Microsomal Oxidases. II. Properties of a Pork Liver Microsomal *N*-Oxide Dealkylase*

J. M. Machinist, W. H. Orme-Johnson, and D. M. Ziegler†

ABSTRACT: Pork liver microsomes can catalyze the relatively rapid dealkylation of a number of closely related *N,N*-dialkylarylamine *N*-oxides. The microsomal enzyme system catalyzing these reactions does not require oxygen, reduced pyridine nucleotides, or other added cofactors. The *N*-oxide dealkylase activity is inhibited by diethylaminoethanol ester (SKF-525A), pyridine, and carbon monoxide. Evidence obtained by treating the particles with detergents, hydrolytic enzymes, and inhibitors demonstrates that the microsomal *N*-oxide dealkylase activity cannot be attributed to nonspecific catalysis of this reaction by hemoproteins present in the preparations. However, a close correlation between the integrity of the membrane-bound carbon monoxide binding pigment and the *N*-oxide dealkylase activity of the preparations has been observed and this hemoprotein may be a component of the microsomal enzyme system catalyzing this reaction.

It has been shown that mammalian hepatic microsomes catalyze the NADPH[1] and oxygen-dependent oxidation of tertiary amines to amine oxides (Baker and Chaykin, 1962; Casida and Stahmann, 1953; Ziegler and Pettit, 1964). Although the *N*-oxides could be terminal products of amine oxidation, they can also be considered as intermediates in metabolic oxidative N-dealkylation reactions as previously suggested (Terayama, 1963; Fish *et al.*, 1955; Oae *et al.*, 1963; Ziegler and Pettit, 1966). If the amine oxides are intermediate oxidation products, then enzymes or enzyme systems capable of catalyzing the dealkylation of these compounds must also be present in microsomes. It has been shown that pork liver microsomes can catalyze the rapid anaerobic demethylation of *N,N*-dimethylaniline *N*-oxide (Pettit and Ziegler, 1963), but the studies of Terayama (1963) have demonstrated that this reaction could also be catalyzed by hemin or by hemoproteins. The possibility that the microsomal *N*-oxide dealkylase activity could be attributed to hemoglobin and other hemoproteins associated with the particles could not be excluded by data previously presented.

The data presented in this report based on the selective action of inhibitors, thermal inactivation, and reaction rates demonstrate that the hepatic microsomal *N*-oxide dealkylase activity is not due to nonspecific hemoprotein catalysis. The substrate specificity and other properties of this enzyme system are also described.

Experimental Section

Materials. Tertiary amine *N*-oxides were synthesized from the parent amines by the method of Chernova and Khokhlov (1960), with the exception of *N,N*-dimethylaniline *N*-oxide which was obtained from K and K Laboratories, Inc. The *N*-oxides were purified by recrystallization from absolute ethanol and identified by their melting points and elemental analysis. Rabbit hemoglobin was obtained from Dr. Boyd Hardesty of this department. Myoglobin (two times recrystallized from horse heart) was a product of the Mann Research Laboratories, Inc. *Naja naja* venom was purchased from the Ross Allen Reptile Institute and purified according to the procedure of Cremona and Kearney (1964). Steapsin (crude pancreatic lipase) and wheat germ lipase were products of the Nutritional Biochemical Corp. and were used without further purification.

Methods. Microsomes were isolated from pork liver by the method outlined by Ziegler and Pettit (1966). In all experiments involving spectrophotometric analysis, the particles were washed twice with 1.15% KCl and resuspended in 0.25 M sucrose. Protein was determined by the Biuret reaction of Gornall *et al.* (1949); formaldehyde was measured by the method of Nash (1953); and total aldehyde was determined as described by Albrecht *et al.* (1962). Total heme as the pyridine hemochromogen, and the carbon monoxide binding pigment, were measured by the methods described by Omura and Sato (1963, 1964).

