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CHANGES OF METAL CONTENTS AND ISOMETALLOTHIONEIN LEVELS IN RAT TISSUES AFTER CADMIUM LOADING

KAZUO T. SUZUKI and MITSURU YAMAMURA
National Institute for Environmental Studies, Yatabe, Tsukuba, Ibaraki 305, Japan

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Abstract—Concentrations of metals (Cd, Zn, Cu and Fe) in serum, liver and kidneys changed with time after a single injection of cadmium chloride into rats. The injected cadmium disappeared from serum within 6 hr and transferred mainly to liver. Distribution patterns of cadmium, zinc and copper in the tissue supernatant fractions were investigated mainly on a SW 3000 column (which was connected to a high speed liquid chromatograph with a flame atomic absorption spectrophotometer) and also on a Sephadex G-75 column. Cadmium in the liver supernatant fractions changed distributions among protein fractions with time and was mostly present as metallothionein 6 hr after the injection. The ratios of both metallothionein-I/-II and Cd/Zn in each isometallothionein changed with time, which suggested active degradation and resynthesis of metallothionein in the liver regardless of the constant metallothionein level. Cadmium in the kidney supernatant fractions increased slowly but consecutively with time and was present mostly as three isometallothioneins on a SW column due to high copper content.

Induction of metallothionein has been well documented in liver and kidneys of laboratory animals after cadmium loading [1–7]. However, there has been no detailed study which examined the time-dependent changes of isometallothioneins in liver and kidneys by analysing not only cadmium, an inducer metal, but also zinc and copper.

Recently we have explored a new analytical method for metallothioneins which made it far easier to investigate metallothioneins at isometallothionein level [8]. The outlet of a high speed liquid chromatograph equipped with a gel permeation column (TSK GEL SW 3000) was directly connected to a nebulizer tube of a flame atomic absorption spectrophotometer (HLC-AAS) and the column was eluted with alkaline buffer solution. Metallothioneins in tissue supernatant fractions were analysed as separate isometallothioneins within an hour at a small sample consumption. The new analytical method was effectively applied to characterize metallothionein dimers [9] and kidney metallothioneins induced by loadings of cadmium-thionein and earthworm cadmium-binding proteins [10, 11].

The present study was intended to investigate the induction of metallothioneins in liver and kidneys at isometallothionein levels by analysing not only cadmium, an inducer metal, but also zinc and copper. At the same time, the changes of metal concentrations induced by cadmium loading were traced in serum for cadmium, copper and iron, and compared with changes in liver and kidneys.

MATERIALS AND METHODS

Induction of metallothionein. Sixty female rats of the Wistar strain (JCL, Clea Japan; 18-week-old; mean body wt ± S.D. 264.4 ± 13.9 g) were injected once intraperitoneally (i.p.) with cadmium chloride at a dose of 1.12 mg Cd/kg body wt. Six animals for each group were killed 1, 3, 6, 12 and 18 hr, 1, 2, 3, 4 and 7 days after the injection by exsanguination under light ether anaesthesia. Control animals (12 rats) were killed without treatment at the middle point of the experiment. Liver and kidneys were removed, washed in chilled Tris–HCl buffer solution (0.1 M, pH 7.4, containing 0.25 M glucose), and stored at −20°.

Preparation of tissue supernatants. A four gram wet weight portion of liver and whole kidneys were homogenized in 3 vol. 0.1 M Tris–HCl buffer solution (pH 7.4, containing 0.25 M glucose) using a polytron homogenizer under ice–water cooling and a nitrogen atmosphere. The homogenates were centrifuged at 170,000 *g* for 60 min at 4° in a Beckman 50 Ti rotor.

Determination of metal concentrations. A one-ml portion of serum was digested with mixed acid (1 ml, $HClO_4$–HNO_3, 1:5 v/v) and diluted to 5 ml with doubly distilled water. Liver and kidney supernatants were diluted 10-fold with doubly distilled water. Concentrations of Cd, Zn, Cu and Fe were determined on an atomic absorption spectrophotometer with deuterium background correction (Shimadzu 640-12).

