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Fate of Kidney Metallothionein intraperitoneally Injected into the Rat

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Rat kidney metallothionein, which contains copper as a major metal, was injected into young female rats to investigate whether the injected kidney metallothionein is degraded and re-synthesis of metallothionein occurs in the kidneys, as in the case of injected liver metallothionein. The changes in the gel filtration profiles of the kidney supernatant fractions with time could be explained by the degradation and re-synthesis of metallothionein in the kidneys. The cadmium and copper liberated from the degraded metallothionein induced the biosynthesis of metallothionein and copper-binding protein (possibly copper-thionein). The re-synthesized metallothionein was rich only in cadmium and zinc, and the half-lives of the two metals were long. On the other hand, the half-life of the copper-binding protein was short. The peak positions of the metallothionein and the copper-binding protein were different on a Sephadex G-75 column.

The renal tubular lining cells were severly damaged by the injection of metallothionein. The necrosis and recovery of the kidneys were confirmed by microscopic examinations and by analysis of urinary glucose.

Keywords——metallothionein; cadmium; zinc; copper; copper-thionein; copper-binding protein; kidney metallothionein; kidney

Cadmium is preferentially accumulated in the kidneys, which are the main target for the adverse effects of the metal.[2)] During the course of investigations on the relationships between the adverse effects and the chemical forms of cadmium in living tissues, we found that copper has a stronger affinity *in vitro* for metallothionein than zinc or cadmium.[3)] In contrast to the very low copper content in rat liver metallothionein induced by cadmium ion injection, kidney metallothionein obtained from the same animal contained copper as the most abundant metal.[3)] The low copper content in liver metallothionein and high copper content in kidney metallothionein are of interest in relation to the adverse effects of cadmium on the kidneys.

To identify the origin of copper in the kidney metallothionein, we have injected liver metallothionein intraperitoneally into rats.[4)] The injected liver metallothionein was mainly transferred to and reabsorbed at the renal tubules without degradation, as observed by Foulkes.[5)] The metallothionein was degraded in the kidneys and the cadmium liberated from the degraded protein stimulated the re-synthesis of metallothionein in the kidneys. However, unlike the kidney metallothionein induced by cadmium ion injection, the re-synthesized metallothionein did not contain copper as a major metal and the relative metal content (Cd/Zn ratio) in the re-synthesized metallothionein was different from that in the injected material.

The present study was intended to clarify whether injected kidney metallothionein is degraded as in the case of injected liver metallothionein, in spite of possible conformational change due to the higher copper content[6)] in the kidney than in the liver metallothionein.

1) Location: *P. O. Yatabe, Ibaraki 300-21, Japan.*
2) L. Friberg, M. Piscator, G.F. Nordberg, and T. Kjellström, "Cadmium in the Environment," CRC Press, Ohio, 1974, p. 101.
3) K.T. Suzuki, K. Kubota, and S. Takenaka, *Chem. Pharm. Bull.* (Tokyo), **25**, 2792 (1977).
4) K.T. Suzuki, T. Maitani, and S. Takenaka, *Chem. Pharm. Bull.* (Tokyo), **27**, 647 (1979).
5) E.C. Foulkes, *Toxicol. Appl. Pharmacol.*, **45**, 505 (1978).
6) K.T. Suzuki and M. Yamamura, *Arch. Environ. Contam. Toxicol.*, **8**, 471 (1979).

The metabolic fate of the injected kidney metallothionein in the kidneys was traced by following the time-dependent changes of the gel filtration profiles of kidney supernatant fractions.

Microscopic examinations of the kidneys and urinary analysis were conducted to determine whether the injected kidney metallothionein is toxic to the kidneys, as in the case of the injected liver metallothionein.

