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METABOLISM OF PHOSPHORUS-CONTAINING COMPOUNDS BY PIG LIVER MICROSOMAL FAD-CONTAINING MONOOXYGENASE

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Abstract—Oxidative desulfuration of phosphonate insecticides such as fonofos (*S*-phenyl ethyl ethylphosphonodithioate) and its analogs is catalyzed by pig liver microsomal FAD-containing monooxygenase, although desulfuration of phosphorodithioates, such as parathion, is not. Substitution of an alkyl group for the remaining alkoxy group, as in *S*-phenyl diethylphosphinodithioate, did not increase its oxidation rate. Diethylphenylphosphine sulfide, containing 3 phosphorus–carbon bonds, was actually a poorer substrate than fonofos. Replacement of the *S*-phenyl group of fonofos with an *O*-phenyl group increased the K_m value above the solubility limit. Trivalent phosphorus-containing compounds were also excellent substrates for this enzyme. Diethylphenylphosphine had a K_m value lower than 2.5 μM. Diethyl phenylphosphonite also appeared to be an excellent substrate but its rapid nonenzymatic hydrolysis and/or oxidation precluded accurate K_m determinations. Stoichiometry studies with diethylphenylphosphine and its sulfide showed that O_2 and NADPH consumption were approximately equal to the substrate consumed. The major metabolite of both diethylphenylphosphine and its sulfide was the phosphine oxide. These results show that microsomal FAD-containing monooxygenase of pig liver has activity as a phosphorus-oxidase, in addition to its well characterized nitrogen- and sulfur-oxidase roles.

Pig liver microsomal FAD-containing monooxygenase [EC 1.14.13.8, dimethylaniline monooxygenase (*N*-oxide forming)] has been purified and characterized extensively by Ziegler and coworkers [1, 2]. It catalyzes the oxidation of nitrogen and sulfur atoms in a wide variety of organic compounds [3].

Sulfur-containing organophosphorous insecticides are substrates of the microsomal FAD-containing monooxygenase [4]. Analysis of the oxidative products of phosphorothioate insecticides show that only thioether sulfur atoms are attacked and the only products are the corresponding sulfoxides. Oxidative desulfuration of phosphorothionate sulfur in these compounds is not catalyzed by the microsomal FAD-containing monooxygenase. However, the thiono group of fonofos (*S*-phenyl ethyl ethylphosphonodithioate) and other organophosphorous compounds containing a phosphorous–carbon bond is metabolized, the major product being the oxon [5].

Rat liver microsomal preparations have been shown to convert diphenylmethylphosphine to diphenylmethylphosphine oxide [6]. Although the activity was attributed to the cytochrome P-450-dependent monooxygenase system, a role for the FAD-containing monooxygenase was not specifically discounted.

Metabolism of fonofos and other phosphonothionates by the microsomal FAD-containing monooxygenase suggests that the enzyme may catalyze oxidation of trialkylphosphines and other phosphorus-containing compounds and that this may be an oxidative attack on the phosphorus atom. In the investigation reported herein, a variety of phosphorus-containing analogs of fonofos as well as a number of trialkyl phosphines have been examined as possible substrates for purified pig liver microsomal FAD-containing monooxygenase.

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MATERIALS AND METHODS

Materials. Pig liver microsomal FAD-containing monooxygenase was purified to homogeneity by the method of Sabourin *et al.* [7]. The specific activity was 950 nmoles·min^{-1}·(mg protein)$^{-1}$. The following chemicals were purchased as indicated: fonofos from Chem Services, Inc. (West Chester, PA), methylphenyl sulfide from the Fairfield Chemical Co. (Blythewood, SC), diethyl phenylphosphonite from the Aldrich Chemical Co. (Milwaukee, WI), and diethylphenylphosphine from the Pressure Chemical Co. (Pittsburgh, PA). N. P. Hajjar provided *S*-phenyl diethylphosphorodithioate and P. Bhatia provided diethylphenylphosphine sulfide and *S*-phenyl diethylphosphinodithioate. Diethylphenylphosphine sulfide was synthesized from the corresponding phosphine [8]. *S*-Phenyl diethylphosphinodithioate and *S*-phenyl diethyl phosphorodithioate were synthesized by reacting thiophenol with the corresponding phosphinothioic or phosphorothioic chlorides by a modification of the method for fonofos synthesis [9]. Synthesized compounds were further purified by preparative TLC, and the structures were confirmed by NMR spectroscopy.

