[Chem. Pharm. Bull. 22(1) 55 — 60 (1974)]

UDC 615.31'1.033.034

Inhibitory Effect of High Dietary Zinc on Copper Absorption in Rats[1]

Taketo Ogiso, Kazuo Moriyama, Sanae Sasaki, Yoshiko Ishimura, and Akira Minato

Faculty of Pharmaceutical Sciences, Chiba University[2]

(Received March 7, 1973)

High dietary zinc resulted in the lowering of growth, hemoglobin concentration, ceruloplasmin activity and copper concentration in liver, kidney and serum with a marked increase in the concentration of zinc in these tissues.

The preliminary experiments were made to determine the sites at which zinc interferes with copper metabolism. When copper was administered to the zinc-fed rats either orally or intraperitoneally, the depression in copper uptake into liver was clearly observed in the former case. Moreover, copper uptake into liver was depressed even when both copper and zinc were administered orally to the normal rats. By the short-period experiments using ^{64}Cu, it was also shown that zinc interferes with the absorption and utilization of copper. These results indicate that the interference of copper metabolism by zinc feeding is mainly due to the inhibition of copper absorption at the intestinal level.

High levels of dietary zinc (0.5 to 1.0%) fed to rats have been reported to result in a depression of growth and the development of hypochromic-microcytic anemia accompanying decreases of both cytochrome oxidase activity and copper content in the liver and heart.[3] The additional supplement of copper has been shown to prevent anemia, increase growth and raise the liver and heart cytochrome oxidase in the zinc-fed rats to normal or greater than normal levels.[3b,c,e]

From these results it has been inferred that zinc may alter the metabolism of copper and that the antagonistic correlation exists between zinc and copper.[4] However, the mechanism by which zinc interferes with the copper metabolism is still poorly understood.

In the previous paper,[5] the authors reported that high zinc intake caused decreases in ceruloplasmin activity and copper content of the rats serum and suggested, in part at least, to be the result of a copper deficient state. The evidence that copper-binding protein of serum, ceruloplasmin (E.C. 1.12.3.1) is a direct molecular link between copper and iron metabolism has been mentioned by Frieden.[6] The present study was undertaken to investigate the inhibitory mechanism of excess zinc against copper metabolism in rats.

Experimental

Animal Experiments——Male Wistar rats weighing approximately 120—130 g were housed in stainless steel wire cages. A commercial diet containing 5 ppm of copper and 80 ppm of zinc (Nihon Clea CE-2) was used as basal diet. Rats of the normal group were free access to the basal diet, and those of the test

1) Presented at the 90th Annual Meeting of Pharmaceutical Society of Japan, Sapporo, July 1970.
2) Location: *1-33, Yayoicho, Chiba.*
3) *a*) W.R. Sutton and V.E. Nelson, *Proc. Soc. Exp. Med.*, **36**, 211 (1937); *b*) S.E. Smith and E.J. Larson, *J. Biol. Chem.*, **163**, 29 (1946); *c*) G.P. Duncan, L.F. Gray, and L.J. Daniel, *Proc. Soc. Exp. Biol. Med.*, **83**, 625 (1953); *d*) R.V. Reen, *Arch. Biochem. Biophys.*, **46**, 337 (1957); *e*) D.R. Grand-Frost and E.J. Underwood, *Australian J. Exp. Biol. Med. Soc.*, **36**, 339 (1958); *f*) A.C. Magee and G. Matrone, *J. Nutr.*, **72**, 233 (1960).
4) *a*) C.H. Hill, G. Matrone, W.L. Rayne, and P.W. Barber, *J. Nutr.*, **80**, 227 (1963); *b*) D.R. Van Campen, *J. Nutr.*, **88**, 125 (1963); *c*) C.T. Settlemire and G. Matrone, *J. Nutr.*, **92**, 159 (1967).
5) A. Minato and T. Ogiso, *Yakugaku Zasshi*, **86**, 521 (1966).
6) *a*) S. Osaki, D.A. Johnson, and E. Frieden, *J. Biol. Chem.*, **241**, 2746 (1966); *b*) R.F. Gould ed., "Bio-

