degradation or metabolism would have been expected for 0-day samples. However, the corresponding RIDA values for extractable avermectin B_1a residues were considerably lower indicating extensive and rapid metabolism and/or degradation. The RIDA values for orange and lemon rind were 0.020 and 0.006 ppm, respectively, or 30 and 16%, respectively, of the sample oxidizer values. Subsequent samples show continuing degradation of the actual avermectin B_1a present.

CONCLUSIONS

It should be noted that application was by dip and not by spray and that this was an experimental formulation not contemplated for commercial development. Thus, both the initial residues and their rates of dissipation may be different from those of other formulations delivered under normal treatment conditions. Assuming for illustration purposes an application of 1,000 gal per acre (93.5 hL/ha), the amount of avermectin B_1a applied would be 11.4 g (0.025 lb) per acre. According to these results, whole fruit residues of avermectin B_1a at harvest would be less than 0.001 ppm. In addition, translocation of avermectin B_1a from the treatment sites would be so low as to be undetectable. Because of its low application rate and short persistence, avermectin B_1a should cause minimal environmental and food contamination.

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Registry No. Avermectin B_1a, 65195-55-3.

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Magnitude of Involvement of the Mammalian Flavin-Containing Monooxygenase in the Microsomal Oxidation of Pesticides

Ronald E. Tynes and Ernest Hodgson*

The oxidation of sulfide-containing organophosphate and carbamate pesticides by the flavin-containing monooxygenase has been measured in mammalian microsomes made devoid of cytochrome P-450-dependent activity, primarily through the use of inhibitory antibodies against NADPH-cytochrome P-450 reductase. Rates of metabolism were determined for mouse, rabbit, and rat liver, lung, and kidney microsomes and for pig liver microsomes. Substrate specificity of the enzyme in different species and tissues is similar. Lung and kidney microsomes have high flavin-containing monooxygenase levels, and this enzyme is important relative to cytochrome P-450 in these tissues. Thioether-containing organophosphates are effective substrates for the flavin-containing monooxygenase in mouse liver microsomes, with K_m values between 3.5 and 36 μM. Thioether-containing carbamates are less effective substrates, having K_m values near 280 μM. Other substances oxidized include (methylthio)phenyl-containing organophosphates, certain phosphonodithioate pesticides, certain dithiocarbamate soil fumigants, ethylenethiourea, nicotine, selenourea, and diethylphenylphosphine.

INTRODUCTION

The flavin-containing monooxygenase (flavin monooxygenase), formerly known as *N,N*-dimethylaniline *N*-oxidase, EC 1.14.13.8, is an enzyme located in the endoplasmic reticulum. This enzyme and the cytochrome P-450-dependent monooxygenase system, also located in microsomes, are the major enzymes catalyzing the oxidation of lipophilic foreign compounds. Hydroxylated products produced by these enzymes have increased water solubility and serve as substrates for conjugative reactions forming glucuronide, glycoside, and sulfate derivatives which are readily excretable.

Techniques for the purification of pig liver flavin monooxygenase were developed some time ago and the capability of this solubilized enzyme to catalyze the oxidation of a wide variety of nucleophilic nitrogen- and sulfur-

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containing compounds has been well established (Ziegler, 1980; Poulsen, 1981). The physiological substrate for this enzyme is believed to be cysteamine, which is oxidized to cystamine providing a thiol oxidant for the synthesis of peptide disulfides (Ziegler and Poulsen, 1977). The flavin monooxygenase appears to be a mammalian enzyme and there is no evidence that this monooxygenase exists in insects.

The major oxidative metabolites of several thioether-containing phosphorodithioate insecticides have been identified in mammals and insects (March et al., 1955; Bowman and Casida, 1958; Bull, 1965; Andrawes et al., 1967). These include sulfoxides, sulfones, and oxons. However, the enzymatic pathways catalyzing the formation of these active metabolites have not been established and in the past have been attributed solely to cytochrome P-450.

Hajjar and Hodgson (1980, 1982a) reported that pig liver flavin monooxygenase could form sulfoxides from thioether-containing organophosphate and carbamate pesticides. More recently, it has been shown that this enzyme can catalyze the oxidative desulfuration of fonofos to form fonofos oxon (Hajjar and Hodgson, 1982b). Both the formation of thioether sulfoxides and oxons represent activation reactions resulting in more potent cholinesterase inhibitors.

It is important to note that the flavin monooxygenase specifically oxidizes sulfides to (*R*)-(+)-sulfoxide enantiomers, while cytochrome P-450-dependent oxidations yield predominantly sulfoxides in the *S*(−) configuration as shown with tolyl ethyl sulfide (Light et al., 1982; Waxman et al., 1982). Similarly, although both cytochrome P-450 and the flavin monooxygenase are believed to be able to catalyze the oxidative desulfuration of fonofos, the two enzymes are thought to form the opposite oxon isomers (Hajjar and Hodgson, 1982b). The two isomers of fonofos have different toxicities in mammals and insects (Lee et al., 1976, 1978a,b). Thus, the potential exists that selective monooxygenation to toxic or nontoxic chiral products could be involved in pesticide selectively.

Since oxidative pesticide metabolism can be carried out by both the flavin monooxygenase and the cytochrome P-450-dependent monooxygenase system, the relative involvement of the two monooxygenases toward the overall metabolism of a particular pesticide is of importance. We have recently described methods that permit measurements of flavin monooxygenase activity in microsomes containing cytochrome P-450 (Tynes and Hodgson, 1984). This report describes the substrate preferences and kinetic characteristics of the flavin monooxygenase in mammalian microsomes with reference to the oxidation of pesiticides and related compounds.

MATERIALS AND METHODS

Chemicals. Phorate, disulfoton, sulprofos, fenthion, methyl carbophenothion, counter, fosthietan, fonofos, aldicarb, methiocarb, sodium metham, nicotine, and α-naphthylthiourea were purchased from Chem Service, West Chester, PA. Croneton, demeton-S-methyl, demetonthione, and demetonthiol were provided by Mobay Chemical Corp., Kansas City, MO. Selenourea, metyrapone, and trypsin inhibitor type II-0 were from Sigma Chemical Co., St. Louis, MO. Methyl phenyl sulfide, dimethyldithiocarbamate, *n*-octylamine, and thiobenzamide were obtained from Aldrich Chemical Co., Milwaukee, WI. Diethylphenylphosphine was obtained from the Pressure Chemical Co., Pittsburg, PA, thiourea from Fisher Scientific CO., Fairlawn, NJ, and parathion from City Chemical Corp., New York, NY. Emulgen 911 was a gift of Kao-Atlas, Tokyo, Japan, *p*-nitroanisole was obtained from Eastman Organic Chemicals, Rochester, NY, and bovine pancreatic trypsin from Nutritional Biochemicals Corporation, Cleveland, OH.