Materials and Methods

All glassware was rinsed three times with doubly distilled water. The animals were maintained on a standard laboratory chow (Clea Japan, Tokyo) and distilled water *ad libitum*. Metal content was determined on a Hitachi 508 atomic absorption spectrophotometer. Absorbances at 254 and 280 nm were recorded on a Hitachi 100-21 spectrophotometer.

Preparation of Kidney Metallothionein——Rat kidney metallothionein was induced in female rats of the Wistar strain (mean body weight, 202 g) by intraperitoneal injections of cadmium chloride (two injections of 1.12 mg Cd^{2+}/kg body weight and one injection of 2.24 mg Cd^{2+}/kg body weight at intervals of three days). Metal contents in the kidney metallothionein were followed for six months. Copper was always accompanied by cadmium in kidney metallothionein, and it increased in amount with time. Copper was present as a major metal even a short time after a single injection of cadmium ions.

Kidney metallothionein used in the present experiments was obtained from twenty rats which were sacrificed six months after the last injection by exsanguination under light ether anaesthesia. The kidneys (41 g) were homogenized using a Teflon homogenizer in four volumes of 0.1 M Tris-HCl buffer solution (pH 7.4) containing 0.25 M glucose, and the homogenate was centrifuged at $105000 \times g$ for 75 min at 2—4°. The supernatant (55 ml/column) was applied to a Sephadex G-75 column (5×80 cm) and the column was eluted with 10 mM Tris-HCl buffer solution (pH 8.6). The metallothionein fractions (monitored by atomic absorption spectrometry of cadmium, copper, and zinc) were concentrated by ultrafiltration on a Diaflo UM-10 membrane (Amicon). The concentrated solution contained cadmium, 24 μg/ml; copper, 18 μg/ml; and zinc, 5.2 μg/ml.

Metabolic Fate of Kidney Metallothionein injectcd into Rats——The metallothionein solution (1 ml/100 g body weight) was injected once intraperitoneally into thirty female rats of the Wistar strain (SLC, Hamamatsu; body weight, 99.9±3.9 g). The animals (6 rats/group) were sacrificed 6 hr, 1, 2, 4, and 7 days after the injection by exsanguination under light ether anaesthesia. The kidneys of three rats were combined and homogenized in four volumes of 0.1 M Tris-HCl buffer solution (pH 7.4) containing 0.25 M glucose, using a Teflon homogenizer under nitrogen gas and with ice-water cooling. The homogenate was centrifuged at $105000 \times g$ for 75 min at 2—4°. The supernatant was applied to a Sephadex G-75 column (2.6×90 cm). The column was eluted with 10 mM Tris-HCl buffer solution (pH 8.6) and the eluate was collected in 5 ml fractions. The metal contents (Cd, Cu, and Zn) and absorbances at 254 and 280 nm were determined for each fraction. The contents of metals in the homogenate and the supernatant fractions were determined after digestion with mixed acids ($HClO_4$, 0.2 ml and HNO_3, 1 ml for 0.5 ml sample) (acids for heavy metal analysis, Wako Pure and Chemical Industries, Ltd., Tokyo).

Histopathological Examinations and Urinary Analysis——For histopathology, the kidneys were removed from the animals along with other organs immediately after death, and fixed in 10% buffered formalin. Preparations, embedded in paraffin, were sectioned at 4 to 5 μm and stained with hematoxylin-eosin and periodic acid-Schiff (PAS) reagent.

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

Results

Figure 1 shows changes of the metal contents in the kidneys and in the supernatant fractions after the injection of kidney metallothionein (Cd, 24 μg; Cu, 18 μg; and Zn, 5.2 μg/100 g body weight). The content of zinc in both the kidneys and the supernatant fractions increased from the control values up to two days after the injection and then remained almost at the same level. The levels for both cadmium and copper increased rapidly from the control values during the first 6 hr after the injection and then decreased to constant low levels by two days.

