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OXIDATION OF THIOBENZAMIDE BY THE FAD-CONTAINING AND CYTOCHROME P-450-DEPENDENT MONOOXYGENASES OF LIVER AND LUNG MICROSOMES

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Abstract—Two distinct microsomal pathways involved in the metabolism of thiobenzamide to thiobenzamide *S*-oxide have been identified and quantitated in the liver and lungs of mice and rats, using a highly inhibitory antibody against NADPH–cytochrome P-450 reductase. Approximately 50 and 65% of the oxidation in mouse and rat liver microsomes, respectively, was due to the FAD-containing monooxygenase, the remainder being catalyzed by cytochrome P-450. In the mouse lung, S-oxidation was predominantly via the FAD-containing monooxygenase while that in the rat lung was about 60% via the FAD-containing enzyme and 40% via cytochrome P-450. Cytochrome P-450-dependent S-oxidation of thiobenzamide was induced in the liver by treatment of mice with phenobarbital and slightly increased by treatment with 3-methylcholanthrene, while in rat liver either of these treatments caused only a small increase in metabolism due to cytochrome P-450. Thermal inactivation of the FAD-containing monooxygenase left the cytochrome P-450 component essentially unchanged. Thermally treated microsomes had a pH activity profile characteristic of cytochrome P-450 and were less inhibited by methimazole and thiourea when compared to untreated microsomes. Female mouse liver microsomes had a much higher, and female rat liver microsomes a lower, ability to S-oxidize thiobenzamide when compared to the males.

Thiobenzamide (C_6H_5-$CSNH_2$) is a hepatotoxicant which is S-oxidized at the thioamide group, as is a structurally similar hepatotoxicant, thioacetamide (CH_3-$CSNH_2$), the latter being a known carcinogen [1]. The oxidation of thiobenzamide and thioacetamide to S-oxides and, probably, a second oxidation to di-S-oxygenated derivatives are thought to be bioactivation steps necessary for the expression of toxicity [2, 3].

In vivo administration of thiobenzamide to the rat results in hepatotoxicity, as evidenced by an increase in plasma bilirubin, plasma glutamic pyruvic transaminase and hepatic triglyceride content [4]. Centribolar necrosis, common to other thione-containing compounds that undergo metabolic activation, has also been reported [5]. Thiobenzamide *S*-oxide induces an earlier and more severe hepatic injury, further implicating S-oxidation as a prerequisite for the expression of hepatotoxicity [6]. Administration of either thiobenzamide or thiobenzamide *S*-oxide *in vivo* results in decreased microsomal cytochrome P-450 content and aminopyrine demethylase activity [5, 6]. Treatment with phenobarbital, SKF-525A, or *N*-octylimidazole alters the hepatotoxicity of thiobenzamide and thiobenzamide *S*-oxide, suggesting that cytochrome P-450 participates in metabolic activation *in vivo* [2–4].

An assay for the oxidation of thiobenzamide to thiobenzamide *S*-oxide has been developed by Cashman and Hanzlik [7]. Using microsomal preparations from rat, rabbit, and mouse, this reaction had many characteristics of those mediated by the FAD-containing monooxygenase, (EC 1.14.13.8), suggesting that this conversion is catalyzed exclusively by this enzyme. We have shown recently [8], however, that thiobenzamide can be S-oxidized by purified, reconstituted cytochrome P-450-dependent systems from mouse liver and rabbit lung, as well as by the purified FAD-containing monooxygenase from mouse liver.

Since both enzymes appear to be involved, it seemed important to define the relative contributions of the FAD-containing and the cytochrome P-450-dependent monooxygenase systems in the overall metabolism of thiobenzamide. The biochemical characteristics of the individual FAD-containing monooxygenase and the cytochrome P-450 pathways described should permit better delineation of the mixed metabolism of other substrates common to both enzymes.

MATERIALS AND METHODS

Materials. $NADP^+$, NADPH, glucose-6-phosphate, glucose-6-phosphate dehydrogenase, metyrapone, butylated hydroxytoluene, methimazole, cytochrome *c*, and goat anti-rabbit whole sera were obtained from the Sigma Chemical Co., St. Louis, MO. Thiobenzamide and *n*-octylamine were

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obtained from the Aldrich Chemical Co., Milwaukee, WI. *n*-Octylamine was used as the hydrochloride, prepared by the equimolar addition of HCl. Thiourea was obtained from the Fisher Scientific Co., Fairlawn, NJ. 3-Methylcholanthrene was obtained from the Eastman Kodak Co., Rochester, NY, and sodium phenobarbital from Gaines Chemical Inc., Pennsville, NJ. All other materials were of the highest quality commercially available.

Preparation of microsomes. Liver and lung microsomes were prepared from 25–30 g male or female Dub:ICR mice and 200–250 g male or female Dub:Sprague–Dawley rats (Dominion Laboratories, Dublin, VA).

Animals were killed by decapitation, and microsomes were isolated by differential centrifugation [9]. The microsomal pellet obtained was resuspended in 100 mM potassium phosphate, 1 mM EDTA, pH 7.6, and centrifuged again before use. Liver microsomes were used fresh, or after storage in 0.25 M sucrose, 50 mM potassium phosphate, and 0.1 mM EDTA, pH 7.5, under N_2 at $-20°$ for up to 4 weeks. No difference in activity was observed between fresh and frozen microsomes. Lung microsomes were used immediately after preparation.

Mice were given intraperitoneal injections of 0.2 ml of saline, corn oil, phenobarbital in saline (80 mg/kg), or 3-methylcholanthrene in corn oil (20 mg/kg) daily for 3 days and were starved for 16–20 hr prior to being killed. In rats, the doses were 75 mg/kg for phenobarbital and 25 mg/kg for 3-methylcholanthrene.

Purification of reductase. NADPH–cytochrome P-450 reductase was purified from phenobarbital-induced mouse liver microsomes using affinity chromatography as developed by Yasukochi and Masters [10] for rat and pig liver microsomes. Microsomes were solubilized with sodium cholate and chromatographed on DEAE-cellulose. The reductase fraction was applied to a 2′5′-ADP Sepharose 4-B affinity column (Pharmacia, Uppsala, Sweden) and eluted with 5 mM 2′-AMP. After concentration over an Amicon PM-30 ultrafiltration membrane, the reductase was passed through an Ultrogel AcA 34 column (LKB Instruments Inc., Rockville, MD) to remove 2′-AMP, Emulgen 911, and several minor bands.