Preparation of Microsomes. Liver, lung, and kidney microsomes were prepared from 25–30-g female Dub:ICR mice and 200–250-g Dub:Sprague Dawley rats (Dominion Laboratories, Dublin, VA). Rabbit microsomes were prepared from adult male New Zealand white rabbits (Dutchland Farms, Denver, PA). Pig livers were obtained from the Jesse Jones sausage plant, Garner, NC, immediately after the pigs were slaughtered. Only livers with high flavin monooxygenase activity (Table IV) were selected for use. Microsomes were isolated by standard differential centrifugation, washed once, and then stored in 0.25 M sucrose under N_2 as described earlier (Tynes and Hodgson, 1983).

Preparation of Antibodies. Antibodies against purified mouse liver NADPH-cytochrome P-450 reductase were elicited in rabbits and purified from serums by ammonium sulfate precipitation and column chromatography on DE52 cellulose (Tynes and Hodgson, 1983). Goat antibodies against rabbit reductase were provided by Dr. R. M. Philpot, NIEHS, Research Triangle Park, NC, and were used in all experiments on rabbit microsomal preparations.

Assays. Rates of NADPH oxidation were determined by using an Aminco DW-2 UV/vis spectrophotometer. NADPH oxidation rates, except those used for the determination of kinetic constants, were obtained in the dual beam mode with both the observation beam at 340 nm and the reference beam at 420 nm passing through a single cuvette. Mouse liver flavin monooxygenase rates used for the determination of kinetic constants were obtained in the split beam mode at 340 nm, with NADPH in the reference cuvette, and with NADPH and microsomal enzyme in the sample cuvette. Measurements obtained in the split beam mode in this manner are identical with those determined in the dual beam mode by using a single cuvette. Kinetic constants were obtained from best fit double-reciprocal plots. Oxygen consumption measurements were determined by using a Clark type electrode.

The standard assay mixture contained 25 mM pyrophosphate, 100 mM alanine, 1.0 mM EDTA, and 0.125 mM NADPH with pH 8.4 at 37 °C with enough microsomal suspension to give a final concentration of 0.1–0.4 mg of microsomal protein/mL in a final volume of 1.0 mL. The flavin monooxygenase was equally active in tricine, tris HCl, or pyrophosphate/phosphate buffers at pH 8.4. Reaction rates at pH 7.6 are about 60% those observed at pH 8.4. For assays of solubilized microsomes, 100 μL/mL of 10% Emulgen 911 was used as suggested by Cavagnaro et al. (1981). Where used, metyrapone was added in 5 μL of acetone, and *n*-octylamine hydrochloride was added in 5 μL of aqueous solution. Antibodies against NADPH-cytochrome P-450 reductase were added to stock microsomal solutions at concentrations listed in the tables and this solution was kept on ice. Equal aliquots of this solution were added to cuvettes containing the standard assay mixture.

Cuvettes were warmed to 37 °C for 2–3 min and then monitored for 1–2 min to record endogenous NADPH oxidase activity. Reactions were initiated by the addition of 5 μL of substrate to the incubation mixture. Absorbance changes were recorded for 2–4 min, and reaction rates were determined by using a molar absorptivity of 6220 M^{-1} cm^{-1} for NADPH. Velocities were determined as sustrate-stimulated minus endogenous rates. The values presented

in Tables III through VII represent the mean plus or minus the standard deviation for three preparations of microsomes.

For trypsin proteolysis of mouse liver microsomes, 5 mg of trypsin was added to 20–25 mL of 100 mM phosphate buffer pH 7.6 which contained about 75 mg of microsomal protein. Digestion proceeded for 2 h at 4 °C after which 10 mg of trypsin inhibitor was added. Microsomes were then sedimented through ultracentrifugation at 1000000g for 1 h and resuspended for use. The flavin monooxygenase of pig liver microsomes is not as stable to proteolysis under these conditions.

Difference spectra for measuring ligand interactions with cytochrome P-450 were determined as described previously (Hodgson and Kulkarni, 1974). p-Nitrophenol formation from p-nitroanisole or parathion was determined at 417 nm by using a molar absorptivity of 14.6 mM^{-1} cm^{-1}. Reactions were conducted in 100 mM phosphate and 1.0 mM EDTA with pH 7.6 at 37 °C. N,N-Dimethylaniline N-oxide concentrations were determined by the method of Ziegler and Pettit (1964). Thiobenzamide S-oxide formation was measured by the procedure of Cashman and Hanzlik (1981). Protein concentrations were determined by the method of Lowry et al. (1951).

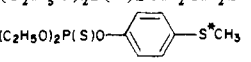
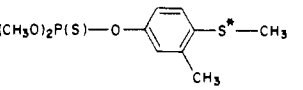
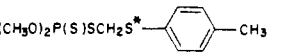
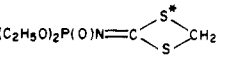
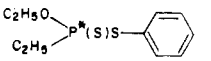
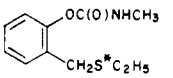
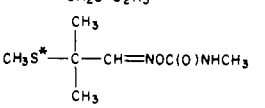
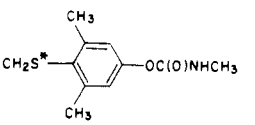
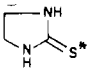
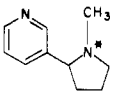
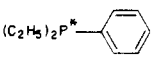
RESULTS AND DISCUSSION

With the exception of fosthietan and sodium metham, the chemicals illustrated in Table I have been demonstrated to be substrates suitable for the flavin monooxygenase isolated from pig liver microsomes (Ziegler, 1980; Poulsen, 1981; Hajjar and Hodgson, 1982a; Smyser and Hodgson, 1985). Unlike metabolism by the cytochrome P-450 family of isozymes, the substrate specificity of the flavin monooxygenase is rather limited, and the products formed are more predictable. The flavin monooxygenase cannot catalyze epoxide formation, O-, N-, or S-dealkylation via carbinol rearrangement of initial α-carbon hydroxylation products, nor will it generally catalyze P=S to P=O conversions, the exception being fonofos and its analogues.

Thioether-containing organophosphates and carbamates are metabolized by the flavin monooxygenase only to the sulfoxide; their is no evidence for the formation of any other products. The sulfoxides formed from these pesticides by the flavin monooxygenase are presumably distinct from those formed by cytochrome P-450 in that they possess the opposite chiral character. Optical rotary dispersion studies have shown that the pig liver flavin monooxygenase forms disulfotonsulfoxide that is optically active, indicating that oxygen addition was, at least in part, stereospecific (Hajjar and Hodgson, 1982a).

