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MECHANISM OF MANGANESE-INDUCED TOLERANCE TO CADMIUM LETHALITY AND HEPATOTOXICITY*

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Abstract—Pretreatment with Mn^{2+} is known to produce tolerance to Cd^{2+}-induced lethality. This study was designed to determine the mechanism of tolerance to Cd^{2+}-induced lethality and hepatotoxicity following Mn^{2+} pretreatment. Rats given 36 μmoles Cd^{2+}/kg, i.v., died within 10–20 hr while only one of nine rats pretreated with Mn^{2+} (250 μmoles/kg, s.c., 48 and 24 hr prior to Cd^{2+} challenge) died. Ten hours after Cd^{2+}, plasma aspartate aminotransferase and sorbitol dehydrogenase activities were elevated markedly, and extensive histopathologic lesions of the liver were evident in control rats but not in Mn^{2+}-pretreated rats. To examine the mechanism of this tolerance, distribution of Cd^{2+} to fourteen organs and the subcellular distribution in six organs were determined in control and Mn^{2+}-pretreated rats. Two hours after challenge (31 μmoles Cd^{2+}/kg, i.v., 0.75 μCi $^{109}Cd^{2+}$/μmole Cd^{2+}), the distribution of Cd^{2+} to liver markedly increased after Mn^{2+} pretreatment with concomitant decreases in other tissues. Mn^{2+} pretreatment also resulted in a marked difference in the hepatic subcellular distribution of Cd^{2+} with more present in cytosol and less associated with organelles. Gel-filtration chromatography indicated that most cytosolic Cd^{2+} was bound to a low molecular weight protein. Isolation and partial characterization of this protein suggest that it is identical to metallothionein (MT); it had a similar relative elution following gel-filtration chromatography, had low absorbance at 280 nm and, after separation into two isoproteins by DEAE A-25 anion exchange chromatography, had the same mobility after electrophoresis on non-denaturing polyacrylamide gels as Cd^{2+}-induced metallothioneins. These data suggest that Mn^{2+} pretreatment reduces Cd^{2+}-induced hepatotoxicity by altering the hepatic subcellular distribution of Cd^{2+} with more Cd^{2+} binding to MT in the cytosol. This decreased hepatotoxicity is probably responsible for tolerance to Cd^{2+}-induced lethality.

Tolerance to cadmium (Cd^{2+}) toxicity following pretreatment with various metals has been widely reported [1–6]. The mechanism of tolerance is most likely due to an altered intracellular distribution of Cd^{2+} in pretreated animals, such that more metal is bound to metallothionein (MT) in the cytosol, the synthesis of which is induced in major organs by various metals [7, 8], and subsequently rendered less toxic. This mechanism has been shown to be responsible for tolerance to Cd^{2+}-induced hepatotoxicity following Cd^{2+} [6, 9] and zinc (Zn^{2+}) [10] pretreatment. Pretreatment with manganese (Mn^{2+}) also protects against a lethal dose of Cd^{2+} [3]; however, while it has been reported that Mn^{2+} induces MT in liver [8, 11], others report that Mn^{2+} is an ineffective inducer of MT [7], and thus it is not known if Mn^{2+} protects by the same mechanism.

The concentration of MT in liver is increased by exposure to Cd^{2+} in rats [12, 13], chickens [14], rabbits [15] and mice [16]. In addition, the proteins induced by zinc, cadmium, mercury, silver and gold in rat liver and kidney have been shown to be MTs [12, 13, 17, 18]. Other metals, such as Mn^{2+} [11], increase formation of low molecular weight metal-binding proteins which are generally assumed to be MT. The protein induced in rat liver by copper is an MT but differs from Cd^{2+}, Zn^{2+}-MT by a number of properties [19]. The Cd^{2+}-binding protein present in molluscs following Cd^{2+} exposure is also different than mammalian MT [20]. Waalkes *et al.* [21] have shown that a low molecular weight Cd^{2+}-binding protein in rat testes assumed to be MT is not MT. Due to the multiplicity of metal-induced low molecular weight proteins, characterization of these proteins induced by other metals is necessary.

Therefore, the purpose of this investigation was (1) to determine the mechanism of tolerance to Cd^{2+}-induced lethality and hepatotoxicity following pretreatment with Mn^{2+}, and (2) to isolate and characterize the low molecular weight Cd^{2+}-binding protein induced by Mn^{2+} pretreatment and compare it to MT.

MATERIALS AND METHODS

Animals. Adult, male Sprague–Dawley rats (Sasco, Omaha, NB, 250–350 g) were housed in plastic cages in groups of four and were exposed to a 12-hr light/dark cycle in an environmentally-controlled room at 23–25°. Food (Purina Laboratory Rodent Chow, Ralston-Purina Co., St. Louis, MO) and water were provided *ad lib.*

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Cadmium and manganese. Metal solutions were prepared by dissolving $CdCl_2$ (99.1% pure, certified A.C.S. grade) and $MnCl_2$ obtained from the Fisher Chemical Co. (Fair Lawn, NJ) in 0.9% NaCl. An aliquot of $^{109}Cd^{2+}$ (carrier-free, New England Nuclear, Boston, MA) was added to a 16 μmole Cd^{2+}/ml solution to obtain a specific activity of 0.75 μCi $^{109}Cd^{2+}$/μmole Cd^{2+}. Cd^{2+} analysis was performed by gamma spectrometry (Packard model 5130, Downers Grove, IL).

Quantitation of metallothionein. To compare the ability of Mn^{2+} to induce hepatic MT with that of Cd^{2+} and Zn^{2+}, hepatic MT following Mn^{2+}, Zn^{2+} and Cd^{2+} pretreatment regimens was quantitated in liver by the method of Onosaka *et al.* [22] as modified by Eaton and Toal [23]. The radiometric method involves the saturation of MT with Cd^{2+} *in vitro* and is described in detail by Waalkes *et al.* [24]. Livers were removed following decapitation from rats which received single injections of either saline (2 ml/kg, s.c.), Mn^{2+} (250 μmoles/kg, s.c.), Zn^{2+} (200 μmoles/kg, s.c.) 48 and 24 hr or Cd^{2+} (18 μmoles/kg, s.c.) 24 hr prior to quantitation of MT.

