Communications to the Editor

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Copper in Cadmium-exposed Rat Kidney Metallothionein

In vitro affinity of cupric ion to metallothionein was found to be stronger than cadmium and zinc ions. The result suggested the passive incorporation of copper into the metallothionein and the possibility of demasking of the masked cadmium toxicity as metallothionein. Copper contents of cadmium-exposed rat kidney metallothionein were analyzed along with cadmium and zinc. The copper in kidney metallothionein was always one of major metals along with zinc and cadmium. On the other hand, the copper in cadmium-exposed liver metallothionein was a minor metal.

Keywords——metallothionein; copper; kidney; cadmium; toxicity; rat; zinc

Since the first isolation and characterization of metallothionein as a unique cadmium-binding protein from an equine kidney,[1] the roles of the protein have been of interest in relation to the homeostasis of zinc[2] or copper,[3] and the detoxification of heavy metals.[4] Many workers have done researches using livers obtained from cadmium-exposed experimental animals. Probably, this is due to the easier induction and availability of a larger amount of metallothionein from the liver than from the kidney. As for the metals in metallothionein obtained from cadmium-exposed experimental animals, only cadmium and zinc have been analyzed. Although copper is reported as one of a minor metal in metallothionein,[1] the metal has never been analyzed in kidney metallothionein obtained from cadmium-exposed experimental animals.

Table I. Contents of Metals in Kidney Metallothionein

a)	Intraperitoneal administration*b*)						Oral administration		
	I	II	III	IV	V	VI	VII*c*)	VIII*c*)	IX*d*)
Zn	3.12	4.78	3.68	6.01	7.54	8.79	0.24	2.26	nd*e*)
Cd	0.98	3.20	3.97	5.86	9.36	17.1	4.12	10.3	nd*e*)
Cu	4.94	8.82	6.10	11.5	13.1	28.3	3.02	9.64	nd*e*)

a) Metals found in metallothionein fractions; $\times 10^{-8}$ mol/g wet tissue.
b) Wistar JCL, female rats (9-week-old, body weight 198 g, S. D. 10.1, three rats/group) were injected intraperitoneally with cadmium ions in saline.
I; injected with 1.12 mg Cd^{2+}/Kg body weight as 2 mM $CdCl_2$ solution and sacrificed 3 days after the injection.
II; injected with 1.12 mg Cd^{2+}/Kg body weight as 2 mM $CdCl_2$ solution two times (three days interval) and sacrificed 3 days after 2nd injection.
III—VI; injected with 1.12 mg Cd^{2+}/Kg body weight as 2 mM $CdCl_2$ solution two times, then 2.24 mg Cd^{2+}/Kg body weight as 4 mM $CdCl_2$ solution once (three days interval) and sacrificed 3, 18, 25, and 32 days after 3rd injection, respectively.
c) Wistar JCL, male rats (three rats/group) were given 0.44 mM $CdCl_2$ in distilled water *ad libitum* for 8 weeks (VII, 4- to 12-week-old) and 16 weeks (VIII, 4- to 20-week-old), respectively.
d) Wistar JCL, male rats (three rats/group) were given distilled water for 16 weeks (4- to 20-week-old).
e) Not detected.

1) J.H.R. Kägi and B.L. Vallee, *J. Biol. Chem.*, **235**, 3460 (1960); *idem*, *ibid.*, **236**, 2435 (1961).
2) M. Webb, *Biochem. Pharmacol.*, **21**, 2751 (1972).
3) G.W. Evans, P.F. Majors, and W.E. Cornatzer, *Biochem. Biophys. Res. Commun.*, **41**, 1244 (1970); G.W. Evans, *Nutr. Rev.*, **29**, 195 (1971); G.W. Evans, *Physiol. Rev.*, **53**, 535 (1973).
4) D.R. Winge and K.V. Rajagopalan, *Arch. Biochem. Biophys.*, **153**, 755 (1972); Z.A. Shaikh and O.J. Lucis, *Fed. Proc.*, **30**, 238 (Abs.) (1971); G.F. Nordberg, M. Piscator, and B. Lind, *Acta Pharmacol. Toxicol.*, **29**, 456 (1971); J.K. Piotrowski, W. Bolanowska, and A. Sopota, *Acta Biochemica. Pol.*, **20**, 207 (1973).

