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PEROXIDASE AS A MODEL FOR REDUCTION OF TERTIARY AMINE OXIDES CATALYZED BY RAT HEPATIC SUPERNATANT AND MICROSOMAL FRACTIONS

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Abstract—Rat hepatic microsomal and 100,000 *g* supernatant fractions catalyzed an NADH- and FMN-dependent reduction of amine oxides. Horseradish peroxidase (HRP) served as a model for the amine oxide reductase located in rat hepatic 100,000 *g* supernatant fraction. The HRP-catalyzed reaction displayed saturation kinetics with respect to NADH and the amine oxide substrate; however, there was an optimum concentration for FMN after which inhibition was observed at increased concentrations of FMN. The reductase in the 100,000 *g* hepatic supernatant fraction closely paralleled HRP-catalyzed amine oxide reduction in coenzyme requirements, sensitivity to inhibitors, and substrate specificity. Moreover, the peroxidase activity of HRP and microsomal and 100,000 *g* supernatant fractions correlated with the NADH- and FMN-dependent amine oxide reductase activities of these enzyme preparations. The NADH- and FMN-dependent amine oxide reductase activity in 100,000 *g* supernatant fractions, however, did not parallel the aldehyde oxidase activity. Thus, the results indicate that there is an amine oxide reductase in rat hepatic 100,000 *g* supernatant fraction with catalytic properties that are modeled well by horseradish peroxidase.

Many drugs and other xenobiotics containing tertiary amines are metabolically converted to the corresponding *N*-oxides. Various other *N*-oxides are either chemically synthesized or isolated from plants, animals, or microorganisms for use as pharmacological agents. Although *N*-oxides can possess unique pharmacological and toxicological properties, many of the biological effects of *N*-oxides are often the result of metabolic transformation [1].

A major route of *N*-oxide metabolism is enzyme-catalyzed reduction to the corresponding amine. While this is a facile chemical transformation, a generally applicable biochemical basis for mammalian *N*-oxide reduction has not been established. Several investigators, however, have described enzyme systems capable of catalyzing reduction of specific *N*-oxides. For example, Murray and Chaykin [2, 3] reported that xanthine oxidase (EC 1.2.3.2) from hog liver catalyzes reduction of nicotinamide *N*-oxide. Dajani *et al.* [4] described (−)-nicotine-1′-*N*-oxide reductase activity in both microsomal and soluble fractions of rat liver. This reductase activity was sensitive to oxygen, inhibited by carbon monoxide and potassium cyanide, and partially inhibited by SKF-525A and EDTA. Reductase activity was supported by either NADH or NADPH, and was enhanced by flavins and phenobarbital. The same authors also demonstrated substantial *in vivo* reduction of (−)-nicotine-1′-*N*-oxide after oral administration to germ-free rats [5]. Sugiura *et al.* [6] demonstrated both NADH-dependent and NADPH-dependent *N*-oxide reductase activity associated with microsomal cytochrome P-450 under anaerobic conditions. Furthermore, Kato *et al.* [7] found that flavins and methyl viologen enhance the rate of microsomal *N*-oxide reductase. Kitamura and coworkers [8–10] reported that rabbit liver aldehyde oxidase (EC 1.2.3.1) has *N*-oxide reductase activity under anaerobic conditions when supplied with suitable electron donors. In addition to these studies on cytosolic and microsomal enzymes, Powis and DeGraw [11] have demonstrated indicine *N*-oxide reduction with reduced cytochrome *c*. Furthermore, Johnson and Ziegler [12] have purified a mitochondrial *N*-oxide reductase which is specific for *N,N*-dimethylaminoazobenzene *N*-oxide.

