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USE OF THIOCARBAMIDES AS SELECTIVE SUBSTRATE PROBES FOR ISOFORMS OF FLAVIN-CONTAINING MONOOXYGENASES

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Abstract—The oxidation of thiourea, phenylthiourea, 1,3-diphenylthiourea, 1,3-bis-(3,4-dichlorophenyl)-2-thiourea and 1,1-dibenzyl-3-phenyl-2-thiourea was measured in reactions catalyzed by purified pig liver flavin-containing monooxygenase (FMO-1) and by microsomal fractions isolated from pig, guinea pig, chicken, rat and rabbit tissues. The reactions, followed by measuring substrate-dependent thiocholine oxidation [Guo and Ziegler, *Anal Biochem* **198**: 143–148, 1991], were carried out in the presence of 2 mM 1-benzylimidazole to minimize potential interference from reactions other than those catalyzed by isoforms of the flavin-containing monooxygenase (FMO). While at saturating substrate concentrations the V_{max} for purified FMO-1 catalyzed oxidation of all five thiocarbamides was essentially constant, velocities for the microsomal catalyzed reactions varied not only with tissue and species but also with the van der Waals' surface area of the thiocarbamide. Rat liver, rat kidney and rabbit liver microsomes failed to catalyze detectable oxidation of thiocarbamides larger than 1,3-diphenylthiourea and lung microsomes from a female rabbit only accepted substrates smaller than 1,3-diphenylthiourea. On the other hand, liver microsomes from chickens, pigs and guinea pigs catalyzed the oxidation of larger thiocarbamides, but the rates decreased with increasing substrate size and chicken liver microsomes showed no detectable activity with the largest thiocarbamide tested. To define more precisely the parameters affecting thiocarbamide substrate specificity of microsomal preparations, activities present in detergent extracts of guinea pig liver microsomes were separated into three distinct fractions. The substrate specificities of these partially purified fractions were different and consistent with the difference observed with microsomal catalyzed reactions. This strongly suggests that thiocarbamides that differ in size may be useful probes for measuring the number of activities of FMO isoforms in crude tissue preparations.

Mammalian tissues contain flavoproteins that catalyze NADPH- and oxygen-dependent oxidation of an exceptional range of xenobiotics, including inorganic as well as organic compounds [1, 2]. Although forms that differ in primary structure have been purified from liver and lung of mouse and rabbit [3–6] and from guinea pig liver [7], all share a mechanism similar to that described for the pig liver microsomal flavin-containing monooxygenase (FMO-1)† [8–10]. The mechanism of these enzymes differs from that of all other monooxygenases in that the oxygenatable substrate is not required for dioxygen activation. These flavoproteins are apparently present within the cell in the 4a-hydroperoxyflavin form and any soft nucleophile that gains access to this potent monooxygenating agent [11] will be oxidized. How access to the enzyme-bound oxidant is controlled is still not clear, but the number and position of charged groups on the nucleophile are important [1, 12]. In addition, a recent report [13] indicates that overall substrate size may be another important factor. The latter parameter appears largely responsible for many (but not all) differences in substrate specificities of the flavoproteins isolated from rabbit lung and pig liver. Access to the 4a-hydroperoxyflavin in the rabbit lung enzyme is restricted to compounds considerably small than those accepted by the pig liver enzyme. For instance, the latter flavoprotein catalyzes at the same V_{max}, the S-oxidation of thiourea, phenylthiourea, 1,3-diphenylthiourea and phenothiazine, but only the first two show significant substrate activity with the rabbit lung monooxygenase. The results obtained with the purified liver and lung flavin-containing monooxygenases (FMOs) suggest that by measuring differences in the S-oxidation of thiocarbamides bearing substituents of increasing size, it may be possible to determine the distribution and activities of isoforms of FMOs in crude tissue preparations.

A selective and sensitive method for measuring FMO-catalyzed oxidation of thiourea described recently [14] makes such studies possible. The experiments described in this report were carried out to explore the possibility of using thiocarbamides that vary in overall size to measure the activity and minimum number of FMO isoforms present in microsomes of tissues from several species.

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† Abbreviations: FMO, flavin-containing monooxygenase; FMO-1 and FMO-2, flavin-containing monooxygenases similar in substrate specificity and structure to the major pig liver and rabbit lung enzymes, respectively; BHT, butylated hydroxytoluene; and DTNB, 5,5′-dithiobis-(2-nitrobenzoic acid).

MATERIALS AND METHODS

The following materials were purchased from the companies indicated: thiourea, 1,3-diphenylthiourea, 1,3-bis(3,4-dichlorophenyl)-2-thiourea, 1,1-dibenzyl-3-phenyl-2-thiourea *N,N*-dimethylaniline, trifluoperazine and *N*-benzylimidazole (Aldrich Chemical Co.); and methimazole (*N*-methyl-2-mercaptoimidazole), $NADP^+$, glucose-6-phosphate, L-mesenteroides glucose-6-phosphate dehydrogenase, catalase, acetylthiocholine hydrochloride, Triton X-45, Triton X-102, Triton X-100 and Cibacron Blue 3GA-Agarose (Sigma Chemical Co.). All other reagents were of the highest grade available from commercial sources. All of the thiocarbamides (except thiourea) were recrystallized several times from ethanol and stored at 0–4°. Because thiocarbamides are recycled continually during activity measurements, it is essential that each substrate is free from any thiocarbamide smaller than the one indicated. Contamination of the larger substrates by a smaller thiocarbamide will give misleading rates and commercial thiocarbamides must be recrystallized and tested for contaminants before use. Aqueous stock solutions (0.1 M) of thiourea and methimazole are stable for several weeks at 0–4°. The other thiocarbamides were less stable in solution and 50 mM solutions in ethanol were prepared daily. Thiocholine was prepared by methanolysis of acetylthiocholine as described previously [14]. While thiocholine is available commercially as the iodide salt, such preparations are not suitable. Iodide is an excellent substrate for FMO and even traces will interfere with the activity measurements. Thiocholine chloride is stable at −20° for months and 20 mM aqueous metal-free solutions can be stored at 0–4° for at least 1 month without undergoing any detectable change. Triton X-45 succinate was prepared by the procedure described previously [15].

