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Effect of Basic Cupric Acetate on the Biochemical Changes in the Liver of the Rat fed Carcinogenic Aminoazo Dye. I. Changes in the Activities of DAB Metabolism by Liver Homogenate

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The effect of basic cupric acetate on the activities of the drug metabolizing enzymes was studied. When basic cupric acetate was administered with and without DAB to the rat, the overall activity of the DAB metabolizing enzyme system was increased remarkably, which was mainly ascribed to the enhancement of the azo-reduction. The activity of azo-reduction was reduced a little by the administration of DAB alone. The activity of N-demethylation was reduced a little by the administration of copper, but by the administration of DAB alone the activity appeared to increase a little. The administration of copper and DAB did not affect the activity of aromatic hydroxylation. One role of copper which retards the hepatic carcinogenesis by DAB might be related to the enhancement of the activity of azo-reduction, which detoxicates the carcinogenic dye to the noncarcinogenic compounds.

It was first demonstrated by Howell that the administration of basic cupric acetate gave a good degree of protection against the hepatic carcinogenesis in the rat by 4-dimethylaminoazobenzene (DAB).[2] This observation has since been confirmed. Subsequent works indicated the effect of copper on certain biochemical changes, such as the contents of RNA, DNA and protein and the activity of succinoxidase, associated with giving DAB.[3] The administration of copper also affects the distribution of copper in the tissues[4] and the contents of bound-dye and bound-copper in the liver of the rat fed DAB.[5]

The bound-dye formation, which might be connected closely with the carcinogenic process and with the metabolic pathway of the dye,[6] was shown to be delayed and reduced by the administration of basic cupric acetate. However, the effect of copper upon the metabolism of DAB administered or upon the drug metabolizing enzyme activities has not hitherto been investigated. The present works attempted to study the effect of basic cupric acetate upon the activities of DAB metabolism, oxidative N-demethylation, azo-reduction and aromatic hydroxylation, during the course of the carcinogenesis by DAB.

1) Location: *a*) *Yayoi, Chiba*; *b*) *Anagawa, Chiba.*
2) J.S. Howell, *Brit. J. Cancer*, **12**, 594 (1958).
3) G. Fare and D.L. Woodhouse, *Brit. J. Cancer*, **17**, 512 (1963).
4) G. Fare and D.L. Woodhouse, *Brit. J. Cancer*, **17**, 775 (1963).
5) G. Fare, *Biochem. J.*, **91**, 473 (1964).
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, N.Y., 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); A. Hanaki, *Chem. Pharm. Bull.* (Tokyo), **15**, 907 (1967).

Experimental

Animal——Four groups of female Wistar rats weighing between 100 and 150 g at the start of the experiment were used. Each group was consisted of 40 rats. One group of animals received 0.09% DAB (1), the second 0.5% basic cupric acetate hexahydrate (2) and the third 0.09% DAB and 0.5% basic cupric acetate hexahydrate (3) in maize diet. The fourth group of animals received maize diet alone (4) and served as a control. Those experimental diets were given *ad libitum* five days a week. In order to provide the necessary vitamins and other growth factors, laboratory chow (CE-2, Nippon Clea Ltd.) was given on Tuesdays and Wednesdays.

Preparation of the Liver Homogenates——In each experimental group, the animals were killed by decapitation at times between 1 and 21 weeks after the start of giving the diet. The livers were immediately perfused with chilled 1.15% KCl, rapidly excised, weighed, and homogenized in 1.15% KCl with a Potter homogenizer having a teflon pestle in crushed ice. Ten per cent liver homogenate thus prepared were used for assay of the activities of DAB metabolism. The contents of aminoazo dyes, DAB, MAB and related compounds, in the liver homogenates were negligibly small in all the groups of rat examined.

Content of Total Copper——Copper in livers was assayed spectrophotometrically with sodium diethyldithiocarbamate.[7] 2 ml of the whole homogenate was mixed with 2 ml of 1N HCl and incubated overnight at 37°. The incubation mixtures, after adding 1 ml of 30% trichloroacetic acid, were centrifuged at 3000 rpm for 20 min, and the supernatant solution was mixed with 1 ml of saturated pyrophosphate, 0.5 ml of concentrated NH_4OH and 1 ml of 0.1% sodium diethyldithiocarbamate. The colored materials were extracted with isoamyl alcohol (6 ml). The content of total copper was estimated by optical absorption at 430 mμ (E_{430}) with a Shimazu–Bausch & Lomb spectrophotometer.