Assay Procedures. The *N*-oxide dealkylase assays

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[1] The abbreviations used are: SKF-525A, diethylaminoethanol ester of diphenylpropylacetic acid; DMAO, *N,N*-dimethylaniline *N*-oxide; DMPTO, *N,N*-dimethyl-*p*-toluidine *N*-oxide; DMNAO, *N,N*-dimethyl-1-naphthylamine *N*-oxide; NADPH, reduced nicotinamide-adenine dinucleotide phosphate; NADH, reduced nicotinamide-adenine dinucleotide.

were usually carried out in test tubes containing the following components: 100 μmoles of potassium phosphate, pH 7.0, 2.0 μmoles of semicarbazide; 5.0 μmoles of *N*-oxide; microsomes; and distilled water to a final volume of 1.0 ml. The reaction was initiated by the addition of either microsomes or substrate and incubated at 38° for 5 min. After 5 min, 0.5-ml aliquots were removed and pipetted into tubes containing sufficient 3.0 M trichloroacetic acid to give a final concentration of 0.36 M. The deproteinized supernatant fractions were then assayed for formaldehyde or acetaldehyde. The formaldehyde rates were corrected for the small amount of formaldehyde produced in the absence of substrate.

Treatment with N. naja Venom, Lipases, and Detergents. A suspension of KCl-washed microsomes (10–12 mg of protein/ml) in 0.1 M Tris–HCl buffer, pH 8.5, was incubated at 0° in the presence of purified phospholipase A (venom:protein ratio = 1:200). Aliquots were removed at regular intervals and assayed for *N*-oxide dealkylase activity and carbon monoxide binding pigment content. The conditions for the steapsin and wheat germ lipase experiments (final lipase concentration = 0.14%) were essentially the same as those described above, except that the microsomes were suspended in 0.1 M phosphate buffer, pH 7.0, and incubated at 38°.

The detergents (the concentrations are listed in the tables) were added to microsomes suspended in 0.25 M sucrose containing 0.1 M phosphate, pH 7.0, at a protein concentration of 25–30 mg of protein/ml, and incubated at 38°. Aliquots were withdrawn at regular intervals and assayed.

Results

The requirement for added nucleotides, oxygen, or other cofactors by the microsomal *N*-oxide dealkylase could not be demonstrated. This activity was also not affected by removing flavin from the particles, as described in the preceding communication (Ziegler and Pettit, 1966). The reaction was proportional to the concentration of microsomes in the range of 0.2–4.0 mg of protein/ml and linear with time for at least 10 min at 38°. The pH optimum of the reaction was between 6.8 and 7.0 for all the substrates tested.

Considerable variation (two- to threefold) in *N*-oxide dealkylase activity was observed among different preparations of pork liver microsomes. There was always a close correlation, however, between this activity and the mixed function tertiary amine oxidase activity also present in these preparations. The *N*-oxide dealkylase activity, like the mixed function oxidase activity, was consistently more concentrated in the "light" than in the "heavy" microsomal fraction (Ziegler and Pettit, 1966) and all of the data presented in this paper was obtained with "light" microsomal fractions.

Substrate Specificity. Of the *N*-oxides tested, only the dialkylarylamine *N*-oxides were catalytically dealkylated by hepatic microsomes. Enzymic demethylation of *N*-oxide derivatives of trialkylamines or morphine could not be detected. *N*-Ethyl derivatives of aniline *N*-oxide were also dealkylated, but at a slower rate than the corresponding methyl derivatives. It would appear that these microsomes could catalyze the dealkylation of only those *N*-oxides in which the nitrogen atom is directly attached to an aromatic ring. However, a much larger group of compounds would have to be tested to clarify this point.

The first four compounds in Table I are listed in order of decreasing lipid solubility. At the concentration tested (5 μmoles/ml), the more lipid-soluble compounds were dimethylated at the fastest rate. However, this was largely due to the much lower Michaelis constant of the more lipid soluble substrates, as shown in Table II. The maximum velocities for the three compounds, calculated from double reciprocal plots of rate *vs.* substrate concentration, are of the same order of magnitude (Table II). The differences in the K_m values for these substrates were significant and suggest that lipid solubility is an important factor influencing the binding of the substrate to the enzyme. The binding of the less lipid-soluble substrates to the enzyme was markedly affected in the presence of *N,N*-dimethylcyclohexylamine. As shown in Table II, the K_m estimated for DMAO was decreased almost 2.5-fold by adding this amine to the assay medium. The maximum velocity of the reaction was not affected, however, nor did this amine appreciably change the Michaelis constant measured for the more lipid-soluble DMNAO. The action of *N,N*-dimethylcyclohexylamine on the demethylation of DMAO could be duplicated by other

TABLE I: Various Amine *N*-Oxides as Substrates for the Microsomal *N*-Oxide Dealkylase.

