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REDUCED NICOTINAMIDE ADENINE DINUCLEOTIDE-DEPENDENT REDUCTION OF TERTIARY AMINE *N*-OXIDE BY LIVER MICROSOMAL CYTOCHROME P-450

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Abstract—NADH-supported reduction of tertiary amine *N*-oxides in rat liver microsomes was investigated with imipramine *N*-oxide, tiaramide *N*-oxide and *N*,*N*-dimethylaniline *N*-oxide as substrate in the presence of NADH. The reductase activity is sensitive to carbon monoxide. NADH-cytochrome b_5 reductase or cytochrome b_5, solubilized by trypsin or subtilisin, showed no *N*-oxide reductase activity. The NADH-dependent reduction of tertiary amine *N*-oxides was markedly inhibited by antibody to NADH-cytochrome b_5 reductase and antibody to cytochrome b_5. These results confirmed that NADH-dependent *N*-oxide reduction was catalyzed by cytochrome P-450 and the reducing equivalent for the *N*-oxide reduction was transferred from NADH to cytochrome P-450 mainly via NADH-cytochrome b_5 reductase and cytochrome b_5 in the microsomal membranes. NADH-dependent *N*-oxide reductase is also sensitive to oxygen and 4 μM oxygen gave 50 per cent inhibition with imipramine *N*-oxide. Kinetic study shows that K_m values for the reduction of imipramine *N*-oxide, tiaramide *N*-oxide and *N*,*N*-dimethylaniline *N*-oxide were 0.05 mM, 0.14 mM and 0.16 mM, respectively. NADH-dependent *N*-oxides reductase activity is affected by azo and nitroso compounds and hydrazide, although the degree of inhibition was rather weak compared with those of NADPH-dependent activity. Furthermore, NADH-dependent reduction of tertiary amine *N*-oxide was only slightly affected by *n*-octylamine, 2,4-dichloro-6-phenylphenoxyethylamine and aniline. NADH-dependent *N*-oxide reductase activity in liver microsomes was less sensitive to phenobarbital or 3-methylcholanthrene pretreatment than NADPH-dependent activity. Some characteristics of NADH-dependent *N*-oxide reductase activity were discussed and compared with those of NADPH-dependent activity.

The carbon monoxide-binding pigment which was called cytochrome P-450 by Omura and Sato [1] is known to play a central role in the hydroxylation of variety of substrates such as steroids, fatty acids, alkanes, polycyclic aromatic hydrocarbons and drugs [2, 3, 4]. In previous papers we have reported that the reduction of tertiary amine *N*-oxides, such as tiaramide *N*-oxide, imipramine *N*-oxide, *N*,*N*-dimethylaniline *N*-oxide to the corresponding tertiary amines are catalyzed by cytochrome P-450 of liver microsomes [5, 6]. These reactions require NADPH as reducing equivalents. While NADH also serves as a cofactor for *N*-oxides reduction of tiaramide *N*-oxide about half as effectively as NADPH [5]. Dajani also reported that in the reduction of nicotine *N*-oxide about one-half of the activity was observed using NADH in place of NADPH [7]. However, only a few investigations on the properties of NADH-dependent mixed function oxidase activities catalyzed by cytochrome P-450 have been reported [8, 9, 10]. Particularly, no information has been obtained on the characteristics of NADH-dependent reductase activity of cytochrome P-450.

In the present studies, we take special interest in the catalytic nature of the NADH-dependent tertiary amine *N*-oxide reduction by microsomal cytochrome P-450, that is: (1) the component of NADH-dependent *N*-oxide reductase enzyme system, (2) the nature of NADH-dependent *N*-oxide reductase activity induced by pretreatment with phenobarbital and 3-methylcholanthrene, (3) sex difference in the reductase activity in rat, and (4) the effects of various drugs and inhibitors on the reduction of tertiary amine *N*-oxides.

MATERIALS AND METHODS

Animals. Seven weeks old male and female rats of a Sprague–Dawley strain were used. Some of these animals were treated intraperitoneally with phenobarbital (90 mg/kg) daily for 3 days or with 3-methylcholanthrene (40 mg/kg) daily for 2 days. Control rats were injected with 0.9% NaCl or with olive oil. All rats treated were sacrificed 24 hr after the last injection.