Tissue preparations. Laboratory animals from commercial sources were killed by decapitation. Tissues, removed as quickly as possible, were transferred to 0.25 M sucrose on ice, minced, rinsed several times to remove excess blood and then homogenized with a glass–teflon homogenizer in 6 vol. of 0.25 M sucrose containing 50 mM potassium phosphate, pH 7.5, and 0.1 mM butylated hydroxytoluene (BHT). Microsomal fractions, separated by differential centrifugation from tissue homogenates, were washed once and resuspended in 0.25 M sucrose containing 0.05 M phosphate, pH 7.5. Microsomes from guinea pig livers purchased from the Rockland Corp., Gilbertsville, PA, were prepared by essentially the same procedure. The livers without gall bladders, shipped overnight on ice, were transferred immediately to 0.25 M sucrose on ice, coarsely minced, and rinsed with two to three changes of 0.25 M sucrose. The tissue from 25 animals was homogenized in 6 vol. of 0.25 M sucrose containing 50 mM phosphate and 0.1 mM BHT, pH 7.5, with the flow-through power homogenizer described previously [15]. The microsomal fraction was separated by differential centrifugation as described above. Isolated microsomes (30–40 mg/mL) stored at −20° were thawed just prior to use. Pig liver microsomes were isolated by the procedure described previously [15]. Protein concentration was determined by the method of Gornall *et al.* [16].

Enzyme purification. FMO-1 was purified to homogeneity from pig liver microsomes by the procedure described earlier [15]. Thiourea-dependent thiocholine oxidase activities of guinea pig liver microsomes were resolved and partially purified by modifications of the method described by Yamada *et al.* [7]. Guinea pig liver microsomes, stored frozen in 0.25 M sucrose containing 50 mM phosphate, were thawed and diluted to 15 mg protein/mL with 0.25 M sucrose containing 10 mM potassium phosphate, pH 7.5, 20% glycerol and 0.1 mM EDTA. The activities were extracted by adding, per 10 mL of the microsomal suspension, 0.9 mL of a mixture of detergents consisting of 5.0 g Triton X-102, 4.0 g Triton X-45 and 1.0 g Triton X-45 succinate dissolved in 100 mL H_2O at pH 7.5. After stirring on ice for 30 min, the suspension was centrifuged at 100,000 *g* for 1.5 hr. The supernatant fraction (10–15 mL) which contained 70–80% of the microsomal thiourea-dependent thiocholine oxidase activity was decanted and loaded immediately onto a Cibacron BLue 3GA-Agarose column (2 × 15 cm) pre-equilibrated with 10 mM potassium phosphate, pH 7.5, containing 0.25 M sucrose, 0.2% Triton X-100, 0.1 mM EDTA and 20% glycerol. After washing the column with 100–150 mL of the same solution to remove unbound proteins, active fractions were diluted with a linear gradient of 0–1 M KCl in the same buffer.

Activity measurements. Thiocarbamide *S*-oxygenase activities of microsomes were measured by following substrate-dependent oxidation of thiocholine at 37°, pH 7.5, by the method described previously [14]. Reaction medium contained 0.1 M potassium phosphate, pH 7.5, 0.25 mM $NADP^+$, 2.5 mM glucose-6-phosphate, 1.5 U glucose-6-phosphate dehydrogenase, 130 mM thiocholine, 100 U catalase, 2 mM benzylimidazole, and 0.4 mM EDTA in a final volume of 2.5 mL. After a 4- to 6-min temperature equilibrium, the microsomal fractions (0.5 to 3 mg protein in no more than 0.2 mL) were added. About 1 min later, the reaction was initiated by adding the thiocarbamide substrate dissolved in water or ethanol. The volume of ethanol added, never more than 15 μL/mL of the reaction mixture, had no detectable effect on the activity measurements. Aliquots (0.4 mL) usually withdrawn at 0, 3, 6, 9 and 12 min were transferred to tubes on ice containing 0.04 mL of 3.0 M trichloroacetic acid. After all aliquots were collected, precipitated protein was separated by centrifugation and 0.35 mL of clear supernatant liquid was transferred to tubes containing 1 mL of 1.0 M phosphate, pH 7.5, 0.6 mL of H_2O, and 0.05 mL of 5,5′-dithiobis-(2-nitrobenzoic acid) (DTNB) (10 mM). A millimolar absorptivity of 13.6 cm^{-1} for 5-thio-2-nitrobenzoate was used to calculate the loss of thiocholine as a function of reaction time.

The oxidation of dodecylamine was measured by minor modifications of the procedure described previously [17]. The reaction medium contained 0.1 M potassium phosphate, pH 7.5, 0.25 mM $NADP^+$, 2.5 mM glucose-6-phosphate, 1.5 U glucose-6-phosphate dehydrogenase, 0.8 to 2.5 mg

of enzyme protein, and 2 mM recrystallized dodecylamine in a final concentration of 3.2 mL. Reactions, carried out in a metabolic shaker bath at 37°, were initiated by adding the substrate. Aliquots (1 mL) of reaction mixture, withdrawn at 0, 5, and 10 min, were mixed vigorously with 1 mL of toluene with a vortex test tube mixer. After phase separation by centrifugation, aliquots (50–100 μL) of the toluene phase were spotted onto high performance thin-layer chromatography plates containing fluorescent indicator. The plates were then developed in chloroform:isopropanol (95:5). After removing the solvent by evaporation, the plates were exposed to iodine vapor for 2 min and illuminated by ultraviolet light. The concentration of dodecycloxime in the chloroform extract was calculated from its density relative to that of standards in adjacent lanes by densitometry.