A specific activity of 36,400 nmoles cytochrome *c* reduced $\cdot$ min^{-1} $\cdot$ $(mg\ protein)^{-1}$ was achieved. The reductase was electrophoretically homogenous, but the samples frequently contained small amounts of the 68,000 mol. wt breakdown product of the native 78,000 mol. wt reductase. Since both forms are immunochemically identical and can produce inhibitory antibodies, they were not separated [11].

Production and isolation of antibody. A primary dose consisting of 630 μg of purified reductase emulsified in 50% Freund's complete adjuvant (Difco Laboratories, Detroit, MI) was administered to adult white New Zealand male rabbits. Doses were equally divided among four intradermal sites along the back and four intramuscular sites along the thighs. Two and four weeks later, 360 μg was administered in Fruend's incomplete adjuvant. Every 4 weeks thereafter, 360 μg was similarly given and sera were collected 8 and 12 days after immunization. Only sera with the highest ability to inhibit microsomal NADPH-dependent cytochrome *c* reduction were further purified.

Purification of the IgG sera fraction was conducted using two ammonium sulfate precipitations followed by column chromatography on DE52 cellulose [12, 13]. The purified fractions are referred to as anti-reductase or preimmune IgG. These contained only the IgG fraction based on their ability to form a single band on immunodiffusion plates when allowed to cross-react with goat anti-rabbit whole sera.

Ouchterlony immunodiffusion. Ouchterlony double immunodiffusion [14] was performed in 0.9% agarose gels containing 100 mM phosphate, pH 7.4, 5% glycerol, 0.02% sodium azide with 0.2% sodium deoxycholate [15]. Immunodiffusion plates (9.5 × 4.5 cm) (Miles Laboratory, Elkhart, IN) were filled with 5.0 ml of solution. Wells, 3 mm in diameter, were cut and filled with either purified enzyme or microsomes solubilized with 1.5 mg sodium deoxycholate/mg protein, and diffusion was allowed to proceed for 36–48 hr at room temperature.

Analytical procedures. Thermal inactivation of the FAD-containing monooxygenase was carried out as described by Poulsen *et al.* [16]. Protein concentration was determined by the method of Lowry *et al.* [17].

Enzyme assays. Thiobenzamide S-oxidation was measured as described by Cashman and Hanzlik [7]. Incubations were monitored at 35° in an Aminco DW-2 UV/vis spectrophotometer in the split beam mode at 370 nm. Standard assay conditions included 100 mM potassium phosphate buffer, 1.0 mM EDTA, pH 7.6, and an NADPH-regenerating system, with enough microsomes to give a final concentration of 0.5 to 0.8 mg/ml. The NADPH-regenerating system contained at final concentration 0.25 mM $NADP^+$, 2.5 mM glucose-6-phosphate and 0.5 units/ml glucose-6-phosphate dehydrogenase.

Anti-reductase or preimmune IgG was preincubated with the microsomes for 5–7 min at 4°. Anti-reductase was used at 6–8 and 4–6 mg IgG per mg microsomal protein for liver and lung microsomes respectively. This corresponded to about 95% inhibition of microsomal NADPH-dependent cytochrome *c* reduction. Preimmune IgG was used at 6 and 4 mg IgG per mg microsomal protein for liver and lung microsomes respectively.

Metyrapone (5 mM final concentration) was added in 5 μl of acetone, and *n*-octylamine hydrochloride (5 mM final concentration) was added in 5 μl of aqueous solution. Inhibitors and preimmune IgG were included in both the sample and reference cuvettes.

Cuvettes were warmed to 35° and monitored for 2–3 min or until a stable baseline was obtained. Reactions were initiated by the addition of 5 μl of thiobenzamide in acetonitrile to the sample cuvette and 5 μl of acetonitrile to the reference cuvette. The initial thiobenzamide concentration in the reaction mixture was 1.0 mM. Absorbance changes were recorded for 5–7 min, and reaction rates were determined using a molar absorptivity of 2930 M^{-1} cm^{-1}.

The competitive inhibition studies with methima-

zole and thiourea were conducted under standard assay conditions, with the competitive inhibitor added simultaneously with the thiobenzamide. Reaction rates were linear for 2–3 min, and initial rates were used.

NADPH-dependent reduction of cytochrome *c* was determined by the method of Masters *et al.* [18] as modified by Vermilion and Coon [19].

Ten male mice or four male rats were treated in each of the following dosage groups; saline, corn oil, phenobarbital, or 3-methylcholanthrene. The microsomes from the pooled livers were assayed twice on different days and the entire experiment was repeated. The data are the mean ± standard deviation of four determinations unless otherwise mentioned. Lung microsomes were assayed only once on the day of preparation, so these data represent two determinations. Identical experiments were conducted on uninduced female mice and rats.

The pH activity profiles were carried out in 100 mM Tris–HCl, 1.0 mM EDTA or 100 mM potassium phosphate, 1.0 mM EDTA. Potassium phosphate was adjusted with potassium hydroxide to achieve the higher pH values.

Purified mouse liver FAD-containing monooxygenase was a gift of Dr. Patrick J. Sabourin, Toxicology Program, North Carolina State University, and the purified pig liver FAD-containing monooxygenase was provided by Professor D. M. Ziegler of the University of Texas at Austin.

RESULTS

Ouchterlony immunodiffusion analysis. Antibody to NADPH–cytochrome P-450 reductase cross-reacted with uninduced, phenobarbital-induced, and 3-methylcholanthrene-induced mouse liver microsomes, as well as with the purified enzyme (Fig. 1). A continuous precipitin line, without spurs, demonstrates that a single, similar antigen was present in each preparation.

