

The Oxidation of Hydrazine Derivatives Catalyzed by the Purified Liver Microsomal FAD-containing Monooxygenase*

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A number of hydrazine derivatives were tested as substrates for the purified liver microsomal FAD-containing monooxygenase and the kinetic properties of the oxidation reactions were partially characterized. Only 1,1-dimethylhydrazine, 1-methyl-1-phenylhydrazine, and the *N*-aminoheterocyclic hydrazines are oxidized as effectively as *N,N*-dimethylaniline, one of the best *N*-methylamine substrates for the enzyme. In addition, the pH-rate profiles for the oxidation of several hydrazines were identical with that noted for *N,N*-dimethylaniline. Studies aimed at defining the stoichiometry of the monooxygenase-catalyzed hydrazine oxidation have indicated that the oxidation reaction is stoichiometric (1:1:1) with regard to NADPH, oxygen, and either 1,1-dimethylhydrazine or 1-methyl-1-phenylhydrazine as substrate. However, the oxidation of the *N*-aminoheterocyclic hydrazines required two molecules each of NADPH and oxygen to metabolize one molecule of *N*-aminopiperidine or *N*-aminohomopiperidine. The formation and stoichiometry of the products obtained from 1,1-dimethylhydrazine strongly support the existence of a diazene intermediate that may result from the dehydration of an *N*-hydroxy metabolite which can tautomerize subsequently under certain conditions to yield a formaldehyde methylhydrazone (possibly via an azomethanimine species).

Inhibitors of the cytochrome P-450-dependent monooxygenase were without effect on the microsomal *N*-demethylation of 1,1-dimethylhydrazine, but methimazole, a substrate for the FAD-containing monooxygenase, strongly inhibited the reaction catalyzed by rat and hamster liver microsomes. This microsomal activity was not induced by animal pretreatment with phenobarbital or 3-methylcholanthrene. These results strongly suggest that the microsomal metabolism of 1,1-dimethylhydrazine, and perhaps other 1,1-disubstituted hydrazines, is principally catalyzed by the FAD-containing monooxygenase.

The existence of several enzymes which catalyze the *N*-oxidation of nitrogenous compounds having carcinogenic and/or toxic potential has prompted the detailed study of the biochemical properties of these proteins. The ability of monooxygenases in the liver to metabolize oxidatively *N*-methyl azo dyes and drugs to yield formaldehyde and an *N*-demethylated amine was noted as early as 1950 (1, 2). These reactions were shown to require molecular oxygen and NADPH and were catalyzed by the liver microsomal fraction of mammals. Subsequently, it has been shown that two major monooxygenases exist in the liver microsomal fraction of mammals: the cytochrome P-450-dependent enzyme system (3) and a flavoprotein (3-6), mixed-function amine oxidase or the FAD-containing monooxygenase (EC 1.14.13.8, dimethylaniline monooxygenase (*N*-oxide forming)).

In 1966, Ziegler described the *N*-oxidation of *N,N*-dimethylaniline by pig liver microsomal fractions and established that this reaction was distinct from the reaction catalyzed by the cytochrome P-450-dependent monooxygenase (4). Subsequently, the FAD-containing monooxygenase was purified, characterized, and shown to have a broad substrate specificity for *N*-methylamine derivatives (5). The enzyme catalyzes the *N*-oxidation of secondary and tertiary *N*-methylamines to yield secondary *N*-methylhydroxylamines or tertiary *N*-methyl-*N*-oxides as products, respectively. In addition, it has been noted that the amine oxidase can also catalyze the *S*-oxidation of thiols and thioureas (6-8) and the *N*-oxidation of certain hydrazine derivatives (9). This flavoprotein is distinct from the mitochondrial amine oxidase and from the flavoprotein reductases involved in the reactions of cytochromes *b*₅ and P-450 (10). Further, the activity of this enzyme is not inducible by barbiturate or carcinogen animal pretreatment and is not adversely affected by many of the inhibitors of the cytochrome P-450 enzyme system, such as metyrapone, SKF 525A, *n*-octylamine, and anti-NADPH-cytochrome *c* (P-450) reductase globulin (11). Although only a few primary amines serve as substrates for the amine oxidase, others such as *n*-octylamine can interact at a regulatory site to enhance the rate of *N*- and *S*-oxidation reactions catalyzed by the enzyme (12). This report will characterize the oxidation of a variety of hydrazine derivatives catalyzed by the purified porcine liver microsomal FAD-containing monooxygenase and will demonstrate that this enzyme is most likely responsible for the oxidative metabolism of 1,1-disubstituted hydrazines in liver microsomal fractions.