Metabolic reduction of amine oxides often yields derivatives with enhanced pharmacological or toxicological activities. For example, naturally occurring pyrrolizidine alkaloid *N*-oxides are hepatotoxic as a result of reduction to the parent amines followed by subsequent metabolism to pyrroles [13]. One of these alkaloids, indicine *N*-oxide, exhibits significant antitumor activity [14]. Conversion of indicine *N*-oxide to indicine is apparently not essential for its antitumor activity [15]; however, it may be the first step in metabolism to more hepatotoxic derivatives [16]. Although the antitumor activity of the heterocyclic indicine *N*-oxide initially led to studies on its metabolic reduction, the present report also extends the applicability of a mammalian *N*-oxide reductase to an alkyl tertiary amine oxide (imipramine *N*-oxide) and an alkyl aryl tertiary amine oxide (*N,N*-dimethylaniline *N*-oxide). A preliminary report on *N*-oxide

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reductase activity in rat hepatic fractions, and its comparison to reduction catalyzed by horseradish peroxidase, has appeared previously [17].

METHODS

Materials. All assay reagents were obtained from commercial sources and were of the highest purity available. All HPLC solvents and recrystallization solvents were of reagent grade or higher purity. Horseradish peroxidase (Type VI) and xanthine oxidase (Grade I) were obtained from the Sigma Chemical Co. (St. Louis, MO). Indicine *N*-oxide was provided by Professor John P. Rosazza of our College.

Synthesis of amines and amine oxides. Indicine *N*-oxide (50 mg) was reduced to indicine using zinc dust (20 mg) in water (10 ml) with 10 mg ammonium chloride [18]. The hydrochloride salt of indicine was twice recrystallized from ethanol/ether. Imipramine *N*-oxide was synthesized from imipramine hydrochloride by the method of Fishman and Goldenberg [19]. *N,N*-Dimethylaniline *N*-oxide was prepared by the following modification of the method of Chernova and Khokhlov [20]: after addition of platinum to destroy excess hydrogen peroxide, the pH was raised to 10 with ammonium hydroxide, the solution was lyophilized, and the resulting solid was suspended in absolute methanol and filtered to remove ammonium chloride. The volume of the filtrate was reduced under vacuum and the product was crystallized from methanol/ether. Melting points and proton NMR spectroscopy were used to verify the structures of indicine, indicine *N*-oxide, imipramine *N*-oxide, and *N,N*-dimethylaniline *N*-oxide.

Animals and tissue preparations. Male Sprague–Dawley rats (BioLabs, St. Paul, MN) weighing 175–250 g were allowed free access to standard food pellets and water. The animals were killed by decapitation, and livers were removed immediately and placed in 0.25 M sucrose at 4°. Livers were blotted dry, weighed, and homogenized in 4 parts (w/v) 0.25 M sucrose at 4°. Subcellular fractions were prepared by differential centrifugation [21]. The microsomal pellet was resuspended in 0.25 M sucrose. All subcellular fractions were kept at 4° until used. Protein concentrations were determined by a modification of the Lowry method [22].

Enzyme incubations. Concentrations of reaction components and incubation conditions for individual experiments are described in the appropriate tables and figures in the Results. All enzymatic reactions were carried out at 37° and pH 7.0 in a total volume of 0.5 ml. Anaerobic incubations were carried out by bubbling argon through reaction mixtures containing all components except NADH and horseradish peroxidase (HRP); NADH and HRP solutions were saturated with argon in separate tubes. NADH was added after a 3-min preincubation at 37°, and each reaction tube was sealed with a rubber septum immediately after addition of HRP. Both aerobic and anaerobic reactions were terminated after 3 min by addition of 0.015 ml of 29% ammonium hydroxide, and each reaction mixture was then extracted three times with 1.0-ml portions of chloroform:methanol (85:15). In several experiments, either microsomes or cytosol was substituted for HRP in the above procedure.

Assay of indicine N*-oxide reduction.* The pooled organic layers from the extraction of incubation mixtures were concentrated to dryness under a stream of nitrogen. The residues were dissolved in 0.1 ml methanol, and HPLC analyses of the resulting solutions were performed on an Alltech 600-SI 10 μm silica column (4.1 mm × 30 cm) with a mobile phase of chloroform:methanol:29% ammonium hydroxide (50:50:1) and UV detection at 254 nm. The concentration of indicine was determined by measuring peak height and comparing to a linear standard curve relating peak height to concentration of indicine injected onto the column; correlation coefficients for standard curves were always greater than 0.99 for HPLC injections containing between 4.8 and 150 nmol of indicine.