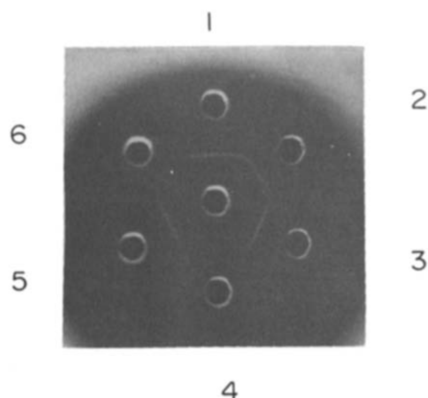


Fig. 2. Ouchterlony double immunodiffusion cross-reactivity of mouse and rat, liver and lung microsomes. The wells contained the following: center well, anti-reductase (400 μg); 1, reductase (8 μg); 2, mouse lung microsomes (130 μg); 3, mouse liver microsomes (330 μg); 4, blank; 5, rat liver microsomes (210 μg); and 6, rat lung microsomes (285 μg).

Microsomes isolated from the mouse lung formed a fused precipitin line with mouse liver microsomes (Fig. 2). Rat liver and lung microsomes also cross-reacted with the antibody developed against mouse liver reductase. The wells containing either rat or mouse microsomes formed precipitin lines that merged, indicating that the rat and mouse reductases are immunochemically similar, if not identical enzymes.

The antibody against purified mouse liver NADPH–cytochrome P-450 reductase did not cause any precipitation of the FAD-containing monooxygenase purified from either mouse liver or pig liver microsomes (Fig. 3).

Antibody inhibition of NADPH–cytochrome c *reductase activity.* The antibody developed against mouse liver NADPH–cytochrome P-450 reductase

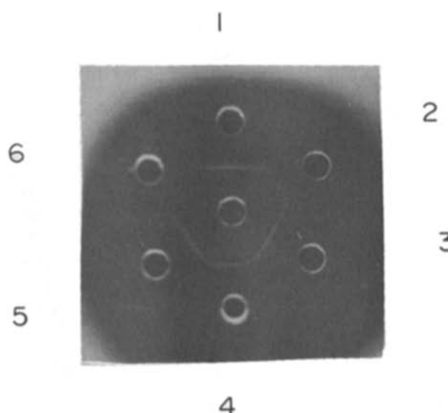


Fig. 1. Ouchterlony double immunodiffusion cross-reactivity of purified NADPH–cytochrome P-450 reductase and mouse liver microsomes. The wells contained the following: center well, anti-reductase (400 μg); 1, reductase (8 μg); 2, uninduced microsomes (330 μg); 3, reductase (8 μg); 4, phenobarbital microsomes (360 μg); 5, 3-methylcholanthrene microsomes (300 μg), and 6, blank.

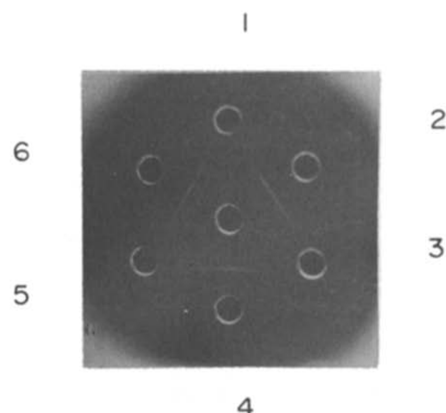


Fig. 3. Ouchterlony double immunodiffusion, lack of cross-reactivity with the purified FAD-containing monooxygenase. The wells contained the following: center well, anti-reductase (400 μg); 1, mouse liver FAD-containing monooxygenase (10 μg); 2, reductase (8 μg); 3, blank; 4, mouse liver microsomes (330 μg); 5, pig liver FAD-containing monooxygenase (10 μg); and 6, reductase (8 μg).

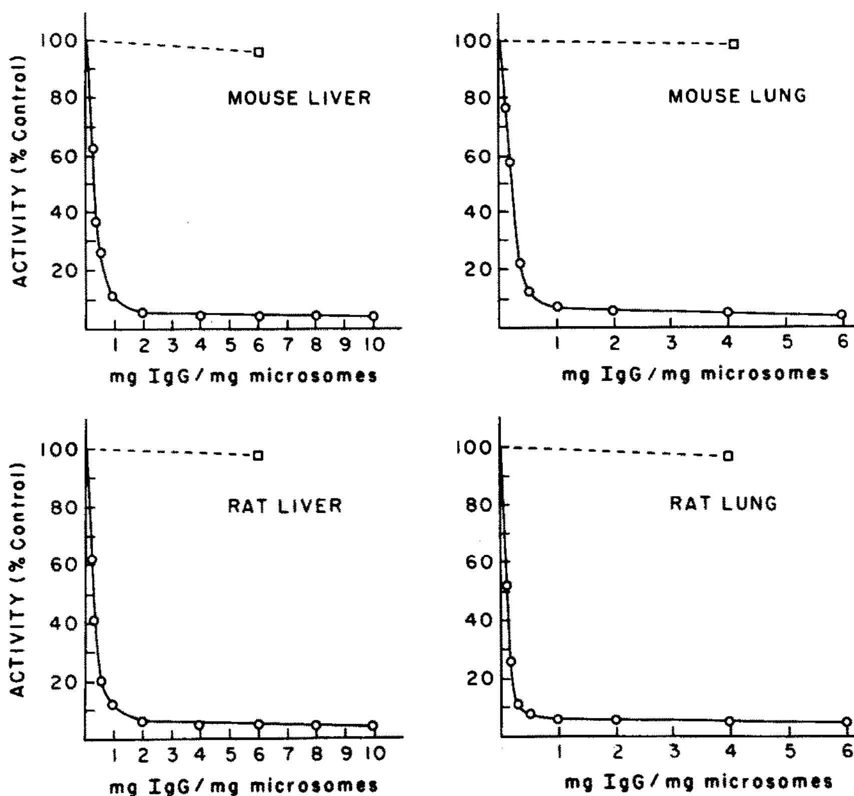


Fig. 4. Inhibition of microsomal NADPH-dependent cytochrome *c* reduction in liver and lung microsomes from mice and rats by anti-reductase. Key: (○——○) effect of anti-reductase; (□– – –□) effect of preimmune IgG. Control microsomal rates were 199, 192, 180 and 78 nmoles cytochrome *c* reduced $\cdot$ min$^{-1}\cdot$mg^{-1} for mouse liver, mouse lung, rat liver and rat lung respectively.

was highly inhibitory against microsomal NADPH-dependent cytochrome *c* reductase activity (Fig. 4). The antibody was capable of inhibiting about 95% of the cytochrome *c* reductase activity in liver and lung microsomes prepared from either the mouse or the rat. The ability to inhibit the microsomal reduction of cytochrome *c* closely corresponds to the ability to inhibit the microsomal reduction of cytochrome P-450 [10]. Anti-reductase did not inhibit thiobenzamide S-oxidation by the purified FAD-containing monooxygenase (data not shown).