group were fed the diet which prepared by addition of zinc in the carbonate form to the basal diet in 1.0%. After feeding for 15 days at longest, the rat in both groups were sacrificed by bleeding from the femoral vein. Whole blood was centrifuged at 3000 rpm for 10 min and serum was obtained. Liver was perfused *in situ* with a cold physiological saline through the portal vein. Liver, kidney and heart were then removed and liver mitochondrial fraction was obtained by the method of Hageboom, *et al.*[7] To ascertain if zinc affects the intestinal absorption of copper, the normal and zinc-fed rats were given orally or intraperitoneally copper alone or copper and zinc in combination and killed at various intervals. Then, livers were removed in the above mentioned manner and the copper content was determined. In the experiment using $^{64}CuCl_2$ (9.8×10^2mCi/g, JAERI), the rats of each group received 35 μCi of ^{64}Cu by a stomach tube and bled from the tail vein at various intervals. After drying each blood sample (0.02 ml) in counting dishes under an infrared lamp, the radioactivity was determined by means of a 2π low background gas flow counter (Aloka, PS 40).

Analytical Methods——About 500 mg of tissue sample in a micro-Kjeldahl flask was oxidized with 2.0 ml of 60% perchloric acid by gentle heating. When charring began, 30% hydrogen peroxide was added dropwise to the flask and heating was continued until a colorless or clear solution was obtained. After standing overnight, ammonium hydroxide was added to adjust pH 1.2—2.8 using a few drop of 0.1% thymol blue as an indicator. Then, 0.5 ml of 0.1% ammonium pyrolidine dithiocarbamate and 5.0 ml of methylisobutylketon (MIBK) were added to the solution and the flask was shaked mechanically for 2 min. After the contents came to equilibrium the upper MIBK layer was subjected to copper analysis by atomic absorption (Hitachi 139—0422). Instrumental settings were as follows: wavelength, 3248 Å; slit range, 0.2 mm; lamp current, 8 mA (200 V); acetylene flow, 10 liter/min; air flow, 2.0 liter/min. The oxidized samples obtained by the above mentioned manner were diluted to 10 ml in a volumetric flask with a distilled water and subjected to zinc analysis in the condition as follows: wavelength, 2139 Å; slit range, 0.3 mm; lamp current, 10 mA (200 V); acetylene flow, 0.7 liter/min; air flow, 7.0 liter/min. Working calibration curves for copper and zinc were prepared from standards analyzed at the same time as the samples. Ceruloplasmin, copper oxidase, was estimated using *p*-phenylendiamine as substrate by the method of Ravin[8] which was modified by Henry.[9] The unit of activity was defined as increase of 0.001 in absorbance at 530 mμ under the incubation for 30 min at 37°. Hemoglobin was determined by the cyanmethemoglobin method[10] and mitochondrial protein content was quantified by the method of Lowry, *et al.*[11] using bovine serum albumin as a standard.

Result and Discussion

Effects of Feeding of 1% Zinc Diet

Table I shows a marked decrease of ceruloplasmin activity in the serum of rats fed 1% zinc diet for 15 days. Slight but significant decreases in body weight agin and hemoglobin value were also observed. The magnitude of decrease of hemoglobin, however, was relatively small in the present study as compared with the value described by Settlemire, *et al.*,[4c] probably due to the larger size of rats and the shorter period of feeding employed in this experiment.

There is no evidence that zinc affects directly the ceruloplasmin activity of normal serum. It is assumed therefore that the depletion of ceruloplasmin in the zinc fed rats is a reflection of some abnormal copper metabolism induced by zinc.

Osaki, *et al.*[6] have proposed a role for ceruloplasmin in promoting the incorporation of ferrous iron into apotransferrin as a ferroxidase. This fact would tend to propose the hypothesis that the anemia induced by excess intake of zinc is consequent on a severe depletion of ceruloplasmin levels in serum. The study on this possibility, that is, effect of zinc on the disturbance of iron metabolism will appear elsewhere.

