[Chem. Pharm. Bull. 27(3) 647—653 (1979)]

UDC 615.916.015.4.076.9 : 546.48.09

Fate of intraperitoneally Injected Liver Metallothionein in Rat Kidney

Kazuo T. Suzuki, Tamio Maitani, and Shinji Takenaka

National Institute for Environmental Studies[1]

(Received August 3, 1978)

Isolated rat liver metallothionein was injected intraperitoneally into female rats to find whether or not the injected metallothionein was recovered from the kidneys with high copper content as in the case of rat kidney metallothionein induced by exposure to inorganic cadmium ion. The distribution patterns of cadmium and zinc among protein fractions in the kidney supernatants changed dramatically within one day after the injection. The changes were explained by the degradation and re-synthesis of the injected metallothionein in the kidneys. Copper was not found as a major metal in any metallothionein fractions obtained from the kidney supernatants. This is in marked contrast to cadmium ion-exposed rat kidney metallothionein. The relationships between the selective and profound toxicity of the injected metallothionein to the tubular lining cells and the changes of the chemical forms of cadmium were also discussed.

Keywords——metallothionein; cadmium-thionein; tubular necrosis; cadmium; zinc; copper; rat

During the course of investigations about the relationships between the adverse effects and the chemical forms of cadmium in living tissue, we have found that cupric ion has a stronger affinity to metallothionein than zinc and cadmium ion, and that it is one of the major metals in cadmium-exposed rat kidney metallothionein.[2] In contrast to the high copper content in the kidney metallothionein of cadmium ion-exposed rats, the liver metallothionein obtained from the same animals contained only a small amount of copper.[2] Although the primary structures of rat metallothioneins have not been determined, the same amino acid compositions have been suggested for liver and kidney metallothioneins.[3] Cadmium is assumed to be transferred from liver to kidney possibly as metallothionein.[4] It is possible that the high content of copper found in the cadmium ion-exposed kidney metallothionein was due to incorporation of copper during transfer of the metallothionein from liver to kidney. To explore this possibility we injected liver metallothionein into rats and traced the metabolic fate of the metallothionein in the kidney. At the same time, histopathological examinations and clinical analyses of urine were conducted to find the relationships between the toxicity of the injected metallothionein and the chemical forms of cadmium in the kidney.

Materials and Methods

Isolation of Liver Metallothionein——The biosynthesis of liver metallothionein was induced in female rats of the Wistar strain (mean body weight, 170 g) by repeated intraperitoneal injections with cadmium chloride (1.12 mg Cd^{2+}/kg body weight, nine times during four weeks). The animals were sacrificed four days after the last injection of cadmium chloride by removing blood under light ether anaesthesia. The livers (100 g) were homogenized using a teflon homogenizer (10—20 g livers/time) in four times the volume of Tris buffer solution (0.1 M, pH 7.4) containing glucose (0.25 M) and the homogenate was centrifuged at 39000 ***g*** for 20 min at 2°. The supernatant was heated at 68° for 2 min under nitrogen gas (100 ml portion in 250 ml flask), cooled in ice–water, then, centrifuged at 39000 ***g*** for 20 min at 2°. The heat-treated super-

1) Location: *P.O. Yatabe, Ibaraki 300-21, Japan.*
2) K.T. Suzuki, K. Kubota, and S. Takenaka, *Chem. Pharm. Bull.* (Tokyo), **25**, 2792 (1977).
3) M. Kimura, N. Otaki, S. Yoshiki, M. Suzuki, H. Horiuchi, and T. Suda, *Arch. Biochem. Biophys.*, **165**, 340 (1974).
4) L. Friberg, M. Piscator, G.F. Nordberg, and T. Kjellström, "Cadmium in the Environment," CRC Press, Ohio, 1974, p. 54.

