG. 4. Relationship between Mt concentration and Cd accumulation in liver and kidneys of rats fed 0.3, 3, 30 and 90 ppm as $CdCl_2$ or CdMt for 10 months. Data-points represent the mean Cd and Mt values of six rats.



Histopathological examination of kidneys after 4 months could not reveal treatment related differences. However, after 10 months of exposure animals fed 30 ppm $CdCl_2$ showed an increase of basophilic tubules in the renal cortex. This effect was more pronounced at the 90 ppm level (Fig 6). Rats fed 90 ppm CdMt also showed an increase in basophilic tubules and there was no significant difference in the incidence of basophilic tubuli between CdMt and $CdCl_2$. In addition, rats exposed to 30 or 90 ppm $CdCl_2$ showed a dose-related increase in signs of glomerular nephrosis (Fig. 6). The effect on the glomerulus was also seen in rats fed 90 ppm CdMt, but much less pronounced than in rats fed $CdCl_2$. One rat fed 90 ppm $CdCl_2$ showed focal peyelitis. In liver no abnormalities were detected in the highest dose groups and moreover plasma ASAT and ALAT enzyme activity appeared to be normal.

DISCUSSION

In a previous 4-week feeding study in rats it was shown that after dietary exposure to CdMt the kidney/liver ratio of the Cd concentration is higher than after $CdCl_2$ intake (Groten et al., 1991a). Moreover, the renal metallothionein and Cd-

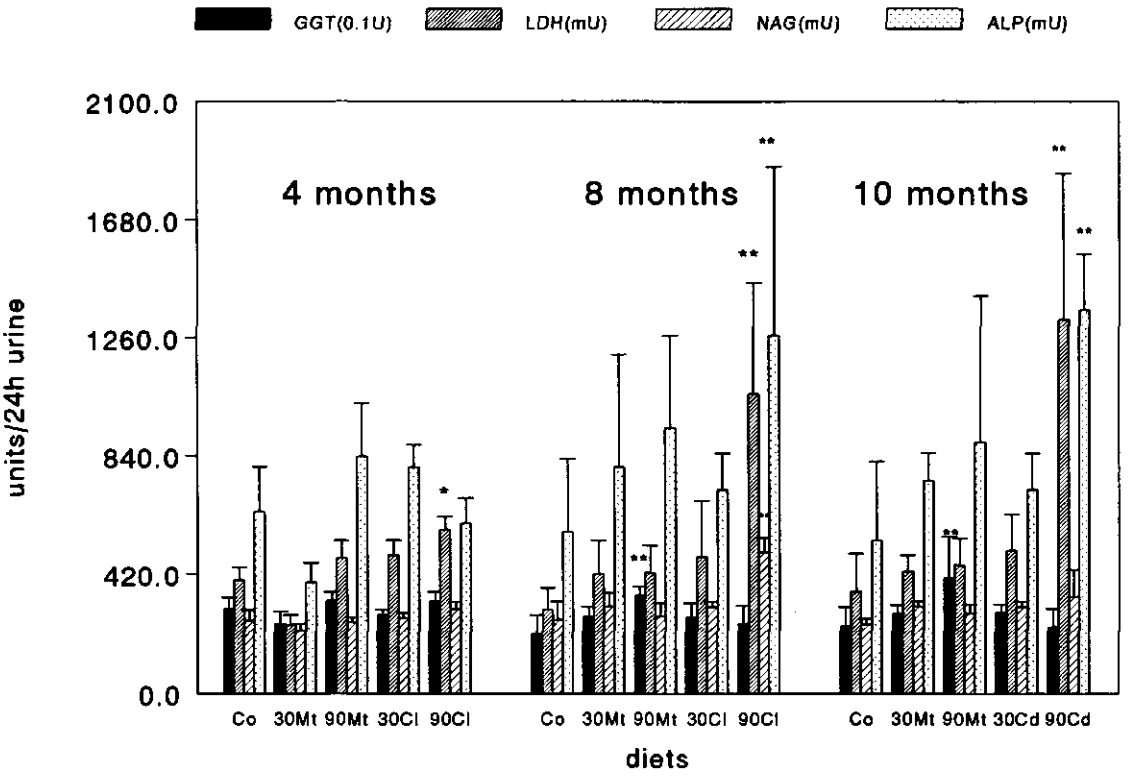
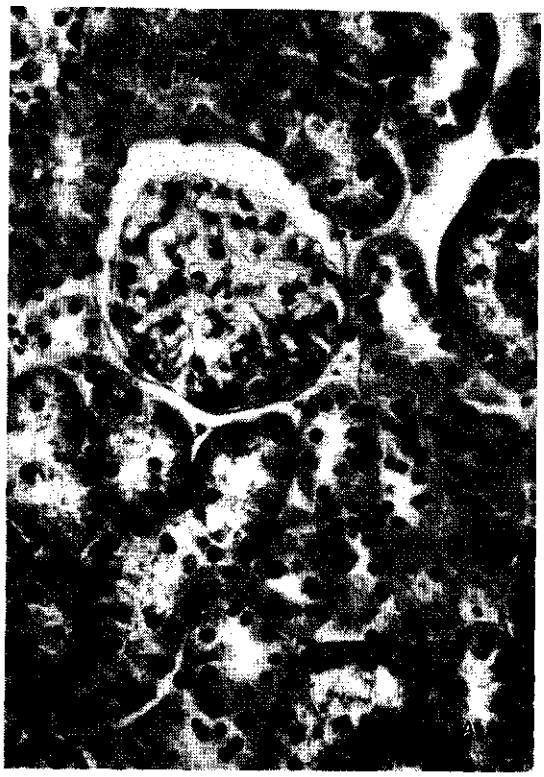
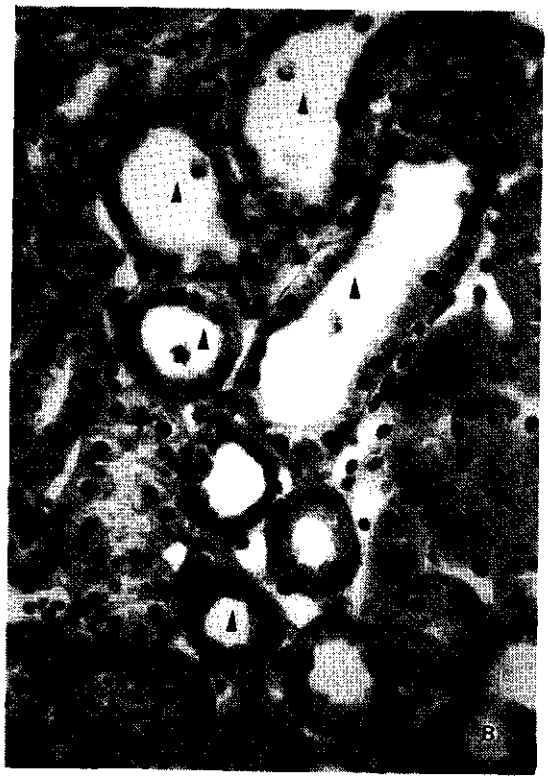


