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Transfer of Cupric Sulfate across Rat Samll Intestine, *in Vitro* and Effect of Chelating Agents on It's Transfer

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Abstract□The transfer of cupric sulfate across the rat small intestine *in vitro* was studied by perfusion method using the segments of everted rat small intestine. Copper transport was approximately propotional to the metal concentration in the mucosal solution and no difference was observed in the metal transport among rat duodenum, jejunum and ileum. It was suggested from these results that copper transport across the rat small intestine would occur by passive diffusion. The effect of various chelating agents on copper transport across the rat small intestine *in vitro* and its uptake by the intestine were also studied. Copper transport was greatly enhanced in the presence of EDTA and NTA. Copper uptake decreased to a greater extent in the presence of EPTA and NTA.

Keywords□copper transport, rat small intestine, perfusion method, passive diffusion, copper uptake, chelating agent, copper-EDTA complex

Copper is widely distributed in the nature and is an essential element of animals and plants. It is one of the important elements of metabolic enzymes in organisms and is also an essential element in cell reproduction.[1,2]

The pathological symptoms appeared in man are concerned with the excessive accumulation of copper. The main disorders caused by copper intoxication are Wilson's disease and Menke's disease which are related to excessive copper accumulation originated in congenital metabolic disorder.[3-5]

It has been reported that copper is mainly absorbed both at stomach and at small intestine in the same rate. Although several kinds of hypotheses were presented about the mechanism of copper absorption, they are still far from the exact absorption mechanism.

Crampton *et al.* had reported that absorption of copper happens by-special mechanism or mechanism dependent on metabolic energy making use of Fomalo golden Hamsters' small intestine. And Starcher had reported that the binding of copper to duodenal protein with approximate molecular weight of 10,000 is an important step in the process of copper absorption.[3,6-13]

Presently, examination about the exact mechanism of copper absorption at gastro-intestinal tract and studies on effects of other substances which have influences to absorption and excretion of copper are not enough.

This paper presents the result of experiment designed to investigate the transport of cupric sulfate across rat small intestine and the effect of chelating agents on its transfer using the circulation apparatus developed by Fisher and Parson and renewed by Smyth *et al.*[14]

EXPERIMENTAL METHOD

1) Transport experiment of cupric sulfate across rat small intestine, in vitro.

The raw was anesthetized with ether. The small intestine was taken out through a midline abdominal incision and washed with saline solution. Mesenterium and fat were removed from it and it was pressed with filter paper to suck up water. It was weighed and everted with glass bar(diameter 2.5 mm) in the saline solution.

As shown in Fig. 1, mucosal side(A) was filled with 110 m*l* of cupric sulfate solution, in advance and was heated up to keep 37 °C, then air was supplied through(C) and the everted intestine was fitted to circulation apparatus. Serosal side(B) was filled with 10m*l* of Krebs-Ringer solution and provided air into(D).

0.5m*l* of mucosal side solution and serosal side solution was sampled from (E) and (F) every 30 minutes, respectively. After sampling, 0.5 m*l* of

Krebs-Ringer solution and saline solution was added into (A) and (B), separately, Experiment was carried out for 3hr and done at every concentration three times.

2) Pretreatment of small intestine with EDTA

As you see Fig. 1, 10ml of Krebs-Ringer solution (pH 7.0) without $CaCl_2$ and M_gSO_4 $7H_2O$ was added into (B) of circulation apparatus fitted with everted intestine and 10ml of EDTA saline solution (10.2mM, pH 5.3) was added into (A). According to the transport experiment, this apparatus was made circulate for 1hr, then mucosal solution was replaced with saline solution for cleaning and serosal fluid was replaced with new Krebs-Ringer solution. Soon after, transport experiment of copper was carried out with this small intestine pretreated.

3) Transport experiment of EDTA across rat small intestine, in vitro.

