Microsomal Oxidases. I. The Isolation and Dialkylarylamine Oxygenase Activity of Pork Liver Microsomes*

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ABSTRACT: The optimum conditions for this isolation of pork liver microsomal fractions that can catalyze the reduced pyridine nucleotide and oxygen-dependent oxidation of dialkylarylamines have been established. Maximum activity was observed with reduced nicotinamide–adenine dinucleotide phosphate (NADPH) which can partially be replaced by reduced nicotinamide–adenine dinucleotide (NADH). The data obtained with *N,N*-dimethylaniline as the substrate indicated that this microsomal tertiary amine mixed function oxidase could be functionally separated into two partial reactions: (1) a reduced pyridine nucleotide and oxygen-dependent *N*-oxide synthetase, and (2) an *N*-oxide dealkylase. The *N*-oxide synthetase activity of microsomal fractions partially depleted of flavin was specifically restored with flavin–adenine dinucleotide (FAD). Flavin mononucleotide did not reactivate the activity of the apoenzyme complex.

The studies of Mueller and Miller (1953) showed that mammalian liver homogenates catalyzed the oxidative demethylation of lipid-soluble *N*-methyl compounds. Subsequent studies by La Du *et al.* (1955) demonstrated that this enzyme system was localized in the microsomal fraction of liver homogenates. The isolated microsomal fraction supplemented with NADPH[1] and oxygen catalyzed the oxidative N dealkylation of a variety of lipid-soluble *N*-alkyl compounds to the aldehyde and corresponding amine (Gillette *et al.*, 1957). This enzyme system appeared to be of the type classified by Mason (1957) as a mixed function oxidase or, according to nomenclature proposed by Hayaishi (1962), as a mixed function oxygenase.

In spite of the extensive studies on microsomal tertiary amine oxygenases, the initial oxygenated product formed during the oxidative N dealkylation of lipid-soluble tertiary amines has not yet been defined. The work of McMahon and Sullivan (1964) suggests a mechanism requiring a direct oxidative attack on the methyl group of *N*-methyl tertiary amines to form the *N*-hydroxymethyl derivative which would readily decompose to the secondary amine and formaldehyde. On the other hand, the experiments of Fish *et al.* (1955) and the subsequent kinetic studies of Ziegler and Pettit (1964) suggest that the initial oxidative attack occurs on the nitrogen atom of the tertiary amine to yield an intermediate *N*-oxide. This oxygenated intermediate is dealkylated by a second enzyme system. The latter investigators demonstrated that under suitable conditions, *N,N*-dimethylaniline is oxidized to the *N*-oxide at a rate compatible with the over-all oxidative N demethylation of the tertiary amine catalyzed by hepatic microsomes. The intermediate *N*-oxide is enzymically dealkylated by hepatic microsome fractions at a rate several times greater than that of the over-all oxidative N dealkylation of the parent amine (Pettit and Ziegler, 1963). Based on these studies, it was suggested (Ziegler and Pettit, 1964) that the microsomal system catalyzing the oxidative N dealkylation of lipid-soluble dialkylarylamines could be separated into two partial reactions (*i.e.*, the *N*-oxide synthesizing and the *N*-oxide dealkylating systems). If the reactions are catalyzed by separate enzymes, or enzyme systems, it would be possible to eventually separate and isolate the enzymes involved.

With few exceptions (Baker and Chaykin, 1962), previous studies designed to isolate a microsomal tertiary amine oxygenase have been confined to hepatic tissue obtained from laboratory animals in which the microsomal fractions were isolated by a minor modification of the method of Hogeboom *et al.* (1948). The small quantity of isolated microsomes that could be conveniently prepared by this technique was a major obstacle in the isolation of an enzyme system as complex and low in activity as a microsomal tertiary amine oxygenase.