Fig. 5. Urinary enzyme excretion of rats fed 0, 30 and 90 ppm CdMt or $CdCl_2$ after 4, 8 and 10 month of exposure. Values represent the mean ± SD of 6 rats. Values marked with asterisk differ significantly from the control group. LDH=Lactate dehydrogenase, GGT=γ-glutamyl transpeptidase, ALP=Alkaline phosphatase, NAG=N-acetyl-β-glucosaminidase. Co=control, 30/90 Mt=30/90 CdMt, 30/90Cl= 30/90 $CdCl_2$.

concentration were almost similar for both $CdCl_2$ and CdMt in spite of the lower intestinal uptake of Cd from CdMt. These findings support the theory (Cherian et al., 1979; Otha and Cherian, 1991) that some of the exogenous CdMt passes the intestinal barrier and reaches the kidneys directly. Since CdMt is a potent nephrotoxic agent when injected intravenously (Nordberg et al., 1975; Squibb et al., 1979), this indicates that dietary CdMt might be a reason for health concern. However, in the same study it was also seen that the differences in renal Cd distribution between CdMt and $CdCl_2$ became smaller at lower oral doses (Groten et al., 1991a). The results of the present study show that the difference in renal disposition between $CdCl_2$ and CdMt is dose-dependent and does not occur at low dietary Cd doses (0.3 and 3 ppm Cd in diet). This is an important result from which the conclusion can be drawn that after long term exposure at environmentally relevant dose levels there will be no difference in accumulation between inorganic and organic Cd. Although the mechanism behind the intestinal uptake of Cd is not clear, it is assumed that at low oral $CdCl_2$ doses, the majority of the Cd will be bound to the endogenous metallothionein of the intestine. This CdMt complex will be released into the systemic circulation and subsequently, similar to intravenously injected CdMt (Tanaka et al., 1975), will reach the kidneys (Lehman and Klaassen, 1986; Ohta and Cherian, 1991). It has been shown that pre-induction of intestinal Mt with zinc indeed causes a higher selective renal Cd accumulation (Min et al., 1991). Moreover, the immunological identification of intestinal metallothionein in the blood plasma (Elsenhans et al., 1991) supports the theory that intestinal Mt is indeed released into the





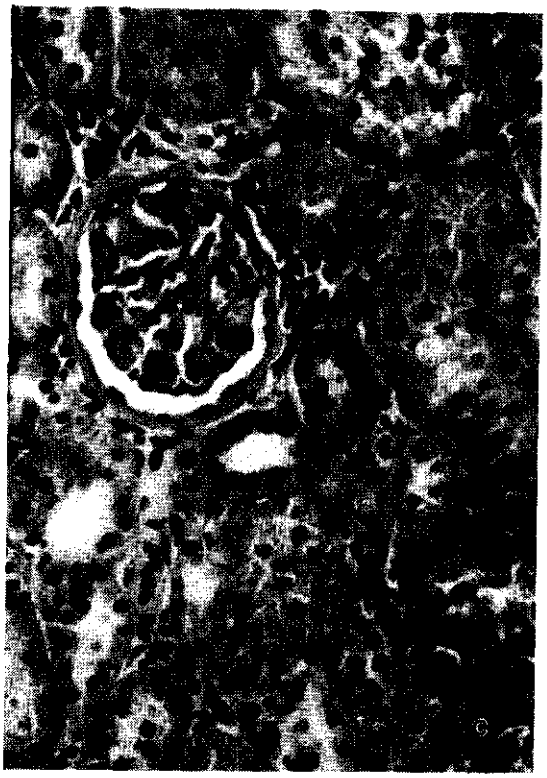


Fig. 6A. Control kidney after 295 days, normal glomerulus and tubules, H&E staining x250

Fig. 6B. Basophilic tubules (arrow heads) and some cellular detritis in lumen of tubules in the medullary of a rat kidney after 295 days of feeding 90 ppm $CdCl_2$.

Fig. 6C. Glomerular nephrosis, consisting of sclerosis of Bowmans capsule and the mesangium of the glomerular tufts, and some basophilic tubules of a rat kidney after 295 days of feeding 90 ppm $CdCl_2$. H&E staining, x250.

systemic circulation. After intake of dietary CdMt part of this metal-protein complex reaches the intestine as a metallothionein-like protein (Cherian, 1979; Crews et al., 1989), where it might be taken up by endocytosis (Ohta and Cherain, 1991). The remainder of the CdMt however, will be degraded (Feldman et al., 1978; Klein et al., 1986) and the Cd will presumably follow the usual metabolic route and becomes bound to endogenous CdMt. Recently it has been shown that after addition of exogenous CdMt to homogenates of the intestine, the cadmium from exogenous CdMt redistributes to endogenous metallothionein. The extent of Cd binding to the two Mt isoforms depends on the ratio of their concentrations (Groten et al., 1992b). This implies that at environmentally relevant Cd doses the transport form of $CdCl_2$ and CdMt across the intestinal wall is similar, which explains the similar Cd disposition observed for both $CdCl_2$ and CdMt in the present study. In addition, it has been shown that iron not only protects against the Cd uptake from $CdCl_2$ (Groten et al., 1991a), but iron is also effective against the intake of Cd from CdMt (Groten et al., 1992a).The fact that iron protects against CdMt indicates that Cd from dietary CdMt shows the same metabolic behaviour as dietary inorganic Cd.

In the present study there is a clear relationship between renal Cd concentration and renal damage. Clear signs of renal damage (i.e. enzymuria, tubular damage and glomerulo nephrosis) were mainly detected in rats exposed to 90 ppm $CdCl_2$ and all effects became more pronounced during the course of the study. Except for the slight increase in urinary GGT all other signs of renal impairment, as observed after $CdCl_2$ treatment, were much less pronounced after feeding of 90 ppm CdMt. The finding that 90 ppm $CdCl_2$ clearly affected the kidneys whereas 90 ppm CdMt does not, can be explained by the fact that the renal Cd concentration was much lower for CdMt than for $CdCl_2$ (after 10 months; 60 and 170 mg/kg tissue respectively). However, the renal Cd concentration due to feeding of 90 ppm CdMt is almost similar to the renal concentration after feeding of 30 ppm $CdCl_2$ (after 10 month; 56 mg/kg tissue) and in both cases similar slight histomorphological changes were observed, but no significant enzymuria. This finding implies that in contrast to intravenous CdMt and $CdCl_2$ (Sendelbach and Klaassen, 1988) renal effects of oral administered CdMt and $CdCl_2$ are dependent on the difference in degree of Cd accumulation in the tissue.