The sulfoxides of phorate, disulfoton, and the carbamate croneton are not detectably further oxidized by purified flavin monooxygenase to their corresponding sulfones (Hajjar and Hodgson, 1980). The pig liver enzyme can form methyl phenyl sulfone from racemic methyl phenyl sulfoxide, although the K_m value for this conversion is large, about 18600 μM (Poulsen, 1981). Light et al. (1982) have observed that in prolonged incubations of the flavin monooxygenase with racemic 4-tolyl ethyl sulfoxide, a preferential oxidation of the (S)-sulfoxide enantiomer to the achiral sulfone occurs. Since the flavin monooxygenase forms only the R enantiomer, incubations containing pure enzyme and the sulfide containing pesticide would not generate the preferred sulfoxide enantiomer suitable for further oxidation. Since cytochrome P-450 does catalyze the formation of (S)-sulfoxides, sequential oxidations by both monooxygenases may be required to form sulfones, a hypothesis currently being explored in this laboratory.

Table I. Structures of Pesticide Substrates and Related Compounds[a]

Organophosphate	
phorate	$(C_2H_5O)_2P(S)SCH_2S^*C_2H_5$
disulfoton	$(C_2H_5O)_2P(S)SCH_2CH_2S^*C_2H_5$
demeton-*S*-methyl	$(CH_3O)_2P(O)SCH_2CHS^*C_2H_5$
demetonthione	$(C_2H_5O)_2P(S)OCH_2CH_2S^*C_2H_5$
demetonthiol	$(C_2H_5O)_2P(O)SCH_2CH_2S^*C_2H_5$
sulprofos	
fenthion	
methyl carbophenothion	
counter	$(C_2H_5O)_2P(S)SCH_2S^*C(CH_3)_3$
fosthietan	
fonofos	
Carbamate	
croneton	
aldicarb	
methiocarb	
Dithiocarbamate	
sodium metham	$CH_3NHC(S^*)SNa$
Thioureylene	
ethylenethiourea	
Tertiary Amine	
nicotine	
Phosphine	
diethylphenylphosphine	

[a] * represents putative atom oxidized.

Fonofosoxon and diethylphenylphosphine oxide have been identified as the major flavin monooxygenase products derived from fonofos and diethylphenylphosphine, respectively (Hajjar and Hodgson, 1982b; Smyser and Hodgson, 1985). Cytochrome P-450 is believed to catalyze oxidative desulfuration via initial attack at the sulfur to form a phosphooxathiiran, which subsequently undergoes a cyclic electron shift with the loss of atomic sulfur, forming the oxon (Neal, 1980). Oxidative attack on the sulfur would lead to a retention of configuration about the phosphorus. The stereospecificity of the oxidative desulfuration of the two chiral isomers of fonofos by mouse liver microsomes has been shown to proceed predominantly with retention of configuration, although inversion occurred to an extent of approximately 25% (Lee et al., 1978a). Inversion of configuration would likely occur by oxidative attack on the backside of the phosphorus atom. Since the flavin monooxygenase can oxidize phosphorus centers (Smyser and Hodgson, 1985), it can be conjectured that the inversion noted by Lee et al. (1978a) occurred as

Table II. Flavin-Containing Monooxygenase Activity in Mouse Liver Microsomes, Stoichiometry between NADPH, Oxygen, and Product Formation[a]

substrates, 1.0 mM (except as noted)	NADPH and oxygen consumption, nmol of NADPH or O_2/min/mg of microsomal protein			product formation, nmol/min/mg of microsomes	
	NADPH	O_2 consumption	NADPH:O_2	thiobenzamide *S*-oxide	dimethylaniline *N*-oxide
phorate (0.5 mM)	11.7 ± 0.3	10.8 ± 0.7	0.9		
disulfoton (0.5 mM)	10.9 ± 1.1	12.7 ± 0.7	1.2		
thiobenzamide	21.2 ± 0.7[b]	24.6 ± 1.8	0.8	20.7 ± 0.8	
dimethylaniline	15.4 ± 0.4	15.5 ± 0.7	1.0		19.0 ± 2.9
diethylphenylphosphine (0.1 mM)	13.4 ± 1.1	16.6 ± 2.0	1.2		
thioanisole	16.9 ± 0.3	18.2 ± 0.9	1.1		
thiourea	20.5 ± 0.7	22.5 ± 0.7	1.1		

[a] Incubations include 4 mg of antireductase IgG/mg of microsomal protein and 3 mM *n*-octylamine. Data represent mean ± standard deviation. [b] NADPH was measured spectrofluorometrically.

a result of catalysis by this enzyme, while cytochrome P-450-dependent catalysis produced the oxon with retained configuration.

For flavin monooxygenase activity in mouse liver microsomes, the consumption of molecular oxygen measured polarographically is equimolar with the oxidation of NADPH measured spectrophotometrically or spectrofluorometrically (Table II). Also, the rate of appearance of oxidized product, either thiobenzamide *S*-oxide, or *N,N*-dimethylaniline *N*-oxide, is equivalent to the rate of disappearance of both NADPH and oxygen. This being the case, NADPH or oxygen consumption measurements provide valid methods for measuring flavin monooxygenase activity. It has been well established for the pig liver enzyme, that in the presence of an oxygenatable substrate, the consumption of NADPH and oxygen is tightly coupled to the generation of oxidized product (Poulsen, 1981; Ziegler, 1980).

Thioether-containing organophosphorus pesticides are sulfoxidized with high affinity by the flavin monooxygenase in mouse liver microsomes (Table III). The K_m value of 3.5 μM places disulfoton among the best known flavin monooxygenase substrates (Ziegler, 1980; Poulsen, 1981). These kinetic constants were obtained in the presence of *n*-octylamine, which increases both K_m and V_{max} values for these pesticides about 25% in mouse liver microsomes. The metabolism of these pesticides in vivo would be expected to compete favorably for the active site of the flavin monooxygenase with the physiological substrate cyteamine, which has a K_m value in mouse liver microsomes of 100 μM and is postulated to be present in hepatocytes at a concentration of about 50 μM (Ziegler et al., 1983).

The carbamates, which are more water soluble than the organophosphates, are metabolized with less affinity by the microsomal flavin monooxygenase. Nicotine is oxidized in a relatively low affinity process. Ethylenethiourea, a breakdown product of ethylenebis(dithiocarbamate) fungicides, is rather efficiently oxidized by this enzyme.

The rate-limiting step of the catalytic cycle of the purified pig liver flavin monooxygenase has been determined to be the breakdown of a product hydroxyflavin pseudobase intermediate (Beaty and Ballou, 1981). Since this step occurs after substrate binding, all substrates should be metabolized at a common maximal velocity upon complete saturation. The maximal common velocity of the flavin monooxygenase in mouse liver microsomes is that shown for ethylenethiourea (24.2 nmol/min/mg of microsomal protein), which is a water soluble substrate capable of fully saturating the monooxygenase. This common maximal velocity was also obtained for other soluble substrates including thiourea, methimazole, cysteamine, and *N,N*-dimethylaniline.