Guinea pig liver microsomes were partially depleted from flavin by a modification of the procedure used with pig liver microsomes [18]. Microsomes, 25–30 mg/mL, were diluted with an equal volume of 3.0 M KCl and incubated for 30 min at 30°. The suspension was then diluted 4-fold with cold (0–4°) 0.25 M sucrose containing 50 mM potassium phosphate, pH 7.5. The particles sedimented by centrifugation at 40,000 rpm for 60 min were resuspended in the same buffer to about 25 mg/mL. Unlike pig liver microsomes, which lost flavin in the presence of KCl only at or near pH 5.5, the flavin in the FMO of the guinea pig liver microsomes is more loosely bound and readily dissociated from the protein by KCl at neutral pH. Because a small (10–15%) but significant amount of protein was lost from the microsomal particles, the control from the same preparation was always carried through the same procedure except that the KCl was replaced by an equal volume of water.

RESULTS

Thiocarbamide-dependent oxidation of thiocholine catalyzed by purified pig liver FMO (Table 1) indicates that the method [14] described for measuring the oxidation of thiourea to formamidine sulfenic acid can also be used to measure the oxidation of other thiocarbamide substrates for this enzyme. All are apparently oxidized to sulfenic acids at essentially the same maximal velocities, indicating that substrate-dependent oxidation of thiocholine appears to give a reliable estimation for the oxidation of these thiocarbamides to their sulfenic acids.

In addition to thiocarbamides, the same method can also be used to measure FMO-1 catalyzed S-oxidation of methimazole, propylthiouracil, or *N,N*-diethyldithiocarbamate (Table 1). The first two are structurally quite similar to thiocarbamides and, like the latter, do not react directly with DTNB but are oxidized to sulfenic acids via FMO-1 [19]. While *N,N*-diethyldithiocarbamate reduces DTNB directly and could therefore interfere with the estimation of thiocholine, it decomposes quantiatively to carbon disulfide and *N,N*-diethylamine in acidic solutions [20], neither of which reacts with DTNB or its reduction product, 5′-thio-2-nitrobenzoate. Thus, acid-labile dithiocarbamates do not interfere with the estimation of thiocholine in aliquots of the reaction medium collected in trichloroacetic acid, and the measurements summarized in Table 1 indicate that activities of compounds bearing thiocarbamide, dithiocarbamate, or mercaptoimidazole functional groups can all be determined by following the substrate-dependent oxidation of thiocholine. However, because thiocarbamides with substituents in the range required for testing the effects of substrate size on activity are available

Table 1. Substrate-dependent oxidation of thiocholine catalyzed by purified hog liver microsomal flavin-containing monooxygenase

Substrate	V_{max} (nmol substrate oxidized/min/mg)
Thiourea	218 ± 18
Phenylthiourea*	222 ± 22
1,3-Diphenylthiourea*	213 ± 3
1,3-Bis(3,4-dichlorophenyl)-2-thiourea*	214 ± 11
1,1-Dibenzyl-3-phenyl-2-thiourea*	214 ± 11
Methimazole	226 ± 11
Propylthiouracil*	211 ± 11
Sodium diethyldithiocarbamate	215 ± 15

Activities were measured at 37° in 0.1 M phosphate, pH 7.5, containing 0.25 mM $NADP^+$, 2.5 mM glucose-6-phosphate, 1.5 U glucose-6-phosphate dehydrogenase, 100 U catalase, 0.4 mM EDTA and 130 mM thiocholine. After 3–4 min temperature equilibration, 0.035 mg FMO was added. One minute later the reaction was initiated by adding substrate. The concentration of thiocholine (DTNB-reducing equivalents) in aliquots withdrawn at regular intervals was measured as described previously [8]. Values are the average ± range of duplicate measurements for each preparation.

* Substrates were added from 50 mM stock solutions in ethanol. All others were added from 0.1 M solutions in water.

Table 2. Thiocarbamide-dependent thiocholine oxidation catalyzed by microsomal flavin-containing monooxygenases in various tissues and species

Species (sex) and tissue	MMI	TU	PTU	DPTU	DCPTU	DBPTU
	(nmol substrate oxidized/min/mg)					
Guinea pig (male)						
25 livers*	8.2 ± 0.1	8.1 ± 0.2	8.5 ± 0.2	6.0 ± 0.1†	4.0 ± 0.1†	2.6 ± <0.05†
Pig (female)						
Liver	9.7 ± 0.2	9.8 ± 0.2	9.5 ± 0.1	8.5 ± 0.1‡	4.9 ± 0.1†	5.0 ± 0.1
Chicken (female)						
Liver	4.4 ± 0.1	4.4 ± 0.1	4.2 ± 0.1	1.5 ± <0.05†	1.6 ± 0.1	NA§
Rat (male)						
Liver	4.5 ± 0.1	4.9 ± 0.2	3.7 ± 0.1‡	3.1 ± <0.05‡	NA	NA
Kidney	2.3 ± 0.1	2.4 ± <0.05	1.6 ± <0.05†	1.0 ± <0.05‡	NA	NA
Rabbit (female)						
Liver	7.5 ± 0.1	7.2 ± <0.05	4.5 ± 0.1†	3.2 ± <0.05†	NA	NA
Lung	2.9 ± 0.1	3.1 ± 0.1	2.5 ± 0.1‡	NA	NA	NA

Activities were measured at pH 7.5 in the presence of saturating NADPH by procedures described in Materials and Methods. Values are the means ± SEM of triplicate measurements for each preparation. Abbreviations: MMI, methimazole; TU, thiourea; PTU, phenylthiourea; DPTU, 1,3-diphenylthiourea; DCPTU, 1,3-bis(3,4-dichlorophenyl)-2-thiourea; and DBPTU, 1,1-dibenzyl-3-phenyl-2-thiourea.

* Microsomal fraction was isolated from the combined homogenates of 25 livers. All other preparations were from individual animals.

†,‡ Significantly different from preceding rate by $\dagger P < 0.0005$ and $\ddagger P < 0.005$, calculated by Student's t-test.

§ NA = no activity.

commercially, most of the measurements described in this report were carried out with thiocarbamides.