DAB Metabolism——For assay of DAB metabolism, the incubation mixtures described by Miller, *et al.*[6] was slightly modified. A reaction mixture contained 0.1 ml of 0.1M $MgCl_2$, 0.2 ml of 0.1M KCl, 0.5 ml of 0.1M phosphate buffer, pH 7.4, 0.2 ml of 0.6M nicotinamide, 0.5 ml of 0.03M glucose-6-phosphate, 0.1 ml of NAD (1 mg/ml), 0.2 ml of NADP (1 mg/ml), 0.1 ml of the ethanol solution of DAB (1 mg/ml) and 2 ml of the homogenate, which was added last. The total volume of the reaction mixture was 3.9 ml. The reaction was started immediately after adding homogenates, and the mixtures were shaken mechanically at 37° in an aerobic condition. The reaction was stopped by adding 0.5 ml of acetone. The dyes were extracted repeatedly with benzene, and the benzene extracts gathered were evaporated under a reduced pressure. The residues were dissolved in light petroleum (bp 50—60°), and applied to the chromatographic separation on an alumina column. DAB and 4-methylaminoazobenzene (MAB) were eluted separately with light petroleum–benzene mixtures.[8] The hydroxylation product, 4′-hydroxy-4-dimethylaminoazobenzene (OH–DAB), which was adsorbed tightly on the top of the column, was eluted with chloroform. Each eluate was evaporated to dryness *in vacuo*, and the residues were dissolved in 2N HCl and applied to the spectrophotometric measurement. The μg absorptivity of the dyes, *i.e.*, E (1 μg dye/1 ml of 2N HCl) were as follows: DAB; 0.200 at 520 mμ, MAB; 0.228 at 510 mμ, OH–DAB; 0.0656 at 547 mμ.

The rate of each metabolic reaction was expressed as follows:
N-Demethylation; MAB produced μg/200 mg liver/10 min.
Aromatic Hydroxylation; OH–DAB produced μg/200 mg liver/10 min.
Azo–Reduction; Decrease of Aminoazo dyes[9] μg/200 mg liver/10 min.

Results

Body Weight

The increase in the body weight of the rats of all the experimental groups except the control was very slow. Especially, in the rats fed copper alone, the body weight could hardly increase within ten weeks from the start. The spleen of the rats fed DAB alone was enlarged enormously, while in the rats fed DAB with copper it was not different significantly from the control. After 20 weeks from the start, the scattered nodules were observed in the livers of the rats fed DAB alone. For assay of the enzyme activities of those livers bearing the nodules, the homogenates of the whole livers were used. In all the cases, any impairment of health was not observed.

7) G.C. Cartwright, P.J. Jones, and M.M. Wintrobe, *J. Biol. Chem.*, **160**, 593 (1945).
8) A. Hanaki and M. Ishidate, *J. Biochem.* (Tokyo), **50**, 519 (1961).
9) Aminoazo dyes involve DAB, MAB and OH–DAB. Then, Decrease of aminoazo dyes = 100 − (MAB × 225/211 + OH–DAB × 225/241 + DAB remained).

Copper Content

The content of total copper in the liver was increased remarkably, more than 40 times, by the administration of copper, and the accumulation of copper was affected a little, usually reduced, by the administration of DAB with copper. The variation in the content of total copper was presented in Fig. 1.

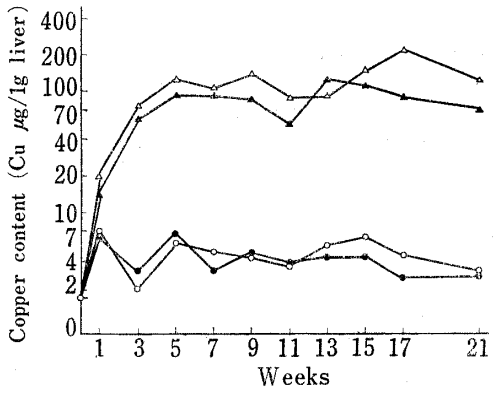

Fig. 1. Accumulation of Copper in the Liver of Rats fed Four Sorts of Diets

maize alone: O—O DAB: ●—●
copper: △—△ DAB and copper: ▲—▲

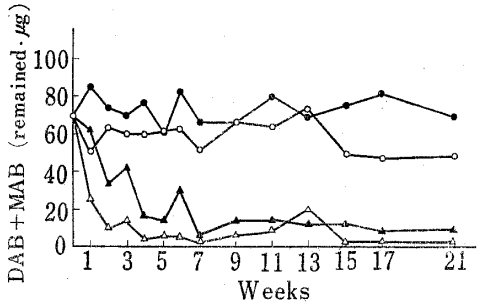

Fig. 2. Over-all Detoxication of the Carcinogen in the Liver Homogenate of the Rats fed Basic Cupric Acetate with and without DAB

maize alone: O—O DAB: ●—●
copper: △—△ DAB and copper: ▲—▲
The amounts of carcinogenic dyes, which contain both DAB and MAB, were determined after the incubation for 10 min.