We are interested in finding out why copper is present in metallothionein,[1] and whether it is present in the kidney metallothionein induced by cadmium-exposure. One of the reasons of the presence may be the passive incorporation of copper to metallothionein. So, we have investigated the *in vitro* binding affinity of copper to metallothionein using divalent copper because divalent copper (cupric ion) is more common in biological systems.[5] At the same time we have analyzed the copper contents of metallothionein in the livers and kidneys from cadmium-exposed rats.

Rats were injected with cadmium chloride (0.9 mg Cd^{2+}/kg body weight) intraperitoneally four times in every two days to induce the biosynthesis of metallothionein. The animals were sacrificed four days after the last injection and the livers were homogenized in four times the volume of 0.1 M Tris-HCl buffer, pH 7.4 containing 0.25 M sucrose. The homogenate was centrifuged at $105000 \times g$ for 60 min. The supernatant was applied to a Sephadex G-75 column (2.6×90 cm) which was pre-equilibrated with 1 mM Tris-HCl, pH 8.6 and the gel filtration was carried out with the same buffer. Absorbances at 254 and 280 nm and contents of zinc, cadmiun, and copper were analyzed in each fraction with a Hitachi 191E spectrophotometer and a Hitachi 508 atomic absorption spectrometer. The amounts of zinc and cadmium in the metallothionein fraction in the original supernatant were found to be 2.96×10^{-7} mol/2 ml and 4.39×10^{-7} mol/2 ml, respectively. The amount of copper was less than 0.09×10^{-7} mol/2 ml (reliable detection limit in this system was more than 0.03 ppm). To know the affinity of zinc, cadmium, and cupric ions to metallothionein, each 2.5 μmol of $ZnCl_2$, $CdCl_2$, and $CuCl_2$ was added to the supernatant (2 ml) and the mixture was allowed to stand for 15 min at room temperature. The solution was, then, applied to the column as above. The contents of zinc, cadmium, and copper were analyzed in each eluate by atomic absorption analysis. The metal found in metallothionein fractions was only copper (1.11×10^{-6} mol); zinc and cadmium were not detected (less than 0.03 ppm in each eluate). The experiment showed that the affinity of cupric ion to metallothionein was stronger than those of zinc and cadmium ions. The result also suggested the possibility that copper could replace zinc and cadmium in metallothionein due to the stronger affinity to metallothionein (passive incorporation).

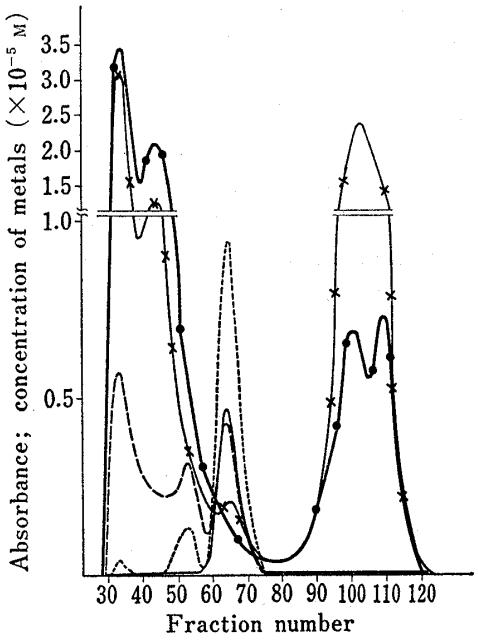

Fig. 1. Sephadex G-75 Elution Pattern of Cadmium-exposed Rat Kidney Supernatant

8.7 ml of the sample IV in the table were applied to the column (2.6×90 cm) and eluted with 1 mM Tris-HCl, pH 8.6. Absorbances at 254 (—×—) and 280 (—●—) and metal contents (Cd, ——; Zn, -------; and Cu, ·····; respectively) by atomic absorption analysis were measured in each eluate (5 ml/tube).