Materials. NADH (grade III), trypsin, soy bean trypsin inhibitor, and cytochrome *c* were purchased from Sigma Chemicals, St. Louis. Subtilisin was purchased from Teikoku Chemical Industry, Osaka. Tiaramide and tiaramide *N*-oxide were synthesized in our Research Laboratories. Imipramine and chlorimipramine were kindly donated by Ciba-Geigy, Basel, and DPEA (2,4-dichloro-6-phenylphenoxyethylamine HCl) by Lilly Research Laboratories, Indianapolis. All other reagents were of the highest grade commercially available. Imipramine *N*-oxide and *N*,*N*-dimethylaniline *N*-oxide were synthesized by the

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methods of Fishman *et al.* [11] and Belov *et al.* [12], respectively.

Preparation of liver microsomes. Microsomal fraction was obtained from perfused rat liver as described previously [5]. Hepatic microsomes were essentially free from hemoglobin and mitochondrial contamination. For the preparation of protease treated microsomes, a suspension of KCl-washed microsomes (10 mg of protein/ml) in 0.1 mM phosphate buffer (pH 7.4) containing 25% glycerol, was incubated at 0° in the presence of purified trypsin or subtilisin for 15 hr anaerobically [13].

Enzyme assays. Tertiary amine *N*-oxide reductase activity by rat liver microsomes was assayed as previously described [6]. The standard assay medium (5 ml) for measuring *N*-oxide reductase activity contained 0.1 M phosphate buffer (pH 7.4), 2 mM NADH, 10 mM $MgCl_2$, 25 mg of microsomal protein and indicated tertiary amine *N*-oxide at a concentration of 1.0 mM. NADH-ferricyanide reductase and NADH-cytochrome *c* reductase were assayed by the methods of Takesue and Omura [14, 15]. Succinate dehydrogenase activity was determined by the method of Pennington [16].

Preparation of antibodies. Antibody to NADH-cytochrome b_5 reductase prepared by the method of Takesue and Omura [15] and antibody to cytochrome b_5 prepared according to Fukushima *et al.* [17] were kindly supplied by Professor T. Omura, Kyushu University.

Analytical methods. Cytochrome P-450 and P-420 were determined from CO difference spectrum of dithionite treated samples as described by Omura and Sato [18]. Protein was determined by the method of Lowry *et al* [19] with bovine serum albumin as standard.

RESULTS

NADH-dependent reduction of tertiary amine N-oxide to tertiary amine in rat liver microsomes. Tertiary amine *N*-oxides were reduced to their corresponding tertiary amines in the presence of hepatic microsomes and NADH (Table 1). In the absence of reduced pyridine nucleotide, reduction of tertiary amine *N*-oxide was almost negligible. As previously shown [5], NADPH appeared to be the preferred reducing agent and the rate observed with saturating concentrations of NADH are about 50 per cent of those obtained with NADPH. Under the gas phase of carbon monoxide, NADH-supported reduction of tertiary amine *N*-oxide was depressed, and the rate was no more than 15 per cent of that observed under the gas phase of nitrogen. When imipramine *N*-oxide or tiaramide *N*-oxide was used as substrate and incubated anaerobically, imipramine or tiaramide were found to be the sole metabolites detectable. When *N*,*N*-dimethylaniline *N*-oxide was used as substrate, *N*,*N*-dimethylaniline was found to be a major metabolite and 10 per cent of monomethylaniline was also detected [20].

Table 1. NADH-dependent *N*-oxide reductase activity in liver microsomes*

Substrate	*N*-oxide reduction rate N_2 (nmoles/mg/min)	*N*-oxide reduction rate CO (nmoles/mg/min)	Inhibition (%)
Imipramine *N*-oxide	0.86	0.06	93
Tiaramide *N*-oxide	0.92	0.05	95
N,*N*-Dimethyl-aniline *N*-oxide	1.49	0.20	87

* Reaction mixtures, prepared as described in Materials and Methods, were incubated at 37° for 10 min under an atmosphere of nitrogen or carbon monoxide. The concentration of the protein in the reaction mixture was 5.0 mg/ml.