Tolerance to Cd^{2+}-induced lethality. Rats received saline (2 ml/kg) or 250 μmoles Mn^{2+}/kg, s.c., 48 and 24 hr prior to administration of a lethal challenge dose of 36 μmoles Cd^{2+}/kg, i.v. Rats were anesthetized with ether, and intravenous injections were given via the saphenous vein. It was necessary to inject the challenge dose over a period of 2–3 min to avoid immediate death of rats. Mortality was recorded 48 hr following challenge.

Tolerance to Cd^{2+}-induced hepatotoxicity. Rats received either saline (2 ml/kg, s.c.) or 250 μmoles Mn^{2+}/kg, s.c., 48 and 24 hr prior to injection of a hepatotoxic dose of 36 μmoles Cd^{2+}/kg, i.v. Ten hours following injection of the challenge dose, blood was withdrawn from ether-anesthetized rats via the dorsal aorta into a heparinized syringe. Immediately following exsanguination the liver was removed and weighed, and a section was excised and fixed in 10% buffered formalin, pH 7.4. Sections were subsequently dehydrated, embedded in paraffin, and stained with hematoxylin and eosin for morphologic evaluation of liver injury.

Biochemical evaluation of liver function was determined by measuring plasma enzyme activities of aspartate aminotransferase (AST) and sorbitol dehydrogenase (SDH). AST activity was determined colorimetrically by the method of Reitman and Frankel [25], using a commercially available kit (Sigma Chemical Co., St. Louis, MO). SDH activity was determined by the method of Asada and Galambos [26].

Organ distribution of Cd^{2+}. The distribution of a challenge dose of Cd^{2+} to various organs was determined in saline- and Mn^{2+}-pretreated rats. Rats received either saline (2 ml/kg, s.c.) or 250 μmoles Mn^{2+}/kg, s.c., 48 and 24 hr prior to injection of 31 μmoles Cd^{2+}/kg, i.v. (0.75 μCi/μmole Cd^{2+}). This dose of Cd^{2+} is not hepatotoxic 2 hr following i.v. injection [27].

Blood and tissues were removed 2 hr following injection of Cd^{2+}. Rats were anesthetized with ether, and blood was withdrawn from the descending aorta into a heparinized syringe. The concentration of Cd^{2+} was determined in liver, kidney, adrenal, spleen, lung, heart, stomach, intestine, muscle, bone, testis, brain, blood and plasma. The femur, soleus and gastrocnemius and the initial 15-cm segment of the evacuated small intestine were used as representative of bone, muscle and intestine respectively.

Subcellular distribution. The subcellular distribution of Cd^{2+} was determined for liver, kidney, intestine, heart, spleen and testis in control and Mn^{2+}-pretreated rats. Rats received saline (2 ml/kg, s.c.) or 250 μmoles Mn^{2+}/kg, s.c., 48 and 24 hr prior to Cd^{2+} challenge (31 μmoles Cd^{2+}/kg, i.v., 0.75 μCi/μmole Cd^{2+}). Two hours after injection of the challenge dose, rats were anesthetized with ether, blood was withdrawn and organs were removed as described above. Tissues were weighed, placed in cold 0.25 M sucrose, and subsequently homogenized after addition of 4 ml isotonic sucrose/g wet weight tissue. Liver, kidney and spleen were homogenized with a Teflon pestle and Potter–Elvehjem glass homogenizer, and intestine, heart and testis with a Polytron (Brinkmann, Luzern, Switzerland). Crude hepatic subcellular fractions were obtained by differential centrifugation [28] at 4°, and the resultant pellets were defined in this study as nuclei (600 *g*, 10 min), mitochondria (10,000 *g*, 10 min), endoplasmic reticulum (100,000 *g*, 60 min) and cytosol (100,000 *g* supernatant fraction). Subcellular fractions for the five other organs (kidney, intestine, heart, spleen and testis) were obtained similarly; however, the three pellets (nuclear, mitochondria and endoplasmic reticulum) were combined to yield the total particulate fraction. The pellets were resuspended in 0.25 M sucrose (2 ml/g wet weight) and aliquots of each fraction, including the initial homogenate and final supernatant fraction, were analyzed for $^{109}Cd^{2+}$.

To determine the effect of Mn^{2+} pretreatment on distribution of Cd^{2+} within the hepatic cytosolic fraction, 5 ml (approximately 1.4 g wet weight equivalents) of liver cytosol was chromatographed on a 70 × 2.5 cm Sephadex G-75 column (Pharmacia Fine Chemicals, Piscataway, NJ) previously equilibrated with 10 mM Tris–acetate (Sigma Chemical Co.) containing 0.2% sodium azide, pH 7.4, at 4°. Elution was performed with the same buffer using a pump-driven positive pressure downward flow system (25 ml/hr), and one hundred 5-ml fractions were collected and analyzed for Cd^{2+}. The Cd^{2+}-containing peak with a retention coefficient (V_e/V_0) from 1.8 to 2.3 was considered to be MT [29].

