Studies on the Mechanism of Nitro Reduction by Rat Liver

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SUMMARY

The reduction of *p*-nitrobenzoic acid by rat liver was studied by measurement of the disappearance of *p*-nitrobenzoic acid and the formation of *p*-hydroxyaminobenzoic acid and of *p*-aminobenzoic acid. The mechanisms by which flavin mononucleotide stimulates, oxygen inhibits, and phenobarbital and methylcholanthrene induce this enzyme system were particularly investigated.

The NADPH-linked and NADH-linked reductases for *p*-nitrobenzoic acid were assumed to be separate enzymes because of differences in their intracellular localization. The rate of the NADH-linked reduction of *p*-nitrobenzoic acid to *p*-hydroxyaminobenzoic acid is negligible in microsomes, whereas the rate of the NADPH-linked reduction of *p*-nitrobenzoic acid to *p*-hydroxyaminobenzoic acid is comparable to that of the reduction of *p*-hydroxyaminobenzoic acid to *p*-aminobenzoic acid.

The reduction of *p*-nitrobenzoic acid to *p*-hydroxyaminobenzoic acid is stimulated by flavin mononucleotide, and the reduced compound accumulates in the incubation mixture. Oxygen strongly inhibits the same step, especially in the microsomal fraction. Phenobarbital treatment stimulates primarily the reduction of *p*-nitrobenzoic acid to *p*-hydroxyaminobenzoic acid by microsomes. *p*-Nitrobenzoic acid reductases of the supernatant fraction and of microsomes display different properties with respect to inhibition by oxygen and stimulation by phenobarbital.

INTRODUCTION

A number of aromatic nitro compounds are reduced to aromatic amines by mammalian liver (1). Fouts and Brodie reported that the nitroreductase is localized mainly in the microsomal and the supernatant fractions (2). Although both NADPH and NADH can serve as electron donors, NADPH is about 4 times as effective as NADH. Microsomal nitroreductase is unusual, however, in that it is active only under anaerobic conditions and its activity is markedly stimulated by large amounts of FAD, FMN, and riboflavin (2).

Later Kamm and Gillette investigated the mechanism of nitro reduction of *p*-nitrobenzoic acid by a solubilized preparation of liver microsomes obtained with purified pancreatic lipase (3). They observed that the reduction of the nitro group seemed to be carried out by NADPH–cytochrome *c* reductase of liver microsomes. Added FAD was reduced by NADPH–cytochrome *c* reductase, and the reduced FAD then nonenzymatically reduced the nitro group to the amine.

Since a variety of aromatic hydroxyamino compounds have recently been recognized as proximate carcinogens, it is important to obtain detailed information on the nitroreductase systems of mammalian liver.

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In nearly all previous studies, measurements were made only of the formation of aromatic amines after the incubation of nitro compounds with tissue components. In the present investigation, the step of nitro reduction was separated into the reduction of the nitro function to the hydroxyamino group and the reduction of the hydroxyamino group to the amine. The following points were emphasized in the present investigation: (*a*) the intracellular distribution of the reductases, (*b*) the cofactor requirement, (*c*) the mechanism of inhibition by oxygen, (*d*) the mechanism of stimulation by FMN and FAD, and (*e*) the mechanism of induction by phenobarbital and methylcholanthrene.[3]

MATERIALS AND METHODS

Male rats of the Wistar strain, weighing about 170–200 g, were used unless otherwise specified. Female rats of the Wistar strain were treated with sodium phenobarbital (70 mg/kg) for 3 days or with methylcholanthrene (25 mg/kg) for 2 days before being killed. Phenobarbital sodium and methylcholanthrene were dissolved in distilled water and olive oil, respectively, and both drugs were given intraperitoneally.

The rats were killed by decapitation, and the livers were rapidly removed, chopped into small pieces, washed thoroughly, and homogenized with 3 volumes of isotonic (1.15%) KCl in a Teflon-glass homogenizer.

Preparation of Supernatant and Microsomal Fractions

The homogenate was centrifuged for 20 min at $9000 \times g$, and the supernatant fluid was then centrifuged for 60 min at $105{,}000 \times g$. The supernatant fluid and microsomal pellet were separated by decantation, and the microsomal pellet was rinsed once and suspended in 1.15% KCl.

[3] A preliminary account of the present communication was presented at the 24th Annual Meeting of the Pharmaceutical Society of Japan (April 1967) and published in brief form in the *Japanese Journal of Pharmacology* [18, 369 (1968)].

Assay of Reducing Activity

A typical incubation mixture contained the following ingredients in a total volume of 5 ml: 2 ml of tissue preparation, equivalent to 500 mg of liver; 0.8 μmole of NADP or NAD; 20 μmoles of glucose 6-phosphate or 40 μmoles of ethanol; 25 μmoles of $MgCl_2$; 50 μmoles of nicotinamide; 1.4 ml of 0.2 M sodium phosphate buffer, pH 7.4; 1.0 ml of 1.15% KCl; and 5 μmoles of *p*-nitrobenzoic acid or *p*-hydroxyaminobenzoic acid. Glucose 6-phosphate dehydrogenase (0.5 unit; 0.5 μmole of NADP reduced per minute) or alcohol dehydrogenase (2 units; 2 μmoles of NAD reduced per minute) was added when microsomes were used. After the addition of the incubation mixture, the flask was sealed with a gum stopper equipped with a double cup and gassed for 30 sec through a needle. The incubation was carried out in duplicate in 20-ml Erlenmeyer flasks under an atmosphere of nitrogen at 37° for 30 min unless otherwise specified.