Gel filtration chromatography on a Sephadex G-75 column. Four millilitre samples of liver and kidney supernatant fractions were pooled in each group and a 10-ml portion of the pooled supernatant fractions was applied to a column (2.6 × 90 cm) of Sephadex G-75 (Pharmacia). The column was eluted with 1 mM Tris–HCl buffer solution, pH 8.6, at a flow rate of 62 ml/hr. Absorbances at 254 and 280 nm and conductivity were continuously recorded on a three-pen recorder (Rikadenki PG-3 with pen gap adjustment memory) using flow cells by a dual wave length u.v. detector (Altex Model 152) and a conductivity meter (M & S Instruments Inc., Model CD-35 M II), respectively. Fractions (5 ml) were collected and

concentrations of cadmium, zinc and copper were determined in each fraction on an atomic absorption spectrophotometer (Hitachi AA 170-50A).

Gel permeation chromatography on a SW 3000 column [8]. The outlet of a high speed liquid chromatograph (Toyo Soda HLC 803A) equipped with a gel permeation column [TSK GEL SW 3000 column, Toyo Soda, 21.5 × 600 mm with a precolumn (21.5 × 100 mm)] was directly connected to a nebulizer tube of a flame atomic absorption spectrophotometer (Hitachi AA 170-50A). A one ml aliquot of the pooled tissue supernatant fractions from each group was applied and the column was eluted with 50 mM Tris–HCl buffer solution (pH 8.6 at 25°, containing 0.1% NaN_3; dissolved gas was removed at 80° under reduced pressure) at a flow rate of 3.7 ml/min. Molecular absorbances at 254 and 280 nm and atomic absorbance of one of the three metals (Cd, Zn and Cu) were recorded on a three-pen recorder.

RESULTS

Cadmium was injected i.p. into rats once at a dose of 1.12 mg Cd/kg body wt as described in Materials and Methods. Concentrations of metals (Cd, Zn, Cu and Fe) in serum and liver and kidney supernatant fractions changed with time after the injection of cadmium as shown in Fig. 1. Cadmium was not detectable in control serum and liver and kidney supernatant fractions. Cadmium found in serum 1 hr after the injection decreased with time and stayed at a low level after 6 hr post-injection. Cadmium in liver increased with time to attain a constant level 6 hr after the injection (data not shown). The concentrations of cadmium in the liver supernatant fractions changed with time and increased to reach their highest level 18 hr after the injection, then remaining at a relatively constant level. The decrease of cadmium and zinc concentrations observed 2 and 3 days after the injection can be explained by the changes of liver weight; namely 8.25 ± 0.24 g (1 day), 9.37 ± 0.41 g (2 days), 10.69 ± 0.84 g (3 days), 9.05 ± 1.09 g (4 days) and 8.32 ± 0.74 g (7 days) (mean ± S.D.). Cadmium in the kidney supernatant fractions continued to increase slowly with time during the experiment.

Zinc in serum decreased to the lowest level (about 25 per cent of the control value) after a temporary increase shortly post-injection, and thereafter started to increase up to 2 days after the injection to reach near the control level. Zinc in the liver supernatant fraction started to increase with time and attained the highest level 18 hr after the injection (as also observed for cadmium) and then stayed at this high level. Although the zinc level in the kidney supernatant fractions fluctuated after the injection, the level increased with the increase of cadmium.

