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DISTRIBUTION OF CADMIUM IN LIVER AND KIDNEYS BY LOADINGS OF VARIOUS Cd-COMPLEXES AND RELATIVE METAL RATIOS IN THE INDUCED METALLOTHIONEINS

KAZUO T. SUZUKI and MITSURU YAMAMURA

Division of Basic Medical Sciences, National Institute for Environmental Studies, P. O. Yatabe, Ibaraki 300-21, Japan

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Abstract—The effects of chelating agents and pre-treatment on the distribution of cadmium in the liver and kidneys and on the relative amounts of cadmium, zinc and copper in the induced metallothioneins were compared by intraperitoneally injecting cadmium with various chelating agents. The co-injections of nitrilotriacetic acid or D-(−)-penicillamine resulted in a distribution of cadmium similar to the injection of the free cadmium ion. The co-injections of 2,3-dimercapto-1-propanol or ethylenediamine tetra-acetate resulted in a distribution intermediate between that of the free cadmium ion and the thionein-bound cadmium. Cadmium was recovered mainly from the kidneys for the injection of cadmium-thionein. The changes in the distributions between liver and kidneys were explained tentatively by the stability constants of the complexes from the distribution patterns of cadmium among protein fractions in serum after adding cadmium *in vitro* with those chelating agents. The copper content in the kidney metallothionein changed depending on the injected form of cadmium and pre-treatment method.

The higher affinity of copper, not only cuprous [1] but also cupric ions [2], to metallothionein than zinc and cadmium *in vitro* and the high content of copper in the kidney metallothionein induced by cadmium ion exposure to rats [2 – 4] are of interest in relation to the adverse effects of cadmium to the kidneys (due to possible replacement of cadmium in metallothionein by copper to release toxic cadmium ion in the kidney). Recently, Nomiyama introduced an *in vivo* relation of copper and kidney dysfunction as an unpublished work of Miyamoto in his review article: namely, urinary copper content has a closer relationship than urinary cadmium content to urinary excretion of β_2-microglobulin, a sensitive and possible marker of renal tubular dysfunction by cadmium exposure, among inhabitants in a cadmium polluted area in Japan [5].

We have been interested to determine whether there are any relationships between high copper content in kidney metallothionein and the adverse effects of cadmium on the kidneys. In order to find the origin of copper in rat kidney metallothionein, we have injected isolated metallothioneins into rats. Unlike the rat kidney metallothionein induced by intraperitoneal injections of cadmium ions, the injections of cadmium as metallothioneins (rat liver Cd-thionein [6, 7], rat liver Cd,Zn-thionein [8] and rat kidney Cd,Cu,Zn-thionein [9]) induced metallothioneins with low copper and high zinc contents in the kidneys.

The different metal ratios (high or low copper content) in the kidney metallothioneins induced by the injections of cadmium as free ion or thionein bound form may give a clue to determine the origin of copper in the kidney metallothionein. Therefore, the present study was intended to investigate why (i) the distribution of cadmium between liver and kidneys and (ii) the relative metal contents in the induced kidney metallothioneins are altered by the injections of different chemical forms of cadmium. The effects of various chelating agents and pre-treatment on the distribution and the relative metal ratios were compared, and the results were tentatively explained by the stability constants of cadmium with chelating agents.

MATERIALS AND METHOD

The reagents used in the present experiment were as follows: cadmium and zinc chlorides (purest grade, Wako), disodium ethylenediamine tetra-acetate (EDTA) (Dojin), D-(−)-penicillamine (Pen) (Aldrich), 2,3-dimercapto-1-propanol (BAL) (Wako), and nitrilotriacetic acid (NTA) (Dojin). Cadmium-thionein was prepared by replacing zinc in rat liver metallothionein (Cd, Zn-thionein) with cadmium as already reported [6]. Cadmium chloride (189 μg Cd/ml, 1.1 mg Cd/kg body weight), zinc chloride (2 mg Zn/ml, 12 mg Zn/kg body weight), cadmium-thionein (30 μg Cd/ml, 0.18 mg Cd/kg body weight) or one to one mixtures (molar ratio, pH was adjusted to 5.5–6.5) of cadmium chloride and EDTA, Pen, BAL, or NTA (0.55 mg Cd/kg body weight) were injected intraperitoneally into rats in the volume of 1 ml/rat.

