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CHARACTERISTICS OF NITRO REDUCTION OF THE CARCINOGENIC AGENT, 4-NITROQUINOLONE *N*-OXIDE*

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Abstract—The reduction of 4-nitroquinoline *N*-oxide (4-NQO), a potent carcinogen, by rat liver has been investigated in comparison with the reduction of *p*-nitrobenzoic acid (*p*-NBA).

The NADPH and NADH-dependent reductions of 4-NQO by supernatant are about twenty-five times and fifteen times, respectively, higher than those by microsomes. The NADPH-dependent reductions of 4-NQO by microsomes, supernatant and microsomes plus supernatant are about 20, 800 and 300 times, respectively, higher than those of *p*-NBA. The reduction of 4-NQO to 4-HAQO (4-hydroxyaminoquinoline *N*-oxide) by microsomes plus supernatant is about 200 times faster than the reduction of 4-HAQO to 4-AQO (4-aminoquinoline *N*-oxide). By contrast, the reduction of *p*-NBA to *p*-HABA (*p*-hydroxyaminobenzoic acid) by microsomes plus supernatant is only about 2 times faster than the reduction of *p*-HABA to *p*-ABA (*p*-aminobenzoic acid). Thus, the large amount of 4-HAQO is rapidly accumulated in the incubation mixture, whereas only small amount of *p*-HABA is found.

The reduction of 4-NQO is not stimulated by FMN, whereas the reduction of *p*-NBA is stimulated about three to four times. The inhibition of 4-NQO reduction by oxygen in microsomes is about 37 per cent, whereas that of *p*-NBA reduction is 82 per cent. The inhibition of 4-NQO reduction by oxygen in supernatant is only 10 per cent, whereas that of *p*-NBA reduction is 45 per cent. Dicoumarol (1×10^{-4} M) inhibits 4-NQO reduction in supernatant by 94 per cent, whereas *p*-NBA reduction is inhibited only by 29 per cent.

The administrations of phenobarbital and methylcholanthrene stimulates the reduction of *p*-NBA by microsomes plus supernatant, whereas the reduction of 4-NQO is not stimulated.

These results suggest that the reduction of 4-NQO to 4-HAQO is carried out mainly by DT diaphorase, but *p*-NBA is reduced by usual nitro reductase. The accumulation of 4-HAQO in tissue, thus, seems to be a responsible factor for 4-NQO carcinogenesis.

4-NQO, SYNTHESIZED first by Ochai and co-workers,[1] has been found to be a potent carcinogenic compounds by Nakahara and co-workers[2] in 1957. Meanwhile, Okabayashi and Yoshimoto[3] observed that 4-NQO was reduced to 4-HAQO and 4-AQO

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by some microbes such as Aspergillus niger, Candia utilis, and *Esherichia coli.* Successively, Endo and Kume[4] and Shirasu[5] observed that 4-HAQO seems to be as active as, if not more so than, 4-NQO. Meanwhile, Hashimoto and co-workers[6,7] observed that 4-NQO was reduced by homogenate and supernatant of several mammalian tissue. On the other hand, Sugimura and co-workers[8] observed that 4-NQO was reduced to 4-AQO by rat liver homogenate and microsomal fraction.

These results indicate that 4-HAQO seems to be a proximate carcinogen. A number of aromatic amines are known as carcinogen and *N*-hydroxyamino compounds are postulate as proximate carcinogens, such as *N*-hydroxyacetylaminofluorene and *N*-hydroxy-*N*-methyl-4-aminoazobenzene.[9–11] On the other hand, it is well known that numerous aromatic nitro compounds are reduced to aromatic amines by mammalian tissues *via* hydroxyamino compounds,[12–15] but only few compounds are known as weak carcinogens with exception of 4-NQO.[10–16]

In the present communication, we investigated the dynamics of the reduction of 4-NQO by rat liver in comparison with that of *p*-NBA, a well known typical substrate for the nitro reduction,[13,14] in relation to the carcinogenic activity of 4-NQO.

EXPERIMENTAL

Male rats of Wistar strain, weighing about 170–250 g were generally used and the rats were maintained with Oriental Laboratory chow (NMF) and water *ad lib.* In some experiments, female rats, weighing about 150–180 g were treated with phenobarbital (80 mg/kg, i.p.) 24, 48 and 72 hr before sacrifice or treated with methylcholanthrene (25 mg/kg, i.p.) 48 and 72 hr before sacrifice. Phenobarbital was dissolved in distilled water, whereas methylcholanthrene was dissolved in corn oil.