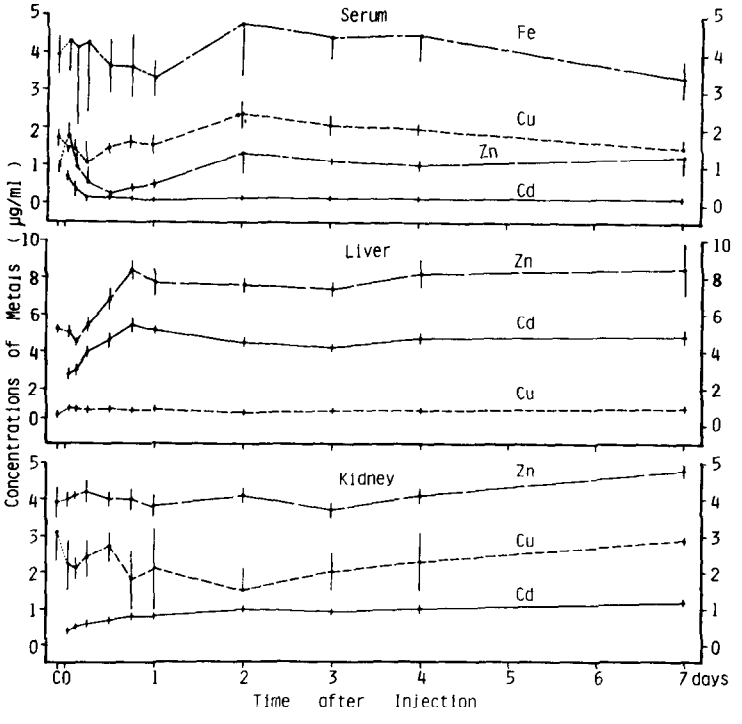

Fig. 1. Changes of metal concentrations in serum and in liver and kidney supernatant fractions after injection of cadmium. Cadmium chloride was injected i.p. into female rats (mean body wt 264 g) at a dose of 1.12 mg Cd/kg body wt. The animals (6 rats/group) were exsanguinated 1, 3, 6, 12 and 18 hr, 1, 2, 3, 4, and 7 days after the injection. Serum was separated by centrifuging blood at 2300 *g* for 10 min. Liver and kidneys were homogenized in three volumes of 0.1 M Tris–HCl buffer solution and the homogenates were centrifuged at 170,000 *g* for 60 min to produce supernatant fractions. Concentrations (μg/ml serum or supernatant fraction) were indicated as mean ± S.D. of six samples in each group. C indicates control values.

Copper in serum decreased for the first 6 hr and then started to increase up to 2 days after the injection. Thereafter, it decreased slowly to level off at a control level. Copper in the liver supernatant fractions stayed almost at constant low level during the experiment. On the other hand, copper in the kidney supernatant fractions fluctuated for the first 2 days (as also observed for zinc) and then started to increase from the lowest level 2 days after the injection. Iron in serum fluctuated without significant changes from the control value.

Distribution patterns of cadmium in the liver supernatant fractions were investigated on a Sephadex G-75 column (data not shown). Cadmium was mostly present in the high molecular weight fraction for the first 3 hr after the injection and then redistributed mostly to the metallothionein fraction 6 hr after the injection on a Sephadex G-75 column, as reported by Nordberg *et al.* [1]. The cadmium redistributed to the metallothionein fraction stayed at the same profile on a Sephadex G-75 column during the experiment. The distribution patterns of cadmium were also investigated on a SW column which was found to have both gel filtration and cation exchange chromatographic properties [8]. A SW 3000 column connected to a high speed liquid chromatograph was eluted with alkaline buffer solution in order to separate metallothionein into isometallothioneins and the outlet of the column was directly connected to a nebulizer tube of a flame atomic absorption spectrophotometer (HLC-AAS) for simultaneous and continuous determination of cadmium concentration in the eluate. The distribution profiles of cadmium were similar to those on a Sephadex G-75 column except for the separation of metallothionein into the two isometallothioneins (metallothionein-I and -II at retention times of 39.6 and 37.2 min, respectively) on a SW 3000 column. As shown in Fig. 2, cadmium was mostly localized in the high molecular weight fraction for the first 3 hr and thereafter was localized in the metallothionein fraction. Although cadmium in the metallothionein fraction was observed as a single peak without any apparent changes on a Sephadex G-75 column during the experiment, the distribution profiles on a SW 3000 column clearly indicated that the ratio of the two isometallothioneins changed with time. The changes of isometallothionein ratios with time without any appreciable changes of metallothionein level indicated the active degradation of metallothionein followed by resynthesis of new metallothioneins with different isometallothionein ratios from the degraded metallothioneins. The ratios of metallothionein-I to -II were unity at 6 hr, less than unity between 12 and 24 hr, again about unity at 2 days, and more than unity for the rest of the experiment.

Distribution profiles of zinc in the liver supernatants were also investigated both on a Sephadex G-75 column and on a SW 3000 column. Zinc in the metallothionein fraction started to increase 6 hr after the injection and then the Zn/Cd ratio stayed at nearly constant level on a Sephadex G-75 column (data not shown). Distributions of zinc on a SW 3000 column (HLC-AAS) also changed with time in the metallothionein fraction, as observed for cadmium, regardless of no appreciable changes on a Sephadex G-75 column. The ratio of metallothionein-I to -II was less than unity from 6 hr to 3 days, unity between

