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Effect of Citric Acid on 3′-Methyl-4-dimethylaminoazobenzene Induced Decrease in Rat Liver pH[1)]

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We studied the effect of citrate on 3′-methyl-4-dimethylaminoazobenzene (3′-Me-DAB)-induced hepatic injury. Examination of the liver of rats receiving a 0.06% 3′-Me-DAB diet with or without simultaneous 1% citrate administration revealed that citrate did not affect the gross findings. In rats receiving a basal diet and 1% citrate solution, no change in the intracellular liver pH (pHi) and blood pH (pHe) was noted. However, in rats on the 3′-Me-DAB diet, simultaneous citrate administration mitigated the 3′-Me-DAB induced decrease of pH. In rats receiving single or continuous 3′-Me-DAB administration, citrate treatment did not recover decreased N-demethylase and azo reductase activities. Time-course examination of protein-bound dyes in the liver revealed that the peak of the amount of bound dyes appeared earlier in rats receiving simultaneous citrate administration than in rats receiving the 3′-Me-DAB diet without simultaneous citrate administration. Fractionation of liver cell sap protein showed that the amount of 3′-Me-DAB bound to each fraction was similar for rats receiving the 3′-Me-DAB diet and citrate and for rats receiving the 3′-Me-DAB diet alone.

Keywords——3′-Me-DAB; intracellular pH; liver damage; citric acid; protein-bound dye

Body tissues are known to maintain a pH value within a limited range and it is believed that some diseases and tissue damage may effect changes in the intracellular environment. We previously used the change in liver pH to study intra- and extracellular changes in the livers of rats fed 3′-methyl-4-dimethylaminoazobenzene (3′-Me-DAB), a hepatotoxic agent, and reported that the pH values in the blood (pHe) and liver (pHi) decreased significantly one week after inception of the 3′-Me-DAB containing diet.[3)] We further found that the feeding of 2-methyl-4-dimethylaminoazobenzene (2-Me-DAB), a non-hepatotoxic agent under normal conditions, did not effect a decrease in pHi or pHe.[3)] In the present investigation we examined whether the pHi decrease is essential for the occurrence of liver damage.

Since citric acid is known to alkalify human tissues,[4)] we investigated whether the simultaneous administration of citrate prevents the 3′-Me-DAB induced decrease in pHe and pHi and inhibits liver damage.

Materials and Methods

162, 7 week-old and 30, 10 week-old male Donryu rats, weighing about 150 g and 200—250 g, respectively, at the beginning of the experiments, were used. The cube diet (Oriental MF) was used as the basal diet, and the 3′-Me-DAB diet contained 0.06% of 3′-Me-DAB in this basal diet. Citric acid was dissolved in water to obtain a concentration of 1%, and $NaHCO_3$ at the same molar concentration was added to the solution. Since the simultaneous administration of 3% citrate solution markedly increased pHe and pHi, especially on the 5th week (Fig. 1), 1% citrate solution was used. The 1% citrate solution was supplied as the drinking water. Diets and water were supplied *ad libitum*.

1) A part of this work was reported at The Eighth Symposium on Drug Metabolism and Action, Hiroshima, Nov. 1976.
2) Location; *5–8, Hatanodai 1 chome, Sinagawa-ku, Tokyo, 142, Japan.*
3) Y. Kitagawa and Y. Kuroiwa, *Life Sci.*, **18**, 441 (1976).
4) S. Akiya and N. Motohashi, *Yakugaku Zasshi*, **76**, 111 (1956); *Idem, ibid.*, **76**, 116 (1956).