Uninduced mouse liver and lung microsomes. In the uninduced mouse liver, the cytochrome P-450 inhibitor, metyrapone, inhibited thiobenzamide S-oxidation by approximately one-third. The net effect of the addition of *n*-octylamine alone was slight inhibition (7.1%).

The addition of anti-reductase to uninduced mouse liver microsomes resulted in 50% inhibition. The activity remaining after inhibition by anti-reductase showed a characteristic property of the FAD-containing monooxygenase, namely, stimulation by *n*-octylamine (+55.2%) (Table 1).

Inhibition by anti-reductase left an FAD-containing monooxygenase activity of about 2.94 nmoles$\cdot$min$^{-1}\cdot$mg^{-1} in mouse liver microsomes. Results from thermally treated microsomes indicate that the cytochrome P-450 component of oxidation was about 2.75 nmoles$\cdot$min$^{-1}\cdot$min^{-1}. In combination, these two activities can account for the total microsomal oxidative activity observed, 5.90 nmoles$\cdot$min$^{-1}\cdot$mg^{-1}.

Mouse lung microsomes oxidized thiobenzamide almost as well as those from mouse liver. Mouse lung, however, had a higher FAD-containing monooxygenase activity toward thiobenzamide than mouse liver (per mg microsomal protein), but had a very low capacity to S-oxidize thiobenzamide via cytochrome P-450. In contrast to the mouse liver, mouse lung was stimulated by the addition of *n*-octylamine alone (+47.0%).

Uninduced rat liver and lung microsomes. Rat liver microsomes oxidized thiobenzamide at 5.65 nmoles$\cdot$min$^{-1}\cdot$mg^{-1} (Table 2), about the same rate as that observed in mouse liver. Metabolism was inhibited somewhat less by metyrapone and anti-reductase, when compared to the mouse liver, indicating that rat liver has a slightly higher FAD-containing monooxygenase level.

Rat lung microsomes had a limited ability to oxidize thiobenzamide (1.54 nmoles$\cdot$min$^{-1}\cdot$mg^{-1}). Metabolism was altered to an extent comparable to the rat liver upon addition of metyrapone, *n*-octylamine, or anti-reductase.

Effects of induction. Phenobarbital treatment increased the oxidation of thiobenzamide by mouse liver microsomes (8.52 compared to 5.44 nmoles$\cdot$min$^{-1}\cdot$mg^{-1}) (Table 3). Metabolism was

Table 1. Thiobenzamide S-oxidation by uninduced mouse liver and lung microsomes

	Liver		Lung	
	Thiobenzamide oxidation (nmoles · min^{-1} · mg^{-1})	Inhibition (%)	Thiobenzamide oxidation (nmoles · min^{-1} · mg^{-1})	Inhibition (%)
Control	5.90 ± 0.94*		4.46 ± 0.65	
+ Metryapone	3.92 ± 0.69*	33.5 ± 3.8	3.56 ± 0.76	20.6 ± 5.5
+ *n*-Octylamine	5.40 ± 1.17*	7.1 ± 8.9	6.56 ± 0.95	+47.0 ± 0.0†
+ Anti-reductase	2.94 ± 0.46*	50.1 ± 2.5	3.54 ± 0.54	20.7 ± 0.5
+ Anti-reductase + *n*-octylamine	4.55 ± 0.75*	+55.2 ± 17.2‡	6.11 ± 1.17	+72.0 ± 7.1‡
+ Preimmune IgG	5.32 ± 0.04	7.4 ± 3.4	4.17 ± 0.64	6.6 ± 0.6
Thermally treated microsomes	2.75 ± 0.23		1.65 ± 0.21	

* N = 5, otherwise N = 2.
† + = Stimulation.
‡ Compared to anti-reductase; other comparisons are made to the control.

Table 2. Thiobenzamide S-oxidation by uninduced rat liver and lung microsomes

	Liver		Lung	
	Thiobenzamide oxidation (nmoles · min^{-1} · mg^{-1})	Inhibition (%)	Thiobenzamide oxidation (nmoles · min^{-1} · min^{-1})	Inhibition (%)
Control	5.65 ± 0.44		1.54 ± 0.18	
+ Metyrapone	4.10 ± 0.53	27.5 ± 6.4	1.20 ± 0.10	21.9 ± 2.1
+ *n*-Octylamine	6.80 ± 0.74	+20.3 ± 9.4*	1.87 ± 0.38	+21.0 ± 11.3
+ Anti-reductase	3.73 ± 0.66	34.3 ± 7.5	0.86 ± 0.03	43.7 ± 4.7
+ Anti-reductase + *n*-octylamine	6.18 ± 1.28	+65.3 ± 12.9†	1.45 ± 0.07	+68.5 ± 2.1†
+ Preimmune IgG	5.19 ± 1.33‡	7.3 ± 13.8	1.46 ± 0.17	4.9 ± 0.1
Thermally treated microsomes	1.91 ± 0.68		0.99 ± 0.66	

* + = Stimulation.
† Compared to anti-reductase; other comparisons are made to the control.
‡ N = 3, otherwise N = 4 for liver and N = 2 for lung.

