[Chem. Pharm. Bull. 23(7)1440—1445(1975)]

UDC 615.276.076.9

Studies on the Induction of DAB Metabolizing Enzyme Activity and Other Microsomal Enzyme Activity in the Liver of Rats fed Copper[1]

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(Received October 22, 1974)

The mode of elevating action of 4-dimethylaminoazobenzene (DAB) metabolizing enzyme activity in the liver of rats fed copper was studied. The increases of the enzyme activity and flavin content were found with that of copper content in the liver homogenates and microsomes of rats fed copper. But addition *in vitro* of cupric ion and/or fravin adenine dinucleotide (FAD) in the reaction mixture involving microsomal preparations did not cause a marked enhancement of the enzyme activity. This fact is thought to indicate the both increases of flavin and apoenzyme.

The concurrent administration of copper and 3′-methyl-4-dimethylaminoazobenzene (3′-Me-DAB) showed additive effect in reduced nicotinamide adenine dinucleotide phosphate (NADPH) cytochrome c reductase activity and competitive effect in DAB azo reductase activity.

It is well known that 4-dimethylaminoazobenzene (DAB) is a very strong chemical hepatocarcinogen in rats. In the liver of rats fed dietary copper the reduced incidence of DAB carcinogenesis has been found.[3] But the suppressive effect of copper on DAB carcinogenesis was not elucidated. In a series of our works[4–7] the mechanism of its inhibitory effect was studied and the enhancement of DAB metabolizing enzyme activities, especially of azo reduction which is an important pathway of DAB metabolism as a detoxicating system, was found in the liver of rats fed copper. These increased enzyme activities have been shown to localize mainly in the microsomal fraction. We have declared in our reports that suppressive effect of copper on DAB carcinogenesis is ascribed to the increased detoxication of rat liver.

In this paper the mode of elevating action of DAB metabolizing enzyme activity was studied in the liver of rats fed copper and at the same time the effect on other microsomal enzyme activity was investigated.

Experimental

Animal——Four-week-old female rats of Wistar strain, obtained from Nippon Rat Co., Ltd., were fed on the commercial diet, CE-2 (CLEA Japan, Inc.) until they were 8 weeks old, and then rats weighing about 120 g were used for the experiments. Control group rats were given the same diet. For the experiments added cupric ion or flavin adenine dinucleotide (FAD) into the reaction mixture *in vitro*, control group rats were used. The experimental diets were prepared by mixing CE-2 with copper and/or 3′-methyl-4-dimethylaminoazobenzene (3′-Me-DAB), and were given to each group. The administration of these experimental

1) Presented in part at the 94th Annual Meeting of the Pharmaceutical Society of Japan, Sendai, Apr., 1974.
2) Location: *1–33 Yayoi, Chiba, 280, Japan.*
3) J.S. Howell, *Brit. J. Cancer*, **12**, 594 (1958); G. Fare and D.L. Woodhouse, *Brit. J. Cancer*, **17**, 512 (1963).
4) Y. Yamane, K. Sakai, I. Uchiyama, M. Tabata, N. Taga, and A. Hanaki, *Chem. Pharm. Bull.* (Tokyo), **17**, 2488 (1969).
5) Y. Yamane, K. Sakai, M. Hayashi, M. Matsuzaki, and A. Hanaki, *Chem. Pharm. Bull.* (Tokyo), **18**, 1050 (1970).
6) Y. Yamane and K. Sakai, *Gann*, **64**, 563 (1973).
7) Y. Yamane and K. Sakai, *Chem. Pharm. Bull.* (Tokyo), **22**, 1126 (1974).

diets to rats did not largely change their body weight and liver weight under the feeding conditions used. The amount of these compounds added into CE-2 and the period of administration are shown in Tables.

Preparation of Rat Liver Microsomes——The animals were killed by striking a blow at them and their livers were perfused with 1.15% KCl and removed immediately. The livers were blotted, weighed, minced and homogenized with 9 vol. 1.15% KCl. The liver homogenate was centrifuged at 9000 ***g*** for 20 min at 0°. The microsomal fraction was prepared by centrifuging the 9000 ***g*** supernatant at 105000 ***g*** for 60 min. The 105000 ***g*** supernatant of control liver was used as the source of glucose-6-phosphate (G-6-P) dehydrogenase.