EXPERIMENTAL PROCEDURES

Materials—NADP⁺ and NADPH were purchased from P-L Biochemicals, Inc., and DL-isocitric acid, isocitrate dehydrogenase (type IV), Trizma base, and Tricine¹ were obtained from Sigma. The hydrazine derivatives used in the study were purchased from either Aldrich, Eastman, K & K Rare and Fine Chemical Co., or Sigma. Procarbazine (*N*-isopropyl- α -(2-methylhydrazino)-*p*-toluamide hydrochloride) and isocarboxazide (5-methyl-3-isoxazolecarboxylic acid 2-benzylhydrazide) were gifts from the Hoffman-LaRoche and Car-

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¹ The abbreviation used is: Tricine, *N*-[tris(hydroxymethyl)methyl]glycine.

bidopa (α -hydrazino-dopa) was a gift from Merck. The purity of the hydrazines was established by Silica Gel G thin layer chromatography using a solvent system consisting of 0.5 M oxalic acid in 1-butanol (1:4, v/v). Most of the compounds elicited a single ninhydrin positive spot on thin layer chromatography; in general, no additional spots were observed after treatment with sulfuric acid, ninhydrin, or UV light. Those which were impure were recrystallized from organic solvent as the hydrochloride or oxalate salt. A number of the hydrazines were maximally stable to air oxidation only as the hydrochloride or oxalate salt. The concentration of most of the hydrazines in buffered solution was determined by adding microliter quantities of the compound to a solution of 1 mM potassium ferricyanide in 0.1 M Tris-HCl at pH 8.5. The reduction of ferricyanide ion by the hydrazines was monitored at 420 nm and the concentration of hydrazine was calculated, assuming an extinction coefficient for ferricyanide ion of $1,040 \text{ M}^{-1} \text{ cm}^{-1}$ and a stoichiometry of reduction of 2 for the hydrazines. In addition, 1,1-dialkylhydrazine concentration could be determined by measuring the amount of formaldehyde-1,1-dialkylhydrazine formed on addition of a 20-fold excess of formaldehyde at pH 7.0, assuming an extinction coefficient of $5.19 \times 10^3 \text{ M}^{-1} \text{ cm}^{-1}$ at 236 nm (13). The concentrations obtained in either manner agreed well with the concentration expected. *N,N*-Dimethylaniline was purified by preparative gas chromatography and *n*-octylamine was purified by distillation. All other reagents were obtained in the highest available purity from commercial sources.

A large portion of the purified FAD-containing monooxygenase used in this study was generously provided by Professor D. M. Ziegler, Department of Chemistry and the Clayton Foundation Biochemical Institute, The University of Texas at Austin. The purified monooxygenase was prepared as described by Ziegler and Poulsen (6, 14) and had an endogenous NADPH "oxidase" rate of approximately 12–15 nmol of NADPH oxidized/min/mg of protein at pH 8.3 and 37 °C.

Preparation of Liver Microsomes—Swine liver tissue was obtained from local slaughter houses, male Golden Syrian hamsters (130–150 g) were purchased from Lakeview Hamster Farms, and Sprague-Dawley rats (150–250 g) were purchased from the Charles River Breeding Laboratories, Inc. The rodents were maintained *ad libitum* on water and laboratory chow. Rodents were injected intraperitoneally with corn oil (0.1 ml), phenobarbital in saline (80 mg/kg), or 5,6-benzoflavone in corn oil (60 mg/kg) daily for 4 days and starved 18 h prior to killing. Pig liver microsomes were prepared as described by Ziegler and Pettit (4) and rodent liver microsomes were prepared as described by Remmer *et al.* (15).

Determination of Protein Concentration—Protein concentration was determined by the method of Lowry *et al.* using bovine serum albumin (Sigma) as a protein standard (16).

Assay of Purified FAD-containing Monooxygenase Activity—The initial rates of hydrazine oxidation were measured by monitoring spectrophotometrically (340 nm) the oxidation of NADPH in a Beckman model 25 spectrophotometer thermostatted at 37 °C. One-ml cuvettes containing either 0.1 M Tris-HCl buffer and 1 mM EDTA or 0.05 M potassium phosphate, 0.05 M Tricine-HCl and 1 mM EDTA were preincubated for 3–5 min at either pH 7.4 or 8.2; 0.02–0.1 mg/ml of purified enzyme and NADPH (0.18–0.25 mM) were added to the sample cuvette. The NADPH "oxidase" rate was measured for 1 min and the substrate of interest was added in a small volume to determine the substrate-dependent NADPH oxidase rate. The rates of metabolism were calculated by subtracting the initial oxidase rate from the substrate-dependent rate of NADPH oxidation. The data obtained for kinetic analysis of 1,1-dimethylhydrazine oxidation (double-reciprocal plots) were obtained by varying the concentrations of both NADPH and hydrazine derivative at 37 °C and pH 7.4. Since the initial rate measurements were constant with respect to NADPH concentrations above 0.15 mM for *N,N*-dimethylaniline, 1,1-dimethylhydrazine, phenylhydrazine, 1-methyl-1-phenylhydrazine, and *N*-aminopiperidine, the substrate specificity studies reported were determined using saturating concentrations of NADPH (0.18 mM) and approximately 0.24 mM oxygen at 37 °C and pH 8.3. Stoichiometry studies were performed by addition of limiting amounts of hydrazine (25–110 μM) and measuring the substrate-dependent extent of either NADPH oxidation or oxygen utilization as described by Poulsen *et al.* (7).

Oxygen uptake was measured with a Clark-type electrode at 37 °C in a thermostatted glass chamber. The electrode response was calibrated with beef heart electron transport particles (17) and NADH. The standard reaction mixture, except enzyme, NADPH, and substrate, were placed in the polarograph chamber and equilibrated at 37 °C. The endogenous rate of NADPH-dependent oxygen consumption

(NADPH oxidase activity) was measured for 1 min after addition of NADPH and pure enzyme in the absence of substrate. The substrate-dependent oxygen consumption was determined by measuring oxygen uptake after addition of substrate; the substrate-dependent rate of oxygen consumption was calculated by subtracting the endogenous rate of oxygen uptake from the substrate-dependent rate of oxygen consumption.