Table 3. Thiobenzamide S-oxidation by phenobarbital and 3-methylcholanthrene induced mouse liver microsomes

	Inhibition* (%)			
	Saline	Phenobarbital	Corn oil	3-Methylcholanthrene
+ Metyrapone	31.7 ± 2.9	42.2 ± 7.0	28.7 ± 1.2	39.7 ± 6.4
+ *n*-Octylamine	5.3 ± 5.7	29.1 ± 5.3	3.7 ± 5.7	21.0 ± 4.2
+ Anti-reductase	50.3 ± 3.4	65.1 ± 3.5	49.1 ± 2.2	54.3 ± 8.0
+ Anti-reductase + *n*-octylamine†	+65.3 ± 12.3‡	+67.0 ± 11.8	+67.8 ± 11.1	+57.5 ± 7.7

* Control rates of thiobenzamide S-oxidation (nmoles·min^{-1}·min^{-1}) were 5.44 ± 0.36, 8.52 ± 0.46, 5.25 ± 0.44 and 6.39 ± 0.38 for saline, phenobarbital, corn oil and 3-methylcholanthrene respectively. Rates of thiobenzamide S-oxidation (nmoles·min^{-1}·mg^{-1}) by thermally treated microsomes were 2.28 ± 0.18, 5.17 ± 1.79, 2.34 ± 0.36 and 3.33 ± 1.25 for saline, phenobarbital, corn oil and 3-methylcholanthrene respectively.

† Compared to anti-reductase; other comparisons are made to the control.

‡ + = Stimulation.

highly inhibited by the cytochrome P-450 pathway inhibitors metyrapone (42.2%) and anti-reductase (65.1%). The addition of *n*-octylamine alone markedly inhibited overall oxidation, further supporting the increased participation of cytochrome P-450.

Based on thermal inactivation, cytochrome P-450 was responsible for the oxidation of about 5.17 nmoles·min^{-1}·mg^{-1}, representing approximately 65% of the total metabolism. Metabolism due to the FAD-containing monooxygenase, as indicated by that activity resistant to inhibition by anti-reductase, was about 2.97 nmoles·min^{-1}·mg^{-1}, comparable to that activity in control microsomes, 2.70 nmoles·min^{-1}·mg^{-1}. 3-Methylcholanthrene treatment also increased cytochrome P-450-dependent metabolism by mouse liver microsomes, but only to a small extent.

Total oxidation in the rat liver did not respond to either of these two cytochrome P-450 inducers to a significant extent (Table 4). Thiobenzamide oxidation by phenobarbital- and 3-methylcholanthrene-induced microsomes was inhibited to a greater extent by metyrapone and anti-reductase when compared to the corresponding saline and corn oil controls. Thermal inactivation data also supported a small increase in the cytochrome P-450 component.

Thiobenzamide S-oxidation in either the mouse lung, which is predominantly through the FAD-containing monooxygenase, or the rat lung, which is 40% cytochrome P-450 catalyzed, was not increased by phenobarbital or 3-methylcholanthrene (Tables 5 and 6).

Effects of competitive inhibitors. Methimazole and thiourea, known to be substrates for the FAD-containing monooxygenase [16], inhibited the participation of this enzyme in the microsomal oxidation of thiobenzamide. At an equimolar concentration (1 mM) of either methimazole or thiourea, little inhibition of activity was observed, suggesting that the lipophilic thiobenzamide is a very good substrate for the microsomal FAD-containing monooxygenase.

At higher concentrations of either methimazole or thiourea, increasing inhibition of thiobenzamide S-oxidation was seen in mouse lung microsomes. Mouse liver, with approximately a 50% cytochrome P-450 contribution, was inhibited to a lesser extent than the lung. Thermally treated mouse liver microsomes, representative of the cytochrome P-450 component of oxidation, were only slightly inhibited by either methimazole or thiourea under these assay conditions (Fig. 5).

Table 4. Thiobenzamide S-oxidation by phenobarbital- and 3-methylcholanthrene-induced rat liver microsomes

	Inhibition* (%)			
	Saline	Phenobarbital	Corn oil	3-Methylcholanthrene
+ Metyrapone	31.4 ± 4.1	40.4 ± 7.1	27.2 ± 5.7	29.3 ± 3.2
+ *n*-Octylamine	+17.3 ± 6.3†	12.1 ± 16.3	+9.8 ± 3.9	1.6 ± 13.0
+ Anti-reductase	39.7 ± 3.3	53.5 ± 9.5	39.7 ± 2.5	49.9 ± 11.4
+ Anti-reductase + *n*-octylamine‡	+61.3 ± 13.9	+51.5 ± 4.9§	+62.0 ± 5.6‖	+55.5 ± 10.6

* Control rates of thiobenzamide S-oxidation (nmoles·min^{-1}·mg^{-1}) were 5.96 ± 0.65, 6.81 ± 0.69, 6.32 ± 0.58 and 6.93 ± 0.72 for saline, phenobarbital, corn oil and 3-methylcholanthrene respectively. Rates of thiobenzamide S-oxidation by thermally treated microsomes (nmoles·min^{-1}·mg^{-1}) were 1.67 ± 0.35, 3.48 ± 0.49, 1.71 ± 0.44 and 2.47 ± 0.24 for saline, phenobarbital, corn oil and 3-methylcholanthrene respectively.

† + = Stimulation.

‡ Compared to anti-reductase; other comparisons are made to the control.

§ N = 2, otherwise N = 4.

‖ N = 3.

Table 5. Thiobenzamide S-oxidation by phenobarbital and 3-methylcholanthrene induced mouse lung microsomes

	Inhibition* (%)			
	Saline	Phenobarbital	Corn oil	3-Methylcholanthrene
+ Metyrapone	16.8 ± 2.3	18.3 ± 3.8	11.0 ± 5.7	11.5 ± 8.7
+ *n*-Octylamine	+56.0 ± 9.9†	+52.0 ± 18.4	+80.0 ± 9.9	+68.5 ± 12.7
+ Anti-reductase	15.8 ± 8.2	11.9 ± 8.4	16.5 ± 5.3	6.6 ± 9.9
+ Anti-reductase + *n*-octylamine‡	+81.0 ± 4.2	+69.0 ± 19.8	+100.5 ± 9.2	+74.0 ± 14.1

* Control rates of thiobenzamide S-oxidation (nmoles·min^{-1}·mg^{-1}) were 4.88 ± 1.12, 4.84 ± 0.90, 4.60 ± 0.93 and 4.49 ± 0.01 for saline, phenobarbital, corn oil and 3-methylcholanthrene respectively.
† + = Stimulation.
‡ Compared to anti-reductase; other comparisons are made to the control.