natant was filtered by Miliporefilter (Φ 0.8 μm) and concentrated to 24 ml by ultrafiltration on a Diaflo UM-10 membrane (Amicon). The concentrated solution (8 ml/column) was applied to a Sephadex G-75 column (2.6×90 cm) and eluted with Tris buffer solution (1 mM, pH 8.6) and collected (70 drops/tube; 5 ml/tube). Light absorbances at 254 and 280 nm were measured with a Hitachi 191E Spectrophotometer and metal contents (Cd, Zn, and Cu) in each eluate were determined with a Hitachi 508 Atomic Absorption Spectrophotometer. The main metallothionein fractions were combined and concentrated by ultrafiltration on a Diaflo UM-10 membrane. The concentrated metallothionein solution contained Cd (31.5 μg/ml), Zn (14.0 μg/ml), Cu (0.7 μg/ml), and protein (1.7 mg/ml, Lowry method[5]).

Injection of Liver Metallothionein——The isolated metallothionein (weight ratio of the metals; Cd:Zn:Cu=45:20:1) was injected intraperitoneally into 72 female rats of the Wistar strain (SLC, Hamamatsu, Japan) (6-weeks-old; mean body weight, 96.5 g; nine rats/group) with 1 ml of the metallothionein solution/100 g body weight. The animals were sacrificed 1, 6, 12 hr, 1, 2, 4 days, 1, and 2 weeks after the injection by removing blood under light ether anaesthesia. Serum was separated by centrifuging blood at 2300 ***g*** for 20 min. Three rats were used for histopathological examinations and six rats were used for metal analysis.

Isolation and Metal Analysis of Metallothionein from the Kidneys——The kidneys of three rats were combined and homogenized in three times the volume of Tris buffer solution (0.1 M, pH 7.4) containing glucose (0.25 M). 0.5 ml of the homogenate was used for measurement of the total metal contents in the kidneys. The rest of the homogenate was centrifuged at 105000 ***g*** for 75 min at 2°. 0.5 ml of the supernatant was used for measurement of metal contents in the supernatant. The measured amount of the supernatant (7—9 ml) was applied to a Sephadex G-75 column (2.6×90 cm), eluted with Tris buffer solution (1 mM, pH 8.6) and collected (5 ml/tube). Light absorbances at 254 and 280 nm, and the metal contents (Cd, Zn, and Cu) were determined as above. The metal contents of tissue homogenate and supernatant were determined after digesting with H_2SO_4 (0.2 ml) and HNO_3 (0.5 ml×2) (acids for metal analysis, Wako Pure and Chemical Industries, Ltd., Tokyo) and diluting to 5 ml with doubly distilled water.

Metal Analysis in Urine——Urine of three rats was collected in one bottle which was cooled in an ice-salt bath. Urine samples were centrifuged at 2300 ***g*** for 10 min to remove non-soluble materials. Urine collected for 12 hr after the injection of metallothionein was used for gel filtration analysis. 5 ml from each bottle were combined and 30 ml (5 ml×6 groups) of the combined samples were concentrated 5-fold by ultrafiltration on a Diaflo UM-10 membrane. The amounts of the three metals in the urine (30 ml) were: Cd, 106 μg; Zn, 96 μg; Cu, 36 μg. The concentrated urine (6 ml) contained 100 μg of Cd, 66 μg of Zn, and 34 μg of Cu.

Histopathological and Clinical Analyses——For histopathology the kidneys were removed from the animals immediately after death, and fixed in 10% buffered formalin. Preparations, embedded in paraffin, were sectioned at 4 to 5 μm and stained with haematoxylin and eosin or periodic acid Schiff (PAS).

Glucose in urine was determined enzymatically with "Eskalb" Bulk Glucose Reagent (SKI) using an autoanalyzer (GEMSAEC III).

Results

Rat liver metallothionein was induced by repeated injections of cadmium chloride and isolated by heat treatment and gel filtration.[6] The metallothionein solution contained cadmium, zinc, and copper in the amount of 31.5, 14.0, and 0.70 μg/ml (weight ratio; 45:20:1), respectively. The solution was injected intraperitoneally into rats in the amount of 1 ml/100 g body weight.