In the present study a series of renal functional tests have been applied, but some of the tests did not reveal renal damage or impairment of the renal function. For instance, low molecular weight proteinuria is one of the most characteristic signs of Cd-induced renal dysfunction (Kjellström et al. 1986). However in animal studies the immmunochemical quantitation of individual urinary proteins such as $ß_2$-microglobulin has rarely been used (Bernard and Lauwerijs, 1991). In the present study it was not possible to measure any increases in $ß_2$-microglobulin due to the high interindividual variation in excretion between male rats of this strain and age. Thus, quantitation of LMW proteinuria seems not be the most efficient approach to detect renal lesions in aged, male rats. In addition, it has been hypothesized that the urinary concentration ability and kidney weight are more sensitive indicators of nephrotoxcity in subacute studies than enzymuria (Kluwe et al., 1986). The results of the present do not support this hypothesis, since the urinary concentration ability of the Cd-treated rats was not affected after 4 months whereas the LDH activity in the urine was significantly increased. The enzymuria in rats fed 90 ppm $CdCl_2$ became more pronounced after 8 and 10 months of exposure as was indicated by a marked increase of urinary LDH, ALP and NAG. The urinary excretion of kidney derived enzymes has proved to be a reliable indicator of kidney toxicity in rat studies (Watanabe et al., 1980; Hofmeister et al., 1986; Stonard et al., 1990). However, there are few data available comparing the histopathological changes of Cd nephropathy with the values of urinary enzyme excretions. In this regard, the oral Cd study of Gatta (et al., 1989) might indicate that changes in urinary enzyme values are in accordance with histological findings.

The rather unspecific distribution of intracellular enzymes such as LDH and NAG along the nephron (Guder and Ross, 1984), makes it difficult to determine the exact site of renal injury, although the pathological changes in the glomerulus indicated that injury was not only restricted to the proximal tubule. In contrast to rats fed 90 ppm $CdCl_2$ the rats fed 90 ppm CdMt did not show increased LDH or NAG activity during that time, but instead these rats showed a very slight, but significant increase in urinary GGT activity. GGT is similar to ALP a brush border enzyme localized on the proximal tubule cell, but in contrast to ALP it is localized more specifically towards the end (straight part) of the

proximal tubule. The effect of oral CdMt on the brush border enzyme in the straight part of the proximal tubule cell seems to differ from the effect of intravenous CdMt since it has been shown that effect intravenous CdMt is more restricted towards the convoluted part of the proximal tubule (Cherian et al., 1976; Webb and Etienne, 1977; Dorian et al., 1992). Based on the pattern of enzymuria and pathology and in agreement with the findings of intravenous CdMt studies, it seems however that the effect of dietary CdMt is more restricted towards the proximal tubule whereas $CdCl_2$ affects both the tubules and the glomeruli.

The most important conclusion which can be drawn from the present study is that there is no indication that CdMt in spite of its higher kidney/liver Cd ratio is more nephrotoxic than $CdCl_2$ at high Cd doses. Furthermore, the selective renal disposition of dietary CdMt does not occur at low dietary doses and the renal Cd levels at low dietary Cd doses are similar for both Cd forms. Therefore dietary intake of CdMt at environmentally relevant doses (<2 ppm in diet) seems not be more of a health risk than the intake of the inorganic form of Cd.

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SUMMARY AND CONCLUDING REMARKS

SUMMARY AND CONCLUDING REMARKS

In **Chapter 1** of this thesis a general introduction is presented with a survey of the literature. It gives a brief overview of the factors involved in the absorption, metabolism and toxicity of Cd after oral intake.

In short, the main source of environmental exposure to cadmium for nonsmokers is food. Oral Cd-studies in rodents have shown that the Cd-dose, the Cd-speciation and the mineral composition of the diet have an enormous impact on the final absorption and organ distribution of Cd. Although most toxicity studies have been performed with inorganic Cd, this is clearly not the chemical form in which Cd occurs in the diet. In animals one of the main Cd-binding ligands is the protein metallothionein, an inducible, cysteine-rich protein of low molecular weight. In plants the main Cd-binding ligands are phytochelatins, proteins wich are functionally analogous to metallothioneins. Metallothioneins can survive, at least partially, the gastro-intestinal digestion. It has been suggested that some of the exogenous Mt passes the intestinal barrier and reaches the kidneys directly. Since CdMt is a potent nephrotoxic agent when injected intravenously, this might indicate that dietary CdMt gives more reason for health concern than inorganic Cd. However, information on the bioavailibility and toxicity of dietary CdMt is currently lacking. Therefore the objectives of this thesis were:

- To compare toxicity of inorganic Cd and CdMt in rats and in cell cultures of target organs,
- To compare the dose-dependent kinetics of Cd-uptake from CdMt and inorganic Cd.
- To establish differences in metabolic pathways between CdMt and $CdCl_2$ to predict disposition and toxicity at environmentally relevant doses.

The studies described in **Chapter 2** and **Chapter 3** examined the toxicity and disposition in rats fed diets containing either pig's liver incorporated Cd or cadmium salt for 4 weeks. For a meaningful interpretation of the results the cadmium-binding ligand in pig's liver was first identified as a mixture of two metallothionein iso forms. The identification was based on molecular weight, Cd-binding properties, heat-stability and spectral analysis. It appeared that over 90% of the Cd present in the pig's liver was bound to metallothionein. It was shown that signs of toxi-city (e.g. anemia and hepatotoxicity) were less pronounced in rats exposed to 30 ppm Cd as CdMt than in rats exposed to a similar dose of inorganic Cd-salt. *The fact that CdMt is less toxic than $CdCl_2$ correlates well with the finding that the intestinal and hepatic uptake of Cd after exposure to 30 ppm Cd as CdMt was lower than after exposure to 30 ppm Cd as $CdCl_2$. However, in spite of the lower total uptake of CdMt, the renal Cd accumulation from CdMt was relatively higher than from $CdCl_2$.* Thus, the kidney to liver ratio of Cd concentration was significantly higher in rats fed CdMt than in the rats fed $CdCl_2$. Since it is well known that CdMt after intravenous administration preferentially accumulates in the kidneys, the results might suggest that CdMt can pass the intestinal barrier and is released in the systemic circulation. This was supported by the fact that the metallothionein concentration in the kidneys in the first week of the experiment was higher after exposure to CdMt than after exposure to $CdCl_2$. However in the introduction (Chapter 1) we have emphasized that rats exposed to low doses of $CdCl_2$ show, similar to dietary CdMt, a selective renal Cd accumulation, which was indicated by their high kidney/liver Cd ratio. Indeed, the study of chapter 3 revealed that at lower dietary Cd levels (1.5 and 8 ppm), relatively more Cd is deposited in the kidneys than at the 30 ppm level. *The difference between the differential disposition of $CdCl_2$ and CdMt between kidneys and liver was less pronounced at the lower doses, but even at these doses the kidney/liver ratio of Cd is still higher with CdMt than with $CdCl_2$.* In the discussion the question was raised of what the impact of Cd redistribution from liver to kidneys will be during long-term oral intake in the final level of renal intoxication. This question was adressed to in chapter 8.