Methods of isolating pork liver microsomal fractions

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[1] The abbreviations used are: DMA, *N,N*-dimethylaniline; SKF-525A, diethylaminoethanol ester of diphenylpropylacetic acid; H-PLM, heavy pork liver microsomes; L-PLM, light pork liver microsomes; NADPH and $NADP^+$, reduced and oxidized nicotinamide–adenine dinucleotide phosphate; NADH and NAD^+, reduced and oxidized nicotinamide–adenine dinucleotide; FAD, flavin–adenine dinucleotide; FMN, riboflavin 5′-phosphate; AMP, adenosine monophosphate; NMN, nicotinamide mononucleotide.

on a relatively large scale were described by Garfinkel (1957) and Baker and Chaykin (1962). In both methods, the liver tissue was homogenized with a Waring blendor. However, in our laboratory, the yield and the DMA[1] oxidase activity of microsomal fractions isolated from homogenates of tissue disrupted with a blendor proved to be much more erratic than that of microsomal fractions isolated from tissue homogenized with a Potter–Elvehjem (glass Teflon) homogenizer. This study, therefore, was undertaken to develop a homogenizer operating on the same principle as the glass Teflon instrument in addition to being capable of disrupting large amounts of tissue. A description of such a homogenizer, a method of isolating relatively large amounts of pork liver microsomes, and the properties of the DMA mixed function oxidase are summarized in this report.

Experimental Section

Reagents. DMA from the Eastman Chemical Co. was distilled under vacuum. Aqueous solutions of 0.05 M DMA were prepared by dissolving the amine in 0.05 M HCl and by adding, if necessary, a slight excess of concentrated HCl to bring the pH down to between 2 and 3. The DMA solution was then stored at −20° until needed.

The nonionic detergent Triton X-100 was supplied by Rohm and Haas Co. The SKF-525A was obtained from Smith Kline and French Laboratories, Philadelphia, Pa. All of the nucleotides and the DL-isocitrate were purchased from Sigma Chemical Co. and were used without further purification. The preparations of isocitrate dehydrogenase, kindly supplied by Dr. Peter Jurtshuk of the Microbiology Department, were partially purified fractions obtained from *A. vinelandii*. The activity of the preparations varied between 12 and 20 μmoles of $NADP^+$ reduced/min mg of protein under the conditions of the DMA oxidase assays.

Methods. The amount of protein was estimated by the method of Gornall *et al.* (1949) and the protein-bound flavin was measured by the method described by De Bernard (1957). The DMA oxidase assays were carried out in open 10-ml erlenmeyer flasks at 38° in a Dubnoff metabolic shaker. The composition of the reaction media is listed in the tables. After preincubation of 4 min, the reaction was started by adding the amine substrate. Aliquots of the reaction mixture were withdrawn at intervals and pipetted into tubes containing sufficient 3.0 M trichloroacetic acid to give a final concentration of 0.3 M. The deproteinized supernatant fractions were assayed for formaldehyde by the method of Nash (1953) and for *N*,*N*-dimethylaniline *N*-oxide by the method previously described (Ziegler and Pettit, 1964). The DMA oxidase rates reported in the tables were corrected for the small amount of formaldehyde produced in the absence of DMA.

The flavin-deficient microsomal fractions were prepared by a modification of the method described by Pettit *et al.* (1964). KCl (220 mg) was added to pork liver microsomes (20 mg of protein/ml) suspended in 0.25 M sucrose. Batches (10 ml) of the suspension were subjected to sonic vibrations for 30 sec with a 20 KC Branson sonifier set at position 8. The pH of the preparation was adjusted to 5.0 by adding 1 M acetic acid and then centrifuged at 50,000 rpm for 15 min. The pellet was resuspended in 3 M KCl, adjusted to pH 5.0 with acetic acid, and resedimented. This step was repeated once more and the final pellet was resuspended in 0.01 M phosphate buffer, pH 7.5. Although this method did not yield a fraction as free from flavin as that obtained with the method described by Pettit *et al.* (1964), it was more reproducible and there was much less irreversible inactivation of the DMA oxidase activity.

Preparation of Pork Liver Microsomes. The livers were collected on the kill floor of the slaughterhouse within 10–15 min after the animals had been killed. The organs were immediately cut into slices not more than 2 cm thick and placed into large polyethylene bags which were flattened and stacked into an ice chest between layers of crushed ice. The temperature in all parts of the tissue had dropped to 5° or less by the time (approximately 20 min) they were transported to the laboratory. All subsequent steps in the preparation were carried out at 0–4°.

The chilled tissue slices were washed three times with approximately equal volumes of 0.25 M sucrose and minced with a meat grinder. The minced tissue (800 g) was suspended in 1 l. of 0.25 M sucrose and passed through the continuous-flow homogenizer shown in Figure 1. The homogenizer was operated at 500 rpm and the clearance between the pestle and the wall of the homogenizer was adjusted to give a flow rate of approximately 300 ml/min.

