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Effect of Copper and Riboflavin on Azo Reductase Activity in the Liver of Rats[1]

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The effects of copper and riboflavin on 4-dimethylaminoazobenzene (DAB) azo reductase activity and 1,2-dimethyl-4-(*p*-carboxyphenylazo)-5-hydroxybenzene (CPA) azo reductase activity in rat liver were investigated.

In the liver microsome both riboflavin content and DAB azo reductase activity were not significantly increased in the groups given the diet supplemented with 20 mg or 50 mg of riboflavin/100 g of diet as a riboflavin-excessive diet, but was significantly increased in the groups given the diet supplemented with copper. In the riboflavin-deficient diet group not only riboflavin but copper was also decreased. DAB azo reductase activity in the groups given the copper-supplemented diet was increased more than that in each group of the same concentration of riboflavin diet.

The increase of CPA azo reductase activity was almost in parallel with that of DAB azo reductase activity in the groups given the diet supplemented with copper.

Keywords——copper; riboflavin; riboflavin deficiency; riboflavin excess; 4-dimethylaminoazobenzene metabolism; 4-dimethylaminoazobenzene azo reductase activity; 1,2-dimethyl-4-(*p*-carboxyphenylazo)-5-hydroxybenzene azo reductase activity; rat liver; microsome

There are reports on effect of riboflavin on the liver microsomal enzyme activities of rats.[3,4] However, there are only few reports on the effect of riboflavin and metals on drugmetabolizing enzyme activities. In a series of our works[5–8] regarding the suppressive effect of copper on 4-dimethylaminoazobenzene (DAB) carcinogenesis, it was found that the enhancement of DAB metabolizing enzyme activities in the liver microsomes of rats fed copper was accompanied with the increase of riboflavin in the liver microsomes.

In the paper the effect of copper and riboflavin on DAB azo reductase activty and 1,2-dimethyl-4-(*p*-carboxyphenylazo)-5-hydroxybenzene (CPA) azo reductase activity in the liver of rats was investigated.

Experimental

Animals and Diets——Four weeks old female rats of Wistar strain, obtained from Nippon Rat Co., Ltd., were fed on the commercial semi-synthetic diet (CE-2, CLEA Japan Inc., Tokyo) until 8 weeks old, and 8 weeks old female rats weighing 120—140 g were used for the experiments. The diet supplemented with 20 or 50 mg of riboflavin/100 g of CE-2 was used as a riboflavin-excessive diet and the commercial synthetic diet (No. 15 Vitamin B_2 Deficient Test Diet, CLEA Japan Inc., Tokyo) was used as a riboflavin-deficient synthetic diet. Content of riboflavin in CE-2 diet was 1.3 mg per 100 g of the diet. Content of riboflavin in the riboflavin-deficient synthetic diet was 0.035 mg per 100 g of the diet. The experimental diet was given to rats for four weeks. The rats were allowed to eat and drink freely.

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Preparation of Rat Liver Microsomes——The livers were perfused with 1.15% KCl, removed immediately and homogenized with Teflon-glass homogenizer in ten volumes of 1.15% KCl. The 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* supernatants of the livers obtained from the rats fed CE-2 diet and the Vitamin B_2 Deficient Test Diet supplemented with 2.0 mg riboflavin/100 g of the diet were used as the source of G-6-P dehydrogenase in each experiment.

Measurement of DAB Metabolizing Enzyme Activity——Measurement of DAB metabolizing enzyme activities, namely azo reductase, N-demethylase and ring hydroxylase activities, was carried out as described in our previous reports.[7,8]

The rate of each metabolic reaction was expressed as follows: N-demethylation; μg 4-methylaminoazobenzene produced/200 mg liver/10 min. Ring hydroxylation; μg 4′-hydroxy-4-dimethylaminoazobenzene produced/200 mg liver/10 min. Azo reduction; μg aminoazo dyes[5] disappeared/200 mg liver/10 min.