Chapter 4 presents a comparative study on the accumulation and toxicity of $CdCl_2$ and two isoforms of CdMt in cell lines and primary

cultures of the intestine, liver and kidneys. Exogenous CdMt added to the culture medium was much less toxic to all cells tested than $CdCl_2$ and no cell type was specifically sensitive to CdMt. Even when the Cd-content of CdMt was artificially raised (5 mol Cd/mol Mt), the cells were not affected by CdMt treatment. *In general the difference in cytotoxicity between CdMt and $CdCl_2$ corresponds well with the difference in cellular uptake of Cd. This is in agreement with the in vivo results described in chapter 2, 3 and 8, showing a difference in toxicity between CdMt and $CdCl_2$ due to a difference in uptake and organ distribution.*

The studies described in **Chapter 5** and **Chapter 6** deal with the influence of minerals on the Cd absorption from $CdCl_2$ and CdMt. Most investigations have focused on single Cd-mineral interactions, and no real attemps have been made to compare the actual mineral status of other trace elements in the same study. Eight minerals were taken into account, all of which had been suggested to interact with the Cd accumulation in the body. The mineral combinations were chosen such that the effect of individual components could be analysed with multiple analyses of variance. The protection against Cd-accumulation and toxicity by mineral combinations was mainly due to the presence of iron (Fe). The question whether the protective effect of iron also applies for organic Cd, was examined in the study of chapter 6. Rats were fed CdMt supplemented with a mineral mixture of Ca/P, Zn and Fe which according to the observations in chapter 5 was the most effective against Cd accumulation. The total uptake of Cd from CdMt was significantly decreased although the protection of the mineral mixture was lower than for $CdCl_2$. Since the Cd accumulation is mainly influenced by Fe and not by other minerals, the studies of chapter 5 and 6 raise the question whether the intestinal transport of Cd in rats is specifically mediated by the Fe transfer system. The fact that the mineral supplement also protects against CdMt, albeit less pronounced than against $CdCl_2$ might be explained by the partial degradation of CdMt in the gastrointestinal tract. Once degraded, Cd from CdMt will show the same metabolic behaviour as inorganic Cd-salt and the uptake in liver and kidneys will be decreased by mineral suppletion. However a small fraction of the ingested CdMt is not degraded and it seems that the mineral supplement does not affect the absorption of the intact exogenous CdMt, which will then reach the kidneys unhampered. This explains why the kidney/liver ratio of Cd concentration was higher in the rats fed a CdMt diet supplemented with Fe in comparison to rats fed a $CdCl_2$ diet supplemented with Fe. *After addition of the mineral supplement, rats fed $CdCl_2$ or CdMt showed relatively more Cd accumulation in the kidneys than in the liver. Since addition of the mineral supplement decreases the Cd absorption, it will prevent to some extent the overload of the intestinal metallothionein pool (see chapter 3) and as a consequence the kidney/liver ratio will increase.*

Chapter 7 deals with the metabolic fate of endogenous (synthesized by tissue) and exogenous (taken up by tissue) CdMt. Purified Mt isoform 1 and 2 from liver and intestine eluted as two single peaks on RP-HPLC. However, both kidney isoforms eluted as one single peak. The fact that renal Mt isoform-2 was not separated on HPLC and the fact that intestinal Mt-isoform 2 is hardly present in control rats, offers the unique possiblity to study the metabolic fate of purified CdMt isoform 1 in intestine and kidneys. A rapid exchange of ^{109}Cd from exogenous CdMt (=hepatic $^{109}CdMt$ isoform 1) towards endogenous CdMt was shown in the kidney and the intestinal mucosa after in vivo and in vitro addition of exogenous CdMt. *The Cd redistribution was dependent on the ratio between the endogenous and exogenous CdMt concentration.* Only if the oral or intravenous exposure to exogenous CdMt is high enough to lead to high exogenous/endogenous CdMt ratio, the ^{109}Cd remains principally bound to exogenous CdMt in the intestinal mucosa and kidney. *If we consider that at environmentally relevant Cd doses (less than 2 ppm in the diet) the exogenous CdMt concentration is much lower than the endogenous CdMt content, this implies that at low dietary CdMt doses Cd will mainly be bound to endogenous, intestinal CdMt and other (high molecular weight) Cd binding proteins.*

Finally, a long-term feeding study was performed and the results are given in **Chapter 8.** It was shown that the kidney/liver ratio of Cd concentration was inversely proportional with dose. Thus as the oral dosage increased, relatively more Cd was found in the liver than in the kidneys. At the dietary concentration of 30 and 90 ppm the Cd ratio was higher in rats fed

CdMt than in rats fed $CdCl_2$. However, at low dietary concentrations (0.3 and 3 ppm) the difference in renal disposition between $CdCl_2$ and CdMt did not occur. The first signs of renal injury indicated by slight signs of enzymuria were seen in rats exposed to $CdCl_2$. Gradually the effects became more severe and histopathological changes were observed (i.e. glomerulonephrosis and an increase in basophilic tubules). The finding that 90 ppm $CdCl_2$ induced slight renal dysfunction whereas 90 ppm CdMt does not can be explained by the fact that the renal Cd concentration after 10 months was much lower for CdMt than for $CdCl_2$ (60 mg/kg tissue and 170 mg/kg tissue respectively). *These findings imply that in contrast to intravenous administered CdMt and $CdCl_2$ renal effects of oral administered CdMt and $CdCl_2$ are dependent on the difference in degree of Cd accumulation in the tissue. Thus, there is no indication that CdMt in spite of its higher kidney/liver Cd ratio at high oral doses is more nephrotoxic than $CdCl_2$.*

To discuss the **consequences for human consumer** it will be essential to take into account all metabolic routes. Figure 1 shows in a simplified model the major metabolic routes of Cd after oral intake of $CdCl_2$ or CdMt. The model is based on the results of this thesis together with available information from the literature. Fig 1A depicts the metabolic pathways after oral exposure to $CdCl_2$. At low oral $CdCl_2$ doses the majority of the Cd will be bound to the endogenous metallothionein of the intestine, which will be released in the systemic circulation and then be deposited in the kidneys. At a high oral $CdCl_2$ dose the available endogenous intestinal Mt pool is overloaded and Cd will be bound to high molecular plasma proteins and then be deposited in the liver. Cd will be bound to hepatic CdMt and with time some of the hepatic CdMt will reach the kidneys.