Figure 2 shows the distribution profiles of the three metals and absorbances at 254 and 280 nm in the control supernatant fraction obtained by homogenizing the kidneys of three non-treated rats in four volumes of the extraction buffer which contained 2 ml of the metal-

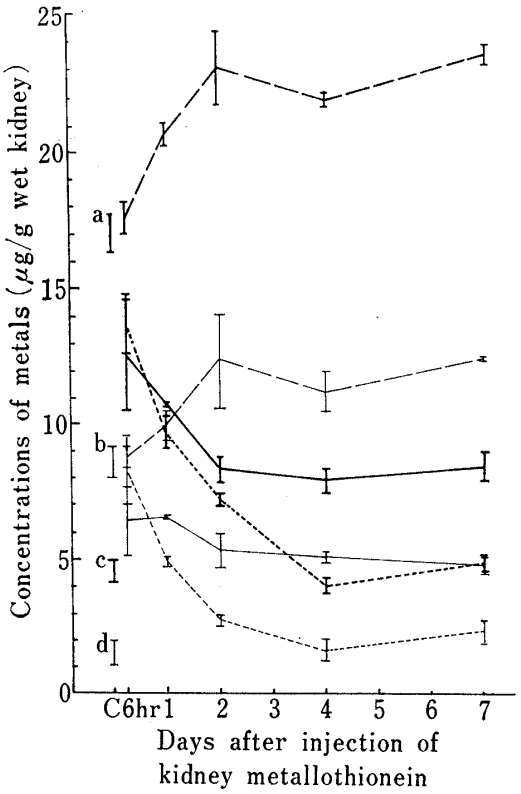

Fig. 1. Contents of Cadmium, Copper, and Zinc in Total Kidneys and in the Supernatant Fractions after Injection of Kidney Metallothionein

Kidneys of three rats were combined and homogenized in four volumes of 0.1 M Tris-HCl buffer solution (pH 7.4) containing 0.25 M glucose. The homogenate was centrifuged at 105000×*g* for 75 min. The homogenates and the supernatants were digested with mixed acids. Values are means of two experiments.
——, Cd; ------, Cu; ——, Zn. Thick and thin lines indicate homogenate and supernatant, respectively. C indicates control values (*a* and *c*, zinc and copper in the homogenates, respectively; *b* and *d*, zinc and copper in the supernatants, respectively). Cadmium was not detected in the control homogenate and supernatant.

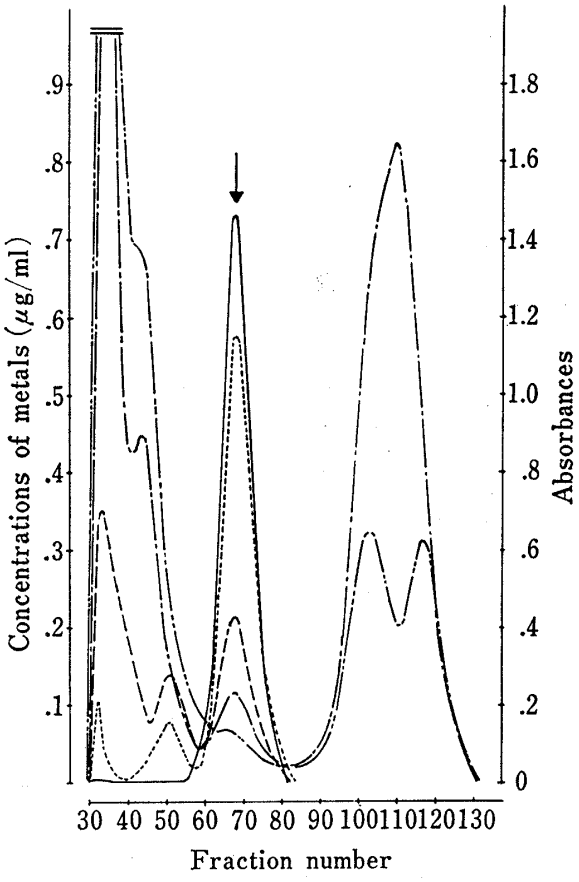

Fig. 2. Sephadex G-75 Elution Profile of Control Supernatant

Kidneys of non-treated rats were homogenized in extraction buffer which contained the kidney metallothionein used for injection, and the supernatant was treated as described in the legend to Fig. 3. ————, Cd; ----------, Cu; — — — —, Zn; ————, absorbance at 254 nm; and —·——·—, absorbance at 280 nm. The arrow indicates the metallothionein fraction.