The amount of methane formed by oxidation of methylhydrazine was measured by gas chromatography as previously described (9, 18). The formaldehyde formed by oxidation of methylhydrazine or 1,1-dimethylhydrazine was measured using the method of Nash (19) and the methylhydrazine formed by oxidation of 1,1-dimethylhydrazine was determined by reacting the methylhydrazine formed with Ehrlich's reagent (*p*-dimethylaminobenzaldehyde) in acid to yield the aldazine derivative which can be measured spectrophotometrically at 465 nm (20) assuming an extinction coefficient of $3,000 \text{ M}^{-1} \text{ cm}^{-1}$. Tetramethyl- and dipiperidyltetrazenes were synthesized according to the method of McBride and Kruse (21). These tetrazenes could be isolated by organic extraction of the aqueous reaction mixtures and quantitated using high pressure liquid chromatography with a μ Bondapak C18 column (Waters Associates, Milford, MA) under isocratic conditions (60% methanol in water, 10 mM ammonium acetate) at a 1 ml/min flow rate (22). The eluate was monitored with a UV detector at 278 nm. Hydrogen peroxide formation was measured using potassium thiocyanate and ferrous ammonium sulfate (23).

The microsomal metabolism of 1,1-dimethylhydrazine was measured using incubation mixtures containing 1 mM 1,1-dimethylhydrazine, 2 mg/ml of microsomal protein, 0.5 mM NADP⁺, 3 mM DL-isocitrate, 5 mM MgSO₄, 0.8 unit/ml of isocitrate dehydrogenase, 1×10^{-3} M EDTA, and 0.05 M potassium phosphate, 0.05 M Tricine HCl buffer at pH 7.4 or 8.3. After a 5-min preincubation at 37 °C, microsomal protein was added and the reaction immediately initiated by addition of 1,1-dimethylhydrazine. One-ml aliquots were removed at 1-min intervals and the amount of formaldehyde or methylhydrazine was determined as described in the previous paragraph. When inhibition experiments were performed, the inhibitor was added prior to the preincubation. Antibody experiments using ammonium sulfate precipitated non-immune and anti-NADPH-cytochrome *c* reductase globulin were performed as reported previously (11).

RESULTS

Stoichiometry of Hydrazine Oxidation Catalyzed by the FAD-containing Monooxygenase—The substrate stoichiometry for the oxidation reaction catalyzed by the purified liver microsomal FAD-containing monooxygenase was determined by measuring the amount of NADPH oxidized or oxygen reduced upon addition of a finite amount of hydrazine substrate. The concentration of hydrazine used was determined chemically and aliquots of the hydrazine substrates were

TABLE I
Stoichiometry of hydrazine oxidation catalyzed by the FAD-containing monooxygenase

The stoichiometry of hydrazine oxidation catalyzed by the purified FAD-containing monooxygenase was measured using the method of Poulsen *et al.* (7) to monitor the extent of reaction. Substoichiometric amounts of the substrate were added and the amount of NADPH or oxygen utilized due to substrate oxidation determined.

Substrate added	Concentration μM	NADPH oxidized μM	Oxygen reduced μM	Stoichiometry ^a
Dimethylaniline	25	24		1.04
	110		104	1.06
Benzphetamine	24	25		0.96
	109		96	1.14
1-Methyl-1-phenylhydrazine	50	53		0.94
	88		86	1.02
<i>N</i> -Aminopiperidine	25	53		0.41
	110		201	0.55
<i>n</i> -Aminohomopiperidine	25	45		0.56
	112		186	0.60

^a The stoichiometry expresses the amount of hydrazine added relative to the amount of either NADPH or oxygen used due to substrate oxidation.

added in substoichiometric amounts relative to oxygen and NADPH. Table I shows the substrate stoichiometry for two tertiary *N*-methylamines and three hydrazines. The stoichiometry of *N*-oxidation is expressed as a ratio of moles of hydrazine added to moles of NADPH oxidized or oxygen utilized. The *tert*-*N*-methylamine compounds had a 1:1:1 stoichiometry for NADPH, oxygen, and amine as reported by Ziegler and Mitchell (5). The methylhydrazine derivatives behaved as expected for a substrate of the monooxygenase, that is, a 1:1:1 stoichiometry was noted for 1-methyl-1-phenylhydrazine (Table I). However, the *N*-aminoheterocyclic hydrazines (*N*-aminopiperidine and *N*-aminohomopiperidine) demonstrated approximately a 2:2:1 stoichiometry for NADPH, oxygen, and substrate, respectively. This result would suggest that the *N*-aminoheterocyclic compounds may undergo two successive *N*-oxidation steps. The first product formed apparently has a high affinity for the monooxygenase and may be metabolized further.

The products of the chemical oxidation of methylhydrazine by sodium bromate were shown to be methane and formaldehyde which were formed in substoichiometric quantities (Table II). As anticipated from other studies (21, 24), the yields of both formaldehyde and methylhydrazine from 1,1-dimethylhydrazine are equal and are stoichiometric with the amount of hydrazine added (approximately 100%, Table II). Upon titration to neutral or slightly alkaline conditions, the 1,1-dimethyldiazonium ion deprotonates to form the 1,1-dimethyldiazene which can dimerize to yield a tetramethyltetrazene with a characteristic absorbance maximum at 278 nm (21). The tetrazene derivative was formed in slightly substoichiometric quantities relative to the amount of parent hydrazine used. The stoichiometry of chemical oxidation shown in Table II is expressed as the amount of product formed by oxidation of a known amount of hydrazine with sodium bromate in acid solution.