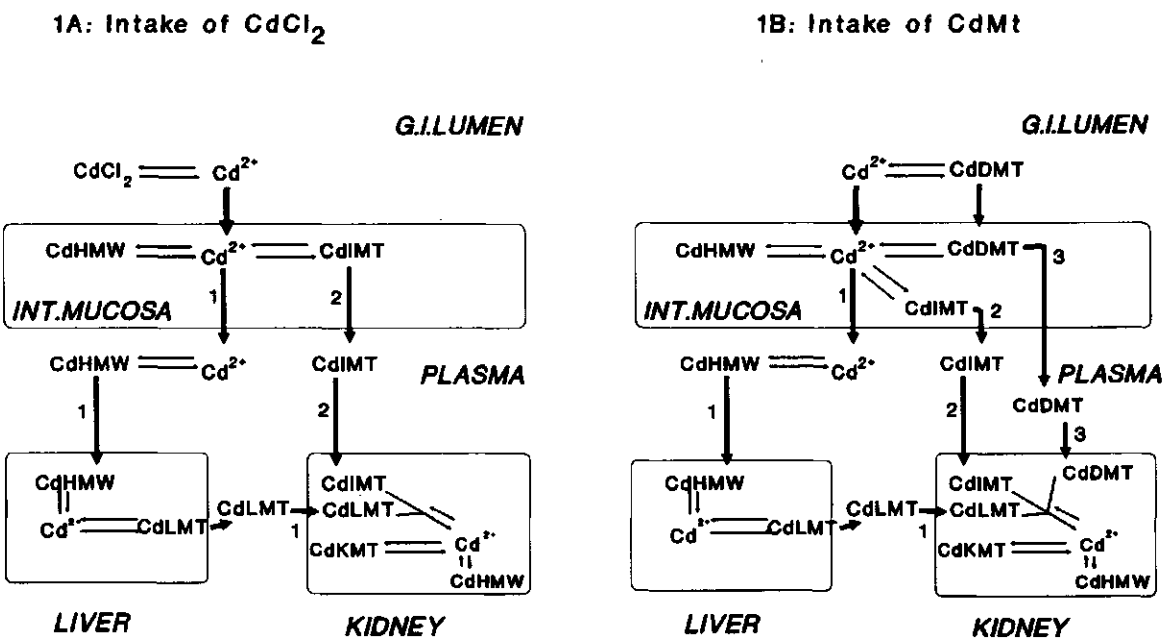

Fig. 1. Simplified presentation of metabolic pathways for $CdCl_2$ and CdMt after oral exposure in rodents as concluded from the studies in this thesis. Arrows marked with a number represent metabolic routes. G.I.LUMEN = gastro-intestinal lumen, INT.MUCOSA=Intestinal mucosa, DMT=Dietary MT, IMT= Intestinal MT, LMT =hepatic MT, KMT= renal MT, HMW= High molecular weight proteins. 1A. Oral exposure to $CdCl_2$: At low dose; route 1< route 2, at high dose; route 1> route 2. 1B. Oral exposure to CdMt: At low dose; route 2> route 1 and route 3 is not present, at high dose; route 1> route 3> route 2.

Fig. 1B. shows the metabolic routes of Cd after oral exposure to CdMt. A large part of the CdMt will not survive the gastro-intestinal digestion and "liberated" Cd will show the same metabolic behaviour as Cd-salts. This explains why Fe interacts with the uptake of CdMt. Moreover at low dietary CdMt doses, the Cd from dietary CdMt which has reached the intestinal mucosa, is quickly redistributed towards endogenous CdMt. This implies that at environmentally relevant Cd doses the transport form of $CdCl_2$ and CdMt across the intestinal wall is similar (cf. Fig 1A). The similar Cd disposition observed for both $CdCl_2$ and CdMt in chapter 8 after long term exposure to low (< 3 ppm) dietary doses is in agreement with this theory. At a high oral dose of CdMt relatively more CdMt will survive the gastro-intestinal digestion and more CdMt will reach the intestine intact. In this case it is plausible that exogenous CdMt is present in excess and Cd will remain bound, at least initially, to the dietary CdMt in the intestinal mucosa. Subsequently, the dietary CdMt will, similar to endogenous CdMt, be released in the systemic circulation and taken up in the kidneys. Thus at high oral Cd doses the metabolic routes of $CdCl_2$ and CdMt will differ.

The critical renal Cd concentration used in the risk assessment of Cd is in part based on laboratory animal experiments with inorganic Cd-salts. However, man is generally exposed to low dietary levels of organic Cd. In terms of risk assessment for the compound CdMt, the present study shows that at low dietary Cd doses, Cd is released from dietary CdMt after passage through the intestinal tract and uptake in the intestinal mucosa. This implies that at environmentally relevant oral Cd doses there is no difference in metabolic pathways between Cd-salt and CdMt. At high doses the metabolic pathways for both Cd-forms slightly differ, but the total Cd-uptake from CdMt at these levels was lower than for $CdCl_2$.

Although CdMt is more nephrotoxic than inorganic Cd after parenteral administration, we found no evidence that CdMt is more nephrotoxic than $CdCl_2$ after oral administration.

Therefore this thesis shows that toxicity data obtained from studies with rodents exposed to low levels of $CdCl_2$ are also applicable for the risk assessment of Cd-intake from CdMt. Another important finding was that Cd accumulation from organic and inorganic Cd is mainly influenced by Fe and not by other minerals. Special consideration should therefore be given to an adequate Fe intake when assessing the health risk of the Cd intake.

SAMENVATTING EN CONCLUSIES

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In **Hoofdstuk 1** van dit proefschrift wordt een algemene inleiding gepresenteerd met hierin een overzicht van de literatuur.

Kort samengevat, de belangrijkste bron van Cd-blootstelling via het milieu voor niet-rokers is voedsel. Orale Cd-studies met knaagdieren hebben aangetoond dat de Cd-dosis, de Cd-speciatie en de mineralen samenstelling van het dieet een enorme invloed hebben op de uiteindelijke absorptie en orgaan distributie van Cd.

Hoewel de meeste toxiciteitsstudies zijn uitgevoerd met anorganisch Cd, is dit niet de chemische vorm waarin Cd aanwezig is in het voedsel. Een van de belangrijkste Cd-bindende liganden in dieren is het eiwit metallothioneïne, een induceerbaar en cysteïne-rijk eiwit met een laag moleculair gewicht. De belangrijkste Cd bindende ligande in planten is het phytochelatine, een eiwit dat functioneel analoog is aan metallothioneïne. Het eiwit metallothioneïne is in staat om tenminste gedeeltelijk intact de maag-darm passage te overbruggen. Gesuggereerd is dat een gedeelte van dit exogene Mt de darm-barriere passeert en vervolgens rechtstreeks in de nier wordt opgenomen. Deze selectieve ophoping van CdMt in de nier vindt ook plaats wanneer CdMt intraveneus wordt toegediend. Omdat CdMt sterk nephrotoxisch is wanneer het intraveneus wordt toegediend, kan dit betekenen dat de aanwezigheid van CdMt in ons voedsel een groter gezondheidsrisico vormt dan tot nu toe voor Cd wordt aangenomen op basis van orale experimenten met anorganisch Cd. Echter er is nauwelijks iets bekend over de biologische beschikbaarheid en de toxiciteit van CdMt, zoals aanwezig in ons voedsel. De doelstellingen van dit proefschrift waren derhalve:

- Het vergelijken van de toxiciteit van anorganisch Cd en CdMt in de rat en in celcultures van de doelwitorganen.
- Het vergelijken van de dosis-afhankelijke kinetiek van Cd opname tussen anorganisch Cd en CdMt.
- Het vaststellen van verschillen in metabole routes tussen CdMt en $CdCl_2$ om zodoende de dispositie en toxiciteit te kunnen voorspellen bij relevante dosis niveau's in het milieu.