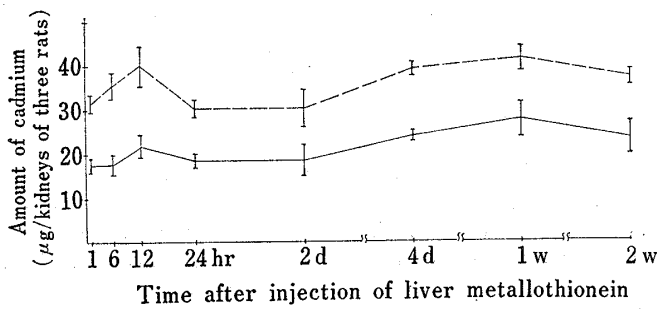

Fig. 1. Time Course of Cadmium Contents in Total Kidneys and Kidney Supernatants after Injection of Liver Metallothionein

Kidneys of three rats were combined. The amount of cadmium in the figure was expressed as a mean value of two experiments (horizontal bars indicate values of each experiment).
----, cadmium in total kidneys; ——, cadmium in supernatants.

Fig. 1 is the time course of cadmium contents in total kidney and kidney supernatants after the injection. The cadmium contents were almost constant in the kidney and supernatant during the experiment reflecting the long half-life in the kidneys. Contrary to the relatively constant amount of cadmium in the kidney, the distribution patterns

5) O.H. Lowry, N.J. Rosebrough, A. Farr, and R.J. Randall, *J. Biol. Chem.*, **193**, 265 (1951).
6) M.G. Cherian, *Biochem. Biophys. Res. Commun.*, **65**, 863 (1974).

