ZINC DISTRIBUTION AND METALLOTHIONEIN IN THE LIVER AND KIDNEY OF MICE TREATED WITH CADMIUM

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Abstract.....Cadmium in the liver and kidney increased with the dosage of this injection. There was a parallel relation between the increase of cadmium in the whole liver and that in the metallothionein. In the liver and kidney, zinc highly increased in the low dose group than in the high dose group. In the 5.0 mg/kg group, zinc in the liver was not different from that of the control group, and about 30% of zinc in the 30,000 g supernatant existed in the metallothionein. By the DEAE-cellulose chromatography of metallothionein, two forms of metallothionein were obtained.

Key words : Cadmium, Zinc, Distribution, Metallothionein

It is well known that cadmium is accumulated as metallothionein induced by this metal, in proportion to the dosage. Kagi and Vallee (1960) demonstrated that zinc also could bind to this protein. Zinc is known to have a protective effect for the cadmium toxicity (Parizek, 1957). It seems reasonable to consider that the incorporation of cadmium affects the physiological zinc, or inversely this zinc influences the cadmium toxicity. Bremner and Davies (1975) reported that the binding of cadmium and mercury in the Zn-thionein may simply be a fortuitous consequence of the chemical similarities between all these elements. In the proceeding study, cadmium of various doses was subcutaneously injected to mice to clarify the effect of cadmium on the physiological zinc in the liver where cadmium primarily accumulated, particularly from the standpoint of a role of metallothionein.

METHODS

Male mice of the ICR strain weighing 30 to 38 g were given 0.5, 1.0, 2.5 and 5.0 mg of Cd/kg by a single sc injection, and sacrificed by bleeding 24 h after the injection. Mice in control group were injected with a physiological saline solution. All groups comprise 9 mice except the 5.0 mg/kg group (10 mice). The liver and kidney were removed from each mouse immediately after sacrifice. About 0.2 g (wet weight) of the liver and kidney were wet-digested with a mixture of nitric acid and perchloric acid (5 : 1) on a hot sand plate. These digestants were diluted with deionized water to 10 ml for metal analysis.

The remaining liver was homogenized with three volumes of a solution of 0.01 M

Tris-HCl buffer (pH 8.0). This homogenate was centrifuged (30 min at 30,000 g) thrice in a refrigerated (2°C) centrifuge. The liver supernatant obtained by the centrifugation was applied to a column (1.8×60 cm), which was packed with Sephadex G-75 (Pharmacia Fine Chemicals, Uppsala, Sweden) and equilibrated with 0.01 M Tris-HCl buffer (pH 8.0). Fractions of 5.0 ml volume were collected at a flow rate of about 12 ml/h. This procedure was carried out in a chamber at 5°C. The absorbance at 280 and 254 nm was recorded for each fraction. Collected fractions were analyzed for cadmium and zinc by the direct aspiration into an atomic absorption spectrometer (Hitachi Model 208). Unless the supernatants were fractionated immediately, the samples were rapidly frozen on dry ice and stored at -20°C.

Fractions (Fraction III containing cadmium) obtained from the Sephadex G-75 column were combined and concentrated by ultrafiltration through a UM-2 membrane in an Amicon Model 52 cell. The concentrated solution was rechromatographed on Sephadex G-75. These fractions containing cadmium were combined and concentrated by UM-2 membrane. This concentrated solution was applied to a column (1.8×60 cm), which was packed with DEAE ion exchange gel (DE-52, Whatman Ltd) and equilibrated with 0.2 M Tris-HCl buffer (pH 8.5). Elution was carried out with the same buffer without gradient. Fractions of 4.0 ml volume were collected at a flow rate of about 10 ml/h. The absorbance at 280 and 254 nm was recorded for the eluates; and, the cadmium and zinc concentration in the eluates were determined directly by atomic absorption.

The final precipitate from the 30,000 g centrifugation was wet-digested, and the digestant was diluted with deionized water to measure cadmium and zinc.

For statistical analyses, a one-way analysis of variance and Student's t-test for comparison of individual mean were employed.