Substrate	Sp Act.[a]
N,N-Dimethyl-1-naphthylamine *N*-oxide	86
N,N-Dimethyl-*p*-toluidine *N*-oxide	56
p-Chlorodimethylaniline *N*-oxide	37
N,N-Dimethylaniline *N*-oxide	12
N-Ethyl-*N*-methylaniline *N*-oxide	9 (4)[b]
N,N-Dimethylbenzylamine *N*-oxide	0
N,N-Dimethylcyclohexylamine *N*-oxide	0
N,N-Dimethyloctylamine *N*-oxide	0
Morphine *N*-oxide	0

[a] Expressed as micromoles of formaldehyde produced per milligram of protein per minute. [b] Expressed as micromoles of acetaldehyde produced per milligram of protein per minute. The concentration of *N*-ethyl-*N*-methylaniline *N*-oxide in the reaction mixture was 10 μmoles/ml. All other substrates were added to give a final concentration of 5.0 μmoles/ml, as described in the section on Methods.

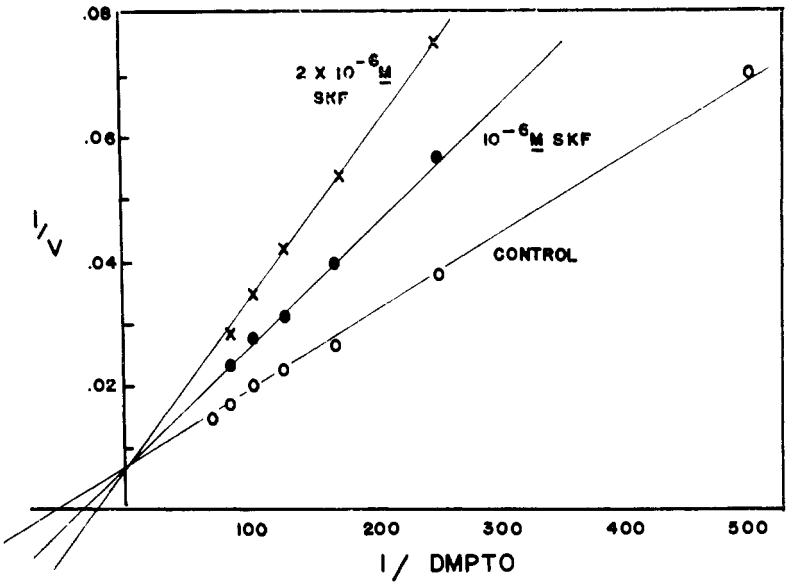

FIGURE 1: Lineweaver–Burk plots showing the competitive inhibition of SKF-525A on the dealkylation of *N,N*-dimethyl-*p*-toluidine *N*-oxide. Assays carried out as described under "Methods."

TABLE II: The Effect of *N,N*-Dimethylcyclohexylamine on the Michaelis Constant and Maximum Velocity of the Microsomal *N*-Oxide Dealkylase with Different Substrates.

	No DMCHA		10^{-3} M DMCHA	
Substrate	K_m[a]	V_{max}[b]	K_m[a]	V_{max}[b]
N,N-Dimethylaniline *N*-oxide	80	167	33	167
N,N-Dimethyl-*p*-toluidine *N*-oxide	20	286	15	286
N,N-Dimethylnaphthylamine *N*-oxide	7	200	6	200

[a] K_m expressed as micromoles of substrate per milliliter. [b] V_{max} expressed as millimicromoles of formaldehyde produced per milligram of protein per minute of infinite substrate concentration.

closely related trialkylamines (*i.e.*, *N,N*-dimethyl derivatives of pentyl-, hexyl-, or octylamine).