Measurement of CPA Azo Reductase Activity——CPA azo reductase activity was measured according to a slight modification of the method of Smith, *et al.* as follows.[9] The incubation mixture consisted of 0.4 ml of 0.2 M potassium phosphate buffer (pH 7.4), 0.3 ml of a mixture containing 25 μmol glucose 6-phosphate 12.5 μmol $MgCl_2$ and 25 μmol nicotinamide, 0.1 ml of NADP (0.8 μmol), 0.5 ml of microsomal suspension (equivalent to 400 mg liver), 1.0 ml of 105000 *g* supernatant and 0.2 ml of CPA (5 μmol). The mixture was gassed for 1 min with nitrogen and incubated for 30 min at 37°. The reaction was terminated by adding 2.5 ml of 10% trichloroacetic acid. The mixture was then centrifuged at 0—4° and 2.5 ml of the supernatant was analyzed for CPA azo reductase activity as follows: 0.5 ml of 0.1% sodium nitrite freshly prepared was added and, after 5 min, 0.5 ml of 0.5% ammonium sulfamate was added to decompose excess sodium nitrite. Approximately 5 min later, 0.5 ml of 0.5% N-(1-naphthyl)ethylenediamine was added and the mixture was allowed to react for 15 min. The solution was swirled vigorously after each addition. The absorbance was measured at 540 nm.

Determination of Copper——The biological sample was digested by heating it in Kjerdahl flasks in the presence of an appropriate amount of 36 N H_2SO_4 and 14 N HNO_3 on an electric burner. After the sample was completely digested, colorimetric method with diethyldithiocarbamate was performed as shown in the previous reports.[6-8]

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. The warm water extract of the tissue was mixed with an equal volume of 1 N NaOH, irradiated for decomposition of riboflavin to lumiflavin, added 0.2 ml of acetic acid and extracted once with 6.0 ml of pure chloroform. The chloroform layer was estimated for intensity of lumiflavin fluorescence produced from riboflavin. At the same time, the additional test of riboflavin was made by the same way, and the amount of flavin was calculated.

Table I. Content of Copper and Riboflavin and Activity of DAB Azo Reductase in the Liver Microsome of Rats fed Riboflavin-excessive Diet with and without Copper

	Group	Copper content (μg/g liver)	Riboflavin content (μg/g liver)	Azo reductase activity
1.	No teratment	0.75±0.23	2.16±0.27	22.61±3.16
2.	20 mg RF[a]	0.65±0.26	2.36±0.24	30.70±6.31
3.	50 mg RF	0.85±0.14	2.42±0.14	24.81±3.05
4.	0.5% BCA[b]	1.59±0.33	3.60±0.41[c]	48.08±4.35
5.	20 mg RF+0.5% BCA	1.36±0.45	4.17±0.39[d]	53.17±1.75
6.	50 mg RF+1.5% BCA	1.48±0.14	5.06±0.30[e]	58.64±3.38

Six rats were used for each group and each value is expressed as the mean±S.E. CE-2 diet was used as a basal diet. The diet supplemented with 20 mg or 50 mg of riboflavin/100 g of CE-2 diet was used as a riboflavin-excessive diet.

a) RF: riboflavin.
b) BCA: basic cupric acetate.
c) Significantly different from group 3 at $p<0.01$.
d) Significantly different from group 4 at $p<0.05$.
e) Significantly different from group 5 at $p<0.01$.

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Results

Content of Copper and Riboflavin in the Liver Microsome of Rats Given the Riboflavin-excessive and/or Copper Diet

As shown in Table I, the content of riboflavin in the liver microsomes was not significantly increased in the groups 2 and 3 given the diet supplmented with 20 mg or 50 mg of riboflavin/100 g of CE-2 diet under the conditions tested, but in all cases of the riboflavin and copper groups 4—6, the content of riboflavin was increased clearly ($p<0.01$) and was increased with increase of amount of riboflavin added to CE-2 diet.

In all of the groups administered copper, the content of copper was increased in the liver microsomes but not affected by the concentration of riboflavin in diet, as shown in Table I.

DAB Azo Reductase Activity in the Liver Microsome of Rats Given the Riboflavin-excessive and/or Copper Diet

DAB azo reductase activity in the liver microsomes was not significantly increased in the groups 2 and 3 of the riboflavin-excessive diet under the conditions tested, as shown in Table I. In the groups 4—6 given the copper-supplemented diet the enzyme activity increased about two times than in the CE-2 groups 1—3 given the diet not supplemented with copper.

The increase of DAB azo reductase activity is in parallel with that of content of riboflavin in the liver microsome. Under the conditions tested, both the content of riboflavin and DAB azo reductase activity of the liver microsome in the groups 2 and 3 given the diet supplemented with 20 mg or 50 mg of riboflavin/100 g of CE-2 diet was lower than those of the group 4 given the diet supplemented with copper to CE-2 diet.