Effect of protease digestion on microsomal NADH-dependent N-oxide reductase activity. Previous studies [8, 21, 22] have shown that treatment of liver microsomes with trypsin or subtilisin solubilizes NADPH-cytochrome *c* reductase from the microsomal membranes but leaves the major portion of NADH-cytochrome b_5 reductase and hemeprotein cytochrome P-450 still intact. The present study has utilized proteolytic digestion for further identifcation

Table 2. Effect of protease digestion on microsomal NADH-dependent *N*-oxide reductase activity*

Treatment		NADH-cytochrome *c* reductase (nmoles/mg/min)	NADH-ferricyanide reductase (μmoles/mg/min)	Cytochrome b_5 content (nmoles/mg)	Cytochrome P-450 content (nmoles/mg)	NADH-dependent† *N*-oxide reductase (nmoles/mg/min)	Protein (%)
Intact microsome		982	4.0	0.42	1.08	0.86	
	Digested	716	3.5	0.32	0.81	0.20	
Trypsin	sup.	119	0.2	0.13	0.00	0.00	60
	Pellet	1216	5.5	0.47	1.36	0.37	40
	Digested	150	3.9	0.36	0.69	0.20	
Subtilisin	Sup.	20	0.3	0.81	0.00	0.00	68
	Pellet	213	5.4	0.06	1.02	0.29	32

* Liver microsomes (10 mg protein/ml) were incubated at 0° with 25 μg trypsin or subtilisin/mg protein for 15 hr anaerobically in 0.05 M sodium phosphate buffer (pH 7.4) containing 25% glycerol. Digestion with trypsin was stopped by addition of soybean trypsin inhibitor (1 mg/mg protein) and the digested microsomes were then spun at 105,000 *g* for 2 hr. The pellets obtained were suspended in 0.05 M sodium phosphate buffer (pH 7.4) and serve for the enzyme assays.

† NADH-dependent reduction of tertiary amine was measured using 1.0 mM imipramine *N*-oxide.

of the microsomal components involved in the NADPH-dependent tertiary amine *N*-oxide reductase enzyme system. The incubation of microsomal fraction at 0° with 25 μg trypsin or subtilisin/mg microsomal protein for 15 hr in the presence of 25% glycerol resulted in a 77 per cent depletion of NADH-dependent *N*-oxide reductase activity (Table 2). No conversion of cytochrome P-450 to P-420 was observed through these procedures, therefore, this depletion of the activity in protease digested microsomes was most probably due to the damage of electron transport caused by protease pretreatment. In agreement with this result, NADH-cytochrome *c* reductase activity was decreased 85 per cent by subtilisin digestion and 27 per cent by trypsin digestion, presumably as a result of the solubilization and distribution of the hydrophobic site of cytochrome b_5 by protease [8]. Both NADH-dependent *N*-oxide reductase activity and cytochrome P-450 were fairly well recovered in the pellet fraction from protease treated digestion, and NADH-ferricyanide reductase activity, a function associated with the enzyme NADH-cytochrome b_5 reductase [14], was hardly affected by protease digestion and remained intact in the pellet fraction. Therefore, these results suggest the involvement of NADH-cytochrome b_5 reductase and cytochrome P-450 in the NADH-dependent *N*-oxide reductase activity. Treatment with subtilisin solubilized about 95 per cent of the cytochrome b_5 and 68 per cent of the total microsomal protein, but NADH-dependent *N*-oxide reductase activity was retained in subtilisin-treated pellet fraction. These observations are well in accord with the result of Oshino [23] on NADH-dependent fatty acid desaturase system which involves cytochrome b_5.

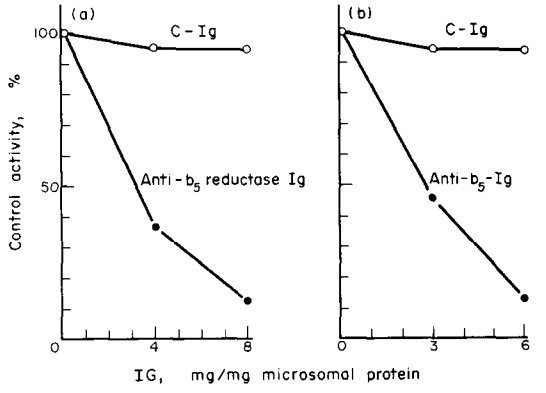

Fig. 1. Inhibition of microsomal NADH-dependent *N*-oxide reductase activity by antibody to NADH-cytochrome b_5 reductase and antibody to cytochrome b_5. Liver microsomes were incubated with indicated amounts of antiserum to NADH-cytochrome b_5 reductase (a) or antiserum to cytochrome b_5 (b) at 37° for 5 min anaerobically in the Thunberg type reaction vessel in a 2-ml solution containing 0.1 M sodium phosphate buffer (pH 7.4). After incubation, NADH was added to make up a total volume of 2.5 ml. For the measurement of NADH-dependent *N*-oxide reductase activity, 2.5 mg microsomal protein and 2.5 μmoles of imipramine *N*-oxide was used. The specific activity in the absence of antiserum of NADH-dependent *N*-oxide reductase system expressed as nmoles imipramine formed per min per mg protein was 0.79. NADH-dependent *N*-oxide reductase: (●) + antibody; (○) + nonimmune globlin.