The rats were separated into 6 groups. Group I (52, 7 week-old rats) received the 3′-Me–DAB diet and the citrate solution, Group II (64, 7 week-old rats) received the same diet and untreated water, Group III (14, 10 week-old rats) received one oral administration of 40 mg/rat 3′-Me–DAB in olive oil solution and untreated water, Group IV (16, 10 week-old rats) received the citrate solution for 1 week prior to oral administration of 40 mg/rat 3′-Me–DAB and untreated water thereafter, Group V (21, 7 week-old rats) received the basal diet and citrate solution, and Group VI (25, 7 week-old rats) served as the control.

pHe was measured at 37° with a Blood Micro System in conjunction with an Acid-Base Analyzer, type PHM 72 (Radiometer, Copenhagen) and pHi was determined by the DMO method.[3]

Estimation of Liver Enzyme Activities——The rats were decapitated and the liver perfused *in situ* with cold 1.15% KCl.

A 20% liver homogenate was prepared in 1.15% KCl, centrifuged at 9000 ***g*** for 20 min and the supernatant was used as the enzyme source. Oxidative N-demethylase and azoreductase activities were determined by the modified method of Mueller and Miller[5] using 3′-Me–DAB in 95% ethanol as substrate. With N-demethylation, the reaction mixture (5.0 ml final volume) contained 50 μmol of nicotinamide, 50 μmol of semicarbazide, 25 μmol of $MgCl_2$, 12.5 μmol of glucose 6-phosphate, 1 μmol of NADP, 2 ml of enzyme solution, and 0.3 M potassium phosphate buffer (pH 7.4). Five μmol of 3′-Me–DAB in 0.1 ml was added last. The reactions were carried out at 37° with mechanical shaking. After 1 hr incubation, the reaction was stopped by the addition of 1.0 ml of 20% $ZnSO_4$ followed by 1.5 ml of saturated $Ba(OH)_2$. The precipitated protein was sedimented by centrifugation at 3000 rpm for 15 min. To the supernatant fluid 2 ml of benzene–acetone (5: 2) mixture was added in order to extract the free aminoazo dyes. Formaldehyde produced in the aqueous phase was measured with an acetylacetone reagent. With azoreduction, the reaction mixture (3.0 ml final volume) contained 0.2 ml of 0.6 M nicotinamide, 0.3 ml of 0.03 M glucose 6-phosphate, 0.1 ml of 0.1% NAD, 0.1 ml of 0.1% NADP, 0.6 ml of 1.15% KCl, 0.1 ml of 0.1 M $MgCl_2$, 0.5 ml of 0.1 M potassium phosphate buffer (pH 7.4), 1.0 ml of enzyme solution, and 0.1 ml of 0.03% 3′-Me–DAB. The reaction was carried out unaerobically at 37° for 50 min and stopped by the addition of 3 ml of 20% trichloroacetic acid in acetone–ethanol (1: 1) solution. After centrifugation at 3000 rpm for 15 min, the optical density of the supernatant was determined at 520 nm. Azoreductase activity was shown by the disappearance of 3′-Me–DAB used as substrate.

Estimation of Polar Dyes——Polar dyes in the livers of rats on the 3′-Me–DAB diet (Groups I and II) were separated and estimated by the method of Terayama.[6] Polar dye concentration was expressed as the absorbancy difference between Groups I and II animals and animals on the basal diet (Groups V and VI) at the maximum absorption wave length (520 nm) in absorption spectra ranging from 620 to 400 nm.

Elution Pattern and Protein-bound Dye of Rat Liver Cell Sap Subjected to Chromatography on CM-cellulose——Preparation of the rat liver cell sap and application to chromatography on CM-cellulose were carried out by the method of Terayama *et al.*[7] Protein was assayed according to Lowry *et al.*[8] The protein-bound dye was estimated spectrophotometrically at 520 nm in concentrated formic acid. The protein was first precipitated with 20% trichloroacetic acid, washed thoroughly with a chloroform–methanol (3: 1) mixture and then dissolved.

Results

Changes in Body and Liver Weight

The body weight of all rats increased during the experiment, however, during the first 5 weeks, animals in Group II gained much less weight than the controls. A significant increase after 17 weeks in the body weight of Group II rats was due to an increase in liver weight. An increase in ascites content was noted and the number of deaths among this group also increased. By week 10, liver weight accounted for 6% of total body weight, by week 15 it was 9% and by week 20, 28%. The percentage of liver weight in the controls was 3 to 3.5 during the course of the experiment. The simultaneous administration of the citrate solution (Group I) had little effect on weight gain, indicating the similar findings to Group II rats.