4-NQO was purchased from Daiichikagaku Co. (Tokyo). 4-NAQO and 4-AQO were synthesized from 4-NQO according to the method of Ochiai and Mitarashi[17] and Ochiai and Naito,[18] respectively. *p*-HABA was synthesized from *p*-NBA according to the method of Bauer and Rosenthal.[19]

Preparation of liver microsomes and supernatant. The animals were decapitated and the livers removed, chopped into small pieces, washed well, and homogenized with 3 volumes of 1·15% (isotonic) KCl solution in a Teflon-glass homogenizer. The homogenate was centrifuged at 9000 *g* for 20 min. The 9000 *g* supernatant was centrifuged at 105,000 *g* for 1 hr. The supernatant was decanted and microsomes were rinsed and suspended in 1·15% KCl solution, and then centrifuged and suspended again.

Assays of drug-reducing activities. The typical incubation mixture for *p*-NBA and *p*-HABA consisted of microsomes, supernatant or microsomes plus supernatant (9000 *g* supernatant) equivalent to 250 mg of liver, 20 μmoles of glucose-6-phosphate, 0·8 μmoles of NADP, 1 U of glucose-6-phosphate dehydrogenase, 50 μmoles of nicotinamide, 50 μmoles of $MgCl_2$, 1·4 ml of 0·2 M sodium phosphate buffer (pH 7·4), 1·5 ml of 1·15% KCl, 5 μmoles of *p*-NBA or *p*-HABA, and water to a final volume of 5·0 ml. When NADH was served as co-factor instead of NADPH, 50 μ-moles of alcohol, 1·0 μmoles of NAD and 3 U of alcohol dehydrogenase were used. The typical incubation mixture for 4-NQO or 4-HAQO are the same as that of *p*-NBA or *p*-HABA excepting the use of phosphate buffer of pH 7·0 instead of pH 7·4. The reduction of 4-NQO to 4-HAQO by the presence of supernatant was extremely rapid, thus the amount of supernatant was reduced to 10 mg equivalent liver. The

incubations were carried out in an atmosphere of nitrogen at 37° for 30 min, unless otherwise specified. The reduction of 4-NQO to 4-HAQO in the presence of supernatant was carried out for 10 min instead of 30 min.

The reduction of 4-NQO to 4-HAQO was investigated by the disappearance of 4-NQO and the formation of 4-HAQO and 4-AQO. The reduction of 4-HAQO to 4-AQO was investigated by the disappearance of 4-HAQO and the formation of 4-AQO. The reduction of *p*-NBA to *p*-HABA was investigated by the disappearance of *p*-NBA and the formation of *p*-HABA and *p*-ABA. The reduction of *p*-HABA to *p*-ABA was investigated by the disappearance of *p*-HABA and the formation of *p*-ABA. All the experiments were run in duplicate.

Chemical assays

(1) *Determination of* 4-*NQO*. Add 0·3 ml of the incubation mixture to 15 ml glass-stoppered centrifuge tube containing 3 ml of 6 N HCl and 5 ml of chloroform and shake for 3 min and centrifuge for 5 min. Read the optical absorbance of chloroform layer at 390 mμ

(2) *Determination of* 4-*HAQO*. Add 1·0 ml of the incubation mixture to 15 ml glass-stoppered centrifuge tube containing 0·25 ml of 20% TCA, 0·25 ml of 1 N HC and 4 ml of chloroform. Shake for 3 min and centrifuged for 5 min. Take 1 ml of water layer and transfer into another centrifuge tube. Then add 3 ml of 0·2 M phosphate buffer (pH 7·0) to bring pH about 3. Cool by ice and add 0·5 ml of 1% $NaNO_2$, stand for 30 sec and add 0·5 ml of 4% ammonium sulfamate. The yellow color developed is extracted with 3 ml of chloroform and read the optical density at 420 mμ

(3) *Determination of* 4-*AQO*. One ml of the incubation mixture is transferred to 15 ml glass-stoppered centrifuge tube containing 2·0 ml of 7·5% TCA and centrifuged for 5 min. Take 2·6 ml of the supernatant and transfer into another glass-stoppered centrifuge tube containing 1·5 ml of 10 N HCl. Cool by ice and add 0·5 ml of 1% $NaNO_2$, stand for 10 min and add 0·5 ml of 4% ammonium sulfamate. Stand for 10 min and add 0·2 ml of 0·5% *N*-(1-naphthyl)-ethylendiamine. Stand for 20 min and read the optical density at 510 mμ.

(4) *Determinations of* p-*NBA*, p-*HABA and* p-*ABA*. The determinations of *p*-NBA, *p*-HABA and *p*-ABA were carried out according to the methods as described in a previous paper.[20]

RESULTS

1. *Reduction of* 4-*NQO by* 9000 g *supernatant of liver microsomes under nitrogen atmosphere*

Since the reduction of 4-NQO has been found mainly in the post-mitochondrial supernatant of liver,[6–8] the reduction of 4-NQO by 9000 *g* supernatant has been comparatively studied by using NADPH or NADH as co-factor. The reduction of 4-NQO by 9000 *g* supernatant in the presence of abundant NADP and glucose-6-phosphate was parallel to the amount of added 9000 *g* supernatant (Figs. 1 A and 2 A). Moreover, the formation of 4-HAQO and 4-AQO were parallel to the amount of added 9000 *g* supernatant. It was clearly observed that the amount of 4-HAQO accumulated was much greater than that of 4-AQO formed. These results, thus, indicate that the reduction of 4-NQO to 4-HAQO is much faster than that of 4-HAQO to 4-AQO. These results were in accordance with previous observations.[6,8]