One hundred and sixty female rats of the Wistar strain (Clea Japan, Tokyo) (body weight 168.7 ± 5.4 g, mean ± S.D.) were divided into 16 groups

(10 rats/group) and were fed on a standard laboratory chow (Clea Japan) and distilled water *ad lib*. Cadmium and/or zinc were injected intraperitoneally into fifteen groups of rats as mentioned in the legend to Fig. 1 and the animals were sacrificed 4 days after the last injection by exsanguination under light ether anaesthesia.

Livers and kidneys of two rats were combined, respectively, and were homogenized using a polytron homogenizer in three volumes of 0.1 M Tris buffer solution (pH 7.4 at 25°, 0.25 M glucose) under nitrogen gas with ice-water cooling. The homogenate was centrifuged at 105,000 *g* for 80 min at 2–4°. The supernatant fraction (1 ml) was diluted 10-fold with doubly distilled water and the contents of cadmium, zinc and copper were determined on a Hitachi 508 atomic absorption spectrophotometer.

Ten ml of the supernatant (2 ml from each supernatant) was applied to a Sephadex G-75 column (2.6 × 90 cm), eluted with 1 mM Tris buffer solution (pH 8.6), and 5 ml fractions were collected. The concentrations of cadmium, zinc and copper, and absorbances at 254 and 280 nm in each eluate were determined on a Hitachi atomic absorption spectrophotometer and on a Hitachi 100-40 spectrophotometer, respectively.

The metallothionein fractions of the eluate from a Sephadex G-75 column were combined and applied to a DEAE Sephadex A-25 column (1.6 × 18 cm) without concentrating the solution. The anion exchange column was washed with 1 m M Tris buffer solution (pH 9.18 at 5°, 25 ml) after the application and two forms of metallothioneins were eluted by a concentration gradient of Tris buffer solution (pH 9.18 at 5°) between 1 mM (100 ml) and 300 mM (300 ml) according to Shaikh and Lucis [10] (Although the concentration of Tris buffer solution was shown as a linear gradient therein, the description indicated that metallothionein was eluted by an exponential gradient of Tris buffer solution as shown in the present study.) The concentrations of the three metals in each eluate (3 ml/tube) were determined as above.

Serum of thirty female rats (14-weeks-old) was pooled and 10 ml portions were used for *in vitro* study. One to one mixtures (molar ratio) of cadmium (20 μg/10 ml serum) and chelating agents were mixed with serum and stood for 5 min at room temperature. Then, the solution was applied to a Sephadex G-75 column (2.6 × 90 cm) (operated at 5°) and processed in the same way as for the tissue supernatants.

RESULTS

Figure 1 shows the distributions of cadmium between liver and kidney supernatants after the injections of cadmium with various complexing agents and/or by different pre-treatments. Figure 1 also shows the changes of copper contents in the liver and kidney supernatants induced by the injection of cadmium and/or zinc. The injected amounts of cadmium depended on the chemical forms of cadmium because of the more toxic effects by the injections of complexed cadmium [6, 11]:namely (i) for $CdCl_2$ only (C, I and K in Fig. 1), 1.1 mg Cd/kg body weight; (ii) for $CdCl_2$ plus complexing agents (D,E,F,L,M,N and O in Fig. 1), 0.55 mg Cd/kg body weight; and (iii) for cadmium-thionein (H, J and P in Fig. 1), 0.18 mg Cd/kg body weight.

The distribution ratios of cadmium between the liver and kidney supernatants changed dramatically for the injections of cadmium with different complexing agents. The injections of cadmium with Pen (E and M in Fig. 1) and NTA (G and O in Fig. 1) resulted in a similar distribution ratio as the injection of free cadmium ion (C and K in Fig. 1).

The injected cadmium as cadmium-thionein was mainly distributed in kidneys as reported already (H, J and P in Fig. 1) [6, 8, 12–15]. Pre-treatment with zinc (J in Fig. 1) or cadmium-thionein (P in Fig. 1) reduced the extent of absorption in the kidney of the second injection of cadmium-thionein. The amount of cadmium in the liver decreased and that in the kidney increased for the injection of cadmium with EDTA (D and L in Fig. 1) and BAL (F and N in Fig. 1). As a result the distribution ratios for D, F, L and N in Fig. 1 resulted in an intermediate value between the above two groups (one group being C, E, G, K, M, and O while the other group is H, J, and P in Fig. 1).