The homogenate[2] was centrifuged for 30 min at 5200 rpm in the 845 head of the International PR-2 centrifuge in order to sediment the nucleic cell debris and most of the mitochondria. The supernatant fraction was decanted through cheese cloth to remove bits of connective tissue and fat. The resulting suspensions, adjusted to pH 5.7 with 1 M acetic acid, was immediately centrifuged at 5200 rpm for 20 min and the well-packed residue was resuspended in about 10 volumes of 0.25 M sucrose. Enough 2 M Tris (free base) was then added to bring the pH to 7.8. The suspension was homogenized with a glass Teflon homogenizer and centrifuged for 10 min at 15,000 rpm in a Spinco Model L centrifuge to remove any mitochondria that might be present. The supernatant fraction was recentrifuged at 30,000 rpm for 40 min to sediment the microsomes. The sediment separated into two distinct layers (previously described by Garfinkel, 1958) which

[2] The pH of the homogenate at this point should have been 6.5 or above. If the pH was below 6.5, the homogenate was discarded since microsome fractions with an active DMA oxidase could not be isolated from such homogenates. In almost every case, when the pH of the homogenate was below 6.5, there had been an unavoidable delay in chilling the tissue after the animal had been killed and some autolysis of the tissue may have occurred.

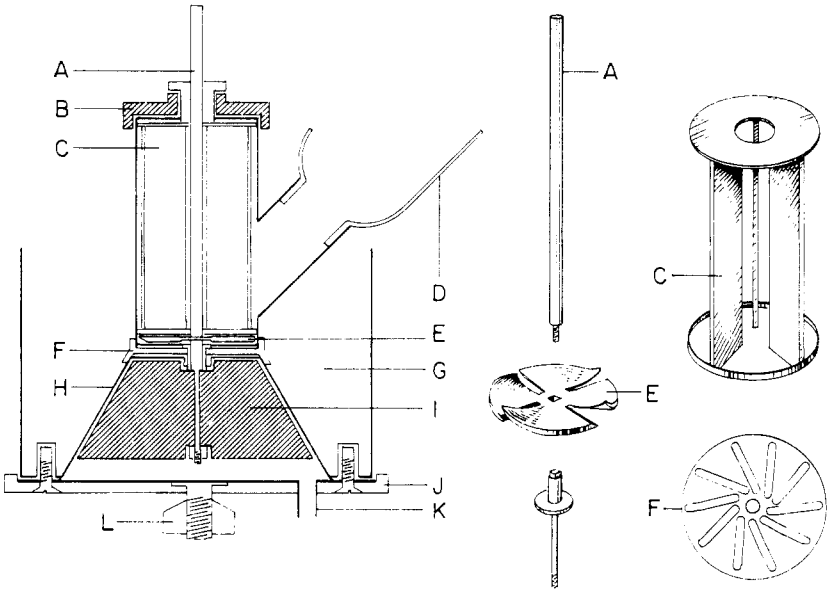


FIGURE 1: Cross section of continuous-flow homogenizer on the left. On the right, projection drawings of parts of the homogenizer, the details of which are not apparent in the drawing shown on the left. In operation, the homogenizer is mounted on a bench model drill press. The bolt that projects from the base plate "J" passes through the center hole of the drill press table and the unit is secured to the table by hand tightening the tapered nut "L." The exit port "K" passes through one of the diagonal slots of the drill press table. The drill press spindle is lowered and the chuck is secured to the homogenzier shaft "A." The clearance between cone "I" and the wall of the homogenizer "H" is adjusted by first moving the drill press spindle up as far as it will go, then depressing it about 0.5–1.0 mm and locking it. There is enough "spring" or give in the impeller blade "E" to permit the slight vertical movement of the homogenizer shaft. If the clearance is not properly set initially, it can readily be adjusted while the unit is in operation. The homogenizer is cooled by filling chamber "C" with ice. The minced tissue, suspended in 0.25 M sucrose, is poured into hopper "D" and the drill press motor is turned on. Cone "I" and the impeller blade "E" (which are secured to the shaft as shown) rotate at about 350 rpm and the impeller forces the tissue through the perforated plate "F" and then between the wall "H" of the homogenizer and the rotating cone "I." The tissue is disrupted by the shearing action exerted on it as it passes between the rotating cone and the wall of the homogenizer. To facilitate the flow of the homogenate, the cone is slotted at the top and on the sides. The slots cut on the sides of the cone are 30° apart and 4 mm deep at the apex, and taper to zero 1 cm above the base. The homogenate flows out through the exit "K" to which is attached a piece of 0.5-in. (od) polyethylene tubing (not shown) leading to a receiving vessel. The top of the receiving vessel is set about 4 in. above that of the homogenizer hopper so that the unit cannot drain during operation. If the unit is kept filled during operation, there is virtually no aeration of the homogenate. The baffles "C" retard the swirling of the tissue suspension in the upper part of the homogenizer which is in contact with air. More detailed drawings of the homogenizer and instructions on the construction of this homogenizer will be supplied upon request.