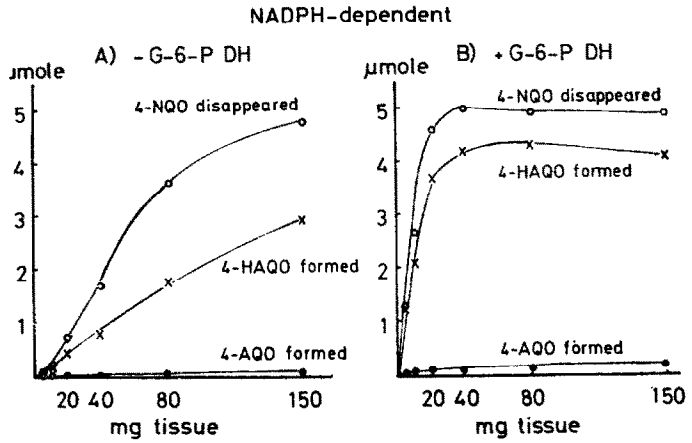

FIG. 1. The reduction of 4-NQO by various amounts of 9000 *g* supernatant in the presence of NADPH. The standard incubation mixture consisted of various amounts of 9000 *g* supernatant, 1·0 μmole of NADP, 20 μmoles of glucose-6-phosphate, 2 U of glucose-6-phosphate dehydrogenase, 25 μmoles of nicotinamide, 25 μmoles of $MgCl_2$, 0·7 ml of 0·2 M sodium phosphate buffer (pH 7·4), 5 μmoles of 4-NQO, various amount of 1·15% KCl and water to a final volume of 2·5 ml. The incubation time was 10 min under nitrogen atmosphere. (A) Glucose-6-phosphate dehydrogenase omitted. (B) Glucose-6-phosphate dehydrogenase added. The results are expressed by the amount of 4-NQO disappeared and 4-HAQO and 4-AQO found in the incubation mixture.

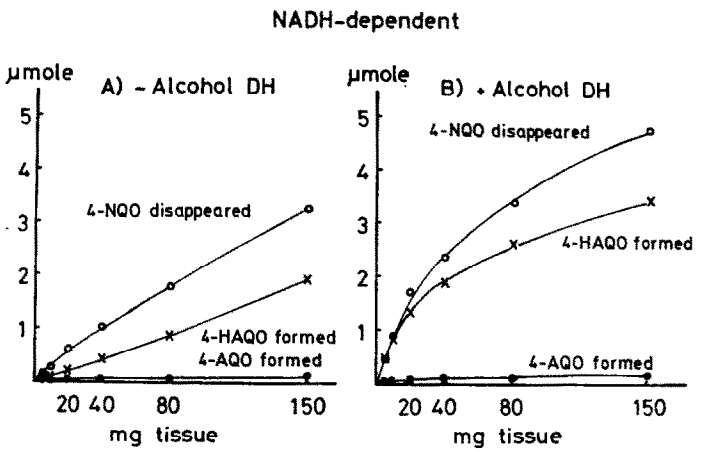

FIG. 2. The reduction of 4-NQO by various amounts of 9000 *g* supernatant in the presence of NADH. 1·0 μmole of NAD, 80 μmoles of alcohol and 5 U of alcohol dehydrogenase were used instead of NADP, glucose-6-phosphate and glucose-6-phosphate dehydrogenase, respectively. The incubation time was 10 min under nitrogen atmosphere. (A) Alcohol dehydrogenase omitted. (B) Alcohol dehydrogenase added.

However, since the reduction of 4-NQO seemed to be much faster than that of NADP or NAD by endogenous dehydrogenases in the incubation mixture, enough of the dehydrogenases were added and the reductive activities were comparatively studied. As shown in Figs. 1 B and 2 B, the rate of the reductions of 4-NQO were increased about 10-fold by the addition of glucose-6-phosphate dehydrogenase. These results indicate that the activities of the endogenous glucose-6-phosphate dehydrogenase and alcohol dehydrogenase are slower than that of 4-NQO reductase. Under the absence of enough regenerating systems for NADPH and NADH the amount of 4-HAQO formed was about 50–60 per cent of 4-NQO reduced. These results suggest that 4-NSQO (4-nitrosoquimolime *N*-oxide) may be accumulated as an intermediate under such condition. The reduction of 4-NQO under nitrogen atmosphere was linear for 20 min, but after then the rate of reduction was decreased probably due to lack of 4-NQO (Fig. 3). 4-HAQO was accumulated in the incubation mixture and the amount of 4-AQO formed was linearly increased for 60 min.