The rates for the oxidation of thiocarbamides and methimazole catalyzed by microsomal preparations from different species and tissues, summarized in Table 2, indicate that both thiourea- and methimazole-dependent thiocholine oxidation measure the same activities. The measurements were quite reproducible and the rates determined with thiourea or methimazole were not significantly different. The rates of phenylthiourea-dependent oxidation of thiocholine catalyzed by liver microsomes from guinea pigs, pigs and chickens were also essentially the same as those measured with thiourea. On the other hand, phenylthiourea-dependent rates catalyzed by microsomes of rat and rabbit tissues were significantly less than those measured with thiourea. The differences become even more pronounced for thiocarbamides with larger substituents. While the 1,3-diphenylthiourea-dependent rates for guinea pigs and pigs were only slightly less (13–26%) than the thiourea-dependent rates, the rates for rat kidney, chicken liver and rabbit liver microsomes were substantially less (56–66%) and 1,3-diphenylthiourea-dependent oxidation of thiocholine could not be detected in reactions catalyzed by rabbit lung microsomes. Oxidation rates for thiocarbamides larger than 1,3-diphenylthiourea were consistently less than either thiourea or 1,3-diphenylthiourea with all preparations tested and the oxidation of 1,3-bis(3,4-dichlorophenyl)-2-thiourea was not catalyzed at all by microsomes from rat or rabbit tissues. Activity with 1,1-dibenzyl-3-phenyl-2-thiourea, the largest substrate tested, was observed only with microsomes from pigs and guinea pigs.

In addition to species differences, the activities of the larger thiocarbamides relative to thiourea also varied somewhat with microsomal preparations from individual pigs and guinea pigs (Table 3). For instance, activity of 1,3-diphenylthiourea relative to thiourea ranged from 70–80% for pigs and 64–78% for guinea pigs in preparations that differed about 2-fold in total activity. Activities of the two largest substrates relative to thiourea were more variable, but oxidation of all thiocarbamides was always detectable in all liver microsomal preparations tested from both pigs and guinea pigs. Individual variations in activities of microsomes from rabbit and rat, although not examined in detail, did not appear as great as that for the pig or guinea pig.

Table 3. Thiocarbamide-dependent oxidation of thiocholine catalyzed by liver microsomes from individual animals

	TU	DPTU	DCPTU	DBPTU
	(nmol substrate oxidized/min/mg)			
Guinea pig				
No. 1	4.2	3.1	1.9	1.4
No. 2	5.1	3.3	2.2	1.5
No. 3	5.1	3.5	1.4	1.2
No. 4	2.7	2.1	1.3	0.6
Pig				
No. 1	6.1	4.9	2.7	1.3
No. 2	6.8	4.9	2.0	2.5
No. 3	2.8	2.2	0.7	0.6
No. 4	3.7	2.5	1.0	0.9

Activities were measured at pH 7.5 in the presence of saturating NADPH by the procedure listed under Materials and Methods. Abbreviations: TU, thiourea; DPTU, 1,3-diphenylthiourea; DCPTU, 1,3-bis(3,4-dichlorophenyl)-2-thiourea; and DBPTU, 1,1-dibenzyl-3-phenyl-2-thiourea.

The lower activities of thiocarbamides with the larger substituents were not due to their inability to

Table 4. Apparent K_m for thiocarbamide-dependent thiocholine oxidation catalyzed by flavin-containing monooxygenases in tissue microsomes from different species

Species and tissue	Apparent K_m (mM)			
	PTU	DPTU	DCPTU	DBPTU
Guinea pig liver	*	5	7	12
Pig liver	—	3	10	20
Chicken liver	<2	5	6	NA†
Rat liver	6	46	NA	NA
Rat kidney	10	20	NA	NA
Rabbit liver	10	28	NA	NA
Rabbit lung	6	NA	NA	NA

Kinetic constants were obtained from reciprocal plots of velocity of thiocarbamide-dependent thiocholine oxidation versus substrate concentrations above and below the concentration required to give half the maximal velocity. Abbreviations: PTU, phenylthiourea; DPTU, 1,3-diphenylthiourea; DCPTU, 1,3-bis(3,4-dichlorophenyl)-2-thiourea; and DBPTU, 1,1-dibenzyl-3-phenyl-2-thiourea.

* A dash indicates that activity was not determined.

† NA = no detectable activity at the highest concentrations of substrate soluble in the reaction medium.

Table 5. Thiocarbamide-dependent oxidation of thiocholine catalyzed by a mixture of rabbit lung and pig liver microsomes

Substrate	Substrate oxidized (nmol/min/mg)	
	Expected	Measured
Methimazole	5.1	5.2 ± 0.3
Thiourea	5.1	5.0 ± <0.05
1,3-Diphenylthiourea	3.4	3.8 ± 0.2
1,3-Bis(3,4-dichlorophenyl)-2-thiourea	1.8	1.9 ± <0.05
1,1-Dibenzyl-3-phenyl-2-thiourea	0.9	0.9 ± 0.1

Pig liver microsomes (40.2 mg; Liver 1, Table 3) were mixed with 18.5 mg of rabbit lung microsomes (Table 2). Thiocarbamide-dependent oxidation of thiocholine was measured as described under Materials and Methods. Values are means ± SEM of triplicate measurements for each preparation.

saturate the microsomal, oxygenases because of decreased water solubility. The kinetic constants (Table 4) determined for all of the thiocarbamides with aromatic substituents indicated that the concentration (500 mM) used to measure activities in Table 2 was sufficient to give rates that were 80–99% of V_{max} for all the preparations tested.

The thiocarbamide specificity of rabbit lung microsomes (Table 2) was similar to that of the major FMO isoform (FMO-2) purified from that tissue [13]. Like purified FMO-2, rabbit lung microsomes did not catalyze the oxidation of thiocarbamides larger than phenylthiourea. To determine whether the activity measurements were sufficiently sensitive to detect the contribution of small amounts of activity with a specificity restricted to relatively small thiocarbamides, the activities of 18.5 mg of lung microsomal protein mixed with 40.2 mg of liver microsomal protein were measured. As shown in Table 5, the measured values were almost identical with expected values calculated from activities of microsomes measured separately. The 1,3-diphenylthiourea relative to the thiourea-dependent rate decreased as expected from 80% in pig liver to 70% in the mixture. The difference, although small, was readily detected and, in these two tissues, the contribution of each of their major isoforms to the oxidation of the different thiocarbamides was readily calculated based on the specificity of the purified enzymes.