With use of Krebs-Ringer solution at serosal side (B) and EDTA dissolved in 0.9% NaCl solution (10.2 mM) at mucosal side (A), experiments were carried out. 0.5 ml of both side fluid was sampled every 30 minutes and the others were performed according to transport experiment of cupric sulfate.

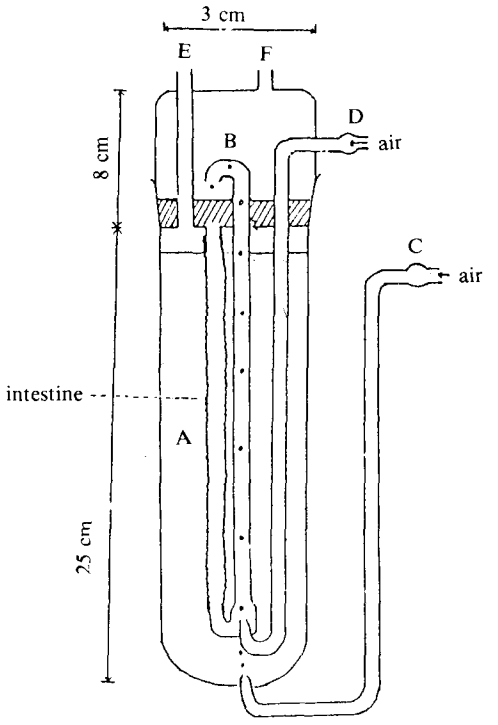

Fig. 1. Schematic diagram of the experimental apparatus.

4) Analytical method

a) Pretreatment of intestine

The intestine used at transport experiment was put into 100 ml Kjeldahl flask and 10 ml of 60% HNO_3 was added, then boiled for 30 minutes and then cool it down. Soon after, 2 ml of 60% $HClO_4$ was added and the mixed solution was discomposed until it slowly began to boil and made white smoke and finally, the solution turned into colorless limpidity. After cooling it down, distilled water was added to make the total volume 100 ml for determination of copper only and to make the total volume 10 ml for determination of copper, calcium and magnesium together.

b) Determination of copper[15]

Definite quantities of each solution sampled from mucosal side and serosal side and of discomposed solution of intestine were determined according to DDTC method.

c) Determination of calcium, magnesium and EDTA

Determined according to chelatemetry

RESULTS AND DISCUSSION

Transport of cupric sulfate across the rat samll intestine, in vitro.

The transport of cupric sulfate across rat small intestine was studied by the perfusion method using the various regions of everted rat small intestine. The upper segment (20cm) of the everted intestine as

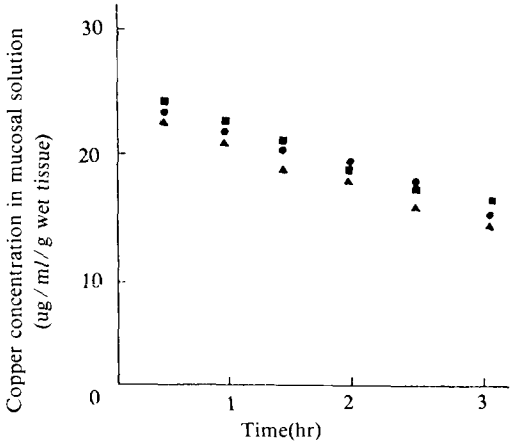

Fig. 2. Mucosal concentration of copper in small intestine.

Initial mucosal concentration of copper was 30 ug/ml. Each value is expressed as the mean of 3 experiments.

■, duodenum; ●, jejunum; ▲, ileum

Table I. Uptake and transport of copper by various regions of rat small intestine

Segmant	Transport (ug/g wet tissue)	Uptake (ug/g wet tissue)
Duodenum	9.89±0.35 (3)	255.86±35.77 (3)
Jejunum	9.52±1.38 (3)	245.57± 7.79 (3)
Ileum	9.00±0.77(3)	219.84±22.36 (3)

Initial mucosal concentration of copper was 30 ug/ml
Each value is expressed as the mean ± standard deviation
Figures in parentheses indicate the number of experiments.

duodenum, lower segment (20cm) as ileum and mid sigment (20 cm) as jejunum were used.

