Flavin Adenine Dinucleotide–Dependent Monooxygenase: Its Role in the Sulfoxidation of Pesticides in Mammals

Abstract. *The flavin adenine dinucleotide–dependent monooxygenase in mammalian hepatic microsomes plays a major role in the oxidative metabolism of thioether-containing pesticides. Thirty-four compounds were tested, and it was determined that organophosphorus insecticides such as disulfoton and phorate are rapidly oxidized by the purified enzyme to their corresponding sulfoxides. The enzyme does not catalyze the oxidation of the thiophosphoryl and thiol sulfur atoms of these or other phosphorothioates and phosphorodithioates, or the oxidation of the sulfoxide to the sulfone. Carbamates aldicarb and Croneton are also oxidized, but at a lower rate.*

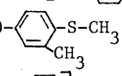
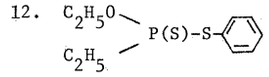
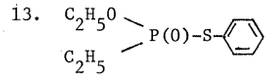
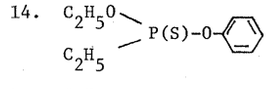
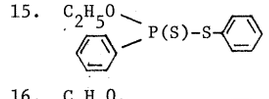
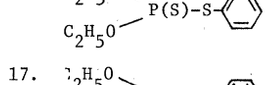
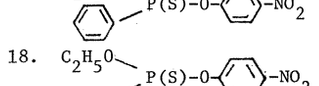
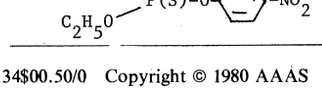
Mammals, insects, and plants are able to oxidize the thioether moiety of certain organophosphorus and carbamate pesticides in vivo. The sulfide is converted to the sulfoxide and subsequently to the sulfone (*1*, *2*). Oxidation of the sulfide increases the water solubility and the anticholinesterase activity of these compounds, and this accounts for their good systemic properties. Increased polarity allows the compounds to be translocated, but their net activity as anticholinesterase inhibitors in mammals and insects depends on the balance between competing activation and detoxification reactions (*1*, *2*).

Incubation of these pesticides with microsomal enzyme preparations and reduced nicotinamide adenine dinucleotide phosphate (NADPH) under aerobic conditions results in the formation of the same metabolites, the sulfoxide being the principal product (*3*). The nature and substrate specificity of the enzymes involved in sulfur oxidation have generally been considered to be the cytochrome P-450–dependent monooxygenase system (*3*, *4*). However, other distinctly different enzymes are present in hepatic microsomes (*4*, *5*). For example, the flavin adenine dinucleotide (FAD)–dependent monooxygenase (E.C. 1.14.13.8) (*5*, *6*) is present in substantial amounts in hepatic microsomes and is a different entity from either of the other two microsomal flavoproteins, NADPH–cytochrome P-450 reductase and NADH–cytochrome b_5 reductase (*4*, *5*). It is, therefore, important to elucidate the function of the different monooxygenases present in order to better understand their individual roles in the overall metabolism of pesticides and other xenobiotics. We find that purified preparations of this FAD-dependent monooxygenase rapidly oxidize the thioether sulfur atom of many organophosphorus and carbamate pesticides to the corresponding sulfoxide.

The oxidation of sulfur-containing pesticides is conveniently measured with the purified enzyme (*7*) by following the substrate-dependent oxidation of NADPH spectrophotometrically at 340 nm under optimum conditions (*8*). Disulfoton and phorate are metabolized rapidly by the monooxygenase (Table 1). From double-reciprocal plots of substrate velocity (V) data, the apparent Michaelis constant (K_m) and V_{max} values were 21.3 and 28.0 μM and 394 and 334 nmole of NADPH per milligram of protein per minute, respectively (*9*), indicating that the compounds are excellent substrates for the enzyme. The addition of *n*-octylamine (3 mM) increases the rate of oxidation two- to threefold for oxidizable substrates, but does not initiate the metabolism of nonsubstrates.