Measurement of DAB Metabolizing Enzyme Activity——Measurement of DAB metabolizing enzyme activities was described in our previous reports.[4-7] The reaction mixture used by Miller, *et al.*[8] was used to be modified slightly. For investigating the effect of cupric ion, FAD, or β-diethylaminoethyldiphenylpropylacetate · HCl (SKF-525A) *in vitro*, the compound dissolved with cofactors in distilled water was added in the reaction mixture. Incubation times were 10 min, during which times reaction rate was linear.

The rate of each metabolic reaction was expressed as follows: N-Demethylation; 4-methylaminoazobenzene produced in μg/200 mg liver/10 min. Ring hydroxylation; 4'-hydroxy-4-dimethylaminoazobenzene produced in μg/200 mg liver/10 min. Azo Reduction; Decrease of Aminoazo Dyes[4] in μg/200 mg liver/10 min.

Measurement of Reduced Nicotinamide Adenine Dinucleotide Phosphate(NADPH) Cytochrome c Reductase Activity——NADPH cytochrome c reductase was measured by the method of Williams and Kamin.[9] The reaction mixture contained 300 mμmoles of NADPH, 102 mμmoles of cytochrome c, and enzyme in 3.3 ml of potassium phosphate, pH 7.7, including 1×10^{-3} 1M cyanide. The reaction was started by the addition of the NADPH and the rate of change in absorption at 550 nm was followed for 3 to 4 minutes while the reaction rate was linear.

Copper Determination of Rat Liver——For the determination of copper content, colorimetric method with diethyldithiocarbamate was performed after preliminary acid decomposition of liver materials, as shown in the previous reports.[6-7]

Determination of Flavin——The total amount of flavins was measured by lumiflavin fluorescence method of Yagi.[10] The microdetermination procedure is reliable to determine flavins in rat liver materials. Warm-water extract of the tissue mixed with equal volume of 1N NaOH was irradiated for decomposition, added 0.2 ml of acetic acid, extracted once with 6.0 ml of pure chloroform and intensity of lumiflavin fluorescence produced from riboflavin of chloroform layer was estimated. At the same time, the additional test of riboflavin was made by the same way, and amount of flavin was calculated.

Measurement of P-450 Content——The content of P-450 was measured by the method of Omura and Sato.[11]

Determination of Protein——Protein was determined by the method of Lowry, *et al.*[12] Crystalline bovine serum was used as the protein standard.

Result

Effect of Cupric Ion Addition *in Vitro* on DAB Azo Reductase Activity

It was observed in our previous reports that the rate of DAB metabolizing enzyme activities was markedly stimulated in the liver of rats fed copper. These increases of enzyme activities were found mainly in the microsomal fraction. Then the experiment added cupric ion *in vitro* in the reaction mixture involving microsomes from control rats was performed. As can be seen in Table I, cupric ion did not stimulate the metabolism *in vitro* by liver microsomal preparations at any concentration studied. At the higher concentration of cupric ion the enzyme activity was decreased.

Effect of FAD Addition *in Vitro* on DAB Azo Reductase Activity

It has been reported that azo reductase is a flavoprotein containing FAD as a prosthetic group. An examination was carried out under our experimental conditions whether a marked enhancement of the enzyme activity was caused by adding FAD *in vitro* to microsomes from

8) J.A. Miller and E.C. Miller, "Advances in Cancer Research," Vol. 1, ed. by J.P. Greenstein and A. Haddow, Academic Press, N.Y., 1953, p. 339.
9) C.H. Willams and H. Kamin, *J. Biol. Chem.*, **237**, 587 (1962).
10) K. Yagi, *J. Biochem.* (Tokyo), **38**, 161 (1951).
11) T. Omura and R. Sato, *J. Biol. Chem.*, **239**, 2370 (1964).
12) O.H. Lowry, N.J. Rosebrough, A.L. Farr, and R.J. Randall, *J. Biol. Chem.*, **193**, 265 (1951).

Table I. Effect of Free Cupric Ion Addition *in Vitro* on DAB Azo Reductase Activity in Rat Liver Microsome

Cu added (μM)	Azo reductase activity (μg/200 mg liver/10 min)	Cu added (μM)	Azo reductase activity (μg/200 mg liver/10 min)
0	12.14±0.15[a]	25	11.28±0.32
1	11.68±0.88	50	8.22±0.45
5	11.86±0.77	100	4.34±0.17
10	11.53±0.29		

Four rats were used and the liver microsomes were prepared from each rat. Cupric sulfate solution was added in the reaction mixture. The azo reductase activity was determined as described in Experimental.
a) mean ±S.E.

control rat liver. As shown in Table II, only a slight increase was found at comparative low concentration studied. At higher concentration the enzyme activity was inhibited.