RESULTS

Cadmium and zinc in the whole tissue: Table 1 shows cadmium and zinc concentration in the liver and kidney. Cadmium concentration in the liver and kidney increased

Table 1. Cadmium and zinc in the liver and kidney

Organ	Metal	Concentration of Cd and Zn ($\mu\text{g/g}$ wet weight)				
		Dose (mg Cd/kg body weight)				
		0	0.5	1.0	2.5	5.0
Liver	Cd	—	4.4±1.0	14.4±2.4	22.1±3.7	32.2±7.0
	Zn	^a 21.7±2.2	^b 31.4±4.6	34.3±4.1	29.1±5.1	^c 21.2±3.7
Kidney	Cd	—	1.2±0.2	3.0±0.8	5.0±0.8	11.8±2.3
	Zn	15.0±2.0	15.7±1.5	^d 19.0±2.8	13.1±2.1	^e 12.7±0.9

Data are presented as mean±SD. Each group comprised 9 mice except the 5.0 mg/kg group (7 mice). ^aF(3, 32)=13.0, $p<0.01$ (from the control group to the 2.5 mg/kg group), ^bF(2, 24)=2.4, $p>0.05$ (in the three groups; 0.5, 1.0 and 2.5 mg/kg group), ^c $p>0.05$, ^d $p<0.01$ and ^e $p<0.05$ respective to control by t-test.

Effect of Cd on Zn distribution

with the dosage of cadmium. Cadmium in the liver and kidney was preferentially located in the 30,000 g supernatant (Table 2). These percentages in both organs were always above 90%, but were not related to the injection dosage of cadmium.

There was a significant difference ($F(3,32)=13.0, p<0.01$) in the liver zinc concentration from the control group to the 2.5 mg/kg group. While, this concentration in the 5.0 mg/kg group was not different from that in the control group. There was no significant difference in the concentration of the liver zinc in the three groups (0.5, 1.0 and 2.5 mg/kg group), and the mean value in the 1.0 mg/kg group was the maximum. Zinc was found in the 30,000 g supernatant at a level of 80-90% in the liver and at a level of 70-80% in the kidney (Table 2). In comparison with cadmium, this proportion of the distribution was somewhat low.

In the kidney, zinc concentration was higher in the 1.0 mg/kg group than in the

Table 2. Cadmium and zinc in the supernatant fraction

Organ	Per cent of Cd and Zn the supernatant fraction									
	Dose (Cd mg/kg)									
	Cd ⁰	Zn	Cd ^{0.5}	Zn	Cd ^{1.0}	Zn	Cd ^{2.5}	Zn	Cd ^{5.0}	Zn
Liver	—	78	91	80	96	88	96	89	94	90
Kidney	—	68	98	85	91	73	94	78	90	82

Each value (snp/sup+ppt×100) represents the mean of 2-3 trials.

Fig. 1. Sephadex G-75 elution profile for Cd and Zn of supernatant fraction from liver (1.0 mg/kg group). The symbols represent the Cadmium (●—●) and zinc (○—○) concentration, respectively. The arrow marks the Cytochrome C.

control group. This concentration was lower in the 2.5 mg/kg group and in the 5.0 mg/kg group than in the control group.

Cadmium and zinc in the 30,000g supernatant proteins: By the separation of the 30,000 g liver supernatant, two peaks and four peaks were obtained by the determination of cadmium and zinc (Fig. 1). Two peaks out of four peaks of zinc were in accordance with the cadmium peaks. The peaks were named F_I, F_{II}, F_{III} and F_{IV} in the order eluted. The molecular weight of the protein in F_{III} as determined by gel filtration was about 10,000.

The proportion of cadmium and zinc in the 30,000 g supernatant proteins are summarized in Table 3. The proportion of cadmium in F_{III} was increased by the injection of cadmium, and these proportion, which always exceeded 90%, were not found in direct relation to the dosage of cadmium. It is reasonable to consider that the increase of cadmium in F_{III} paralleled the elevation of cadmium in the whole liver, since cadmium also increased in the supernatant fractions with the dosage. Such observation had been also reported by Piotrowski et al. (1974).

The proportion of zinc was markedly increased in F_{III} by the injection of cadmium in comparison with control group, and the maximum value in the four groups (0.5, 1.0, 2.5 and 5.0 mg/kg group) was 36% in the 1.0 mg/kg group. These percentages were found in relation to the cadmium concentration in the whole liver, but not in relation to the dosage of cadmium. In the 5.0 mg/kg group, about 30% of zinc in the supernatant was found in F_{III}; nevertheless, zinc concentration in this group was not different from that in the control group (Table 1). This indicates that zinc in F_I and F_{II} (especially, F_I) decreased. This value of about 36% may be a threshold value under the condition in the present study. Suzuki and Yoshikawa (1972) reported that about 72% of zinc in the liver supernatant was found in the metallothionein from rats received repeatedly cadmium. The differences of these percentages may be due to the total dose of cadmium or the duration of exposure to cadmium.