Table 3. Kinetic parameters of imipramine *N*-oxide, tiaramide *N*-oxide and *N*,*N*-dimethylaniline *N*-oxide reduction in liver microsomes*

Substrate	K_m (mM)	V_{max} (nmoles/mg/min)
Imipramine *N*-oxide	0.05	1.3
Tiaramide *N*-oxide	0.14	1.2
N,*N*-Dimethylaniline *N*-oxide	0.16	2.2

* K_m values for tertiary amine *N*-oxide and the V_{max} values were obtained from double reciprocal plots of the reaction velocities against substrate concentrations in the presence of excess NADH. Other experimental conditions were the same as described in Materials and Methods.

Inhibition of NADH-dependent N*-oxide reductase activity by antibody to NADH-cytochrome* b_5 *reductase and antibody to cytochrome* b_5. To evaluate further the role that NADH-cytochrome b_5 reductase and cytochrome b_5 play in NADH-dependent tertiary amine *N*-oxide reduction in liver microsomes, effect of antibody to NADH-cytochrome b_5 reductase and antibody to cytochrome b_5 was examined. The results are illustrated in Fig. 1a and 1b. At the antibody to NADH-cytochrome b_5 reductase: protein ratio of about 7:1, NADH-dependent *N*-oxide reductase activity was inhibited about 80 per cent. Non-immune globlin at this ratio had no appreciable effect on the NADH-dependent *N*-oxide reductase activity. Similar inhibition of activity by the antibody to cytochrome b_5 was also observed at the antibody to cytochrome b_5: protein ratio of about 5:1.

These results confirmed that the reducing equivalent for *N*-oxide reduction is transferred from NADH to cytochrome P-450 mainly via NADH-cytochrome b_5 reductase and cytochrome b_5 in the microsomal membranes, as was the case in NADH-dependent oxidation of benzo(a)pyrene with reconstituted microsomal electron transport system [9].

Kinetic studies on NADH-dependent N*-oxide reductase activity in liver microsomes.* Using these *N*-oxides as substrate, the reaction rate showed saturation kinetics with increasing concentration of substrates. Michaelis–Menten kinetics was, therefore, applied for the NADH-dependent reduction of imipramine *N*-oxide, tiaramide *N*-oxide and *N*,*N*-dimethylaniline *N*-oxide, and the apparent K_m values for each were estimated to be 0.5×10^{-4} M, 1.4×10^{-4} M and 1.6×10^{-4} M, respectively (Table 3). The apparent K_m value for imipramine *N*-oxide was considerably smaller than those observed with tiaramide *N*-oxide and *N*,*N*-dimethylaniline *N*-oxide.

Inhibition of NADH-dependent N*-oxide reduction by oxygen and carbon monoxide.* Oxygen and carbon monoxide are ligands which interact with reduced cytochrome P-450 in its heme region as the sixth ligand, and have been reported to inhibit cytochrome P-450-dependent reduction of nitro and azo groups in liver microsomes [4]. Our present results (Fig. 2) demonstrate that oxygen and carbon monoxide are

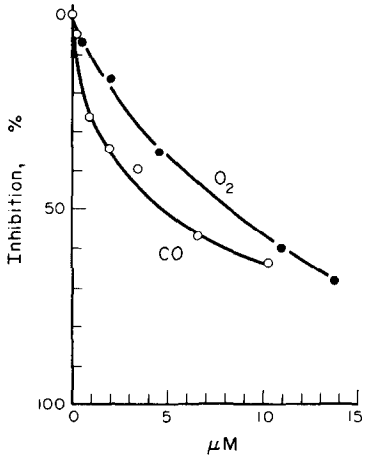

Fig. 2. Effect of oxygen and carbon monoxide on NADH-dependent *N*-oxide reductase activity. NADH-dependent *N*-oxide reductase activity from liver microsomes were assayed in the presence of 10 mM $MgCl_2$, 2 mM NADH, 5 mg microsomal protein/ml, 0.1 M phosphate buffer (pH 7.4) and 1.0 mM imipramine *N*-oxide under indicated concentration of oxygen (●) or carbon monoxide (○). Various composition of O_2–N_2 and CO–N_2 mixtures were prepared in gas burettes and passed into Thunberg type incubation vessels containing the enzyme preparations as described above. The concentration of carbon monoxide and oxygen in the aqueous phase was calculated from the partial pressure of carbon monoxide and oxygen and temperature. Simultaneous control reactions were carried out under N_2 atmosphere.

potent inhibitors of NADH-dependent *N*-oxide reduction, half-maximal inhibition of the reaction rate occurring at an oxygen concentration of about 8 μM and a carbon monoxide concentration of about 4 μM.

