Chem. Pharm. Bull. 27(2) 515-521 (1979)

UDC 547.96'546.47.04.08:615.916.011.5.033.076.9

Inhibitory Effect of High Dietary Zinc on Copper Absorption in Rats. 1) II. Binding of Copper and Zinc to Cytosol Proteins in the Intestinal Mucosa

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(Received June 24, 1978)

Earlier studies showed that high dietary zinc interfered with copper absorption at the intestinal level. The data presented here demonstrated that there was a significant decrease in copper content with a concomitant increase in zinc content of the intestinal segment of the zinc-fed rats. Gel filtration analysis on Sephadex G-75 of the cytosol fraction from the mucosa of the zinc-fed rats demonstrated that major parts of zinc in the cytosol bind to the protein with a molecular weight of about 10000, that is, the zinc-thionein. The simultaneous administration of copper and zinc to the normal rats also caused an incorporation of copper into this protein. These results suggested that the interference of copper absorption by zinc may be due to the inductive effect of zinc on the synthesis of the zinc-thionein, which sequesters copper available for absorption.

Keywords—copper absorption; inhibitory effect of zinc; intestinal mucosa; gel filtration; elution pattern; metal binding proteins; metallothionein

In the previous paper,¹⁾ we have reported that high dietary zinc resulted in lowering of growth, hemoglobin level, ceruloplasmin (ferroxidase, E.C. 1.12.3.1) activity and copper content in rat tissues and that these disturbances of copper metabolism appeared to be mainly due to the inhibition of copper absorption at the intestinal level.

Van Campen, et al.³⁾ first demonstrated that the absorption of copper from an isolated intestinal segment was inhibited by zinc. Starcher⁴⁾ confirmed these findings in the chick and identified a protein with a low molecular weight in the intestinal mucosa, suggesting that zinc acts as a copper antagonist in a binding capacity to displacing copper from this mucosal protein. Evans, et al.⁵⁾ also demonstrated that the metal-binding protein with a molecular weight of about 10000 in the duodenum, liver and kidney of rats was similar to metallothionein, which was originally isolated and characterised as cadmium-, zinc- and mercury-binding protein by Kagi, et al.⁶⁾ Bremner, et al.⁷⁾ have shown that metallothionein which contains no cadmium or mercury, but only zinc and copper occurs in rat liver by zinc injection and have suggested that this protein is involved in the normal metabolism of zinc, perhaps in some temporary storage or detoxication capacity. Additionally, Cousins, et al.⁸⁾ have suggested that the intestinal zinc-binding protein, zinc-thionein, induced by zinc administra-

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⁷⁾ a) I. Bremner and N.T. Davies, Biochem. J., 146, 733 (1975); b) I. Bremner, Brit. J. Nutr., 35, 245 (1976).

⁸⁾ a) M.P. Richard and R.J. Cousins, Biochem. Biophys. Res. Commun., 64, 1215 (1975); b) Idem, ibid., 75, 286 (1977); c) Idem, Proc. Soc. Exp. Biol. Med., 153, 52 (1976); d) M.P. Richards and R.F. Cousins, J. Nutr., 106, 1991 (1976).

tion may function in a mechanism that regulates zinc absorption by acting as an intracellular agent.

There is limited information about copper absorption in response to changes in the level of mucosal zinc-binding proteins. In an attempt to gain more understanding of the mechanism by which zinc interferes with copper absorption, we examined the changes in gel filtration patterns of copper and zinc in the cytosol fraction of mucosa of rats after administration of these metals.

Experimental

Animal Experiments—Male rats of the Wistar strain, weighing about 150—200 g, were maintained on the basal diet (Nihon Clea, CE-2), which contained 7 ppm of copper and 50 ppm of zinc, whereas those of the test group were fed the diet which was prepared by addition of zinc in the carbonate form to the basal diet in 1%. After feeding for 8 days, rats in both groups were killed by bleeding from the femoral vein under ether anesthesia. The stomach, duodenum, the first 10 cm of jejunum and of ileum were removed, opened and washed with cold physiological saline. Metal contents in these tissues were determined by the method described previously.¹⁾ In some experiments, rats were fasted overnight and then administered 25.2 mg of zinc (as ZnCl₂) and/or 0.4 mg of copper (as CuSO₄·5 H₂O) per 100 g of body weight. Control rats received 1 ml of saline solution per 100 g of body weight. After specified periods, rats were killed by the manner mentioned above.