Comparing the distribution ratios of cadmium between liver and kidney supernatants derived from singly and doubly injected rats, the effects of pre-treatment on the distribution was divided into two groups. For one group (C and K, $CdCl_2$ only; D and L, $CdCl_2$ + EDTA; E and M, $CdCl_2$ + Pen; and G and O, $CdCl_2$ + NTA), the distribution of cadmium did not depend on the pre-treatment. In the other group (F and N, $CdCl_2$ + BAL) the distribution ratio changed with pre-treatment. The induction of the biosynthetic system for metallothionein by pre-treatment with zinc also affected the distribution pattern of cadmium (C and I in Fig. 1).

Although the amounts of copper in the liver supernatants were affected only slightly by the injections of all kinds of cadmium complexes, those in the kidney supernatants were considerably affected by the injections. Injections of zinc also affected the amount of copper in the kidney supernatant (B in Fig. 1). The amount of copper (in the cadmium-thionein injected kidney supernatants) increased significantly for pre-treatment with zinc, but not for pre-treatment with cadmium-thionein (compare H, J and P in Fig. 1).

Figure 2 shows the elution profiles of representative kidney supernatants on a Sephadex G-75 column. Cadmium was mainly found in the metallothionein fraction for all samples. The increased amount of copper in the supernatants by the injections was also found in the metallothionein fraction where a small amount of zinc and copper was found in control supernatant.

Almost all cadmium was also found in the metallothionein fraction along with increased zinc in any liver supernatants, but copper did not increase in any metallothionein fractions as expected from the values in the supernatants (Fig. 1) (gel filtration profiles for liver supernatants were not shown).

Figure 3 shows the elution profiles of representative kidney metallothionein fractions on a DEAE Sephadex A-25 column. The distribution pattern of the three metals in Fig. 3-K was similar to that

already reported for the copper-containing kidney metallothionein obtained by the intraperitoneal injection of cadmium ion (where a recovery of metallothionein-II was low) and was different from that of the liver metallothionein (which contains copper only as a trace metal and a recovery of the two forms of metallothionein was comparable on a DEAE Sephadex A-25 column.) [6, 16]. The elution profile of the three metals in Fig. 3-N was similar to that of Fig. 3-K. On the other hand, the distribution profile of the three metals in Fig. 3-P (where the relative copper ratio was low) was different from those of Figs 3-K and -N (where the relative copper ratio was high). The elution patterns of cadmium and zinc in Fig. 3-P were similar to the liver metallothionein and that of copper was similar to the kidney metallothionein induced by the injection of cadmium ion, indicating that the distribution patterns of the metals in Fig. 4-P were an intermediate between typical metallothioneins with low and high copper contents.

Figure 4 shows the distribution patterns of cadmium after adding cadmium without or with chelating agents *in vitro* into rat serum. The distribution patterns of cadmium for the addition of Cd–Pen and Cd–NTA were similar to that of Cd^{2+}, reflecting the