Effects of pH. Liver microsomes from the uninduced mouse had a dominant pH peak in the higher pH range, typical of the purified pig liver FAD-containing monooxygenase [20], supporting the high involvement of this enzyme in thiobenzamide S-oxidation (Fig. 6).

Thermally treated microsomes had lost the high pH peak and instead had a broad pH peak from 7.0 to 7.8. Liver microsomes obtained from phenobarbital-treated mice had a pH activity profile containing a peak from pH 7.0 to 7.8.

Similar pH activity profiles between thermally treated and untreated microsomes were observed in the rat liver. Female mice and mouse lung microsomes had pH optima greater than 9.0, probably reflecting the true pH activity peak of the mouse microsomal FAD-containing monooxygenase.

Sex differences in metabolism. Female mouse liver microsomes were much more active in thiobenzamide oxidation than male mouse liver microsomes (9.38 compared to 5.90 nmoles·min^{-1}·mg^{-1}) (Table 7). Microsomal thiobenzamide oxidation in female mouse liver microsomes was decreased only to a small extent by the cytochrome P-450 pathway inhibitors metyrapone (17.1%) and anti-reductase (27.0%).

The activity resistant to inhibition by anti-reductase suggests that, compared to the male mouse, the female mouse liver has a more than 2-fold higher level of FAD-containing monooxygenase. There does not appear to be any quantitative sex difference in the cytochrome P-450 component of oxidation. Data obtained from female mouse lung microsomes were identical to those obtained from male mouse lung microsomes (Table 1).

Female rat liver microsomes did not have the increased ability to oxidize thiobenzamide that was characteristic of female mouse liver microsomes. In fact, the female rat liver had a lower FAD-containing monooxygenase activity when compared to the male rat liver. Similar to the mouse lung, female rat lung microsomes were identical to male rat lung microsomes (Table 2).

DISCUSSION

It has become increasingly evident that some microsomal oxidations can be carried out by at least two enzymes, namely, cytochrome P-450 and the FAD-containing monooxygenase. The wide variety of substrates oxidized by the FAD-containing monooxygenase overlaps those already known to be metabolized by the cytochrome P-450 monooxygenase system. The FAD-containing monooxygenase is now known to oxidize organic nitrogen, sulfur, selenium [20, 21] and, most recently, even organophosphorous substrates [22].

The relative contributions of the FAD-containing and cytochrome P-450 monooxygenases to the metabolism of common substrates *in vivo* are difficult to determine but can be approximated by studying selective inhibition of microsomal systems, which contain accessory proteins and enzymes involved in other pathways. A highly potent and selective inhib-

Table 6. Thiobenzamide S-oxidation by phenobarbital and 3-methylcholanthrene induced rat lung microsomes

	Inhibition* (%)			
	Saline	Phenobarbital	Corn oil	3-Methylcholanthrene
+ Metyrapone	28.5 ± 4.9	16.2 ± 7.7	25.9 ± 1.6	16.4 ± 10.9
+ *n*-Octylamine	+13.5 ± 13.4†	+33.0 ± 4.2	+13.0 ± 2.8	+45.0 ± 25.5
+ Anti-reductase	39.0 ± 5.7	37.7 ± 4.1	42.7 ± 1.9	34.8 ± 14.1
+ Anti-reductase + *n*-octylamine‡	+61.5 ± 3.5	+76.5 ± 29.0	+65.0§	+55.0 ± 26.9

* Control rates of thiobenzamide S-oxidation (nmoles·min^{-1}·mg^{-1}) were 1.61 ± 0.12, 1.72 ± 0.19, 1.69 ± 0.24 and 1.53 ± 0.11 for saline, phenobarbital, corn oil and 3-methylcholanthrene respectively.
† + = Stimulation.
‡ Compared to anti-reductase; other comparisons are made to the control.
§ N = 1, otherwise N = 2.

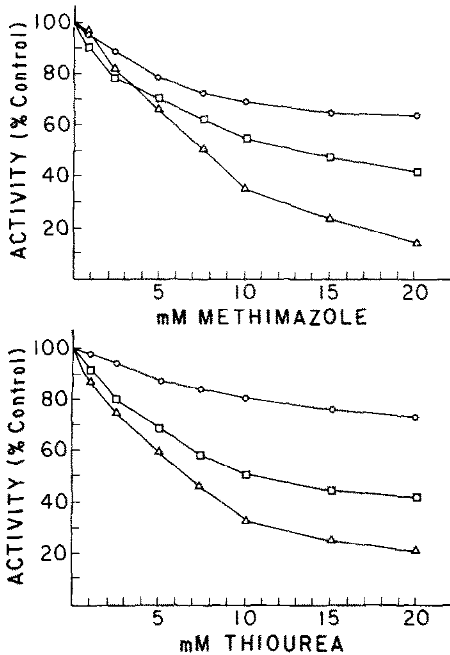


Fig. 5. Inhibition of thiobenzamide S-oxidation by methimazole and thiourea. Key: (○——○) thermally treated mouse liver microsomes; (□——□) mouse liver microsomes and (△——△) mouse lung microsomes.

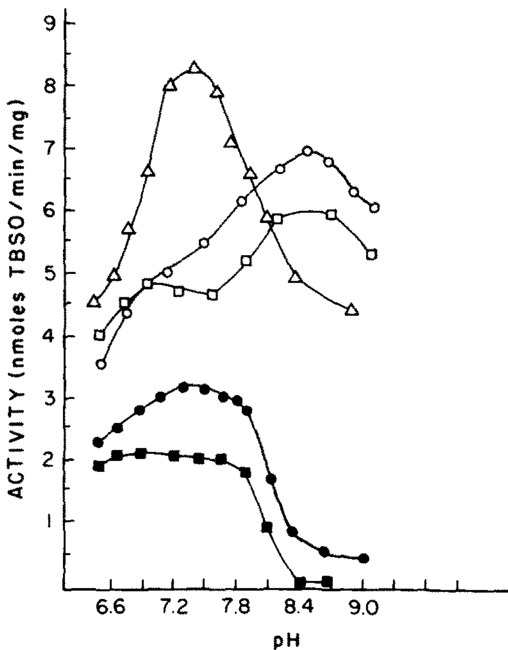


Fig. 6. Effect of pH on thiobenzamide S-oxidation by mouse liver microsomes. Key: (○——○) uninduced microsomes in potassium phosphate; (□——□) uninduced microsomes in Tris–HCl; (△——△) phenobarbital-induced microsomes in Tris–HCl; (●——●) thermally treated uninduced microsomes in potassium phosphate; and (■——■) thermally treated uninduced microsomes in Tris–HCl.