During the enzymatic oxidation of methylhydrazine catalyzed by the flavoprotein, both formaldehyde and methane were formed at combined rates which approximately equal the amount of NADPH oxidized (Table II). The NADPH-dependent oxidation of 1,1-dimethylhydrazine yielded both formaldehyde and methylhydrazine in equimolar concentrations (based on rate of oxidation) as acid-stable products. The stoichiometry for the enzymatic reaction is expressed as the rate of product formation relative to the rate of NADPH

TABLE II

Identification of the products of hydrazine oxidation by potassium bromate and by enzymatic monooxygenation

Substrate	Product	Stoichiometry	
		Chemical ^a	Enzymatic ^b
CH ₃ NHNH ₂	CH ₄	0.04	0.01
	HCHO	0.40	1.04
(CH ₃) ₂ NNH ₂	HCHO	0.95	0.90
	CH ₃ N ₂ H ₃	1.14	0.83
	Tetrazene	0.40 ^c	0.00
<i>N</i> -Aminopiperidine	Tetrazene	0.45	0.03

^a The chemical oxidation was performed using a modification of the method of McBride *et al.* (21) as described under "Experimental Procedures." The stoichiometry was determined by quantitation of the amount of product formed by oxidation of a known amount of hydrazine by sodium bromate and is expressed as moles of product formed/mol of hydrazine added.

^b The enzymatic *N*-oxidation was determined by measuring the rate of product formation and the rate of NADPH oxidation. The stoichiometry is expressed as the rate of product formation divided by the rate of NADPH oxidation.

^c McBride *et al.* (21) reported yields of 80–90%.

TABLE III

Michaelis-Menten parameters for the oxidation of hydrazines catalyzed by the FAD-containing monooxygenase

Substrate ^a	<i>K_m</i>	<i>V_{max}</i> ^b
	mM	nmol/min/mg
Dimethylaniline	0.02	950
Methylhydrazine	35.0	1370
Ethylhydrazine	40.0	1350
<i>n</i> -Propylhydrazine	15.0	1520
Isopropylhydrazine	8.3	603
Butylhydrazine	6.9	1480
Phenylhydrazine	3.0	890
Benzylhydrazine	7.0	880
1,2-Dimethylhydrazine	12.0	644
1-Methyl-2-benzylhydrazine	2.0	1260
Procarbazine	5.7	560
β-Ethylphenylhydrazine	3.3	1250
1,1-Dimethylhydrazine	0.43	890
1-Methyl-1-phenylhydrazine	0.08	1130
1,2-Dimethylphenylhydrazine	0.38	895
<i>N</i> -Aminopyrrolidine	0.10	960
<i>N</i> -Aminomorpholine	0.61	950
<i>N</i> -Aminopiperidine	0.03	960
<i>N</i> -Aminohomopiperidine	0.17	878

^a Nonsubstrates: Carbidopa, Hydralazine, Isoniazide, Iproniazide, and Isocarboxazide.

^b Experiments were performed using purified monooxygenase in 0.05 M Tricine buffer, pH 8.2, at 37 °C.

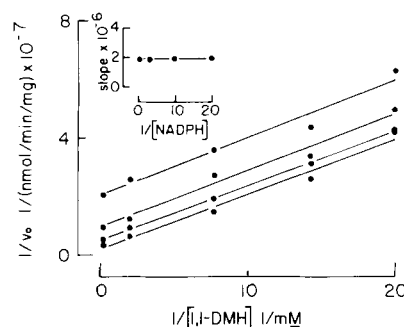


FIG. 1. The double reciprocal plots of initial velocities of 1,1-dimethylhydrazine oxidation. The reaction conditions were as described under "Experimental Procedures." In the plot, 1,1-dimethylhydrazine concentrations were varied and the oxygen concentration initially was 0.24 mM. The concentrations of NADPH from bottom to top are 250, 30, 10, and 5 μ M. The inset shows the replots of the slopes versus NADPH concentration. 1,1-DMH, 1,1-dimethylhydrazine.

oxidation. The rates of oxygen reduction and NADPH oxidation were equal in all cases. In addition, ethyl acetate extracts of the reaction mixture terminated with base were analyzed using high performance liquid chromatography; no detectable amounts of tetramethyltetrazene were detected. The high pressure liquid chromatography system developed could have detected levels of tetramethyltetrazene produced at 0.1–0.5% of the rate of NADPH oxidation. When *N*-aminopiperidine was utilized as a substrate, substoichiometric amounts of dipiperidyltetrazene was formed (approximately 3%), and to date, the other metabolites formed have not been isolated or characterized.

Since nitrosamines can be detected in aged aqueous solutions of hydrazine,² we used thermoelectron activation analysis (25) to test whether the FAD-containing monooxygenase could enzymatically convert the 1,1-disubstituted hydrazines

² R. N. Hines and R. A. Prough, unpublished results.

