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Effect of Basic Cupric Acetate on Biochemical Changes in the Liver of the Rat fed Carcinogenic Aminoazo Dye. III.[1] Effect of Copper compared with Some Other Metals, Phenobarbital and 3-Methylcholanthrene on the Metabolism of 4-Dimethylaminoazobenzene[2]

YASUHIRO YAMANE and KAZUO SAKAI

Faculty of Pharmaceutical Sciences, Chiba University[3]

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The effect of copper on the metabolism of 4-dimethylaminoazobenzene (DAB) in rat liver was examined, comparing with those of manganese, nickel, and zinc. Copper had the highest effect, followed by manganese, and the effect of nickel was slightly higher than or almost comparable to that of the control group. The effect of zinc gave almost the same or slightly lower activity as the control group.

The effect of copper on the activity of DAB metabolism was observed in the microsomal fraction of the rat liver, and the elevation of activity was found with the increase of copper content in the microsomes. The fact seems to indicate that copper plays some role in elevating the metabolic activity. It was also found that the effect of copper on DAB metabolism concerned the elevations of azo reductase activity and ring hydroxylase activity.

4-Dimethylaminoazobenzene (DAB) is used as a chemical carcinogen in carcinogenesis experiments, chiefly in rat liver, and its carcinogenic mechanism is being elucidated.

In the previous report,[4] we found that azo reduction of DAB was markedly enhanced in the liver of the rat fed dietary copper. In the present paper, the effect of copper salt administered was compared with those of manganese, nickel, and zinc using the whole homogenate of rat liver to examine the specificity of the effect of copper. The effect of copper was compared with those of phenobarbital (PB) and 3-methylcholanthrene (3-MC), the representative inducers of drug metabolizing enzymes. The concentration of copper administered and kinds of copper salt used were examined in the rat liver microsomes.

Experimental

Animals——Female rats (100—120 g) of the Wistar strain were used and they were divided into the following ten groups.

Group 1: Control, given the commercial diet, CE-2 (CLEA Japan, Inc.).
Group 2: Copper group, given 0.5% $CuAc_2 \cdot CuO \cdot 6H_2O$ in CE-2 diet.
Group 3: Manganese group, given 0.5% $MnAc_2 \cdot 4H_2O$ in CE-2 diet.
Group 4: Nickel group, given 0.1% $NiAc_2 \cdot 4H_2O$ in CE-2 diet.
Group 5: Zinc group, given 0.5% $ZnAc_2 \cdot 2H_2O$ in CE-2 diet.
Group 6: Phenobarbital, 0.06%, in CE-2 diet.[5]
Group 7: 3-Methylcholanthrene, 0.0067%, in CE-2 diet.[5]
Group 8: Copper group, given 0.25% $CuAc_2 \cdot CuO \cdot 6H_2O$ in CE-2 diet.
Group 9: Copper group, given 0.1% $CuAc_2 \cdot CuO \cdot 6H_2O$ in CE-2 diet.
Group 10: Copper group, given 0.5% $CuSO_4 \cdot 5H_2O$ in CE-2 diet.

1) Part II: Y. Yamane, K. Sakai, M. Hayashi, M. Matsuzaki, and A. Hanaki, *Chem. Pharm. Bull.* (Tokyo), **18**, 1050 (1970).
2) Presented at the 92th Annual Meeting of the Pharmaceutical Society of Japan at Osaka, Apr. 1972.
3) Location: *1–33 Yayoi, Chiba, 280, Japan.*
4) Y. Yamane, K. Sakai, I. Uchiyama, M. Tabata, N. Taga, and A. Hanaki, *Chem. Pharm. Bull.* (Tokyo), **17**, 2488 (1969).
5) M. Ishidate, M. Watanabe, and S. Odashima, *GANN*, **58**, 267 (1967).

Preparation of Enzyme Sample——The rats were killed by striking a blow on their head and the livers were immediately perfused with 1.15% KCl, excised and prepared as a 10% homogenate, using 1.15% KCl, with ice cooling. The homogenate was centrifuged at 9000 *g* for 20 min at 0°, and its supernatant was centrifuged at 105000 *g* for 60 min at 0°. The pellet thus obtained was taken as the microsomal fraction.

As the source of glucose-6-phosphate (G-6-P) dehydrogenase, the supernatant of 105000 *g* for 60 min centrifugation of the control liver homogenate was used.