Chemical Assays

Determination of p-nitrobenzoic acid. Transfer 1 ml of the incubated mixture into 15-ml glass-stoppered centrifuge tubes containing 1 ml of 1 N HCl and 10 ml of chloroform. Shake for 5 min and centrifuge for 5 min. Thansfer a 2-5 ml aliquot of the chloroform layer to another centrifuge tube containing 2.5 ml of 0.5 N NaOH. Shake for 5 min and centrifuge for 5 min. Transfer 1 ml of the NaOH layer to a test tube containing 3 ml of 1 N HCl. Read the optical density at 263 mμ and 288 mμ.

Determination of p-hydroxyaminobenzoic acid. Transfer the incubation mixture (3 ml) to 50-ml glass-stoppered centrifuge tubes containing 1 ml of 2 N HCl and 2.5 g of NaCl. Add 25 ml of ethyl acetate containing 10% *n*-hexane, shake for 15 min, and centrifuge for 5 min. Transfer 20 ml of the solvent phase to another centrifuge tube containing 4.5 ml of 5% sodium acetate and 0.3 ml of 2 N NaOH. Shake for 3 min and centrifuge for 3 min. Transfer a 3-ml aliquot of the aqueous phase to a test tube containing 0.2 ml of 0.3% pentacyanoaquaferroate (4). Allow to stand for

20 min, and read the optical density at 535 mμ. Since *p*-hydroxyaminobenzoic acid is oxidized to *p*-nitrosobenzoic acid in this procedure, this method determines both compounds.

Determination of p-aminobenzoic acid. *p*-Aminobenzoic acid was determined according to a slight modification of the method of Fouts and Brodie (2). This method has been used as a specific procedure for the determination of *p*-aminobenzoic acid, but in the present study it was found that *p*-hydroxyaminobenzoic acid also formed the same diazo compound (5), in an average yield of about 8% of *p*-aminobenzoic acid. This evidence was confirmed by two-dimensional thin layer chromatography as described below. In the present communication, therefore, the amount of *p*-aminobenzoic acid was corrected according to the amount of *p*-hydroxyaminobenzoic acid in the incubation mixture.

Preparation of p-Hydroxyaminobenzoic Acid

p-Hydroxyaminobenzoic acid was prepared by the reduction of *p*-nitrobenzoic acid according to the method described by Bauer and Rosenthal (5).

Identification of Metabolites

The metabolites were identified by thin layer chromatography. The thin layer plate with silica gel (Tokyo Kasei) was developed in benzene–acetic acid–ethanol (90:5:5 by volume). The spots were detected by their ultraviolet absorption and the diazo reaction. The main metabolites of *p*-nitrobenzoic acid were identified as *p*-hydroxyaminobenzoic and *p*-aminobenzoic acids. However, it is likely that small amounts of *p*-hydroxyaminobenzoic acid were transformed into less polar compounds on thin layer chromatography, which have not yet been identified.

Determination of Protein

The supernatant and microsomal protein contents were determined according to the method of Lowry *et al.* (6).

Source of Materials.

NADP, NAD, NADPH, NADH, FAD, FMN, riboflavin, glucose 6-phosphate, glucose 6-phosphate dehydrogenase, and alcohol dehydrogenase (yeast) were purchased from Boehringer; other chemicals were reagent grade. All chemicals were used without further purification. The nitrogen gas was purchased from Suzuki Shokan Company, Tokyo, and was not further deoxygenated.

RESULTS

Intracellular distribution and cofactor requirement for activities of p-nitrobenzoate and p-hydroxyaminobenzoate reductases. *p*-Nitrobenzoic acid disappeared progressively with time of incubation when the incubation mixture contained the 9000 × *g* supernatant fraction and NADPH, and *p*-aminobenzoic acid was formed progressively (Fig. 1). The amount of *p*-hydroxyaminobenzoic acid in the incubation mixture increased in proportion to the incubation time up to about 20 min, after which the concentration declined gradually. These results indicate that *p*-hydroxyaminobenzoic acid is a principal intermediate in the reduction of *p*-nitro-

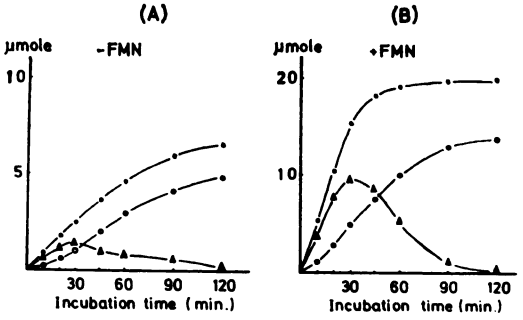

FIG. 1. *Disappearance of p-nitrobenzoic acid and formation of p-hydroxyaminobenzoic acid and p-aminobenzoic acid by microsomes plus supernatant in the presence of NADPH*

A. Without FMN. B. With FMN (5×10^{-4} M). ●——●, Amount of disappearance of *p*-nitrobenzoic acid; ▲——▲, amount of formation of *p*-hydroxyaminobenzoic acid; ○——○, amount of formation of *p*-aminobenzoic acid. Instead of the standard incubation mixture, 2 μmoles of NADP, 80 μmoles of glucose 6-phosphate, and 20 μmoles of *p*-nitrobenzoic acid were used.