Table III. Michaelis–Menton Constants for the Flavin-Containing Monooxygenase in Mouse Liver Microsomes[a]

pesticide substrate (range, μM)[b]	K_m, μM	V_{max}, nmol/min/mg of microsomal protein
	Organophosphate	
phorate (5.5–66.6)	13.7 ± 2.5	11.8 ± 1.1
disulfoton (5.5–66.6)	3.5 ± 0.7	14.5 ± 2.8
demeton-*S*-methyl (5.5–166)	34.5 ± 4.9	15.9 ± 3.9
demetonthione (5.5–66.6)	14.8 ± 3.2	14.4 ± 3.7
demetonthiol (5.5–166)	36.1 ± 4.8	13.1 ± 2.8
sulprofos (5.5–33.3)	3.2 ± 1.3	6.7 ± 0.6
fenthion (5.5–33.3)	3.4 ± 0.6	6.2 ± 2.4
fosthietan (16.6–333)	80.8 ± 12.6	19.4 ± 5.6
fonofos (11.1–100)	13.7 ± 2.8	5.6 ± 0.8
	Carbamate	
aldicarb (66.6–500)	279 ± 67	7.9 ± 0.8
croneton (66.6–500)	288 ± 38	15.1 ± 2.7
	Tertiary Amine	
nicotine (250–2000)	720 ± 210	10.6 ± 2.0
	Thioureylene	
ethylenethiourea (100–2000)	253 ± 28	24.2 ± 3.0

[a] Assays included 5 mg of antireductase IgG/mg of microsomal protein and 3.0 mM *n*-octylamine. [b] The range represents the lowest and highest substrate concentrations employed for the kinetic determinations. The highest values listed for the organophosphates represent approximately the solubility limits for these compounds.

The organophosphorus pesticides, restrained by their solubility limits, cannot physically saturate the microsomal flavin monooxygenase. For the less water soluble pesticides, apparent V_{max} values are lower than theoretical. The apparent V_{max} values for the more soluble substrates, such as demeton-*S*-methyl and fosthietan, more closely approach the theoretical common V_{max} as estimated by ethylenethiourea.

A second factor contributing to the low apparent V_{max} values is the nonlinear kinetics frequently observed at high substrate concentrations for lipophilic compounds such as thiobenzamide (Figure 1). A possible explanation is that the monooxygenase in microsomes has a population of low affinity substrate binding sites that require inordinately high substrate concentrations to saturate. Extrapolation of double-reciprocal plots at low thiobenzamide concentrations (dotted line) gives a V_{max} value below that which would be obtained upon extrapolation at high substrate concentrations. For example, since phorate is not soluble at high substrate concentrations, only a low V_{max} can be observed.

Rates of pesiticide metabolism by the flavin monooxygenase of mouse and pig liver microsomes are shown in Table IV. Estimates of substrate affinity can be made by comparing rates at different substrate concentrations, where measured, and by comparison with the maximal

Table IV. Rates of Pesticide Metabolism by the Flavin-Containing Monooxygenase in Mouse and Pig Liver Microsomes

substrate	concn, mM	activity, nmol of NADPH/min/mg of microsomal protein: mouse liver, plus antireductase[a]	mouse liver, plus 1% Emulgen[b]	mouse liver, trypsin treated		pig liver[c] plus *n*-octylamine
		Organophosphate				
phorate	0.1	7.4 ± 1.0	1.8 ± 0.3	1.8 ± 0.2	10.1 ± 3.9	22.3 ± 4.9
	0.5		7.7 ± 1.3			
disulfoton	0.1	9.0 ± 1.4	11.4 ± 3.5	5.1 ± 1.7	10.6 ± 3.6	24.8 ± 6.5
	0.5		15.3 ± 2.7			
demeton-*S*-methyl	0.1	10.6 ± 2.5	6.3 ± 1.9		11.6 ± 2.9	23.1 ± 6.2
	0.5	13.9 ± 2.1	13.1 ± 4.9		11.8 ± 2.6	23.6 ± 3.9
demetonthione	0.1	10.5 ± 0.8	7.7 ± 1.6		11.2 ± 2.9	25.9 ± 6.6
	0.5	13.9 ± 0.4	13.5 ± 0.7		11.9 ± 1.9	26.2 ± 6.3
demetonthiol	0.1	9.6 ± 1.6	7.4 ± 1.9		12.4 ± 3.0	24.9 ± 8.0
	0.5	12.6 ± 2.1	14.0 ± 3.3		12.5 ± 2.8	25.4 ± 7.4
sulprofos	0.1	6.6 ± 1.5	1.4 ± 0.8		8.7 ± 3.3	10.6 ± 3.7
	0.5		3.4 ± 0.8			
fenthion	0.1	6.5 ± 1.4	0.8 ± 0.2	0.9 ± 0.2	8.9 ± 2.1	16.4 ± 5.8
	0.5		3.3 ± 1.1			
methyl phenyl sulfide	0.1	11.1 ± 1.4	6.1 ± 1.0	7.0 ± 2.4		
methyl carbophenothion	0.1	7.8 ± 1.0	2.6 ± 0.9	1.2 ± 0.4	8.0 ± 1.1	10.7 ± 2.7
	0.5		9.3 ± 1.8			
counter	0.1	1.1 ± 0.5	0.7 ± 0.4		3.9 ± 1.5	3.3 ± 1.5
	0.5		1.1 ± 0.4			
fosthietan	0.1	9.8 ± 0.7	3.8 ± 1.6		8.2 ± 2.2	18.1 ± 5.6
	0.5	17.1 ± 2.1	6.6 ± 2.7			
fonofos	0.1	2.4 ± 0.3	1.0 ± 0.3		6.6 ± 3.7	11.4 ± 4.0
	0.5		1.8 ± 0.5			
		Carbamate				
aldicarb	0.1	2.1 ± 0.5	0.7 ± 0.2	<0.4	5.5 ± 1.4	3.9 ± 0.7
	0.5	3.8 ± 0.7	1.7 ± 0.4	1.1 ± 0.7	6.9 ± 1.6	8.6 ± 2.0
croneton	0.1	5.1 ± 1.9	2.1 ± 0.9	1.0 ± 0.4	7.9 ± 2.1	10.8 ± 4.2
	0.5	8.5 ± 1.7	6.0 ± 1.8	3.0 ± 0.9	10.5 ± 3.7	16.7 ± 5.6
methiocarb	0.1	2.1 ± 0.7	0.5 ± 0.3		5.0 ± 1.7	4.9 ± 1.0
	0.5	3.7 ± 0.7	1.2 ± 0.4		5.7 ± 0.8	7.0 ± 1.7
		Dithiocarbamate				
sodium metham	1.0	3.9 ± 1.3	5.4 ± 2.3		4.1 ± 1.9	8.6 ± 4.8
dimethyldithiocarbamate	1.0	3.9 ± 0.8	4.9 ± 1.6		2.2 ± 1.1	6.4 ± 1.8
		Thioureylene				
α-naphthylthiourea	0.1	8.4 ± 0.7	17.7 ± 1.8	9.8 ± 2.0	7.9 ± 0.9	21.6 ± 3.0
ethylenethiourea	1.0	16.3 ± 2.4	22.1 ± 1.1		11.2 ± 3.3	25.3 ± 4.2
thiourea[d]	1.0	23.6 ± 5.6	32.9 ± 5.2	22.2 ± 3.6	9.9 ± 1.7	24.6 ± 2.3
		Tertiary Amine				
nicotine	1.0	3.6 ± 1.5	4.0 ± 0.6	0.8 ± 0.2	8.6 ± 2.0	17.1 ± 4.0
		Phosphine				
diethylphenylphosphine	0.1	9.4 ± 0.8	20.8 ± 3.0	10.1 ± 2.6	9.4 ± 1.9	24.2 ± 6.2
endogenous NADPH oxidase rate		3.2 ± 0.7	3.2 ± 0.3	1.4 ± 0.4	4.0 ± 0.5	3.5 ± 0.9