Measurement of DAB Metabolic Activity——For the measurement of DAB metabolic activity, the reaction mixture used by Miller, *et al.*[6] was slightly modified. The reaction solution contained 0.3 ml of 0.2 M phosphate buffer (pH 7.4), 0.1 ml of 0.2 M KCl, 0.1 ml of 0.1 M $MgCl_2$, 0.06 ml of 3.4 mM NADP, 0.04 ml of 3.2 mM NAD, 0.1 ml of 1.0 M nicotinamide, 0.2 ml of 0.05 M G-6-P, 0.1 ml of ethanolic solution of DAB (1 mg/ml) and 2 ml of the liver homogenate or 1 ml of microsomal fraction (corresponding to 200 mg of liver) suspended in 1.15% KCl solution and 1 ml of the supernatant. Its total volume was 3.0 ml. This solution was incubated at 37° for 10 min, the reaction was stopped by adding 0.5 ml of acetone, and DAB and its metabolites, in the reaction solution, were exhaustively extracted with benzene. The benzene extracts gathered were evaporated to dryness under a reduced pressure, and the residues were dissolved in petroleum ether. They were applied to the chromatographic separation on an alumina column. The chromatographic separation and calculation of the metabolic activity were described in the previous report.[4]

Measurement of Copper Content——The liver were hydrolyzed with HCl or decomposed with H_2SO_4–HNO_3 and the solution obtained was submitted to colorimetry with diethyldithiocarbamate.[7]

Result

Examination with Whole Homogenate

Effect of Various Metal Salts on DAB Metabolic Activity——In order to examine the effect of various metal salts on DAB metabolism, experimental diets containing manganese, zinc, and nickel, besides copper, were administered for 2 weeks. As shown in Table I, N-demethylation activity was somewhat lower in the group given copper or manganese than the control. Ring hydroxylation activity was higher in the group given copper. In the azo reduction

Table I. DAB Metabolism in the Liver Homogenate from Rats fed Some Metal-containing Diets[a]

Group	DAB metabolizing enzyme activity			Copper content (μg/g liver)
	Azo reduction	N-demethylation	Ring hydroxylation	
Control (CE-2)	28.5±1.83	3.89±0.30	4.28±0.26	4.43
Basic cupric acetate[b]	57.8±0.61[c]	2.49±0.19[d]	4.97±0.15[d]	115.7
Manganese acetate	44.4±2.61[c]	2.88±0.34[d]	3.90±0.15	4.48
Nickel acetate	33.3±1.22	4.27±0.30	4.19±0.43	4.40
Zinc acetate	24.0±1.57	4.99±0.22[d]	3.23±0.16[d]	4.08

a) The experimental diets were continuously given for 2 weeks. Six rats were used for each group and each activity is expressed as the mean±S.E. Each value of copper content is the average of 2 samples. 1 sample consists of the combined tissue of three rats.
b) 0.5% mixture (Group 2)
c) significantly different from control (CE-2) at $p < 0.01$
d) significantly different from control (CE-2) at $p < 0.05$
the rate of azo reduction: decrease of aminoazo dyes in μg/200 mg liver/10 min
the rate of N-demethylation: MAB produced in μg/200 mg liver/10 min
the rate of ring hydroxylation: OH-DAB produced in μg/200 mg liver/10 min

6) J.A. Miller and E.C. Miller, "Advances in Cancer Research," Vol. 1, ed by J.P. Greenstein and A. Haddow, Academic Press, New York, 1953, p. 339; H. Terayama, M. Ishidate, and A. Hanaki, *Nature*, **184**, 1460 (1959); T. Higashinakagawa, M. Matsumoto, and H. Terayama, *Biochem. Biophys. Res. Commun.*, **24**, 811 (1966).
7) G.C. Cartwright, P.J. Jones, and M.M. Wintrobe, *J. Biol. Chem.*, **160**, 593 (1945).

activity, the effect of copper was the most marked, followed by that of manganese and nickel, and the activity in the group given zinc was same or slightly lower than that of the control group.

Copper content in the liver was elevated in the group given copper salt, but not increased in the group given manganese, nickel, or zinc.

Correlation between Increase in Copper Content and Elevation of Azo Reduction Activity ——Correlation between the increase in copper content and elevation of azo reduction activity in the liver was examined, because accumulation of copper in the liver was found in the group given copper diet. Both the copper content and azo reduction activity in Group 2 were measured 1, 2, and 4 weeks after the start of the experiment. The activity was elevated with increased content of copper (Table II).