TABLE 1

Intracellular distribution and cofactor requirement in reduction of p-nitrobenzoic acid and p-hydroxyaminobenzoic acid

Each experiment was done with fractions obtained from the pooled livers of three to six rats and was run in duplicate. The results are expressed as average ± standard error. The numbers in parentheses indicate the number of experiments. Results are expressed on the basis of 1 g of liver, wet weight.

Tissue and cofactor	NBA[a] reduction		ABA formed from HABA
	HABA formed	ABA formed	
	μmoles/g/30 min		*μmoles/g/30 min*
Microsomes			
NADPH	0.91 ± 0.08 (8)	0.74 ± 0.05 (12)	1.48 ± 0.12 (7)
NADH	0.08 ± 0.02 (8)	0.03 ± 0.01 (10)	1.09 ± 0.08 (6)
Supernatant			
NADPH	0.72 ± 0.05 (8)	0.09 ± 0.02 (12)	0.64 ± 0.04 (7)
NADH	0.65 ± 0.05 (8)	0.07 ± 0.01 (10)	0.54 ± 0.05 (6)
Microsomes + supernatant			
NADPH	1.43 ± 0.12 (8)	1.02 ± 0.05 (12)	2.17 ± 0.17 (7)
NADH	0.81 ± 0.08 (8)	0.42 ± 0.03 (10)	1.57 ± 0.09 (6)

[a] In this and the following tables, where necessary, the abbreviations used are: NBA, *p*-nitrobenzoic acid; HABA, *p*-hydroxyaminobenzoic acid; ABA, *p*-aminobenzoic acid.

benzoic acid. The reduction of the nitro group to the hydroxyamino group seems to be a rate-limiting step, and this step is probably inactivated more easily than the reduction of the hydroxyamino group to the amine. However, the amount of *p*-hydroxyaminobenzoate plus *p*-aminobenzoate formed was usually slightly less than that of the *p*-nitrobenzoate that disappeared. NADH-dependent nitro reduction was slower than NADPH-dependent nitro reduction, but *p*-hydroxyaminobenzoate remained longer in the incubation mixture, as shown in Fig. 2. The studies with microsomes and supernatant fraction revealed that the activity of NADPH-linked *p*-nitrobenzoate reductase was localized in microsomes, and only weak activity was found in the supernatant fraction (Table 1). In contrast, the NADH-linked activity was very low in microsomes, and only weak activity was observed in the supernatant. NADPH-linked *p*-hydroxyaminobenzoate reductase activity was found in both microsomes and supernatant, being greater in the microsomes than in the supernatant. In contrast to NADH-linked *p*-nitrobenzoic acid-reducing activity, the NADH-linked *p*-hydroxyaminobenzoic acid-reducing activity was found in both microsomes and supernatant fractions. The microsomal NADH-linked *p*-hydroxyaminobenzoic acid-reducing activity was about 70% of the microsomal NADPH-linked activity. However, the influence of

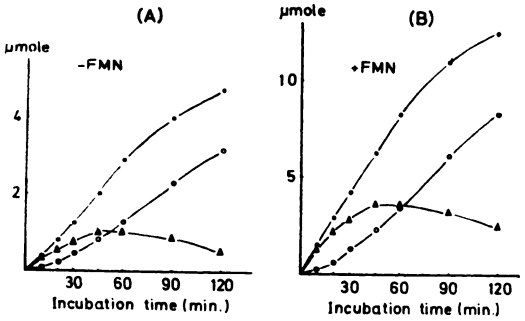

FIG. 2. *Disappearance of p-nitrobenzoic acid and formation of p-hydroxyaminobenzoic acid and p-aminobenzoic acid by microsomes plus supernatant in the presence of NADH*

A. Without FMN. B. With FMN (5×10^{-4} M). ●——●, Amount of disappearance of *p*-nitrobenzoic acid; ▲——▲, amount of formation of *p*-hydroxyaminobenzoic acid; ○——○, amount of formation of *p*-aminobenzoic acid. Instead of the standard incubation mixture, 2 μmoles of NAD, 150 μmoles of ethanol, and 20 μmoles of *p*-nitrobenzoic acid were used.

insufficient generation of NADH on the reduction rate cannot be excluded. The activity of NADPH-linked *p*-hydroxyaminobenzoate reductase was only slightly lower than that of NADPH-linked *p*-nitrobenzoate reductase; therefore, small amounts of *p*-hydroxyaminobenzoic acid accumulated in the incubation mixture. *p*-Nitrobenzoic acid was fairly well reduced to *p*-aminobenzoic acid by microsomes plus supernatant fraction in the presence of NADH. These results suggest that *p*-nitrobenzoic acid is reduced principally by the supernatant, and *p*-hydroxyaminobenzoic acid mainly by the microsomes, in the presence of NADH, and that consequently the ability of microsomes plus supernatant to form *p*-aminobenzoate from *p*-nitrobenzoate is increased.