Diethylphenylphosphine oxide was prepared by reacting the phosphine (1.0 g in 50 ml $CHCl_3$) with 3-chloroperbenzoic acid (1.22 g/40 ml $CHCl_3$) at 0°. After stirring for 5 hr at room temperature, the solution was extracted with equal volumes of 5% $NaHCO_3$ solution and then with equal volumes of saturated NaCl solution. The $CHCl_3$ phase was dried with anhydrous sodium sulfate and evaporated, and the oxide was crystallized from ethyl ether–petroleum ether. Identity was confirmed by NMR spectroscopy and elemental analysis.

Enzyme assays. FAD-containing monooxygenase activity was measured by following substrate-dependent O_2 uptake or NADPH oxidation at 37° according to the procedure of Poulsen and Ziegler [1] with the following modification. The reaction media contained 0.1 M potassium phosphate, pH 7.6, 3 mM *n*-octylamine, and either 0.1 mM NADPH or an NADPH-generating system [7]. Reactions were started by adding substrate. Kinetic constants were obtained using the KINFIT program of Knack and Rohm [10] which uses curve-fitting techniques to determine the kinetic constants which best fit the data to the Michaelis–Menten equation. In stoichiometry studies NADPH or O_2 consumption rates prior to addition of substrate were extrapolated to the time of reaction completion. These values were then subtracted from total NADPH or O_2 consumed [11].

Metabolite production. Incubation mixtures contained 100 μM substrate in 5 ml reaction medium. Reactions were carried out in open 25-ml Erlenmeyer flasks shaken in a 37° water bath. Control incubations contained enzyme previously inactivated by heat treatment at 50° for 2.5 min. To determine reaction completion times, a parallel incubation was carried out in which NADPH oxidation was monitored. Reactions were complete within 15 min for diethylphenylphosphine and within 30 min for diethylphenylphosphine sulfide.

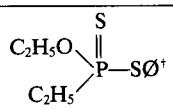
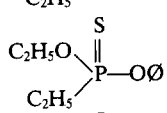
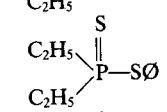
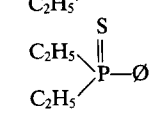
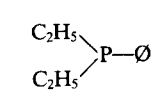
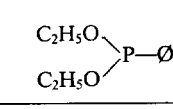
Isolation of diethylphenylphosphine oxide. After completion of the reaction, incubation mixtures were applied to C_{18}-Sep-pak cartridges. The Sep-pak was washed with 5 ml water previously passed through a 0.45 μm filter and then eluted with 5 ml of a high performance liquid chromatography (HPLC) grade methanol. The methanol fraction contained the oxide.

HPLC parameters. Samples were analyzed at 215 nm (0.02 AUFS) on a Beckman model no. 334 HPLC with an Ultrasphere ODS, 5 μm, 25 cm × 4.6 mm column. The solvent system was 50% aqueous methanol with a flow rate of 1 ml/min. A standard diethylphenylphosphine oxide concentration curve was plotted. At concentrations less than 1 mM, diethylphenylphosphine reacted with the column to give a nonlinear concentration curve, but quantitation of the oxide was unaffected.

RESULTS AND DISCUSSION

Several phosphorus-containing compounds were examined as possible substrates for pig liver microsomal FAD-containing monooxygenase. The series consisted of fonofos analogs and related trivalent phosphorus compounds, in order to study structure–activity relationships (Table 1). Enzyme activity was measured spectrophotometrically by monitoring NADPH oxidation.

Table 1. Structure–activity relationships of phosphorus-containing substrates for the pig liver microsomal FAD-containing monooxygenase*

Compound no.	Structure	Name	K_m (μM)
1.		Fonofos	33 ± 2
2.		Ethyl phenyl ethylphosphonothionate	K_m > Solubility limit of ≅125
3.		*S*-Phenyl diethylphosphinothiolothionate	48 ± 3
4.		Diethylphenylphosphine sulfide	99 ± 3
5.		Diethylphenylphosphine	<2.5
6.		Diethylphenylphosphonite	Not determined

* Kinetic constants were determined as described in Materials and Methods. Values are means ± S.D.
† Ø represents a phenyl group.

Table 2. Stoichiometry of diethylphenylphosphine and diethylphenylphosphine sulfide*

Substrate added (μM)		NADPH oxidized (μM)	O_2 consumed (μM)
C_2H_5, C_2H_5, P—Ø†	50	35	36
	100	62	66
C_2H_5, S, C_2H_5, P—Ø	50	68	42
	100	121	83
CH_3—S—Ø	50	42	50
	100	87	97

* Values represent means of duplicate determinations. The variation in all cases was less than 5%.
† Ø represents a phenyl group.