Assay of imipramine N*-oxide reduction.* Assay of imipramine *N*-oxide reduction was similar to that of indicine *N*-oxide reduction except that 0.035 ml of 29% ammonium hydroxide was added to the incubation mixture after 3 min, and another 0.01 ml of 29% ammonium hydroxide was added before the second extraction. HPLC was carried out with a 4.1 mm × 30 cm Alltech C-6000 10 μm phenyl bonded silica column and a mobile phase of acetonitrile:0.1 M monobasic potassium phosphate (3:2) [9]. Imipramine concentration was determined by comparison of HPLC peak heights to a linear standard curve for imipramine; correlation coefficients for standard curves were always greater than 0.99 for HPLC injections containing between 0.8 and 80 nmol of imipramine.

Assay of N,N*-dimethylaniline* N*-oxide reduction.* Assay of *N,N*-dimethylaniline *N*-oxide reduction was similar to the method described for imipramine *N*-oxide reduction, with the following modifications. After addition of ammonium hydroxide and extraction with chloroform:methanol:29% ammonium hydroxide (50:50:1), the pooled organic layers were extracted three times with equal volumes of 1.0 M acetic acid. The aqueous layers were combined and an aliquot was subjected to HPLC analysis. The HPLC column was the same as that used above for imipramine, with a mobile phase of 0.1 M monobasic potassium phosphate:acetonitrile (3:2). The concentration of dimethylaniline was determined by HPLC peak area and comparison to a linear standard curve; correlation coefficients for standard curves were always greater than 0.99 for HPLC injections containing between 0.3 and 3.0 nmol of *N,N*-dimethylaniline.

Assay of pyrogallol oxidation. Oxidation of pyrogallol catalyzed by peroxidase and subcellular fractions was determined by observation of purpurogallin absorbance at 420 nm [23].

Assay of aldehyde oxidase activity. Increase in absorbance at 300 nm was monitored to determine the oxidation of *N*-methylnicotinamide to the 6-pyridone [24]. Assays were carried out in room air at pH 7.0 and 37°; molecular oxygen was the electron acceptor.

Determination of oxygen concentration. Oxygen concentration was determined polarographically at

Table 1. Reduction of indicine *N*-oxide by peroxidase and by rat liver supernatant and microsomal fractions

Assay	Indicine formed (nmol/min/mg protein)		
	Supernatant	Microsomes	Peroxidase
Complete*	143 ± 41	61 ± 13	2750 ± 1200
−NADH	0	5	0
−FMN	0	6	0
−Enzyme	0	0	0
−NADH + NADPH (20 mM)	129	59	1605 ± 63
+Argon†	115 ± 23	62 ± 8	5930 ± 590‡
PM-10 Filtrate§	0	0	0
+Sodium azide (5 mM)	15 ± 2‡	4 ± 1‡	322 ± 120‡
+Aminotriazole (5 mM)	109 ± 27	43 ± 1	3040 ± 480
+CO†	5 ± 1‡	8 ± 2‡	160 ± 1‡
+*n*-Octylamine (5 mM)	102 ± 11	28 ± 1‡	1820 ± 360

* Assay mixtures contained 20 mM NADH, 3 mM indicine *N*-oxide, 1 mM FMN, 0.05 M potassium phosphate at pH 7.0, and the indicated enzyme preparation. Values are either a single determination or the means ± SD of at least three determinations.

† These assay mixtures were anaerobic and were saturated with the indicated gas.

‡ Significantly different ($P < 0.01$) from complete control assay of the same enzyme preparation.