Metabolism of DAB and Its Related Compounds in the Liver Homogenates

The metabolism of DAB, which was measured in the liver homogenates, was enhanced as a whole by the administration of basic cupric acetate. The liver homogenates of the rat which had been fed DAB appeared to rather reduce the detoxication of this carcinogen, while the administration of copper stimulated markedly the detoxication of this carcinogen (Fig. 2).

As shown in Fig. 2, the amounts of the carcinogenic dyes, *i.e.*, DAB and MAB, recovered from the incubation mixtures were found to decrease in the liver of the rat fed basic cupric acetate. This fact means that the administration of copper accelerates the detoxication of the carcinogen taken in the liver cell and thereby the duration of the contact between the carcinogen and the cell is shortened.

DAB undergoes the oxidative N-demethylation, the aromatic hydroxylation and the reductive cleavage of azo bond in the liver homogenates of the rat.[10] The N-demethylation product, MAB, having one metabolizable N-methyl group, was oxidized further to 4-amino-azobenzene(AB), which was detected and identified chromatographically on an alumina column. MAB might be also splitted reductively to aniline and N-methylphenylenediamine, which was not examined. The hydroxylation product, OH–DAB, having two N-methyl group, did resist to the N-demethylation and the reduction as shown in Table I.

Table I. Metabolism of OH–DAB with Liver Homogenate

Incubation time (min)	Dye recovered (μg)	
0	16.0	16.6
5	16.3	16.2
10	16.1	15.9

OH–DAB (17.0 μg) was incubated aerobically at 37° in the reaction mixtures described in the experimental part.

10) G.C. Mueller and J.A. Miller, *J. Biol. Chem.*, **180**, 1125 (1949).

Azo-Reduction

Since the liver preparation contains several enzymes concerning the metabolism of DAB, the activity of the individual enzymes, *i.e.*, azo-reductase, N-demethylase and aromatic hydroxylase, could not be estimated exactly and simultaneously by using the substrate like DAB. However, in the initial stage of the reaction where the substrate is present excessively as compared with the metabolite, the apparent rate of each reaction may be expressed by the amounts of the individual metabolite in a certain period. In this paper, the activity of azo-reduction was expressed as the decrease in the amounts of the aminoazo dyes within 10 min.

The reductive cleavage of the azo bond was a main pathway for the metabolism of DAB. The activity of azo-reduction in the liver homogenates was enhanced remarkably by feeding basic cupric acetate, and reduced slightly by feeding DAB alone. After two weeks from the start, the activity reached to a maximum level, which was approximately three times higher than that of the control and continued during the experiment. The administration of DAB seemed to reduce the activity of azo-reduction. When the rat was administered DAB with basic cupric acetate, the activity was also elevated markedly, though slightly lower than that of the liver of the rat fed copper alone. The variation in the activities of azo-reduction, which were measured as the decrease of the aminoazo dyes within 10 min, were presented in Fig. 3.

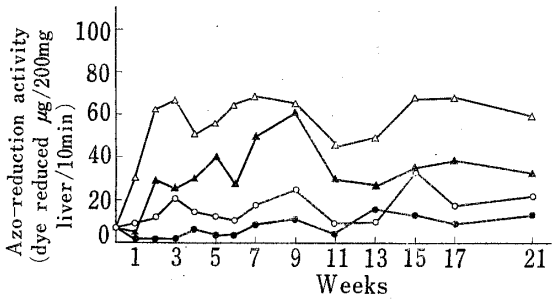

Fig. 3. Effect of Feeding Basic Cupric Acetate on the Activity of Azo-reduction in the Liver Homogenates of Rat

The reaction mixtures were incubated at 37° for 10 min.
maize alone: O—O DAB: ●—●
copper: △—△ DAB and copper: ▲—▲

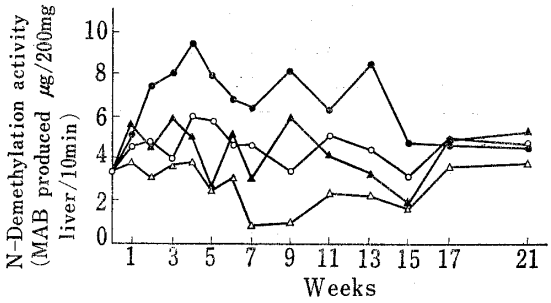

Fig. 4. Effect of Feeding Basic Cupric Acetate on the Activity of N-Demethylation in the Liver Homogenates of Rat

The reaction mixtures were incubated at 37° for 10 min.
maize alone: O—O DBA: ●—●
copper: △—△ DAB and copper: ▲—▲

As mentioned above, the livers of the rats fed copper with and without DAB contain large amounts of copper, which was expressed as the total copper, as compared with the control animals. The enhancement of the azo-reduction activity might be related to the accumulation of free or bound coppers. However, the possibility that the enhancement is due to the presence of free copper ion in the liver homogenates can be expelled, because free copper ion added into the reaction mixtures could not elevate enzyme activity as shown in Table II.

