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Zinc Accumulation and Succinate Dehydrogenase Activation in Hepatic Mitochondria of Rats orally Administered Zinc Sulfate

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The changes of enzyme activities in subcellular fractions of rat liver were investigated after a single oral administration of zinc sulfate (20 mg Zn/100 g body weight). Zinc contents in the fractions of plasma membrane, nuclei, mitochondria, microsomes and cytosol were significantly increased 3 h after zinc administration. On the other hand, a significant decrease of the microsomal glucose-6-phosphatase activity and a remarkable elevation of the mitochondrial succinate dehydrogenase activity were caused by zinc administration, while the activities of the plasma membrane Ca^{2+}—adenosine triphosphatase(ATPase), nuclear ribonucleic acid (RNA) polymerase and cytosolic acid phosphatase were not significantly altered. At the lowest dose (5 mg Zn/100 g) used in this experiment, the microsomal glucose-6-phosphatase activity was not significantly decreased, while the mitochondrial succinate dehydrogenase activity was markedly increased. Also, activation of succinate dehydrogenase in the liver mitochondria was observed at 12 h after zinc administration (10 mg Zn/100 g). The present study indicates that zinc taken up by the liver cells accumulates in the mitochondria and the metal may have an effect on the mitochondrial functions.

Keywords——zinc; succinate dehydrogenase; glucose-6-phosphatase; microsomes; mitochondria; rat liver

Zinc is essential for the proper growth and development of man and many animals.[1] Supplemental zinc in a diet markedly accumulates in the liver of animals.[2,3] Zinc accumulated in the liver causes an increase in metallothionein in the cytosol of liver cells.[4,5] It is uncertain, however, whether zinc taken up by liver binds to the subcellular organelles and affects the cellular metabolic systems.

Recently, we have reported that the liver is a target organ of zinc orally administered to rats.[6] The present study was therefore undertaken to investigate zinc accumulation and its action on the enzymes in the hepatic subcellular fractions of rats orally administered zinc sulfate. We found that zinc accumulated in the mitochondria and markedly raised the succinate dehydrogenase activity in rat liver.

Materials and Methods

Male Wistar rats, weighing approximately 100—120 g, were obtained commercially (Nippon Bio Supp. Center Co., Tokyo). The animals were fed commercial lab. chow and distilled water freely until use. Zinc sulfate was dissolved in distilled water to final concentrations of 5, 10 and 20 mg as Zn^{2+}/ml. These solutions were orally administered to rats fasted for 2 h before the experiments. The animals were bled by cardiac puncture 3, 12 and 24 h after zinc administration. The livers were perfused with cold 0.25 M sucrose solution, cut into small pieces, suspended 1 : 4 in 0.25 M sucrose solution and homogenized in a Potter–Elvehjem homogenizer with a Teflon pestle. The homogenate was spun at $120\times g$ in a refrigerated centrifuge for 10 min and the supernatants were spun at $400\times g$ (ppt; plasma membrane fractions), $1100\times g$ (ppt; nuclei fraction), and $10800\times g$ (ppt; mitochondria fraction) for 10 min. The precipitate from each centrifugation was collected, and the $10800\times g$ supernatant was spun at $105000\times g$ for 60 min. The precipitate (microsome fraction) and the supernatant (cytosol fraction) were separated. The amount of zinc in each fraction was determined by atomic absorption spectrophotometry after digestion with nitric acid.

To assay Ca^{2+} —adenosine triphosphatase (ATPase) activity in the plasma membrane fraction, the reaction mixture contained, in a final volume of 1.5 ml, 30 mM Tris–HCl buffer (pH 7.4), 10 mM $CaCl_2$, 5 mM

ATP and plasma membrane protein. Incubation was carried out at 37 °C for 10 min. The reaction was then stopped by chilling the tubes in an ice-bath and adding 1 ml of 15% (w/v) $HClO_4$. ATP hydrolysis was measured in terms of the release of inorganic phosphate as determined by the method of Nakamura and Mori.[7] Ca^{2+}—ATPase activity was expressed as nmol Pi released per min per mg protein. The protein content was determined according to Lowry *et al.*[8]