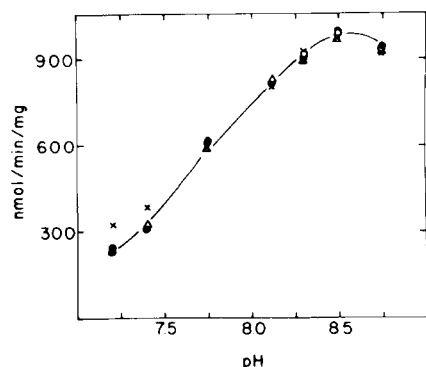


FIG. 2. The pH-rate profiles for hydrazine oxidation catalyzed by the purified FAD-containing monooxygenase. ○, *N,N*-dimethylaniline; ●, 1,1-dimethylhydrazine; △, phenylhydrazine; ×, *N*-aminopiperidine.

TABLE IV

Influence of substrates on hydrogen peroxide production by the microsomal FAD-containing monooxygenase

Addition ^a	H ₂ O ₂ production nmol/min/mg	NADPH oxidation nmol/min/mg
None	147	169
Dimethylaniline	129	876
<i>N</i> -Aminopiperidine	57	645

^a The reaction was performed using 0.05–0.1 mg/ml of pure enzyme at 37 °C and pH 8.2. The oxygen concentration was 0.24 mM and the substrate concentration was 0.25 mM. The preparation of enzyme had a higher NADPH “oxidase” rate than normal, but addition of substrate did cause increased NADPH and oxygen consumption. *N*-Aminopiperidine caused a time-dependent decrease in the standard curve for the hydrogen peroxide assay. The lower values obtained may be due in part, to an error in the assumed extinction coefficient of the inorganic complex formed in the presence of this hydrazine.

to their corresponding nitrosamine derivatives. No significant concentration of nitrosamine above the control experiment (minus enzyme) was found when the enzyme, NADPH, oxygen, and either 1 mM 1,1-dimethylhydrazine or 0.25 mM *N*-aminopiperidine were incubated for 10 min at 37 °C. A sample of authentic *N*-nitrosopiperidine at concentrations expected from the putative enzymatic reaction was tested; the sample had amounts of the corresponding nitrosamine in levels which were easily quantitated.

The total stoichiometry for 1,1-dimethylhydrazine oxidation measured as the extent of reaction could not be obtained by addition of substoichiometric amounts of this hydrazine due to its higher apparent K_m value. In such experiments, the amount of NADPH utilized was on the order of 0.6 mol/mol of 1,1-dimethylhydrazine added. However, the stoichiometry could be obtained by comparing the rates of NADPH or oxygen consumption and product formation. Based on the rate of substrate utilization and product formation (Table II), the reaction required 1 mol of NADPH and 1 mol of oxygen to form 1 mol of either acid-stable product (formaldehyde or methylhydrazine). These results suggest that when methyl- or 1,1-dimethylhydrazine are used as substrates, the rate of substrate utilization equals the rate of product formation. Based on these findings, the characterization of the enzymatic oxidation of a large number of hydrazine derivatives was performed using the substrate-dependent oxidation of NADPH. For a number of hydrazine derivatives, the rate of metabolism was measured using both NADPH oxidation and oxygen utilization; in all cases the two methods gave identical results.

Hydrazine Substrate Specificity for Microsomal FAD-con-

taining Monooxygenase—The apparent Michaelis constants and maximal velocities of oxidation were obtained from the initial rates of NADPH oxidation in the presence of 0.18 mM NADPH and 0.24 mM oxygen and compared to those for *N,N*-dimethylaniline *N*-oxidation (Table III). Upon comparison of the K_m values of the various hydrazines, it can be discerned that the 1,1-disubstituted hydrazines are the best substrates for the monooxygenase. However, it can be seen that a number of mono- and 1,2-disubstituted hydrazines have higher maximal velocities (10–70%) than do the 1,1-disubstituted hydrazines. A similar result has been shown by Poulsen *et al.* (26) for 2-naphthylamine and the larger V_{max} value has been interpreted to be due possibly to the ability of some substrates to serve as allosteric activators of the enzyme, as well as a substrate. A number of therapeutic hydrazines and hydrazides were tested as substrates for the enzyme; only procarbazine, a 1-benzyl-2-methylhydrazine, was a substrate. It should be noted that, since the K_m for procarbazine is lower than that noted for either benzyl- or methylhydrazine, the activity most likely was not due to contamination of the procarbazine sample with either methylhydrazine or the benzylhydrazine analogue.

Initial velocity patterns are shown for 1,1-dimethylhydrazine oxidation in Fig. 1. The measurement was made at pH 7.4 since atmospheric oxygen concentrations at 37 °C (approximately 0.24 mM) would not be expected to saturate the enzyme at pH 8.5 (27). As can be seen in Fig. 1, the plots are similar to those shown by Poulsen and Ziegler (27) for trimethylamine and methimazole, suggesting that the kinetic mechanism of hydrazine oxidation for the 1,1-disubstituted hydrazines is apparently identical with the other *N*- and *S*-oxidation reactions studied to date.