The progressive decrease in thiocarbamide oxidation rates as substituents increased in size was particularly striking in reactions catalyzed by guinea pig liver microsomes. To define more precisely the nature of the enzyme(s) involved, microsomes were partially freed from FAD by the procedure described under Materials and Methods. While the total amount of acid-extractable flavin decreased by only 28%, thiourea- and 1,3-diphenylthiourea-dependent thiocholine oxidase dropped 70–75% and the activity was restored specifically by FAD (Table 6). The activity loss with the two largest substrates, which could not be significantly restored by FAD, was less (43–46%) than that with the two smallest substrates. Although the reason for the difference in response of the enzymes catalyzing the oxidation of the two largest substrates is not known, the data clearly show that these enzymes differ in stability from those catalyzing the oxidation of thiourea and 1,3-diphenylthiourea. FMN either alone or in combination with FAD had no effect on these activities (data not shown).

To test whether the S-oxidation of thiocarbamides is catalyzed by more than one enzyme, the activities present in guinea pig liver microsomes were extracted and fractionated as described under Materials and Methods. While the detergent inactivated or failed to extract about 30% of the microsomal activity, the thiourea-dependent thiocholine oxidative activities in the extract were separated into three fractions (Fig. 1), with distinct but overlapping substrate specificities after one pass through the column (Table 7). While the enzyme(s) in peak II accepted all of the thiocarbamides tested, those in peaks I and III excluded substrates larger than 1,3-diphenylthiourea and 1,3-bis(3,4-dichlorophenyl)-2-thiourea, respectively. In addition to these differences, the enzymes in these fractions responded differently to *n*-dodecyclamine. Dodecylamine stimulated the thiourea-dependent oxidation of thiocholine catalyzed by both peak I and peak II by 118 and 31%, respectively, while it inhibited that catalyzed by peak III by 64% (Fig. 2). The inhibition of the activity in peak III appeared to be competitive (data not shown), which suggested that this fraction, like FMO-2 (from rabbit lung microsomes), catalyzed the N-oxygenation of primary alkylamines. This was confirmed by measuring the oxidation of dodecylamine to dodecyloxime. Only the enzyme(s)

Table 6. Effect of flavin depletion on activities of guinea pig liver microsomes

Preparation*	TU†	DPTU	DCPTU	DBPTU
	(nmol substrate oxidized/min/mg)			
Control	3.9 ± 0.2	3.2 ± 0.3	2.3 ± 0.1	1.5 ± 0.3
Pretreated with 1.5 M KCl	1.2 ± 0.2	0.8 ± 0.3	1.0 ± 0.2	0.4 ± 0.1
Pretreated with 1.5 M KCl + 50 mM FAD	3.0 ± 0.1	2.3 ± 0.3	1.0 ± 0.3	0.7 ± 0.2

* Microsomes were partially depleted of flavin by preincubation with 1.5 M KCl as described under Materials and Methods. The acid-extractable flavin was decreased 28–30% for the preparation of microsomes used for these measurements. All values are the means ± SEM of triplicate measurements for each preparation.

† Abbreviations: TU, thiourea; DPTU, 1,3-diphenylthiourea; DCPTU, 1,3-bis(3,4-dichlorophenyl)-2-thiourea; and DBPTU, 1,1-dibenzyl-3-phenyl-2-thiourea.

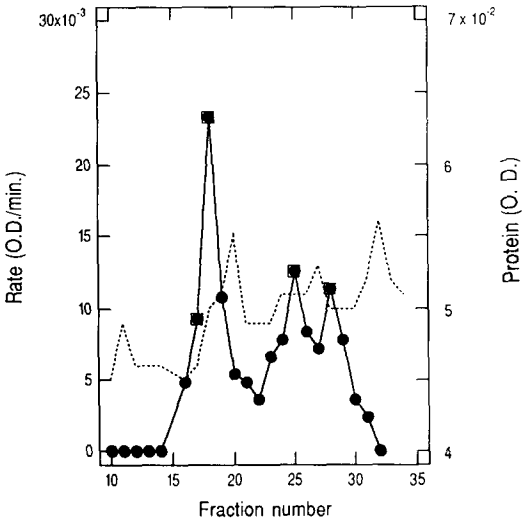

Fig. 1. Profiles of fractions from column chromatography used for the separation of FMO isoforms in detergent extracts of guinea pig livers. The solubilized protein was fractionated on a Cibacron Blue 3GA-Agarose column by the procedure described under Materials and Methods. Activities of thiourea-dependent NADPH oxidation were followed at 340 nm, in medium containing 0.1 M phosphate, pH 7.5, 1 mM EDTA, 0.2 mM NADPH, 1 mM thiourea and 0.04 to 0.4 mg of protein. Circles are the measured rates (O.D./min, left axis) for different fractions. Squares indicate the fraction(s) in each peak chosen for measurements of thiocarbamide-dependent oxidation of thiocholine. The dashed line indicates protein concentration (O.D. at 280 nm, right axis).

Table 7. Thiocarbamide-dependent oxidation of thiocholine catalyzed by the guinea pig liver fraction(s) separated by Cibacron Blue 3GA-Agarose column chromatography

	Substrate oxidized (nmol/min/mg)		
Substrate*	Peak I (fraction 17–18)†	Peak II (fraction 25)	Peak III (fraction 28)
		24 ± 1	
TU	45 ± 2		17 ± 1
PTU	42 ± 2	24 ± 1	17 ± 1
DPTU	42 ± 2	23 ± 1	15 ± 1
DCPTU	4.8 ± 0.1	21 ± 1	17 ± 1
DBPTU	4.9 ± 0.1	17 ± 1	6.6 ± 0.2

Activities were measured at pH 7.5 in the presence of saturating NADPH by the procedure described in Materials and Methods. Values are the average ± range of duplicate measurements.