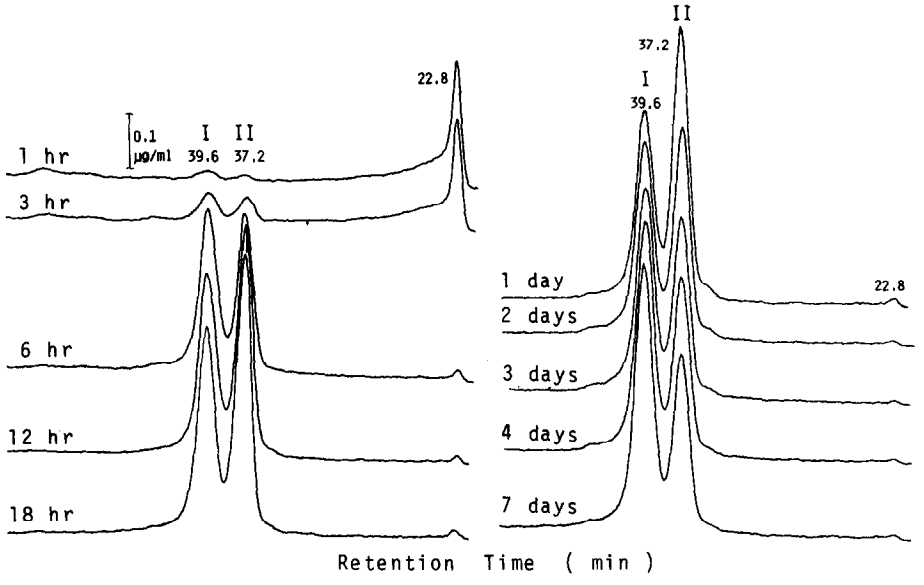

Fig. 2. Gel permeation–cadmium atomic absorption chromatograms of liver supernatant fractions after injection of cadmium. A 1-ml portion of the pooled supernatant fractions from each group was applied to a high speed liquid chromatograph equipped with a gel permeation column [TSK GEL SW 3000, 21.5 × 600 mm with a precolumn (21.5 × 100 mm)]. The column was eluted with 50 mM Tris–HCl buffer solution (pH 8.6 at 25°) at a flow rate of 3.7 ml/min. Atomic absorbance of cadmium was continuously monitored by directly connecting the outlet of the column to a nebulizer tube of a flame atomic absorption spectrophotometer. The detector level of the spectrophotometer was set as indicated with a vertical bar. I and II indicate metallothionein-I and -II, respectively.

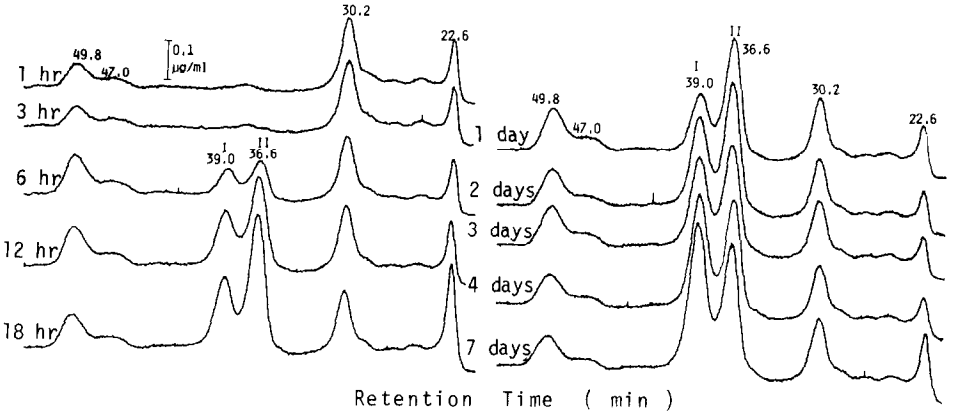

Fig. 3. Gel permeation–zinc atomic absorption chromatograms of liver supernatant fractions after injection of cadmium. A 1-ml portion of the pooled liver supernatant fractions from each group was applied to a high speed liquid chromatograph as indicated in the legend of Fig. 2. Atomic absorbance of zinc was continuously monitored, instead of cadmium as in Fig. 2. The detector level of an atomic absorption spectrophotometer was set as indicated by a vertical bar. I and II indicate metallothionein-I and -II, respectively.

3 and 4 days, and more than unity 7 days after the injection. The ratios of metallothionein-I to -II in Fig. 3 changed differently from those of Fig. 2 (the ratios monitored by zinc changed with time delay from those monitored by cadmium) and thus the Cd/Zn ratios in each isometallothionein were also found to change with time. Zinc peaks other than metallothionein peaks remained at constant pattern without any appreciable changes during the experiment. Although zinc peaks at retention times of 47.0 and 49.8 min were not identified, those peaks were found to originate from the high molecular weight fraction on a Sephadex column and to be unstable to heat treatment (80° for 10 min) [13].