Inhibition of NADH-dependent N*-oxide reductase activity by primary amines.* It is well known that type I and type II substrates interact with microsomal cytochrome P-450 to produce characteristic spectral changes [24] and modify the reduction rate of cytochrome P-450 [25]. Type II compounds such as aniline, *n*-octylamine and DPEA, which are assumed to interact with cytochrome P-450 directly in its heme region and decrease NADPH-dependent reduction rate of cytochrome P-450 [26, 27] and of tertiary amine *N*-oxides [6]. In the present studies, the effect of aniline, n-octylamine and DPEA on the reduction of NADH-supported *N*-oxide reductase activities was investigated (Table 4). The reduction of imipramine *N*-oxide, tiaramide *N*-oxide and *N*,*N*-dimethylaniline *N*-oxide was, however, hardly affected by these primary amines, in contrast with the previous observation on the reduction of NADPH-dependent tertiary amine *N*-oxides. It is, therefore, of considerable interest to examine whether primary amines can modify the NADH-dependent reduction rate of cytochrome P-450 in liver microsomes. It is also noted that NADH-dependent *N*-oxides reduction was inhibited by other teriary amine *N*-oxides (Table 5). NADH-dependent imipramine *N*-oxide reduction was inhibited by the addition of 1 mM of *N*,*N*-dimethylaniline *N*-oxide and tiaramide *N*-oxide, 38% and 14%, respectively. It is also observed that addition of azo compound to the incubation mixture markedly reduced NADH-dependent *N*-oxide reduction. These results are interesting enough, since cytochrome P-450 in liver microsomes was also involved in the reduction of azo compound [4]. Furthermore, hydrazide and cumene hydroperoxide, which are known to interact with cytochrome P-450 [26], were found to be effective inhibitors for NADH-dependent *N*-oxides reductase in liver microsomes.

Table 5. Effect of various compounds on NADH-dependent *N*-oxide reductase activity in liver microsomes*

Compounds	Inhibition of activity (%)
N,*N*-Dimethylaniline *N*-oxide	38
Tiaramide *N*-oxide	14
α-Nitroso-β-naphtol	67
p-Dimethylaminoazobenzene	41
Cumene hydroperoxide	52
Benzylhydrazine	36

* NADH-dependent *N*-oxide reductase activity was determined in the presence of 1 mM of indicated compounds using imipramine *N*-oxide as substrate. The control activity for imipramine *N*-oxide reduction was 0.79 nmoles/mg/min.

Effect of phenobarbital or 3-methylcholanthrene pretreatment on microsomal NADH-dependent N*-oxide reductase activity.* Pretreatment of animals with phenobarbital or polycyclic hydrocarbons such as 3-methylcholanthrene results in the increased hepatic microsomal levels of cytochrome P-450 and in the induction of the hepatic microsomal oxidations of certain drugs [4]. Therefore, the effect of phenobarbital or 3-methylcholanthrene pretreatment on microso-

Table 4. Effect of Type II ligands on NADH-dependent *N*-oxide reductase activity in liver microsomes*

Type II ligand	Imipramine *N*-oxide	Tiaramide *N*-oxide	*N*,*N*-Dimethyl-aniline *N*-oxide
		Inhibition (%)	
Aniline (1×10^{-3} M)	2	2	5
n-Octylamine (1×10^{-3} M)	22	17	8
DPEA (1×10^{-4} M)	22	12	16

* NADH-dependent *N*-oxide reductase activity was determined in the presence of specified concentration of Type II substances. The control activities for imipramine *N*-oxide, tiaramide *N*-oxide and *N*,*N*-dimethylaniline *N*-oxide were 0.79, 1.05 and 1.65 nmoles/mg/min, respectively.