Inhibition of reduction of p-nitrobenzoate and p-hydroxyaminobenzoate by oxygen. The NADPH-linked reduction of *p*-nitrobenzoic acid to *p*-hydroxyaminobenzoic acid by liver microsomes was decreased by 82% in the presence of oxygen, whereas the decrease in the supernatant activity was less than 50% (Table 2). The NADH-linked and NADPH-linked activities in the supernatant fraction were decreased by a similar extent. On the other hand, the NADPH-linked reduction of *p*-hydroxyaminobenzoic acid in both the supernatant and microsomes was inhibited only slightly by the presence of oxygen. These results indicate that the inhibition by oxygen in the reduction of *p*-nitrobenzoic acid to *p*-aminobenzoic acid takes place mostly in the reduction of *p*-nitrobenzoate to *p*-hydroxyaminobenzoate, and are in accord with the view that the inhibitory effect of oxygen is probably due to the oxidation of *p*-hydroxyaminobenzoic acid to *p*-nitrosobenzoic acid (7).

Stimulation of reduction of p-nitrobenzoate and p-hydroxyaminobenzoate by FMN and FAD. It is clear from Figs. 1 and 2 that the rate of NADPH-linked reduction of *p*-nitrobenzoic acid by microsomes plus supernatant is markedy stimulated by FMN. *p*-Hydroxyaminobenzoic acid accumulated in proportion to the incubation time, but after 20 min the rate of accumulation became slower and the amount of *p*-hydroxyaminobenzoate subsequently declined. Indeed, after 60 min of incubation, *p*-nitrobenzoate and *p*-hydroxyaminobenzoate almost disappeared from the incubation mixture. FMN had similar effects on the NADH-dependent reduction, but *p*-hydroxyamino-

TABLE 2

Inhibition of reduction of p-nitrobenzoic acid and p-hydroxyaminobenzoic acid by oxygen

The incubation was carried out under air instead of nitrogen, and the results are given as a percentage of inhibition of reduction by oxygen. The numbers in parentheses indicate the number of determinations. Pooled livers from three to six rats were used for each determination.

Tissue and cofactor	Inhibition by oxygen		
	NBA reduction		ABA formed from HABA
	HABA formed	ABA formed	
	%	%	%
Microsomes			
NADPH	82 ± 4 (6)	95 ± 4 (6)	25 ± 4 (5)
NADH	85 ± 6 (6)	96 ± 3 (6)	18 ± 3 (5)
Supernatant			
NADPH	46 ± 3 (5)	57 ± 5 (5)	15 ± 3 (4)
NADH	38 ± 4 (5)	51 ± 6 (5)	13 ± 4 (4)
Microsomes + supernatant			
NADPH	69 ± 5 (8)	90 ± 2 (8)	22 ± 4 (6)
NADH	63 ± 4 (8)	82 ± 4 (8)	15 ± 3 (6)

TABLE 3

Stimulation of reduction of p-nitrobenzoic acid and p-hydroxyaminobenzoic acid by FMN

The results are given as a percentage of the stimulation of reduction by FMN. The numbers in parentheses indicate the number of determinations. Pooled livers from three to six rats were used for each determination.

Tissue and cofactor	Stimulation by FMN		
	NBA reduction		ABA formed from HABA
	HABA formed	ABA formed	
	%	%	%
Microsomes			
NADPH	315 ± 25 (6)	368 ± 21 (6)	65 ± 4 (5)
NADH	286 ± 28 (6)	329 ± 17 (6)	21 ± 5 (4)
Supernatant			
NADPH	268 ± 31 (5)	307 ± 15 (6)	42 ± 3 (5)
NADH	259 ± 25 (5)	255 ± 16 (6)	18 ± 4 (4)
Microsomes + supernatant			
NADPH	288 ± 19 (8)	351 ± 20 (8)	51 ± 4 (5)
NADH	302 ± 24 (8)	253 ± 14 (8)	23 ± 5 (4)

benzoic acid seemed to accumulate and disappear more slowly than in the NADPH-dependent reduction. These results indicate that FMN stimulates mainly the reduction of *p*-nitrobenzoate to *p*-hydroxyaminobenzoate. The effect of FMN was dependent on its concentration; maximal stimulation was obtained with 1.0 mM FMN, whereas 0.1 mM FMN produced only 2–3-fold stimulation. The NADPH-linked reduction of *p*-nitrobenzoic acid by microsomes was markedly stimulated by FMN, while its

TABLE 4

Effect of FMN on inhibition of reduction of p-nitrobenzoic acid reduction by oxygen

NADPH was the cofactor. The numbers in parentheses indicate the number of determinations. The results are given as average ± standard error. Pooled livers from three to six rats were used for each determination.

Tissue and addition	NBA reduction			
	HABA formed	Inhibition	ABA formed	Inhibition
	μmoles/g/20 min	%	*μmoles/g/20 min*	%
Microsomes				
No addition	0.63 ± 0.08 (4)		0.50 ± 0.05 (4)	
Oxygen	0.12 ± 0.03 (4)	81.0	0.03 ± 0.01 (4)	94.0
FMN	2.89 ± 0.29 (4)		2.47 ± 0.18 (4)	
Oxygen + FMN	1.01 ± 0.14 (5)	65.1	0.66 ± 0.11 (5)	73.3
Supernatant				
No addition	0.48 ± 0.06 (4)		0.06 ± 0.02 (4)	
Oxygen	0.30 ± 0.04 (4)	37.5	0.03 ± 0.01 (4)	50.0
FMN	1.76 ± 0.24 (4)		0.25 ± 0.04 (4)	
Oxygen + FMN	1.21 ± 0.15 (5)	31.2	0.12 ± 0.04 (5)	52.0
Microsomes + supernatant				
No addition	0.98 ± 0.10 (5)		0.69 ± 0.08 (5)	
Oxygen	0.46 ± 0.07 (5)	53.1	0.07 ± 0.02 (5)	89.9
FMN	3.81 ± 0.35 (5)		3.48 ± 0.39 (5)	
Oxygen + FMN	1.81 ± 0.19 (6)	52.5	1.02 ± 0.09 (6)	70.7