§ Peroxidase and hepatic fractions were each subjected to filtration on an Amicon PM 10 ultrafiltration membrane, and the filtrate was used in a complete assay.

37° with a Clarke oxygen electrode in a closed, stirred, and thermostated 3.8-ml reaction vessel.

RESULTS

N-Oxide reductase activity in rat hepatic microsomal and 100,000 g *supernatant fractions.* As seen in Table 1, rat liver supernatant and microsomal fractions catalyzed reduction of indicine *N*-oxide in a reaction requiring a reduced pyridine nucleotide (either NADH or NADPH) and flavin mononucleotide (FMN). Furthermore, the requirements for a reduced pyridine nucleotide and FMN were maintained when the assays were carried out under anaerobic conditions (Table 2). The rate of reaction with either hepatic fraction was unaffected by saturation of all solutions with argon before assay, and all activity was retained on ultrafiltration membranes that retain molecules of 10,000 molecular weight or greater. *N*-Oxide reductase activity in both subcellular fractions was inhibited significantly by sodium azide and carbon monoxide. However, the two fractions differed in their susceptibilities to inhibition by *n*-octylamine; supernatant activity in the presence of *n*-octylamine was not significantly different from the control, whereas microsomal *N*-oxide reductase activity was decreased to 46% of that determined in the absence of *n*-octylamine.

Since previous investigators have implicated aldehyde oxidase in the anaerobic reduction of tertiary amine oxides [8–10], we compared the effects of the peroxidase inhibitor azide and the aldehyde oxidase inhibitor estradiol on reduction of indicine *N*-oxide catalyzed by rat hepatic supernatant fraction. The results shown in Table 3 indicated that the effects of azide and estradiol on the *N*-oxide reductase were not consistent with involvement of aldehyde oxidase in the NADH- and FMN-requiring reaction.

N-Oxide reductase activity of horseradish peroxidase. Horseradish peroxidase (HRP) catalyzed reduction of tertiary amine oxides in a reaction with coenzyme requirements and inhibitor sensitivity similar to that observed with hepatic supernatant and microsomal fractions. As seen in Table 1, the reduction of indicine *N*-oxide catalyzed by HRP paralleled that observed with hepatic 100,000 *g* supernatant, with the exception that an increased rate was seen after argon saturation. In addition to differences in rates obtained under an argon atmosphere, there was also a difference between HRP and the rat liver microsomal preparation in their responses to *n*-octylamine.

Further experiments comparing peroxidase activity and *N*-oxide reductase activity of HRP and hepatic fractions are summarized in Table 4. Peroxidase activity, as measured by pyrogallol

Table 2. Requirement for NADH and FMN in reduction of indicine *N*-oxide catalyzed by rat liver supernatant and microsomal fractions

Assay	Indicine formed (nmol/min/mg protein)	
	Supernatant	Microsomes
Complete*	115	62
−NADH	2.0	6.5
−FMN	0	4.5

* All assays were carried out at 37° under anaerobic conditions (saturation with argon). Complete assays contained 20 mM NADH, 3 mM indicine *N*-oxide, 1 mM FMN, 0.05 M potassium phosphate at pH 7.0 and the indicated hepatic fraction. All values are the average of two determinations.

Table 3. Effects of azide and estradiol on *N*-oxide reductase and aldehyde oxidase activities in rat liver supernatant fraction

Assay	Aldehyde oxidase* nmol Pyridone (min) (mg protein)	Relative activity	*N*-Oxide reductase† nmol Indicine (min) (mg protein)	Relative activity
Complete	1219 ± 72‡	100	98 ± 7‡	100
+Azide	1190 ± 59	98	22 ± 7	22
−Substrate	238 ± 33	20	0	0
Complete	1696 ± 400	100	56 ± 3	100
+Estradiol	752 ± 100	44	67 ± 2	120
−Substrate	496 ± 28	29	0	0

* Assay is described in Methods. The concentrations of sodium azide and estradiol were 5 and 0.25 mM respectively.