In de studies die in **Hoofdstuk 3** en **4** worden beschreven, wordt de toxiciteit en dispositie bestudeerd in ratten, die gedurende 4 weken gevoerd werden met een dieet waarin ofwel varkenslever-geincorporeerd Cd aanwezig was ofwel Cd zout. Voor een juiste interpretatie van de resultaten werd de Cd bindende ligande in de varkenslever allereerst geidentificeerd als een mengsel van 2 metallothioneïne isovormen. Deze identificatie was gebaseerd op het molecuul gewicht, de Cd-bindende eigenschappen, de hitte-resistentie en de spectrale analyse. Meer dan 90% van het Cd in de varkenslever bleek gebonden te zijn aan dit metallothioneïne.

Aangetoond werd dat de toxiciteitsverschijnselen (o.a. anemia en hepatotoxiciteit) minder ernstiger waren in de ratten blootgesteld aan CdMt dan in de ratten blootgesteld aan een vergelijkbare dosis anorganisch Cd. *De lagere toxiciteit na blootstelling aan CdMt in vergelijking met $CdCl_2$, correleert goed met het feit dat de opname van Cd in de darm en lever na blootstelling aan 30 ppm Cd in de vorm van CdMt lager was dan na blootstelling aan 30 ppm Cd in de vorm van $CdCl_2$. Echter, ondanks de lagere totale opname van CdMt, was de Cd stapeling in de nier relatief hoger na inname van CdMt dan na inname van $CdCl_2$.* Dus de verhouding van de Cd concentratie tussen nier en lever was significant hoger in ratten die gevoerd waren met CdMt dan in de ratten, die gevoerd waren met $CdCl_2$. Omdat het bekend is dat CdMt na intraveneuze toediening voornamelijk ophoopt in de nieren, zouden de resultaten suggereren dat CdMt de darm barriere kan passeren en vervolgens wordt afgegeven aan de systemische circulatie. Dit werd ondersteund door het feit dat de Mt concentratie in de nieren in de eerste week van het experiment hoger was na blootstelling aan CdMt dan na blootstelling aan $CdCl_2$. Echter, in de inleiding hebben we benadrukt dat ratten die zijn blootgesteld aan lage dosis $CdCl_2$, net als na blootstelling aan CdMt, een selectieve accumulatie van Cd vertonen in de nier. De studie van hoofdstuk 3 laat inderdaad zien dat bij lage Cd levels in het dieet (1.5 en 8 ppm) relatief meer Cd in de nier terechtkomt dan bij een hoge orale dosis van 30 ppm. *Het verschil in de*

differentiele dispositie van $CdCl_2$ en CdMt tussen lever en nier was kleiner bij lage doses, maar zelfs bij die doses is de verhouding tussen de Cd concentratie in nier en lever nog steeds hoger na CdMt dan na $CdCl_2$ blootstelling. In de discussie werd de vraag opgeroepen welk gevolg de Cd redistributie vanuit de lever naar de nieren na langdurige orale blootstelling zou hebben voor het uiteindelijk niveau van nierschade dat bereikt zal gaan worden. Deze vraag werd behandeld in hoofdstuk 8.

Hoofdstuk 4 behandelt een vergelijkende studie van de accumulatie en toxiciteit van $CdCl_2$ en 2 isovormen van CdMt in cellijnen en primaire celcultures van de dunne darm, lever en nier.
Exogeen CdMt toegevoegd aan het kweek medium was minder toxisch voor alle geteste celtypen dan $CdCl_2$ en geen celtype was speciaal gevoelig voor CdMt. Zelfs als het Cd-gehalte van CdMt kunstmatig werd verhoogd naar 5 mol Cd/mol Mt, beinvloedde dit de viabiliteit van de cellen maar nauwelijks. *In het algemeen correspondeert het verschil in toxiciteit tussen CdMt en $CdCl_2$ goed met het verschil in cellulaire opname van Cd. Dit is in overeenstemming met de in vivo resultaten zoals beschreven in hoofstuk 2, 3 en 8 waarin is aangetoond dat het verschil in toxiciteit tussen CdMt en $CdCl_2$ een gevolg is van het verschil in opname en orgaan distributie.*

De studies beschreven in **Hoofdstuk 5** en **6** behandelen de invloed van mineralen op de absorptie van Cd na blootstelling aan $CdCl_2$
of CdMt. De meeste onderzoeken in de literatuur zijn tot nu toe steeds gericht op enkelvoudige Cd-mineraal interacties en er zijn geen echte pogingen gedaan om meer elementen uit de mineralen-huishouding mee te vergelijken in dezelfde studie. Acht mineralen werden onder loep genomen, waarvan gesuggereerd was dat ze interactie vertonen met de Cd accumulatie in het lichaam. De mineralen combinaties werden zo gekozen dat het effect van de individuele componenten geanalyseerd kon worden met behulp van multi-variantie analyses. De bescherming tegen de accumulatie en toxiciteit van Cd ten gevolge van mineralen mengsels bleek voornamelijk te danken te zijn aan de aanwezigheid van ijzer (Fe). De vraag of ijzer ook beschermt tegen de opname van organisch Cd, werd onderzocht in de studie beschreven in hoofdstuk 6. Ratten werden hierbij gevoerd met CdMt aangevuld met een extra mineralen mengsel bestaande uit Ca/P, Zn en Fe. Dit mengsel was volgens de bevindingen van hoofdstuk 5 het meest effectief tegen Cd accumulatie. De totale Cd opname na blootstelling aan CdMt was significant verlaagd door de aanwezigheid van het mineralen supplement, hoewel de bescherming van het supplement lager was dan na blootstelling aan $CdCl_2$. Omdat de Cd accumulatie voornamelijk beinvloed wordt door Fe en niet door andere mineralen, roept het onderzoek van hoofdstuk 5 en 6 de vraag op in hoeverre het transport van Cd over de dunne darm specifiek gemedieerd wordt door het transport systeem voor Fe. Het feit dat het mineralen supplement ook beschermd tegen CdMt, hoewel minder dan tegen $CdCl_2$ zou verklaard kunnen worden door het feit dat CdMt voor een gedeelte wordt afgebroken gedurende de maag-darm passage. Eenmaal gedegradeerd zal Cd uit CdMt hetzelfde metabole gedrag vertonen als anorganisch Cd zout en zal door toevoeging van extra mineralen de accumulatie in lever en nieren verlaagd worden. Echter een klein gedeelte van het opgenomen CdMt wordt niet afgebroken en het lijkt dat het mineralen supplement de opname van dit intact exogeen CdMt niet beïnvloedt waardoor dit CdMt ongehinderd de nieren kan bereiken. Dit verklaart waarom de verhouding tussen de Cd concentratie in de nier en lever hoger was in ratten die gevoerd werden met CdMt en Fe dan in ratten die gevoerd werden met $CdCl_2$ en Fe. *Na toevoeging van het mineralen mengsel vertonen de ratten, die gevoerd werden met $CdCl_2$ of CdMt een relatief hogere Cd accumulatie in de nier dan in de lever. Omdat de toevoeging van het mineralen suplement aan het dieet tot een lagere Cd absorptie leidt, zal dit gedeeltelijk voorkomen dat de hoeveelheid metallothioneine, die voorradig is in de dunne darm mucosa, wordt overladen. Als gevolg steeg de nier/lever Cd ratio.*