effect on the supernatant activity seemed to be slightly smaller (Table 3). FMN stimulated the microsomal and supernatant NADH-linked activities to approximately the same extent as the NADPH-linked activities. Similar effects were observed when FAD was used instead of FMN. These results indicate that the stimulatory effects of both FMN and FAD on the formation of *p*-aminobenzoic acid in microsomes and supernatant are localized in the reduction of *p*-nitrobenzoic acid to *p*-hydroxyaminobenzoic acid. Thus our observations accord with the view of Kamm and Gillette that FAD stimulates the disappearance of *p*-nitrobenzoic acid in the presence of NADPH and microsomes (3).

Effect of FMN on inhibition of reduction of p-nitrobenzoic acid by oxygen. Since oxygen inhibited the reduction of *p*-nitrobenzoate to *p*-hydroxyaminobenzoate and FMN stimulated this process, the effect of FMN on the inhibitory action of oxygen was investigated. FMN only partially overcame the inhibitory action of oxygen (Table 4). The degree of inhibition of the reduction of *p*-nitrobenzoic acid to *p*-aminobenzoic acid by the microsomal fraction

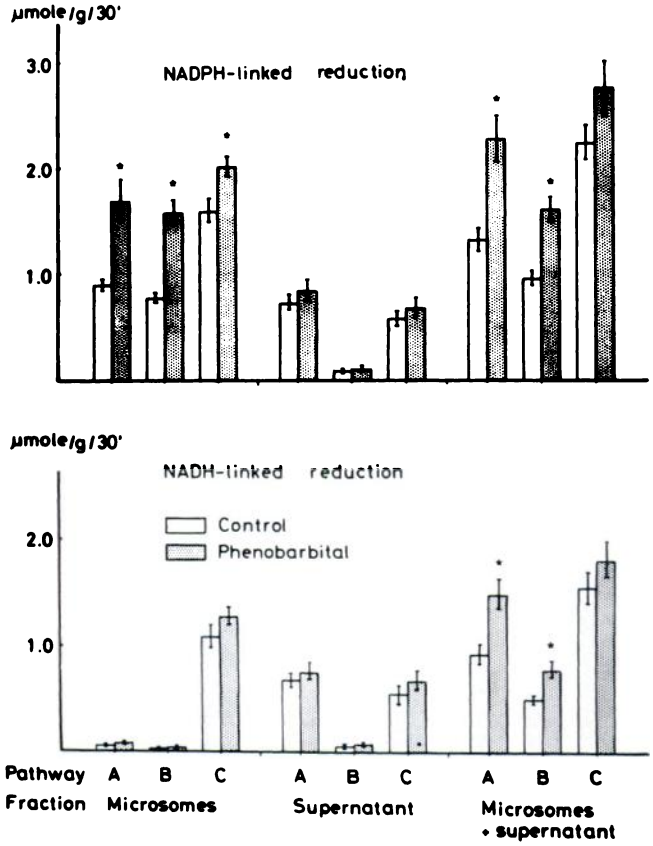

FIG. 3. *Effect of phenobarbital treatment on reduction of p-nitrobenzoic acid and p-hydroxyaminobenzoic acid*

The rats were treated with phenobarbital (80 mg/kg intraperitoneally) for 3 days. The activities are expressed as micromoles of *p*-hydroxyaminobenzoic acid and *p*-aminobenzoic acid formed by the tissue obtained from 1 g of liver in 30 min. The results are the average ± standard error of three or four determinations. Pooled livers from three to five rats were used for each determination. Pathway *A*, reduction of *p*-nitrobenzoic acid to *p*-hydroxyaminobenzoic acid; pathway *B*, reduction of *p*-nitrobenzoic acid to *p*-aminobenzoic acid; pathway *C*, reduction of *p*-hydroxyaminobenzoic acid to *p*-aminobenzoic acid. Asterisks indicate significant difference ($p < 0.05$) from controls.