Isolation and characterization of hepatic metal-binding protein. Metal-binding proteins were isolated for comparative purposes from Cd^{2+}- and Mn^{2+}-pretreated rats. Rats were pretreated with either Cd^{2+} (18 μmoles/kg, s.c.) 24 hr prior to termination or Mn^{2+} (250 μmoles/kg, s.c.) 48 and 24 hr prior to termination. Thirty minutes prior to termination, rats were injected with 31 μmoles Cd^{2+}/kg, i.v. (0.75 μCi $^{109}Cd^{2+}$/μmole Cd^{2+}). Approximately 40 g of pooled liver was obtained from rats of each pretreatment group following decapitation and was homogenized (1/1, w/v) in 10 mM Tris–acetate buffer, pH 7.4, containing 1.0 mM dithiothreitol (DTT) as a reducing agent. Each homogenate was

centrifuged at 10,000 *g* for 20 min and the supernatant fraction was further centrifuged at 100,000 *g* for 60 min. The cytosol was applied to a 70 × 2.5 cm Sephadex G-75 column equilibrated with 10 mM Tris–acetate buffer, pH 7.4, containing 0.2% sodium azide and 1.0 mM DTT at 4°. Elution was performed with the same buffer at 25 ml/hr and seventy 5-ml fractions were collected. The concentration of Cd^{2+} and the absorbance at 280 nm were determined in the eluate in a gamma spectrometer and a Beckman DU-8 spectrophotometer respectively.

Eluted fractions which contained a low molecular weight Cd^{2+}-binding species ($V_e/V_0 = 1.8$ to 2.4) were pooled and further purified on a 40 × 2.5 cm DEAE Sephadex A-25 anion exchange column equilibrated with 10 mM Tris–acetate buffer, pH 7.4, containing 0.2% sodium azide and 1.0 mM DTT at 4° [30]. After the pooled sample (approximately 100 ml) was applied to the column, a linear gradient of Tris–acetate buffer (10–240 mM, pH 7.4) was initiated at a flow rate of 25 ml/hr and one hundred 5-ml fractions were collected. The eluate was analyzed for $^{109}Cd^{2+}$ by gamma spectrometry, and the conductivity of the effluent was determined with a YSI model 31 conductivity bridge (Yellow Springs Instrument, Yellow Springs, OH).

The two major protein subfractions isolated by ion exchange chromatography were characterized by non-denaturing polyacrylamide-gel electrophoresis as described by Davis [31] and Brewer *et al.* [32]. Approximately 25 μg protein from each sample was applied to 7.5% acrylamide cylindrical gels. Electrophoresis was performed in a Tris–glycine buffer, pH 8.3, at 2.5 mA/gel using Bio-Phore tracking dye (Bio-Rad, Richmond, CA). The protein present in gels was stained with 0.25% Coomassie brilliant blue R-250, and excess stain was removed with 25% isopropanol-10% acetic acid in a charcoal diffusion destainer (Bio-Rad). The gels were scanned at 650 nm using a Hitachi model 100–60 spectrophotometer.

Statistics. All data were compared using a one-way analysis of variance for repeated measures followed by Duncan's multiple range test [33] except the tissue distribution data that were compared by Student's *t*-test and the mortality data by the Chi-square test [34]. The level of significance for all tests was $P < 0.05$.

Table 1. Hepatic metallothionein concentrations following Mn^{2+}, Zn^{2+} and Cd^{2+} pretreatments

Pretreatment	Metallothionein (μg MT/g liver)
Saline*	12.4 ± 1.7† (6)
Mn^{2+}*	153 ± 9 (2)
Zn^{2+}*	363 ± 59 (2)
Cd^{2+}‡	303 ± 12 (4)

* Saline (2 ml/kg, s.c.), Mn^{2+} (250 μmoles/kg, s.c.) and Zn^{2+} (200 μmoles/kg, s.c.) were injected 48 and 24 hr prior to MT quantitation [22, 23].

† Values represent mean ± S.E. (N).

‡ Cd^{2+} (18 μmoles/kg, s.c.) was injected 24 hr prior to MT quantitation.

RESULTS

A comparison of the capacity of several metals to induce synthesis of MT in rat liver is shown in Table 1. Pretreatment with these metals as described in Methods resulted in a substantial increase in the concentration of MT in the liver following injection of Mn^{2+} (12-fold), Zn^{2+} (29-fold) and Cd^{2+} (24-fold).

Pretreatment with Mn^{2+} was effective in protecting against the lethal effect of a subsequent dose of Cd^{2+} (36 μmoles/kg, i.v.) injected 24 hr later. All control rats died (10/10) between 10 and 20 hr following injection of the lethal dose, while 11% pretreated with Mn^{2+} died (1/9).

The effects of Mn^{2+} pretreatment on Cd^{2+}-induced increases in AST and SDH activities in plasma are illustrated in Fig. 1. AST and SDH activities in rats receiving Mn^{2+} pretreatment plus saline challenge were not different from controls. Ten hours after Cd^{2+} challenge in control rats, the activities of AST and SDH were 90 and 210 times higher, respectively, than those of untreated controls. Following Mn^{2+} pretreatment, the elevated plasma activities of AST and SDH produced by the Cd^{2+} treatment were significantly lower than with Cd^{2+} alone; however, AST and SDH activities were 22 and 34 times higher than untreated control values respectively.

Liver morphology was altered markedly following Cd^{2+} injection. Compared to controls (Fig. 2a), severe liver injury was evident 10 hr following a challenge dose of 36 μmoles Cd^{2+}/kg, i.v. Changes included parenchymal cell swelling, congestion, cyto-

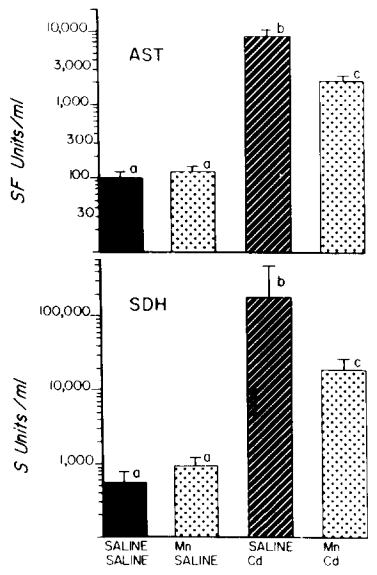

Fig. 1. AST (Sigma–Frankel Units/ml) and SDH (Sigma Units/ml) activities in rats 10 hr after receiving the following treatments: saline pretreatment (2 ml/kg, s.c.), saline challenge (2 ml/kg, i.v.); Mn^{2+} pretreatment (250 μmoles Mn^{2+}/kg, s.c.), saline challenge; saline pretreatment, Cd^{2+} challenge (36 μmoles Cd^{2+}/kg, i.v.); and Mn^{2+} pretreatment, Cd^{2+} challenge. Rats were pretreated both 48 and 24 hr prior to injection of the challenge dose. Values represent mean ± S.E. (N = 4–8). Values with no letters in common are significantly different ($P < 0.05$).