Contents of Copper and Riboflavin in the Liver of Rats Giben the Riboflavin-deficient Diet with and/or without Copper

Although the contents of riboflavin and copper were not significantly different among the groups 8—10 given the diet supplemented with 0.1 mg, 0.5 mg or 2.0 mg of riboflavin/100 g of the diet, the content of copper in the group 7 decreased signicantly in the whole liver and slightly in the liver microsome as shown in Table II, but in the riboflavin content of the microsome there was no difference between the group 7 and the groups 8 and 11, as shown in Table II. The riboflavin content in the livers of groups 11—14 was more than that in the groups

Table II. Content of Copper and Riboflavin in the Whole Liver or Liver Microsome of Rats fed Riboflavin-deficient Diet with and without Copper

Group		Copper		Riboflavin	
		Whole liver (μg/g liver)	Microsome (μg/g liver)	Whole liver (μg/g liver)	Microsome (μg/g liver)
7.	No treatment	3.11±0.62[a)]	0.66±0.04[a)]	6.33±0.47[c)]	1.43±0.07
8.	0.1 mg RF	4.48±0.54	0.90±0.07	14.67±1.20	1.39±0.16
9.	0.5 mg RF	3.52±0.68	0.93±0.10	17.63±1.98	1.92±0.25
10.	2.0 mg RF	4.92±0.56	1.02±0.12	18.83±2.06	2.14±0.28
11.	0.5% BCA	222.7 ±38.3[b)]	1.90±0.25[b)]	10.88±0.56[d)]	1.53±0.22
12.	0.1 mg RF+0.5% BCA	381.2 ±31.8	2.37±0.31	17.33±1.42	1.65±0.29
13.	0.5 mg RF+0.5% BCA	448.5 ±47.8	2.09±0.23	22.67±3.15	2.29±0.27
14.	2.0 mg RF+0.5% BCA	403.4 ±48.8	2.85±0.44	27.83±4.52	3.10±0.25

Six rats were used for each group and each value is expressed as the mean±S.E. The riboflavin-deficient synthetic diet (no treatment) was used as a basal diet. The diet supplemented with 0.1 mg, 0.5 mg or 2.0 mg of riboflavin/100 g of the basal diet with and without copper was used.

a) Significantly different from group 10 at $p<0.01$.
b) Significantly different from group 14 at $p<0.01$.
c) Significantly different from group 8 at $p<0.01$.
d) Significantly different from group 12 at $p<0.01$.

7—10 given the same diet not supplemented with copper. The copper content in groups 12—14 was not clearly different.

DAB Metabolizing Enzyme Activities in the Liver Microsome of Rats Given the Riboflavin-deficient Diet with and without Copper

DAB azo reductase activity was increased more in the group given the diet supplemented with higher concentration of riboflavin and the enzyme activity in the groups 11—14 given the copper-supplemented diet was increased more than that in the groups 7—10 in the same concentration of riboflavin diet without copper, even in the case of the group 11 ($p<0.05$), as shown in Table III.

Table III. DAB Metabolizing Enzyme Activities in the Liver Microsome of Rats fed Riboflavin-deficient Diet with and without Copper

Group	Azo reductase activity	N-Demethylase activity	Ring hydroxylase activity
7. No treatment	15.94±1.05	0.43±0.03	0.84±0.15
8. 0.1 mg RF	22.36±1.67	0.38±0.05	0.88±0.16
9. 0.5 mg RF	26.03±2.33	0.31±0.02[a]	1.25±0.25[a]
10. 2.0 mg RF	30.06±1.23	0.35±0.06[a]	1.36±0.18[a]
11. 0.5% BCA	19.33±2.21[a]	0.60±0.09	1.26±0.12[a]
12. 0.1 mg RF+0.5% BCA	27.88±1.91	0.54±0.06	1.01±0.14
13. 0.5 mg RF+0.5% BCA	41.35±1.73	0.36±0.05[b]	1.51±0.36
14. 2.0 mg RF+0.5% BCA	46.42±4.39	0.37±0.07[b]	1.72±0.25[b]

Six rats were used for each group and each value is expressed as the mean±S.E. The riboflavin-deficient synthetic diet (no treatment) was used as a basal diet. The diet supplemented with 0.1 mg, 0.5 mg or 2.0 mg of riboflavin/100 g of the basal diet with and without copper was used.
a) Significantly different from group 7 at $p<0.05$.
b) Signifitantly different from group 11 at $p<0.05$.