† Assay mixtures contained 40 mM NADH, 3.0 mM indicine *N*-oxide, 0.5 mM FMN, and 0.05 M potassium phosphate at pH 7.0. Inhibitor concentrations were identical to the aldehyde oxidase assays. Reactions were incubated under anaerobic conditions (saturation with argon) for 3.0 min at 37°; HPLC analysis of indicine is described in Methods.

‡ Values are means ± SD, N = 3.

oxidation, paralleled *N*-oxide reductase activity with HRP, rat liver microsomes, and 100,000 *g* supernatant.

The increase in rate of indicine *N*-oxide reduction when reaction mixtures were saturated with argon led to an investigation of the role of oxygen in the reduction. As seen in Fig. 1, oxygen concentration and product formation were monitored simultaneously in a sealed reaction vessel equilibrated with room air; indicine was formed only after all oxygen was removed. Further oxygen uptake studies indicated that the initial decrease in oxygen concentration was independent of enzyme and was not inhibited by sodium azide or *n*-octylamine (data not shown). Similarly, sodium azide and *n*-octylamine failed to inhibit the rate of decrease in oxygen concentration in the presence of either microsomal or supernatant fractions. This rapid decrease in oxygen concentration reflected the previously characterized reduction of molecular oxygen by reduced flavin [25]. Since the resulting superoxide anion radical might have been an intermediate reductant of the HRP, an alternate superoxide generating system [26] was employed. In this experiment, xanthine and xanthine oxidase, as a source of superoxide anion radical, did not replace NADH and FMN in the HRP-catalyzed reduction of indicine *N*-oxide (data not shown).

Kinetic characteristics of the HRP-catalyzed anaerobic reduction of indicine *N*-oxide are shown in Figs 2–4. Initial rates of indicine *N*-oxide reduction were dependent on NADH concentration (Fig. 2), and the enzyme was saturable with respect to NADH. As seen in Fig. 3, there was an optimal concentration of FMN of 0.5 to 1.0 mM, with higher concentrations yielding reduced rates of indicine *N*-oxide reduction. The reaction was saturable with indicine *N*-oxide (Fig. 3), with an apparent K_m of 9.0 mM and V_{max} of 50 μmol amine formed/min/mg HRP.

Substrate specificity of amine oxide reduction catalyzed by HRP and by rat hepatic 100,000 g *supernatant fraction.* Both HRP and rat hepatic 100,000 *g* supernatant fraction catalyzed reduction of the alkyl tertiary amine oxide, imipramine *N*-oxide, and the alkyl aryl tertiary amine *N*-oxide, *N,N*-dimethylaniline *N*-oxide. A comparison of reduction rates for indicine *N*-oxide, imipramine *N*-oxide, and *N,N*-dimethylaniline *N*-oxide is seen in Table 5. *N,N*-

Table 4. Comparison of peroxidase and *N*-oxide reductase activities

	Pyrogallol oxidation* (mmol/min/mg protein)	Indicine *N*-oxide reduction† (nmol/min/mg protein)
Peroxidase	2800 ± 290	7600 ± 310
Supernatant fraction	37 ± 1	180 ± 10
Microsomal fraction	9 ± 2	36 ± 4

* The rate of peroxidation of 50 mM pyrogallol with 100 mM H_2O_2 was determined at pH 7.0 in 25 mM potassium phosphate at 37°. Molar absorptivity at 420 nm for purpurogallin under these conditions was 3430. Results are means ± SD of three determinations.

† Indicine *N*-oxide reduction was determined under anaerobic conditions (saturation with argon) at pH 7.0 and 37° with 40 mM NADH, 0.5 mM FMN, and 3 mM indicine *N*-oxide. Results are means ± SD of three determinations.