Gross Findings in the Liver

On the 5th week, the livers of Groups I and II rats showed some small white nodules. These increased in number and size with prolongation of the feeding period. On the 15th

5) G.C. Mueller and J.A. Miller, *J. Biol. Chem.*, **202**, 579 (1953); *Idem*, *ibid.*, **180**, 1125 (1949).
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7) T. Sugimoto and H. Terayama, *Biochim. Biophys. Acta*, **214**, 533 (1970).
8) O.H. Lowry, N.J. Rosenbrough, A.L. Farr and R.T. Randall, *J. Biol. Chem.*, **193**, 265 (1951).

week, the livers showed irregular surfaces with multiple large, yellowish nodules up to 2 cm in diameter. On the 20th week, the liver lobes adhered to one another, to the digestive tract and to the diaphragm. In some animals, metastasis from liver neoplasms were noted in the omentum, mesentric lymph nodes and diaphragm. Citrate administration did not affect the gross findings.

Effect of Citrate on pHe and pHi in Rats on the 3′-Me-DAB Diet

The basal diet plus 1% citrate (Group V) did not change pHe or pHi which were in the normal range during the entire experimental period (Fig. 1). Rats in Group I maintained

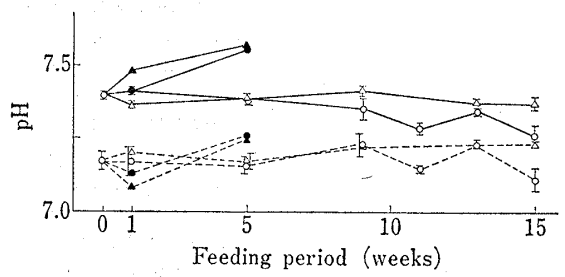

Fig. 1. Effect of Citrate on pHe and pHi in Rats fed 3′-Me-DAB Containing or Basal Diets

——; pHe ----; pHi.
○; group fed 3′-Me-DAB diet+1% citrate solution (Group I), ●; group fed 3′-Me-DAB diet+3% citrate solution, △; group fed the basal diet+1% citrate solution (Group V), ▲; group fed the basal diet+3% citrate solution.
Each point represents the mean±S.E. for more than 3 rats.

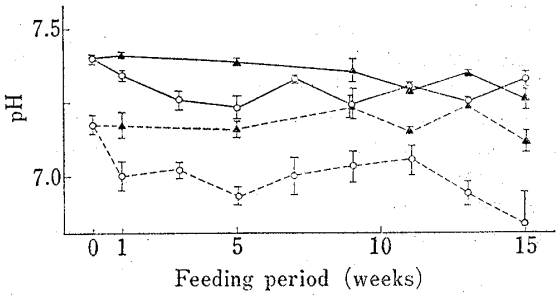

Fig. 2. Change in pHe and pHi in Rats fed 3′-Me-DAB Containing Diet with and without Citrate

——; pHe ----; pHi.
▲; 1% citrate solution (Group I), ○; water (Group II)
Each point represents the mean value±S.E. for more than 3 rats.

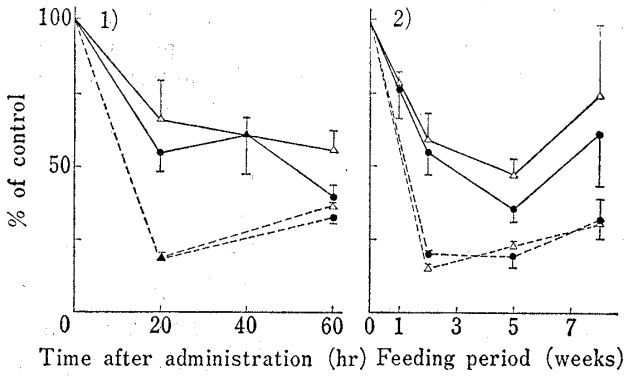

Fig. 3. Change in 3′-Me-DAB Metabolizing Enzyme Activities in Livers of Rats treated with 3′-Me-DAB