Table II. Effect of Free Copper Ion on the Metabolism of DAB

Cu added (μg)	Activity (μg/200 mg liver/10 min) Azo-reduction	N-Demethylation	Aromatic hydroxylation
0	17.3±2.8[a)]	5.4±0.2	5.0±0.1
5	18.0±2.3	4.9±0.4	5.3±0.4
15	12.0±0.4	2.7±0.7	4.8±0.8
30	11.4±0.7	<0.1	3.2±0.5
50	4.5±0.2	<0.1	2.1±0.5

For assay of the enzyme activities, 5 rats were used and the liver homogenates were prepared from each rat. The detailed procedure for aassy was described in experimental part.
a) standard deviation from mean value

N-Demethylation

The activity of N-demethylation of the liver homogenates of the rat fed DAB alone showed a highest value, while the activity was significantly reduced by the administration of copper. The N-demethylation activity of the liver homogenates of the rats fed copper with and without the dye was shown in Fig. 4.

Aromatic Hydroxylation

The activity of the aromatic hydroxylation, lying between 4 and 8 μg/200 mg liver/10 min, was not apparently affected by the administration of copper and/or DAB. The results were shown in Table III.

Table III. Effect of Feeding Basic Cupric Acetate on the Activity of Aromatic Hydroxylation in the Liver Homogenates of Rat

Group	Diet	*p*-Hydroxylation activity (OH–DAB produced μg/200 mg liver/10 min) Week						
		0	3	6	9	13	17	21
1	DAB in maize		5.67	4.24	4.34	3.84	6.71	7.44
2	Cu in maize		5.86	5.34	5.86	6.76	8.13	6.44
3	DAB+Cu in maize		4.27	7.81	6.69	5.22	7.82	7.66
4	maize (control)	7.94	5.00	5.59	6.74	4.39	8.47	7.03

Discussion

It has been shown that, when basic cupric acetate is administered to the rat with hepatocarcinogenic aminoazo dyes, both the hepatic carcinogenesis by the dye and the formation of the protein bound dye are markedly delayed. As for significance of the bound–dye formation to the carcinogenesis, it has been postulated that the liver proteins linked to the metabolites of the carcinogenic dye, named the primary carcinogen, play key roles in the response of the cell to its intrinsic growth controls and to the extrinsic growth controls exercised by the rest of the organism. The aminoazo dye having an ability to produce the bound–dye possesses at least one metabolizable N-methyl group, and its aminoazobenzene skeleton should be conserved in the bound dye. Therefore, the delay of the bound–dye formation by the administration of basic cupric acetate is expected to be due to the enhancement of the metabolic transformation of the dye, especially of the decomposition of the aminoazobenzene skeleton.

The oxidative N-demethylation, the aromatic hydroxylation at 4′-position and the reductive cleavage of the azo bond are participated in the detoxication of the carcinogenic aminoazo dyes. Among those three detoxicating reaction, the last was stimulated extraordinarily by the administration of basic cupric acetate. The administration of the copper compound for a long period is expected to affect other metabolic reaction, especially the oxidative N-demethylation. However, since the reduction is catalyzed very rapidly in the liver homogenates and the N-demethylation appears to be a minor pathway for the detoxication of DAB, the elevation of the activity of N-demethylation by feeding copper could not be detected, even if copper stimulates this enzyme. Free copper ion added into the homogenate did not elevate the activity of azo–reduction. This fact may indicate that copper ion does not stimulate directly the enzyme activity itself but the electron-transfer system concerning the reduction of azo bond. A single injection of an aqueous solution of copper salt could not generally elevate the enzyme activity within 24 hr.[11] The duration of the contact between copper ion

11) Y. Yamane, K. Sakai, and A. Hanaki, *Chem. Pharm. Bull.* (Tokyo), in preparation.

and the liver cell may be necessiated for the stimulation of the reductive activity and for the protection from the chemical carcinogenesis by the dye. Those findings described above suggest that one role of copper concerning the retardation of the hepatic carcinogenesis by DAB might be related to the enhancement of the activity of azo-reduction, which decomposes the carcinogenic dye to the noncarcinogenic compounds.