lothionein solution. If the injected metallothionein was recovered unaltered from the injected rats, the distribution profiles would be similar to those in Fig. 2.

Figure 3 shows the Sephadex G-75 elution profiles of the three metals in the supernatant fractions of kidneys of rat obtained 6 hr, 1, 2, and 4 days after the injection of kidney metallothionein. In contrast to the control distribution pattern in Fig. 2, cadmium in Fig. 3 at 6 hr was not confined to the metallothionein fraction, but was distributed throughout the fractions. Copper was also distributed throughout the fractions at 6 hr. Cadmium and copper in the metallothionein fraction appeared at the same elution volume, as in Fig. 2.

The distribution pattern of the three metals one day after the injection showed that cadmium and copper were again mostly localized in the metallothionein fraction, and zinc in the metallothionein fraction increased (Fig. 3 at 1 day). Although the positions of cadmium and zinc were identical, they were different from that of copper.

The copper content in the metallothionein fraction decreased in the elution profile of the supernatant obtained two days after the injection (Fig. 3 at 2 days). On the other hand, the content of zinc in the metallothionein fraction increased. Again, the positions of cadmium

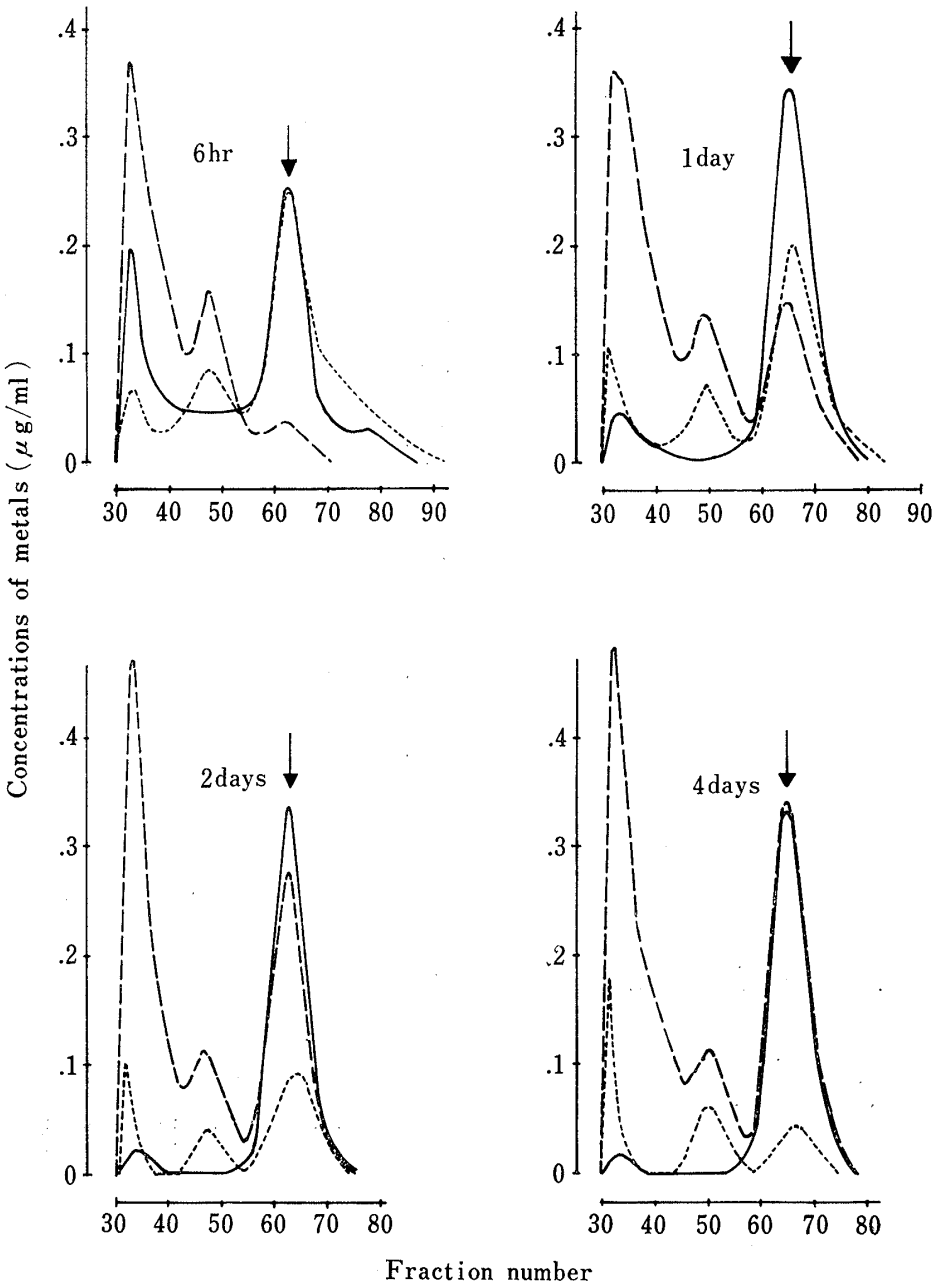

Fig. 3. Sephadex G-75 Elution Profiles of Kidney Supernatants after Injection of Kidney Metallothionein

Kidneys of three rats which were injected intraperitoneally with kidney metallothionein were obtained 6 hr, 1, 2, and 4 days after the injection and homogenized. Each supernatant was applied to a Sephadex G-75 column (2.6×90 cm) and eluted with 10 mM Tris-HCl buffer solution (pH 8.6.). Five ml fractions were collected. ————, Cd; – – – – –, Cu; and — — — —, Zn.

and zinc were identical, but were different from that of copper. The increase of zinc and the decrease of copper in the metallothionein fraction were more marked in the supernatant fractions obtained 4 and 7 days after the injection (Fig. 3 at 4 days and Fig. 4).

When the distribution profile of the control supernatant (Fig. 2) is compared with that of the supernatant obtained 7 days after the injection (Fig. 4), it is clear that the metal ratios in the metallothionein fraction were completely different in spite of the similar distribution patterns of the three metals in other fractions and of the absorbances at 254 and 280 nm throughout the fractions. The metal contents in the metallothionein fractions of Fig. 3 at 4 days and Fig. 4 resemble those of the supernatants obtained 4 and 7 days after the injection