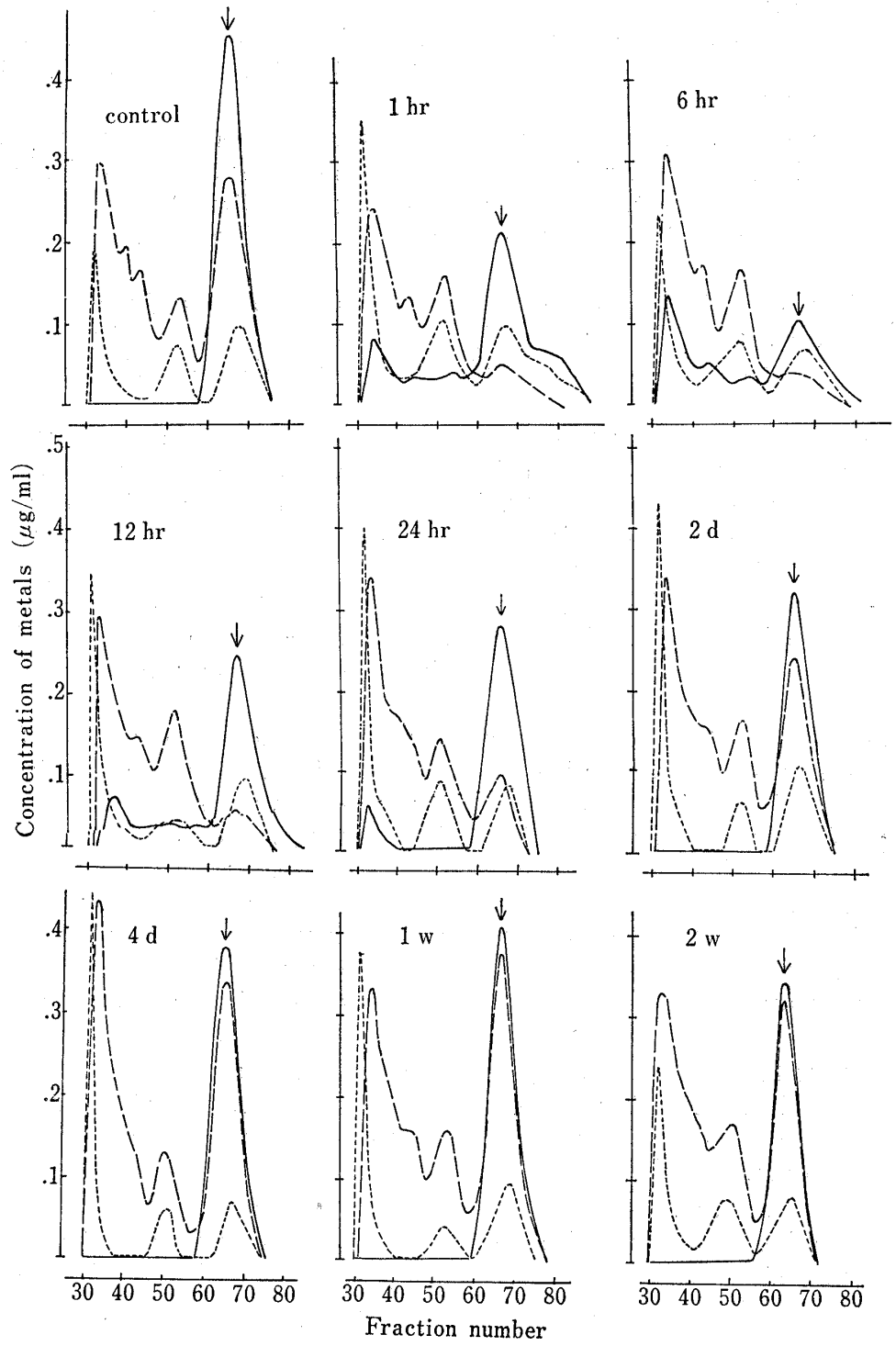

Fig. 2. Changes of Sephadex G-75 Elution Profiles of Kidney Supernatants after Injection of Liver Metallothionein

The curves are as follows: ——, Cd; – – –, Zn; – – –, Cu. The arrow indicates the metallothionein fraction.

of cadmium in the supernatant fractions of the kidneys changed dramatically within several days after the injection as shown in Fig. 2.

Fig. 2–control shows the distribution patterns of cadmium, zinc, and copper on a Sephadex G-75 column in the control supernatant. The control supernatant was obtained by homogenizing the kidneys of three non-treated control rats in three times the volume of Tris buffer solution (0.1 M, pH 7.4, 0.25 M glucose) which contained 1 ml of the liver metallothionein solution used for the injection.

Fig. 2–1 hr is the distribution pattern of the three metals in the supernatant of the kidneys obtained one hour after the injection. Cadmium was not confined to the metallothionein fraction in contrast to Fig. 2–control, but was distributed throughout the protein fractions. The amount of zinc in the metallothionein fraction was far lower than in the control.

Fig. 2–6 hr is the distribution pattern of the three metals in the supernatant of the kidneys obtained six hours after the injection. The amount of cadmium in the high molecular weight protein fractions increased at the expense of cadmium in the metallothionein fraction.

Fig. 2–12 hr shows the distribution pattern of the three metals in the supernatant of the kidneys obtained twelve hours after the injection. Contrary to the changes up to six hours the amount of cadmium in the metallothionein fraction increased and that of cadmium in the high molecular weight protein fractions decreased. The amount of zinc in the metallothionein fraction still remained low.

Fig. 2–24 hr is the distribution pattern of the three metals in the supernatant of the kidneys obtained one day after the injection. Cadmium in the metallothionein fraction increased more than that in Fig. 2–12 hr and cadmium in the high molecular weight protein fractions decreased.

Fig. 2–2 d is the distribution pattern of the three metals in the supernatant of the kidneys obtained two days after the injection. The amount of zinc in the metallothionein fraction increased and cadmium in the high molecular protein fractions was not detected.

Fig. 2–4 d, –1 w, and –2 w illustrate the distribution patterns of the three metals in the supernatants of the kidneys obtained 4 days, 1, and 2 weeks after the injection and the patterns are very similar. Copper in the metallothionein fraction was always present as a minor metal and the copper was eluted a little slower than cadmium and zinc for the metallothionein fraction. The low content of copper and the slow elution rate of copper on a Sephadex G-75 column were in contrast to the kidney metallothionein obtained by exposure to inorganic cadmium ion.[2)]

Fig. 3 shows the time course of the metal contents in serum. Cadmium was found only in serum obtained one hour after the injection. A decrease of copper in serum obtained one and six hours after the injection and a decrease of zinc in serum obtained six hours after the injection were observed.