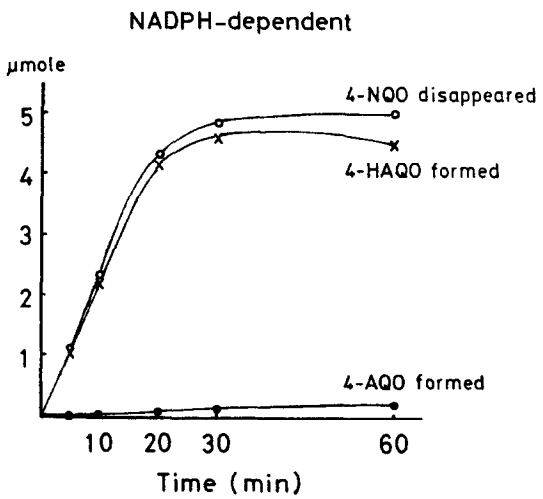

FIG. 3. Time course study on the reduction of 4-NQO and formations of 4-HAQO and 4-AQO by 9000 *g* supernatant in the presence of NADPH. The composition of the incubation mixture was the same as in Fig. 1 B. The amount of 9000 *g* supernatant was 10 mg liver. The results are expressed by the amount of 4-NQO disappeared and 4-HAQO and 4-AQO found in the incubation mixture.

2. *Reduction of* 4-*NQO*, 4-*HAQO*, p-*NBA and* p-*HABA by liver microsomes and supernatant under nitrogen atmosphere*

4-NQO was rapidly reduced to 4-HAQO by microsomes and supernatant in the presence of NADPH (Table 1). However, the activity of supernatant was about twenty times higher than that of microsomes. Whereas, the reduction of 4-HAQO to 4-AQO by microsomes and supernatant was much slower than that of 4-NQO to 4-HAQO, and the activity of 4-HAQO reduction was somewhat higher in microsomes than in supernatant. Thus, the rate of reduction of 4-NQO to 4-AQO by supernatant was similar to that by microsomes and the activity of microsomes plus supernatant was higher than the sum of microsomes and supernatant. In most experiments, the amount

of the disappearance of 4-NQO was almost similar to that of the formation of 4-HAQO plus 4-AQO, but the amount of the disappearance of 4-HAQO was always a little smaller than that of the formation of 4-AQO.

On the other hand, the reduction of *p*-NBA to *p*-HABA by microsomes and supernatant was much slower than that of 4-NQO to 4-HAQO. The *p*-NBA reduction, especially in supernatant was about 0·1 per cent of the 4-NQO reduction. Similar results were observed when NADH was used as co-factor instead of NADPH.

TABLE 1. REDUCTION OF 4-NQO, 4-HAQO, *p*-NBA AND *p*-HABA BY LIVER MICROSOMES AND SUPERNATANT UNDER THE PRESENCE OF NADPH GENERATING SYSTEM

Substrate	determination	Reductive activity (μmole/g liver/min)		
		Microsomes	Supernatant	Microsome + Supernatant
4-NQO	4-NQO disappearance	1·210 ± 0·085	28·498 ± 2·181	30·021 ± 3·515
	4-HAQO formation	1·124 ± 0·108	28·241 ± 1·952	29·388 ± 2·712
	4-AQO formation	0·031 ± 0·002	0·036 ± 0·003	0·098 ± 0·012
4-HAQO	4-HAQO disappearance	0·045 ± 0·008	0·035 ± 0·005	0·088 ± 0·012
	4-AQO formation	0·042 ± 0·005	0·029 ± 0·002	0·007 ± 0·005
p-NBA	*p*-NBA disappearance	0·059 ± 0·005	0·037 ± 0·003	0·093 ± 0·009
	p-HABA formation	0·032 ± 0·003	0·030 ± 0·005	0·058 ± 0·007
	p-ABA formation	0·024 ± 0·002	0·002 ± 0·001	0·029 ± 0·002
p-HABA	*p*-HABA disappearance	0·033 ± 0·004	0·029 ± 0·004	0·054 ± 0·009
	p-ABA formation	0·028 ± 0·002	0·021 ± 0·003	0·048 ± 0·005

Twenty μmoles of glucose-6-phosphate, 0·8 μmole of NADP and 1U of glucose-6-phosphate dehydrogenase were used. The incubation was run in duplicate in an atmosphere of nitrogen for 30 min with the microsomes and supernatant equivalent to 250 mg liver, excepting the reduction of 4-NQO to 4-HAQO under the presence of the supernatant in which the tissues equivalent to 10 mg liver were used and incubated for 10 min. The results are given the amount of the substrate disappeared or the amount of the metabolites formed by g wet weight of liver and expressed as the average ± S.E. of four to six determinations. The pooled liver from three to five rats was used and the determinations were run in duplicate.