were collected separately: the well-packed residue or heavy fraction (H-PLM) that constituted 50–60% of the total microsomal pellet and the loosely sedimented fraction, or the light fraction (L-PLM). Both fractions were resuspended in about 10 volumes of 0.25 M sucrose and resedimented at 30,000 rpm for 1 hr. The fractions were resuspended in 0.25 M sucrose and stored overnight at −20°.

The following day, the thawed fractions were diluted with an equal volume of 0.25 M sucrose to give approximately 30 mg of protein/ml, and then resedimented at 30,000 rpm for 1 hr in order to remove as much soluble protein as possible. The washed pellets were then resuspended in a minimal volume of 0.25 M sucrose and stored at −20°. The fractions can be stored at this temperature for at least 6 months with little or no loss in DMA oxidase activity. Although the yield and the ratio of light to heavy microsome fractions varied considerably, the average amount of each fraction obtained from 800 g (wet weight) of the minced tissue was approximately 5 g of microsomal protein.

Results

The L-PLM fraction was consistently more active than the H-PLM fraction in catalyzing the oxidation of DMA (Table I). This was not surprising since the reports of Fouts (1962) and Orrenius *et al.* (1965) demonstrated that the oxidative N-dealkylation enzymes were more concentrated in the slower sediment-

TABLE I: DMA Oxidase Activities of Pork Liver Microsomal Fractions.[a]

Microsomal Prepn	No. of Prepn	mμmoles of DMA Oxidized[b]/ min mg of Protein
Female H-PLM	48	3.6 (1.9–7.0)
L-PLM	48	5.2 (2.7–9.5)
Male[c] H-PLM	16	3.7 (2.6–4.4)
L-PLM	16	5.0 (2.7–8.0

[a] The assay medium contained in micromoles per milliliter: potassium phosphate, pH 7.6, 100; semicarbazide, 1.0; $NADP^+$, 1.0; DL-isocitrate, 5.0; $MgCl_2$, 5.0; DMA, 5.0; and sufficient isocitric dehydrogenase to reduce 1 μmole of $NADP^+$/min. The concentration of microsomal protein was 2.5–4 mg/ml; incubation time, 10 min; temperature, 38°. [b] The numerical average of the activities obtained with preparations from the number of different animals indicated. The highest and lowest activities included in the average are shown in parenthesis. [c] Microsomal fractions isolated from hepatic tissue of castrated males. Hepatic tissue from normal males was not available since boars are not slaughtered locally.

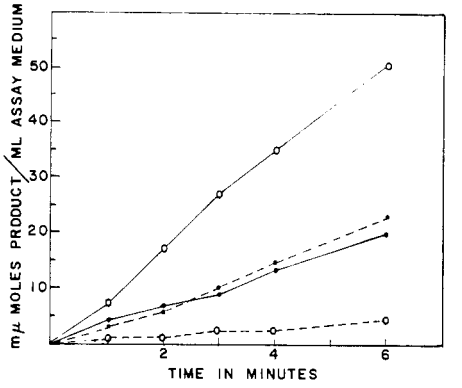


FIGURE 2: The rates of formaldehyde and *N*-oxide production in the presence of either NADH or NADPH. Assay conditions as described in Table II. O—O, millimicromoles of formaldehyde with NADPH; ●—●, millimicromoles of *N*-oxide with NADPH; O---O, millimicromoles of formaldehyde with NADH; ●---●, millimicromoles of *N*-oxide with NADH.

ing, smooth-surfaced microsomal particles than in the faster sedimenting, rough-surfaced microsomal particles. The H-PLM fraction contained quite variable but measurable amounts of succinic dehydrogenase and cytochrome *c* reductase activities. These activities could not, however, be detected in the L-PLM fraction.