The copper content of kidney metallothionein obtained from cadmium-exposed rats was analyzed along with the zinc and cadmium contents to check the possibility suggested from the *in vitro* experiment. Cadmium chloride was injected intraperitoneally to rats and the kidneys were processed in the same way as mentioned in the *in vitro* experiment except that three times the volume of the buffer was used for the extraction. Copper was one of the major metals in metallothionein fractions along with cadmium and zinc regardless of the number of times of injections (I—III) and days after the last injection (III—VI) as shown in

5) R. Malkin, "Inorganic Biochemistry," Vol. 2, G.L. Eichhorn ed., Elsevier, Amsterdam-Oxford-New York, 1973.

the table. Cadmium chloride was also given in drinking water and the metals in metallothionein fractions were analyzed in the same way. Copper was again one of the major metals (VII—VIII in the table), but none of the three metals was found in metallothionein fractions in the case of control animals (IX in the table). Figurelis a typical Sephadex G-75 elution pattern of the kidney supernatant from the sample IV in the table. Thus, the content of copper in kidney metallothionein was found extremely high regardless of the methods of cadmium exposure. This is the marked difference from the result of equine kidney metallothionein,[1] an example of environmental cadmium exposure. The metal contents of liver metallothionein fractions of experiments I to VIII in the table were also analyzed in the same way. Although zinc and cadmium were found as major metals in every experiment, copper was a minor metal (less than 2—3% of cadmium content by weight per cent) in liver metallothionein fractions. As a typical example, the metal contents of liver metallothionein in experiment IV were shown as following; zinc 2.08×10^{-7}, cadmium 1.63×10^{-7}, and copper 0.02×10^{-7} mol/g wet tissue, respectively.

Our results can be summarized as follows. i) The cupric ion has stronger affinity to metallothionein than zinc and cadmium ions. ii) The copper content in cadmium-exposed rat kidney metallothionein fractions is very high. iii) On the other hand, the copper content in cadmium-exposed rat liver metallothionein is very low.

These results suggest that much attention should be focused on the toxicity of metallothionein in kidney in relation to copper, especially the possible demasking effects of copper to the masked cadmium toxicity as metallothionein.

National Institute for Environmental Studies
P.O. Yatabe, Tsukuba, Ibaraki, 300-21, Japan

KAZUO T. SUZUKI
KENTARO KUBOTA
SHINJI TAKENAKA

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Syntheses of Heterocycles *via* Intramolecular Cycloaddition of Azahexatrienes. Photochemical Cyclization of 5-Arylazo-6-dimethylaminomethyleneamino-1,3-dimethyluracils

Photochemical cyclization of 5-arylazo-6-dimethylaminomethyleneamino-1,3-dimethyluracils (I), 1,2,5-triazahexatriene-type precursors, under aerobic condition resulted in the formation of 6-aryl-1,3-dimethyl-6,7-dihydro-6-azalumazine-7-ones. On the other hand, the photolysis of I under anaerobic condition gave 8-arylaminotheophyllines.

Keywords——Intramolecular cycloaddition; azahexatriene; photolysis; 6-azalumazine-7-one; 8-arylaminotheophylline; 5-arylazo-6-dimethylaminomethyleneamino-1,3-dimethyluracil

Recent investigations in this laboratory have established the novel synthetic method for preparation of heterocycles such as purines,[1] pteridines[2,3] and pyrazolo[3,4-*d*]pyrimidines[1,4] by intramolecular cycloaddition of aza analogs of hexatriene. This paper describes

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3) F. Yoneda, M. Higuchi, and M. Kawamura, *Heterocycles*, **4**, 1659 (1976).
4) F. Yoneda, T. Nagamatsu, T. Nagamura, and K. Senga, *J. C. S. Perkin I*, **1977**, 765.