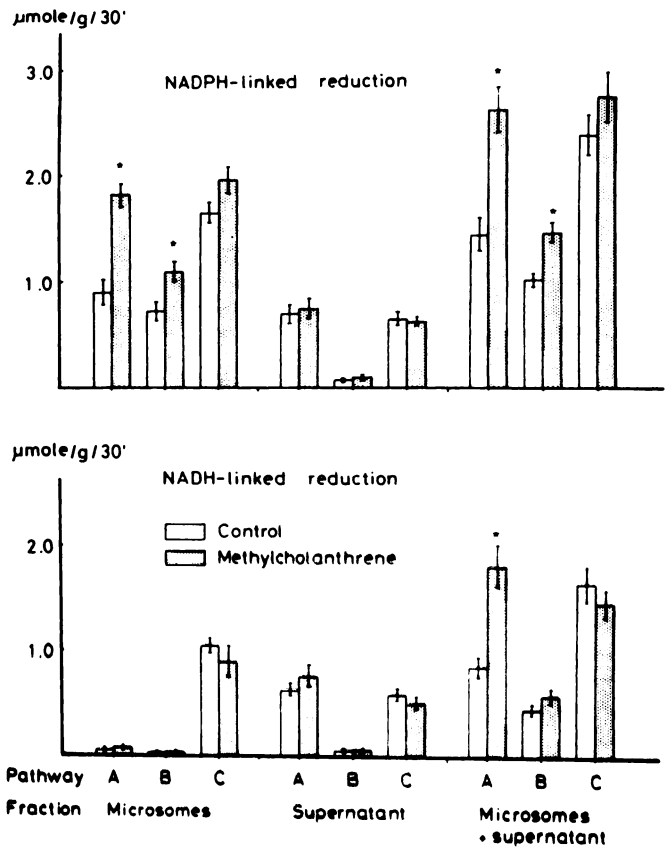

FIG. 4. *Effect of methylcholanthrene treatment on reduction of p-nitrobenzoic acid and p-hydroxyaminobenzoic acid*

The rats were treated with methylcholanthrene (25 mg/kg intraperitoneally) for 2 days. For details, see the legend to Fig. 3.

was 94% in the absence of FMN and 73% in its presence.

Effect of phenobarbital or methylcholanthrene treatment on reduction of p-nitrobenzoate and p-hydroxyaminobenzoate. It has been reported that the administration of phenobarbital and methylcholanthrene increases the formation of *p*-aminobenzoate from *p*-nitrobenzoate by liver microsomes in the presence of NADPH (8, 9). As shown in Fig. 3, the administration of phenobarbital stimulated the NADPH-linked reduction of *p*-nitrobenzoate to *p*-hydroxyaminobenzoate and *p*-aminobenzoate by liver microsomes; in contrast, the supernatant activities were not significantly stimulated. The NADH-linked activities in both microsomes and supernatant separately were not significantly stimulated by phenobarbital treatment, but they were significantly stimulated when the microsomes and supernatant were combined. In addition, the NADPH-linked reduction of *p*-hydroxyaminobenzoate in microsomes was slightly stimulated by phenobarbital; the NADH-linked reduction was not significantly stimulated. These results indicate that the mechanism of phenobarbital stimulation of nitro reduction is concentrated mainly in the conversion of *p*-nitrobenzoic acid to *p*-hydroxyaminobenzoic acid in microsomes. The administration of methylcholanthrene stimulated the NADPH-linked reduction of *p*-nitrobenzoate to *p*-hydroxyaminobenzoate and *p*-aminobenzoate by microsomes,

but the supernatant activities were not stimulated (Fig. 4). The reduction of *p*-nitrobenzoate to *p*-hydroxyaminobenzoate was stimulated more than that of *p*-nitrobenzoate to *p*-aminobenzoate. On the other hand, the NADH-linked activities in both microsomes and supernatant were not significantly stimulated. However, NADH-linked reduction of *p*-nitrobenzoate to *p*-hydroxyaminobenzoate in the combined fractions was stimulated more markedly by methylcholanthrene than by phenobarbital. In contrast, the NADH-linked reduction of *p*-nitrobenzoate to *p*-aminobenzoate by the combined microsomes plus supernatant was not significantly stimulated. Neither the NADPH- nor the NADH-linked reduction of *p*-hydroxyaminobenzoate by microsomes and supernatant was significantly stimulated; in fact, the NADH-linked reduction of *p*-hydroxyaminobenzoate seemed to be slightly decreased. These results indicate that the mechanism of methylcholanthrene stimulation of nitro reduction is due mainly to stimulation of the reduction of *p*-nitrobenzoate to *p*-hydroxyaminobenzoate in microsomes, and thus *p*-hydroxyaminobenzoic acid accumulates. This tendency was observed more clearly in the NADH-linked nitro reduction.

Stoichiometry of the disappearance of p-nitrobenzoate and formation of p-hydroxyaminobenzoate and p-aminobenzoate. Table 5 shows the effects of various experimental conditions on the reduction of *p*-nitrobenzoate to *p*-hydroxyaminobenzoate and *p*-aminobenzoate after incubation for 30 min. The ratios of *p*-hydroxyaminobenzoate plus *p*-aminobenzoate formed to *p*-nitrobenzoate disappearing were between 0.85 and 0.95 under all conditions tested. These results clearly indicate that FMN stimulates the reduction of *p*-nitrobenzoate to *p*-hydroxyaminobenzoate and consequently increases the formation of *p*-amino-

TABLE 5

Relationship between disappearance of p-nitrobenzoic acid and formation of p-hydroxyaminobenzoic acid and p-aminobenzoic acid under various experimental conditions

Two milliliters of the combined microsomal and supernatant fractions, equivalent to 500 mg of liver, were used as the enzyme source. Instead of 5 μmoles, 10 μmoles of *p*-nitrobenzoic acid were used. The incubation time was 30 min. The numbers in parentheses indicate the number of determinations. The results are given as average ± standard error. Pooled livers from three to six rats were used for each determination.