The distribution profiles (the cadmium profile increased consecutively in the kidney supernatant fractions after the injection) were also investigated both on a Sephadex G-75 column (data not shown) and on a SW 3000 column. In contrast to the distribution profiles of cadmium in the liver supernatant fractions, cadmium in the kidney supernatant fractions was mainly present in the metallothionein fraction even 1 hr after the injection. The cadmium added to the kidney supernatant fractions with time was found in the metallothionein fraction and the cadmium in the metallothionein was eluted at the same rate with zinc and copper on a Sephadex G-75 column, as already reported [12]. The distribution profiles of cadmium in the kidney supernatant fractions were different from those of liver supernatant fractions on a SW column. Cadmium in the metallothionein fraction was separated into three peaks as shown in Fig. 4. The first peak, which corresponds to metallothionein-II, was eluted at a retention time of 36.8 min and was far smaller than the second one; the second peak, which corresponds to metallothionein-I, was eluted at a retention time of 39.2 min; and the third peak, with a retention time of 43.0 min, was present as a third isometallothionein peak in the kidney supernatant fractions. The third peak has been observed only when copper content is high in the metallothionein fraction as observed for cadmium ion loadings [13] and *in vitro* replacement of zinc and/or cadmium with cupric ion in liver metal-

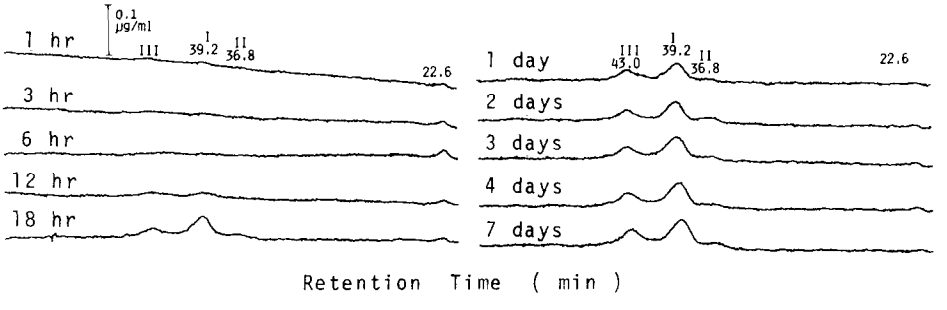

Fig. 4. Gel permeation-cadmium atomic absorption chromatograms of kidney supernatant fractions after injection of cadmium. A 1-ml portion of the pooled kidney supernatant fractions from each group was applied to a SW 3000 column as indicated in Fig. 2. The detector level of an atomic absorption spectrophotometer was set as indicated by a vertical bar. I, II and III indicate metallothionein-I and -II and the third peak, respectively.

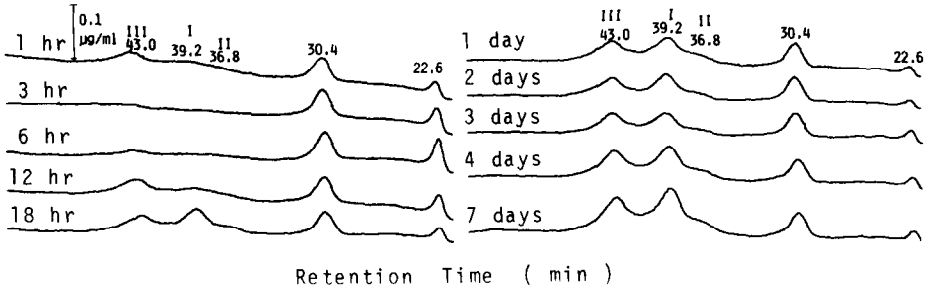

Fig. 5. Gel permeation–zinc atomic absorption chromatograms of kidney supernatant fractions after injection of cadmium. A 1-ml portion of the pooled kidney supernatant fractions from each group was applied to a SW 3000 column as indicated in Fig. 2. The detector level of an atomic absorption spectrophotometer was set as indicated by a vertical bar. I, II and III indicate metallothionein-I and -II, and the third peak, respectively.

lothionein (liver metallothionein is low in copper content [7]) [14].

Figure 5 shows the distribution profiles of zinc in the kidney supernatants on a SW 3000 column. Zinc peak at a retention time of 30.4 min corresponds to superoxide dismutase. Zinc in the metallothionein fraction was separated into three peaks as observed for the distribution profiles of cadmium in Fig. 4. The three isometallothionein peaks continued to increase with the increase of cadmium peaks in Fig. 4. Zinc peaks with larger retention times than isometallothionein peaks were not observed in the kidney supernatant fractions.