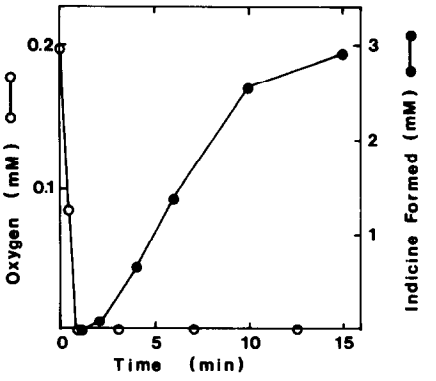

Fig. 1. Simultaneous determination of oxygen and indicine concentrations. The reaction mixture contained 20 mM NADH, 1 mM FMN, 3 mM indicine *N*-oxide, and 0.19 mg horseradish peroxidase at pH 7.0 and 37° in a final volume of 3.8 ml. Oxygen concentration was monitored continuously with a Clark oxygen electrode, and aliquots were taken at the indicated times for determination of indicine concentration.

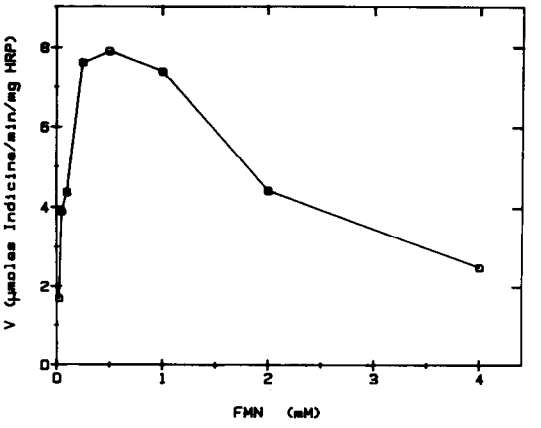

Fig. 3. HRP-catalyzed indicine *N*-oxide reduction as a function of FMN concentration. Reaction mixtures contained 40 mM NADH, 3 mM indicine *N*-oxide, 0.025 mg horseradish peroxidase, and the indicated concentration of FMN in a final volume of 0.5 ml. Reactions were carried out under anaerobic conditions (saturation with argon) at pH 7.0 and 37°.

Dimethylaniline *N*-oxide was reduced at a higher rate than indicine *N*-oxide, which was reduced at a much higher rate than imipramine *N*-oxide.

DISCUSSION

Microsomal and 100,000 *g* supernatant fractions from rat liver catalyzed the NADH- and FMN-dependent reduction of *N*-oxides. This reduction was similar in many respects to the NADH- and FMN-dependent reduction of *N*-oxides catalyzed by horseradish peroxidase (HRP). Reduction of indicine *N*-oxide was inhibited by sodium azide and carbon monoxide with all three enzyme preparations, as might be expected due to relatively non-specific binding of these inhibitors to hemoproteins. However, *n*-octylamine, a more specific inhibitor of cytochrome P-450 enzymes, illustrated that at least some of the microsomal reductase activity may be associated with these enzymes. A substantial portion of the activity in microsomes, as well as all of the activity with 100,000 *g* supernatant fraction, was not affected by *n*-octylamine. This activity closely paralleled HRP in coenzyme requirements, inhibitor sensitivity, and substrate specificity. Furthermore, peroxidative activity with subcellular fractions and HRP correlated with *N*-oxide reductase activity in these enzyme preparations.

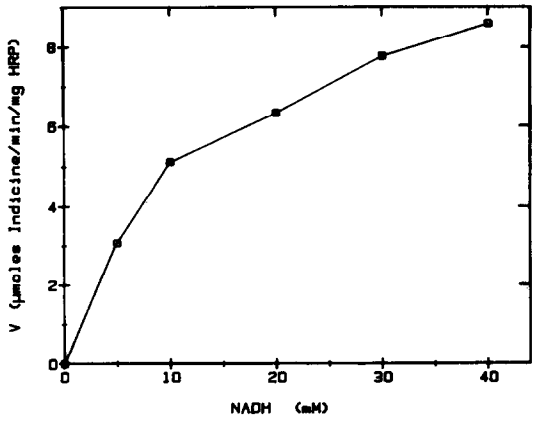

Fig. 2. HRP-catalyzed indicine *N*-oxide reduction as a function of NADH concentration. Reaction mixtures contained 3 mM indicine *N*-oxide, 1 mM FMN, 0.025 mg horseradish peroxidase, and the indicated concentration of NADH in a final volume of 0.5 ml. Reactions were carried out under anaerobic conditions (saturation with argon) at pH 7.0 and 37°.