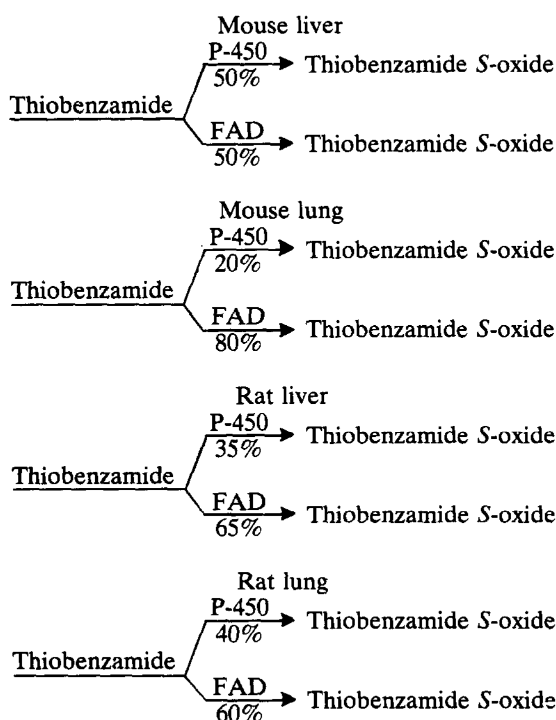
Table 7. Thiobenzamide S-oxidation by female mice liver and female rat liver microsomes

	Mouse		Rat	
	Thiobenzamide oxidation (nmoles·min^{-1}·mg^{-1})	Inhibition (%)	Thiobenzamide oxidation (nmoles·min^{-1}·mg^{-1})	Inhibition (%)
Control	9.38 ± 0.42		4.22 ± 0.31	
+ Metyrapone	7.76 ± 0.12	17.1 ± 4.2	2.90 ± 0.15	31.1 ± 2.8
+ *n*-Octylamine	11.04 ± 0.33	+18.0 ± 6.6*	4.37 ± 0.33	+3.5 ± 5.6
+ Anti-reductase	6.86 ± 0.28	27.0 ± 4.8	2.69 ± 0.17	36.2 ± 1.2
+ Anti-reductase + *n*-Octylamine	9.92 ± 0.83†	+48.0 ± 12.0‡	4.02 ± 0.42	+54.5 ± 8.5‡
Thermally treated microsomes	2.09 ± 0.20		1.96 ± 0.34	

* + = Stimulation.
† N = 3, otherwise N = 4.
‡ Compared to anti-reductase; other comparisons are made to the control.

itor of the microsomal cytochrome P-450 pathway is an antibody to NADPH–cytochrome P-450 reductase [23].

Using anti-reductase, metyrapone, *n*-octylamine, and differential thermal inactivation, thiobenzamide was found to be S-oxidized to a significant extent by both of these two monooxygenases in mouse and rat microsomes. Considering the cytochrome P-450 component of S-oxidation to be that activity which is inhibited by anti-reductase, and the residual activity to represent the FAD-containing monooxygenase component, the relative participations under standard assay conditions are approximately as follows:



Selective thermal inactivation, which irreversibly denatures the FAD-containing monooxygenase and removes its corresponding oxidative activity [16], leaves a heat stable activity that corresponds closely in magnitude to that estimated to be catalyzed by cytochrome P-450 based on inhibition by anti-reductase.

Metyrapone, which is thought to block the oxygen-binding site on cytochrome P-450 [24], inhibited thiobenzamide S-oxidation to an extent consistent with the nearly quantitative inhibition obtained with anti-reductase. When metyrapone was added to microsomes incubated with anti-reductase (data not shown), only slightly more inhibition was obtained, suggesting that anti-reductase removes almost all cytochrome P-450-dependent activity, and that the FAD-containing monooxygenase is not much inhibited by the presence of this cytochrome P-450 inhibitor.

n-Octylamine, another inhibitor of cytochrome P-450 [25], was also a positive effector of the FAD-containing monooxygenase, presumably by interaction at a regulatory site distinct from the catalytic site [26]. When added alone, *n*-octylamine inhibited thiobenzamide oxidation in mouse liver microsomes, but the net effect was stimulation of S-oxidation in mouse lung, rat liver, and rat lung microsomes, although to differing extents. Primary alkylamines have been reported to activate the microsomal FAD-containing monooxygenase of hog, hamster, and guinea pig, but not of rat or rabbit [27].

There are two explanations for the varying effects of *n*-octylamine on thiobenzamide S-oxidation. First, there may be inherent enzyme differences among species and tissues in responsiveness to positive effectors or, second, cytochrome P-450, which would be inhibited by *n*-octylamine, may have contributed to the microsomal oxidation.

For thiobenzamide, the variations in the effect of *n*-octylamine reflect the extent to which cytochrome P-450 participates in the metabolism. After cytochrome P-450-dependent oxidation was inhibited by anti-reductase, stimulation by *n*-octylamine, in the mouse and rat, and in both tissues examined, was consistently between 1.5- and 2.0-fold. Thiobenzamide is, potentially, a positive effector of its own oxidation, possibly explaining why stimulation was not 2.0 to 2.5-fold, as has been observed in the pig for other substrates [20].

Mouse lung, based on thiobenzamide S-oxidation, has a high level of microsomal FAD-containing monooxygenase and, in this respect, resembles rabbit lung [15, 28]. Rat lung, however, has only a limited ability to S-oxidize thiobenzamide. Although toxic effects of thiobenzamide on the lung have not been reported, a similar compound, α-naphthylthiourea, is a known pulmonary toxin, and metabolic S-oxidation, probably by the same enzyme systems, is thought to be responsible for the expression of its toxicity [29].