Disulfoton and phorate have three sulfur atoms each: the thiophosphoryl or thiono, the thiol, and the thioether—each of which could, potentially, be oxidized. We find, however, that on incubation of either of these substrates with the enzyme, the thioether sulfur is the only sulfur atom oxidized and the corresponding sulfoxides are the only products formed (*10*). The reaction stoi-

Table 1. Oxidation of sulfur-containing pesticides and related compounds (*11*) by the FAD-dependent monooxygenase from pig liver microsomes. Activity is measured as nanomoles of NADPH per nanomole of enzyme per minute (± standard error).

	Substrate	Common name	Activity
1.	$(C_2H_5O)_2$ P(S)-S-CH_2-CH_2-S-C_2H_5	Disulfoton	11.51 ± 0.42
2.	$(C_2H_5O)_2$ P(S)-O-CH_2-CH_2-S-C_2H_5	Demeton O (Thiono)	6.98 ± 0.59
3.	$(C_2H_5O)_2$ P(O)-S-CH_2-CH_2-S-C_2H_5	Demeton S (Thiol)	4.24 ± 0.84
4.	$(CH_3O)_2$ P(O)-S-CH_2-CH_2-S-C_2H_5	Demeton S methyl	2.61 ± 0.40
5.	$(C_2H_5O)_2$ P(S)-S-CH_2-S-C_2H_5	Phorate	9.58 ± 0.90
6.	$(C_2H_5O)_2$ P(S)-S-CH_2-S-C $(CH_3)_3$	Counter	3.77 ± 0.41
7.	$(C_2H_5O)_2$ P(S)-S-CH_2-S-  -Cl	Carbophenothion	1.45
8.	$(CH_3O)_2$ P(S)-O-  -S-CH_3	Fenthion	7.54 ± 0.32
9.	$\left[(CH_3O)_2 \text{ P(S)-O-}\right]_2$S	Abate	0.00
10.	$(C_2H_5O)_2$ P(S)-S-CH_2-CH_2-SO-C_2H_5	Disulfoton sulfoxide	0.00
11.	$(C_2H_5O)_2$ P(S)-S-CH_2-SO-C_2H_5	Phorate sulfoxide	0.00
12.		Fonofos	6.45 ± 0.30
13.		Fonofos oxon	0.00
14.			0.00
15.			10.36
16.			0.00
17.		EPN	0.00
18.		Parathion	0.00

0036-8075/80/0905-1134$00.50/0

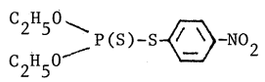
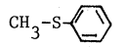
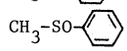
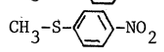
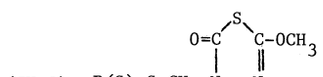
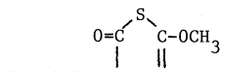
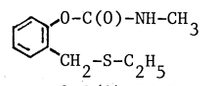
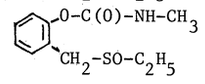
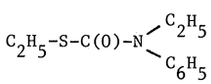
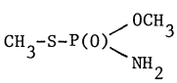
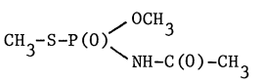
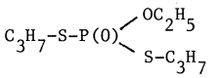
chiometries indicate that, throughout the reactions, 1 mole of NADPH is oxidized per mole of sulfoxide formed. [This ratio remained constant over a tenfold (5 to 50 μM) range of substrate concentration.] Although the thiono and thiol sulfur atoms are not oxidized, they affect the rate at which the thioether is attacked. The structure-activity relations indicate that substitution of either or both of these with oxygen reduces the activity, possibly because of a difference in electronegativity between the sulfur and oxygen atoms (Table 1) (*11*). This effect is further enhanced by substituting the *O,O*-diethyl groups with *O,O*-dimethyl. Structural changes in the thioether moiety that affect the nucleophilicity or increase the steric hindrance of the sulfur atom apparently decrease enzyme-substrate binding and thus affect the rate of oxidation. This is best illustrated by the sulfoxides of disulfoton and phorate and by abate, which are not substrates.

The sulfoxides of disulfoton, phorate, the carbamate Croneton, and methyl-phenyl sulfoxide (*12*) are not substrates, indicating that the FAD-dependent monooxygenase does not catalyze the further oxidation of sulfoxides to sulfone. This further oxidation may be due to another oxidase or, in some cases, may be nonenzymatic.