The reductions of 4-NQO to 4-HAQO and 4-AQO and of 4-HAQO to 4-AQO in the presence of NADH were slower than those in the presence of NADPH (Table 1 and 2) and the reduction in microsomes was especially slow. As reported in a previous paper[20] the activity of *p*-NBA reduction to *p*-HABA in the presence of NADH was extremely low in microsomes, thus negligible amount of *p*-ABA was formed from *p*-NBA. Moreover, a fairly large amount of 4-NQO was reduced by liver mitochondria in the presence of NADPH or NADH, but the reduction of *p*-NBA was completely absent (Kato *et al.*, unpublished observation).

3. *Stimulatory effect of FMN on the reductions of* 4-*NQO*, 4-*HAQO*, p-*NBA and* p-*HABA by liver microsomes and supernatant*

Since the reduction of *p*-NBA by liver microsomes is markedly stimulated by flavin adenine dinucleotide (FAD), flavin mononucleotide (FMN) and riboflavin,[13,14,20] the effect of the flavins on the reduction of 4-NQO, 4-HAQO, *p*-NBA and *p*-HABA by microsomes, supernatant and microsomes plus supernatant was comparatively

TABLE 2. REDUCTION OF 4-NQO, 4-HAQO, *p*-NBA AND *p*-HABA BY LIVER MICROSOMES AND SUPERNATANT UNDER THE PRESENCE OF NADH GENERATING SYSTEM

Substrate determination		Reductive activity (μmole/g liver/min)		
		Microsomes	Supernatant	Microsomes + Supernatant
4-NQO	4-NQO disappearance	0·758 ± 0·052	11·507 ± 1·102	12·112 ± 0·985
	4-HAQO formation	0·731 ± 0·071	10·518 ± 0·953	11·046 ± 1·121
	4-AQO formation	0·013 ± 0·002	0·025 ± 0·002	0·073 ± 0·009
4-HAQO	4-HAQO disappearance	0·031 ± 0·005	0·025 ± 0·006	0·064 ± 0·007
	4-AQO formation	0·029 ± 0·003	0·020 ± 0·003	0·052 ± 0·006
p-NBA	*p*-NBA disappearance	0·004 ± 0·001	0·035 ± 0·003	0·050 ± 0·004
	p-HABA formation	0·003 ± 0·001	0·028 ± 0·004	0·031 ± 0·003
	p-ABA formation	0·001 ± 0·001	0·002 ± 0·001	0·012 ± 0·002
p-HABA	*p*-HABA disappearance	0·030 ± 0·004	0·030 ± 0·006	0·049 ± 0·006
	p-ABA formation	0·028 ± 0·002	0·021 ± 0·003	0·040 ± 0·003

50 μmoles of alcohol, 1·0 μmole of NAD and 3 U of alcohol dehydrogenase were used. See the legends for Table 1.

TABLE 3. EFFECT OF FMN ON THE REDUCTION OF 4-NQO, 4-HAQO, *p*-NBA AND *p*-HABA BY LIVER MICROSOMES AND SUPERNATANT UNDER THE PRESENCE OF NADPH GENERATING SYSTEM

Substrate	Enzyme	Relative reductive activity (control = 100)		
		$NO_2 \rightarrow NHOH$	$NO_2 \rightarrow NH_2$	$NHOH \rightarrow NH_2$
4-NQO or 4-HAQO	Microsomes	102 ± 5 (5)	175 ± 9 (5)	170 ± 12 (5)
	Supernatant	95 ± 3 (4)	108 ± 5 (4)	135 ± 5 (4)
	Mic. + Super.	96 ± 6 (5)	138 ± 4 (5)	158 ± 7 (5)
p-NBA or *p*-HABA	Microsomes	415 ± 25 (6)	468 ± 21 (6)	165 ± 4 (5)
	Supernatant	368 ± 31 (5)	407 ± 15 (6)	121 ± 5 (4)
	Mic. + Super.	388 ± 19 (8)	451 ± 20 (8)	142 ± 3 (5)

The concentration of FMN in the incubation mixture was 5×10^{-4} M. The reductive activity is given by the formation of the metabolites indicated in the Table. The results are expressed as relative values to control and given as average ± S.E. The figures in the parentheses indicate the number of the determination. The pooled liver from three to five rats were used and the determinations were run in duplicate.

studied. The reduction of *p*-NBA to *p*-HABA by microsomes and supernatant was markedly stimulated by FMN. Whereas the reduction of 4-NQO to 4-HAQO was not significantly affected (Table 3). On the other hand, the reductions of *p*-HABA to *p*-ABA and of 4-HAQO to 4-AQO by microsomes and supernatant were stimulated in similar extent by FMN. Similar results were obtained by FAD and riboflavin. Moreover, similar tendency was observed when NADH was served as co-factor instead of NADPH.

4. *Inhibitory effect of oxygen on the reductions of* 4-*NQO*, 4-*HAQO*, p-*NBA and* p-*HABA by liver microsomes and supernatant*

Since the reduction of *p*-NBA is inhibited in the presence of oxygen,[13,14,20] the

effect of oxygen on the reduction of 4-NQO, 4-HAQO- *p*-NBA and *p*-HABA by microsomes and supernatant was comparatively studied by the incubation under air.