* Abbreviations: TU, thiourea; PTU, phenylthiourea; DPTU, 1,3-diphenylthiourea; DCPTU, 1,3-bis(3,4-dichlorophenyl)-2-thiourea; and DBPTU, 1,1-dibenzyl-3-phenyl-2-thiourea.

† Fractions were separated by chromatography—see Fig. 1.

in peak III catalyzed the N-oxidation of this amine at a significant rate (Fig. 3).

DISCUSSION

The structure–activity studies described in this report show that microsomal preparations from different species and tissues catalyzed the NADPH- and oxygen-dependent oxidation of thiourea and a number of its derivatives to thiol reactive metabolites (Table 2). Measurements carried out with purified FMO-1 (Table 1) indicate that substrate-dependent oxidation of thiocholine accurately measured the oxidation of thiocarbamides, 2-mercaptoimidazoles or 2-mercaptopyridines to sulfenic acids—the known initial oxidation products of FMO-catalyzed oxidation of compounds bearing these functional groups [19]. The close correlation of thiourea S-oxidation with both methimazole S-oxidation (Table 2) and *N*,*N*-dimethylaniline N-oxygenation [14] indicates that all three activities are catalyzed by the same enzymes. Because the oxidation of the latter two substrates is usually considered as characteristic activity markers for flavin-containing monooxygenases, thiourea- and perhaps other *N*-

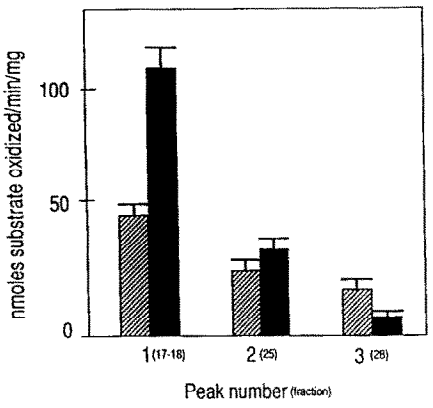

Fig. 2. Effect of dodecylamine on thiourea-dependent oxidation of thiocholine catalyzed by a fraction(s) separated on the Cibacron Blue 3GA-Agarose column. Thiourea-dependent oxidation of thiocholine activities catalyzed by different peak fractions was measured as described under Materials and Methods at 37° with (■) and without (▨) 2 mM dodecylamine. Numbers in parentheses represent the fraction used for activity measurements. Error bars represent the standard error for triplicate assays.

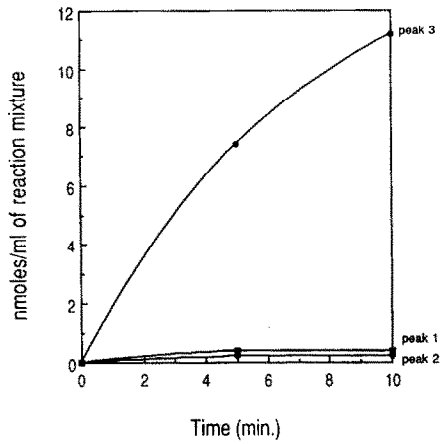

Fig. 3. Time-dependent dodecyloxime formation catalyzed by enzyme fraction(s) separated on a Cibacron Blue 3GA-Agarose column. The dodecyloxime formation catalyzed by hepatic guinea pig fraction(s) separated on the Cibacron Blue 3GA-Agarose column was measured by the procedure described under Materials and Methods. Oxime formation by fraction 28 in peak III; oxime formation by fractions 17 and 18 in peak I; oxime formation by fraction 25 in peak II.

benzylimidazole-insensitive thiocarbamide-dependent oxidations of thiocholine are catalyzed primarily by flavin-containing monooxygenases. This conclusion is also supported by the observation that the loss of thiourea- and 1,3-diphenylthiourea-dependent thiocholine oxidase activity in flavin-depleted microsomes was restored by FAD (Table 6).

The sulfur atom in the thiocarbamides used is relatively close to the bulky substituents on the nitrogens and differences in the van der Waals' surface area appear to reflect the effect of size on substrate accessibility to the active site. The dimensions of the computerized space-filling models of methimazole and five thiocarbamides at minimum energies, determined by a software program (PC model), are shown in Fig. 4. With all the microsomal preparations tested, the velocity of thiocarbamide-dependent thiocholine oxidation decreased with increasing substrate size (Table 2). Without exception, microsomal preparations which do not show activity toward a thiocarbamide with intermediate size will not catalyze the oxidation of larger thiocarbamides while smaller analogs are readily accepted.

If we assume that for a specific isoform of FMO, the V_{max} for the oxidation of all thiocarbamides accepted as substrates is the same, the differences in rates can be used to determined the minimum number of isoforms and their relative activity. For example, the isoforms present in all the microsomal preparations tested readily accepted thiourea and other substrates not much larger than methimazole, but only microsomes from guinea pig, pig and chicken livers catalyzed the oxidation of phenylthiourea at the same rate as thiourea. The relatively lower rate for phenylthiourea than for thiourea observed with microsomes from rat and rabbit tissues suggests that these tissues contain a form which excludes substrates with a van der Waals' surface area larger than 180 Å. A similar analysis of the differences in rates as a function of substrate size suggests that the microsomal preparations tested contained a minimum of five isoforms with the specificities summarized in Table 8 and with the species and tissue distribution shown in Table 9. Form E apparently excludes all thiocarbamides much larger than methimazole while at the other extreme form A accepts all thiocarbamides equal to or smaller than 1,1-dibenzyl-3-phenyl-2-thiourea.