Fig. 4 shows the time course of the three metals in urine. Cadmium was excreted in urine within twelve hours along with zinc. The distribution pattern of the three metals in urine was analyzed as follows. A urine sample (30 ml) collected for twelve hours after the injection was concentrated to 6 ml by ultrafiltration on a Diaflo UM-10 membrane. A considerable amount of zinc was filtered out during the concentration step, but only small amounts of cadmium and copper were filtered out. This means that metal ion or metal bound to smaller molecules were filtered out from the membrane. The distribution pattern of the three metals is shown in Fig. 5. Almost all the cadmium but only a small amount of zinc was found in the metallothionein fraction. The rest of the zinc was found in the low molecular weight fractions, and this result explained why a considerable amount of zinc was removed during filtration. Almost all the copper was found in the metallothionein fraction. This is due to the stronger affinity of cupric ion to metallothionein relative to zinc and cadmium ion.[2)]

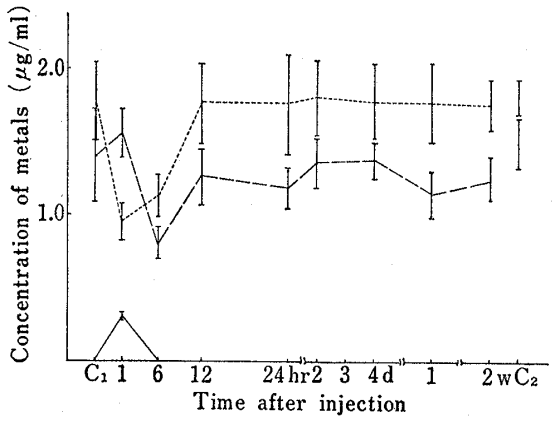

Fig. 3. Changes of Metal Contents in Serum after Injection of Liver Metallothionein

C_1 and C_2 indicate the control values at the beginning and at the end of experiment (rats were sacrificed at one day and two weeks), respectively. The values indicate the mean±S.D. (n=9). The curves are as follows: ——, Cd; – – –, Zn; ------, Cu.

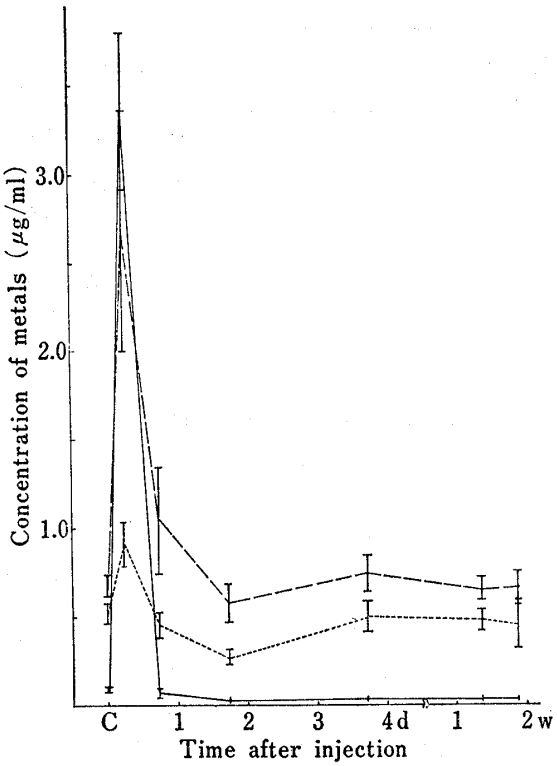

Fig. 4. Changes of Metal Contents in Urine after Injection of Liver Metallothionein

Urine of three rats was collected in one bottle for 12 hr at various time intervals after the injection of metallothionein. C indicates control values before the injection. The values indicate the mean±S.D. (n=6 or 9). The curves are as follows: ——, Cd; – – –, Zn; ------, Cu.