The reduction of *p*-NBA to *p*-HABA by microsomes was markedly inhibited in the presence of oxygen, whereas the reduction of 4-NQO to 4-HAQO was less sensitively affected (Table 4).

The reduction of *p*-NBA to *p*-HABA by supernatant was inhibited by oxygen, whereas the reduction of 4-NQO to 4-HAQO was only slightly inhibited. The detailed mechanism of the oxygen inhibition on the reduction of *p*-NBA and *p*-HABA was not yet elucidated, but it has been supposed that *p*-HABA is oxidized by atmospheric

TABLE 4. EFFECT OF OXYGEN ON THE REDUCTION OF 4-NQO, 4-HAQO, *p*-NBA AND *p*-HABA BY LIVER MICROSOMES AND SUPERNATANT UNDER THE PRESENCE OF NADPH GENERATING SYSTEM

Substrate	Enzyme	Relative reductive activity (control = 100)		
		$NO_2 \rightarrow NHOH$	$NO_2 \rightarrow NH_2$	$NHOH \rightarrow NH$
4-NQO or 4-HAQO	Microsomes	63 ± 5 (6)	30 ± 1 (5)	52 ± 3 (4)
	Supernatant	90 ± 3 (6)	88 ± 4 (5)	72 ± 4 (4)
	Mic. + Super.	87 ± 2 (6)	62 ± 2 (5)	66 ± 4 (4)
p-NBA or *p*-HABA	Microsomes	18 ± 2 (5)	3 ± 1 (6)	76 ± 4 (4)
	Supernatant	55 ± 4 (5)	44 ± 3 (6)	86 ± 8 (4)
	Mic. + Super.	36 ± 2 (5)	8 ± 1 (6)	77 ± 3 (4)

The incubation was run under air instead of an atmosphere of nitrogen. The reductive activity is given by the formation of the metabolites indicated in the table. The results are expressed as relative values to control values and given as average ± S.E. The figures in the parentheses indicate number of the determination. The pooled liver from three to five rats were used and the determinations were run in duplicate.

oxygen to *p*-NSBA, which is supposed to be an intermediate of the reduction of *p*-NBA to *p*-HABA and the accumulation of *p*-NSBA may inhibit the reduction of of *p*-NBA.[14,20,21]

It is, therefore, possible to speculate that the reduction of 4-NQO to 4-HAQO is extremely rapid, thus the reductive activity may overcome the autoxidation of 4-HAQO to 4-NSQO. Alternatively, it is also possible to suppose that the oxidation of 4-HAQO by oxygen is more resistant than that of *p*-HABA.

5. *Inhibitory effect of dicoumarol on the reductions of* 4-*NQO*, 4-*HAQO*, p-*NBA and* p-*HABA by liver microsomes and supernatant*

Sugimura *et al.*[21] reported that the reduction of 4-NQO by a partial purified enzymes from liver homogenate was inhibited by dicoumarol. Thus the effect of dicoumarol on the reduction of 4-NQO and *p*-NBA by microsomes and supernatant was comparatively studied.

The reduction of 4-NQO to 4-HAQO in microsomes was markedly inhibited by dicoumarol, whereas the reduction in supernatant was only partially inhibited, thus the reduction by microsomes plus supernatant was inhibited about 60 per cent (Table 5).

The inhibition in the reduction of 4-HAQO to 4-AQO by dicoumarol in supernatant was less than that in the reduction of 4-NQO to 4-HAQO. On the other hand, the

TABLE 5. EFFECT OF DICOUMAROL ON THE REDUCTION OF 4-NQO, 4-HAQO, *p*-NBA AND *p*-HABA BY LIVER MICROSOMES AND SUPERNATANT UNDER THE PRESENCE OF NADPH GENERATING SYSTEM

Substrate	Enzyme	Relative reductive activity (control = 100)		
		$NO_2 \rightarrow NHOH$	$NO_2 \rightarrow NH_2$	$NHOH \rightarrow N_2$
4-NQO or 4-HAQO	Microsomes	75 ± 3 (5)	82 ± 3 (5)	55 ± 3 (4)
	Supernatant	6 ± 3 (5)	48 ± 2 (5)	71 ± 4 (4)
	Mic. + Super.	22 ± 4 (5)	62 ± 3 (5)	61 ± 4 (4)
p-NBA or *p*-HABA	Microsomes	97 ± 2 (4)	92 ± 2 (4)	88 ± 3 (4)
	Supernatant	51 ± 4 (4)	44 ± 4 (4)	71 ± 4 (4)
	Mic. + Super.	95 ± 2 (4)	90 ± 3 (4)	85 ± 2 (4)

The concentration of dicoumarol in the incubation mixture was 1×10^{-4} M. The reductive activity is given by the formation of the metabolites indicated in the table. The results are expressed as relative values to control values and given as average ± S.E. The figures in the parentheses indicate the number of the determination. The pooled liver from three to four rats were used and the determinations were run in duplicate.