Differences in rates as a function of substrate size (Table 2) suggest that guinea pig liver microsomes contain the most, perhaps as many as four, different isoforms of FMO. Three of these forms in detergent extracts were readily resolved by one pass through a Cibacron Blue 3GA-Agarose column. Forms C, A and B were most concentrated in peaks I, II and III, respectively. Form D was apparently lost during extraction or during chromatography on Agarose. The substrate activity of form B in guinea pig liver for primary alkylamines resembled that of form D in rabbit lung, but it appeared to accept larger substrates than purified FMO-2 from rabbit lung. Also, the stability of enzymes catalyzing the oxidation of the two largest substrates, which are probably forms A and B, was quite different from those catalyzing the oxidation of thiourea and 1,3-diphenylthiourea, forms C and D (Table 6). Previous studies by Brodfuehrer and Zannoni [27] have also shown that ascorbic acid-deficiency significantly reduces activities of guinea pig liver FMOs, particularly the form with the greatest affinity for FAD.

Form B is qualitatively similar to one of the FMO isoforms purified to homogeneity from guinea pig liver by Yamada *et al.* [7], but there are some quantitative differences in the relative rates with

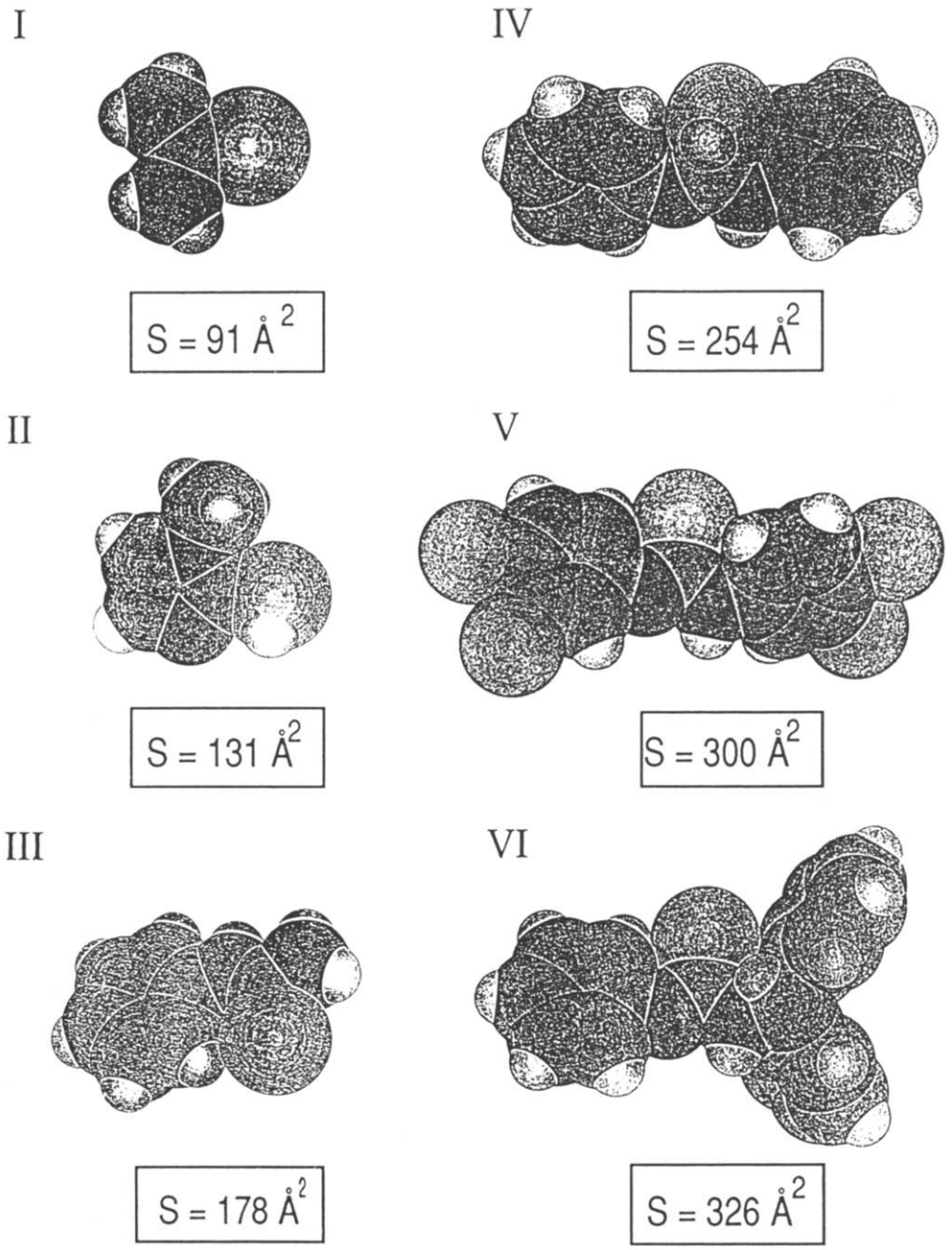

Fig. 4. Space-filling models of thiocarbamides at a minimum energy level determined by the PC model software program. S is the van der Waals' surface area. Thiourea (I) and phenylthiourea (III) are in the ketone form, whereas methimazole (II), 1,3-diphenylthiourea (IV), 1,3-bis(3,4-dichlorophenyl)-2-thiourea (V) and 1,1-dibenzyl-3-phenyl-2-thiourea (VI) are in their imine forms.

thiourea, methimazole and phenylthiourea. For instance, the low rate for the oxidation of methimazole relative to thiourea reported by Yamada *et al.* [7] is not consistent with the measurements with microsomes (Table 2) or with fractions resolved on the Agarose column (Table 7). Whether these differences are real or only reflect differences in the procedures used to measure activities is not known at present.

The preceding analysis of difference in rates of thiocarbamide-dependent thiocholine oxidation as a function of FMO isoforms relies heavily on the assumption that V_{max} for the S-oxidation of all thiocarbamides catalyzed by a specific isoform is the same. This is certainly true for the major forms purified to homogeneity from pig liver and rabbit lung [13]. All thiocarbamides that gain access to this oxidant are oxidized by oxygen transfer from the

Table 8. Thiocarbamide substrate for FMO isoforms

Isoform	Substrate*				
	TU	PTU	DPTU	DCPTU	DBPTU
A	+	+	+	+	+
B	+	+	+	+	−†
C	+	+	−	−	−
E	+	−	−	−	−

Abbreviations: TU, thiourea; PTU, phenylthiourea; DPTU, 1,3-diphenylthiourea; DCPTU, 1,3-bis(3,4-dichlorophenyl)-2-thiourea; and DBPTU, 1,1-dibenzyl-3-phenyl-2-thiourea.