Experimental group and cofactor	NBA reduction			Ratio of HABA formed to NBA disappeared	Ratio of HABA formed to ABA formed
	NBA disappeared	HABA formed	ABA formed		
	mμmoles	*mμmoles*	*mμmoles*	%	%
Control					
NADPH	1386 ± 72 (5)	685 ± 49 (5)	552 ± 25 (5)	49.4	124.1
NADH	778 ± 35 (5)	421 ± 27 (5)	265 ± 18 (5)	54.1	158.9
FMN stimulation					
NADPH	7421 ± 305 (4)	4448 ± 120 (4)	2851 ± 99 (4)	59.9	156.0
NADH	3278 ± 249 (4)	2001 ± 87 (4)	1207 ± 55 (4)	61.0	165.8
Oxygen inhibition					
NADPH	305 ± 25 (5)	201 ± 27 (5)	53 ± 5 (5)	65.9	379.2
NADH	184 ± 23 (5)	125 ± 13 (5)	37 ± 7 (5)	67.9	337.8
Phenobarbital					
NADPH	2234 ± 123 (5)	1253 ± 85 (5)	886 ± 52 (5)	56.1	141.4
NADH	1247 ± 83 (5)	758 ± 56 (5)	382 ± 31 (5)	60.8	198.4
Methylcholanthrene					
NADPH	2158 ± 180 (4)	1321 ± 109 (4)	689 ± 51 (4)	61.2	191.7
NADH	1213 ± 62 (4)	863 ± 88 (4)	301 ± 31 (4)	71.1	286.7

benzoate. In addition, oxygen inhibits the reduction of *p*-nitrobenzoate to *p*-hydroxyaminobenzoate and consequently decreases the formation of *p*-aminobenzoate. It has been observed that methylcholanthrene causes accumulation of *p*-hydroxyaminobenzoate through stimulation of its formation from *p*-nitrobenzoate; thus the ratios of *p*-hydroxyaminobenzoate to *p*-aminobenzoate formed were 1.92 and 2.87, respectively, for the NADPH- and NADH-linked reductions. On the other hand, phenobarbital stimulates the reduction both of *p*-nitrobenzoate to *p*-hydroxyaminobenzoate and of the latter to *p*-aminobenzoate; thus the ratios of *p*-hydroxyaminobenzoate to *p*-aminobenzoate formed were 1.41 and 1.98, respectively, for the NADPH- and NADH-linked reductions. Moreover, the ratios of *p*-hydroxyaminobenzoate formed to *p*-nitrobenzoate that disappeared were 0.56 and 0.60, respectively, for the NADPH- and NADH-linked reductions.

DISCUSSION

The experiments described in this paper show that there are two different enzymatic systems for the reduction of *p*-nitrobenzoic acid in rat liver. The activity of the NADPH-linked reduction of *p*-nitrobenzoate to *p*-hydroxyaminobenzoate is higher in microsomes than in the supernatant fraction, while NADH-linked reduction is present predominantly in the supernatant fraction (Table 1). These results suggest that the NADPH- and NADH-linked nitroreductase activities in microsomes may be those of different enzymes, but it cannot be concluded from the present studies whether or not both enzymes in the supernatant fraction are identical. Oxygen markedly inhibits the activities of both reductases in microsomes, but to a much smaller extent in the supernatant fraction (Table 2). Both reductase activities in microsomes are stimulated by treatment with phenobarbital or methylcholanthrene, while the supernatant activities are not significantly affected. Kamm and Gillette have assumed that the reduction of aromatic nitro compounds is at least partially mediated by the microsomal NADPH–cytochrome *c* reductase (3). While this manuscript was in preparation, Gillette *et al.* reported the possibility that cytochrome P-450 is involved in the reduction of *p*-nitrobenzoate to *p*-aminobenzoate by liver microsomes (10). Since the activity of NADPH–cytochrome *c* reductase and the content of cytochrome P-450 are increased by phenobarbital or methylcholanthrene treatment (11, 12), the present observations are in accord with these views. The activity of NADPH-linked reduction of *p*-hydroxyaminobenzoate to *p*-aminobenzoate is present mainly in microsomes, with only one-third of the total being found in the supernatant fraction. The distribution of the corresponding NADH-linked activity is similar. In contrast to the reduction of *p*-nitrobenzoate to *p*-hydroxyaminobenzoate, the reduction of the latter to *p*-aminobenzoate in microsomes is only slightly stimulated by FMN and is slightly inhibited by oxygen. Moreover, the reduction of *p*-hydroxyaminobenzoate is stimulated only slightly by phenobarbital and essentially unaffected by methylcholanthrene administration. The stoichiometric studies on the disappearance of *p*-nitrobenzoate and *p*-aminobenzoate show that about 85–95% of the *p*-nitrobenzoate which disappears is converted to *p*-hydroxyaminobenzoate plus *p*-aminobenzoate under various experimental conditions. These findings are consistent with the results of thin layer chromatography. Although the NADH-linked reduction of *p*-nitrobenzoic acid to *p*-aminobenzoic acid is negligible in microsomes and barely detectable in the supernatant fraction, the activity in the combined fractions is relatively high. These results suggest that *p*-nitrobenzoate is reduced to *p*-hydroxyaminobenzoate principally by the supernatant and that the *p*-hydroxyaminobenzoate formed is then reduced to *p*-aminobenzoate mainly by the microsomes. The stimulatory effect of phenobarbital administration on the NADH-linked reduction of *p*-nitrobenzoate to *p*-aminobenzoate may be accounted for

