Oxidative Chemistry and Toxicology of *S,S,S*-Tributyl Phosphorotrithioate (DEF Defoliant)[†]

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Treatment of $(BuS)_2P(O)SBu$ (DEF defoliant) with *m*-chloroperoxybenzoic acid in chloroform yields products tentatively assigned as DEF sulfoxide and sulfone and in methanol gives $(BuS)_2P(O)OMe$ probably via the very reactive $(BuS)_2P(O)S(O)Bu$. This chemical model for DEF as an oxidative phosphorylating agent is applicable to the mouse liver mixed-function oxidase (MFO) system as the oxidant and cholinesterase as the trapping agent for the reactive intermediate(s) with 1000- and 10-fold metabolic activation for inhibition of acetylcholinesterase (AChE) and butyrylcholinesterase (BuChE), respectively. AChE is phosphorylated by MFO-activated [^{3}H]DEF. Piperonyl butoxide blocks these in vitro bioactivations but not the DEF-induced in vivo inhibition of erythrocyte AChE and plasma BuChE. Mouse liver microsomal and cytosolic esterases are also very sensitive to DEF. The metabolites of DEF in the mouse liver MFO system (examined with [^{3}H]DEF) and in the urine of treated rats (investigated by ^{31}P NMR) include $(BuS)_2P(O)SH$ and $(BuS)_2P(O)OH$ probably formed via $(BuS)_2P(O)SCH(OH)Pr$ and $(BuS)_2P(O)S(O)Bu$, respectively.

INTRODUCTION

S,S,S-Tributyl phosphorotrithioate, $(BuS)_2P(O)SBu$, with the trademark DEF defoliant, is the principal defoliant for cotton in the United States and many other parts of the world. It also has several other types of biological activity including initial cholinergic action and late or delayed acute effects in rats and hens (Abou-Donia et al., 1979a,b, 1984; Murphy and DuBois, 1959), delayed neurotoxicity and inhibition of neuropathy target esterase (NTE) in hens (Abou-Donia et al., 1979a; Baron and Johnson, 1964; Casida et al., 1963; Johnson, 1975; Lapadula et al., 1984), more potent inhibition of plasma butyrylcholinesterase (BuChE) than of brain acetylcholinesterase (AChE) (Murphy and DuBois, 1959), pronounced effects on bone and cartilage in fingerling trout and catfish (Cleveland and Hamilton, 1983), and synergism of malathion and selected pyrethroids under laboratory conditions (Casida et al., 1963; Gaughan et al., 1980).

Studies on the chemical and biological oxidation of DEF suggested that at least a portion of its biological reactions and effects are initiated by activation on sulfoxidation. Thus, DEF is sulfoxidized by *m*-chloroperoxybenzoic aicd (MCPBA) (Wu et al., 1991, 1992). On incubation with the mixed-function oxidase (MFO) system of liver microsomes, DEF is converted to a more potent inhibitor of AChE (Habig and Di Giulio, 1988; Wing et al., 1983, 1984) and NTE (Chow et al., 1986), which is possibly the sulfoxide, $(BuS)_2P(O)S(O)Bu$. Metabolism of DEF in the hen gastrointestinal tract yields butyl mercaptan which may contribute to its toxicological profile (Abou-Donia et al., 1979a,b). These reactions are shown in Figure 1.

About 40 of the current pesticides have a phosphorothiolate linkage (Worthing, 1987). The metabolic fate and toxicology of these compounds are related in part to their

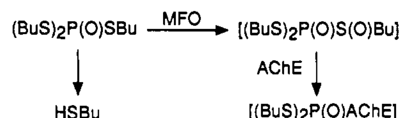

Figure 1. Previously proposed metabolic reactions of DEF.

biological sulfoxidation. The relation of the oxidative chemistry to the toxicology is established here for DEF as an example of this large number of phosphorothiolate pesticides. Three approaches are used: examine the chemical and biological oxidation of DEF with trapping agents for the reactive phosphorylating intermediates; establish the relevance of the results to esterase inhibition in treated rats and mice; identify several oxidatively formed metabolites.