Characteristics of the Oxidation of Hydrazines by Purified FAD-containing Monooxygenase—The pH-rate profiles for three hydrazines are contrasted to that of *N,N*-dimethylaniline (Fig. 2). The profile was obtained by measuring the initial rate of NADPH oxidation with 0.18 mM NADPH and 0.24 mM oxygen at 37 °C and at pH values between pH 7.0–9.0. The pH-rate profile for all three hydrazines were identical to that of *N,N*-dimethylaniline with a maximum near pH 8.4. It is interesting that the rate at pH 7.4 is approximately 40% of that seen at pH 8.4. The rate measurements may not be maximal since the K_m for oxygen at pH 8.5 is large enough that 0.24 mM oxygen will not completely saturate the enzyme (27). The effect of octylamine on the oxidation of hydrazines catalyzed by the pure mammalian liver FAD-containing monooxygenase was compared to that of *N,N*-dimethylaniline. Five hydrazines, *n*-butyl-, 1-methyl-2-benzyl-, 1,1-dimethyl-, and 1-methyl-1-phenylhydrazine, as well as *N*-ami-

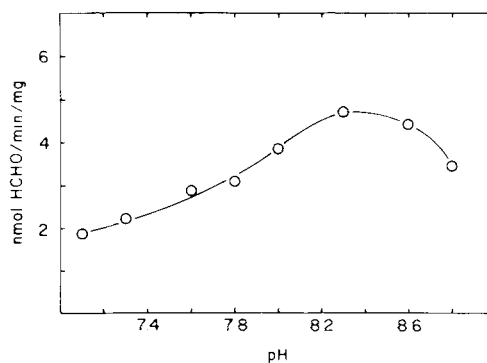


FIG. 3. The pH-rate profiles for demethylation of 1,1-dimethylhydrazine by hamster liver microsomes. The metabolism of the hydrazine was measured as formaldehyde formed as described under “Experimental Procedures.”

nopiperidine, are stimulated in a manner identical (150–190% of control) with other known substrates of the enzyme (12).

Since the hydrazines are relatively good reducing agents and could react enzymatically with oxygen to form hydrogen peroxide, we used the method of Hildebrandt *et al.* (23) to measure the rate of hydrogen peroxide formation by the pure enzyme in the absence and presence of substrates (Table IV). The endogenous rate yields stoichiometric amounts of hydrogen peroxide but, on the addition of a substrate, *N,N*-dimethylaniline, there is no further increase in peroxide production. When *N*-aminopiperidine was added, there was a slightly decreased formation of hydrogen peroxide suggesting that this hydrazine behaves in a manner identical to other nitrogen substrates for the enzyme.

Microsomal Metabolism of 1,1-Dimethylhydrazine—Since 1,1-dimethylhydrazine is an excellent substrate for the FAD-containing monooxygenase and yields formaldehyde as a stable degradation product, we decided to investigate whether mammalian liver microsomes could demethylate 1,1-dimethylhydrazine. Fig. 3 shows the pH-rate profile for the microsomal demethylation of 1,1-dimethylhydrazine. It is of interest that the pH optimum is near pH 8.4. As noted previously, the *sec*- and *tert*-*N*-methyl amine *N*-demethylation reactions catalyzed by microsomal cytochrome P-450 have pH maxima at or near pH 7.6, while *tert*-*N*-methylamine *N*-oxidation reactions of pure amine oxidase or liver microsomes have pH maxima near pH 8.4 (9).

TABLE V
Liver microsomal Demethylation of 1,1-dimethylhydrazine

Enzyme source	Rate ^a nmol/min/mg
Hamster	
Control	2.33 ± 0.30 (<i>n</i> = 6)
PB-treated	2.50 ± 0.50 (<i>n</i> = 7)
MC-treated	2.40 ± 0.43 (<i>n</i> = 4)
Rat	
Control	1.56 ± 0.21 (<i>n</i> = 5)
PB-treated	1.60 ± 0.35 (<i>n</i> = 3)
MC-treated	1.35 ± 0.45 (<i>n</i> = 3)
Pig	4.60 ± 0.50 (<i>n</i> = 2)

^a Experiments were performed using 0.05 M potassium phosphate, 0.05 M Tricine-HCl buffer, pH 7.6, at 37 °C. The rate of metabolism was measured as the amount of formaldehyde detected using the method of Nash (19). PB, phenobarbital; MC, methylcholanthrene.

TABLE VI
Effect of inhibitors on the microsomal demethylation of 1,1-dimethylhydrazine

Inhibitor ^a	Source of enzyme			
	Purified enzyme	Pig microsomes	Hamster microsomes ^b	Rat microsomes ^b
	% control			
None	100	100	100	100
<i>n</i> -Octylamine	188	212	145	169
Methimazole	15	33	32	24
Immune globulin	— ^c	—	78	77
Nonimmune globulin	—	—	83	80

^a The inhibitors were incubated with the pure enzyme or microsomal protein as described under "Experimental Procedures." The methimazole and *n*-octylamine concentrations were 2 mM. The ratio of globulin to microsomal protein was 4:1; this amount of anti-NADPH-cytochrome *c* reductase globulin was sufficient to inhibit the microsomal NADPH-cytochrome *c* reductase activity of hamster and rat liver by 80–90%.

^b These experiments were also performed with liver microsomes from hamsters or rats pretreated with either phenobarbital or 5,6-benzoflavone. The results were identical with those shown above for liver microsomes from untreated animals.

^c —, not determined.