Table II. Effect of FAD[a] on the Metabolism *in Vitro* of DAB by Rat Liver Microsome

FAD added (M)	Azo reductase activity (μg/200 mg liver/10 min)
0	14.80 ± 1.25[b]
10^{-6}	17.00 ± 0.13[c]
10^{-5}	17.06 ± 0.22[c]
10^{-4}	14.80 ± 2.87
10^{-3}	7.17 ± 2.10[d]

Each value is the mean from four samples. The azo reductase activity was determined as described in Experimental.
a) FAD: flavin adenine dinucleotide
b) S.E.
c) significantly different from the sample without addition of FAD at $p < 0.10$
d) significantly different from the sample without addition of FAD at $p < 0.05$

Correlation between Increase of Copper Content and That of Flavin Content in the Liver of Rats fed Copper

Although addition of FAD to microsomes from control rat liver did not show the marked increase of enzyme activity under our experimental conditions, it was investigated whether flavin content in the liver of rats fed copper was increased or not. As shown in Table III at first, the increase of flavin content was found with that of copper content in the whole homogenate of rats fed copper.

Table III. Content of Copper and Riboflavin in the Liver of Rats fed Dietary Copper

Group	Content in whole homogenate	
	Copper (μg/g liver)	Riboflavin (μg/g liver)
control (CE-2)	3.95 ± 0.23	36.95 ± 2.35
0.5% basic cupric acetate	106.3 ± 16.84[a]	54.38 ± 4.36[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.
a) significantly different from control (CE-2) at $p < 0.01$
b) significantly different from control (CE-2) at $p < 0.05$

As can be seen in Table IV, the increase of flavin was also found with that of copper in the liver microsome of rats fed copper and, at the same time, DAB metabolizing enzyme activities without N-demethylase activity were striking enhanced.

Table IV. Amount of Copper and Riboflavin and Activity of DAB Metabolizing Enzyme in the Liver Microsome of the Rat fed the Diet containing Copper

Group	Copper (μg/g liver)	Riboflavin (μg/g liver)	DAB metabolizing enzyme activity (μg/200 mg liver/10 min)		
			Azo-reduction	N-Demethylation	Ring hydroxylation
Control	0.89 ± 0.16	2.32 ± 0.28	24.8 ± 0.52	3.32 ± 0.05	3.33 ± 0.09
Cu	2.00 ± 0.10[a)]	4.35 ± 0.58[a)]	58.6 ± 6.07[b)]	2.67 ± 0.43	6.90 ± 0.84[a)]

The experimental diet was continuously given for 4 weeks. Four rats were used for each group and each value is expressed as the mean ± S. E. Each activity was determined as described in Experimental.
a) significantly different from control (CE-2) at $p < 0.05$
b) significantly different from control (CE-2) at $p < 0.01$
control: CE-2 Cu: 0.5% basic cupric acetate

Effect of SKF-525A Addition *in Vitro* on DAB Metabolizing Enzyme Activities in the Liver of Rats fed Copper

The effect of SKF-525A on DAB metabolizing enzyme activities in the liver microsome of rats fed copper was compared with that of control. As can be seen in Table V, the addition of SKF-525A caused a striking decrease in ring hydroxylase activity, but no significant decrease was seen in N-demethylase activity and azo reductase activity under our experimental conditions. Excellent paralleism was seen in the effects of SKF-525A on DAB metabolizing enzyme activities in liver microsomes of both normal and copper treated rats.