Several laboratories have shown (see review by Brodie, 1958; Schuster, 1964) the oxidative N-dealkylation enzymes to be more concentrated in hepatic tissue from males than from females. Nevertheless, as shown in Table I, there was no significant difference in the levels of the DMA oxidase in pork liver tissue obtained from females or castrated males, although the activity of this enzyme system would probably have been greater in livers obtained from normal males. The enzyme activities reported in Table I were measured in microsomes isolated from liver tissue of healthy, adult (250–300 lb) animals. However, it should be noted that the animals were not selected according to breed and there was considerable variation in the length of time the animals were kept without food before slaughtering, factors which may partially account for the variation in activity observed in different preparations.

The optimum conditions for the oxidation of DMA by pork liver microsomes are given in Table I. The oxidation of DMA catalyzed by pork liver microsomes was previously shown to be dependent on oxygen and reduced pyridine nucleotide (Ziegler and Pettit, 1964). In the presence of both NADPH and oxygen, the oxidation of DMA was linear with time for at least 6 min (Figure 2) and was also a linear function of enzyme concentration, between 1 and 5 mg of microsomal protein/ml. There was no measurable reduction or dealkylation of the free *N*-oxide at the levels produced in the short reaction times used in these assays. The DMA oxidase activity of the microsomes preincubated at 38° for 10 min in the presence of NADPH was not diminished but, in its absence, about one-half of the activity was lost in 4 min. The concentration of NADPH (1 μmole/ml) used was approximately four times that required to saturate the enzyme (F. F. Sun, W. H. Orme-Johnson, and D. M. Ziegler, in preparation) and since hepatic microsomes reportedly contain dinucleotide pyrophosphatases (Jacobson and Kaplan, 1957), this level was routinely used to ensure that the reduced nucleotide did not become limiting during the reaction.

Pyridine Nucleotide Specificity of DMA Oxidase. In contrast to freshly prepared rat liver microsomes (Ziegler and Pettit, 1964), significant amounts of the tertiary amine *N*-oxide were always formed during the NADPH-dependent oxidation of DMA (Table II) catalyzed by pork liver microsomes. The ratio of *N*-oxide to formaldehyde was quite variable with different microsomal preparations, but with freshly prepared L-PLM fractions the rate of formaldehyde production was usually 1.5–3 times greater than the rate of *N*-oxide synthesis and both products were synthesized at rates linear with time during the first 6 min of the reaction (Figure 2).

Maximum DMA oxidase activities were always obtained in the presence of NADPH (Table II), although this enzyme system did not appear to be absolutely specific for this nucleotide. A small but definite rate was usually observed when NADH was substituted for NADPH, and the addition of both reduced nucleotides did not increase the activity over that obtained with NADPH alone. NAD^+ or the mononucleotides AMP and NMN had no effect on the tertiary amine oxygenase activity of the microsomes in the presence

TABLE II: Reduced Pyridine Nucleotide Requirements of Pork Liver DMA Oxidase.

Pyridine Nucleotides Added[a]	mμmoles of Product/min mg of Protein	
	N-Oxide	Formaldehyde
$NADP^+$	0.0	0.0
NADPH	1.3	2.7
NAD^+	0.0	0.0
NADH	0.9	0.3
NADPH + NADH	1.5	2.6
NADPH + NAD^+	1.4	2.8
NADH + $NADP^+$	1.3	2.6

[a] The assay conditions were as given in Table I, except the isocitrate–NADPH generating system was omitted. The nucleotides were added as indicated to give a final concentration of 1 μmole/ml; microsomal preparation, L-PLM. Quantitatively similar results were obtained with the DMA oxidase of rat liver microsomes (D. M. Ziegler and F. H. Pettit, unpublished observation).

TABLE III: Flavin Nucleotide Requirements of the Microsomal DMA Oxidase.