Fig. 2 shows intestinal mucosal copper concentration versus time. The disappearance of mucosal copper is linear for every segment of small intestine. And the disappearance of copper from the mucosal solution of rat duodenum, jejunum and ileum followed apparent first-order kinetics.

The variation of serosal copper concentration transported from small intestinal mucosa vs. time is shown in Fig. 3. Copper transported from mucosal fluid and its uptake into intestinal mucosal tissue are shown in Table I. As-shown Fig. 3 and Table I, the amount of copper transported from mucosal to serosal of rat duodenum, jejunum, ileum increased with the passage of time.

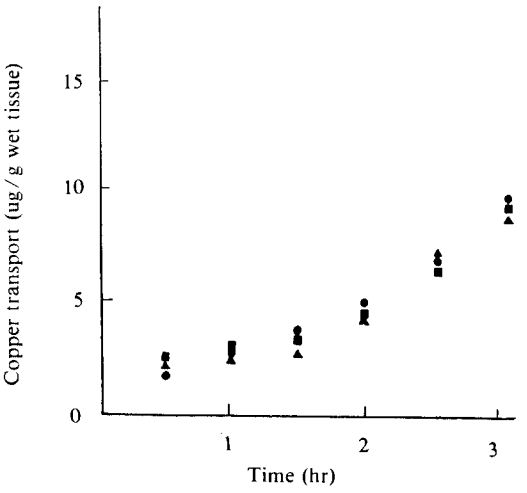

Fig. 3. Transport of copper by various regions of rat small intestine.
Initial mucosal concentration of copper was 30 ug/ml. Each value is expressed as the mean of 3 experiments.
■, duodenum; ●, jejunum; ▲, ileum.

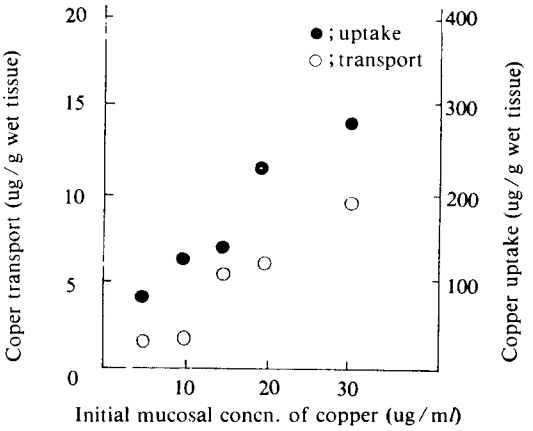

Fig. 4. Transport and uptake of copper in various mucosal concentrations
Rat jejunum was used. Each value is expressed as the mean of 3 experiments.

But it was small in amount and a large portion of the copper in the mucosal solution was taken up into the intestinal tissue. And no difference was observed in the copper transport among rat duodenum, jejunum, ileum. However, as shown in Fig. 2 and Fig. 3, copper transported from the mucosal of ileum was small as compared with those of duodenum and jejunum. In addition, the disappearance rate of copper from mucosal fluid of ileum was small as against those of duodenum and jejunum.

Fig. 4 shows the amount of copper transported from mucosal fluid at every 5 ug, 10 ug, 15 ug, 20 ug and 30 ug Cu/ml intial copper concentration. As shown in Fig. 4, copper transport was approximately proportional to the copper concentration in the mucosal solution and its uptake into the intestinal tissue increased according to the initial copper concentration. These results suggest that copper transport across the rat small intestine should occur by passive diffusion.

Effect of various chelating agents on rat small intestinal transport of cupric sulfate, in vitro.