Inhibitors. As shown in Table III, the *N*-oxide dealkylase activity of microsomes was inhibited by compounds that are frequently used to inhibit oxidative N-dealkylation reactions; pyridine and SKF-525A were the most effective inhibitors tested. Both compounds, at a final concentration of 10^{-4} M, almost completely inhibited the demethylation of all the *N*-oxides tested. Both compounds appeared to be competitive with substrate and gave similar K_i values (2×10^{-6} M). A double reciprocal plot of the enzymic demethylation of DMPTO in the presence of two levels of SKF-525A is illustrated in Figure 1.

The microsomal *N*-oxide dealkylase activity was also inhibited by carbon monoxide, but only if the particles were reduced with NADH or NADPH. Unlike SKF-525A or pyridine, carbon monoxide did not, however, affect this activity of the fully oxidized particles. Since it would appear from other studies (Omura *et al.*, 1965) that the microsomal, carbon monoxide binding pigment combines only with the reduced hemoprotein, these data suggest that this hemoprotein may be involved in the anaerobic dealkylation of *N*-oxides. However, DMPTO (up to 20 μmoles/ml) did not alter the spectrum of this pigment obtained upon the addition of NADH or NADPH and carbon monoxide.

Effect of Detergents and Lipases. Omura and Sato (1964) reported that in microsomes treated with detergents, phospholipase A, or steapsin the absorption maximum of the carbon monoxide binding pigment shifted from 450 to 420 mμ. The experiments summarized in Table IV showed that the *N*-oxide dealkylase activity of microsomes was decreased in particles treated with these reagents. The enzymic activity of the treated particles declined at a somewhat faster rate than that at which the carbon monoxide binding pigment, measured at 450 mμ, was destroyed. However, destruction of the 450-mμ carbon monoxide binding pigment without loss of *N*-oxide dealkylase activity was never observed. Treatment of the particles with wheat germ lipase did not destroy a significant amount of the *N*-oxide dealkylase activity nor did this treatment alter the spectrum of the carbon monoxide binding pigment.

In particles treated with moderate levels of potassium

TABLE III: Effect of Inhibitors on the Microsomal *N*-Oxide Dealkylase Activity.

Compd Added to Assay Medium[a]	Concn (M)	mμmoles of Formaldehyde/min mg of Protein
None	...	49
Sodium azide	10^{-3}	50
Sodium cyanide	10^{-3}	48
Pyridine	10^{-4}	5
SKF-525A	10^{-4}	3
NADPH	5×10^{-5}	48
CO	100% CO in gas phase	49
CO + NADPH (5×10^{-4} M)	100% CO in gas phase	9
CO + NADH (5×10^{-4} M)	100% CO in gas phase	10
N_2 + NADPH (5×10^{-4} M)	100% N_2 in gas phase	44
CO + O	$[CO]/[O_2] = 4$ in gas phase	18

[a] Assays carried out as described in "Methods." Substrate used was DMPTO. The additions were made to the assay medium at the concentrations indicated. In the experiments where the gas phase was different from air, the reactions were carried out in Warburg vessels. The vessels were gassed for 5 min with the gas mixtures indicated and the reactions were started by adding substrate from the side arm.

TABLE IV: Effect of Lipases and Deoxycholate on the Carbon Monoxide Binding Pigment and *N*-Oxide Dealkylase Activity of Microsomes.

Pretreatment of Particles[a]		Per Cent of Untreated Particles[b]	
Reagent	Min at 38°	CO-Binding Pigment (450 mμ)	*N*-Oxide Dealkylase Act.
Wheat germ lipase	15	100	93
	30	93	91
Steapsin	15	84	60
	30	75	42
Phospholipase	10	80	56
	20	53	37
	30	48	20
Deoxycholate	10	55	36
	20	45	22
	30	42	15
	40	37	14
	60	31	2

[a] Conditions as described in Methods. Concentration of deoxycholate, 0.3 mg/mg of protein. [b] The values are expressed as the percentage of the original carbon monoxide binding pigment, measured at 450 mμ, and the DMPTO dealkylase activity remaining in the treated preparations.

deoxycholate at 38°, both the *N*-oxide dealkylase activity and the 450-mμ carbon monoxide binding pigment were destroyed (Table IV). Concentrations of this detergent (1.0 mg/mg of protein) sufficient to completely "solubilize" the particles destroyed both the *N*-oxide dealkylase activity and the 450-mμ carbon monoxide binding pigment in approximately 1 hr at 0°.