As shown in Table II, high zinc intake for 6 days caused already significant decreases in copper content of the liver, kidney and serum, except heart. The copper content of liver mitochondria also decreased to a value less than half the normal level. During 15 days, there were more decreases in copper content of liver, kidney and serum, while the change in copper

7) G.H. Hageboom, W.C. Schneider, and G.E. Pallade, *J. Biol. Chem.*, **127**, 619 (1948).
8) H.A. Ravin, *Lancet*, **270**, 726 (1956).
9) R.H. Henry, N. Chiamori, S.L. Jacobs, and M. Segalone, *Proc. Soc. Exp. Biol. Med.*, **104**, 620 (1960).
10) W.H. Crosby, J.I. Munn, and F.W. Furth, *U. S. Armed Forces M. J.*, **8**, 693 (1954).
11) O.H.N. Lowry, J. Rosebrough, A.L. Parr, and R.J. Randall, *J. Biol. Chem.*, **193**, 265 (1951).

Table I. Effect of High Dietary Zinc on Body Weight, Hemoglobin Level and Ceruloplasmin Activity in Rats

	Weight (g)				15 days	
	0 day	6 days	15 days	gain	Hb g/100 ml	Cp unit
Normal diet	130.9	146.2	171.6	40.7±2.3	14.90±0.15	278.0±9.7
1% Zn diet	132.2	144.4	155.8	23.6±1.8[a)]	12.17±0.93[a)]	19.4±3.2[a)]

Six rats in each group were maintained with the normal diet or 1% Zn diet for 15 days. Values represent mean standard error.
a) significantly less than normal values at $p < 0.01$

content of heart was relatively small. At that time, the copper content of liver mitochondria did not show any significant decrease from that of the zinc-fed rats for 6 days.

Owen, *et al.*[12] have reported that ceruloplasmin is synthesized in the liver, and that plasma level of ceruloplasmin is approximately proportional to the copper level in the liver. This strongly suggests that the observed depletion of hepatic copper in the zinc-fed rats may result in the decrease in ceruloplasmin activity. The depletion of copper in serum also indicates that the copper content in serum parallels with the serum ceruloplasmin activity. Equally, the observation on copper content of liver mitochondria is compatible with the fact that cytochrome oxidase activity was lower in the zinc intoxication[3c)] and lost in copper deficient state when the copper content in liver was about half the control level.[13]

Table II. Effect of High Dietary Zinc on Copper Concentration in Various Organs

	Normal	1% Zn diet for	
		6 days	15 days
Liver[a)]	3.06±0.44	2.69±0.32[b)]	1.16±0.23[c)]
Kidney[a)]	6.45±0.50	3.72±0.40[b)]	2.25±0.21[c)]
Heart[a)]	3.88±0.14	3.43±0.37	2.31±0.23
Serum[d)]	0.67±0.11	0.27±0.09[b)]	0.16±0.02[c)]
Liver Mit.[e)]	19.41±3.85	7.28±2.56[c)]	9.13±2.10[c)]

Six rats in each group were fed with the normal or 1% Zn diet and sacrificed at 6 and 15 days. Values represent mean±standard error.
a) μg/fresh tissue (g), *b*) significantly less than normal values at <0.05 *c*) $p < 0.01$
d) μg/serum (ml), *e*) μg/protein (g)

As shown in Table III, the liver, kidney and serum concentration of zinc increased in the zinc fed-rats as the time of feeding passed, while the heart concentration remained unchanged throughout the feeding period of 15 days. It seems possible therefore that the decreases in copper content of the liver, kidney and serum in the zinc-fed rats occur as a consequence of the accumulation of zinc and that there are competitive correlation between copper and zinc in these tissues.

Hepatic Accumulation of Administered Copper

As can be seen in Fig. 1, the normal rats receiving orally 4 mg/100 g of copper demonstrated a rapid increase in the copper content of the liver reaching the maximum 12 hr later followed by a gradual decrease. In contrast, the oral administration of the same dose of

12) *a*) J.B. Hazelrig, C.A. Owen, and E. Ackerman, *Am. J. Physiol.*, **211**, 1075 (1966); *b*) C.A. Owen and J.B. Hazelrig, *ibid.*, **211**, 210, (1966).
13) C.H. Gallagher, J.P. Judah, and K.R. Rees, *Proc. Royal. Soc.* (London), *Ser. B.*, **145**, 134 (1956).