TABLE 6. EFFECT OF PHENOBARBITAL TREATMENT ON THE REDUCTION OF 4-NQO, 4-HAQO, *p*-NBA AND *p*-HABA BY LIVER MICROSOMES AND SUPERNATANT UNDER THE PRESENCE OF NADPH CENERATING SYSTEM

Substrate	Enzyme	Relative reductive activity (control = 100)		
		$NO_2 \rightarrow NHOH$	$NO_2 \rightarrow NH_2$	$NHOH \rightarrow NH$
4-NQO or 4-HAQO	Microsomes	132 ± 8 (6)	129 ± 7 (5)	115 ± 6 (4)
	Supernatant	103 ± 5 (6)	105 ± 6 (5)	99 ± 5 (4)
	Mic. + Super.	110 ± 4 (6)	108 ± 7 (5)	110 ± 3 (4)
p-NBA or *p*-HABA	Microsomes	183 ± 8 (6)	213 ± 10 (6)	121 ± 5 (4)
	Supernatant	112 ± 6 (5)	108 ± 5 (5)	107 ± 4 (4)
	Mic. + Super.	169 ± 7 (6)	181 ± 5 (6)	117 ± 6 (4)

The rats were treated with phenobarbital (80 mg/kg, i.p.) 72 hr, 48 hr and 24 hr before sacrifice. The reductive activity is given by the formation of the metabolites indicated in the Table. The results are expressed as relative values to control values and given as average ± standard error. The pooled liver from three to four rats were used and the determinations were run in duplicate.

reduction of *p*-NBA to *p*-HABA in microsomes was not inhibited by dicoumarol, but the reduction in the supernatant was inhibited by 50 per cent. The reduction of *p*-HABA to *p*-ABA was slightly inhibited by dicoumarol, especially in supernatant and the magnitude of the inhibition was similar to that of the reduction of 4-HAQO to 4-AQO.

These results indicate that the reduction of 4-NQO to 4-HAQO and a part of the reduction of *p*-NBA to *p*-HABA in the supernatant is mediated through dicoumarol sensitive pathway.

6. *The effect of phenobarbital or methylcholanthrene treatment in the reductions of 4-NQO, 4-HAQO, p-NBA and p-HABA by liver microsomes*

In previous paper the stimulation of the reduction of *p*-NBA to *p*-HABA and

p-ABA by the treatment with phenobarbital or methylcholanthrene was reported.[20] Thus the effects of phenobarbital or methylcholanthrene treatment on the reductions of 4-NQO and 4-HAQO were studied.

The reduction of 4-NQO to 4-HAQO in microsomes was slightly increased by the phenobarbital treatment (Table 6), whereas the reduction of 4-HAQO to 4-AQO was practically not stimulated. Similarly, the phenobarbital treatment stimulated the reduction of *p*-NBA to *p*-HABA in microsomes.

It was also observed that the treatment with methylcholanthrene slightly stimulated the reductions of 4-NQO to 4-HAQO and *p*-NBA to *p*-HABA in microsomes.

DISCUSSION

In the present studies, unusual nitro reduction of 4-NQO has been demonstrated by the comparison of the nitro reduction of *p*-NBA, a typical substrate for nitro reductase.

p-NBA is reduced mainly by microsomes under presence of NADPH, while 4-NQO is very rapidly reduced by supernatant and more than 95 per cent activity is found in supernatant. The NADPH-dependent reduction of *p*-NBA to *p*-HABA is somewhat slower than that of *p*-HABA to *p*-ABA, thus only small amount of *p*-HABA is found in the incubation mixture. By contrast, the reduction of 4-NQO to 4-HAQO is about fifteen times faster in microsomes and 1000 times faster in the supernatant that that of 4-HAQO to 4-AQO.

Moreover, the NADH-dependent reduction of *p*-NBA by microsomes is extremely slow, whereas a higher activity of the NADH-dependent reduction of 4-NQO to 4-HAQO is found in microsomes.

The reduction of *p*-NBA to *p*-HABA is stimulated by FMN, while the reduction of 4-NQO to 4-HAQO is not stimulated. Furthermore, the reduction of *p*-NBA to *p*-HABA is markedly inhibited under atmosphere of air, but the reduction of 4-NQO to 4-HAQO is not practically inhibited. These results indicated that the reduction of *p*-NBA to *p*-HABA was mainly exerted by the nitro reductase of liver microsomes, but that of 4-NQO to 4-HAQO was exerted by other enzymes. These results are in accord with the recent observations of Sugimura *et al.*[27] that 4-NQO is reduced by partially purified DT diaphorase, but 4-nitroquinoline is not reduced by this enzyme.