MATERIALS AND METHODS

Spectroscopy and Chromatography. ^{1}H and ^{31}P NMR spectra, the latter with and without ^{1}H decoupling, were recorded with a Bruker WM-300 spectrometer at 300 and 121.5 MHz, respectively. Chemical shifts are referenced to internal 3-(trimethylsilyl)propionic-*2,2,3,3-d*$_4$ acid, sodium salt, or tetramethylsilane for ^{1}H spectra in D_2O and $CDCl_3$, respectively. External trimethyl phosphate in the appropriate solvent was the reference for ^{31}P spectra. For aqueous solutions ~10% D_2O was added as the locking solvent. Chemical shifts are reported for solutions in $CDCl_3$ unless specifically noted otherwise. Quantitative measurements utilized an inverted gate heteronuclear decoupling pulse sequence (without nuclear Overhauser effect) and a 10-s interpulse delay time.

Thin-layer chromatography (TLC) was carried out on silica gel F_{254} chromatoplates (0.25- and 2-mm gel thickness for analysis and preparative isolation, respectively).

Chemicals. DEF supplied by Mobay Chemical Corp. (Kansas City, MO) was a single component (>99%) on the basis of ^{31}P NMR (δ 62.4 in $CDCl_3$) following purification by preparative TLC (hexane–ethyl acetate 4:1, R_f = 0.63). Other chemicals used were the MFO inhibitor piperonyl butoxide (PB) from Fluka Chemie AG (Buchs, Switzerland), AChE (type V-S from electric eel, 1000–2000 units/mg of protein), and BuChE (from horse serum, 500–1000 units/mg of protein) from Sigma Chemical Co. (St. Louis, MO).

Synthesis of [^{3}H]DEF (Figure 2). *$(KS)_2P(S)OMe$.* A mixture of triethylamine (0.09 mol) and MeOH (0.09 mol) was

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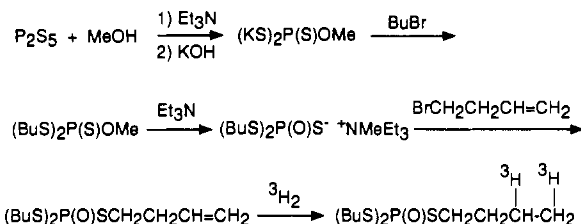

Figure 2. Radiosynthesis of [^{3}H]DEF.

added to P_2S_5 (0.015 mol) in ether (15 mL) over a period of 1.5 h. The solution was then heated at 40 °C for 10 min, and the lower layer obtained was mixed with KOH (0.1 mol) in MeOH (30 mL). The resulting white crystals were recovered by filtration and dried in vacuum.

(BuS)$_2$P(S)OMe. (KS)$_2$P(S)OMe (7 g) was reacted with 1-bromobutane (13 mL) at 65 °C for 12 h and then at 25 °C for 2 days. The mixture was filtered and excess bromobutane evaporated. The product was purified by TLC (hexane–ether 40:1): ^{1}H NMR δ 0.94 (t, J = 7.3 Hz, 6 H, $SCH_2CH_2CH_2CH_3$), 1.44 (m, J = 7.7 Hz, 4 H, $SCH_2CH_2CH_2CH_3$), 1.72 (m, J = 7.5 Hz, 4 H, $SCH_2CH_2CH_2CH_3$), 2.94 (2 t, J = 7.3, 16 Hz, 4 H, $SCH_2CH_2CH_2CH_3$), 3.77 (d, $^3J_{HP}$ = 16 Hz, 3 H, OCH_3); ^{31}P NMR δ 111.2.

(BuS)$_2$P(O)S$^-$ $^+$NMeEt$_3$. (BuS)$_2$P(S)OMe was heated in excess triethylamine at 70 °C overnight. ^{31}P NMR showed ~90% of the desired demethylation product (75.5 ppm) and ~10% of the rearranged product (BuS)$_2$P(O)SMe (64.6 ppm). The excess triethylamine was evaporated and the residue used directly for the reaction below.

(BuS)$_2$P(O)SCH$_2$CH$_2$CH=CH$_2$. (BuS)$_2$P(O)S$^-$ $^+$NMeEt$_3$ was mixed with excess 4-bromobutene, and the mixture was stirred at 65 °C for 8 h and then at 25 °C overnight. The product was purified by TLC (hexane–ether 5:1): ^{1}H NMR δ 0.94 (t, J = 7.3 Hz, 6 H, $SCH_2CH_2CH_2CH_3$), 1.43 (m, J = 7.6 Hz, 4 H, $SCH_2CH_2CH_2CH_3$), 1.74 (m, J = 7.5 Hz, 4 H, $SCH_2CH_2CH_2CH_3$), 2.50 (2 t, J = 8.5, 6.8 Hz, 2 H, $SCH_2CH_2CH{=}CH_2$), 3.01 (m, 6 H, $SCH_2CH_2CH_2CH_3$, $SCH_2CH_2CH{=}CH_2$), 5.10 (m, 2 H, $SCH_2CH_2CH{=}CH_2$), 5.78 (m, 1 H, $SCH_2CH_2CH{=}CH_2$); ^{31}P NMR δ 62.1.