Differences between Hemoprotein and Microsomal N-Oxide Dealkylase Activities. As shown in Table V, hemoglobin and myoglobin can catalyze the dealkylation of DMPTO at a significant rate. However, the rate (per unit of heme) obtained with the hemoproteins was 50–100 times slower than the rate of the reaction catalyzed by microsomes. Furthermore, the *N*-oxide dealkylase activity of the hemoproteins, in contrast to the microsomal activity, was not destroyed by heat, detergents, or phospholipase treatment. The hemoprotein-catalyzed reaction was also insensitive to pyridine and SKF-525A at concentrations that inhibited the microsomal activity about 90%. Both the microsomal and the hemoglobin *N*-oxide dealkylase activities were inhibited by carbon monoxide to approximately the same extent, whereas the myoglobin-catalyzed reaction was somewhat less sensitive to this inhibitor.

Discussion

The data presented in this report demonstrate that pork liver microsomes can catalyze the dealkylation of a number of different but closely related *N*,*N*-dialkylarylamine *N*-oxides. It is evident from the experiments summarized in Table V that the microsomal *N*-oxide dealkylase activity cannot be attributed to nonspecific catalysis of this reaction by hemoproteins present in these particles. However, the hemoglobin-catalyzed reaction may, as proposed by Oae *et al.* (1963) and Terayama (1963), serve as a useful and valuable model for the microsomal enzyme system catalyzing the dealkylation of *N*-oxides.

Although the microsomal *N*-oxide dealkylase is inhibited by compounds (SKF-525A and carbon monoxide) used to inhibit mixed function oxidases (Brodie, 1958; Omura *et al.*, 1965), this enzyme system cannot be considered an oxygenase. A requirement for either oxygen or reduced pyridine nucleotides by the *N*-oxide dealkylase cannot be demonstrated. This enzyme system, however, may catalyze the last step in the oxidative dealkylation of tertiary amines catalyzed by microsomes, as previously suggested.

A close correlation is consistently observed between the *N*-oxide dealkylase activity and the integrity of the membrane-bound carbon monoxide binding pigment.

TABLE V: Microsomal- and Hemoprotein-Catalyzed Dealkylation of DMPTO under Different Conditions.

Pretreatment of Prepn	Addn to Assay Medium[a] (M)	mμmoles of Formaldehyde/min mμmoles of Heme[b]		
		Microsomes	Hemoglobin	Myoglobin
None	...	45	0.7	0.3
None	SKF-525A (10^{-4})	2	0.7	0.3
None	Pyridine (10^{-4})	8	0.9	0.2
None	100% CO in gas phase; NADH (10^{-3}) in medium	18	0.3	0.2
Heated at 100° for 3 min	...	0.0	3.5	1.1
Incubated with phospholipase at 38° for 20 min	...	12	0.8	0.3
Deoxycholate[c]	...	1.5	2.7	2.9
Triton X-100[c]	...	0	9.2	2.9

[a] The concentration of DMPTO used in these experiments was 10 μmoles/ml. Assays carried out as described in Methods. [b] The microsomes contained 1.2 μmoles of heme/mg of protein, measured as the pyridine hemochromogen. The concentrations of carbon monoxide binding pigment (450 mμ) and cytochrome b_5 were 0.49 and 0.52 mμmole/mg of protein, respectively. The concentration of the hemoproteins in the assays was 1.2–3.0 mμmoles of heme/ml. [c] Detergent (12 mg) added/ml of the preparations containing 24 mμmoles heme, and then incubated at 38° for 5 min.

Reagents known to alter the spectrum of this hemoprotein (Omura and Sato, 1964) also inactivate the *N*-oxide dealkylase activity of the preparations (Table IV). In addition, the *N*-oxide dealkylase activity of the reduced particles is inhibited by carbon monoxide (Table III). Although this evidence is only indirect, it indicates that the carbon monoxide binding pigment may be a component of the microsomal enzyme system catalyzing the anaerobic dealkylation of *N,N*-dialkylarylamine *N*-oxides.

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