Comparison of the Administration of PB, 3-MC, and Copper Salt on DAB Metabolic Activity ——The effect by a short-term administration, for 1 week, of dietary copper was examined. At the same time, the effect of PB and 3-MC was comparatively examined under the same conditions. Variations in the activities of azo reduction, N-demethylation, and ring hydroxylation are shown in Table III. When PB was given, the activities increased as a whole, and the elevation of ring hydroxylation activity was the most marked. In the case of 3-MC, both the azo reduction and N-demethylation activities were elevated. In contrast, effect of copper was the elevation of azo reduction and ring hydroxylation. Copper content in the

Table II. Azo Reduction Activity and Copper Content in the Liver Homogenate from Rats fed the Diet containing Basic Cupric Acetate[a]

	Administration period		
	1[b] (week)	2[c] (week)	4[d] (week)
Copper content (μg/g liver)			
Control	3.28±0.26	3.23± 0.26	2.95± 0.81
Cu	66.8 ±5.35	118.3 ±10.61[b]	152.8 ±10.77[d]
Azo reduction activity			
Control	33.38±1.38	31.02± 1.17	32.68± 1.86
Cu	50.28±3.10	63.98± 3.08[c]	75.72± 3.14[d]

a) Eight rats were used for each group and each value was expressed as the mean±S.E. The rate of azo reduction activity is the same in Table I.
e) significantly different from *b*) to *c*) at $p<0.01$
f) significantly different from *b*) to *c*) at $p<0.05$
g) significantly different from *c*) to *d*) at $p<0.05$
control: Group 1 Cu: Group 2

Table III. Comparison of the Administration of Phenobarbital, 3-Methylcholanthrene and Basic Cupric Acetate on DAB Metabolic Activity by the Rat Liver Homogenate[a]

Group	DAB metabolizing enzyme activity		
	Azo reduction	N-Demethylation	Ring hydroxylation
Control (CE-2)	30.1±0.85	3.72±0.13	4.12±0.34
Phenobarbital	61.6±1.45[b]	6.31±0.35[b]	17.68±1.06[b]
3-Methylcholanthrene	65.4±3.96[b]	6.32±0.40[b]	5.32±0.61
Basic cupric acetate[c]	56.8±3.22[b]	2.03±0.19[b]	6.33±0.77[d]

a) The experimental diets were continuously given for 1 week. Four rats were used for each group and each value was expressed as the mean±S.E. Each rate of metabolic reaction is the same in Table I.
b) significantly different from control (CE-2) at $p<0.01$
c) 0.5% mixture (Group 2)
d) significantly different from control (CE-2) at $p<0.05$

Table IV. Copper Content in the Liver of Rats in the Experiment shown in Table III

Group	Copper content (μg/g liver)
Control (CE-2)	4.12±0.33[a]
Phenobarbital	3.97±0.51
3-Methylcholanthrene	4.33±0.33
Basic cupric acetate	68.96±6.72

a) the mean±S.E.

liver of rats in this experiment, as shown in Table IV, was not increased except in the animals given copper salt.

Examination with Microsomal Fraction

Effect of Copper Salt on DAB Metabolic Activity——Effect of copper on DAB metabolism was examined with microsomal fraction. The results showed that the increases of azo reductase activity and ring hydroxylase activity in the liver of rats fed copper was mainly obtained in the microsomal fraction (Table V).

Table V. Intracellular Distribution of Azo Reductase Activity and Ring Hydroxylase Activity[a]

Fraction	Azo reductase relative activity		Ring hydroxylase relative activity	
	Control (Group 1)	Copper (Group 2)	Control (Group 1)	Copper (Group 2)
Soluble fraction (1)	0	0	0	0
(1)+(2)[b]	100[c]	100[d]	100[e]	100[f]
(1)+(3)	5.6	3.5	3.6	2.8
(1)+(4)	4.1	1.0	4.6	1.8
(1)+(5)	4.5	3.0	3.6	1.8

(1): soluble fraction: supernatant at 105000 *g* for 60 min
(2): precipitate at 105000 *g* for 60 min with supernatant of (3)
(3): precipitate at 9000 *g* for 20 min with supernatant of (4)
(4): precipitate at 5000 *g* for 10 min with supernatant of (5)
(5): precipitate at 600 *g* for 10 min with whole homogenate

a) Four rats were used for each group and the experimental diet was continuously given for 4 weeks.
b) The various fractions from 200 mg liver were used for the measurement of activity and each value expressed as the mean±S.E. of (1)+(2) was as follows: *c*)=13.09±0.19. *d*)=28.81±0.62. *e*)=1.81±0.27. *f*)=3.53±0.36. The activity was determined as described in Experimental and the rate is the same as described in Table I.