[3,4-^{3}H$_2$-butyl]DEF. In preliminary studies the butenyl thiolate above was subjected to catalytic hydrogenation under H_2 (balloon) with different catalysts, solvents, and reaction times, determining the ratio of conversion by ^{31}P NMR integration and HPLC analysis (see below). Under similar conditions, PtO_2 gave 23 times more DEF than Pt/C. Ethyl acetate was better than EtOH with DEF yields of 92% and 49%, respectively. A higher yield was obtained with a larger amount of PtO_2 and a shorter reaction time (95%, 25 mg of PtO_2, 4 h vs 92%, 8 mg of PtO_2, 16 h). In all cases, DEF was the only product.

For radiolabeling, (BuS)$_2$P(O)SCH$_2$CH$_2$CH=CH$_2$ (50 mg) and PtO_2 (50 mg) were mixed in ethyl acetate (4 mL). The mixture was exhaustively degassed in a microhydrogenation apparatus by the application of three freeze–pump–thaw cycles. Tritium gas was admitted to a pressure of 710 mmHg, and the substrate was thawed. The reaction mixture was then stirred at room temperature; the $^3H_2$ pressure dropped to 528 mmHg in 30 min and, after that, remained constant. After 4.5 h, the mixture was frozen (liquid N_2) and the residual $^3H_2$ pumped away. The flask was extensively flushed with N_2, and MeOH (2 mL × 2) was added to the mixture to remove any labile or dissolved tritium. After the MeOH and ethyl acetate were pumped away, benzene (5 mL) was added and the solution filtered and concentrated under N_2. The ^{1}H NMR spectrum of the residue showed that about 80% of the starting material was reduced. The crude product was purified by HPLC (μPorasil column) monitoring by UV (254 nm) and radioactivity and using hexane–ethyl acetate (95:5) as the mobile phase [R_t = 15.5 (for starting material) and 18.0 min (for [^{3}H]DEF)]. The pure product showed almost the same proton NMR pattern as DEF, and further, the two ^{3}H signals appeared at the same chemical shifts as the methyl and 3-methylene protons, i.e., ^{3}H NMR (C_6D_6) 0.71 and 1.17 ppm vs ^{1}H NMR (C_6D_6) 0.70 and 1.15 ppm, respectively. The total radioactivity was 1.78 Ci with a specific activity of 27.5 Ci/mmol.

Peracid Oxidation. DEF (0.2 mmol) in $CDCl_3$ (dried by distillation from P_2O_5) (0.5 mL) in an NMR tube was placed in an acetone–dry ice bath (–60 °C) for 10 min, and then *m*-chloroperoxybenzoic acid (MCPBA) (99%) (1.5 molar equiv) was added. Alternatively, DEF (0.1 mmol) in CD_3OD (0.5 mL) was cooled to 0 °C prior to addition of MCPBA (2.0 molar equiv). In each case, the tube was taken from the coolant and shaken vigorously; the ^{31}P spectrum was recorded 10 ($CDCl_3$) or 20 min (CD_3OD) later following warming to 20 °C. This methodology for the study of reactive intermediates on oxidation of phosphorothioates is adapted from that of Segall and Casida (1983) and Wu et al., (1991, 1992).

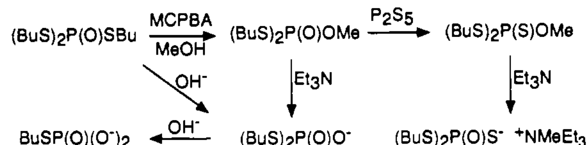

Figure 3. Synthesis of candidate metabolites.