Cadmium and zinc in the metallothionein fraction: By the DEAE-cellulose chro-

Table 3. Cadmium and zinc in the liver 30,000 g supernatant proteins

Fraction	Per cent of Cd and Zn in fraction I-IV									
	Dose (Cd mg/kg)									
	0		0.5		1.0		2.5		5.0	
	Cd	Zn	Cd	Zn	Cd	Zn	Cd	Zn	Cd	Zn
I	—	69	8	57	2	51	5	48	8	50
II		25		15		12		15		18
III	—	5	92	26	98	36	95	35	92	31
IV		1		2		1		2		1

Each value represents the mean of 2-5 experiments. In estimating the value in each fraction, the metal content of the border fraction of Fraction I and II, Fraction II and III, and Fraction III and IV is equally divided and then added to each fraction.

Effect of Cd on Zn distribution

Fig. 2. DEAE ion exchange chromatographic pattern of a Cd, Zn-containing fraction, Fraction III, from the liver in the 1.0 mg/kg group. Peak I, II and III corresponds to the tube number 22 to 30, 31 to 42 and 43 to 53, respectively. The symbols represents the cadmium (●—●) and zinc (○—○) concentration.

matography of metallothionein (F_{III}) which separated by rechromatography of Sephadex G-75, three peaks were obtained (Fig. 2). All proteins in the three peaks associated with cadmium and zinc. The UV spectrum and the ratio of A_{250} to A_{280} of these proteins are shown in Fig. 3 and Table 4. The protein in Peak I was not metallothionein, although the molecular weight was the same as that of the metallothionein determined by the behaviour of Sephadex G-75. The protein in Peak II and III may be corresponding to metallothionein which was already reported by others (Shaikh and Lucis, 1971 ; Nordberg et al., 1972). The proportion of both metals in Peak I, II and III is summarized in Table 5. More than 90% of cadmium and zinc in the metallothionein obtained by Sephadex G-75 was recovered in Peak II and III. In the cadmium-treated groups, the proportion of cadmium and zinc was slightly higher in Peak II than in Peak III. The ratio of A_{250}/A_{280} and Cd/Zn increased with the dosage of cadmium. The coefficient between

Fig. 3. Absorption spectra of metallothionein. Protein concentration in Peak I, II and III was 0.75, 0.14 and 0.15 mg/ml, respectively. The proteins were dissolved in 0.01 M Tris-HCl buffer (pH8.5).

Table 4. Ratio of cadmium to zinc and of A_{250} to A_{280} in Peak I-III

Dose (Cd mg/kg)	Peak	Cd/Zn	A_{250}/A_{280}
0.5	I	1.0	0.6
	II	0.6	1.9
	III	0.5	3.0
1.0	I	3.1	0.8
	II	2.0	3.1
	III	1.6	4.1
2.5	I	1.8	0.7
	II	1.8	5.7
	III	1.6	4.2
5.0	I	3.2	1.1
	II	4.1	7.2
	III	3.4	3.8

Ratio of cadmium to zinc is expressed as moles per mole. Absorbance at 250 and 280 nm was measured using 0.01 M Tris-HCl buffer (pH8.5).

Effect of Cd on Zn distribution

Table 5. Cadmium and zinc in DEAE-cellulose column separation

Peak	Per cent of Cd and Zn in Peak I-III							
	Dose (Cd mg/kg)							
	Cd ^{0.5}	Zn	Cd ^{1.0}	Zn	Cd ^{2.5}	Zn	Cd ^{5.0}	Zn
I	9	8	10	6	4	1	14	9
II	60	55	61	57	58	60	56	54
III	31	37	29	37	38	39	30	37

Details were shown in Fig. 2.

two ratios was 0.72 and this value was significant ($p < 0.05$).

DISCUSSION

Cadmium which accumulated in the liver induced the synthesis of metallothionein, and this accumulation was related to the dosage of cadmium. Under the conditions in the present study, the presence of metallothionein in the liver may reflect the presence of cadmium.