Table III. Effect of High Dietary Zinc on Zinc Concentration in Rats

	Normal	1% Zn diet for	
		6 days	15 days
Liver[a)]	32.21±2.92	134.41±14.15[b)]	159.89±13.10[b)]
Kidney[a)]	24.51±0.61	77.27± 5.22[b)]	105.46± 4.43[b)]
Heart[a)]	20.01±0.85	22.91± 3.49	20.04± 1.15
Serum[c)]	2.13±0.44	15.43± 1.67[b)]	20.40± 1.86[b)]

Conditions were the same as described in Table II.
a) μg/fresh tissue (g), *b*) significantly idfference at $p < 0.01$,
c) μg/serum (ml)

Table IV. Effect of Routes of Copper Administration on Copper Uptake in Liver

	Normal (Cu μg/fresh tissue g)	1% Zn diet	Uptake inhibition (%)
PO	20.2±1.3	3.5±2.0	82.7
IP	38.8±2.3	22.4±1.5	42.3

PO=per os, IP=intraperitoneal; six rats in each group fed with 1% Zn diet for 5 days were sacrificed 12 hours after the oral or in traperitoneal dose of 4 and 0.25 mg of copper/100 g body weight respectively.

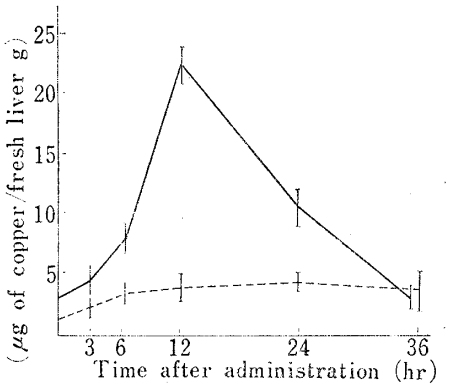

Fig. 1. Effect of Dietary Zinc on Liver Retention of Copper at Various Time after the Oral Administration of Copper

Copper of 4 mg/100 g of body weight was given orally to six rats in one group fed the normal (—) or 1% zinc diet (---) for 5 days. Data are presented as mean standard error.

copper to the rats fed 1% zinc diet for 5 days failed to increase the hepatic copper content over the normal level. The possible mechanism by which zinc interferes with the uptake of administered copper to the liver can be presumed at least in the process of the intestinal absorption or the incorporation of copper into hepatic cells.

To testify this assumption, the effect of copper dosing routes on copper uptake into the liver was compared as shown in Table IV. The zinc-fed rats demonstrated an uptake inhibition of intraperitoneally injected copper to the liver as compared with the normal. However, the uptake inhibition percent was markedly lower in the intraperitoneal group than in the oral group of the zinc-fed rats.

Subsequently, copper was administered to the normal rats simultaneously with zinc by oral route or by intraperitoneal route. As can be seen in Table V, the accumulation of copper in the normal rat liver was reduced by the simultaneous administration of zinc regardless of the route of administration. Nevertheless, it was clearly noted that the reduction of copper uptake was larger in the oral experiment than in the intraperitoneal experiment.

These results suggest strongly that excess intake of dietary zinc may cause a depletion of hepatic copper mainly by reducing the intestinal absorption of copper.

Time-Course of Blood Levels of ^{64}Cu

As illustrated in Fig. 2, a "zig-zag" pattern of blood concentration of radioactivity was observed in the normal rats following an oral administration of ^{64}Cu. In contrast, an approximately linear increase of blood ^{64}Cu was noted in both groups of the rats given 1 mg of zinc and those fed 1% zinc diet for 9 days. Blood radioactivity of these zinc-treated rats, however, was much less in magnitude than that of the zinc-untreated controls.

Several workers[14,15] have pointed out that the first peak of radioactivity in the blood of the normal rats following an oral administration of ^{64}Cu is mainly due to a cupric ion which is bound loosely to plasma albumin, while the second rise of ^{64}Cu in the blood may be owing to the metal bound tightly to a protein, ceruloplasmin, which is synthesized in the liver.

14) H. Scheinberg and I. Sternlieb, *Pharmacol. Rev.*, **12**, 355 (1960).
15) *a*) J.A. Bush, J.P. Mahony, H. Markwitz, C.J. Gubler, G.E. Cortwright, and M.M. Wintrobe, *J. Clin. Invest.*, **34**, 1766 (1955); *b*) S.B. Osborn and J.M. Walshe, *Lancet*, **1**, 1 (1956).