Ring hydroxylase activity was also increased in the groups 9 and 10 given the diet supplemented with 0.5 mg or 2.0 mg of riboflavin/100 g of the diet and more increased in the group 14 given the diet supplemented with copper and 2.0 mg of riboflavin/100 g of the diet, while N-demethylase activity was slightly decreased in the groups 9 and 10 given the diet supplemented with 0.5 mg or 2.0 mg of riboflavin or in the groups 13 and 14 given the diet supplemented with copper and 0.5 mg or 2.0 mg of riboflavin.

Table IV. CPA Azo Reductase Activity in the Liver Microsome of Rats fed Riboflavin-deficient Diet with and without Copper

Group	CPA azo reductase activity (n mol/400 mg liver/30 min)
7. No treatment	19.3±3.8
8. 0.1 mg RF	30.4±3.7
9. 0.5 mg RF	33.0±4.3
10. 2.0 mg RF	35.1±2.0
11. 0.5% BCA	23.8±2.7[a]
12. 0.1 mg RF+0.5% BCA	38.0±3.6
13. 0.5 mg RF+0.5% BCA	50.5±3.4
14. 2.0 mg RF+0.5% BCA	61.3±5.8

Six rats were used for each group and each value is expressed as the mean±S.E. The riboflavin-deficient synthetic diet(no treatment) was used as a basal diet. The diet supplemented with 0.1 mg, 0.5 mg or 2.0 mg of riboflavin/100 g of the basal diet with and without copper was used.
a) Significantly different from group 7 at $p<0.05$.

CPA Azo Reductase Activity in the Liver Microsome of Rats Given the Riboflavin-deficient Diet with and without Copper

CPA azo reductase activity was significantly decreased in the group 7 given the vasal diet, compared with the groups 8—10 given the riboflavin-supplemented diet. The enzyme activity in the groups 11—14 given the copper-supplemented diet was higher than that in the groups 7—10 given the same diet not supplemented with copper. Not only DAB azo reductase activity but CPA azo reductase activity was also enhanced in the groups given the diet supplemented with copper.

As shown in Tables III and IV, the increase of CPA azo reductase activity was almost in parallel with that of DAB azo reductase activity in each group studied.

Discussion

It is well known that riboflavin is a factor which affected the level of azo reductase and controlled at the same time the carcinogenic potency of the azo dyes.[11,12] Copper is one of metals which is able to form the complex with riboflavin and flavin nucleotides.[13] Therefore, it is possible that copper affects the action of flavins by the formation of complex in rats, riboflavin passing through the intestinal mucosa, being carried in blood, being transfered into the liver cell or being accumulated in the liver cell.

As shown in Table I, copper showed the ability to increase the content of riboflavin in the liver microsome and DAB azo reductase activity in the group 4 given the copper-supplemented diet (to CE-2 diet) was increased more than that in the group 3 given the diet supplemented with 50 mg of riboflavin/100 g of CE-2 diet. From the fact shown in Table I, it is suggested that copper may play roles in carrying riboflavin into liver cells from blood through intestinal mucosa and/or accumulating riboflavin in the liver cell.

In the group 7 given the lowest ribofiavin diet, the content of copper decreased clearly in comparison with those of the groups 8—10 given the riboflavin-supplemented diet. In the group 11 given the lowest riboflavin diet with copper, the content of copper also decreased in comparison with those of the groups 12—14 given the diet supplemented with riboflavin and copper. As contrasted with the result that content of copper was not affected by the concentration of riboflavin in diet as shown in Table I, this fact shows that of by low concentration of riboflavin diet the accumulation of copper both in the whole liver and in the liver microsome was affected.

For the explanation that suppressive effect of copper on DAB carcinogenesis in rats is primarily ascribed to the enhancement of DAB metabolism, especially of azo reduction, DAB was used as the substrate for measuring azo reductase activity in a series of our works. Futheremore, we tried to examine whether in the different structure of azo dye the enhancement of azo reductase activity in the liver microsome of rats fed copper is found or not. CPA was chosen as the substrate for measuring the enzyme activity; a product, *p*-aminobenzoic acid, is routinely measured by the procedure initially described by Bratton and Marshall.

As a result it was found that, in the groups given the diet supplemented with copper, the activity CPA azo reductase was increased as much as that of DAB azo reductase.

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