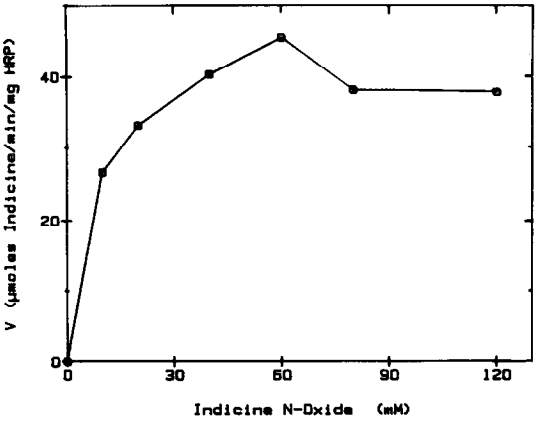

Fig. 4. HRP-catalyzed indicine *N*-oxide reduction as a function of *N*-oxide concentration. Reaction mixtures contained 40 mM NADH, 0.5 mM FMN, 0.025 mg horseradish peroxidase, and the indicated concentration of indicine *N*-oxide in a final volume of 0.5 ml. Reactions were carried out under anaerobic conditions (saturation with argon) at pH 7.0 and 37°.

Table 5. Reduction of imipramine *N*-oxide, *N,N*-dimethylaniline *N*-oxide, and indicine *N*-oxide catalyzed by rat hepatic 100,000 *g* supernatant fraction and by horseradish peroxidase

Enzyme preparation	Rate of reduction* (nmol product formed/min/mg protein)		
	Imipramine *N*-oxide	*N,N*-Dimethylaniline *N*-oxide	Indicine *N*-oxide
Supernatant	10.5 ± 0.3	434 ± 17	96 ± 6
HRP	117 ± 24	36,600 ± 2,100	7,500 ± 740

* Assays were conducted under anaerobic conditions (saturation with argon) at pH 7.0 and 37° with 40 mM NADH, 0.5 mM FMN, and a 3.0 mM concentration of the indicated *N*-oxide. Rates are means ± SD of at least three determinations.

Although the rate of indicine *N*-oxide reductase activity with HRP was stimulated by argon to a significant extent, this was most likely due to the fact that the reaction in Table 1 was actually anaerobic after the first 45 sec (Fig. 1). The greater than 10-fold difference in absolute rates, with the same time required for NADH and FMN to remove oxygen from the reaction, accounts for the apparent difference in the effect of argon on rat hepatic fractions and HRP. Therefore, all subsequent *N*-oxide reductase assays were carried out under anaerobic conditions.

While the NADH- and FMN-dependent *N*-oxide reductase activity in 100,000 *g* supernatant fraction correlated with oxidative and reductive activities of peroxidase, it did not parallel the aldehyde oxidase activity in this fraction. So, while aldehyde oxidase may contribute to *N*-oxide reduction under other conditions [8–10], it did not appear to be associated with the NADH- and FMN-dependent *N*-oxide reductase in 100,000 *g* supernatant fraction from rat liver.

Initial experiments toward determining the substrate specificity of *N*-oxide reductase showed qualitative similarities between 100,000 *g* supernatant and HRP in reduction of three *N*-oxides. The structural differences between these *N*-oxides are seen in Fig. 5. The aryl alkyl *N*-oxide, *N,N*-dimethylaniline *N*-oxide, was reduced at the highest rate by both HRP and 100,000 *g* supernatant fraction. The heterocyclic bridgehead alkyl *N*-oxide, indicine *N*-oxide, was reduced at an intermediate rate, while the alkyl *N*-oxide imipramine *N*-oxide was reduced at the lowest rate of the three.