Ribonucleic acid (RNA) polymerase activity in the nuclear fraction was measured in reaction mixture (final volume, 0.5 ml) containing 100 mM Tris–HCl buffer (pH 7.5), 4 mM $MnCl_2$, 0.6 mM ATP, guanosine triphosphate (GTP) and cytidine triphosphate (CTP), 5 μM uridine triphosphate (UTP) and 1 μCi [5-^{3}H]UTP, 0.05 ml of a saturated solution of $(NH_4)_2SO_4$ (pH 7.5) and nuclear suspension (100—200 μg deoxyribonucleic acid (DNA)).[9] The reaction mixture was preincubated without [5-^{3}H]UTP at 37°C for 15 min, and it was further incubated by adding [5-^{3}H]UTP and UTP at 37°C for 45 min. The reaction was then stopped by adding cold 0.5 ml of 10% trichloroacetic acid containing 0.04 M sodium pyrophosphate. The reaction mixture was filtered through Whatman glass paper, which was washed with cold 5% trichloroacetic acid containing 0.02 M sodium pyrophosphate. The glass paper was dried and transferred into vials. The radioactivity of the glass paper was measured in a liquid scintillation spectrometer. The enzyme activity was expressed as cpm per min per mg DNA. DNA was determined according to Dische and Schwarz.[10]

Succinate dehydrogenase activity in mitochondria was measured by incubation for 15 min at 37°C in a medium (1.0 ml) that contained 50 mM potassium phosphate buffer (pH 7.4), 0.1% 2-(*p*-iodophenyl)-3-(*p*-nitrophenyl)-5-phenyltetrazolium chloride, 50 mM sodium succinate and 25 mM sucrose.[11] Trichloroacetic acid (10%, 1 ml) was added and the formazun was extracted with 4 ml of ethyl acetate and its extinction was measured at 490 nm. Enzyme activity was expressed as absorbance at 490 nm per min per protein.

Glucose-6-phosphatase activity in the microsome fraction was determined in a system containing 40 mM glucose-6-phosphate, 7 mM histidine, and 1 mM ethylenediamine tetraacetic acid (EDTA), pH 6.5, for 20 min at 37°C.[12] Glucose-6-phosphatase activity was expressed as nmol of an inorganic phosphate released per min per mg protein.

Acid phosphatase activity in the cytosol fraction was determined by a method based on the release of *p*-nitrophenol from *p*-nitrophenylphosphate.[13] Acid *p*-nitrophenylphosphate was assayed in a total volume of 1.2 ml in the presence of 5.5 mM *p*-nitrophenylphosphate and 50 mM citrate buffer, pH 5.0. The reaction was stopped by the addition of 4.0 ml of 0.1 N NaOH. Enzyme acitvity was expressed as nmole *p*-nitrophenol liberated per min per mg protein.

The data were subjected to an analysis of variance, and standard error (SE) was calculated from the residual error term. The significance of differences was evaluated by means of Student's *t*-test.

Results

The changes of zinc contents in the subcellular fractions of the liver after a single oral administration of zinc sulfate (5, 10 and 20 mg Zn/100 g) to rats are shown in Table I. At 3 h after the administration of zinc, the dose of 5 mg Zn/100 g caused a significant increase of

Table I. Changes of Zinc Content in the Subcellular fractions of Rat Liver after a Single Oral Administration of Zinc Sulfate

Subcellular fraction	Zinc content (μg/g wet tissue) Dose of zinc (mg/100 g)			
	Control	5	10	20
120×*g*, ppt. (Cell debris)	6.45±0.35	6.59±0.12	7.51±0.32[a]	8.29±0.24[a]
400×*g*, ppt,. (Plasma membrane)	1.26±0.05	1.59±0.11[a]	1.80±0.19[a]	2.29±0.11[a]
1100×*g*, ppt. (Nuclei)	1.15±0.08	1.43±0.25	1.43±0.07	1.79±0.23[a]
10800×*g*, ppt. (Mitochondria)	1.69±0.10	2.01±0.25	2.26±0.04[a]	2.45±0.06[a]
105000×*g*, ppt. (Microsome)	3.20±0.22	3.65±0.27	4.31±0.43[a]	4.44±0.33[a]
105000×*g*, sup. (Cytosol)	10.1 ±0.37	13.8 ±0.48[a]	14.2 ±0.36[a]	14.8 ±0.86[a]

Zinc was administered orally, and rats bled 3 h later.
Each value represents the mean±S.E. for 5 animals.
a) $p<0.01$ as compared with control.

Table II. Time Course of Zinc Contents in the Subcellular Fractions of Rat Liver after a Single Oral Administration of Zinc Sulfate

Subcellular fraction	Zinc content (μg/g wet tissue)			
	0 h	3 h	12 h	24 h
Cell debris	6.45±0.35	8.29±0.24[a)]	6.80±1.01	6.77±0.20
Plasma membrane	1.26±0.05	2.29±0.11[a)]	1.49±0.26	1.07±0.12
Nuclei	1.15±0.08	1.79±0.23[a)]	1.07±0.09	0.90±0.03
Mitochodria	1.69±0.10	2.45±0.06[a)]	2.36±0.24[a)]	1.47±0.11
Microsome	3.20±0.22	4.44±0.33[a)]	3.68±0.26	2.90±0.02
Cytosol	10.1 ±0.37	14.8 ±0.86[a)]	12.8 ±0.40[a)]	12.4 ±1.64

Zinc (20 mg/100 g) was administered orally, and the rats were killed after various times. Each value represents the mean±S.E. for 5 animals.
a) $p<0.01$ as compared with zero time.