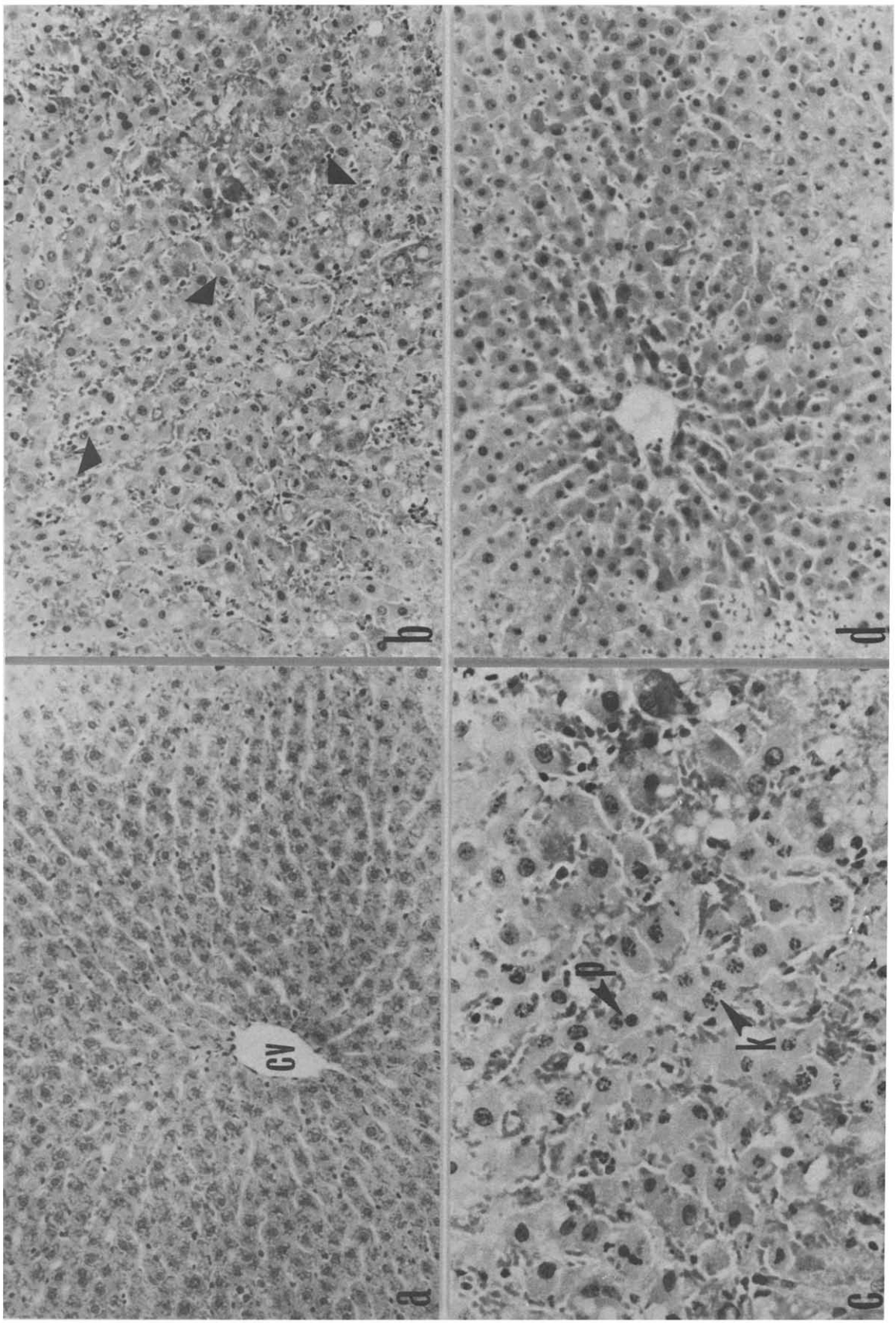
a
CV
b
c
p
k
d

Table 2. Effect of Mn^{2+} pretreatment on tissue concentration of Cd^{2+}

Tissue	Cd^{2+} concentration (μg/g) Control	Mn^{2+} pretreatment*
Liver	48.4 ± 1.66†	87.0 ± 3.72‡
Kidney	16.6 ± 0.95	16.1 ± 0.89
Intestine	18.3 ± 1.42	15.9 ± 1.30
Adrenal	7.41 ± 0.32	8.03 ± 1.29
Stomach	5.13 ± 0.48	3.02 ± 0.29‡
Heart	4.18 ± 0.22	5.42 ± 0.14‡
Spleen	4.19 ± 0.37	2.73 ± 0.16‡
Bone	2.77 ± 0.15	2.19 ± 0.13‡
Lung	2.57 ± 0.01	2.30 ± 0.05‡
Testis	0.78 ± 0.02	0.40 ± 0.01‡
Blood	0.77 ± 0.07	0.88 ± 0.02
Plasma	0.58 ± 0.05	0.36 ± 0.05‡
Muscle	0.37 ± 0.03	0.24 ± 0.03‡
Brain	0.15 ± 0.01	0.14 ± 0.01

* Mn^{2+} (250 μmoles/kg, s.c.) 48 and 24 hr prior to Cd^{2+} challenge (31 μmoles/kg, i.v.); tissues were removed 2 hr following Cd^{2+} administration.

† Each value represents mean ± S.E. (N = 4 or 5).

‡ Significantly different from control by Student's *t*-test, $P < 0.05$.