Gel Filtration--Starting at the pylorus, the first 10 cm of the intestinal segment was removed, rinsed thoroughly with 30 ml of ice-cold saline, placed on an ice-cold glass plate and then split lengthwise. Mucosal cells were collected by gently scraping off the intestine with a microscope slide. The mucosal cells obtained from 2 rats were homogenated in 5 volumes of a solution of 50 mm KCl in 1 mm Tris-HCl buffer, pH 8.6, with a glass-Teflon homogenizer. The homogenate was centrifuged at $140000 \times g$ for 1 hr at 0°. The resulting cytosol fraction was concentrated at 7 ml by lyophilization. An aliquot of 5 ml was taken for gel filtration and 2 ml of the residue was subjected to metal analysis by the method described previously.1) Gel filtration was carried out on a column (2.6×60 cm) of Sephadex G-75 (grade of superfine, Pharmacia Fine Chem.) equilibrated with the Tris-HCl buffer at 4°. The column was eluted with the same buffer at a flowrate of 10 ml per hr and each fraction of 5 ml was collected. Zinc contents in each fraction were determined directly by an atomic absorption spectrophotometer (Jarrel Ash, Model 200-20) and copper determination was carried out by a flameless atomic absorption spectrophotometer (Jarrel Ash, Model FLA-10). Protein contents were measured by ultra-violet absorption at 250 nm with a spectrophotometer (Hitachi, Model 200-20). Molecular weight was estimated by the modified method of Andrews,9) using a Sephadex G-75 column (2.6 × 60 cm) equilibrated with the Tris-HCl buffer. Blue dextran 2000 (Pharmacia Fine Chem.), ribonuclease A, myoglobin, cytochrome c and trypsin (Sigma Chem. Co. Ltd.) were used as the appropriate reference substances.

Table I. Effect of High Dietary Zinc on Metal Contents in Gastrointestinal Segments of Rats

	Normal		1% zinc-diet	
	Zn	Cu	Zn	Cu
Stomach	20.71 ± 0.30	2.11 ± 0.33	136.11 ± 13.95^{a}	1.28 ± 0.04^{b}
Duodenum	18.31 ± 0.69	1.14 ± 0.08	151.83 ± 16.12^{a}	0.47 ± 0.06^{b}
Jejunum	20.71 ± 0.84	1.04 ± 0.06	97.49 ± 8.12^{a}	0.62 ± 0.01^{b}
Ileum	14.83 ± 0.48	0.69 ± 0.01	109.57 ± 6.11^{a}	0.35 ± 0.09^{b}

Six rats in one group were maintained on normal- or 1% zinc-diet for 8 days. Values represent mean \pm standard error.

a) significantly higher than normal at p < 0.01.

b) significantly less than normal at p < 0.01.

⁹⁾ P. Andrews, Biochem. J., 91, 22 (1964).

Results

Metal Contents in Gastrointestinal Segments

Table I shows a marked increase in zinc content accompanying a significant decrease in copper content of the stomach and duodenum from the rats fed 1% zinc-diet for 8 days. In these experiments, about 85% of total zinc in the duodenum was detected in the mucosal layer. Subfractionation of the mucosal homogenate showed that about 70% of zinc in the mucosa was associated with the cytosol fraction (data not shown). Campen, et al. 10) using in vivo ligated segment of the intestinal tracts of rats, have shown that both copper and zinc were absorbed from the duodenum. Therefore, the first 10 cm of intestinal segment including the duodenum was used for further study.