[a] Incubations included 5 mg of antireductase IgG/mg of microsomal protein and 3.0 mM *n*-octylamine. Velocities represent substrate-stimulated minus endogenous rates. Rates are mean ± SD. [b] Assays contained 1% Emulgen 911 and 3.0 mM *n*-octylamine. [c] Pig liver microsomes contained 3.0 mM metyrapone or 3.0 mM *n*-octylamine. [d] Thiourea completely saturates the enzyme at a concentration of 1.0 mM; these rates represent approximately the maximal velocities of these microsomal preparations.

common velocity of the microsomal preparation as approximated by the thiourea rate. The mouse liver microsomal rates were obtained in the presence of *n*-octylamine, which activates the mouse liver monooxygenase only about 25%. Throughout this paper, the endogenous NADPH oxidase rates have been subtracted from the substrate-stimulated rates. Since a portion of the endogenous rate may become coupled to substrate oxidation in the presence of substrate, these NADPH rates may slightly underestimate product formation rates.

Most of the substrates are oxidized by the flavin monooxygenase to products that cannot undergo further oxidation by the monooxygenase. Fosthietan, presumably, can form sulfoxides at both of its sulfur atoms. Thioureylenes can be monooxygenated to formamidinesulfenic acids, and then can be dioxygenated to the foramidinesulfinic acid. The K_m value for the formation of thioureasulfenic acid from thiourea is about 23 μM (Poulsen, 1981) and for the formation of thioureasulfinic acid from the sulfenic acid is about 140 μM. Since only initial velocity measurements were obtained, the velocities shown represent only the rate of conversion of the parent compound to its initial oxygenated product.

Estimates of saturation cannot be obtained from simple rate measurements for diethylphenylphosphine due to its unique kinetic behavior with the flavin monooxygenase. The highly localized electron density on the trivalent phosphorus of diethylphenylphosphine makes it an excellent flavin monooxygenase substrate, having a K_m value of less than 2 μM in mouse liver microsomes. However, at concentrations above 5–10 μM, rates are decreased relative to rates obtained at lower substrate concentrations, and rates at 100 μM are much less than the thiourea oxidation rates. Apparently, the flavin monooxygenase is sensitive to inhibition by excess phosphine substrate.

Selenourea, at a concentration of 1.0 mM, was metab-

Table V. Rates of Pesticide Metabolism by the Flavin-Containing Monooxygenase in Mouse Lung and Kidney Microsomes

substrate	concn, mM	activity, nmol of NADPH/min/mg of microsomal protein: mouse lung, plus antireductase[a]	mouse lung, plus 1% Emulgen 911	mouse kidney plus antireductase[a]
		Organophosphate		
phorate	0.1	10.7 ± 0.9	8.5 ± 1.0	4.4 ± 0.9
disulfoton	0.1	11.1 ± 1.4 (14.0 ± 0.5)[b]	11.3 ± 0.3	5.9 ± 2.0
fenthion	0.1	4.1 ± 0.8	2.0 ± 1.5	2.7 ± 0.1
methyl carbophenothion	0.1	7.8 ± 1.7	6.0 ± 0.2	2.8 ± 0.9
		Carbamate		
aldicarb	0.1	2.1 ± 0.5	0.6 ± 0.2	1.3 ± 0.4
	0.5	3.7 ± 0.4	1.3 ± 0.4	2.2 ± 1.0
croneton	0.1	4.4 ± 0.9	1.2 ± 0.5	2.4 ± 0.6
	0.5	6.8 ± 0.7	2.8 ± 0.6	
		Other		
α-naphthylthiourea	0.1	6.8 ± 1.6	6.9 ± 0.7	
methyl phenyl sulfide	0.1	9.2 ± 2.4 (13.9 ± 0.3)	12.6 ± 3.8	
nicotine	1.0	3.2 ± 0.8	1.5 ± 0.7	2.3 ± 0.2
diethylphenylphosphine	0.1	4.9 ± 1.2 (7.5 ± 1.6)	12.6 ± 2.8	
thiourea[c]	1.0	12.3 ± 1.6 (18.7 ± 0.8)	19.4 ± 4.9	6.7 ± 2.6
endogenous NADPH oxidase rate		1.4 ± 0.4 (3.0 ± 1.0)	2.1 ± 0.6	1.2 ± 0.2

[a] Incubation mixtures included 5 mg of antireductase IgG/mg of microsomal protein; kidney microsomes also contained 3.0 mM *n*-octylamine. Velocities represent substrate-stimulated minus endogenous rates. Rates are the mean ± SD. [b] These rates were obtained in the presence of 3.0 mM *n*-octylamine. Values in this column for other substrates were nearly identical with rates obtained in the absence of *n*-octylamine. [c] Thiourea completely saturates the enzyme of a concentration of 1.0 mM; these rates represent approximately the maximal velocities of these microsoaml preparations.