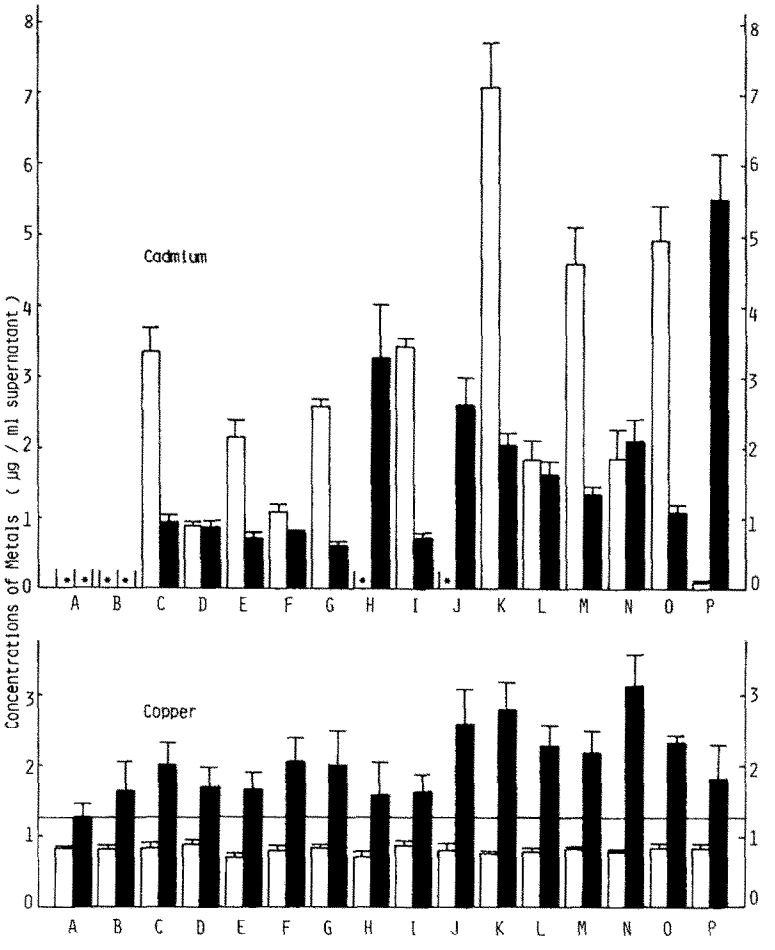

Fig. 1. Concentrations of cadmium and copper in the liver and kidney supernatant fractions after loadings of cadmium-complexes. Ten female rats (two rats/group, n = 5) in each group (15 groups, 150 rats in total) were injected intraperitoneally with metal solution (as described below) and were sacrificed 4 days after the last injection. The livers and kidneys of the two rats were combined and homogenized in three volumes of 0.1 M Tris buffer solution (pH 7.4, 0.25 M glucose). The homogenates were centrifuged at 105,000 *g* for 80 min. Concentrations of the metals were expressed as μg/ml supernatant (mean ± S.D.). Open and solid squares indicate the concentrations of metals in the liver and kidney supernatants, respectively. The amounts of metals used for injections are as follows: $ZnCl_2$ (12 mg Zn/kg body weight); $CdCl_2$ (1.1 mg Cd/kg body weight); $CdCl_2$ + chelator (1 : 1, molar ratio; 0.55 mg Cd/kg body weight); and Cd–thionein (0.18 mg Cd/kg body weight).

A: control, *B*: $ZnCl_2$ once, *C*: $CdCl_2$ once, *D*: $CdCl_2$ + EDTA once, *E*: $CdCl_2$ + Pen once, *F*: $CdCl_2$ + BAL once, *G*: $CdCl_2$ + NTA once, *H*: Cd-thionein once, *I*: $ZnCl_2$ once then after 4 days $CdCl_2$ once, *J*: $ZnCl_2$ once then after 4 days Cd–thionein once, *K*: $CdCl_2$ twice every 2 days, *L*: $CdCl_2$ + EDTA twice every 2 days, *M*: $CdCl_2$ + Pen twice every 2 days, *N*: $CdCl_2$ + BAL twice every 2 days, *O*: $CdCl_2$ + NTA twice every 2 days, *P*: Cd–thionein twice every 2 days. Asterisk indicates that cadmium was not detectable.