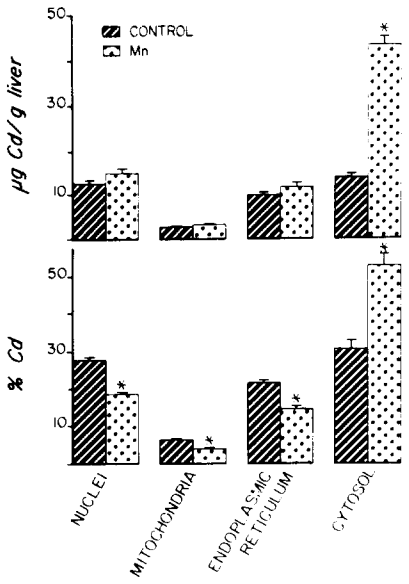

Fig. 3. Effect of Mn^{2+} pretreatment (250 μmoles Mn^{2+}/kg, s.c., 48 and 24 hr prior to Cd^{2+} challenge) on the hepatic subcellular distribution of Cd^{2+} 2 hr after injection of 31 μmoles Cd^{2+}/kg, i.v., 0.75 μCi/μmole Cd^{2+}. Recovery of whole homogenate $^{109}Cd^{2+}$ following fractionation ranged from 83 to 92%. Results in the lower panel are expressed as the percentage of total $^{109}Cd^{2+}$ in the homogenate. Values represent mean ± S.E. (N = 4 or 5). Asterisks indicate significant difference from the corresponding value in control rats ($P < 0.05$).

plasmic eosinophilia, pyknosis, karyorrhexis and diffuse necrosis (Fig. 2, b and c). In Mn^{2+}-pretreated rats given the same challenge dose of Cd^{2+}, occasional pyknotic nuclie and areas of vacuolization were visible, but sinusoids were patent (Fig. 2d). Liver morphology of rats receiving Mn^{2+} pretreatment plus saline challenge was indistinguishable from controls.

Distribution of Cd^{2+} to various tissues 2 hr after its injection is shown in Table 2. Pretreatment with Mn^{2+} resulted in an 80 and 30% increase in the concentration of Cd^{2+} in liver and heart, respectively, and decreases in testis (49%), stomach (41%), plasma (27%), muscle (35%), spleen (35%), bone (21%) and lung (10%).

The subcellular distribution of Cd^{2+} in liver 2 hr following Cd^{2+} challenge is shown in Fig. 3. The concentration of Cd^{2+} did not change in the nuclear, mitochondrial or endoplasmic reticulum fractions as a result of Mn^{2+} pretreatment. In contrast, Mn^{2+} resulted in a tripling in the distribution of Cd^{2+} to cytosol. When the data were expressed as percent of the total Cd^{2+} within liver, Mn^{2+} pretreatment resulted in a significant decrease in the proportion of Cd^{2+} in the nuclear, endoplasmic reticulum and mitochondrial fractions while a marked increase was observed in cytosol.

The distribution of Cd^{2+} to particulate and cytosolic fractions of liver, kidney, intestine, heart, spleen and testis 2 hr following Cd^{2+} challenge is shown in Fig. 4. In most tissues the amounts of Cd^{2+} in the particulate fraction and cytosol were approximately equal, but in heart the Cd^{2+} concentration was 5–6 times higher in the particulate fraction than in cytosol. The distribution of Cd^{2+} to the particulate fraction did not change in Mn^{2+}-pretreated rats in liver, kidney, and intestine, decreased in spleen and testis, and increased in heart. The distribution of Cd^{2+} to the cytosol increased in liver and heart following Mn^{2+} pretreatment, decreased in kidney, spleen and testis, but did not change in intestine.

Pretreatment with Mn^{2+} also altered the dis-

Fig. 2. Photomicrographs of rat liver sections 10 hr following Cd^{2+} administration. Sections were stained with hematoxylin and eosin. Magnification was approximately 100× unless noted otherwise. (a) Control (2.0 ml/kg saline, i.v.). Sinusoidal spaces are visible and parenchymal cell size is normal. The mottled appearance of the cytoplasm is indicative of typical staining. CV = central vein. (b) Cd^{2+} (36 μmoles/kg, i.v.). Foci of necrotic parenchymal cells (arrows) are evident and the large numer of erythrocytes in the sinusoids is indicative of congestion. The pale appearance of the cytoplasm left of center is due to the high degree of eosinophilia imparted by the loss of basophilic staining cellular components such as ribonucleoprotein. (c) Cd^{2+} (36 μmoles/kg, i.v.) Higher magnification (approximately 200×) of the center of the field in Fig. 2b. Pyknotic (p) and karyorrhectic (k) nuclei are present in addition to necrosis and congestion. (d) Mn^{2+} pretreatment (250 μmoles Mn^{2+}/kg, s.c., 48 and 24 hr prior to Cd^{2+}) plus 36 μmoles Cd^{2+}/kg, i.v. Nuclei are intact and similar to control but a few are pyknotic. Sinusoids are generally patent. Vacuolization is visible in the upper left and lower right corners.

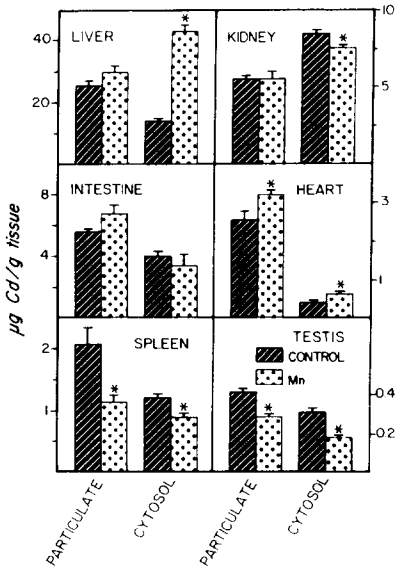

Fig. 4. Effect of Mn^{2+} pretreatment (250 μmoles Mn^{2+}/kg, s.c., 48 and 24 hr prior to Cd^{2+} challenge) on the subcellular distribution of Cd^{2+} to particulate and cytosolic fractions of liver, kidney, intestine, heart, spleen and testis 2 hr after injection of 31 μmoles Cd^{2+}/kg, i.v., 0.75 μCi/μmole Cd^{2+}. Recovery of $^{109}Cd^{2+}$ following fractionation ranged from 76 to 99%. Values represent mean ± S.E. (N = 4 or 5). Asterisks indicate a significant difference from controls ($P < 0.05$).