Fraction	Flavin (mμmoles/mg of Protein)	Additions of Medium[a]		DMA Oxidized[b]/min mg of Protein
		Pyridine Nucleotide	Flavin Nucleotide	
L-PLM	0.34	NADPH	None	5.1
			FAD	5.1
			FMN	4.9
		NADH	None	2.2
			FAD	2.1
			FMN	2.1
Acid–salt treated L-PLM	0.23	NADPH	None	1.8
			FAD	4.8
			FMN	1.9
		NADH	None	0.9
			FAD	1.9
			FMN	0.7

[a] Assay conditions as given in Table I, except NADPH-generating system was omitted. The concentration of pyridine nucleotides was 1 μmole/ml and the flavin nucleotides, 10 mμmoles/ml. [b] Activities calculated by summation of both *N*-oxide and formaldehyde formed. After acid–KCl treatment, the *N*-oxide is the principal product formed.

of saturating levels of NADPH. In the majority of preparations studied, NADH was from 20 to 30% as effective as NADPH. However, considerable variation in this ratio was observed with different preparations of microsomes; in one preparation, NADH was 90% as effective as NADPH, and in two other preparations NADH-dependent DMA oxidase activity could not be detected. The two nucleotides also yielded a quantitative difference in the ratio of *N*-oxide to aldehyde (Figure 2). In the presence of NADH, the tertiary amine *N*-oxide was the principal product obtained during the first few minutes of the reaction.

In spite of the great variation observed in the ratio of NADH- to NADPH-dependent DMA oxidase activities among different preparations of microsomes, these activities do not appear to be due to entirely separate enzyme systems. Both pyridine nucleotide dependent DMA oxidase activities of flavin-deficient microsomes were specifically restored by FAD (Table III) to approximately the same extent. Flavin mononucleotide or other nucleotides (NMN and AMP) did not restore either the NADPH- or NADH-dependent DMA oxidase activities of the apoenzyme.

Factors Affecting the Oxidation Products Produced by the DMA Oxidase. The data presented in Table IV demonstrate that the ratio of *N*-oxide to formaldehyde formed during the oxidation of DMA is markedly affected by pretreatment of the microsomes. The DMA oxidase of particles disrupted mechanically by repeated freezing and thawing or by sonic irradiation catalyzes the production of more *N*-oxide than formaldehyde. Similar results are obtained by fragmenting the microsomes with detergents (Table IV). The concentration of detergents (0.3 mg/mg of protein) used produces a marked decrease in the turbidity of the suspension, but this level is not sufficient to completely "solubilize" the particles. At higher concentrations (1 mg/mg of protein) of either detergent, the DMA oxidase activity is virtually destroyed after incubation at 0° for 1 hr. However, under the conditions described in Table IV, the DMA oxidase activity (expressed as the sum of the ratio of formaldehyde and *N*-oxide production) of the treated particles is about the same as that of the untreated particles.

The effect of several different oxidase inhibitors on the DMA oxidase activity of microsomes is shown in Table V. In contrast to the alcohol mixed function oxidase also present in these particles (Orme-Johnson and Ziegler, 1965), the DMA oxidase is not inhibited by either azide or cyanide. The last two compounds listed (SKF-525A and carbon monoxide), at the lower concentrations tested, also do not inhibit the oxygenation of DMA, as shown by the increased rate of *N*-oxide synthesis in the presence of these compounds. However, both SKF-525A and CO appear to inhibit the subsequent dealkylation of the *N*-oxide to the secondary amine and aldehyde since the rate of formaldehyde formation is decreased by these inhibitors. These results are consistent with the observation (Machinist *et al.*, 1966) that the anaerobic dealkylation of substrate amounts of *N*,*N*-dialkylarylamine *N*-oxides,

TABLE IV: Effect of Different Pretreatments on the DMA Oxidase Activity of Microsomes.[a]

Microsomal Prepn	Oxidation Products in mμmoles/min mg of Protein: *N*-Oxide	Formaldehyde
Freshly prepared L-PLM	1.5	2.7
L-PLM frozen and thawed each day for 5 days	2.2	1.0
L-PLM subjected to sonic vibrations[b]	2.3	0.8
L-PLM treated with Triton X-100[c]	3.0	0.6
L-PLM treated with potassium cholate[c]	2.8	0.3

[a] Assay conditions as described in Table I. [b] The microsomal suspension (15 ml, 40 mg of protein/ml) was adjusted to pH 9.0 with KOH and subjected to sonic vibration for four 15-sec intervals with a Branson 20KC sonifier set at position 8. [c] The microsomes (40 mg of protein/ml) were suspended in 0.21 M sucrose and 0.02 M glycine buffer, pH 9.5. Triton X-100 or cholate was added to give a final concentration of 12.0 mg/ml and the suspension was incubated 1 hr at 0° before assaying.