Gel Filtration Patterns of Cytosol Fraction from Zinc-fed Rats

A typical separation on Sephadex G-75 of cytosol fraction from the rats fed the zinc-diet for 8 days is shown in Fig. 1 (b). Three main peaks of zinc-binding components were obtained. Peak I eluted at the void volume of the column and represented proteins having high molecular weight larger than 50000 of the exclusion limit of Sephadex G-75. The most obvious effect of zinc-feeding was to increase markedly the amount of zinc and to some extent of copper in peak II. Estimated molecular weight for the protein in this peak was about 10000, which corresponded to metallothionein or zinc-thionein previously found in the liver, kidney and intestinal mucosa.^{6,7)} Absorbance at 280 nm was scarcely observed in the peak II but a characteristic maximum of absorbance at 250 nm due to the metallothionein was clearly visible. About 85% of zinc in the cytosol fraction was found in the peak II. A minor zinc-containing fraction of peak III corresponds to low molecular weight compounds, that is, zinc chelate complexes that have been proposed to function in intestinal zinc absorption.^{8c)} As shown in Fig. 1 (a), in the cytosol fraction from the normal rats, neither zinc nor copper was detected in the fraction corresponding to the zinc-thionein. These results indicated that the relative content of copper tends to decline in the fraction of high molecular weight proteins and elevate concomitantly in the zinc-thionein fraction in response to feeding of the high zinc-diet.

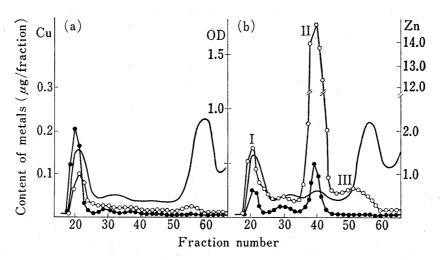


Fig. 1. Fractionation on Sephadex G-75 of Cytosol Fraction from Intestinal Mucosa of Normal (a) and 1% Zinc-fed Rats for 8 Days (b)

The curves indicate the following; ———: copper, ———: zinc, ———: absorbance at 250nm.

¹⁰⁾ a) D.R. Campen and E.A. Mitchell, J. Nutr., 86, 120 (1965); b) D.R. Campen, ibid., 88, 125 (1966).

Subsequently, copper was given orally to the zinc-fed rats and changes in the elution pattern of cytosol fraction were examined. As shown in Fig. 2, in the zinc-fed rats, a rapid accumulation of copper occurred in the zinc-thionein fraction within 4 hr after the administration of copper. In the normal rats, however, the administered copper caused a marked increase in copper content in the fraction of high molecular weight proteins and no peak of copper was observed in the region where the zinc-thionein would be expected to occur. These results indicated that the zinc-thionein induced by high zinc-intake may function to bind the administered copper.

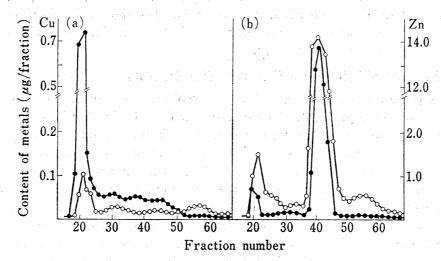


Fig. 2. Fractionation on Sephadex G-75 of Cytosol Fraction from Intestinal Mucosa of Normal (a) and 1% Zinc-fed Rats (b) 4 hr after Oral Administration of Copper (0.4 mg/100 g)

---; copper, ---; zinc.

Gel Filtration Pattern of Cytosol Fraction from Normal Rats after Administration of Metals

As shown in Fig. 3, the rats receiving a single administration of zinc demonstrated that the majority of zinc were eluted in the fraction of low molecular weight compounds during the first 4 hr, although a slight peak of zinc-thionein was detected. After 14 hr, however, zinc dosing resulted in an increase of zinc content in the zinc-thionein fraction, in which a small amount of copper was detected. This alteration was accompanied with a marked

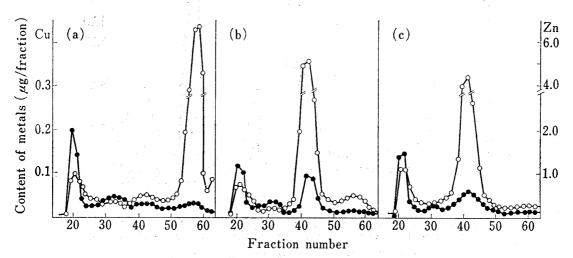


Fig. 3. Fractionation of Sephadex G-75 of Cytosol Fraction from Intestinal Mucosa of Rats (a) 4, (b) 14 and (c) 24 hr after Oral Administration of Zinc (25.2 mg/100g)