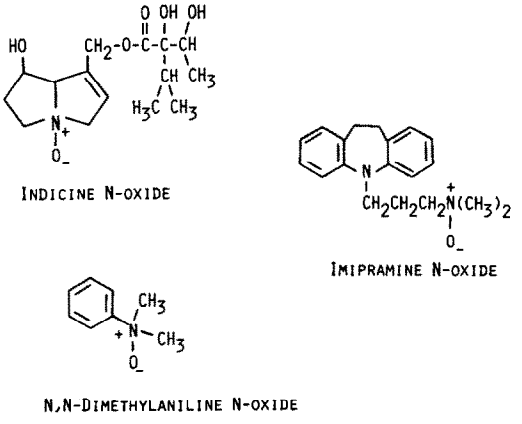

Fig. 5. Tertiary amine oxide substrates for reduction catalyzed by hepatic 100,000 *g* supernatant fraction and HRP.

While these results are consistent with an *N*-oxide reductase with a rather broad substrate specificity, the physiological significance of this reaction is as yet unknown. However, studies with germ-free rats [5] do indicate that amine oxide reduction occurs within mammalian tissues *in vivo*. Furthermore, various cell fractions from rat liver (whole homogenate, 10,000 *g* supernatant, microsomal, and 100,000 *g* supernatant) all catalyze amine oxide reduction under anaerobic conditions [4]. In studies relevant to our results on the reductase activities of microsomes and cytosol, Sugiura *et al.* [6] have presented evidence that the NADH-supported reduction of imipramine *N*-oxide, tiaramide *N*-oxide, and *N,N*-dimethylaniline *N*-oxide catalyzed by rat liver microsomes is dependent on a different pathway from the NADPH-dependent microsomal reduction of tertiary amine oxides. The NADPH-dependent microsomal reduction has been linked to cytochromes P-450 [7]. Further evidence for the presence of two distinct pathways for microsomal reduction of amine oxides has been provided by Powis and Wincentsen [27] using indicine *N*-oxide as a substrate. Our results are also consistent with two pathways for *N*-oxide reduction catalyzed by rat liver microsomes, one dependent on cytochromes P-450 and another that is more similar to a reductase also found in 100,000 *g* supernatant fraction.

Although our results indicate that the *N*-oxide reductase in rat hepatic 100,000 *g* supernatant fraction has catalytic properties similar to those of horseradish peroxidase, the molecular mechanisms for these reductions are not yet clear. One possibility that is consistent with our data includes an initial reduction of the Fe^{3+}HRP to Fe^{2+}HRP. This is a well documented reaction for cytochrome *c* [25] and ferritin [28, 29], in which an Fe^{3+} complex is reduced to an Fe^{2+} complex by the combination of NADH and FMN. The steps whereby the amine oxide would be reduced by the Fe^{2+}HRP are, however, more elusive. One possible intermediate in the reaction might be an oxoiron(IV) hemoprotein. Evidence in favor of this type of intermediate comes from recent

studies on the transfer of oxygen from an amine oxide to model Fe^{2+} porphyrins [30, 31]. Even if this type of oxoiron(IV) intermediate is shown to be present in the HRP and the cytosolic *N*-oxide reductase, further mechanistic details regarding the reduction of such an intermediate will remain to be determined.

Elucidation of the complete mechanistic details for the NAD(P)H- and FMN-dependent reaction will await further studies on the HRP-catalyzed amine oxide reduction, and purification of the peroxidase-like *N*-oxide reductase from rat hepatic 100,000 *g* supernatant fraction. Likewise, investigations into the exact nature of the biological reductant(s), functionally equivalent to the NADH and FMN used in these studies, will be facilitated by purification of the reductase. However, the use of NADH and FMN as a reducing system, and HRP as a model, should be useful in further investigations on the enzymology of *N*-oxide reduction in mammalian tissues.

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