The FAD-containing monooxygenase is not inducible by phenobarbital or 3-methylcholanthrene [30]. In fact, treatment of mice with phenobarbital significantly reduces the specific content of the liver microsomal FAD-containing monooxygenase [31], presumably caused by proliferation of endoplasmic reticulum proteins. However, it is difficult to explain how any activity specifically attributed to the FAD-containing monooxygenase can be increased (per mg microsomal protein) in animals treated with cytochrome P-450 inducers, other than that the increase in oxidative activity is, in fact, due to the cytochrome P-450-dependent system. Thus, although the oxidative activity of microsomes isolated from phenobarbital- and 3-methylcholanthrene-treated mice and rats was not strikingly higher, a shift in metabolism from the FAD-containing monooxygenase to cytochrome P-450 was apparent.

The FAD-containing monooxygenase showed a pH activity peak at high pH values, while cytochrome P-450-dependent metabolism appeared to have a broad pH maximum from pH 7.2 to 7.8, a more physiological pH range. The contributions of these

two enzymes to microsomal thiobenzamide oxidation will vary with pH. The cytochrome P-450 component of oxidation diminished greatly near the pH optimum of the FAD-dependent monooxygenase, and we have found (data not shown) that the oxidation of thiobenzamide provided a nearly specific microsomal assay for the FAD-containing monooxygenase, provided assays are run at pH 8.5.

FAD-containing monooxygenase activities are higher in female mouse liver and lower in female rat liver compared to males. Not only does the mouse offer the opportunity for genetic manipulation of responsiveness to cytochrome P-450 induction [32], but also has sex differences in FAD-containing monooxygenase levels, which can be controlled by changing the level of sex hormones [33]. If toxicity could be correlated to levels of FAD-containing monooxygenase, then one could potentially define the role of this enzyme in the *in vivo* expression of toxicity.

Common to several other hepatotoxic thione-containing compounds [29], thiobenzamide administration *in vivo* results in decreased hepatic microsomal cytochrome P-450 levels and corresponding oxidative activity. Studies specifically dealing with alterations in FAD-containing monooxygenase levels after toxic insult remain to be done. Considering the decrease in cytochrome P-450 levels under such circumstances, the relative role of the FAD-containing monooxygenase in activation and/or detoxication reactions will probably assume primary importance.

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REFERENCES

1. E. A. Barker and E. A. Smuckler, *Molec. Pharmac.* **8**, 318 (1972).
2. R. P. Hanzlik, J. R. Cashman and G. J. Traiger, *Toxic. appl. Pharmac.* **55**, 260 (1980).
3. M. C. Dyroff and R. A. Neal, *Molec. Pharmac.* **23**, 219 (1983).
4. R. P. Hanzlik, K. P. Vyas and G. J. Traiger, *Toxic. appl. Pharmac.* **46**, 685 (1978).
5. E. Chieli, G. Malvaldi and R. Tongiani, *Toxicology* **13**, 101 (1979).
6. E. Chieli, G. Malvaldi and D. Segnini, *Toxic. Lett.* **7**, 175 (1980).
7. J. R. Cashman and R. P. Hanzlik, *Biochem. biophys. Res. Commun.* **98**, 147 (1981).
8. P. Levi, R. Tynes, P. Sabourin and E. Hodgson, *Biochem. biophys. Res. Commun.* **107**, 1314 (1982).
9. A. P. Kulkarni, R. B. Mailman and E. Hodgson, *J. agric. Fd Chem.* **23**, 177 (1975).
10. Y. Yasukochi and B. S. S. Masters, *J. biol. Chem.* **251**, 5337 (1976).
11. B. S. S. Masters, *Meth. Enzym.* **52**, 240 (1978).
12. P. E. Thomas, A. Y. H. Lu, S. B. West, J. Kawalek and W. Levin, *J. biol. Chem.* **251**, 1385 (1976).
13. C. A. Williams and M. W. Chase, *Meth. Immun. Immunochem.* **1**, 54 (1968).
14. O. Ouchterlony, *Acta path. microbiol. scand.* **26**, 507 (1949).
15. C. J. Serabjit-Singh, C. R. Wolf and R. M. Philpot, *J. biol. Chem.* **254**, 9901 (1979).
16. L. L. Poulsen, R. M. Hyslop and D. M. Ziegler, *Archs Biochem. Biophys.* **198**, 78 (1979).
17. O. H. Lowry, N. J. Rosebrough, A. L. Farr and R. J. Randall, *J. biol. Chem.* **193**, 265 (1951).
18. B. S. S. Masters, C. H. Williams, Jr. and H. Kamin, *Meth. Enzym.* **10**, 565 (1967).
19. J. L. Vermilion and M. J. Coon, *Biochem. biophys. Res. Commun.* **60**, 1315 (1974).
20. D. M. Ziegler, in *Enzymatic Basis of Detoxication* (Ed. W. B. Jakoby), Vol. 2, p. 201. Academic Press, New York (1980).
21. L. L. Poulsen, *Rev. biochem. Toxic.* **3**, 33 (1981).
22. E. Hodgson, *J. Toxicol. clin. Toxicol.* **19**, 609 (1983).
23. R. A. Prough and D. M. Ziegler, *Archs Biochem. Biophys.* **180**, 363 (1977).
24. K. J. Netter, *Drug Metab. Dispos.* **1**, 162 (1973).
25. C. R. E. Jefcoate, S. L. Gaylor and R. L. Calabrese, *Biochemistry* **8**, 3455 (1980).
26. D. M. Ziegler, L. L. Poulsen and E. M. McKee, *Xenobiotica* **1**, 523 (1971).
27. L. L. Poulsen, R. M. Hyslop and D. M. Ziegler, *Biochem. Pharmac.* **23**, 3431 (1974).
28. G. A. Dannan and F. P. Guengerich, *Molec. Pharmac.* **22**, 787 (1982).
29. R. A. Neal, *Rev. biochem. Toxic.* **2**, 131 (1980).
30. M. D. Burke and R. T. Mayer, *Drug Metab. Dispos.* **2**, 583 (1974).
31. P. J. Wirth and S. S. Thorgeirsson, *Biochem. Pharmac.* **27**, 601 (1978).
32. D. W. Nebert and J. S. Felton, *Fedn Proc.* **35**, 1133 (1976).
33. M. W. Duffel, J. M. Graham and D. M. Ziegler, *Molec. Pharmac.* **19**, 134 (1981).