Sulfide-containing methyl carbamates such as aldicarb and Croneton are not oxidized as readily as the organophosphorus pesticides, while the herbicides Eptam and cycloate are not oxidized to any measurable extent (Table 1). It is known, however, that their sulfoxides are formed in appreciable amounts in vivo and in vitro (*13*).

Our findings indicate that, for phosphorodithioate pesticides, the FAD-dependent monooxygenase is specific for the thioether sulfur atom. In agreement with this, we find that parathion, its dithio analog, and *O,O*-diethyl, *S*-phenyl phosphorodithioate are not substrates for this enzyme. Parathion and other phosphorothioates are known to be metabolized to their oxygen analogs by the cytochrome P-450–dependent monooxygenase system (*14*). The phosphonodithioate pesticide fonofos and its phenyl analog (compound 15) may represent yet another group of pesticides that are also metabolized by the FAD-dependent monooxygenase (Table 1).

The role of the FAD-dependent monooxygenase from mammalian hepatic microsomes in the oxidative metabolism of pesticides has thus been demonstrated, and it is apparent that it may provide a mechanism of considerable importance. Methods currently available for the purification of a number of microsomal enzymes should be useful in further defining their function and relative importance in vivo.

	Substrate	Common name	Activity
19.			0.00
20.		Methyl phenyl sulfide	18.26
21.		Methyl phenyl sulfoxide	0.00
22.			18.45
23.		Methidathion	0.00
24.			1.37 ± 0.14
25.	$CH_3-S-C(CH_3)_2-CH=N-O-C(O)-NH-CH_3$	Aldicarb	1.15 ± 0.12
26.	$CH_3-S-C(CH_3)=N-O-C(O)-NH-CH_3$	Methomyl	0.00
27.		Croneton	3.16 ± 0.20
28.		Croneton sulfoxide	0.00
29.	$C_2H_5-S-C(O)-N\ (C_3H_7)_2$	Eptam	0.00
30.		Cycloate	0.00
31.	$3(H_2O)\cdot Na-S-C(S)-N-(C_2H_5)_2$	Diethyl dithiocarbamate	0.28
32.		Methamidophos	0.00
33.		Acephate	0.00
34.		MOCAP	0.00

NICOLAS P. HAJJAR
ERNEST HODGSON
Toxicology Program, Department of Entomology, North Carolina State University, Raleigh 27650

References and Notes

1. M. Eto, *Organophosphorus Pesticides: Organic and Biological Chemistry* (CRC Press, Cleveland, 1974); R. J. Kuhr and H. W. Dorough, *Carbamate Insecticides: Chemistry, Biochemistry and Toxicology* (CRC Press, Cleveland, 1976).
2. J. J. Menn and G. G. Still, *Crit. Rev. Toxicol.* **5**, 1 (1977); T. R. Fukuto and R. L. Metcalf, *Ann. N.Y. Acad. Sci.* **160**, 97 (1969); J. E. Casida and L. Lykken, *Annu. Rev. Plant Physiol.* **20**, 607 (1969).
3. T. Nakatsugawa and M. A. Morelli, in *Insecticide Biochemistry and Physiology*, C. F. Wilkinson, Ed. (Plenum, New York, 1976), p. 61; A. P. Kulkarni and E. Hodgson, *Pharmacol. Ther.* **8**, 379 (1980).
4. R. W. Estabrook and J. Werringloer, in *The Induction of Drug Metabolism*, R. W. Estabrook and E. Lindenlaub, Eds. (Schattauer, Stuttgart, 1979), p. 187; V. Ullrich, Ed., *Microsomes and Drug Oxidation* (Pergamon, Elmsford, N.Y., 1977).
5. D. M. Ziegler, in *The Molecular Biology of Membranes*, S. Fleisher *et al.*, Eds. (Plenum, New York, 1978), p. 193.
6. This flavoprotein was originally characterized as a relatively nonspecific amine oxidase. Subsequent studies demonstrated that it also catalyzes the oxidation of sulfur-containing compounds (such as thiourea, cysteamine, and methimazole) and thus might be more accurately described as a sulfur oxidase [L. L. Poulsen and D. M. Ziegler, *J. Biol. Chem.* **254**, 6449 (1979); L. L. Poulson, R. M. Hyslop, D. M. Ziegler, *Arch. Biochem. Biophys.* **198**, 78 (1979)].
7. D. M. Ziegler and L. L. Poulsen, *Methods Enzymol.* **52**, 142 (1978).
8. The reaction medium contained 0.05*M* potassium phosphate buffer (*p*H 7.4) and 0.1 m*M* NADPH in a total volume of 3.0 ml. Absorbance was measured by a spectrophotometer (Aminco DW-2 UV-Vis). After 2 to 3 minutes of in-