——: copper, ——; zinc.

decrease of zinc in the fraction of low molecular weight compounds. After 24 hr, the elution pattern was essentially the same as that obtained from the rats 14 hr after dosing, except a slight decrease of zinc content in both the zinc-thionein fraction and the fraction of low molecular weight compounds. These results indicated that the zinc-thionein in the intestinal mucosa was induced by zinc intake with a lag phase of at least 4 hr and that endogenous copper in the mucosa was bound by this protein.

The elution patterns of cytosol 4 hr after dosing of copper to the zinc-pretreated rats are shown in Fig. 4 (a), demonstrating that copper was associated with the zinc-thionein fraction and the fraction with high molecular weight proteins. In the cytosol fraction from the rats 14 hr after the pretreatment with zinc, by which the zinc-thionein has been induced as shown in Fig. 3 (b), the administered copper was incorporated into the zinc-thionein at higher levels than that in the cytosol 4 hr after dosing of copper alone to the normal rats (Fig. 2a). At the same time, this incorporation of copper into the zinc-thionein caused a marked increase in the zinc content of fraction containing low molecular weight compounds. The elution pattern of cytosol 14 hr after dosing of copper to the zinc-pretreated rats is shown in Fig. 4 (b), which demonstrates that zinc content of the zinc-thionein fraction increases again with decreasing the zinc content in the fraction containing low molecular weight compounds. These results indicated that the administered copper may competitively bind to the zinc-thionein by displacing zinc from this protein.

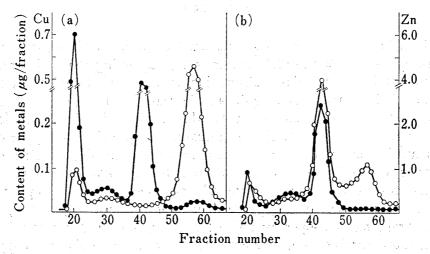


Fig. 4. Effect of Pre-treatment with Zinc on Accumulation of Copper in Cytosol Fraction of Rat Intestinal Mucosa

Copper was given orally to rats (0.4 mg/100 g) 14 hr after an oral administration of zinc (25.2 mg/100 g). Cytosols prepared at 4 (a) and 14 hr (b) after the administration of copper were fractionated on Sephadex G-75 as described in the text.

———; copper, ——; zinc.

Binding of Copper and Zinc to Cytosol Proteins after Simultaneous Administration

In the previous paper,¹⁾ we have reported that copper absorption was greatly depressed when copper and zinc were simultaneously administered to the normal rats. As shown in Fig. 5, a rapid accumulation of copper also occurred in the zinc-thionein fraction 4 hr after the simultaneous administration of two metals, followed by a gradual decrease in copper content with time. No peak of zinc was detected in the region corresponding to the zinc-thionein during 14 hr and major parts of zinc were associated with the fraction eluted late from the column. After 24 hr, however, the peak of zinc-thionein containing a large amount of copper appeared in the elution profile, while the peak of zinc attributed to the fraction of low molecular weight compounds became very small, probably because of the incorporation of zinc into the zinc-thionein.

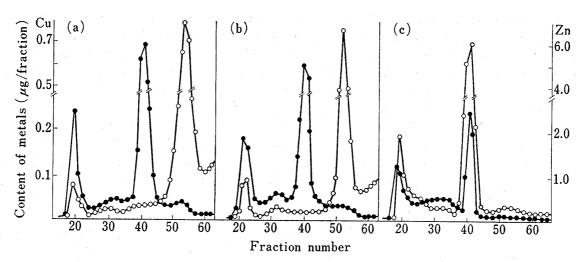


Fig. 5. Fractionation on Sephadex G-75 of Cytosol Fraction from Intestinal Mucosa of Rats (a) 4, (b) 14 and (c) 24 hr after Simultaneous Administration of Copper (0.4 mg/100 g) and Zinc (25.2 mg/100 g)