The supernatant fraction at 105000 *g* for 60 min had no azo reductase and ring hydroxylase activities as shown in Table V. However, it was considered that the supernatant fraction from the copper diet group might effect the activities in microsomes, and the effect of the supernatant fraction from the control and copper diet groups was examined (Table VI). There was no difference between the two in the effect on the DAB metabolic activity. Therefore, as a source of G-6-P dehydrogenase, the supernatant from centrifugation at 105000 *g* for 60 min of the liver from untreated control rats was used.

Effect of Copper Salt in Elevating DAB Metabolic Activity, and Comparison with PB and 3-MC——DAB metabolizing activity was comparatively examined in the liver microsomal fractions of rats given copper, PB or 3-MC. As shown in Table VII and Table VIII, marked elevation in ring hydroxylase activity by administration of PB, marked elevation of N-demethylase activity by that of 3-MC, and marked elevation of azo reductase activity by the

Table VI. Effect of the Supernatant[a] on the Liver Microsome of the Rat fed the Diet containing Copper[b]

Group		DAB metabolism		
Sup.	Mic.	Azo reduction	N-Demethylation	Ring hydroxylation
Cont.	Cont.	9.88±1.00	2.28±0.06	1.54±0.43
Cu	Cont.	8.77±1.50	2.48±0.33	1.61±0.16
Cont.	Cu	21.34±2.66	2.83±0.21	3.28±0.33
Cu	Cu	19.05±1.57	3.26±0.34	3.14±0.32

Sup.=supernatant Mic.=microsome Cont.=control (Group 1)
Cu=0.5% basic cupric acetate (Group 2)
a) The supernatant centrifuged at 105000 *g* for 60 min.
b) The experimental diet was continuously given for 2 weeks. Four rats were used for each group and each value is expressed as the mean±S.E. Each rate of metabolic reaction is the same as shown in Table I.

Table VII. DAB Metabolism in the Liver Microsome from Rats fed Phenobarbital, 3-Methylcholanthrene and Basic Cupric Acetate[a]

Group	DAB metabolizing enzyme activity		
	Azo reduction	N-Demethylation	Ring hydroxylation
Control (CE-2)	9.32±0.69	2.38±0.15	1.57±0.19
Phenobarbital	15.41±0.91[b]	9.17±0.16[b]	13.51±0.40[b]
3-Methylcholanthrene	12.05±0.30[c]	7.66±0.35[b]	1.91±0.04
Basic cupric acetate[d]	20.19±1.42[b]	3.05±0.20	3.21±0.19[b]

a) The experimental diets were continuously given for 2 weeks. Four rats were used for each group and each value is expressed as the mean±S.E. Each rate of metabolic reaction is the same in Table I.
b) significantly different from control (CE-2) at $p<0.01$
c) significantly different from control (CE-2) at $p<0.02$
d) 0.5% mixture (Group 2)

Table VIII. DAB Metabolism and Copper Content in the Liver Microsome from Rats fed Phenobarbital, 3-Methylcholanthrene and Basic Cupric Acetate[a]

Group	DAB metabolizing enzyme activity			Copper content (μg/g liver)
	Azo reduction	N-Demethylation	Ring hydroxylation	
Control (CE-2)	12.22±1.69	2.55±0.20	2.49±0.22	0.60±0.02
Phenobarbital	18.08±1.37[b]	8.16±0.27[c]	17.58±1.58[c]	0.89±0.11[b]
3-Methylcholanthrene	26.82±3.64[b]	12.79±1.65[c]	5.77±1.13[b]	0.73±0.06[d]
Basic cupric acetate[e]	35.64±2.52[c]	3.38±0.23[b]	6.26±0.59[c]	1.55±0.21[c]

a) The experimental diets were continuously given for 4 weeks. Four rats were used for each group and each value is expressed as the mean±S.E. Each rate of metabolic reaction is the same as shown in Table I.
b) significantly different from control (CE-2) at $p<0.05$
c) significantly different from control (CE-2) at $p<0.01$
d) significantly different from control (CE-2) at $p<0.1$
e) 0.5% mixture (Group 2).

administration of copper diet were mainly found. In the copper group, the increase of copper content in the microsomes was in parallel with the elevation in azo reductase activity (Table VIII).