Fonofos (Compound 1) had a K_m value of 33 μM and, as seen previously [5], showed curvilinear reaction kinetics. In comparison to fonofos, ethyl phenyl ethylphosphonothionate (Compound 2) was a less effective substrate with a K_m value greater than its aqueous solubility limit. Thus, replacement of the *S*-phenyl group in fonofos with an *O*-phenyl group dramatically decreased its ability to act as a substrate. The affinity of the enzyme for *S*-phenyl diethylphosphinothiolothionate (Compound 3) was approximately equal to that for fonofos. In this case, replacement of an ethoxy group with an ethyl group had little effect on enzyme affinity. In contrast, replacement of the ethyl group in fonofos with an ethoxy group as in *S*-phenyl diethyl phosphorodithioate completely abolishes enzyme activity [5]. Diethylphenylphosphine sulfide (Compound 4) was also a substrate for the FAD-containing monooxygenase. Its relatively high K_m value, 99 μM, may be due to hindrance by the phenyl group adjacent to the thiophosphorus bond.

In the above cases, it cannot be determined from the kinetic data whether the enzyme catalyzes oxidative attack on the phosphorus or the sulfur atom. Therefore, diethylphenylphosphine (Compound 5), an analog without sulfur atoms, was tested as a substrate. The phosphine was the best substrate of the series, with a K_m lower than 2.5 μM. The K_m was too low to be determined under the stated experimental conditions due to analytical limitations. In contrast to most enzyme-catalyzed reactions, the rate of NADPH oxidation increased as the phosphine was consumed. Previous studies have shown that the k_{cat} for all nitrogen- and sulfur-containing substrates is constant. Diethylphenylphosphonite (Compound 6) (50 μM) gave rates equivalent to V_{max} although total NADPH consumption, when run to completion, was only 3 μM. It appears that the phosphonite is an excellent substrate but that its rapid nonenzymatic hydrolysis and/or oxidation prohibit calculation of stoichiometric and kinetic values.

Previous work with thioether-containing organophosphates indicates that 1 mole of NADPH is oxidized per mole of substrate as the sulfide is converted to the sulfoxide [11]. The stoichiometry of substrate addition, NADPH oxidation and O_2 consumption was analyzed for the phosphine, the phosphine sulfide, and methylphenylsulfide (Table 2). Stoichiometry studies with methylphenylsulfide, an excellent substrate [3], showed that quantitation methods for O_2 consumption and NADPH oxidation are valid.

With diethylphenylphosphine as substrate, equivalent amounts of NADPH and O_2 were consumed but this amount was significantly lower than the amount of phosphine added. The discrepancy was first attributed to the susceptibility of phosphines to nonenzymatic oxidation [12] by molecular oxygen but later discounted. If oxygen dissolved in the buffer were responsible for nonenzymatic phosphine oxidation, O_2 consumption would be expected to equal the substrate added while NADPH oxidation would be lower. Furthermore, there was no significant O_2 consumption in the absence of the enzyme but in the presence of diethylphenylphosphine. The possibility was examined that H_2O_2, produced by preincubating the enzyme with NADPH in the absence of substrate, is responsible for phosphine oxidation. Pretreatment with catalase for 2, 5, or 10 min had no effect on the ratio of NADPH oxidized to substrate added (Table 3).

The stoichiometry of the more stable diethylphenylphosphine sulfide was closer than expected (Table 2). In the absence of substrate, the FAD-containing monooxygenase slowly oxidizes NADPH using O_2 and producing $NADP^+$ and H_2O_2 [1]. With

Table 3. Effect of catalase on NADPH oxidation with 50 μM diethylphenylphosphine as substrate*

Time of catalase pretreatment (min)	Total NADPH oxidation (μM)	
	(− Catalase)	(+ Catalase)
2	28	30
5	29	28
10	25	29

* Total NADPH oxidation was determined according to the stoichiometry procedure described in Materials and Methods. Catalase, 600 nmoles/min, was added with enzyme for specified times before substrate addition.