——; N-demethylase, ----; azo reductase.
1) Single oral administration of 40 mg/rat 3′-Me-DAB.
△; pretreated with 1% citrate solution for 1 week (Group IV),
●; non-treated with citrate (Group III).
2) Continuous feeding of 3′-Me-DAB diet.
△; with 1% citrate solution (Group I),
●; without citrate (Group II).
Activities are expressed in % against the activities of non-treated rat livers.
Each point is the average±S.E. of the activities for 3 or 4 rats.
activities in controls,
N-demethylase; 5.1±0.74 μg HCHO/ml of 9000 g sup/hr (100±14.5%),
azo reductase; 19.9±1.39 μg 3′-Me-DAB reduced/ml of 9000 g sup/50 min (100±7.0%).

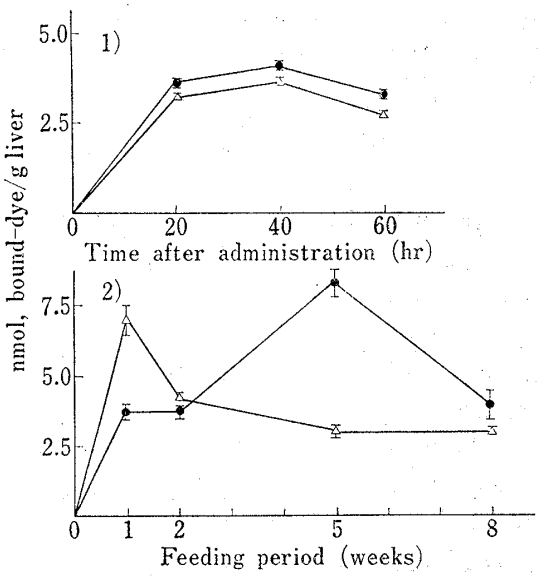

Fig. 4. Change in Protein-bound Dye Levels in Livers Rats treated with 3′-Me-DAB with and without Citrate

1) Single oral administration of 40 mg/rat of 3′-Me-DAB.
△; pretreatment with 1% citrate solution for 1 week (Group IV),
●; non-treated with citrate (Group III).
2) Continuous feeding of 3′-Me-DAB diet.
△; with 1% citrate solution (Group I),
●; without citrate (Group II).

normal pH values until the 9th week, indicating that citrate prevented a decrease in pHe and pHi (Fig. 2). However, after the 11th week, pH values in both blood and liver showed a tendency to decrease.

Effect of Citrate on the Activities of 3′-Me-DAB Metabolizing Enzymes in the Liver of Rats on the 3′-Me-DAB Diet

The activities of N-demethylase and azoreductase to 3′-Me-DAB decreased in Groups I—IV and N-demethylase activity was somewhat higher in Groups I and IV than in Groups II and III. However, azoreductase activity was not affected. The time-course for the activities of the above enzymes was similar, irrespective of citrate treatment (Fig. 3).

TABLE I. Distribution of Proteins and Protein-bound Dye in Heat-treated Rat Liver Cell Sap among Fraction Groups separated by Chromatography on CM-Cellulose

1) Single Administration of 40 mg/rat of 3′-Me-DAB, 40 hr

Fraction group	Protein (%)			Protein-bound dye (%)		Bound-dye nmol/mg protein	
	Normal	(−) [a]	(+)	(−)	(+)	(−)	(+)
Heated cell sap	100 (618) [c]	100 (690)	100 (660)	100 (119)	100 (79)	0.17	0.12
A	72.9	67.3	69.4	52.3	40.6	0.13	0.07
B	4.9	5.9	4.7	—	—	—	—
C	1.4	1.8	2.0	—	—	—	—
D	2.6	5.0	4.4	10.2	15.3	0.34	0.42
E	1.9	1.6	1.9	—	—	—	—
F	9.0	2.0	1.1	1.6	0.9	0.13	0.10
G		1.4	2.1	0.3	1.4	0.04	0.08
Sum	92.7	85.0	85.7	64.4	58.9		