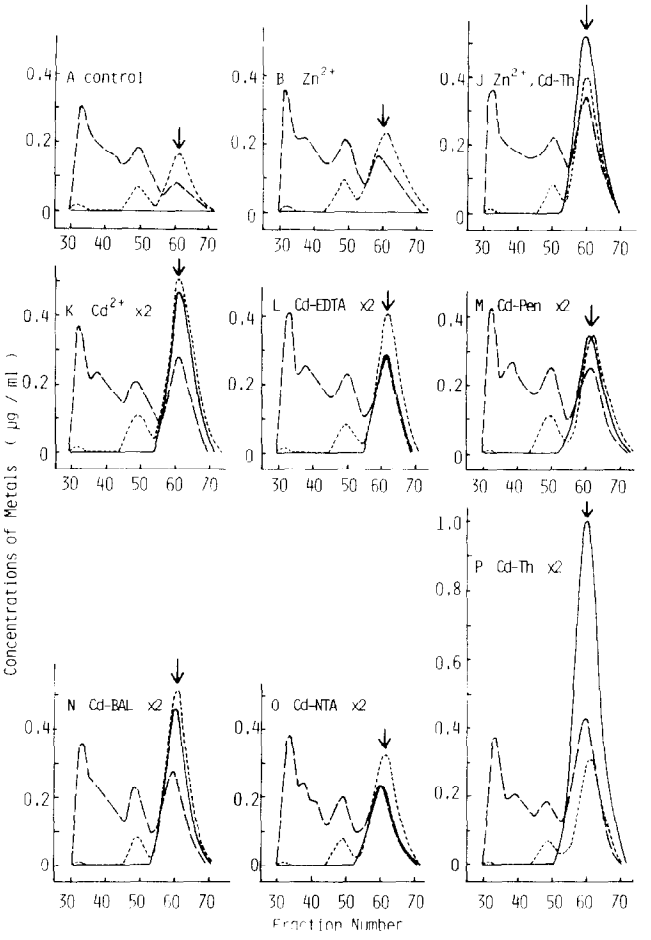

Fig. 2. Sephadex G-75 gel filtration profiles of representative kidney supernatants. Ten millilitres of each kidney supernatant was applied to a Sephadex G-75 column (2.6 × 90 cm), eluted with 1 mM Tris buffer solution (pH 9.18 at 5°), and 5 ml fractions were collected. The letters in the figures correspond to those in Fig. 1. The curves are as follows: ——, Cd; – – –, Zn; and ·····, Cu. The arrow indicates the metallothionein fraction.

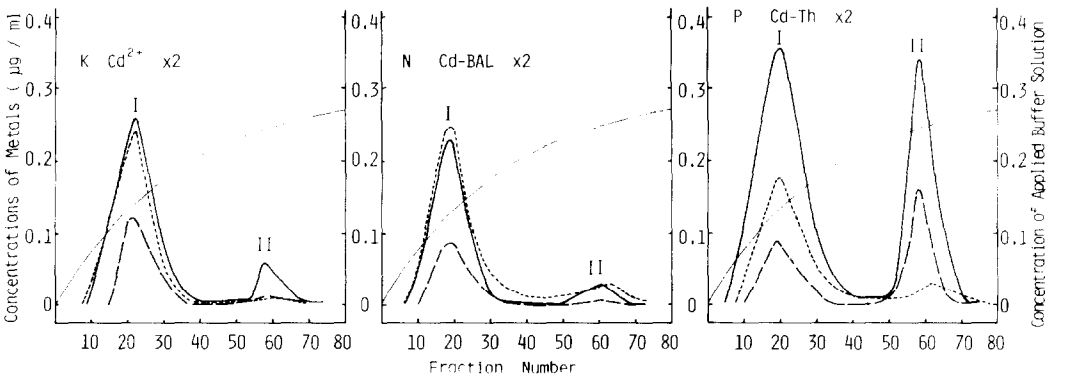

Fig. 3. DEAE Sephadex A-25 ion exchange profiles of representative kidney metallothioneins. Main metallothionein fractions on a Sephadex G-75 column were combined and applied to a DEAE Sephadex A-25 column (1.6 × 18 cm) without concentrating the solution. Two forms of metallothioneins were eluted by a concentration gradient of Tris buffer solution (pH 9.18 at 5°) between 1 mM (100 ml) and 300 mM (300 ml) and 3 ml fractions were collected. The letters in the figures correspond to those in Fig. 2. The curves are as follows: ——, Cd; – – –, Zn; ·····, Cu; and — · —, concentration of applied buffer solution. I and II indicate metallothionein-I and -II fractions, respectively.