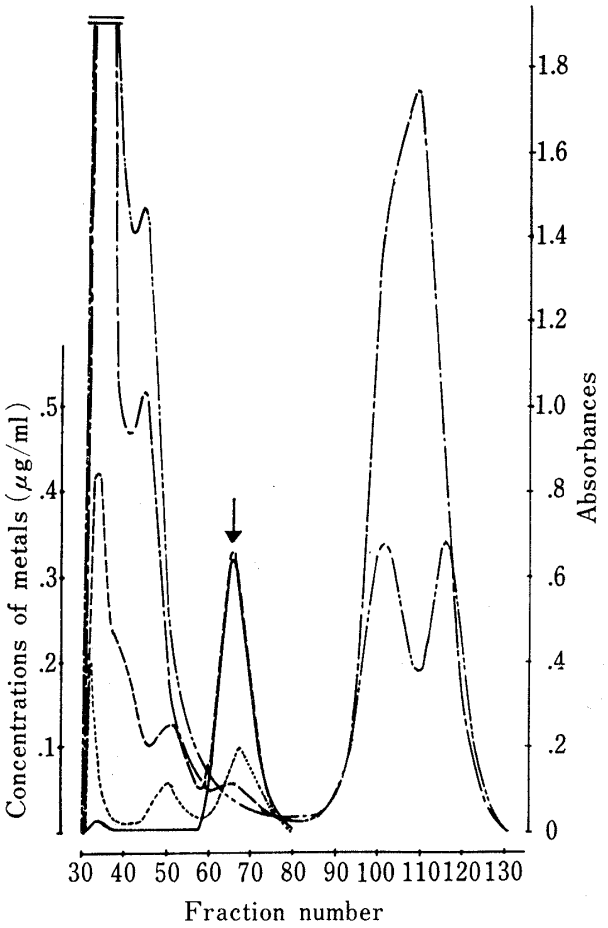

Fig. 4. Sephadex G-75 Elution Profile of the Supernatant obtained 7 Days after Injection of Kidney Metallothionein

The supernatant was gel-filtered as described in the legend to Fig. 3.
———, Cd; - - - - - -, Cu; ———, Zn; ———, absorbance at 254 nm: and ———, absorbance at 280 nm. The arrow indicates the metallothionein fraction.

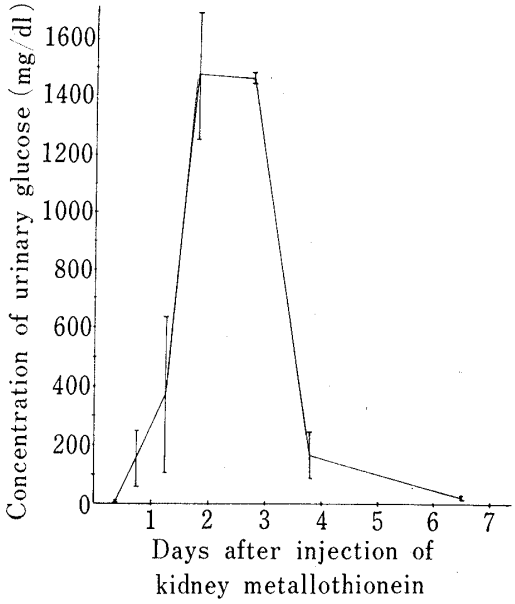

Fig. 5. Urinary Glucose Concentration following Metallothionein Injection

Urine of six rats which had been injected with kidney metallothionein was collected using a metabolic cage. The values (mg/100 ml urine) are the means ± S.D. of six samples.

of liver metallothionein,[4] although the Zn/Cd ratio in the present experiments was a little higher than that in the latter case.

Histopathological examinations were performed for kidneys obtained 1, 2, 4, and 7 days after the injection. The renal tubular lining cells of the rats obtained one and two days after the injection were severely damaged. Recovery of the kidneys was observed 4 days after the injection as already reported in a study on the toxicities of metallothionein with various Cd/Zn ratios to the renal tubular lining cells.[7]

Changes of glucose contents in the urine with time are shown in Fig. 5. The urinary glucose was selected as an indicator of kidney dysfunction in the present experiments because its change was most dramatic among those previously noted in experiments with liver metallothionein (unpublished observation). Figure 5 indicates that kidney dysfunction was caused by the injection of metallothionein, and recovery of the kidneys started shortly after the damage.

Discussion

The higher affinity of metallothionein *in vitro* for copper than cadmium or zinc, and the high copper content in kidney metallothionein of the rat induced by cadmium ion exposure may be related to the adverse effects of cadmium on the kidneys (due to possible *in vivo* replacement of cadmium in metallothionein by copper to release cadmium ions, which damage the kidneys).[3,8] We therefore investigated the origin of copper in the kidney metallothionein (when, where, and how the copper is incorporated into the protein).