Table V. Effect of SKF-525A Addition *in Vitro* on DAB Metabolism by the Liver Microsome of Rats fed Dietary Copper

Group	SKF-525A (M)	DAB metabolizing enzyme activity (μg/200 mg liver/10 min)		
		Ring hydroxylation	N-Demethylation	Azo reduction
Control	0	2.64 ± 0.27	2.63 ± 0.23	12.2 ± 0.61
	2×10^{-4}	0.80 ± 0.14	2.57 ± 0.14	11.5 ± 0.52
Cu	0	4.09 ± 0.29	3.69 ± 0.14	26.2 ± 1.51
	2×10^{-4}	1.17 ± 0.08	2.99 ± 0.12	25.3 ± 2.11

Each value is the mean from four samples. Each metabolizing enzyme activity was determined as described in Experimental.
control: CE-2 Cu: 0.5% basic cupric acetate

Comparison of NADPH Cytochrome c Reductase Activity and DAB Azo Reductase Activity in the Liver Microsome of Rats Fed Copper and/or 3′-Me-DAB

It was reported by Hernandez, *et al.*[13] that azo reductase and NADPH cytochrome c reductase activities showed similar inhibition by sulfhydryl group inhibitors and similar heat stability, but in intact microsomes the reductase activity might not be entirely attributed to NADPH cytochrome c reductase. In this experiment the activities of both enzymes were compared in the liver microsome of rats fed copper and/or 3′-Me-DAB. As shown in Table VI, treatment of rats with copper caused increases in NADPH cytochrome c reductase activity and DAB azo reductase activity, but in the treatment with 3′-Me-DAB the former activity increased while the latter activity decreased. The concurrent administration of copper and 3′-Me-DAB showed additive effect in NADPH cytochrome c reductase activity and competitive effect in DAB azo reductase activity. Apparent differences in the two inducing

13) P.H. Hernandez, J.R. Gillette, and P. Mazel, *Biochem. Pharmacol.*, **16**, 1859 (1967).

Table VI. Activities of NADPH-Cytochrome c Reductase and Azo Reductase in the Liver Microsome of the Rat fed 3′-Me-DAB and/or Copper

Group	NADPH-cytochrome c reductase activity (unit/50 mg liver/min)	Azo reductase activity (μg/200 mg liver/10 min)
Control (CE-2)	0.062 ± 0.003	11.27 ± 1.00
0.5% basic cupric acetate	0.113 ± 0.013[a]	26.96 ± 2.69[a]
0.06% 3′-Me-DAB	0.104 ± 0.012[a]	5.57 ± 0.07[b]
Cu+3′-Me-DAB	0.131 ± 0.017[a]	12.13 ± 0.84

The experimental diet was continuously given for 4 weeks. Four rats were used for each group and each value is expressed as the mean ±S.E. Each activity was determined as described in Experimental. One unit of NADPH-cytochrome c reductase activity is defined as that amount of enzyme which causes an absorbancy change of 1.0 per minute with a 1 cm light path.
a) significantly different from control at $p < 0.01$
b) significantly different from control at $p < 0.05$

agents were recognized when NADPH cytochrome c reductase activity and DAB azo reductase were compared.

Effect of Copper Administration on Cytochrome P-450 Content of Rat Liver Microsomes

Cytochrome P-450 content was measured in the liver microsome of rats fed copper and at the same time measurement of DAB metabolizing enzyme activities was also performed. As can be seen in Table VII, treatment of rats with copper caused parallel increases in cytochrome P-450 content and DAB ring hydroxylase activity. The activity of azo reductase increased with the increase of cytochrome P-450 content but such apparent parallelism was not found.

Table VII. Level of P-450 and Activity of DAB Metabolizing Enzyme in the Liver Microsome of the Rat fed the Diet containing Copper

Group	P-450 (nmol/mg protein)	DAB metabolizing enzyme activity (μg/200 mg liver/10min)		
		Ring hydroxylation	N-Demethylation	Azo reduction
Control	0.77 ± 0.018	2.87 ± 0.29	1.97 ± 0.11	21.3 ± 1.09
2W Cu	1.02 ± 0.093[a]	3.81 ± 0.22[a]	2.29 ± 0.09	43.7 ± 4.66[a]
4W Cu	1.24 ± 0.081[a]	4.85 ± 0.48[a]	1.84 ± 0.25	51.6 ± 4.91[b]

Four rats were used for each group and each value is expressed as the mean ±S.E. Each metabolizing enzyme activity was determined as described in Experimental.
a) significantly different from control at $p < 0.05$
b) significantly different from control at $p < 0.01$
control : CE-2
Cu : 0.5% basic cupric acetate
2W Cu : The experimental diet was continuously given for 2 weeks.
4W Cu : The experimental diet was continuously given for 4 weeks.