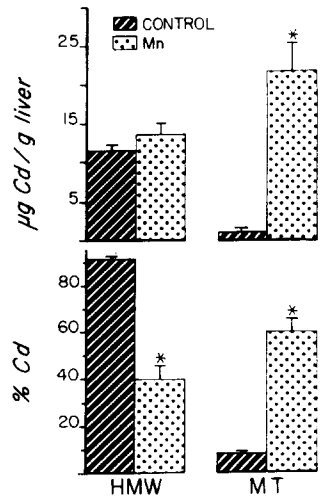

Fig. 6. Distribution of Cd^{2+} in hepatic cytosol chromatographed on Sephadex G-75 2 hr after injection of Cd^{2+} (31 μmoles/kg, i.v., 0.75 μCi/μmole Cd^{2+}) in control and Mn^{2+}-pretreated rats. Results in the lower panel are expressed as percentage of total $^{109}Cd^{2+}$ recovered following elution. Recovery of cytosolic $^{109}Cd^{2+}$ in the two major peaks (HMW and MT) following elution ranged from 64 to 95%. Values represent mean ± S.E. (N = 4 or 5). An asterisk indicates a significant difference from controls ($p < 0.05$). HMW = high molecular weight proteins, and MT = metallothionein.

tribution of Cd^{2+} within hepatic cytosol (Fig. 5). In control animals the majority of Cd^{2+} localized within cytosol was associated with high molecular weight proteins (V_e/V_0 = 1.0 to 1.4) In contrast, with Mn^{2+} pretreatment, the majority of Cd^{2+} eluted with a low molecular weight protein (V_e/V_0 = 1.8 to 2.3) which was assumed to be MT.

Compilation of gel-filtration data is illustrated in Fig. 6. Pretreatment with Mn^{2+} did not alter the distribution of Cd^{2+} to high molecular weight proteins but markedly increased its binding to the low molecular weight protein (MT). When expressed as a proportion of total Cd^{2+} within the cytosol, 92% was associated with high molecular weight proteins

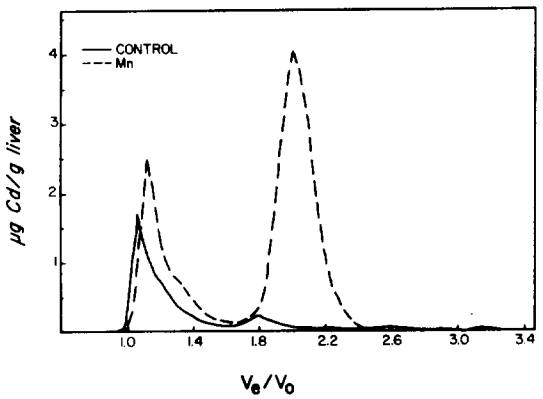

Fig. 5. Representative gel filtration (Sephadex G-75) elution profiles of $^{109}Cd^{2+}$ in hepatic cytosol 2 hr after injection of Cd^{2+} (31 μmoles/kg, i.v., 0.75 μCi/μmole Cd^{2+}) in control and Mn^{2+}-pretreated rats. Elution of $^{109}Cd^{2+}$ with retention coefficients (V_e/V_0) of 1.0 to 1.4 and 1.8 to 2.3 was considered bound to high molecular weight proteins and MT respectively.

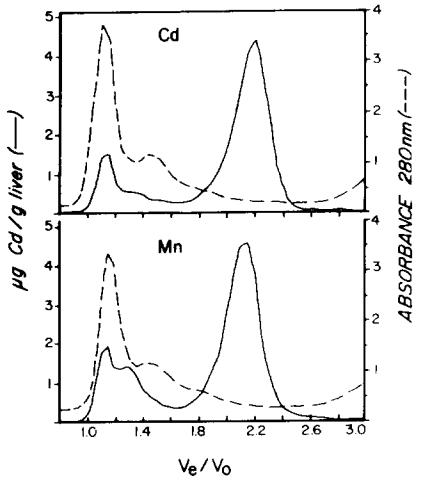

Fig. 7. Comparison of gel filtration (Sephadex G-75) elution profiles of protein-bound Cd^{2+} in hepatic cytosol 30 min after injection of Cd^{2+} (31 μmoles/kg, i.v., 0.75 μCi/μmole Cd^{2+}) in rats pretreated with Cd^{2+} (18 μmoles/kg, s.c., 24 hr prior) and Mn^{2+} (250 μmoles/kg, s.c., 48 and 24 hr prior to Cd^{2+}). Both major Cd^{2+}-binding peaks eluted at a V_e/V_0 of approximately 2 and had characteristic low absorbance at 280 nm.

and only 8% was bound to MT in control animals. In contrast, Mn^{2+} pretreatment resulted in only 40% of the Cd^{2+} being associated with high molecular weight proteins while 60% was bound to MT.

To determine if the Mn^{2+}-induced low molecular weight metal-binding protein was MT, cytosol of livers of Cd^{2+}- and Mn^{2+}-pretreated rats was chromatographed on Sephadex G-75 gel-filtration columns. A major Cd^{2+}-containing peak was evident at V_e/V_0 of approximately 1.8 to 2.3 in liver from Cd^{2+}-pretreated rats (Fig. 7, upper panel), which has been extensively characterized as MT [12, 13, 17]. This major Cd^{2+}-binding protein had a low absorbance at 280 nm which is characteristic of MT. The elution profile of hepatic cytosol from livers of Mn^{2+}-pretreated rats (Fig. 7, lower panel) closely resembles that of the Cd^{2+}-pretreated rats, i.e. a major Cd^{2+}-binding peak was also evident at $V_e/V_0 = 1.8$ to 2.3 with a low absorbance at 280 nm.