Copper reacts with EDTA, NTA and CyDTA in the same form as cadmium, nickel and cobalt to produce water soluble chelate compounds. It has been known that organic acids such as citric acid, succinic acid and tartaric acid existed in the nature react with copper to form metal chelate compounds. It is suggested that such chelating agents influence the movement of copper in the living body.

Thus, the effect of chelating agents such as citric acid, succinic acid, glycine, EDTA and NTA on rat

small intestinal transport of cupric sulfate, *in Vitro* were investigated. EDTA, NTA, succinic acid, citric acid and glycine were added to saline solution of cupric sulfate and the intestinal transport experiment was carried out by the method described in experimental. And the copper transported from mucosal of rat jejunum and its uptake into the intestinal tissue wefe calibrated. These results are shown in Table II.

As shown in Table II, the amount of copper transported from mucosal to serosal side increased up to 20-25 times of that of control by EDTA and NTA and increased up to 3-7 times of that of control by citric acid and succinic acid. These results suggest that copper transport be facilitated by copper chelate formation and the degree of their effect is dominated by the large of stability constant of chelate compounds (log K).[16]

In addition to, it has been reported that lipid insoluble organic compounds such as heparin, mannit, inulin etc. are absorbed only a little into rat intestine but their absorption is promoted by concomitant oral administration with EDTA.[17,18]

Absorption facilitation by EDTA has been explained as follows. EDTA removes calcium ion and magnesium ion existed in intestinal epithelial membrane. So, membrane pore size and epithelial gaps become large. As the result of it, material transport characteristics of intestinal epithelium increase.

To investigate the effect of calcium ion and magnesium ion on copper transport, experiment was carried out with cupric sulfate solution containing EDTA (10.2 mM). After 3 hr. experiment, the intestine used in experiment was wet-ashed and the amount of calcium and magnesium was determined. These results are shown in Table III. The contents of calcium and magnesium in intestine of rat jejunum decreased by half in comparison with control.

And the mucosal side of rat jejunum was pretreated with EDTA saline solution for 1 hr. Soon after, it was washed with saline solution for 15 min. With this intestine pretreated, transport experiment of cupric sulfate was done.

As shown in Table IV, the copper uptake of intestine pretreated with EDTA increased very much in contrast with concomitant EDTA presence, but its transport increased likewise. In addition, the contents of calcium and magnesium in the intestine of rat jejunum decreased as compared with those of concomitant EDTA use.

On the other hand, control experiment was done with the intestine pretreated with saline solution. Its results are shown in Table IV. The contents of calcium and magnesium in intestine decreased in comparison with concomitant EDTA use.

Results obtained from EDTA transport experiment on rat jejunum and from intestinal transport experiment of cupric sulfate and cupric sulfate in the presence of EDTA on rat jejunum are shown in comparison in Fig. 5. As shown in Fig. 5, transport rates (%) of EDTA were much larger than those of cupric sulfate. And the transport rates of cupric sulfate in the presence of EDTA were similar to those of EDTA.

Table II. Effects of various chelating agents of rat jejunum uptake and transport of copper, *in vitro*.

	Stability constant of copper chelate compound(log K)	Copper	
		Uptake (ug/g wet tissue	Transport(ug /g wet tissue
Control	—	206.77 ± 44.08	1.69 ± 0.39
EDTA	18.79	7.41 ± 0.89	28.99 ± 1.60
NTA	13.16	8.49 ± 0.86	22.16 ± 2.54
Succinic acid	2.6	76.46 ± 4.60	7.59 ± 0.46
Citric acid	5.2	46.64 ± 2.95	11.26 ± 0.90
Glycine	8.62	130.83 ± 4.44	4.67 ± 0.15

Initial mucosal concentration were: Cupric sulfate, 10ug Cu/ml; EDTA, NTA and Glycine, 10.2 mM; Citric acid, 17.8 mM; Succinic acid, 32.2 mM.
Each value is expressed as the mean ± standard deviation followed by 3 experiments.