7) K.T. Suzuki, S. Takenaka, and K. Kubota, *Arch. Environ. Contam. Toxicol.*, **8**, 85 (1979).
8) K.T. Suzuki, *Arch. Environ. Contam. Toxicol.*, **8**, 225 (1979).

The first approach was the injection of copper into rats which had been pre-injected with cadmium.[6,9] Independent formation of copoer-binding protein (possibly copper-thionein[10]) from the pre-formed metallothionein in both the liver and kidneys of rats was observed when cupric chloride was subsequently injected. The injected cupric ions were not incorporated into the pre-formed metallothionein in both the liver and kidneys.

The second approach was the injection of liver metallothionein to trace its metabolic fate in the kidneys.[4] The isolated liver metallothionein, which contained copper only as a minor metal, was injected to determine whether copper was incorporated during the transfer of metallothionein from the liver to the kidneys. The injected liver metallothionein was mainly transferred to the kidneys and degraded in the kidneys. The cadmium liberated from the degraded protein induced the biosynthesis of metallothionein in the kidneys. The re-synthesized metallothionein did not contain copper as a major metal, unlike the kidney metallothionein induced by the injection of cadmium chloride.[3]

Conformational changes of metallothionein with high copper content have been suggested in view of its different chromatographic properties (on Sephadex gel and anion exchange columns) from metallothioneins with low or no copper content.[6] Therefore, kidney metallothionein (metallothionein with high copper content) may be metabolically different from liver metallothionein (metallothionein with low copper content). The present study was intended to determine the metabolic fate of metallothionein with high copper content (namely, kidney metallothionein) in the kidneys as a third approach.

The changes in the distribution profiles of cadmium and zinc with time were similar to those already reported for injected liver metallothionein[4] and cadmium-thionein-I and -II.[11] The changes can be explained in terms of the degradation of the injected kidney metallothionein and the re-synthesis of metallothionein induced by the cadmium liberated from the degraded protein. The time-dependent changes of distribution patterns of copper after the injection were different from those after the injection of liver metallothionein. The changes can be explained by the liberation of copper from the injected kidney metallothionein and by the independent induction of copper-thionein compared to cadmium-containing metallothionein. Unlike copper in the injected kidney metallothionein, copper in the re-synthesized metallothionein fraction was concluded not to be bound to the same molecule as cadmium for the following reasons; i) cadmium and zinc were eluted at the same elution volume but copper appeared at a different elution volume; ii) although cadmium in the metallothionein fraction stayed at a constant level, the amount of copper in the metallothionein fraction decreased with time. If the three metals are bound to the same molecule, they are eluted at the same rate on a Sephadex G-75 column and copper does not disappear from the metallothionein fraction with time, as observed for the kidney metallothionein induced by exposure to cadmium ions.[3,8]

Thus, although metallothionein with a high copper content (namely, kidney metallothionein) was transferred mainly to the kidneys and reabsorbed at the renal tubules, it was degraded, and the cadmium and copper liberated from the degraded protein independently induced the biosynthesis of cadmium- and copper-containing metallothionein in the kidneys, respectively. The metallothionein re-synthesized in the kidney after the injection of kidney metallothionein was again as low in copper content as the re-synthesized kidney metallothionein induced by the injection of liver metallothionein. The origin of copper in the kidney metallothionein induced by cadmium ion exposure still remains unresolved.

Acknowledgement We thank Dr. K. Kubota for encouragement.

9) K.T. Suzuki, T. Maitani, K. Kubota, and S. Takenaka, presented at the 4th Symposium on Environmental Pollutants and Toxicology, Toyama, Oct. 1977.
10) I. Bremner and B.W. Young, *Biochem. J.*, **157**, 517 (1976).
11) K.T. Suzuki and M. Yamamura, *Biochem. Pharmacol.*, **28**, 1806 (1979).