2) Continuous Feeding of 3′-Me-DAB Diet

Fraction group	Feeding period (week)	Protein (%)		Protein-bound dye (%)		Bound-dye nmol/mg protein	
		(−) [b]	(+)	(−)	(+)	(−)	(+)
Heated cell sap	1	100 (621) [c]	100 (660)	100 (129)	100 (87)	0.21	0.13
	5	100 (659)	100 (706)	100 (63)	100 (75)	0.19	0.11
A	1	69.1	62.2	63.5	37.9	0.19	0.08
	5	68.3	79.3	43.5	37.0	0.12	0.05
B + C	1	8.3	10.8	—	—	—	—
	5	6.4	3.3	—	—	—	—
D	1	5.9	7.0	14.8	15.0	0.53	0.24
	5	5.8	5.6	13.1	8.8	0.43	0.18
E	1	0.8	1.6	—	—	—	—
	5	1.1	0.3	0.2	4.9	0.12	0.70
F + G	1	3.3	3.5	2.5	4.0	0.15	0.29
	5	4.9	3.8	6.0	7.8	0.71	0.45
Sum	1	86.8	85.1	80.8	58.5		
	5	86.5	92.3	62.8	58.7		

a) 3′-Me-DAB was administered orally following 1 week pretreatment with citrate (+; Group IV), or without citrate pretreatment (−; Group III).
b) 1% citrate solution was given as the drinking water during the administration of the 3′-Me-DAB diet (+; Group I, −; Group II).
c) Figures in parentheses indicate the amount of protein (mg) and protein-bound dye (nmol) applied to chromatography.

Effect of Citrate on the Amount of Protein-bound Dye

During the experiment period as a whole, the amount of bound dye formed in the livers of Group IV animals was somewhat less than in Group III animals. However, the time course of dye-protein binding in both groups was almost the same. Maximal binding was observed at 40 hr in Groups III and IV and the amount of bound dye reached the maximum level after approximately 1 week in Group I and on about the 5th week in Group II. After passing the peak, dye-protein binding decreased to half of the maximum in Groups I—IV (Fig. 4).

Chromatography of Heat-treated Cell Sap on CM-cellulose

The distribution of protein and protein-bound dye among fractionated protein groups is summarized in Table I and Fig. 5. In Groups I—IV, the band containing the largest amount of protein was observed at the first peak (Fraction A), two bands had a small amount of protein in the 0.01 M and 0.02 M NaCl eluates (Fractions B and C), the second largest amount of protein was found in the 0.02 M NaCl eluates (Fraction D), and two bands had a small amount of protein in the 0.05 M NaCl eluates (Fractions F and G). As shown in Table I, protein-bound dye was primarily located in Fractions A (37—64%) and D (9—15%). In Group IV, citrate pretreatment decreased the protein-bound dye in Fraction A and increased it in Fraction D. In Group II, continuous feeding of 3′-Me-DAB decreased the protein-bound dye in Fractions A and D, but it increased in Fractions E, F and G with feeding time. In Group I, simultaneous administration of citrate decreased the bound dye in Fractions A and D, and it increased significantly in Fractions E, F and G.