Table III. Effects of EDTA on rat small intestinal uptake and transport of copper and on contents of calcium and magnesium in small intestine, *in vitro*

	Copper (ug/g wet tissue)		Contents in intestine (ug/g wet tissue)	
	Uptake	Transport	Calcium	Magnesium
Control	138.41 ± 11.41	2.66 ± 0.19	5.78 ± 2.38	67.64 ± 1.69
EDTA	5.63 ± 0.40	24.13 ± 1.01	3.87 ± 1.50	34.78 ± 2.37

Initial mucosal concentrations were: Cupric sulfate, 10ug Cu/ml; and EDTA, 10.2 mM.
Each value is expressed as the mean ± standard deviation followed by 3 experiments.

Table IV. Transport and uptake of copper and contents of calcium and magnesium in rat small intestine pretreated with EDTA, *in vitro*

	Copper (ug/g wet tissue)		Contents in intestine (ug/g wet tissue)	
	Uptake	Transport	Calcium	Magnesium
Control	155.63 ± 9.26	3.07 ± 0.17	3.53 ± 0.32	33.61 ± 2.14
EDTA	70.29 ± 3.20	23.26 ± 0.42	1.46 ± 0.21	14.59 ± 1.58

Rat jejunum was used. Initial mucosal concentrations: cupric sulfate, 10ug Cu/ml; and EDTA, 10.2 mM.
Control experiment was carried out using the rat jejunum preperfused with saline solution for 1 hr. Each value is expressed as the mean ± standard deviation followed by 3 experiments.

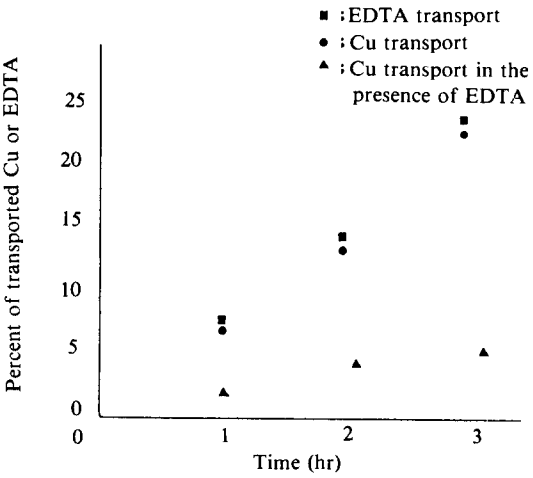

Fig. 5. Comparison of transported rates of copper and EDTA in rat small intestine, *in vitrol*
Rat jejunum was used. Initial mucosal concentrations were; cupric sulfate 10 ug Cu/ml and EDTA 10.2 mM.

$$\text{percent of transported Cu or EDTA} = \frac{\text{Total amount of Cu or EDTA in serosal side/g wet tissue}}{\text{Initial total amount of Cu or EDTA in mucosal side}} \times 100$$

From all the results of experiments carried out, it can be said that EDTA changes the structure of membrane by decreasing the amount of calcium and magnesium in the intestinal epithelial membrane. But this is hardly said to be basic factor in transport promotion of copper across intestinal membrane. As for the promotive effect of EDTA on the transport of copper across the intestinal membrane, it can be explained that EDTA prevents copper from binding to intestinal mucosal tissue and forms stable chelate complex with copper.

It is assumed that this chelate transports across the intestinal tissue. Now, it can be said that the promotive effect of copper transport by the organic acids such as citric acid and succinic acid have same mechanism as EDTA.

CONCLUSION

1. The disappearance of copper from the mucosal solution of rat duodenum, jejunum and ileum followed apparent first-order kinetics and copper transported from the mucosal to the serosal side of each region of the intestine was small in amount and a large portion of the copper in the mucosal solution was taken up by the intestinal tissue.
2. Copper transport was approximately proportional to the copper concentration in the mucosal solution and no differences were observed in the copper transport among rat duodenum, jejunum and ileum.
3. Copper transport was greatly enhanced in the presence of EDTA and NTA, to a lesser degree, in the presence of citric acid and succinic acid. Copper uptake decreased to a great extent in the presence of EDTA and NTA.
4. The effect of EDTA on the intestinal transfer of copper was not due to a modification of intestinal permeability characteristics but possibly due to the enhanced transport of copper across the intestine in the form of copper-EDTA complex.