Hoofdstuk 7 behandelt het metabole lot van endogeen (gesynthetiseerd door weefsel) en exogeen (geabsorbeerd door weefsel) CdMt. Gezuiverde Mt isovorm 1 en 2 afkomstig uit darm en lever elueren als 2 afzonderlijke pieken op RP-HPLC. De beide isovormen uit de nier elueren echter als één piek. Het feit dat in de nier Mt isovorm-2 niet kon worden gescheiden van isovorm 1 en het feit dat Mt-isovorm 2
nauwelijks aanwezig is in de darm, biedt de unieke mogelijkheid om het metabole lot van gezuiverde Mt-isovorm 1 te bestuderen in de darm en in de nier. Na in vivo en in vitro toe-

diening van exogeen CdMt-1 werd in de nier en darm een snelle uitwisseling aangetoond van ^{109}Cd van exogeen CdMt (=lever $^{109}CdMt$ isovorm 1) naar endogeen CdMt. De Cd redistributie was afhankelijk van de verhouding tussen de endogene en exogene Mt concentratie. Alleen wanneer de orale of de intraveneuze blootstelling aan exogeen CdMt hoog genoeg is om tot een hoge ratio tussen exogeen en endogeen CdMt te leiden, zal ^{109}Cd in principe aan CdMt gebonden blijven.
Als we er vanuit gaan dat bij milieu-relevante blootstellingsniveau's (minder dan 2 ppm in het voedsel) de exogene CdMt concentratie veel lager is dan de endogene CdMt concentratie dan betekent dit dat bij lage CdMt doseringen via het voedsel, Cd voornamelijk gebonden zal zijn aan endogeen Mt en andere (hoog moleculaire) Cd bindende eiwitten.

Tenslotte werd in dit project een langdurige voedingsstudie uitgevoerd waarvan de resultaten in **Hoofdstuk 8** zijn weergegeven. Aangetoond werd dat de nier/lever ratio van de Cd concentratie omgekeerd evenredig is met de dosis. Dus, wanneer de orale dosis toeneemt, zal relatief meer Cd in de lever worden teruggevonden dan in de nieren. Bij een Cd-dieet concentratie van 30 en 90 ppm was de Cd ratio hoger bij de dieren, die blootgesteld waren aan CdMt dan bij dieren die waren blootgesteld aan $CdCl_2$. Echter bij lage Cd concentraties in het dieet (0.3 en 3 ppm) is er geen verschil in Cd stapeling in de nier tussen $CdCl_2$ en CdMt. De eerste verschijnselen van nier toxiciteit, aangegeven door een lichte vorm van enzymuria werden waargenomen in ratten die 4 maanden waren blootgesteld aan 90 ppm $CdCl_2$. Geleidelijk werden de effecten ernstiger en kon ook histopathologische veranderingen worden waargenomen (o.a. glomerulonephrosis en een toename in basofiele tubuli). De bevinding that 90 ppm $CdCl_2$ aanleiding geeft tot duidelijke verschijnselen van nierdysfunctie terwijl 90 ppm CdMt dat minder laat zien, kan verklaard worden door het feit dat de Cd concentratie in de nier lager was na 10 maanden blootstelling aan CdMt (60 mg/kg weefsel bij 90 ppm CdMt en 170 mg/kg bij 90 ppm $CdCl_2$). *Deze resultaten betekenen dat in tegenstelling tot intraveneus toegediend $CdCl_2$ of CdMt, de effecten op de nier van oraal toegediend CdMt of $CdCl_2$ voornamelijk afhankelijk zijn van het verschil in Cd accumulatie in het weefsel. Dus er is geen aanwijzing dat CdMt ondanks de hogere nier/lever Cd ratio bij hoge doseringen meer niertoxisch is dan $CdCl_2$.*

Om de **consequenties voor de humane consument** goed te kunnen inschatten is het essentieel om met alle metabole routes rekening te houden. Figuur 1 toont in een vereenvoudigd schema de belangrijkste metabole routes van Cd na orale blootstelling aan $CdCl_2$ of CdMt. Het model is gebaseerd op de resultaten van dit proefschrift samen met beschikbare gegevens uit de literatuur. Fig. 1A toont de metabole routes na orale blootstellling aan $CdCl_2$. Bij een lage orale dosis van $CdCl_2$ zal het merendeel van het Cd gebonden worden aan endogeen Mt in de dunne darm. Dit Mt komt terecht in de systemische circulatie en wordt vervolgens opgenomen in de nieren. Bij blootstelling aan een hoge orale dosis aan $CdCl_2$ wordt de beschikbare voorraad endogeen Mt overladen. Cd zal dan ook gebonden worden aan hoog moleculaire plasma eiwitten en komt vervolgens in lever terecht. Cd zal worden gebonden aan Mt in de lever en mettertijd zal een gedeelte van het CdMt uit de lever in de nieren terecht komen.
Fig. 1B toont de metabole routes voor Cd na orale blootstelling aan CdMt. Een groot gedeelte van het CdMt zal de maag-darm passage niet overleven en het vrij gekomen Cd zal hetzelfde metabole gedrag vertonen als de Cd zouten. Dit verklaart waarom Fe interactie vertoont met de opname van CdMt. Bovendien zal bij lage CdMt levels in het dieet snel sprake zijn van redistributie van Cd vanuit exogeen CdMt naar endogeen Mt. Dit betekent dat bij relevant blootstellings niveau's in het milieu de Cd-transportvorm voor CdMt gelijk zal zijn aan $CdCl_2$. Deze theorie wordt ondersteund door het feit dat de Cd dispositie voor beide Cd vormen vergelijkbaar was in hoofdstuk 8 na langdurige blootstelling aan lage (< 3ppm) dosis in het dieet. Bij een hoge orale dosis aan CdMt zal relatief meer CdMt de vertering overleven en zal meer CdMt de dunne darm intact bereiken. In dat geval is het mogelijk that er een overmaat exogeen CdMt in de darm mucosa aanwezig is en dat Cd aanvankelijk gebonden blijft aan exogeen CdMt. Vervolgens zal dit CdMt afkomstig uit het voedsel worden afgegeven aan de systemische circulatie en vandaaruit de nier bereiken. Dus bij een hoge orale Cd dosis zullen de metabole routes voor $CdCl_2$ en CdMt verschillen. *De kritische Cd concentratie in de nier, die gebruikt wordt in de risico evaluatie van Cd, is gedeeltelijk gebaseerd op*