According to Ernster *et al.*,[23] liver DT diaphorase is mainly present in supernatant and some activity is found in microsomes. On the other hand, Sato[24] has observed only negligible DT diaphorase activity in liver microsomes by the fractionation with isotonic KCl.

In the present experiments, 1×10^{-4} M dicoumarol inhibits the activity of 4-NQO reduction by 94 per cent in supernatant and decrease of 49 per cent is observed in the reduction of *p*-NBA. The reduction of 4-NQO to 4-HAQO by microsomes is slightly inhibited by dicoumarol, while the reduction of *p*-NBA to *p*-HABA by microsomes is not practically inhibited by dicoumarol.

These results indicate that the reduction of *p*-NBA to *p*-HABA by supernatant is partially exerted by DT diaphorase and microsomes is probably contaminated by DT diaphorase. However, nonspecific inhibitory action of such relatively high concentration of dicoumarol could not be excluded.

Moreover, the pretreatment with phenobarbital increased the accumulation of *p*-HABA by microsomes, and microsomes plus supernatant, but the accumulation of

4-HAQO was slightly increased by microsomes and practically not increased by microsomes plus supernatant.

These results suggest that 4-NQO is rapidly reduced to 4-HAQO by DT diaphorase, but other nitro compounds are slowly reduced only by so-called nitro reductase. A large amount of 4-HAQO, therefore, may be accumulated *in vivo* after the administration of 4-NQO, but hydroxyamino derivatives of other nitro compounds are probably not accumulated as the case of *p*-NBA.

A number of hydroxyamino compounds are known as proximate carcinogens and it is supposed that the formation of the hydroxyamino compounds *in vivo* is the important process for the carcinogenic action.[10,11] Numerous aromatic amines are known as potent carcinogen and they are hydroxylated to proximate carcinogen and accumulated *in vivo* in some extent.[10] On the other hand, none of the aromatic nitro compounds, excepting 4-NQO, are known as potent carcinogen, because probably they are not rapidly reduced and may be not accumulated *in vivo* as demonstrated in the present studies under presence of oxygen. In fact, 2-nitrofluorene is reduced very slowly to 2-hydroxyaminofluorene. Therefore, the former is known as a non-carcinogen, nevertheless the latter is known as a very potent carcinogen.[10,25]

REFERENCES

1. E. Ochiai, M. Ichikawa and S. Sai, *Yakugaku Zasshi* **63**, 280 (1943).
2. W. Nakahara, F. Fukuoka and T. Sugimura, *Gann* **48**, 129 (1957).
3. T. Okabayashi and A. Yoshimoto, *Chem. Pharm. Bull. (Tokyo)* **16**, 1211 (1962).
4. H. Endo and F. Kume, *Gann* **54**, 443 (1963).
5. Y. Shirasu, *Gann* **54**, 487 (1963).
6. Y. Hashimoto, M. Terasawa and N. Toriyama, *Seikagaku* **36**, 557 (1964).
7. N. Karasuyama, *Nichidai Igaku Zasshi* **24**, 423 (1965).
8. T. Sugimura, K. Okabe and H. Endo, *Gann* **56**, 489 (1965).
9. E. C. Miller, J. A. Miller and H. A. Hartmann, *Cancer Res.* **21**, 815 (1961).
10. E. C. Miller and J. A. Miller, *Pharmac. Rev.* **18**, 805 (1966).
11. L. A. Poirier, J. A. Miller, E. C. Miller and K. Sato, *Cancer Res.* **27**, 1600 (1967).
12. R. T. Williams, *Detoxication Mechanisms*, Chapman and Hall, London (1959).
13. J. R. Fouts and B. B. Brodie, *J. Pharmac. exp. Ther.* **119**, 197 (1957).
14. J. R. Gillette, *Progress in Drug Research* (Ed. E. Jucker), vol. 6, p. 13, Birkhauer, Basel (1963).
15. R. Kato and T. Oshima, *Jap. J. Pharmac.* **18**, 369 (1968).
16. J. A. Miller and E. C. Miller, *Lab. Invest.* **15**, 217 (1966).
17. E. Ochiai and H. Mitarashi, *Ann. Rep. Itsuu Lab.* **13**, 22 (1963).
18. E. Ochiai and T. Naito, *Yakugaku Zasshi* **64**, 206 (1944).
19. H. Bauer and S. M. Rosenthal, *J. Am. Chem. Soc.* **66**, 611 (1944).
20. R. Kato, T. Oshima and A. Takanaka, *Molec. Pharmac.* in press.
21. H. Uehleke, *Naturwissenschaften* **50**, 335 (1963).
22. T. Sugimura, K. Okabe and M. Nagao, *Cancer Res.* **26** 1717 (1966).
23. L. Ernster, L. Danielson and M. Ljunggren, *Biochim. biophys. Acta* 58, 171 (1962).
24. R. Sato, Personal communication.
25. P. D. Lotikar, E. C. Miller, J. A. Miller and A. Maagreth, *Cancer Res.* **25**, 1743 (1965).