Effect of Concentration and Kind of Copper Salts——Effect of copper administration, 0.5, 0.25, or 0.1% of basic cupric acetate, or 0.5% cupric sulfate, was examined. As shown in Table IX, significant elevation ($p<0.01$) of azo reductase and ring hydroxylase activities

Table IX. DAB Metabolism in the Liver Microsome from Rats fed the Diet containing Different Concentration of Copper[a]

Group	DAB metabolizing enzyme activity			Copper content
	Azo reduction	N-Demethylation	Ring hydroxylation	(μg/g liver)
Control (CE-2)	17.88±1.17	2.60±0.23	2.65±0.25	0.54±0.10
0.5% Basic cupric acetate	34.57±0.95[b]	2.30±0.09	4.42±0.27[b]	1.12±0.12[c]
0.25% Basic cupric acetate	18.38±3.15	2.74±0.21	2.50±0.38	0.65±0.06
0.1% Basic cupric acetate	17.36±0.67	2.78±0.15	2.35±0.20	0.60±0.03
0.5% Cupric sulfate	26.33±2.37[b]	2.60±0.26	3.55±0.31[b]	0.92±0.10[c]

a) The experimental diets were continuously given for 4 weeks. Four rats were used for each group and each value is expressed as the mean±S.E. Each rate of metabolic reaction is the same as described in Table I.
b) significantly different from control (CE-2) at $p<0.01$
c) significantly different from control (CE-2) at $p<0.05$

was seen in the group fed 0.5% basic cupric acetate, and increase in copper content of the microsomes was also significant ($p<0.05$). The metabolic activities and copper content in microsomal fraction from the liver of rats fed 0.25% and 0.1% basic cupric acetate were not significantly elevated as compared with the control. Significant elevation ($p<0.01$) of azo reductase and ring hydroxylase activities was seen in the group fed 0.5% cupric sulfate, with significant increase ($p<0.05$) in microsomal copper content.

Discussion

It is interesting that the difference in the elevation of azo reduction activity by different metal salts became very apparent. The effect of copper was the most marked, followed by that of manganese and nickel, and the effect of zinc was not different from the control. The concentration of nickel salt administered was made 0.1% because of its high toxicity.

Hernandez and others[8] reported that azo reductase is located in the microsome of rat liver. We observed that azo reduction activity was markedly elevated and ring hydroxylation activity was significantly elevated in the liver microsomal fraction of rats fed copper. These characteristic effect by copper appears to be different from that in the case of PB or 3-MC.

It was found in the microsomal preparation that, by administration of 0.5% basic cupric acetate, the azo reductase and ring hydroxylase activities were elevated in the same degree, 2—3 times, as compared with the control (Group 1).

Close correlation between the elevations in azo reductase and in microsomal copper content suggests that copper in microsomes plays a role in elevating the activity.

The copper content in the microsomal fraction was significantly higher than that of the control as shown in Table VIII, though copper content in the whole homogenate of the liver after administration of PB or 3-MC was not different from that of the control as shown in Table IV. Copper content in the liver of rats given other metal salts was not different from that of the control using the whole homogenate, as shown in Table I.

It has been reported that a long-term administration of copper increases copper content in the liver.[9] In 4-week administration of 0.25 or 0.1% basic cupric acetate, no statistically

8) P.H. Hernandez, J.R. Gillette, and P. Mazel, *Biochem. Pharmacol.*, **16**, 1859 (1967).
9) B.D. Milne and P.H. Weswig, *J. Nutr.*, **95**, 429 (1968); E.J. Underwood, "Trace Elements in Human and Animal Nutrition," 3rd ed., Academic Press, New York, 1970, p. 57.

significant increase in copper content as well as in metabolic activity was observed. However, we found that copper content and DAB metabolic activity in the liver microsome from rats given a 0.25% basic cupric acetate for 3 months increased.[10]

It is clear that the enhantement of DAB metabolism by copper is mainly ascribed to that of azo reduction, the main metabolic route of DAB, and the detoxication of DAB is accelerated thereby.

Consequently, we consider[11] that the marked elevation of azo reductase activity in rat liver by concurrent administration of aminoazo dye and copper salt is a main factor for suppressing[12] aminoazo dye carcinogenesis.

10) Y. Yamane and K. Sakai, *Chem. Pharm. Bull.* (Tokyo), "In preparation."
11) Y. Yamane and K. Sakai, *GANN*, **64**, 563 (1973).
12) J.S. Howell, *Brit. J. Cancer*, **12**, 594 (1958); G. Fare, and D.L. Woodhouse, *Brit. J. Cancer*, **17**, 512 (1963); G. Fare, and D.L. Woodhous, *Brit. J. Cancer*, **17**, 775 (1963).