cubation at 37°C, with continuous stirring, the reaction was started by adding enzyme solution to a final concentration of approximately 1 nmole/ml; the oxidation rate in the absence of the substrate was recorded for 30 to 40 seconds. The substrate (25 or 50 μM) was then added in 5 μl of acetone, and the oxidation of NADPH was recorded for 30 to 60 seconds.

9. These values are comparable to those determined for dimethylaniline (15 μM), the substrate commonly used for determining the amine oxidase activity of this enzyme and methimazole (6.7 μM) (*6, 7*), the substrate used for assaying its sulfur oxidase activity. Since, at saturation, all substrates for this monooxygenase appear to be oxygenated at the same velocity (*6*), the rates listed in Table 1 presumably reflect differences in K_m rather than V_{max}. In some cases, velocity is probably limited by solubility.
10. Product identification was carried out by first oxidizing 50 μM disulfoton, phorate, or phorate fortified with 1.7×10^5 dis/min of methylene-[^{14}C]phorate (specific activity, 9.7 mCi/mmole) under optimum conditions. In the presence of NADPH, the reaction was essentially complete in 2 to 3 minutes, and the reaction mixture was immediately extracted twice with chloroform. The product was identified by one- and two-dimensional thin-layer chromatography on silica gel, with development in five different solvent systems.
11. Compounds 1, 5 through 9, 23, 25, 26, 29, 30, and 32 through 34 were purchased from Chem Service; compounds 1, 5, and 7, from City Chemical Co.; compounds 20 and 21, from Fairfield Chemical Co.; and compound 31, from Matheson, Coleman and Bell. Compounds 2 through 4, 27, and 28 were donated by Mobay Chemical Corp.; compounds 12 and 13, by the Environmental Protection Agency. Compounds 10, 11, 14 through 19, 22, and 24 were synthesized according to established procedures.
12. This is a metabolite of fonofos in vivo [P. W. Lee, R. Allahyari, T. R. Fukuto, *Pestic. Biochem. Physiol.* **9**, 23 (1978)].
13. J. E. Casida, R. A. Gray, H. Tilles, *Science* **184**, 573 (1974); J. P. Hubbell and J. E. Casida, *J. Agric. Food Chem.* **25**, 404 (1977); J. E. Casida, E. C. Kimmel, H. Ohkawa, R. Ohkawa, *Pestic. Biochem. Physiol.* **5**, 1 (1975); Y. S. Chen and J. E. Casida, *J. Agric. Food Chem.* **26**, 263 (1978).
14. R. A. Neal, *Rev. Biochem. Toxicol.* **2**, 131 (1980).
15. Supported by grants ES-07046 and ES-00044 from the National Institute of Environmental Health Sciences. We thank W. C. Dauterman and A. A. Nomeir for their advice and for providing compounds 17 through 19, 22, and 24. We also thank D. M. Ziegler, who provided all the purified enzyme used in these studies. This is paper No. 6341 of the Journal Series of the North Carolina Agricultural Research Service.