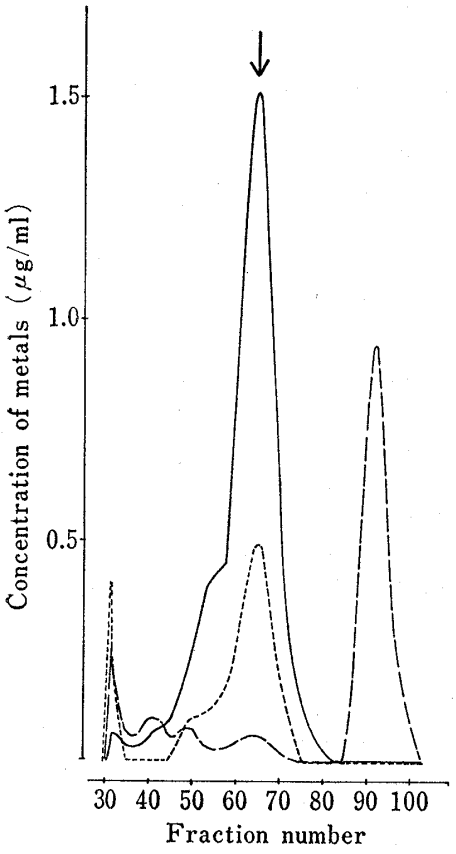

Fig. 5. Sephadex G-75 Elution Profiles of Urine

Urine of eighteen rats which was collected for 12 hr after the injection of metallothionein was concentrated 5-fold by ultrafiltration. The concentrated solution (6 ml) was applied to a Sephadex G-75 column. The curves are as follows: ——, Cd; – – –, Zn, ------, Cu. The arrow indicates the metallothionein fraction.

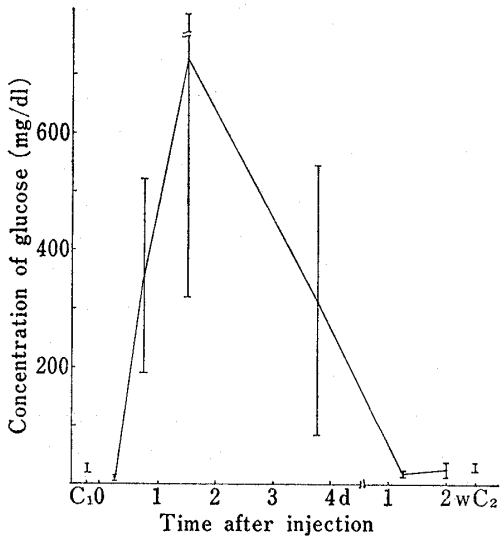

Fig. 6. Time Course of Glucose Content in Urine

Urine of three rats was collected in one bottle for 12 hr. The values indicate mean±S.D. (n=6 or 3). C_1 and C_2 show the control values at the beginning and at the end of the experiment (n=4), respectively.

Histopathological examinations of the kidneys were carried out. The injection of metallothionein caused massive tubulonecrosis at the proximal renal tubules on the next day as reported by other authors.[7]

On the second day, though the tubulonecrosis remained, frequent mitosis appeared at the residual epithelial cells of the renal tubules.

On the fourth day, may regenerated epithelial cells appeared in the region of the proximal tubules.

Fig. 6 shows the change of glucose content in urine after the injection of metallothionein.

Discussion

It is clear that for all of the distribution profiles in Fig. 2 the recovered metallothionein did not contain copper as a major metal in contrast to the kidney metallothionein induced by exposure to inorganic cadmium ion.[2]

The increased amount of cadmium bound to proteins other than metallothionein and the decreased amount of cadmium in the metallothionein fraction suggested the degradation of the injected metallothionein and the re-distribution of the cadmium liberated from the degraded metallothionein to other proteins. The degradation must have occurred in the kidneys from the following reasons. If the degradation occurred during circulation, the liberated cadmium (from the degraded protein) would be partly bound to intact metallothionein by replacing zinc and partly bound to high molecular weight proteins in serum. The cadmium rebound to intact metallothionein would be carried to the kidneys and the cadmium bound to high molecular weight proteins would be carried to the liver as in the case of the injected inorganic cadmium ion or bound cadmium to albumin.[7c] The low distribution of cadmium to the liver was observed in the present study (less than 0.1 ppm in the liver supernatant obtained from any livers after the injection of metallothionein) as already reported by other authors[7] and the observation suggested that the degradation during circulation or in the liver did not occur or occurred only to a small extent.