Table III. Changes of Enzyme Activities in the Subcellular Fractions of Rat Liver after a Single Oral Administration of Zinc Sulfate

Subcellular fraction	Enzyme	Control	Zinc
Plasma membrane	Ca^{2+}-ATPase[a)]	39.3± 2.03	42.1± 2.82
Nuclei	Mn^{2+} -$(NH_4)_2SO_4$-Activated RNA polymerase[b)]	278±25.6	257±21.3
Mitochondria	Succinate dehydrogenase[c)]	8.8± 0.31	12.6± 0.84[d)]
Microsome	Glucose-6-phosphatase[a)]	204.8± 4.4	180.3± 2.0[d)]
Cytosol	Acid phosphatase[a)]	27.6± 1.54	29.6± 0.17

Zinc (20 mg/100 g) was administered orally, and the rats were bled 3 h later.
Each value represents the mean±S.E. for 5 animals.
Enzyme activities were expressed as: *a*) nmoles/min/mg protein; *b*) cpm/45 min/mg DNA; *c*) $10^{-2}\times$ absorbance at 490 nm units /min/mg protein.
d) $p<0.01$ as compared with control.

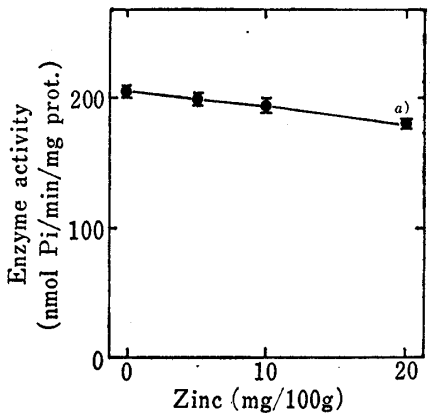

Fig. 1. Change of Glucose-6-phosphatase Activity in the Microsomal Fraction of Rat Liver after a Single Oral Administration of Zinc Sulfate

Zinc was administered orally, and the rats were bled 3 h later. Each point represents the mean of 5 animals. Vertical lines represent the S.E.
a) $p<0.01$ as compared with zero point.

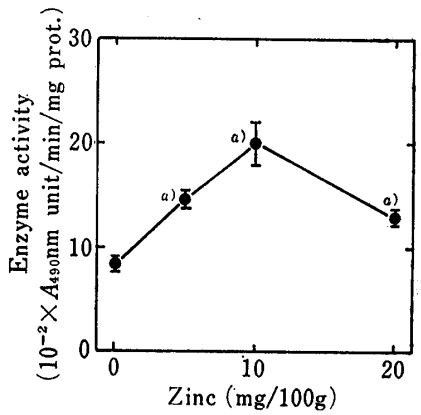

Fig. 2. Change of Succinate Dehydrogenase Activity in the Mitochondrial Fraction of Rat Liver after a Single Oral Administration of Zinc Sulfate

Zinc was administered orally, and the rats were bled 3 h later. Each point represents the mean of 5 animals. Vertical lines represent the S.E.
a) $p<0.01$ as compared with zero point.

zinc content in the plasma membrane and cytosol fractions, while the contents in the fractions of nuclei, mitochondria and microsomes were not significantly higher. At the dose of 20 mg Zn/100 g, the zine contents in all the fractions increased significantly. The greatest zinc content was found in the cytosol.

The time course of zinc accumulation in subcellular fractions after the administration of zinc (20 mg Zn/100 g) is shown in Table II. At 3 h after zinc administration, the zinc contents in all the fractions had increased significantly. At 12 h later, significant elevation of zinc contents in the mitochondria and cytosol fractions was still observed, although the contents in the nuclei and microsome fractions had returned to control levels. In all the fractions at 24 h after the administration, the zinc contents were not significantly different from the control values.