Many investigators have reported that zinc increased by the injection of cadmium in the liver may be due to the metallothionein induced by cadmium (Suzuki and Yoshikawa, 1972; Colucci et al., 1975; Kimura and Otaki, 1975; Stonard and Webb, 1976). In the present study, by the cadmium injection, the zinc in the whole liver and in the 30,000 g supernatant was increased, but no significant dose-related increase of zinc was observed. The increased zinc in the liver may also be due to the metallothionein induced by cadmium. However, the appearance of metallothionein did not always increase the zinc in the liver (5.0 mg/kg group). These results suggest the following two possibilities:

(1) metallothionein which was induced by the low dose of cadmium is not saturated with cadmium, and zinc binds to the remaining sites of this protein in consequence of the chemical similarities between two metals, (2) when metallothionein is induced by the high dose of cadmium, this protein must associate with most of the cadmium, thus resulting in a decrease of zinc. Indeed, in F_{III}, cadmium and zinc content, which were calculated as the product of three values (metal content in the whole liver, proportion of metal in the supernatant, and proportion of metal in F_{III}), were about 6 μ g and 14 μ g in the 0.5 mg/kg group; and, about 54 μ g and 13 μ g in the 5.0 mg/kg group. Suzuki and Yoshikawa (1974) reported that when male rats were pretreated with a small amount of cadmium (0.3 mg/kg, ip) at 24 h before injection with a challenge dose of cadmium (3.0 mg/kg, ip), a large amount of cadmium was also found in the low molecular weight protein, whereas zinc in this protein disappeared at 2 h after the challenge injection. Leber and Miya (1976) observed the same phenomenon in vitro.

Winge et al. (1975) noted accumulation of zinc in rat liver when animals were exposed to cadmium. They attributed the accumulation of zinc, in part, to a depletion of

blood zinc. Faeder et al. (1977) also reported that the increasing accumulation of zinc paralleled the increasing accumulation of cadmium in the liver. However, they relied on increased dietary uptakes as an explanation of the high zinc content in the liver because the liver zinc originated from the blood scarcely contributed to the increased zinc in the liver.

These observations containing the observation of present study seem to be contradictory to the previous reports that the injection of cadmium brought the symptoms of zinc deficiency (Supplee, 1961 ; Supplee, 1963). In addition, Powell et al. (1964) reported that the symptoms of zinc deficiency was found, though zinc increased in the liver. It is reasonable to consider that zinc associated with the metallothionein, namely the forming of the inactivated zinc, results in the symptoms of zinc deficiency. Therefore, it seems likely that the zinc increased in the liver dispersedly come from the other tissues, but not concentratedly.

Webb (1972) reported the existence of a low molecular weight Zn-containing protein in the livers of intact female rats, but not male rats. In the present study, however, about 5 % of zinc in the liver supernatant fraction was found in F_{III} (Zn-thionein) of the control male mice.

In the 5.0 mg/kg group, two mice out of ten died 24 after the cadmium injection. This indicates that the dose (5.0 mg/kg) was unbearable to mice. However, the ability of metallothionein synthesis is not thought to be markedly decreased because the proportion of cadmium in the high molecular weight protein (F_I) did not increase.

Zinc content in F_I was lower in the 5.0 mg/kg group than in the other cadmium-treated groups. In all probability, cadmium also affected protein synthesis of F_I which plays a major role as a store of zinc in the liver, thus resulting in no change of zinc concentration. Webb (1972) reported that the Zn-contents of the other proteins of the cell sap decreased with the uptake of zinc by the Cd-binding component when rats were injected with $CdCl_2$ ($2.2 \mu\text{mole}/100 \text{ g body weight} \approx \text{about } 2.5 \text{ mg/kg body weight as Cd}$). It seems that zinc metabolism is affected not only by the appearance of the metallothionein, but also by the lack of zinc binding to F_I .

In the cadmium-treated groups, two forms of metallothionein (Peak II and III) were obtained by DEAE-cellulose chromatography. The increase of zinc bound to the metallothionein in the low dose group indicates that this protein has plenty of reserve power to further associate with cadmium. In addition, it might explain why mice previously exposed to a low dose cadmium will survive longer than animals not exposed to the low dose, when challenged with a high dose.