The change of the distribution patterns of cadmium and zinc in the interval from 6 hr to 4 days after the injection is similar to the change observed in liver after the injection of cadmium ion.[8] Therefore, the change in the interval from 6 hr to 4 days after the injection can be best explained by the synthesis of metallothionein in the kidneys.

Further experimental evidence supporting the degradation and the re-synthesis of the injected metallothionein in the kidneys was obtained for an experiment where injection of isolated cadmium–thionein-I or -II resulted in the recovery of both forms of metallothionein.[9] The injection of single form of metallothionein with cadmium but with a trace amount of zinc and the recovery of both forms of metallothionein with cadmiun and zinc from the kidneys indicated that the apoprotein part of the injected metallothionein was degraded and the re-synthesis of the two forms occurred *de novo*.

The necrotic lesion of renal tubular lining cells was induced shortly after the injection of metallothionein and the restoration of the tissue started two days after the injection in spite of the presence of almost same amount of cadmium in the kidneys. The changes in chemical forms of cadmium during the period between necrosis and commencement of restoration of the kidneys was evident from the changes of the gel filtration profiles as shown in Fig. 2. Cadmium was liberated from the degraded metallothionein in the kidneys shortly after the injection and the metal was bound to high molecular weight protein fractions. Later cadmium

7) *a*) G.F. Nordberg, R.A. Goyer, and M. Nordberg, *Arch. Pathol.*, **99**, 192 (1975); *b*) M.G. Cherian, R.A. Goyer, and L.D. Richardson, *Toxicol. Appl. Pharmacol.*, **38**, 399 (1976); *c*) M. Webb and A.T. Etienne, *Biochem. Pharmacol.*, **26**, 25 (1977).
8) G.F. Nordberg, M. Piscator, and B. Lind, *Acta Pharmacol. Toxicol.*, **29**, 456 (1971).
9) K.T. Suzuki and M. Yamamura, *Biochem. Pharmacol.*, in press.

was become bound to metallothionein. This result explained since the lesion induced by the injection of metallothionein is due not to metallothionein itself but to the liberated cadmiun from the degraded protein. The changes of glucose content in urine as a marker of kidney dysfunction also confirmed the renal lesion and the restoration (Fig. 6).

The distribution profile of the three metals among urine (Fig. 5) indicated that zinc in metallothionein was replaced by cadmium and copper due to its (*i.e.* zinc) weaker affinity to the protein. This result also indicates that when only a small amount of metallothionein is present in urine, the amount of copper in urine is sufficient to replace completely zinc and cadmium in the metallothionein. Therefore, cadmium and zinc will not be found in the metallothionein fraction of urine and only copper will be found in the fraction when only a small amount of metallothionein is excreted in urine.

Our results can be summarized as follows: 1) The distribution patterns of cadmium and zinc among protein fractions in the kidney supernatants changed dramatically within one day after the injection. The changes were explained by the degradation and re-synthesis of the injected metallothionein in the kidneys. 2) The re-synthesized metallothionein does not contain copper as a major metal and it contrasts to the kidney metallothionein induced by exposure to inorganic cadmium ion. 3) The renal lesion induced by the injection of metallothionein is probably due not to metallothionein itself but to the cadmium liberated from the degraded protein.

The present study posed the following questions: 1) why the injected liver metallothionein was degraded in the kidneys in spite of the same amino acid compositions as that of the kidney metallothionein when induced by exposure to inorganic cadmium ion, 2) why the metal contents were different for kidney metallothionein induced by exposure to inorganic cadmium ion and for kidney metallothionein induced by the injection of metallothionein, 3) when, and where the copper in kidney metallothionein was incorporated by exposure to inorganic cadmium ion.

Acknowledgement We thank Dr. K. Kubota for encouragement and Mrs. Y. Shinozaki for clinical data.