Table 6. Effect of phenobarbital and 3-methylcholanthrene pretreatment on NADH-dependent *N*-oxide reductase activity and cytochrome P-450 content of liver microsomes*

Treatment	Imipramine *N*-oxide reduction (nmoles/mg/min)	Tiaramide *N*-oxide reduction (nmoles/mg/min)	*N*,*N*-Dimethylaniline *N*-oxide reduction (nmoles/mg/min)	Cytochrome P-450 (nmoles/mg)
Saline	0.89 ± 0.06	1.09 ± 0.08	1.49 ± 0.06	1.01 ± 0.03
Phenobarbital† (90 mg/kg, 3 days)	0.99 ± 0.04	1.59 ± 0.10§	1.58 ± 0.06	1.62 ± 0.07§
Olive oil	0.70 ± 0.03	1.10 ± 0.15	1.76 ± 0.13	1.11 ± 0.05
3-Methylcholanthrene‡ (40 mg/kg, 2 days)	0.75 ± 0.06	1.19 ± 0.12	1.53 ± 0.08	1.43 ± 0.06§

* The results were obtained from at least three rats expressed as the mean ± S.E.M.
† Phenobarbital treated rats were injected i.p. with 90 mg/kg, 72, 48 and 24 hr before sacrifice.
‡ 3-Methylcholanthrene treated rats were injected with 40 mg/kg, 48 and 24 hr before sacrifice.
§ $P < 0.05$ compared with saline or olive oil control.

mal NADH-dependent *N*-oxide reductase activity and other microsomal components is investigated (Table 6). It should be pointed out that pretreatment of rats with phenobarbital produced only slight elevation in the specific activity of the reduction of imipramine *N*-oxide, tiaramide *N*-oxide, and *N*,*N*-dimethylaniline *N*-oxide, whereas cytochrome P-450 showed a 2-fold increase in specific content by phenobarbital treatment. A similar lack of enhancement in NADH-dependent reduction of tertiary amine *N*-oxides was observed in 3-methylcholanthrene pretreated rat liver microsomes.

Sex difference in NADH-dependent N*-oxide reductase activity in liver microsomes.* It is widely established that NADPH-dependent oxidation of certain drugs and reduction of azo and nitro compounds in male rat liver microsomes proceeded more rapid than that observed in female rat [28, 29]. In a previous paper [6], we reported the sex difference in NADPH-dependent *N*-oxide reductase activity in which reducing equivalent was transferred to cytochrome P-450 via NADPH-cytochrome *c* reductase. As illustrated in Table 7, the sex difference in the NADH-dependent *N*-oxide reductase enzyme system, which was now established to be composed of NADH-cytochrome b_5 reductase, cytochrome b_5, and cytochrome P-450, was found to be insignificant with these substrates used. The ratio of NADH-dependent reduction rate of tertiary amine *N*-oxide varies from 1.7-fold to 1.1-fold with substrates. These results indicated that sex difference in NADH-dependent *N*-oxide reductase activity is less significant than observed in NADPH-dependent reduction [6].

Acceleration of NADH-dependent N*-oxide reductase activity by flavins.* The addition of FMN, FAD and riboflavin to microsomes at a concentration of 50 μM enhanced *N*-oxide reductase activity about 10-fold and this enhanced *N*-oxide reductase activity was almost completely blocked under the gas phase of carbon monoxide (Table 8). It is feasible that the flavins added exogenously are transhydrogenated by NADH and then accelerate the reduction rate of cytochrome P-450 in the microsomes, followed by the enhancement of *N*-oxide reduction. In connection with these observations, it is of considerable interest to note that the addition of methylviologen (0.1 mM) to microsomal fraction fortified with NADH resulted in a marked enhancement of the reduction of tertiary amine *N*-oxide to tertiary amine, and carbon monoxide almost completely inhibited *N*-oxide reduction by this system.

Table 7. NADH-dependent *N*-oxide reductase activity in male and female rat liver microsomes*

Substrate	NADH-dependent *N*-oxide reductase activity (nmoles/mg/min) Male (A)	Female (B)	Ratio (A/B)
Imipramine *N*-oxide	0.89 ± 0.06	0.53 ± 0.03	1.7
Tiaramide *N*-oxide	1.09 ± 0.08	0.82 ± 0.08	1.3
N,*N*-Dimethylaniline *N*-oxide	1.49 ± 0.06	1.40 ± 0.05	1.1

* Reaction mixtures, prepared as described in Materials and Methods, were incubated at 37° for 10 min under an atmosphere of nitrogen. The results were obtained from at least seven rats and expressed as the mean ± S.E.M. The cytochrome P-450 concentrations of male and female rat liver microsomes were found to be 1.01 ± 0.03, 0.68 ± 0.02 nmoles/mg protein, respectively.