in a similar manner. In this case neither the microsomal nor the supernatant activity is significantly stimulated by phenobarbital, but both factors may potentiate the over-all reaction (Fig. 3). On the other hand, methylcholanthrene stimulated only the NADH-linked reduction of *p*-nitrobenzoate to *p*-hydroxyaminobenzoate, not the reduction of *p*-nitrobenzoate to *p*-aminobenzoate (Fig. 4). Thus, the accumulation of *p*-hydroxyaminobenzoate as compared with the disappearance of *p*-nitrobenzoate is higher with the livers obtained from methylcholanthrene-treated rats than from phenobarbital-treated rats. The microsomal reduction of *p*-hydroxyaminobenzoate to *p*-aminobenzoate is only slightly inhibited by oxygen; thus the inhibition of *p*-nitrobenzoate reduction to *p*-aminobenzoate by oxygen is due to the reduction of the former to *p*-hydroxyaminobenzoate. These results are consistent with the suggestion by Gillette that oxygen may reoxidize the *p*-hydroxyaminobenzoate to *p*-nitrosobenzoate (7). However, the reason for the difference between the microsomes and supernatant in the inhibition of *p*-nitrobenzoate reduction remains obscure. In connection with these results, it is worthwhile to note the recent suggestion of Gillette *et al.* (10) that the inhibition of *p*-nitrobenzoate reduction may be due to the reoxidation of cytochrome P-450 by oxygen. Since the reduction of *p*-hydroxyaminobenzoate to *p*-aminobenzoate by microsomes and supernatant is only slightly stimulated by FMN, the stimulation of the reduction of *p*-nitrobenzoate to *p*-aminobenzoate by FMN is due to the reduction of the former to *p*-hydroxyaminobenzoate. The partial prevention by FMN of the inhibition of *p*-nitrobenzoate reduction by oxygen is still unexplained, but it is probable that *p*-hydroxyaminobenzoate is oxidized to *p*-nitrosobenzoate and the accumulation of the latter may inhibit the reduction of *p*-nitrobenzoate (7, 13). The excess of FMN therefore accelerates the reduction of *p*-nitrosobenzoate to *p*-hydroxyaminobenzoate or antagonizes the inhibition of the reduction of *p*-nitrobenzoate by accumulated *p*-nitrosobenzoate.

Kamm and Gillette (3) observed that FAD was reduced by purified NADPH–cytochrome *c* reductase and that FADH reduced *p*-nitrobenzoate nonenzymatically. On the other hand, Otsuka (14) observed that a purified *p*-nitrophenol reductase from swine liver homogenates reduced FAD and FMN, but only FADH could reduce *p*-nitrophenol; thus, only FAD could stimulate *p*-nitrophenol reductase. However, the purified *p*-nitrophenol reductase was NADH-dependent, and only negligible activity was observed with NADPH (14). Consequently, the enzyme system of Otsuka may be different from that of Kamm and Gillette and that described in the present study. The detailed biochemical mechanism of the stimulation by excess FMN of the reduction of *p*-nitrobenzoate in microsomes and supernatant, therefore, is still unknown. Recently, Yoshida[4] has observed that *p*-nitrobenzoate is reduced by purified NADPH–cytochrome *c* reductase whereas *p*-hydroxyaminobenzoate is reduced by both NADPH–cytochrome *c* and NADPH–cytochrome b_5 reductases in liver microsomes.

In the present study we did not succeed in separately determining *p*-nitrosobenzoate and *p*-hydroxyaminobenzoate because of their instability. Therefore, separate determinations of these compounds and studies with purified *p*-nitrobenzoate reductase and *p*-hydroxyaminobenzoate reductase are required for elucidation of the biochemical mechanism of inhibition by oxygen and stimulation by FMN.

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REFERENCES

1. R. T. Williams, "Detoxication Mechanisms." Chapman and Hall, London, 1959.

[4] Y. Yoshida, personal communication.

2. J. R. Fouts and B. B. Brodie, *J. Pharmacol. Exp. Ther.* **119,** 197 (1957).
3. J. J. Kamm and J. R. Gillette, *Life Sci.* **4,** 254 (1963).
4. S. Okuma, *Yakugaku Zasshi* **80,** 487 (1960).
5. H. Bauer and S. M. Rosenthal, *J. Amer. Chem. Soc.* **66,** 611 (1944).
6. O. H. Lowry, N. J. Rosebrough, A. L. Farr and R. J. Randall, *J. Biol. Chem.* **193,** 265 (1951).
7. J. R. Gillette, *Progr. Drug Res.* **6,** 13 (1963).
8. A. H. Conney, *Pharmacol. Rev.* **19,** 317 (1967).
9. R. Kato and M. Takayanagi, *Jap. J. Pharmacol.* **16,** 380 (1966).
10. J. R. Gillette, J. J. Kamm and H. A. Sasame, *Mol. Pharmacol.* **4,** 541 (1968).
11. S. Orrenius and L. Ernster, *Biochem. Biophys. Res. Commun.* **16,** 60 (1964).
12. L. Ernster and S. Orrenius, *Fed. Proc.* **24,** 1190 (1965).
13. H. Uehleke, *Naturwissenschaften* **50,** 335 (1963).
14. S. Otsuka, *J. Biochem. (Tokyo)* **50,** 85 (1961).