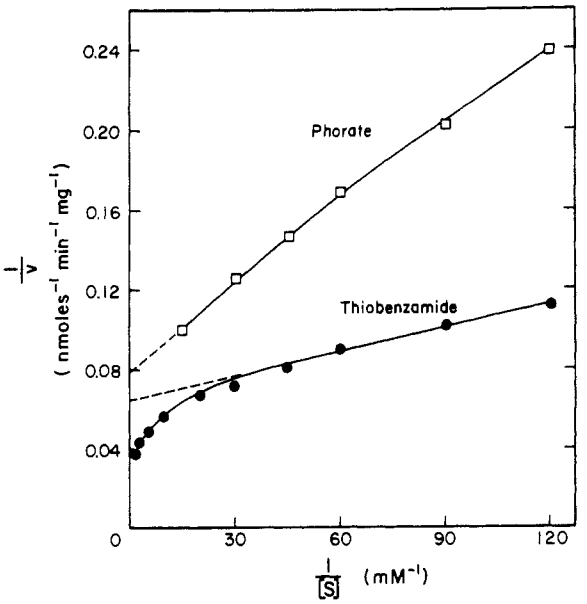


Figure 1. Double-reciprocal plots of thiobenzamide and phorate oxidation by the flavin monooxygenase in mouse liver microsomes. The phorate assays included 5 mg of antireductase IgG/mg of microsomal protein and 3.0 mM *n*-octylamine. The thiobenzamide assays contained 3.0 mM *n*-octylamine.

olized by antireductase inhibited mouse liver microsomes at a rate of about 18 nmol/min/mg of microsomal protein, indicating that this substrate is not quite as good a substrate as its sulfur analogue, thiourea, which is metabolized at about 23 nmol/min/mg of microsomal protein. Selenourea undergoes rapid nonenzymatic oxidation in the presence of molecular oxygen precluding detailed kinetic analyses. The following pesticides were not detectably metabolized at concentrations of 0.1 mM by the flavin monooxygenase in mouse liver microsomes: profenofos, thiophonate methyl, parathion, fenitrothion, monitor, dimethrimol, methomyl, acephate, cycloate, and dimethoate.

Trypsin treatment effectively denudes microsomal vesicles of NADPH-cytochrome P-450 reductase but leaves the flavin monooxygenase functionally intact. Thiourea oxidase rates with trypsin treated microsomes are approximately the same as those rates obtained in antireductase inhibited microsomes. However, trypsin treatment increases K_m values for substrates of the microsomal monooxygenase. For instance, methimazole has a K_m value in untreated microsomes of 17 μM, but in treated microsomes has a K_m value of 125 μM. For this reason, organophosphates such as phorate, fenthion, and methyl carbophenothion are turned over slowly in trypsin treated as compared to antireductase inhibited microsomes.

The pig liver flavin monooxygenase in microsomes appears more readily saturable by substrates than the mouse liver enzyme. In fact, phorate, disulfoton, and the demetons are metabolized at concentrations of 0.1 mM as well as saturating concentrations of thiourea. Characteristic 2.5-fold stimulation by *n*-octylamine is apparent for most of the better substrates. Dithiocarbamates are rather poor substrates for both the pig and mouse liver flavin monooxygenases.

Thiourea oxidase rates in mouse lung microsomes are slightly lower than thiourea oxidase rates in mouse liver microsomes (Table V). The flavin monooxygenase found in mouse lung microsomes has recently been shown to be a unique form of flavin monooxygenase which is not present in mouse liver microsomes (Tynes et al., 1985). The high endogenous rate of NADPH oxidation obtained in the presence of *n*-octylamine reflects the ability of the mouse pulmonary flavin monooxygenase form to apparently utilize *n*-octylamine as a hydroxylatable substrate. Also, *n*-octylamine is a positive effector of the mouse lung enzyme, increasing thiourea oxidase rates about 1.5-fold. Solubilization with 1% Emulgen 911 increases thiourea oxidase rates in mouse lung microsomes to an extent similar to that observed for mouse liver microsomes.

Phorate and disulfoton at concentrations of 0.1 mM almost fully saturate the flavin monooxygenase in mouse lung microsomes, unlike mouse liver microsomes, and in antireductase inhibited lung microsomes are metabolized at rates approaching those of thiourea. Methyl phenyl sulfide is also efficiently metabolized by the enzyme in mouse lung microsomes.

Table VI. Rates of Pesticide Metabolism by the Flavin-Containing Monooxygenase in Rabbit Liver, Lung, and Kidney Microsomes

substrate	concn, mM	activity, nmol of NADPH/min/mg of microsomal protein: liver, anti-reductase[a]	liver, plus 1% Emulgen 911[b]	lung, anti-reductase[a]	lung, plus 1% Emulgen 911	kidney
		Organophosphate				
phorate	0.1	3.6 ± 0.7	0.8 ± 0.5	12.4 ± 3.5	3.0 ± 1.2	0.6 ± 0.2
disulfoton	0.1	5.6 ± 0.5	3.1 ± 0.9	12.8 ± 3.1	3.6 ± 0.3	1.0 ± 0.1
fenthion	0.1	4.0 ± 0.4	<0.4	2.6 ± 0.7	<0.8	
methyl carbophenothion	0.1	4.6 ± 0.3	1.2 ± 0.1	10.9 ± 4.0	6.4 ± 2.6	
		Carbamate				
aldicarb	0.1	1.5 ± 0.2	0.7 ± 0.4	1.2 ± 0.5	<0.8	0.7 ± 0.1
	0.5	3.2 ± 0.2	1.7 ± 0.9	1.6 ± 0.9	<0.8	1.5 ± 0.3
croneton	0.1	2.8 ± 0.6	1.1 ± 0.3	4.1 ± 0.7	<0.8	
	0.5	5.6 ± 0.7	3.2 ± 0.6	6.3 ± 0.9	<0.8	
		Other				
α-naphthylthiourea	0.1	7.0 ± 1.2	13.0 ± 3.9	2.1 ± 1.2	1.1 ± 0.7	
methyl phenyl sulfide	0.1	8.2 ± 2.5	10.8 ± 2.9	10.3 ± 0.9	23.4 ± 5.1	
nicotine	1.0	3.2 ± 0.4	2.5 ± 0.4	1.3 ± 1.1	<0.8	
diethylphenylphosphine	0.1	10.5 ± 2.4	14.4 ± 3.6	10.7 ± 2.1	15.7 ± 1.8	
thiourea[c]	1.0	14.6 ± 3.7	25.8 ± 1.6	11.9 ± 3.5	28.8 ± 2.7	2.9 ± 0.7
endogenous NADPH oxidase rate		1.4 ± 0.2	1.6 ± 0.4	1.5 ± 1.0	3.1 ± 0.4	0.9 ± 0.5

[a] Incubations included 5 mg of antireductase IgG/mg of microsomal protein and 3.0 mM *n*-octylamine, except for the rabbit lung assays which were run in the absence of *n*-octylamine. Velocities represent substrate-stimulated minus endogenous rates. Rates are the mean ± SD. [b] Assays include 1% Emulgen 911 and 3.0 mM *n*-octylamine. [c] Thiourea completely saturates the enzyme at a concentration of 1.0 mM; these rates represent approximately the maximal velocities of these microsomal preparations.

The mouse pulmonary flavin monooxygenase can also oxidize nicotine, presumably forming the *N*-oxide, a metabolite identified as being produced by liver, lung, and kidney microsomes (Hill et al., 1972). Nicotine *N*-oxide formation is probably dominant over cotinine formation as an oxidative route of metabolism in mouse lung microsomes since lung microsomes contain relatively little cytochrome P-450. The oxidation of tertiary amines by the flavin monooxygenase is stereospecific and produces *N*-oxides of distinct chiral character (Ziegler et al., 1973).