This major Cd^{2+}-binding protein from both Cd^{2+}- and Mn^{2+}-pretreated rats was further purified by anion-exchange chromatography (Fig. 8). The protein from Cd^{2+}-pretreated rats separated into two major isoproteins as observed by others [12, 17]. The Cd^{2+}-binding isoproteins from Mn^{2+}-pretreated rats exhibited a similar profile and eluted at the same conductivity as those from Cd^{2+}-pretreated rats; MT-1 eluted in Tris–acetate buffer having a conductivity of 1.5 to 1.7 mMho/cm and MT-2 eluted at a buffer conductivity of 2.75 to 2.95 mMho/cm. Despite these similarities, differences in peak height of the two isoproteins from the two pretreatment groups were observed. While the amounts of the two isoproteins from Cd^{2+}-pretreated rats were nearly equal, the first peak isolated from Mn^{2+}-pretreated rats was less than the height of the second peak. The smaller Cd^{2+}-containing peak eluting between the two major isoproteins was of such small proportion that further

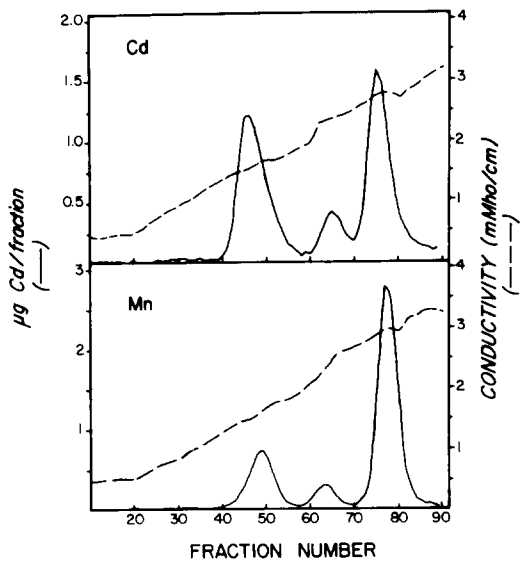

Fig. 8. Comparison of the low molecular weight Cd^{2+}-binding proteins isolated after gel-filtration chromatography from rats pretreated with Cd^{2+} (upper panel) or Mn^{2+} (lower panel) by DEAE A-25 anion exchange chromatography.

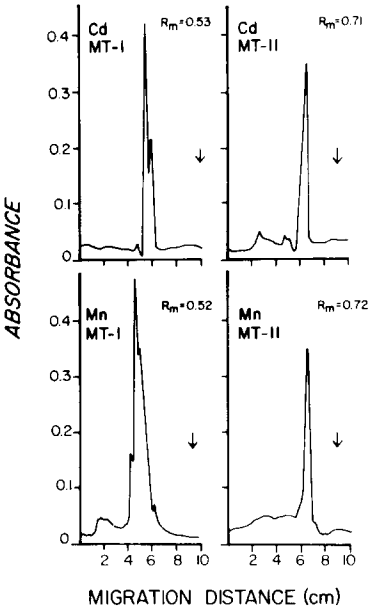

Fig. 9. Comparisons of MT-1 and MT-2 by non-denaturing polyacrylamide gel electrophoresis isolated from liver of Cd^{2+}- and Mn^{2+}-pretreated rats by gel filtration and ion exchange chromatography as described in Methods. The gels were scanned at 650 nm. The arrows represent the mobility of the tracking dye. R_m = relative mobility.

studies on this protein were not performed [18]. To determine the recovery of the low molecular weight protein during various isolation steps, three isolations were peformed. Eighty-two percent of the Cd^{2+} in the homogenate eluted bound to the low molecular weight protein following G-75 chromatography, while subsequent purification by anion exchange chromatography gave a final average yield of 50% of the Cd^{2+} in the two major peaks.

To further characterize the two isoproteins, polyacrylamide gel electrophoresis of MT-1 and MT-2 from livers of both Cd^{2+}- and Mn^{2+}-pretreated rats was performed (Fig. 9). The electrophoretic mobility of MT-1 relative to the tracking dye was 0.53 and 0.52 for Cd^{2+}- and Mn^{2+}-pretreated rats, respectively; however, some contamination was evident. The electrophoretic mobility of MT-2 was 0.71 and 0.72 for Cd^{2+}- and Mn^{2+}-pretreated rats, respectively, and only one band per gel was visible.

DISCUSSION

The results of this investigation confirm the observation that pretreatment with Mn^{2+} produces tolerance to an acute lethal dose of Cd^{2+} [3]. Tolerance was evident as only one of nine rats died which had been pretreated with Mn^{2+} 48 and 24 hr prior to injection of a lethal dose of Cd^{2+}.

Previous reports have suggested that tolerance to Cd^{2+} toxicity following pretreatment with metals results from an altered organ distribution of Cd^{2+} due to preformed MT sequestering a greater percentage of the toxic dose in liver, and subsequently decreasing the amount available for distribution to

other target organs of toxicity such as kidney [35–37]. However, in previous investigations we did not observe this altered distribution of Cd^{2+} following pretreatment with Cd^{2+} [6] but did show an increase in distribution of Cd^{2+} to liver following Zn^{2+} pretreatment [10]. In the present study, there was a significant increase in distribution of Cd^{2+} to liver following Mn^{2+} pretreatment. Liver accumulated 51 and 79% of the dose of Cd^{2+} in control and Mn^{2+}-pretreated rats respectively (Table 2). Additionally, while the distribution of Cd^{2+} to heart increased, decreases in Cd^{2+} distribution to stomach, spleen, bone, lung, testis, plasma and muscle were observed in Mn^{2+}-pretreated rats. Thus, this altered tissue distribution of Cd^{2+} does not appear to be a primary mechanism for tolerance because liver is a target organ of acute Cd^{2+} toxicity.