______; copper. _____; zinc.

Discussion

The zinc-thionein has been previously reported to be induced in the liver, kidney and intestine of rats by feeding of the high dietary zinc, suggesting that this protein may play a role in zinc metabolism, particularly absorption, storage and detoxication of zinc.⁸⁾ The present data showed that there was a significant decrease in the copper content of the intestinal segments of the zinc-fed rats, but the induced zinc-thionein accumulated a certain amount of copper (Table I and Fig. 1). The observed depression of copper content in the intestinal segments is consistent with the view that the high dietary zinc results in the decreases of copper content in various tissues, probably due to long periods of feeding of the zinc-diet.¹⁾ These results suggested that the adverse effect of zinc on copper absorption as reported previously¹⁾ may be caused by the depression of copper uptake by the intestinal wall and /or the trapping of copper available for absorption by the zinc-thionein.

The present data, furthermore, demonstrated that the administered copper was incorporated into the zinc-thionein in the zinc-fed and zinc-pretreated rats (Fig. 2 and 4), suggesting that inhibitory effect of zinc on copper absorption is possibly correlated to changes in the amount of zinc-thionein, by which copper is sequestered and thus the mobilization of copper into the circulation is blocked. Some investigators^{4,5)} have speculated that the metallothionein may have a role in copper metabolism by providing binding sites with which copper forms a mercaptide and that copper dissociated from this protein diffuses directly into plasma in the form of a copper-amino acid complex or ionic copper.

In the present experiments, however, copper in the fraction corresponding to the metallothionein was not detectable in significant quantities in the elution patterns obtained from the normal rats after the administration of copper (Fig. 2a). In all cases tested, the administration of zinc caused the increase in the amount of copper associated with the zinc-thionein. The simultaneous administration of copper and zinc to the normal rats resulted in the increase of copper content in the zinc-thionein within 4 hr, whereas the major portion of zinc were associated with the fraction eluted late from the column (Fig. 5). These results suggested that copper binding to the zinc-thionein is due to the replacement of zinc by copper from the metal-binding sites of zinc-thionein and thus the induced zinc-thionein is capable of sequestering copper.

The possible replacement of zinc by metals from the metallothionein is supported by several findings that the order of the relative affinity for SH group as binding sites is as follows; Cu>Cd>Hg>Zn, which is confirmed experimentally in vitro by the displacement of metallothionein-bound metals. (6b,11) Little is known about the inductive mechanism of the zinc-thionein in the intestine. However, the present results suggest that the synthesis of this protein takes place within at least 4 hr after the oral administration of zinc. Synthesis of the hepatic zinc-thionein has been reported to be stimulated with a maximum at 5—6 hr after the parenteral administration of zinc. (12)

The administration of zinc to rats has demonstrated that zinc absorption is not correlated with the serum zinc content but is related to the metallothionein synthesis in the mucosa and directly related to the amount of zinc associated with the low molecular weight compounds. In our experiments, zinc associated with the low molecular weight compounds has a tendency to bind to the zinc-thionein following an oral administration of zinc (Fig. 3 and 5). These results suggested that zinc may primarily combine with low molecular weight compounds in the mucosal cells, after which zinc is transfered to the plasma or incorporated to the apo-thionein induced by zinc.

Several workers^{5b)} have suggested that copper may be transported across the intestinal wall as copper-amino acid complexes or ionic copper. It appears that zinc-thionein induced by zinc intake competes with these copper and that zinc-thionein in the mucosa leads to a reduction of the amount of copper available for transfer to the circulation. Copper that remains bound to the thionein is probably unavailable for utilization and excreted by shedding of the epithelial cells in a manner analogous to the excretion of ferritin.¹³⁾

Davis, et al.¹⁴⁾ have recently presented an evidence that the inhibition of copper absorption by cadmium is associated with an increase in the amount of copper bound to the cadmium-thionein. Trapping of metals by metallothionein may function as a mucosal block to protect against the absorption of toxic level of copper as well as zinc, cadmium and mercury. Further experiments on the mechanism involved in the synthesis and degradation of this protein in the intestine are required to substantiate this proposed mechanism.

Acknowlegement The authors are indebted to Miss M. Hirano for her technical assistance in parts of this work.

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