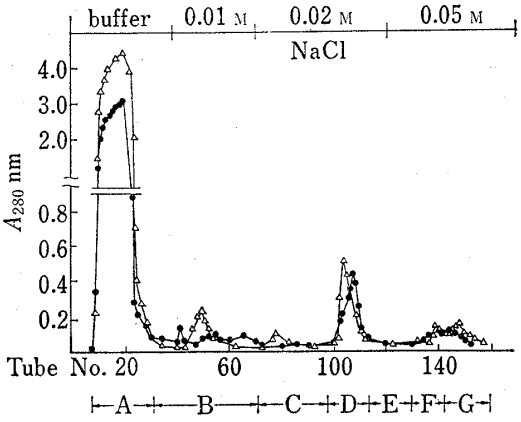

Fig. 5. Elution Pattern of Heat-treated (55°, 3 min) Rat Liver Cell Sap Proteins Subjected to Chromatography on CM-Cellulose

The liver cell sap was prepared 40 hr after oral administration of mg/rat of 3′-Me-DAB with (△; Group IV) and without (●; Group III) pretreatment with 1% citrate for 1 week. Stepwise elution was carried out by running 0.01 M Tris-HCl buffer (pH 7.0) 0.01, 0.02 and 0.05 M NaCl in the same buffer successively. Fractions of 5.5 ml effluent were collected. A_{280} nm as a rough estimate of proteins.

Discussion

3′-Me-DAB is known to be hepatotoxic, while its analog, 2-Me-DAB, is not hepatotoxic. We reported earlier[3] that the pHi of 2-Me-DAB fed rats was not decreased. In the present investigation we examined whether simultaneous administration of citrate prevents a pHe and pHi decrease in rats on a 3′-Me-DAB diet. In rats administered with 3′-Me-DAB and citrate (Group I), no drop in liver pHi was observed, suggesting that citrate mitigates the 3′-Me-DAB induced liver insult. We found that simultaneous administration of 1% citrate solution maintained pHe and pHi levels within the normal range. On the other hand, administration of 3% citrate solution increased pHe and pHi levels to above normal range and a marked weight loss was noted in the animals by the 5th week after inception of the 3′-Me-DAB feeding (Fig. 1), possibly due to a high metabolism rate. In Groups I and II gross findings in the livers were similar. No inhibition of liver damage by citrate was noted microscopically[9] and the fact that the degree of 3′-Me-DAB induced liver damage was greater in Group I than in Group II, indicates that citrate accelerated liver cell metabolism.

9) Unpublished data.

Furthermore, cholangiofibrosis developed more often in Group I than in Group II. The citrate-induced recovery of pH values did not mitigate the hepatotoxic action of 3′-Me–DAB. Well known reducing agents of azo dye carcinogenicity such as 3-methylcholanthrene (3MC) and phenobarbital (PB)[10] prevented the pHe and pHi decrease in rats on the 3′-Me–DAB diet.[11] Simultaneous administration suppresses the 3′-Me–DAB induced decrease of the activities of N-demethylase and azoreductase, both of which play a role in the metabolism of azo dyes.[12] On the other hand, simultaneous administration of PB increases N-demethylation and decreases azo reduction.[12] In both single and continuous administration of 3′-Me–DAB, the inhibition of azo reductase activity was greater than that of N-demethylase, and was not affected by the administration of citrate. However, in continuous 3′-Me–DAB administration, the simultaneous administration of citrate (Group I) recovered N-demethylase activity faster and increased it by 15% on the 5th and 8th weeks as compared to Group II. On the other hand, citrate did not affect the azo reduction of 3′-Me–DAB which is a main route of detoxication. The amount of liver-bound dye was similar in Groups III and IV. In Group I the peak for protein-bound dye appeared early, possibly due to a fast liver protein turnover. However, the amount of protein-bound dye at the peak was similar for Groups I and II, suggesting that the degree of 3′-Me–DAB induced liver damage was similar in these two groups. The amount of protein-bound dye in liver cell sap had a tendency to decrease in the nonbasic protein (Group I, Fraction A) and to increase in the basic protein (Fractions E, F and G). Since 2-Me–DAB is bound largely to the basic protein fractions,[7] citrate may not play a major role in the reduction of 3′-Me–DAB induced hepatic injury.

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11) Y. Kitagawa and Y. Kuroiwa, *Chem. Pharm. Bull.*, (Tokyo), in press.
12) K. Takamiya, S.H. Chen, and H. Kitagawa, *Gann*, **64**, 363 (1973).