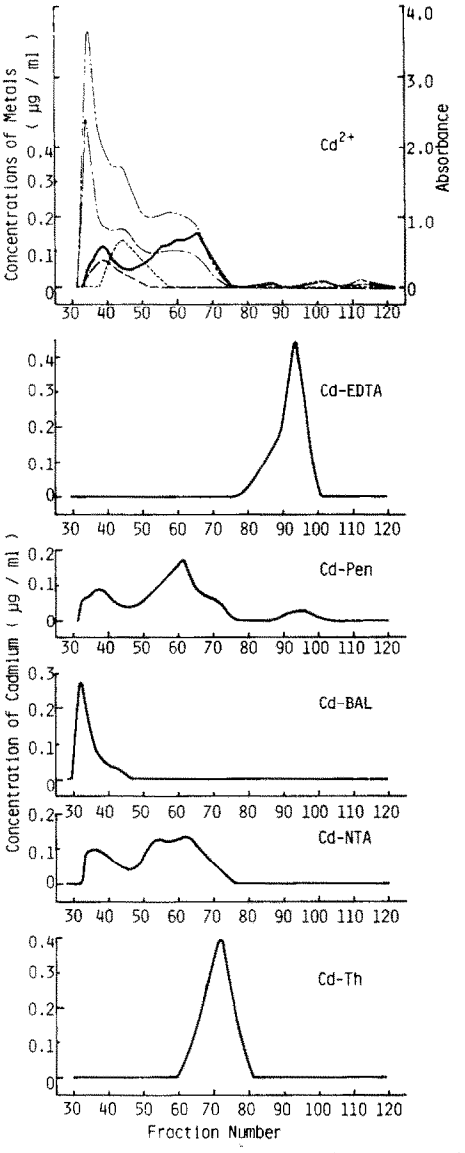

Fig. 4. Sephadex G-75 elution profiles of rat serum mixed with various cadmium complexes. Free or complexed cadmium (1 : 1 molar ratio) was added to rat serum (20 μg Cd/10 ml serum), and the solution was mixed and stood for 5 min at room temperature (20°). Then, the solution was applied to a Sephadex G-75 column (2.6 × 90 cm, operated at 5°), eluted with 1 mM Tris buffer solution and 5 ml fractions were collected. Cd^{2+}, Cd–EDTA, Cd–Pen, Cd–BAL, Cd–NTA and Cd–Th in the figures indicate that free cadmium ion, one to one mixture of cadmium and corresponding agents or Cd-thionein was added to the serum, respectively. The curves indicate as follows: ——, Cd; – – –, Zn; --------, Cu; — · —, absorbance at 254 nm; and — -- —, absorbance at 280 nm.

similar distributions of cadmium between the liver and kidneys in Fig. 1. Cadmium was located in the metallothionein fraction for the addition of cadmium-thionein. On the other hand, cadmium was mainly located in the low molecular weight fraction for the addition of Cd–EDTA and in the high molecular weight fraction for the addition of Cd–BAL. Cadmium was probably present as Cd–EDTA complex (1 : 1) for the former case, but cadmium was assumed to be present as a polymeric form of Cd–BAL for the latter case because cadmium was eluted at a void volume (faster than any serum proteins).

DISCUSSION

Cadmium in metallothionein is know to be coordinated with three mercapto groups (estimated stability constant, log β_3, is 25.5 [17]). When cadmium bound to metallothionein was injected, cadmium was recovered mainly from the kidneys as reported already [6, 8, 12–15] and cadmium was also found in urine as metallothionein [8]. On the other hand, injected cadmium without chelating agent was found mainly in the liver and not in the urine. The injected cadmium as the albumin complex has been reported to distribute in the same way as the free cadmium ion [15].

Therefore, the extreme difference observed in the distributions by the injections of cadmium as metallothionein or the free ion is probably due to the following reasons. Cadmium bound to metallothionein is not dissociated to give cadmium-high molecular weight protein (mainly albumin) complexes in circulating fluid and will be carried as metallothionein to the kidneys where metallothionein is filtered from glomeruli. The filtered metallothionein is mainly reabsorbed into the renal tubular linings when the amount of the injection is low and the rest of the metallothionein is recovered in the urine, especially when the injected amount is high [8, 18]. Unlike cadmium bound to metallothionein, the injected free ion is probably bound to high molecular weight protein (mainly albumin) and transferred to the liver.

In order to test the above mentioned possibility, cadmium complexes of various degrees of stability were tentatively selected, and the chelator dependent changes of distribution of cadmium between liver and kidneys and the metal contents in the induced metallothionein were investigated. The stability constants of the complexes, log K_1, are approximately as follows : Cd–Pen (11), Cd–NTA (10), Cd–EDTA (16), but that for Cd–BAL has not been reported [19]. From the similarity of the chemical nature of cadmium and zinc, the stability constant for Cd–BAL was estimated to be of the order 13–14 from the following data: Zn–Pen (9.5), Zn–NTA (10.4), Zn–BAL (13.5), and Zn–EDTA (16.3) (stability constants mentioned above were not measured at the same conditions). Cd–Pen and Cd–NTA belong to complexes with relatively low stability constant and the distributions of cadmium injected as those complexes were similar to that of the free cadmium ion. Cd–EDTA and Cd–BAL belong to complexes with relatively high stability constant and the cadmium injected as those complexes was distributed in the liver and kidneys at an intermediate ratio between the free and the thionein-bound cadmium. Low total recovery of the latter group (Cd–EDTA and Cd–BAL) is probably due to low reabsorption into renal tubular linings.