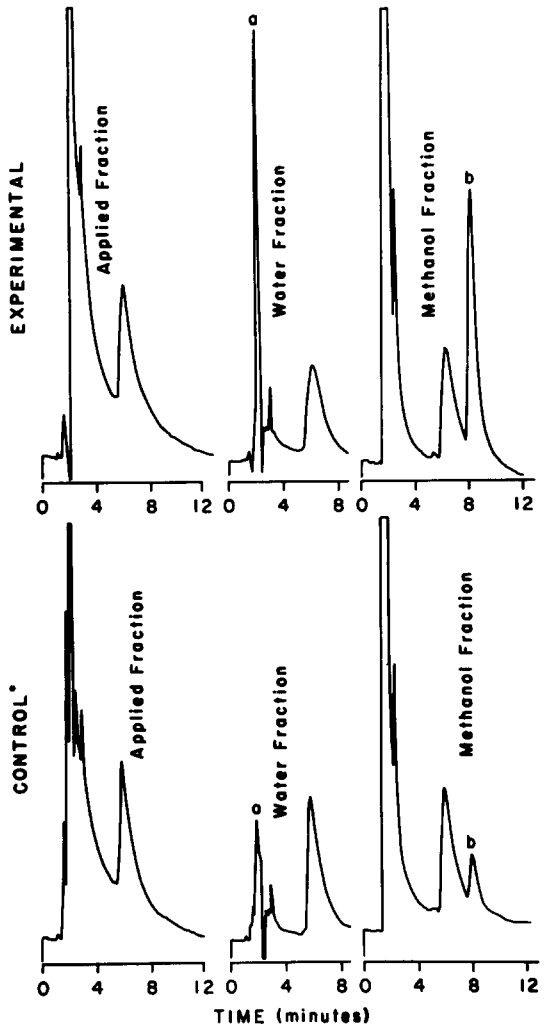


Fig. 1. High-performance liquid chromatograms of 100 μM diethylphenylphosphine which had been incubated with pig liver microsomal FAD-monooxygenase. Incubation conditions, fractionation procedure, and HPLC column parameters are described in Materials and Methods. Key: (*) control incubations contain heat-inactivated enzyme, (a) unidentified minor metabolite, and (b) diethylphenylphosphine oxide.

relatively poor substrates such as the phosphine sulfide, the low background oxidation rate becomes significant, making it difficult to determine when substrate-dependent oxygen consumption and NADPH oxidation reach completion. Despite the quantitation difficulties, O_2 consumption and NADPH oxidation were shown to be approximately equal to the substrate added.

High performance liquid chromatography was employed for metabolite identification and quantitation. Ultraviolet-absorbing peaks eluting near the solvent front were detected in all Sep-pak fractions with diethylphenylphosphine (Fig. 1) or diethylphenylphosphine sulfide (Fig. 2) as substrates. With diethylphenylphosphine two peaks in the incubation mixtures with active enzyme were significantly higher than the peaks in the control incubation mixtures. These peaks were due to phosphine oxide, with a retention time of 8 min, in the methanol fraction and a minor unidentified peak with a retention time of 2 min (solvent front) in the water fraction. With diethylphenylphosphine sulfide as substrate, only the phosphine oxide peak in the methanol fraction was significantly higher in the enzyme incubations versus the control incubations. The peak with a retention time of 6 min is an unknown impurity found in all samples including pure HPLC-grade methanol, water and acetonitrile. Diethylphenylphosphine sulfide eluted with a retention time of 23–24 min.

Diethylphenylphosphine oxide was the major metabolic product of diethylphenylphosphine oxidation and the only product of the oxidation of diethylphenylphosphine sulfide (Table 4). The recovery of 100 μM diethylphenylphosphine oxide following incubation and Sep-pak cleanup was 90%. The phosphine and the phosphine sulfide were first analyzed for oxide impurity. One hundred micromolar phosphine sulfide solution in methanol contained no detectable oxide. Solutions of the less stable phosphine were slowly oxidized at room temperature. HPLC analysis of phosphine solutions contained 6 μM or less oxide impurity after 30 min at room temperature. Control incubation mixtures, containing heat-inactivated enzyme with the phosphine, had higher levels of the oxide (15.4 ± 3.5 μM). This increase was due to the susceptibility of trialkylphosphines to oxidation [6, 12]. Incubation mixtures with active enzyme had much higher (72.8 ± 5.8 μM) oxide levels. The amount of oxide produced by the enzyme, total oxide minus the initial oxide impurity, was approximately equivalent to the O_2 and NADPH consumed. The oxide was not detected in diethylphenylphosphine sulfide control incubation mixtures. Incubation mixtures of diethylphenylphosphine sulfide with active enzyme contained 88.2 ± 1.6 μM oxide, showing that the sulfide was also a substrate of the FAD-containing monooxygenase.

The kinetic studies with these phosphorus compounds show that P=S containing compounds are substrates for the pig liver microsomal FAD-containing monooxygenase. It cannot be determined from these data whether the enzyme catalyzes oxidative attack on the sulfur or the phosphorus atoms. However, with diethylphenylphosphine and diethyl phenylphosphonite as substrates, the oxidative attack must occur on the phosphorus atom. Thus, the FAD-containing monooxygenase of pig liver must have phosphorus oxidase activity in addition to its better known nitrogen and sulfur oxidase ability. It is apparent that the cytochrome P-450-dependent monooxygenase system is not the only microsomal oxidase able to metabolize organophosphorus xenobiotics and that in future studies the role of both enzymes must be evaluated.