TABLE V: Effect of Inhibitors on the Microsomal DMA Oxidase.

Addn to the Basic Compd	Assay Media[a] Concn (M)	Oxidation Products in mμmoles/min mg of Protein: *N*-Oxide	Formaldehyde
None	...	2.1	2.4
Sodium azide	10^{-3}	2.0	2.4
Sodium cyanide	10^{-3}	2.2	2.3
SKF-525A	1×10^{-4}	2.7	1.5
	9×10^{-4}	3.2	0.9
	1.8×10^{-3}	3.3	0.5
	Ratio of CO/O_2 in Gas Phase		
Carbon monoxide[b]	2.3	2.4	2.2
	4.0	2.8	1.1
	9.0	2.2	0.9

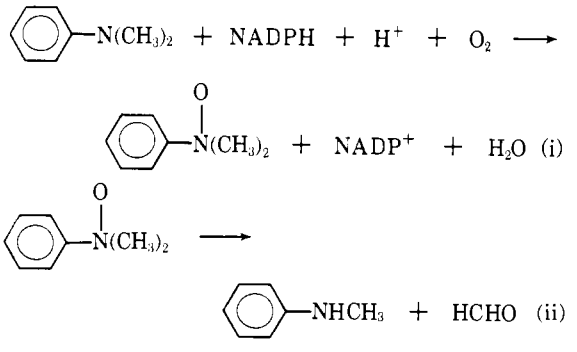
[a] The assay conditions were as described in Table I. The L-PLM fraction used in these experiments had been frozen and thawed three times over a 1-week period. [b] These experiments were carried out in Warburg vessels. The vessels were gased with prepared CO–O_2 mixture for 5 min and then the reaction was started by adding substrate from the side arm. After 5 min, TCA was added to stop the reaction.

catalyzed by microsomes, is inhibited by these two compounds.

Discussion

The determination of the optimum conditions for the preparation of hepatic microsomes in relatively large quantities is a necessary prerequisite for later studies aimed at the extraction of a tertiary amine oxygenase from these particles. The use of the continuous-flow homogenizer that operates on the same principle as the Potter–Elvehjem homogenizer yields liver microsome fractions with tertiary amine oxygenase activity comparable to that of hepatic microsomes isolated on a small scale from laboratory animals. Although this study was restricted to the use of a single lipid-soluble tertiary amine substrate, the data obtained with DMA might be extended to other *N*,*N*-dialkylaryl compounds. The studies of Gillette *et al.* (1957), Axelrod (1956), and McMahon (1961) indicate that the microsomal oxidative N-dealkylation systems are relatively nonspecific and can oxidize a wide variety of drugs and other foreign compounds containing *N*-alkyl groups. However, the work of Gillette *et al.* (1957), based on the ability of certain inhibitors to differentially affect the oxidation of different substrates, suggests that more than one enzyme system may be involved.

The experiments summarized in Table V provide additional evidence that the pork liver microsomal DMA oxidase can be separated into the following two partial reactions:



In reaction i, the "oxygenation" step, the amine is oxidized to the *N*-oxide. The intermediate *N*-oxide is subsequently dealkylated by a second enzyme system, as shown in reaction ii. The observation that, in the presence of SKF-525A or carbon monoxide, the oxidation of DMA proceeds only to the *N*-oxide (Table II) is consistent with this mechanism, since it has been shown (Machinist *et al.*, 1966) that the enzymic dealkylation of the *N*-oxide is inhibited by both SKF-525A and carbon monoxide.

The data given in Table IV support the hypothesis that the microsomal enzymes are protected by a lipoidal barrier that is penetrated only by fat-soluble compounds (Gaudette and Brodie, 1959). In keeping with this model, the lipid-soluble tertiary amine can penetrate to the DMA oxidase where it is oxidized to the polar *N*-oxide. Because of its low lipid solubility, the *N*-oxide cannot readily escape from the lipoidal compartment and is therefore dealkylated to the less polar secondary amine. However, if this lipoidal barrier is disrupted, the polar *N*-oxide could leave the site of synthesis and, because of its low affinity for the *N*-oxide dealkylase (Pettit and Ziegler, 1963), accumulate in the media. As shown by the data, this is precisely what occurs when the microsomes are disrupted by sonic vibrations, by repeated freezing and thawing, or by treatment with detergents.

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