12 March 1980; revised 21 April 1980

Serial Female Sex Changes After Simultaneous Removal of Males from Social Groups of a Coral Reef Fish

Abstract. *The simultaneous removal of three to nine males from large social groups of* Anthias squamipinnis *led to close to a one-to-one replacement of the removed males by sex-reversing females. The females changed sex serially within each group with a mean interval between successive onset times of 1.9 days. The timing of sex change is thus not independent for each fish but is influenced by the events surrounding other sex reversals within the group.*

Female-to-male sex reversal can be initiated in several species of marine fish by the removal of a male either from spatially well-defined, bisexual social groups (*1–3*) or from less tightly structured aggregations (*4*), depending on the species. This phenomenon has been examined carefully in small, single-male groups where the removal of one male has led to the sex reversal of one female (*5, 6*). The sexually dichromatic, serranid fish *Anthias squamipinnis* (Peters) is a protogynous hermaphrodite that lives in sedentary, bisexual social groups (*2, 7, 8*). When females of this species reverse sex, their coloration, behavior, and gonadal histology change in well-defined sequences (*5, 9*). This report shows (i) that the simultaneous removal of N males from large *A. squamipinnis* social groups leads to the sex reversal of N females, that is, there is close to a one-to-one replacement of sex-reversing females for removed males and (ii) that the multiple sex reversals initiated within a social group by the simultaneous removal of multiple males do not begin at the same time but are evenly spaced in onset, approximately 2 days apart, that is, the timing of sex change is not independent for each fish but is influenced by other sex reversals or by the events initiating other sex reversals within the group.

Forty-eight social groups (*7, 10*) of *A. squamipinnis* were identified with numbered floats and visually censused for the number of adult males and females, at a water depth of less than 15 m on the northeast reef of Apo Island, near Dumaguete City, Negros Oriental, Philippines, in July and August 1978. From this sample, 15 control groups and 11 experimental groups were selected and matched for approximate similarity in size and adult composition. Groups ranged from 2 adult males and 13 adult females to 50 males and 294 females, with a median group containing 10 males and 61 females. In general, from each control group one male was removed. From each experimental group three to nine males were removed (*11*) within an 8-hour period. After the males were removed, the females of each group were observed daily to within 2 m and scrutinized for the earliest changes in coloration indicative of sex reversal. Previous laboratory and fieldwork on 44 socially initiated sex reversals had specified the precise sequence of color changes to be expected in five body regions (*2, 9*). The first day of recognizable color change was recorded as the day of onset of sex change. The sex-reversing individuals were distinguished within each group primarily by the progress and degree of advancement of their respective coloration changes. Experimental groups were observed daily for 15 to 25 days and control groups for 12 to 24 days after male removals. A female was said to have changed sex if her color changed from characteristically female to typically male. This criterion was thought sufficient because 96.9 percent of individuals ($N = 130$) in an earlier study showed full correspondence between the gender of external color pattern and the histologically determined gonadal gender and because 100 percent of individuals ($N = 45$) that were observed to change from female to male coloration contained testes with histological evidence of a prior ovarian state (*3*).

After 58 males were removed from the experimental groups, 57 females changed sex. In each group, very close to the same number of females changed sex as the number of males removed (Fig. 1). Similarly, in most of the control groups a single female changed sex after the removal of one male, resulting in a mean of 1.17 sex reversals per male removed. The median onset day for sex reversals was day 3 after male removal, with a range of 1 to 7, in the control groups, and day 5, with a range of 1 to 16, in the experimental groups. The variance in day of onset for the 57 sex reversals in the experimental groups was 6.7 times as great as the variance in the control groups (variance ratio test, $P < .001$, two-tailed) and the onset day for experimental groups was significantly higher than the onset day for control groups (Mann-Whitney U test, $P < .001$, two-tailed). In the experimental groups, when the first, second, third, fourth, and so on successive sex reversals within each group were examined separately, the onset day varied directly with the order of sex reversal within the group (Fig. 2). The sex reversals from the control and experimental groups were unlikely to have come from a single, homogeneous population (Fig. 2) (Kruskal-Wallis one-way analysis of variance, $P < .001$; single factor analysis of variance, $P < .001$, two-tailed). The mean onset day for the one sex reversal in the control groups was significantly lower than the mean onset day for the third, fourth, fifth, sixth, and seventh plus sex reversals in the experimental groups

0036-8075/80/0905-1136$00.50/0