The effect of animal pretreatment with either phenobarbital or 3-methylcholanthrene on the 1,1-dimethylhydrazine demethylase activity of hamster or rat liver microsomes was studied. Rodent pretreatment with either phenobarbital or 3-methylcholanthrene had little or no effect on the demethylase activity (Table V). Using pig liver microsomes, parallel experiments designed to measure any methylhydrazine formed during this reaction gave rates of aldazine formation equal to that measured for formaldehyde production. The highest activity was seen using pig liver microsomes.

Relative to non-immune globulin, the specific antibody elicited toward NADPH-cytochrome P-450 (*c*) reductase had little effect on the 1,1-dimethylhydrazine demethylase activity of hamster or rat liver microsomes (Table VI). In contrast, methimazole, a competitive substrate for the FAD-containing monooxygenase which does not yield formaldehyde as a product, caused a 65–75% inhibition of the activity. In addition, *n*-octylamine increased the microsomal activity by 50% when pig liver microsomes were used as a source of enzyme.

DISCUSSION

It is well established that hydrazine derivatives produce tumors in laboratory rodents (28) and produce a number of toxic reactions in mammals (29). Miller and Miller (30) and Magee and Barnes (31) have conceptualized the need for metabolism to form proximate or ultimate carcinogens and toxins from relatively stable molecules. The deleterious effects of the hydrazines also argue for activation of these compounds by metabolic reactions.

Recently, we have studied the metabolism of the 1,1-disubstituted hydrazines by rodent liver microsomes and have noted that they form unique spectral intermediates with microsomal cytochrome P-450 (32, 33). The formation of this cytochrome P-450-metabolite complex greatly affected cytochrome P-450-dependent monooxygenase function *in vitro* and *in vivo*. The formation of this complex appeared to require a cytochrome P-450-catalyzed process and effectors of the FAD-containing monooxygenase did not affect complex formation. Since the formation of this abortive metabolite complex with cytochrome P-450 precluded its involvement in the metabolism of 1,1-disubstituted hydrazines, we directed our attention to the possible role of the liver microsomal FAD-dependent monooxygenase in the oxidative metabolism of these compounds.

Ziegler and co-workers (4–8, 10–12, 14, 26, 27, 34–36) have provided most of the information which is available regarding the characteristics of the purified microsomal FAD-dependent monooxygenase of mammalian liver. These studies have included the detailed description of the *N*-oxidation products of the *tert*- and *sec*-*N*-methylamines (5, 11) and of the *S*-oxidation products of a number of thiols and thioureylenes (7, 8). With several of the substrates, it was noted that the *N*-hydroxy (34, 35) and sulfenic acid metabolites (36) can be subsequently oxidized by the enzyme to form secondary products, *i.e.* nitrones and sulfinic acid derivatives, respectively. In these cases, the first oxidation products have a high affinity for the enzyme (*i.e.* low apparent *K_m* values). A similar observation is reported in this publication for the oxidation of the *N*-aminoheterocyclic hydrazines, such as *N*-aminopiperidine, by the purified microsomal FAD-dependent monooxygenase.

Lemal has reviewed the chemical mechanism for the oxidation of 1,1-disubstituted hydrazines to yield 1,1-dialkyldiazene (13). The early work of McBride *et al.* (21, 24) showed that the chemical oxidation of 1,1-dimethylhydrazine under strongly acidic conditions leads to the formation of a diazenium ion which can be detected by its unique decomposition products (Fig. 4). For clarity, 1,1-dimethylhydrazine is used as

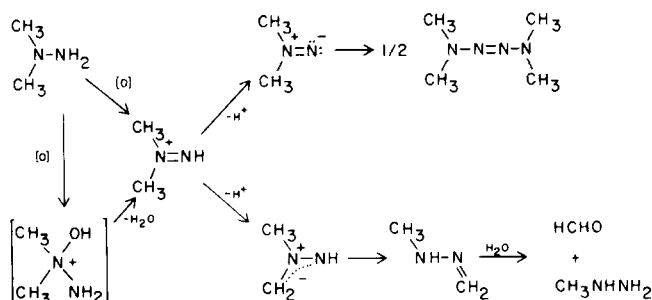


FIG. 4. Scheme for chemical and enzymatic oxidation of 1,1-dimethylhydrazine and subsequent degradation products.

a representative 1,1-disubstituted hydrazine throughout this discussion. One major product formed from the diazene at neutral pH is the tetramethyltetrazene. However, in hydroxylic solvents there is considerable evidence that another tautomer of the diazene, an azomethanimine intermediate, exists which can rearrange to form a formaldehyde methylhydrazine or its hydrolysis products (13). Indeed, as seen in Table II, one can obtain stoichiometric amounts of either formaldehyde and methylhydrazine or tetrazene depending on the conditions applied following the oxidation of 1,1-dimethylhydrazine by sodium bromate. It should also be noted that the dimerization reaction, *i.e.* tetrazene formation, is sensitive to solvent polarity and pH and since it involves a bimolecular process, is also dependent upon free diazene concentration. The products of *N*-aminopiperidine chemical oxidation are not as well described as those for 1,1-dimethylhydrazine. To date, we have only noted a substoichiometric formation of dipiperidyltetrazene upon neutralization of the oxidation products of *N*-aminopiperidine. The chemical oxidation of methylhydrazine gave substoichiometric quantities of formaldehyde as a major product and methane as a minor product (Table II).