Although the amount of copper in the liver supernatants was not dependent on the injected form

of cadmium, copper in the kidney supernatant fractions increased after the injection of cadmium. The increased amount of copper was found in the metallothionein fraction for any treatment. The relative amount of cadmium to the increased amount of copper in the kidney supernatant fraction depended upon the chelating agents and the pre-treatment. The relative ratio of increased copper to cadmium in the kidney supernatants of cadmium-thionein-injected rats (H and P in Fig. 1) was far lower than those for other treatments. Pre-treatment with zinc however enhanced the relative copper content in cadmium-thionein-injected kidney supernatant (J in Fig. 1). The higher copper content in the cadmium-thionein injected kidney supernatant by the pre-treatment with zinc than with cadmium-thionein may indicate that the induction of metallothionein or the altered metabolism of zinc in the liver affects the amount of available copper in the kidneys.

The cadmium and increased amount of copper in any of the liver and kidney supernatants were almost confined to the metallothionein fraction (Fig. 2). Two types of elution profiles on an anion exchange column for kidney metallothioneins were observed. One was of a typical pattern for cadmium ion-exposed rat kidney metallothionein (which contains copper as a major metal) [16]; namely, (i) metallothioneins were separated into two forms. (ii) Type I metallothionein contains three metals (Cd, Cu and Zn) and those are eluted at the same buffer concentration. (iii) Recovery of type II metallothionein is lower compared to Type I metallothionein and copper is eluted at a slightly higher buffer concentration than cadmium and zinc. The other is of an intermediate pattern between the typical cadmium ion-exposed rat kidney profile and the liver metallothionein profile. A typical liver metallothionein which contains copper as a trace metal is separated into type I and type II metallothioneins and recovery of both types is comparable on a DEAE Sephadex A-25 column [6, 10]. Fig. 3-P can be explained as a mixture of typical liver and kidney metallothionein types.

The distribution patterns of cadmium indicated that cadmium added *in vitro* with chelating agents of low stability constants (Cd–Pen and Cd–NTA) was promptly distributed among serum proteins resulting in similar distribution patterns for the addition of free cadmium ion. This may explain why cadmium was distributed in a similar ratio between liver and kidneys for the intreperitoneal injections of cadmium without and with NTA or Pen. The distribution pattern of cadmium added as Cd–EDTA complex indicated that the stability for Cd–EDTA complex is high enough not to be redistributed among serum proteins within a short time after the addition. In contrast to Cd–EDTA complex, the addition of Cd–BAL complex resulted in an elution of cadmium mainly at a void volume, indicating that Cd–BAL complex was present as a highly polymerized form (probably not bound to serum proteins because cadmium was eluted faster than main serum proteins). Although it is not clear, at the present time, why cadmium was distributed in a similar way between the liver and kidneys for the injections with EDTA and BAL, the *in vitro* distribution pattern of cadmium among serum proteins indicated that complexes of high stability constants (including metallothionein) may not be distributed among serum proteins within a short time after the injection. The *in vitro* experiment suggested that the distributions of cadmium injected as the complexes with different stability constants may also be different in *in vivo* experiment and the stability constant seems to explain partly the results of *in vivo* experiment.

Injected metallothionein is promptly transferred to and degraded in the kidneys, and thus, a relatively higher amount of cadmium may be available at once for the induction of kidney metallothionein than the amount of cadmium for the injection of cadmium ion. The capacity for metallothionein biosynthesis probably depends on the amount of inducing metal (cadmium) ion and the ratio of the inducing metal to the other metals (zinc and copper) in the metallothionein probably depends on the induced capacity of the biosynthetic system and the available metals. Although further experimental evidences are required, the present experiment suggested that cadmium complexes of high stability constants have more chances to be transferred to the kidneys than those of low stability constants, but the rate of transfer or reabsorption of those complexes (available amount of cadmium for the induction) is not so fast (high) except for that of metallothionein: namely, stability of cadmium-complexes seems to explain the distribution between the liver and kidneys, and available amounts of metals at the biosynthetic sites seems to explain the metal ratio in the induced metallothionein.

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