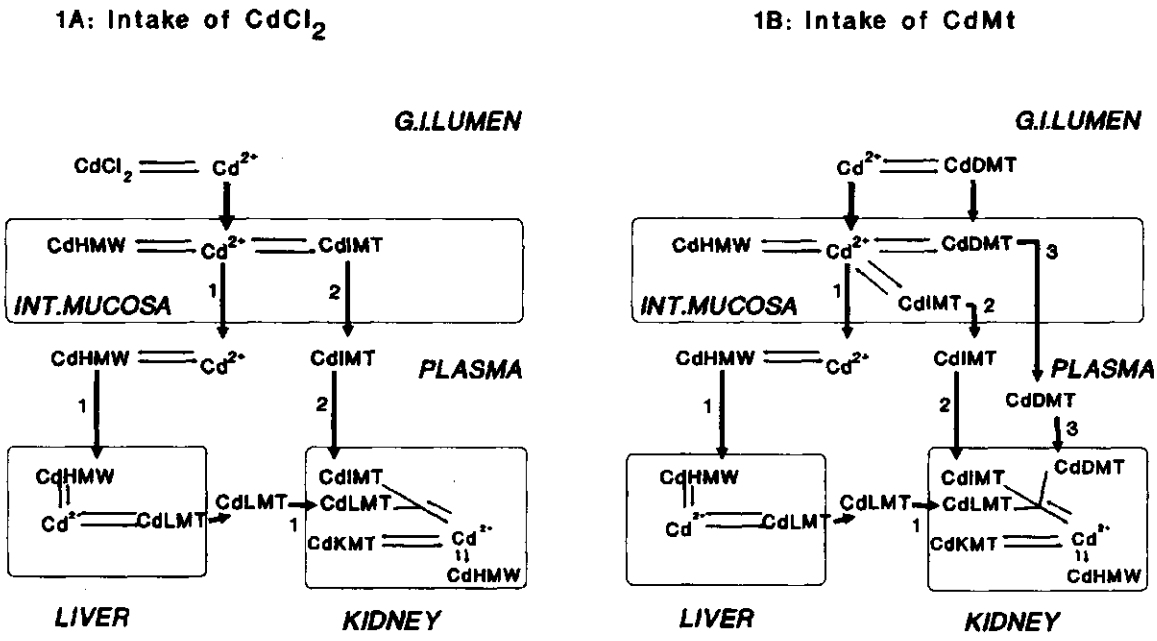

Fig. 1 Vereenvoudigde weergave van metabole routes voor $CdCl_2$ en CdMt na orale blootstelling in knaagdieren zoals geconcludeerd uit de studies van dit proefschrift. De pijlen die voorzien zijn van een nummer geven de verschillende metabole routes weer. G.I.LUMEN= lumen van maag/darm, INT.MUCOSA= Dunne darm mucosa, IMT=Mt dunne darm, LMT=lever Mt, KMT=nier Mt, HMW=eiwit van hoog moleculair eiwit. 1A. Orale blootstelling aan $CdCl_2$: Bij lage dosis; route 1< route 2, bij hoge dosis; route 1> route 2. 1B Orale blootstelling aan CdMt: Bij lage dosis; route 2> route 1 en route 3 is niet aanwezig, bij hoge dosis; route 1> route 3> route 2.

proefdier experimenten met een hoge dosis aan $CdCl_2$. Echter, de mens wordt voornamelijk blootgesteld aan een lage dosis organisch Cd. In termen van risico-evaluatie voor de verbinding CdMt kan met behulp van dit proefschrift de conclusie worden getrokken dat bij een lage Cd dosis in het dieet Cd wordt vrijgemaakt uit CdMt gedurende de maag-darmpassage en na opname in de darm mucosa. Dit betekent dat bij relevante orale doses vanuit de omgeving, er geen verschil bestaat in metabole routes tussen CdMt en $CdCl_2$. Bij een hoge dosis zullen de metabole routes voor beide Cd-vormen weliswaar iets verschillen, maar de totale Cd-opname na inname van CdMt is bij die dosis lager dan na inname van $CdCl_2$.

Hoewel na parenterale toediening CdMt meer nephrotoxisch is dan anorganisch Cd, hebben wij geen aanwijzingen gevonden dat oraal toegediend CdMt ook meer nephrotoxisch zou zijn dan $CdCl_2$. Dit proefschrift laat derhalve zien dat de toxiciteits gegevens, die gebaseerd zijn op studies met knaagdieren, blootgesteld aan lage doseringen $CdCl_2$, ook toepasbaar zijn voor de risico-evaluatie na Cd blootstelling via CdMt. Een andere belangrijke bevinding is het feit dat de Cd accumulatie van organisch en anorganisch Cd voornamelijk wordt beïnvloed door Fe en niet door andere mineralen. Derhalve dient speciale aandacht te worden gegeven aan de Fe opname wanneer we het gezondheidsrisico van de inname van Cd op een juiste wijze willen inschatten.

DANKWOORD

Eind 1987 werd ik op de op SETAC meeting in Florida op een voor mij onverklaarbare wijze zeer geboeid door een aantal voordrachten die handelden over rol van metallothioneine binnen de ecotoxicologie van zware metalen. Tot dan toe was het terrein van de zware metalen ergens in een donker hoekje in mijn hersenen weggemoffeld. Dit moest een voorteken zijn, want een dag na terugkomst van de SETAC werd ik in Bellingham opgebeld en polste men mijn interesse voor een cadmium project. En zie daar, met wat water en wat extra mest ontlook uit die donkere hoek een mooie bloem. Vanaf nu zijn de zware metalen op de grijze massa van mijn cortex ingepriemd en vormen een kleine gazon dat niet meer zal/mag verdrogen...
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CURRICULUM VITAE

Johan Peter (John) Groten werd geboren op 18 juni 1963 te Kerkrade. In 1981 behaalde hij het diploma gymnasium ß aan het Gymnasium Rolduc te Kerkrade en in datzelfde jaar begon hij zijn studie Biologie aan de Landbouwuniversiteit te Wageningen. In de doctoraalfase bewerkte hij drie hoofdvakken: Experimentele Pathologie aan de Rijksuniversiteit te Utrecht, Toxicologie bij de Lanbouwuniversiteit en bij het RIKILT te Wageningen en tenslotte Celbiologie wederom bij de Landbouwuniversiteit te Wageningen. Na de doctoraalfase verrichtte hij tijdens zijn stage gedurende 7 maanden onderzoek bij het Institute of Wildlife Toxicology van de Western Washington University in Bellingham, Washington, USA. De biologie studie werd in april 1988 cum laude afgerond.
In maart 1988 begon hij als toegevoegd onderzoeker bij de vakgroep Toxicologie van de Landbouwuniversiteit te Wageningen aan het in dit proefschrift beschreven onderzoek. Het leeuwendeel van dit onderzoek werd verricht als gastmedewerker van de afdeling Biologische Toxicologie van het TNO Instituut voor Toxicologie en Voeding te Zeist. Vanaf maart 1992 is hij als toxicoloog in dienst bij TNO-Voeding te Zeist.

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