The changes of enzyme activities in the subcellular fractions of the liver after a single oral administration of zinc sulfate to rats are shown in Table III. The rats were killed 3 h after the administration of zinc (20 mg Zn/100 g). The activities of plasma membrane Ca^{2+}—ATPase, nuclear Mn^{2+}–$(NH_4)_2SO_4$–activated RNA polymerase, and cytosolic acid phosphatase were not significantly altered by zinc administration, while a significant decrease of microsomal glucose-6-phosphatase activity and a marked elevation of mitochondrial succinate dehydrogenase activity were observed. Then, the dose–effect relation for zinc administration in terms of the activities of microsomal glucose-6-phosphatase and mitochondrial succinate dehydrogenase was examined 3 h after the administration of zinc (5, 10 and 20 mg Zn/100 g). The microsomal glucose-6-phosphatase activity was not significantly altered by doses of 5 and 10 mg Zn/100 g (Fig. 1). On the other hand, the mitochondrial succinate dehydrogenase activity was linearly increased by doses of up to 10 mg Zn/100 g (Fig. 2). The time course of increase in mitochondrial succinate dehydrogenase activity after the administration of zinc (10 mg Zn/100 g) was examined. Figure 3 shows that, as early as 3 h after zinc administration, there was an increase in succinate dehydrogenase activity in the mitochondria. The enzyme activity continued to increase, reaching a maximum at 12 h, and then began to decrease at 24 h.

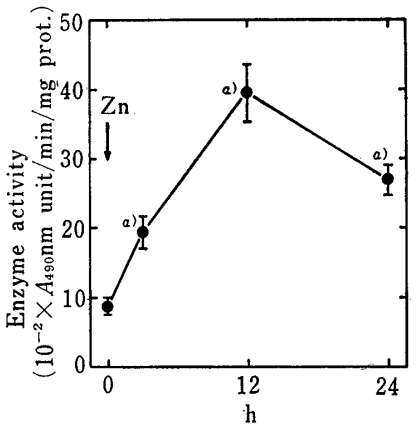

Fig. 3. Time Course of Succinate Dehydrogenase Activity in the Mitochondrial Fraction of Rat Liver after a Single Oral Administration of Zinc Sulfate

Zinc (10 mg/100 g) was administered orally, and the rats were bled after various times. Each point represents the mean of 5 animals. Vertical lines represent the S.E.
a) $p<0.01$ as compared with zero time.

Discussion

Recently we have reported that the liver is a target organ of zinc orally administered to rats.[6] In the present study, the changes of zinc contents in the subcellular fractions of rat liver were examined after a single oral administration of zinc sulfate to rats. The zinc contents in the fractions of plasma membrane, nuclei, mitochondria, microsome and cytosol of liver cells were significantly increased 3 h after the administration of zinc (20 mg/100 g). However, zinc was accumulated in the mitochondria and cytosol for a longer time than in other fractions.

On the other hand, glucose-6-phosphatase activity in the microsome fraction was significantly decreased and succinate dehydrogenase activity in the mitochondria was markedly increased 3 h after the administration of zinc (20 mg/100 g). However, at a lower dose of zinc (10 mg/100 g), the metal had a greater effect on the mitochondrial succinate dehydrogenase

activity than on the microsomal glucose-6-phosphatase activity. Presumably, zinc taken up by the liver cells may affect the mitochondrial function of rat liver.

Previously it was reported that zinc ions affect the biochemical function in mitochondria isolated from liver cells of rats.[14,15] Concentrations lower than 4 μM Zn^{2+} cause a respiratory stimulation in coupled mitochondria isolated from rat liver.[14] Conversely, zinc ions are effective inhibitors of the respiratory chain of mitochondria. The inhibition site by 1—10 μM zinc ions is between cytochromes b and c_1, while ATPase is not inhibited.[15] Thus, the *in vitro* effect of zinc ions on the mitochondrial function in rat liver has not been fully resolved. Also, the *in vivo* effect of zinc on the liver mitochondria is little understood.

Succinate dehydrogenase is located in the inner membrane of mitochondria and participates in the electron transport system. The present findings that succinate dehydrogenase activity in rat liver mitochondria was markedly increased by zinc administration suggest that zinc may influence the mitochondrial function of rat liver. Zinc may stimulate the electron transport and oxidative phosphorylation systems in the mitochondria, but this remains to be elucidated.

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Effect of Modification of Albumin on Tolbutamide–Serum Albumin Binding[1a,b]

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The binding of tolbutamide, a representative sulfonylurea-related drug, to bovine serum albumin (BSA) modified completely or partially with three chemicals (*p*-chloromercuribenzoic acid, *p*-aminophenylmercuric acetate and *p*-hydroxynitrobenzyl bromide) was investigated by means of an equilibrium dialysis method. Scatchard plots of the data indicated that: (1) free sulfhydryl groups in the BSA molecule are not important binding sites for tolbutamide, (2) two tryptophan residues in the BSA molecule form a hydro-