The products formed by the enzymatic oxidation of the hydrazines were distinct from those obtained from the chemical oxidation reactions. Formaldehyde was formed stoichiometrically from methylhydrazine by the FAD-dependent monooxygenase with only minor amounts (3–5%) of methane formed, as compared with either NADPH or oxygen utilization. No tetramethyltetrazene was formed during the metabolism of 1,1-dimethylhydrazine but, relative to NADPH oxidation, stoichiometric quantities of both methylhydrazine and formaldehyde were measured in the acid-quenched reaction mixtures containing the flavoprotein. These results (*i.e.* formation of products which would be derived from hydrolysis of hydrazone intermediates) suggest that an azomethanimine intermediate is formed and its rearrangement/hydrolysis products are measured using our analytical techniques. Further, the formation of only minor amounts of methane from methylhydrazine, the lack of tetramethyltetrazene from 1,1-dimethylhydrazine, and the small amount of dipiperidyltetrazene from *N*-aminopiperidine indicates that the product of enzymatic oxidation is unstable and forms preferentially an azomethanimine intermediate rather than a diazene (Fig. 4). Since the dimerization reaction leading to tetrazenes is very concentration-dependent, the low amounts of diazene formed enzymatically would favor the tautomerization reaction. To date, our results can not distinguish between the formation of an *N*-hydroxy (or *N*-oxide) metabolite which can easily eliminate water or a direct dehydrogenation of the hydrazine molecule by the flavin-hydroperoxide form of the enzyme. Regardless of the mechanism of *N*-oxidation, the metabolism of 1,1-dimethylhydrazine by the purified or membrane-bound enzyme can be sensitively measured by monitoring either NADPH or oxygen utilization and by monitoring the forma-

tion of formaldehyde or methylhydrazine. The analysis of the mechanism and products of *N*-oxidation of *N*-aminopiperidine is complicated by the occurrence of a second oxidation step and studies are currently in progress to resolve this problem.

Poulsen and Ziegler have also presented the steady state mechanism of the *N*-oxidation of trimethylamine and the *S*-oxidation of 1-methyl-2-mercaptoimidazole (27). In both cases, the kinetic analysis of the initial rates of the reaction is most consistent with an ordered sequential ter-bi mechanism with the order of substrate addition being NADPH, oxygen, and substrate, and the order of product release being hydroxylated substrate and NADP^+ . The enzyme irreversibly forms a stable peroxyflavin intermediate upon reaction with NADPH and oxygen which can only be rapidly discharged by addition of a hydroxylatable substrate. There exists a small rate of oxygen reduction to hydrogen peroxide due to the decomposition of the reduced-flavin, oxygen-bound form of the enzyme and under anaerobic conditions, the reduced enzyme can pass electrons to acceptors such as dichlorophenolindophenol, potassium ferricyanide, and cytochrome *c*.

The partial analysis of the initial rates of 1,1-dimethylhydrazine oxidation by this enzyme indicates that the kinetic mechanism of hydrazine oxidation is very similar to that of trimethylamine or 1-methyl-2-mercaptoimidazole. However, the complete kinetic analysis involving variation of the oxygen concentration was not completed due to the limited amount of enzyme available and the technical difficulties involved in precisely varying oxygen concentration. In addition, it was noted that the pH-rate profiles for oxidation of *N,N*-dimethylaniline and four hydrazine substrates are nearly identical and that octylamine, a known positive effector of the enzyme, also stimulates hydrazine oxidation.

A survey to determine which hydrazine derivatives serve as substrates for the purified FAD-containing monooxygenase was performed to evaluate which structural features are required in order for hydrazines to be *N*-oxidized by the enzyme. At 0.18 mM NADPH and 0.24 mM oxygen, the apparent V_{max} and K_m values were determined for comparison. Nearly all of the mono- and disubstituted hydrazines had V_{max} values near 1000 nmol of product formed/min/mg of protein at pH 8.3 and 37 °C. The mono- and 1,2-disubstituted hydrazines were poor substrates based on their apparent K_m values. However, the 1,1-disubstituted hydrazines are metabolized as efficiently as either *N,N*-dimethylaniline or 1-methyl-2-mercaptoimidazole. Hydrazones and hydrazides were not substrates for the enzyme.

The metabolism of 1,1-dimethylhydrazine was studied using microsomal fractions to establish the relative participation of the two monooxygenases in the reaction. The pH-rate profile for formaldehyde formation had an optimum at pH 8.2, which is similar to that seen with the purified FAD-containing enzyme, but is significantly different from the pH-optimum noted for the cytochrome P-450-dependent monooxygenase reactions (pH 7.6). Animal pretreatment with either phenobarbital or 3-methylcholanthrene had no effect on the *in vitro* metabolism of 1,1-dimethylhydrazine by liver microsomal fractions from hamster or rat liver. The inhibitory antibody against NADPH-cytochrome P-450 reductase did not affect the reaction and *n*-octylamine, a potent inhibitor of cytochrome P-450 and activator for the FAD-containing monooxygenase, stimulated formaldehyde formation by 50%. However, methimazole, which is an alternate substrate for the flavoprotein that does not yield formaldehyde as a product, inhibited the reaction by 70–80%. These results suggest that for 1,1-dimethylhydrazine and perhaps other 1,1-disubstituted hydrazines, the FAD-containing monooxygenase of liver is the principle enzyme involved in the first step of metabolism of

hydrazines and can account for the first step in its *in vivo* metabolism to carbon dioxide (37) possibly involving formaldehyde as an intermediate.

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