Table V. Effect of Zinc Administration on Copper Uptake in Liver of the Normal Rats

Dose mg/100 g body Wt.	Cu μg/g fresh liver	uptake inhibition (%)
Oral administration		
Cu: 4 mg	20.2±1.3	
Cu: 4 mg+Zn: 8 mg	14.7±1.6	27.3
Cu: 4 mg+Zn: 24 mg	8.1±0.6	60.0
Intraperitoneal administration		
Cu: 0.25 mg	38.8±2.3	
Cu: 0.25 mg+Zn: 0.5 mg	34.7±2.3	10.6
Cu: 0.25 mg+Zn: 1.5 mg	27.0±2.8	30.4

Six rats in one group were sacrificed 12 hours after the simultaneous administration of copper and zinc orally or intraperitoneally.
Values represent mean±standard error.

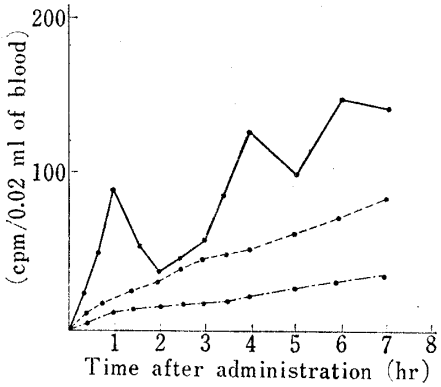

Fig. 2. Activity of ^{64}Cu in Blood after an Oral Administration of Copper in Rats

——: 35 μCi of ^{64}Cu was given to the normal rats
——: 35 μCi of ^{64}Cu and 1 mg of zinc was given simultaneously to the normal rats
——: 35 μCi of ^{64}Cu was given to the rat fed 1% zinc diet for 9 days
Each point represents the mean value of two observations.

The additional evidence obtained by these short-period experiments also confirmed the early findings that zinc interferes with copper absorption at the intestinal level.

Excretion of Copper

Table VI shows that the biliary excretion of copper was less than 4.0 μg/100 ml in the rats fed 1% zinc diet for 3 and 6 days respectively, in contrast to 40.3 μg/100 ml in normal controls, although the urinary excretion was approximately the same in both groups. It is known that the bile is the major pathway of copper excretion.[16] The present data appear thus to support the idea that the intestinal absorption of copper may be very poor in the zinc-fed rats and that the progressive zinc accumulation in tissues of the zinc-fed rats as shown in Table III does not result in the increase of the copper excretion.

Table VI. Effect of Feeding of Excess Dietary Zinc on Copper Excretion in Bile and Urine

	Copper in bile μg/100 ml	Copper in urine	
		μg/day	μg/100 ml
Control	40.3	0.36	6.96
Zn-diet for 3 days	<4.0	0.26	7.33
Zn-diet for 6 days	<4.0	0.39	9.89

Bile was obtained by cannulation of the common bile duct, and urine was collected separately in a metabolic cage.
Values represent mean of 6 rats.

Crampton, *et al.*,[17] using sacs of hamster small intestine *in vitro*, have shown that copper absorption involves a special mechanism dependent on metabolic energy. Furthermore, Starcher[18] and Evans, *et al.*[19] have suggested that the binding of copper to duodenal sul-

16) C.L. Comar and F. Bronner, "Mineral Metabolism," Vol. II, Academic Press, New York and London, 1962, p. 385.
17) R.F. Cramptom, D.M. Mattheus, and R. Poisner, *J. Physiol.*, **178**, 111 (1965).
18) B.C. Starcher, *J. Nutr.*, **97**, 321 (1969).
19) G.W. Evans, P.E. Majors, and W.E. Cornatzer, *Biochem. Biophys. Res. Commun.*, **40**, 1142 (1970).

fhydryl-rich protein, metallothionein, is an important step in the process of copper absorption. The inhibition of copper absorption by zinc, therefore, could be elucidated by either of these mechanism or a combination of them. The present data provide no information on which one of these mechanism may be responsible for the inhibition of intestinal copper absorption by zinc. Other studies bearing on this and other related questions are under investigation.