LITERATURE CITED

1. Hill, C.H., Martrone, G., Payne, W.L. and Barber, C.W.: *In vivo* interaction of Cd with Cu, Zn and Fe. *J. Vutr.* **80,** 227 (1963).
2. Van Campen, D.R. and Darrel, R.: Effect of Zn, Cd, Ag and Hg on the absorption and distribution of copper-64 in rats. *Ibid.* **88,** 125 (1966).
3. Crampton, R.F., Matthew, D.W. and Poisner, R.: Observations on the mechanism of absorp-

tion of copper by the small intestine. *J. Physiol.* **178,** 111 (1965).
4. Tanaka, Y., Tanaka, R. and Kashimoto, T.: Effects of cysteine on the biological actions of Cd in rats. effects of Cd on the fates of Cu and Zn. *J. Food and Hyg.* **26,** 411 (1985).
5. Klassen amdur Doull. *et al.: Casarett and Doull's Toxicology,* p. 612, (1986).
6. Kojima, S. and Kiyozumi, M.: Transfer of $CdCl_2$ across rat small intestine *in vitro* and effect of chelating agents on its transfer. *Yakugaku Zasshi,* **94,** 695 (1974).
7. Kojima, S., Kiyozumi, M., Matsmoto, S., Yamamoto, M., Nakamura, C. and Nino, K.: Effect of chelating agents gastrointestinal absorption, distribution and excretion of $CdCl_2$ in rats. *Eisei Kagaku,* **23,** 43 (1977).
8. Kojima, S., Kiyozumi, M. and Kamiya, M.: Effect of proteins and amino acids on the small intestinal absorption of Cd in Rats. *Ibid.* **25,** 245 (1979).
9. Aburaya, K., Kojima, S. and Furuya, K.: Effects of some fibers on the small intestinal absorption of Cd in rats. *Ibid.* **26,** 267 (1980).
10. Kiyozumi, M., Mizunaga, F., Mishima, S., Nakgamura, M., Noda, S., Miyata, K. and Takashi, Y.: Effects of dietary fibers on absorption of Cd in rats. *Chem. Pharm. Bull.* **30,** 4494 (1982).
11. Kojima, S., Kiyozumi, M., Mishima, M., Honda, T. and Nakgawa, M.: Effects of three proteins on absorption of Cd in rats. *Toxicology,* **34,** 161 (1985).
12. Kojima, S., Honda, T., Moriyama, Y. and Shimizu, T.: Effect of Cd on small intestinal absorption of L-histidine in rats. *Chem. Pharm. Bull.* **34,** 372 (1986).
13. Kiyozumi, M., Yamane, M., Shimizu, T. and Honda, T.: Effects of chelating agents on inhibition of L-histidine absorption with Cd in rats. *Eisei Kagaku,* **32,** 312 (1986).
14. Smyth, D.H., and Whaler, B.C.: *Apparatus* for the *in vitro* study of intestinal *absorption. J. Physiol.* **121,** 29 (1953).
15. The Pharmaceutical Society of Japan, *Standard Methods of Analysis for Hygienic Chemists,* p. 24, (1980).
16. Sakaguchi, T. *et al.*: *"Metal Chelate (III)"*, Appendix, Tokyo, (1965).
17. Tidball, C.S. *et al.*: Mg and Ca as regulators of intestinal permeability. *Am. J. Physiol.* **206,** 243 (1964).
18. Cassidy, M.M. and Tidball, C.S.: Cellular mechanism of intestinal permeability alterations produced by chelation depletion. *J. Cell. Biol.* **32,** 685 (1967).