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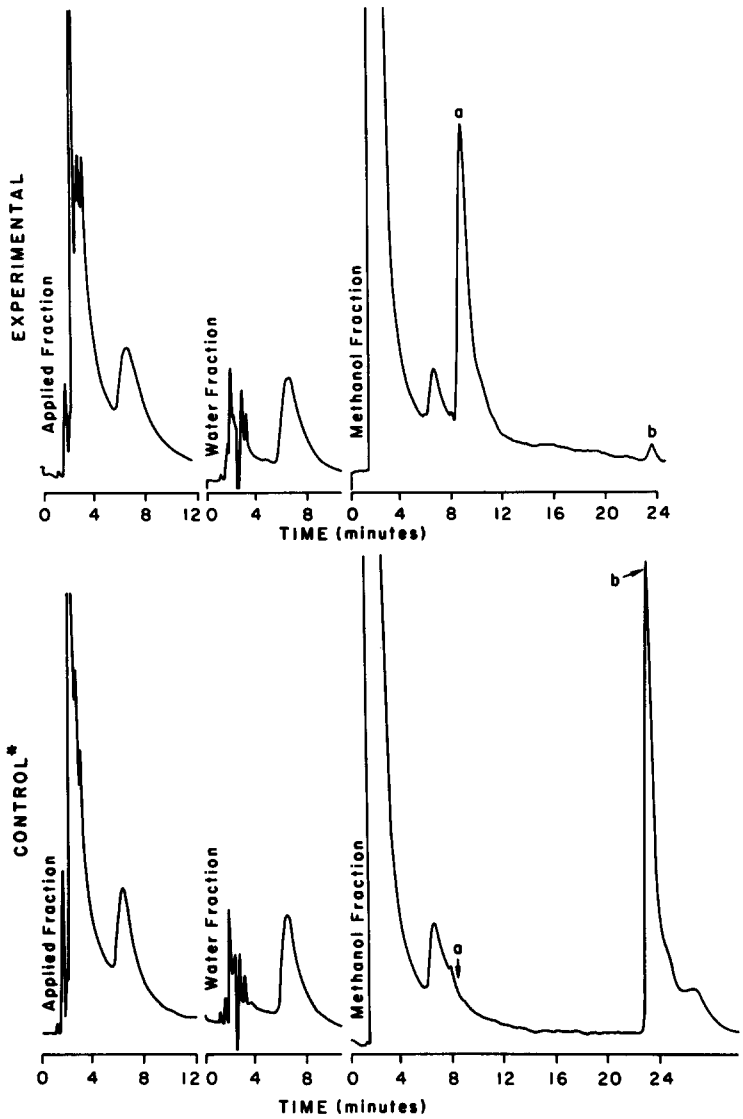

Fig. 2. High-performance liquid chromatograms of 100 μM diethylphenylphosphine sulfide which had been incubated with pig liver microsomal FAD-monooxygenase. Incubation conditions, fractionation procedure, and HPLC column parameters are described in Materials and Methods. Key: (*) control incubations contain heat-inactivated enzyme, (a) diethylphenylphosphine oxide, and (b) diethylphenylphosphine sulfide.

Table 4. Quantitation and identification of diethylphenylphosphine and diethylphenylphosphine sulfide metabolites by HPLC

Sample	Oxide detected* (μM)
100 μM $(C_2H_5)_2P\text{Ø}$† (Purity check)	4.6 ± 1.9
100 μM $(C_2H_5)_2P\text{Ø}$ Control incubation‡	15.4 ± 3.5
100 μM $(C_2H_5)_2P\text{Ø}$ Incubation	72.8 ± 5.8
100 μM $(C_2H_5)_2P(O)\text{Ø}$ Control incubation‡	92.8 ± 10.4
100 μM $(C_2H_5)_2P(S)\text{Ø}$ (Purity check)	0 ± 0
100 μM $(C_2H_5)_2P(S)\text{Ø}$ Control incubation‡	0 ± 0
100 μM $(C_2H_5)_2P(S)\text{Ø}$ Incubation	88.2 ± 1.6

* Each value represents the mean ± S.D. of four duplicate experiments.
† Ø represents a phenyl group.
‡ Control incubation mixtures contained heat-inactivated enzyme.

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