Table 8. Effect of FMN on NADH-dependent *N*-oxide reductase activity in rat liver microsomes*

Substrate	*N*-oxide reduction rate (nmoles/mg/min) −FMN N_2	+FMN N_2	+FMN CO
Imipramine *N*-oxide	0.86	7.02	0.22
Tiaramide *N*-oxide	0.94	9.91	0.06
N,*N*-Dimethylaniline *N*-oxide	1.32	8.56	0.10

* Reaction mixtures, prepared as described in Materials and Methods, were incubated at 37° for 10 min under an atmosphere of nitrogen or carbon monoxide in the presence or absence of 50 μM FMN. The concentration of the protein in the reaction mixture was 5.0 mg/ml.

DISCUSSION

Our previous work [5, 6] demonstrated that cytochrome P-450 is involved in the reduction of tertiary amine *N*-oxide by liver microsomes. In these reactions, NADPH was a preferred reducing equivalent and considerable activity was also observed when NADH was used as an electron donor. Usually, in the NADH-dependent oxidative metabolism of drugs, polycyclic hydrocarbons and steroids, the activities were only about 10–15 per cent of those in the NADPH-dependent activities and few reports on the characteristics of NADH-dependent activities have appeared [8, 9, 10]. In the meanwhile, about one-half of reductase activity in the NADPH-dependent *N*-oxide reduction was detected in the NADH-dependent tertiary amine *N*-oxide reduction and now it became possible to ascertain the nature of NADH-dependent reductive reaction catalyzed by cytochrome P-450.

Recently, studies on the characteristics of the NADH-dependent oxidative activity were performed with 3,4-benzo(a)pyrene hydroxylase [9] and cumene hydroperoxide peroxidase [10] and these studies revealed that both NADH-cytochrome b_5 reductase and cytochrome b_5 were involved in these reactions. A similar result was also obtained from the present study using antibody to cytochrome b_5 and NADH-cytochrome b_5 reductase; a flavoprotein, and involvement of cytochrome b_5 in the NADH-dependent *N*-oxide reductase activity was established.

Figure 3 shows the proposed scheme of NAD(P)H-dependent tertiary amine *N*-oxide reduction in liver microsomes. It is suggested that NADH supplies reducing equivalents to cytochrome P-450 via the flavoenzyme NADH-cytochrome b_5 reductase and cytochrome b_5. Reduced cytochrome P-450 then acts as a microsomal *N*-oxide reductase, converting tertiary amine *N*-oxide to the corresponding tertiary amine. Cytochrome b_5 can also mediate the channeling of electrons from NADH-cytochrome b_5 reductase to fatty acid desaturase, called the "cyanide sensitive factor (CSF)," and hydroxylamine reductase [23, 30–32].

To summarize the characteristic nature of NADH-dependent *N*-oxide reductase which involves cytochrome P-450 as a key participant, the properties of NADH-dependent *N*-oxide reductase activity are compared with those of NADPH-dependent *N*-oxide reductase activity [6]. First, both the NADH and NADPH-dependent *N*-oxide reductase activities are sensitive to carbon monoxide and oxygen, and K_m values of substrate for NADH and NADPH-dependent *N*-oxide reductase activity are in the order of 10^{-5} to 10^{-6} M. Secondly, in contrast to NADPH-dependent *N*-oxide reductase activity which was enhanced 80 per cent to 110 per cent by phenobarbital pretreatment, the enhancement of NAD-dependent *N*-oxide reductase activity was slight (10–45 per cent). Third, the influence of the modification of cytochrome P-450 by various reagents such as "Type II" reagent, azo and hydrazide on NADH-dependent enzyme system was less significant than that observed with NADPH-dependent enzyme system. These results are suggestive of the presence of multiple forms of cytochrome P-450 in liver microsomes. Recently, data has been accumulated showing the presence of multiple forms of cytochrome P-450, such as phenobarbital induced cytochrome P-450 and methylcholanthrene induced cytochrome P-450, in liver microsomes [33, 34, 35]. These results are, therefore, indicative of the presence of one species among them which is more reducible by NADH via NADH-cytochrome b_5 reductase and cytochrome b_5 and plays a central role in NADH-dependent reactions.