Mouse kidney microsomes have about one-third the thiourea oxidase activity of mouse liver microsomes. The effects of *n*-octylamine and solubilization with 1% Emulgen 911 are similar to those observed for mouse liver microsomes. Relative to thiourea, phorate and disulfoton are oxidized efficiently by the flavin monooxygenase in mouse kidney microsomes.

Although rabbit liver microsomes have considerable flavin monooxygenase activity toward thiourea, they slowly catalyze the oxidation of phorate and disulfoton (Table VI). In contrast, the flavin monooxygenase in unsolublized rabbit lung microsomes appears to be fully saturated by 0.1 mM concentrations of phorate or disulfoton. The rabbit pulmonary flavin monooxygenase is also well saturated by methyl phenyl sulfide, especially in the presence of 1% Emulgen 911. However, for reasons not readily apparent, the rabbit lung enzyme has a marked inability to oxidize α-naphthylthiourea efficiently.

Rat liver and lung microsomes have lower flavin monooxygenase activity than liver or lung microsomes from the mouse or rabbit (Table VII). In contrast to the mouse and rabbit, the activity of the rat lung flavin monooxygenase is qualitatively similar to the activity of the rat liver enzyme. Rat kidney microsomes have high thiourea oxidase activity although they do not efficiently oxidize phorate or disulfoton. Solubilization with 1% Emulgen 911 or the addition of *n*-octylamine to rat microsomes does not significantly increase activity.

Pesticide inhibition of cytochrome P-450-dependent *p*-nitroanisole O- demethylation implies interaction of the pesticide with cytochrome P-450 (Table VIII). In the case

Table VII. Rates of Pesticide Metabolism by the Flavin-Containing Monooxygenase in Rat Liver, Lung, and Kidney Microsomes

substrate	concn, mM	activity, nmol/min/mg of microsomal protein[a]: liver	lung	kidney
	Organophosphate			
phorate	0.1	3.4 ± 0.6	1.4 ± 0.5	1.6 ± 0.7
disulfoton	0.1	4.3 ± 0.6	1.8 ± 0.5	2.4 ± 0.2
fenthion	0.1	2.4 ± 1.2	0.8 ± 0.3	
methyl carbophenothion	0.1	2.5 ± 1.2	1.0 ± 0.4	
	Carbamate			
aldicarb	0.1	1.9 ± 0.4	0.3 ± 0.1	0.7 ± 0.1
	0.5	2.5 ± 1.4	0.8 ± 0.4	1.5 ± 0.3
croneton	0.1	3.2 ± 1.5	1.2 ± 0.2	
	0.5	3.6 ± 1.7	1.3 ± 0.2	
	Other			
α-naphthylthiourea	0.1	4.6 ± 0.7	1.4 ± 0.4	
methyl phenyl sulfide	0.1	5.4 ± 0.3	1.9 ± 0.2	
nicotine	1.0	2.7 ± 1.1	0.8 ± 0.3	
diethylphenylphosphine	0.1	4.0 ± 1.4	0.8 ± 0.2	
thiourea[b]	1.0	8.3 ± 1.3	2.8 ± 0.6	9.1 ± 1.7
endogenous NADPH oxidase rate		2.9 ± 0.4	0.6 ± 0.3	1.5 ± 0.6

[a] All incubations included 5 mg of antireductase IgG/mg of microsomal protein and 3.0 mM *n*-octylamine. Velocities represent substrate-stimulated minus endogenous rates. Rates are the mean ± SD. [b] Thiourea completely saturates the enzyme at a concentration of 1.0 mM; these rates represent approximately the maximal velocities of these microsomal preparations.

of phorate, initial velocity measurements analyzed on double-reciprocal plots show that this interaction is of a competitive type, indicating that phorate competes for the catalytic site of cytochrome P-450. Dixon plots give a K_i value of about 12 μM for phorate. *p*-Nitroanisole has a K_m value of 33 μM in the linear concentration range at 33.3–200 μM. Presumably, the interactions of the other pesticides with cytochrome P-450 are also of a competitive nature with their magnitude of inhibition a direct reflection

Table VIII. Pesticide Inhibition of Cytochrome P-450-Dependent *p*-Nitrophenol Formation from *p*-Nitroanisole and Parathion

	concn, mM	*p*-nitroanisole[a] O-demethylation, % control (range)	parathion[b] O-dearylation, % control (range)
phorate	0.1	35 (31–49)	64 (63–66)
disulfoton	0.1	38 (31–52)	59 (54–64)
sulprofos	0.1	34 (32–37)	68 (58–77)
fonofos	0.1	40 (32–45)	44 (40–47)
aldicarb	0.1	95 (90–101)	83 (78–87)
	1.0	58 (51–64)	67 (64–69)
diethylphenyl-phosphine	0.1	<10	35 (32–38)

[a] Assays contained 100 μM *p*-nitroanisole, pH 7.6. Control rates averaged 1.63 nmol of *p*-nitrophenol/min/mg of microsomal protein. [b] Assays contained 100 μM parathion, pH 7.6. Control rates averaged 2.30 nmol of *p*-nitrophenol/min/mg of microsomal protein.

Table IX. Type I Spectral Binding of Pesticides to Oxidized Cytochrome P-450[a]

substrate	concn, μM	10^2A (peak to trough)[b]
phorate	5	1.3
	100	3.4
disulfoton	5	1.3
	100	2.7
sulprofos	5	1.9
	100	3.3
fonofos	5	1.0
	100	2.9
aldicarb	1000	<0.25
diethylphenylphosphine[c]	5	4.8
	100	10.8
parathion	5	1.4
	100	3.4

[a] Assays contained 1.75 mg of mouse liver microsomal protein/mL which contained 1.66 nmol of cytochrome P-450/mL. [b] Type I spectra obtained had peaks at 375–388 nm and troughs at 416–421 nm. [c] Diethylphenylphosphine also bound to reduced cytochrome P-450 to form a type III spectra, K_s = 3.6 μM for 460-nm peak.

of their binding affinities for the cytochrome.

These pesticides also inhibit the cytochrome(s) P-450 responsible for parathion metabolism, although they inhibit parathion metabolism less than *p*-nitroanisole metabolism. This is consistent with parathion having a lower K_m value of about 19 μM in mouse liver microsomes.

Pesticide-induced type I spectral changes with cytochrome P-450 are believed to represent the formation of enzyme–substrate complexes at the substrate binding site of cytochrome P-450 (Table IX). Aldrin, malathion, and parathion are metabolized by cytochrome P-450 and also produce type I spectra (Hodgson and Kulkarni, 1974).