† A dash indicates that the isoform *did not* oxidize that compound.

Table 9. Percent of thiourea oxidase activity associated with different FMO isoforms

Tissues	FMO isoform				
	A	B	C	D	E
Guinea pig liver	32	17	25	26	—*
Pig liver	35	—	42	23	—
Chicken liver	—	39	61	—	—
Rat liver	—	—	66	13	21
Rat kidney	—	—	42	25	33
Rabbit liver	—	—	43	18	39
Rabbit lung	—	—	—	82	18

* A dash indicates that this isoform was not detected.

hydroperoxyflavin to the sulfur, and this reaction is probably never rate limiting. In addition, there is no evidence that thiocarbamides free from polar or ionic groups affect any other steps, especially a rate-limiting step, in the catalytic cycle of any FMO isoform examined to date.

Because of the sensitivity and simplicity of the thiocarbamide-dependent thiocholine oxidase assay system, the activities determined with thiocarbamides that differ in size may be a useful tool for measuring the number and activities of FMO isoforms in crude tissue preparations.

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REFERENCES

1. Ziegler DM, Flavin-containing monooxygenases: Catalytic mechanism and substrate specificities. *Drug Metab Rev* **19**: 1–32, 1988.
2. Ziegler DM, Flavin-containing monooxygenases: Enzymes adapted for multisubstrate specificity. *Trends Pharmacol Sci* **11**: 321–324, 1990.
3. Williams DE, Hale SE, Meurhoff AS and Masters BSS, Rabbit lung flavin-containing monooxygenase: Purification, characterization, and induction during pregnancy. *Mol Pharmacol* **28**: 381–390, 1984.
4. Tynes RE, Sabourin PJ and Hodgson E, Identification of distinct hepatic and pulmonary forms of microsomal flavin-containing monooxygenase in the mouse and rabbit. *Biochem Biophys Res Commun* **126**: 1069–1075, 1985.
5. Ozols J, Liver microsomes contain two distinct NADPH-monooxygenases with NH_2-terminal segments homologous to the flavin containing NADPH monoxygenase of *Pseudomonas fluorescens*. *Biochem Biophys Res Commun* **163**: 49–55, 1989.
6. Lawton MP, Gasser R, Tynes RE, Hodgson E and Philpot RM, The flavin-containing monooxygenase enzymes expressed in rabbit liver and lung are products of related but distinctly different genes. *J Biol Chem* **265**: 5855–5861, 1990.
7. Yamada H, Yuno K, Oguri K and Yoshimura H, Multiplicity of liver microsomal flavin-containing monooxygenase in the guinea pig: Its purification and characterization. *Arch Biochem Biophys* **280**: 305–312, 1990.
8. Poulsen LL and Ziegler DM, The liver microsomal FAD-containing monooxygenase. *J Biol Chem* **254**: 6449–6455, 1979.
9. Beaty NB and Ballou DP, The reductive half-reaction of liver microsomal FAD-containing monooxygenase. *J Biol Chem* **256**: 4611–4618, 1981.
10. Beaty NB and Ballou DP, The oxidative half-reaction of liver microsomal FAD-containing monooxygenase. *J Biol Chem* **256**: 4619–4625, 1981.
11. Ball S and Bruice TC, Oxidation of amines by a 4a-hydroperoxyflavin. *J Am Chem Soc* **102**: 6498–6503, 1980.
12. Taylor KL and Ziegler DM, Studies on substrate specificity of the hog liver flavin-containing monooxygenase: Anionic organic sulfur compounds. *Biochem Pharmacol* **36**: 141–146, 1987.
13. Nagata T, Williams DE and Ziegler DM, Substrate specificities of rabbit lung and porcine liver flavin-containing monooxygenases: Differences due to substrate size. *Chem Res Toxicol* **3**: 372–376, 1990.
14. Guo WXA and Ziegler DM, Estimation of flavin-containing monooxygenases activities in crude tissue preparations by thiourea-dependent oxidation of thiocholine. *Anal Biochem* **198**: 143–148, 1991.
15. Ziegler DM and Poulsen LL, Hepatic microsomal mixed-function amine oxidase. *Methods Enzymol* **52**: 142–151, 1978.
16. Gornall AC, Bardawill CJ and David MM, Determination of serum protein by means of the biuret reaction. *J Biol Chem* **177**: 751–766, 1949.
17. Poulsen LL, Taylor K, William DE, Masters BSS and Ziegler DM, Substrate specificity of the rabbit lung flavin-containing monooxygenase for amines: Oxidation products of primary alkylines. *Mol Pharmacol* **30**: 680–685, 1986.
18. Pettit FH, Orme-Johnson W and Ziegler DM, The requirement for flavin adenine dinucleotide by a liver microsomal oxygenase catalyzing the oxidation of alkylaryl amines. *Biochem Biophys Res Commun* **16**: 444–448, 1964.
19. Poulsen LL, Hyslop RM and Ziegler DM, S-Oxygenation of N-substituted thioureas catalyzed by the pig liver microsomal FAD-containing monooxygenase. *Arch Biochem Biophys* **198**: 78–88, 1979.
20. Ziegler DM, Functional groups bearing sulfur. In: *Metabolic Basis of Detoxication* (Eds. Jakoby B, Bend JR and Caldwell J), pp. 171–184. Academic Press, New York, 1980.
21. Brodfuehrer JI and Zannoni VG, Flavin-containing monooxygenase and ascorbic acid deficiency. Qualitative and quantitative differences. *Biochem Pharmacol* **36**: 3161–3167, 1987.