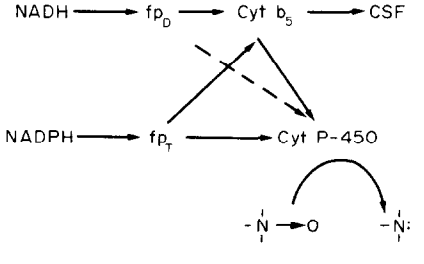

Fig. 3. Scheme of NAD(P)H-dependent tertiary amine *N*-oxide reduction in liver microsomes.

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REFERENCES

1. T. Omura and R. Sato, *J. biol. Chem.* **237,** 1375 (1962).
2. A. Y. H. Lu and M. J. Coo., *J. biol. Chem.* **243,** 1331 (1968).
3. A. H. Conney, *Pharmac. Rev.* **19,** 317 (1967).
4. J. R. Gillette, *Adv. Pharmac.* **4,** 219 (1966).
5. M. Sugiura, K. Iwasaki, H. Noguchi and R. Kato, *Life Sci.* **15,** 1433 (1974).
6. M. Sugiura, K. Iwasaki and R. Kato, *Molec. Pharmac.* **12,** 322 (1976).
7. R. M. Dajani, J. W. Gorrod and A. H. Beckett, *Biochem. Pharmac.* **24,** 109 (1975).
8. E. G. Hrycay and P. J. O'Brien, *Archs Biochem. Biophys.* **160,** 230 (1974).
9. S. B. West, W. Levin, D. Ryan, M. Vore and A. Y. H. Lu, *Biochem. biophys. Res. Comm.* **58,** 516 (1974).
10. E. G. Hrycay and R. A. Prough, *Archs Biochem. Biophys.* **165,** 331 (1974).
11. V. Fishman, H. Goldenberg, *J. Pharm. Pharmac.* **14,** 187 (1962).
12. V. N. Belov and K. K. Savich, *J. gen. Chem. U.S.S.R.* **17,** 257 (1947).
13. E. G. Hrycay and P. J. O'Brien, *Archs Biochem. Biophys.* **157,** 7 (1973).
14. S. Takesue and T. Omura, *J. Biochem.* **67,** 259 (1970).
15. S. Takesue and T. Omura, *J. Biochem.* **67,** 267 (1970).
16. R. J. Pennington, *Biochem. J.* **80,** 649 (1961).
17. K. Fukushima, A. Ito, T. Omura and R. Sato, *J. Biochem.* **71,** 447 (1972).
18. T. Omura and R. Sato, *J. biol. Chem.* **239,** 2370 (1964).
19. O. H. Lowry, N. J. Rosebrough, A. L. Farr and R. J. Randall, *J. biol. Chem.* **193,** 265 (1951).
20. J. M. Machinist, W. H. Orme-Johnson and D. M. Ziegler, *Biochemistry* **5,** 2939 (1966).
21. H. Nishibayashi and R. Sato, *J. Biochem.* **63,** 766 (1968).
22. S. Orrenius, A. Berg and L. Ernster, *Eur. J. Biochem.* **11,** 193 (1969).
23. N. Oshino and T. Omura, *Archs Biochem. Biophys.* **157,** 395 (1973).
24. J. B. Schenkman, H. Remmer and R. W. Estabrook, *Molec. Pharmac.* **3,** 113 (1967).
25. P. L. Gigon, T. E. Gram and J. R. Gillette, *Molec. Pharmac.* **5,** 109 (1969).

26. C. R. E. Jefcoate and J. L. Gaylor, *Biochemistry* **8,** 3464 (1969).
27. E. G. Hrycay and P. J. O'Brien, *Archs Biochem. Biophys.* **147,** 14 (1971).
28. R. Kato, *Drug Metab. Rev.* **3,** 1 (1974).
29. R. Kato and J. R. Gillette, *J. Pharmac. exp. Ther.* **159,** 279 (1965).
30. N. Oshino, *Archs Biochem. Biophys.* **149,** 378 (1972).
31. F. F. Kadlubar and D. M. Ziegler, *Archs Biochem. Biophys.* **162,** 83 (1974).
32. F. F. Kadlubar, E. M. Mckee and D. M. Ziegler, *Archs Biochem. Biophys.* **156,** 47 (1973).
33. K. Comai and J. L. Gaylor, *J. biol. Chem.* **248,** 4947 (1973).
34. W. Levin, D. Ryan, S. West and A. Y. H. Lu, *J. biol. Chem.* **249,** 1747 (1974).
35. T. A. Hoeven and M. J. Coon, *J. biol. Chem.* **249,** 6302 (1974).