The hepatic subcellular distribution of Cd^{2+} after acute exposure to Cd^{2+} is well documented [6, 10, 29, 38–41], and the distribution reported herein is in agreement with the earlier findings. Following Mn^{2+} pretreatment, however, a higher percentage of Cd^{2+} in liver distributed to cytosol with less in particulate fractions. A similar alteration in the subcellular distribution of Cd^{2+} has been observed following pretreatment with Cd^{2+} [6, 29, 42] and Zn^{2+} [10].

While the most striking change in subcellular distribution of Cd^{2+} was in liver, the present data indicate that the subcellular distribution of Cd^{2+} is altered following Mn^{2+} pretreatment in several other organs, although none paralleled the changes observed in liver. Of the five extrahepatic tissues investigated, altered distribution of Cd^{2+} was most evident in spleen and testis; however, less Cd^{2+} was found in both the particulate and cytosol fractions following Mn^{2+} pretreatment. A slight decrease in distribution of Cd^{2+} to kidney cytosol was also observed. Heart was the only organ other than liver in which an increase in distribution of Cd^{2+} to cytosol occurred. The lack of any marked change in subcellular distribution of Cd^{2+} in the extrahepatic organs may be due to lack of or poor induction of MT by Mn^{2+} in these tissues. Mogilnicka *et al.* [7] reported that Mn^{2+} does not induce MT in liver or kidney, while Eaton *et al.* [11] showed that Mn^{2+} induces MT rather poorly in liver but not at all in kidney. In a more comprehensive study, Waalkes and Klaassen [8] reported that, of ten organs, Mn^{2+} induces MT only in liver and to a lesser extent than does Cd^{2+} or Zn^{2+}.

To explore the possibility that MT was responsible for altering the hepatic subcellular distribution of Cd^{2+} in Mn^{2+}-pretreated rats, hepatic cytosol was fractionated by gel-filtration chromatography. The results revealed that more Cd^{2+} was bound to a low molecular weight protein, presumably MT, in the hepatic cytosol of Mn^{2+}-pretreated rats, thus accounting for the altered subcellular distribution of Cd^{2+} which has also been demonstrated following Cd^{2+} [6, 29, 42] and Zn^{2+} [10] pretreatment.

As noted above, a major portion of the challenge dose of Cd in Mn^{2+}-pretreated rats was associated with a low molecular weight protein (Fig. 3) assumed to be MT; however, a gel-filtration elution profile similar to that of MT ($V_e/V_0 = 2$) is not definitive identification for MT [19–21]. More comprehensive criteria are required to identify such a protein as actually being MT. Therefore, the low molecular weight protein present in liver after Mn^{2+} pretreatment was compared to the previously characterized MT induced following Cd^{2+} treatment [13, 16, 17] by employing a number of criteria commonly used to identify MT.

Similar retention coefficients after gel-filtration chromatography suggested that the two proteins had similar Stoke's radii and likely are of the same molecular weight. Both the Cd^{2+}-induced MT and the Mn^{2+}-induced protein had a low absorbance at 280 nm, also characteristic of MT [43]. Further evidence suggested that the two proteins possessed similar ionic properties. First, each protein separated into two major isoproteins (MT-1 and MT-2) following anion exchange chromatography and the corresponding isoproteins eluted in buffer of similar conductivity (Fig. 8). Second, the isoproteins from Mn^{2+}-pretreated rats exhibited the same electrophoretic mobilities as the corresponding isoproteins of Cd^{2+}-pretreated rats after electrophoresis on polyacrylamide gels (Fig. 9). The electrophoretic mobilities of the two major isoproteins isolated from liver of Cd^{2+}- and Mn^{2+}-pretreated rats in this study (approximately 0.53 and 0.71) were similar to those of the two isoforms of MT isolated from liver of rats treated with Cd^{2+} [12, 17] or Zn^{2+} [18, 44]. Thus, an increase in the concentration of MT in the liver of rats after Mn^{2+} treatment has been established, similar to what has earlier been shown for Cd^{2+} and Zn^{2+}.

The altered subcellular distribution of Cd^{2+} in liver is extremely important because liver has been shown to be a target organ of toxicity after acute exposure to Cd^{2+} [9, 27, 45, 46]. The diffuse hepatic necrosis produced by Cd^{2+} in these studies is of such magnitude that death results, at least in part, from hepatic failure. In the present study, morphologic and biochemical evidence demonstrated that Mn^{2+} pretreatment prevented the massive hepatic necrosis produced by Cd^{2+}. Cell swelling, slight vacuolization, a minor degree of pyknosis and cytoplasmic eosinophilia were the predominant lesions observed in Mn^{2+}-pretreated, Cd^{2+}-challenged rats; cell swelling is generally thought to be a reversible effect [47]. Although the induction of MT in rat liver by Mn^{2+} was marked (Table 1), the fact that Mn^{2+} did not induce MT to the extent that Zn^{2+} or Cd^{2+} induced MT might account for the slight hepatotoxicity observed in Mn^{2+}-pretreated, Cd^{2+}-challenged rats (Figs. 1 and 2).

This investigation has shown that Mn^{2+} pretreatment (1) prevented Cd^{2+}-induced lethality and hepatotoxicity, (2) increased the distribution of Cd^{2+} to liver and generally decreased the distribution to other organs, and (3) markedly altered the hepatic subcellular distribution of Cd^{2+} with more localizing in the cytosol bound to a low molecular weight protein, which appears to be MT. These data suggest that tolerance to Cd^{2+}-induced hepatotoxicity following Mn^{2+} pretreatment appears to be due, at least in part, to a higher percentage of Cd^{2+} distributing to cytosol, where it binds to MT, the levels of which are increased by Mn^{2+} pretreatment. This mechanism is probably also responsible for tolerance to

Cd^{2+}-induced lethality following Mn^{2+} pretreatment and further illustrates the involvement of MT in liver as a critical factor in tolerance to Cd^{2+} toxicity.

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