Figure 6 illustrates the distribution profiles of copper in the kidney supernatant fractions on a SW 3000 column. Copper in the metallothionein fraction was again separated into three peaks and showed similar changes of elution profiles to those of cadmium and zinc. Copper peak at a retention time of 30.4 min corresponds to superoxide dismutase.

DISCUSSION

Metallothionein is known to be a mixture of two isometallothioneins with different isoelectric points and is separable into the two isoproteins by ion exchange column chromatography [15], column and flat-bed electrofocusing [16] and electrophoresis [3]. Although ion exchange column chromatography is a preferable procedure due to easy detection of metals in solution, the analytical procedure is time-consuming and often lacks reproducibility. Therefore, metallothionein has not been routinely separated into isometallothioneins. Recently we have explored a new analytical method for metallothioneins which made it possible to analyse metallothioneins routinely at isometallothionein level: direct connection of a high speed liquid chromatograph equipped with a gel permeation column (TSK GEL SW 3000) and a flame atomic absorption spectrophotometer (HLC-AAS), and elution of the column with alkaline buffer solution [8].

Separations of metallothionein into isometallothioneins were demonstrated to be highly effective for the understanding of dynamic aspects of metallothionein. The present results for the changes of both Cd/Zn ratios in each isometallothionein and ratios between metallothionein-I and -II support the results for active degradation and resynthesis of metallothionein obtained by using labeled amino acid [5]. Although the reasons why metallothionein of different isometallothionein ratios and different Cd/Zn ratios are biosynthesized with time in liver after cadmium loading are not known, these results have not so far been recognized. The changes might be related to different stability of isometallothioneins to proteolytic enzymes and stability constants between metals (Cd and Zn) and iso-thioneins.

In contrast to the dramatic changes of isometal-

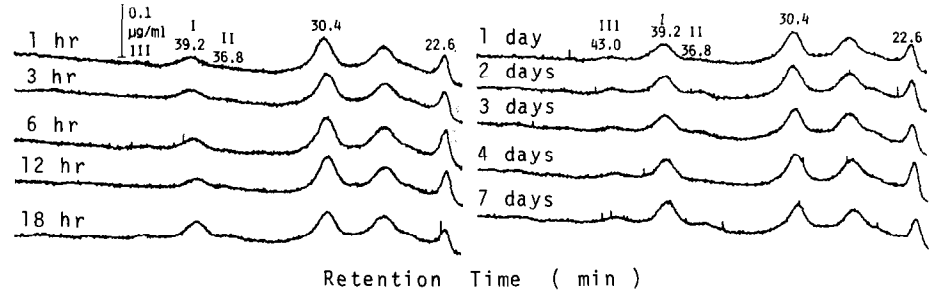

Fig. 6. Gel permeation–copper atomic absorption chromatograms of kidney supernatant fractions after injection of cadmium. A 1-ml portion of the pooled kidney supernatant fractions from each group was applied to a SW 3000 column as indicated in Fig. 2. The detector level of an atomic absorption spectrophotometer was set as indicated by a vertical bar. I, II and III indicate metallothionein-I and -II and the third peak, respectively.

lothionein and metal ratios with time in the liver supernatant fractions, isometallothioneins in the kidney supernatant fractions remained at constant profiles. The latter result also contrasted with the dramatic changes observed for kidney supernatant fractions after induction of kidney metallothionein by the injection of cadmium-thionein [10]. The different isometallothionein profiles observed in the kidney supernatant fractions are related to the chemical forms of injected metal, cadmium ion or cadmium-thionein. In the case of injection of cadmium ion reported in the present paper, cadmium is transferred consecutively but very slowly into the kidneys. A small amount of metallothionein (Cu, Zn-thionein) is always present in the kidneys of adult rats, and the metallothionein is assumed to be actively degraded and resynthesized. Therefore, the slow transfer of cadmium may not stimulate a further induction of mRNA for metallothionein biosynthesis. On the other hand, rapid transfer of cadmium into the kidneys may induce a further transcription of the specific mRNA. Induction of metallothionein in kidneys by cadmium ion injection (present result) is probably the former case and inductions of metallothionein in liver by cadmium ion injection (present result) and in kidneys by cadmium-thionein injection [10] are probably the latter case.

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