The organophosphates phorate, disulfoton, sulprofos, and fonofos all interact with cytochrome P-450 in such a way as to markedly inhibit the metabolism of *p*-nitroanisole and parathion, both of which have rather high affinities for cytochrome P-450. The organophosphates also form large type I spectra with cytochrome P-450. These observations suggest that cytochrome P-450 and the flavin monooxygenase both have the potential to participate in the oxidation of the same pesticide.

The carbamate aldicarb, which is not a particularly good substrate for the flavin monooxygenase, also appears to be a poor substrate for cytochrome P-450. This could be a reflection of the hydrophilic character of this compound. Diethylphenylphosphine, an excellent substrate for the flavin monooxygenase, is a potent inhibitor of *p*-nitroanisole metabolism and forms a large type I spectra. Wiley et al. (1972) have provided evidence for cytochrome P-450 participation in the oxidation of trivalent phosphines by rat liver microsomes.

In conclusion, based on flavin monooxygenase kinetic constants and microsomal pesticide oxidation rates, the flavin monooxygenase undoubtedly can provide a major oxidative route of pesticide biotransformation in mammals.

If the typical mouse liver contains about 40 mg of microsomal protein/g of wet liver weight (Estabrook et al., 1971), it can be calculated from a turnover rate for phorate of only 2 nmol/min/mg of microsomal protein that a mouse liver weighing 2 g could potentially catalyze the oxidation of about 9.6 μmol of phorate/h (equal to 2.4 mg of phorate/h). Since a 0.12-mg phorate dose to a 30 g mouse would be a 50% lethal dose (LD_{50} = 4 mg/kg), the liver microsomal flavin monooxygenase could oxidize a 50% lethal dose of phorate in just 3 min. Although this does not consider potential pharmacokinetic limitations, it does illustrate the potential significance of the flavin monooxygenase as a metabolic route of pesticide oxidation in vivo.

Registry No. Ethylenethiourea, 96-45-7; nicotine, 54-11-5; selenourea, 630-10-4; diethylphenylphosphine, 1605-53-4; sodium metham, 137-42-8; methiocarb, 2032-65-7; aldicarb, 116-06-3; croneton, 29973-13-5; fonofos, 944-22-9; fosthietan, 21548-32-3; counter, 13071-79-9; methyl carbophenothion, 953-17-3; fenthion, 55-38-9; sulprofos, 35400-43-2; thiobenzamide, 2227-79-4; dimethylaniline, 121-69-7; phorate, 298-02-2; disulfoton, 298-04-4; demetonthione, 298-03-3; demetonthiol, 126-75-0; demeton-*S*-methyl, 919-86-8; methyl phenyl sulfide, 100-68-5; thiourea, 62-56-6; α-naphthylthiourea, 86-88-4; dimethyldithiocarbamate, 79-45-8; parathion, 56-38-2; thioanisole, 100-68-5; NADH-cytochrome P 450 reductase, 9039-06-9; monooxygenase, 9038-14-6.

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Analysis of the Herbicide Sulfometuron Methyl in Soil and Water by Liquid Chromatography

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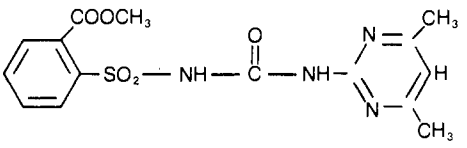
An analytical method based on the use of a liquid chromatograph and a photoconductivity detector is described for sulfometuron methyl, methyl 2-[[[[(4,6-dimethyl-2-pyrimidinyl)amino]carbonyl]-amino]sulfonyl]benzoate, previously known as DPX-T5648, which is the active ingredient in Du Pont "Oust" herbicide. As little as 100 pg can be detected and measured after passage through the chromatographic column. Coupled with suitable extraction, cleanup, and isolation procedures, the method provides a means of determining sulfometuron methyl in soil and water at levels as low as 200 pg/g (0.2 ppb).

Du Pont "Oust" herbicide is effective in controlling many annual and perennial grasses and broad-leafed weeds on noncropland areas such as airports, fence rows, highways, lumber yards, petroleum tank farms, pipeline and utility rights-of-way, pumping installations, railroads, storage areas, and plant sites.

The active ingredient, sulfometuron methyl, methyl 2-[[[[(4,6-dimethyl-2-pyrimidinyl)amino]carbonyl]-amino]sulfonyl]benzoate is a sulfonylurea.



Sulfometuron Methyl

"Oust" may be applied under either pre- or postemergence conditions by using a wide variety of application rates (70–840 g/ha). The decomposition rate of the active ingredient, sulfometuron methyl, is very rapid under field conditions. Consequently, the methods developed to measure sulfometuron methyl in soil and water have a very low detection limit (0.2 ppb) to insure that the quantities of sulfometuron methyl which might be present are not sufficiently large to be injurious to agricultural crops. Derivatization of sulfometuron methyl is not required, and the operating conditions are sufficiently mild that decomposition is avoided.

A literature search revealed a number of methods that can be used for the analysis of sulfonylureas. If gas chromatography is to be used for the analysis, the sulfonylureas must be derivatized to more volatile and stable compounds by reacting the polar NH groups with dimethyl sulfate, methyl iodide, or diazomethane. Derivatization with diazomethane has been reported by Braselton et al. (1975, 1976, 1977), Midha et al. (1976), Taylor (1972), and Taylor et al. (1977). Maeda et al. (1981) have demonstrated that sulfonylureas can be determined by methylation with diazomethane followed by acylation with heptafluorobutyric anhydride. The use of dimethyl sulfate is described by Kleber et al. (1977), Prescott and Redman (1972), Sabih and Sabih (1970), and Simmons et al. (1972). An extractive methylation involving methyl iodide in methylene chloride is given in the paper by Hartvig et al. (1980).

A radioimmunoassay technique has been reported by Kajinuma et al. (1982) for the analysis of a sulfonylurea in serum.

Huck (1978) has developed a method in which sulfonylureas are hydrolyzed, converted to the dansyl derivative, separated by thin-layer chromatography, and detected by fluorescence.

A comparative study of gas chromatography and liquid chromatography has been made by Kimura et al. (1980) who found comparable sensitivity and reproducibility.

Methods for sulfonylureas based on liquid chromatography have been reported by Beyer (1972), Harzer (1980), Molins et al. (1975), Raghow and Meyer (1981), Reinauer et al. (1980), Robertson et al. (1979), Sved et al. (1976), Tsugi and Binns (1982), Uihlein and Sistovaris (1982), Waahlin-Boll and Melander (1979), and Weber (1976). Both normal and reverse-phase systems have been used, and it is not necessary to form derivatives since sulfonylureas generally give adequate response with ultraviolet absorbance detectors. Besenfelder (1981) has reported an improvement in sensitivity based on precolumn derivati-

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