SUMMARY

Male mice of the ICR strain were given 0.5, 1.0, 2.5 and 5.0 mg of Cd/kg by a single sc injection. Cadmium in the liver and kidney increased with the dosage of injection. There was a parallel relation between the increase of cadmium in the whole liver and that in the metallothionein. In all probability, the increase of cadmium in both

organs is thought to be due to the appearance of metallothionein. In the liver and kidney, zinc highly in the low dose group than in the high dose group. In the 5.0 mg/kg group, zinc in the liver was not different from that of the control group, and about 30% of zinc in the 30,000 g supernatant existed in the metallothionein. This indicates that the appearance of metallothionein does not always bring the increase of zinc in the liver. By the DEAE-cellulose chromatography of the metallothionein, which was separated by Sephadex G-75, two forms of metallothionein were obtained. There was a significant correlation between the ratio of Cd/Zn and the ratio of A_{250}/A_{280} ($r=0.72$, $p<0.05$) in the metallothionein. It seems likely that the zinc-rich metallothionein was synthesized in the low dose group.

REFERENCES

- Bremner, I. and Davies, N. T. (1975). The induction of metallothionein in rat liver by zinc injection and restriction of food intake. *Biochem. J.* **149**, 733-738.
- Colucci, A. V., Winge, D. and Krasno, J. (1975). Cadmium accumulation in rat liver. *Arch. Environ. Health* **30**, 153-157.
- Faeder, E. J., Chaney, S. Q., King, L. C., Hinners, T. A., Bruce, R. and Fowler, B. A. (1977). Biochemical and ultrastructural changes in livers of cadmium-treated rats. *Toxicol. Appl. Pharmacol.* **39**, 473-487.
- Kagi, J. H. R. and Vallee, B. L. (1960). Metallothionein: a cadmium- and zinc-containing protein from equine renal cortex. *J. Biol. Chem.* **235**, 3460-3465.
- Kimura, M. and Otaki, N. (1975). Cadmium accumulation in liver and kidney of rabbit with single injection. *Ind. Health* **13**, 203-210.
- Leber, A. P. and Miya, T. S. (1976). A mechanism for cadmium- and zinc-induced tolerance to cadmium toxicity: involvement of metallothionein. *Toxicol. Appl. Pharmacol.* **37**, 403-414.
- Nordberg, G. F., Nordberg, M., Piscator, M. and Verterberg, O. (1972). Separation of two forms of rabbit metallothionein by electric focusing. *Biochem. J.* **126**, 491-498.
- Parizek, J. (1957). The destructive effect of cadmium ion on testicular tissues and its prevention by zinc. *J. Endocrinol.* **15**, 56-63.
- Piotrowski, J. K., Trojanowska, B. and Sapota, A. (1974). Binding of cadmium and mercury by metallothionein in the kidneys and liver of rats following repeated administration. *Arch. Toxicol.* **32**, 351-360.
- Powell, G. W., Miller, W. J., Morton, J. D. and Clifton, C. M. (1964). Influence of dietary cadmium level and supplemental zinc on cadmium toxicity in the bovine. *J. Nutr.* **84**, 205-214.
- Shaikh, Z. A. and Lucis, O. J. (1971). Isolation of cadmium-binding proteins. *Experientia* **27**, 1024-1025.
- Stonard, M. D. and Webb, M. (1976). Influence of dietary cadmium on the distribution of the essential metals copper, zinc and iron in tissues of the rat. *Chem.-Biol. Interactions* **15**, 349-363.
- Supplee, W. C. (1961). Production of zinc deficiency in turkey poults by dietary cadmium. *Poultry Sci.* **40**, 827-828.
- Supplee, W. C. (1963). Antagonistic relationship between dietary cadmium and zinc. *Science*. **139**, 119-120.
- Suzuki, Y. and Yoshikawa, H. (1972). Effects of cadmium injection on intracellular distribution of essential metals in rat liver. *Ind. Health* **10**, 93-106.
- Suzuki, Y. and Yoshikawa, H. (1974). Role of metallothionein in the liver in protection against cadmium toxicity. *Ind. Health* **12**, 141-151.

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- Webb, M. (1972). Binding of cadmium ions by rat liver and kidney. *Biochem. Pharmacol.* **21**, 2751-2765.
- Winge, D, R., Premakumar, R, and Rajagopalan, K. V. (1975). Metal-induced formation of metallothionein in rat liver. *Arch. Biochem. Biophys.* **170**, 242-252.