Discussion

A variety of factors affecting carcinogenesis by DAB have been investigated by many workers and shown to involve the drug metabolizing enzyme inducers such as phenobarbital[14]

14) M. Ishidate, M. Watanabe, and S. Odashima, *Gann*, **58**, 267 (1967); L.G. Hart, R.H. Adamson, D.L. Dixon, and J.R. Fouts, *J. Pharmacol. Exptl. Therap.*, **137**, 103 (1962); R. Kato and J.R. Gillette, *Federation Proc.*, **23**, 538 (1964); R. Kato, *Japan J. Pharmacol.*, **17**, 181 (1967).

and 3-methylcholanthrene,[15] or nutritional compounds such as proteins and vitamins.[16] DAB metabolizing enzyme activities were found to be enhanced in the liver of rats fed copper as shown in our previous papers. Authors suggested that these activities made more specific when the copper is incorporated in a protein to form a copper enzyme. However, addition of cupric ion to rat liver microsomes did not increase the activities. In this respect Peters and Fouts[17] also reported that cupric ion did not stimulate the metabolism *in vitro* of benzphetamine and aniline by liver microsomal preparations. The observation of authors that DAB metabolizing enzyme activities were enhanced in the liver of rats fed copper cannot be ascribed to the sole increase of copper content in the liver microsomes of rats.

It was clear from our unpublished data that a marked enhancement of DAB metabolizing enzyme activity was not found by the addition of flavin mononucleotide (FMN), riboflavin, or copper and flavin to the reaction mixture. The DAB metabolizing enzyme activity was slightly enhanced by adding FAD into the reaction mixture *in vitro* under our experimental conditions as shown in Table II, and it is not thought that the marked enhancement of DAB metabolizing enzyme activity is attributed to the only increase of flavin content in the liver microsomes of rats. Accordingly, the findings that the increases of the enzyme activity and flavin content were shown with that of copper content in the liver homogenates and microsomes of rats fed copper are considered as indicating the both increases of flavin and apoenzyme.

The similar inhibitory rate in the liver microsomes from both no treated control and copper treated rats was seen in the effect of SKF-525A on ring hydroxylase activity in DAB metabolism. This fact suggests that the mechanism of the enzyme enhanced in the liver of rats fed copper may be similar to that of normal rats.

It is interesting that the increase in DAB azo reductase activity roughly parallels the increase in NADPH cytochrome c reductase activity by the administration of copper but reverse effect on the respective enzymes is seen by 3′-Me-DAB. Authors are convinced that the suppressive effect of copper on liver carcinogenesis by 3′-Me-DAB is largely attributed to the competitive effect in DAB azo reductase activity by the concurrent administration of copper and 3′-Me-DAB.

The increase of cytochrome P-450 content by copper administration may be stimulated with the increase of DAB metabolizing enzymes.

The role of copper in the mode of elevating action of DAB metabolizing enzyme activities is not adequately clear in this paper. Moffitt, *et al.*[18] have recently reported that some drug metabolizing hydroxylase activities were increased with the increase of some metals involving copper in the liver microsomes of rats by the administration of phenobarbital or benzpyrene. Accordingly, it should be in detail investigated whether copper might play a role in the mechanism of drug metabolizing enzyme induction by inducers such as phenobarbital and 3-methylcholanthrene.

Acknowledgement We are grateful to Prof. H. Kitagawa and Dr. T. Kamataki of our faculty for a generous gift of SKF-525A and measurement of P-450 content. We are also grateful to J. Ito, J. Ito, M. Shibata, and T. Toriuchi for excellent technical assistance.

15) E.C. Miller, J.A. Miller, and R.R. Brown, *Cancer Res.*, **12**, 282 (1952); A.H. Conney, E.C. Miller, and J.A. Miller, *Cancer Res.*. **16**, 450 (1956); J.W. Cramer, J.A. Miller, and E.C. Miller, *J. Biol. Chem.*, **235**, 250 (1960).
16) C.J. Kensler, K. Sugiura, N.F. Young, C.R. Halter, and C.P. Rhoads, *Science*, **93**, 308 (1941); D.L. Miner, J.A. Miller, C.A. Baumann, and H.P. Rusch, *Cancer Res.*, **3**, 269 (1943).
17) M.A. Peters, and J.R. Fouts, *Biochem. Pharmacol.*, **19**, 533 (1970).
18) A.E. Moffitt, Jr., J.R. Dixon, F.C. Phipps, H.E. Stokinger, *Cancer Res.*, **32**, 1148 (1972).