Synthesis of Candidate Metabolites (Figure 3). *(BuS)$_2$P(O)OMe.* MCPBA (0.04 mol) was added slowly to a stirred solution of DEF (0.02 mol) in MeOH (100 mL), and stirring was continued for 16 h at 25 °C. Following solvent evaporation the residue was dissolved in CH_2Cl_2, which was washed with saturated $NaHCO_3$ and dried (Na_2SO_4) prior to purification by preparative TLC (hexane–ethyl acetate 3:1, R_f = 0.45): ^{1}H NMR δ 0.94 (t, J = 7.3 Hz, 6 H, $SCH_2CH_2CH_2CH_3$), 1.43 (m, J = 7.3 Hz, 4 H, $SCH_2CH_2CH_2CH_3$), 1.71 (m, J = 7.3 Hz, 4 H, $SCH_2CH_2CH_2CH_3$), 2.93 (m, J = 7.3 Hz, 4 H, $SCH_2CH_2CH_2CH_3$), 3.82 (d, $^3J_{HP}$ = 13.8 Hz, 3 H, OCH_3); ^{31}P NMR δ 56.7.

(BuS)$_2$P(O)OH. (BuS)$_2$P(O)OMe (0.5 g) was heated at 60 °C in triethylamine (3 mL) for 16 h. After evaporation of excess triethylamine, the residue was acidified with dilute HCl and extracted with CH_2Cl_2, and the organic phase was dried (Na_2SO_4) and evaporated to obtain the desired (BuS)$_2$P(O)OH: ^{1}H NMR δ 0.91 (t, J = 7.4 Hz, 6 H, $SCH_2CH_2CH_2CH_3$), 1.42 (m, J = 7.4 Hz, 4 H, $SCH_2CH_2CH_2CH_3$), 1.67 (m, J = 7.2 Hz, 4 H, $SCH_2CH_2CH_2CH_3$), 2.84 (m, J = 7.6 Hz, 4 H, $SCH_2CH_2CH_2CH_3$); ^{31}P NMR δ 36.5 (quintet, $^3J_{PH}$ = 11.6 Hz).

(BuS)$_2$P(O)SH. (BuS)$_2$P(O)OMe (514 mg, 2 mmol) was treated with P_2S_5 (88.8 mg, 0.08 mmol) in toluene (20 mL) at reflux for 16 h. The sulfuration product (BuS)$_2$P(S)OMe was purified by TLC (hexane–ethyl acetate 3:1): ^{1}H NMR δ 0.93 (t, J = 6.9 Hz, 6 H, $SCH_2CH_2CH_2CH_3$), 1.44 (m, J = 6.7 Hz, 4 H, $SCH_2CH_2CH_2CH_3$), 1.70 (m, J = 6.8 Hz, 4 H, $SCH_2CH_2CH_2CH_3$), 2.96 (m, J = 6.9, 15.7 Hz, 4 H, $SCH_2CH_2CH_2CH_3$), 3.76 (d, $^3J_{HP}$ = 15.9 Hz, 3 H, $POCH_3$); ^{31}P NMR δ 111.7. (BuS)$_2$P(S)OMe was demethylated by triethylamine, and the desired (BuS)$_2$P(O)SH was obtained in a similar way as described above: ^{1}H NMR δ 0.91 (t, J = 7.4 Hz, 6 H, $SCH_2CH_2CH_2CH_3$), 1.46 (m, J = 7.4 Hz, 4 H, $SCH_2CH_2CH_2CH_3$), 1.68 (m, J = 7.6 Hz, 4 H, $SCH_2CH_2CH_2CH_3$), 2.89 (m, J = 7.5 Hz, 4 H, $SCH_2CH_2CH_2CH_3$); ^{31}P NMR δ 83.2 (quintet, $^3J_{PH}$ = 11.7 Hz).

BuSP(O)(OH)$_2$. DEF was refluxed in 5 N NaOH for 4 h, and then the mixture was cooled and acidified with concentrated HCl. BuSP(O)(OH)$_2$ was formed in 60% yield: ^{31}P NMR (10% D_2O) δ 13.4 (t, $^3J_{PH}$ = 9.2 Hz). The other two products were HOP(O)(OH)$_2$ (16%) and HP(O)(OH)$_2$ (24%). BuSP(O)(OMe)$_2$ was obtained for characterization by treating the reaction mixture with diazomethane and purifying by TLC (hexane–ethyl acetate 3:1): ^{1}H NMR δ 0.93 [t, J = 7.4 Hz, 3 H, $(CH_2)_3CH_3$], 1.42 [m, J = 7.4 Hz, 2 H, $(CH_2)_2CH_2CH_3$], 1.67 (m, J = 7.5 Hz, 2 H, $CH_2CH_2CH_2CH_3$), 2.84 [m, J = 7.5 Hz, 2 H, $CH_2(CH_2)_2CH_3$], 3.80 [d, J = 12.6 Hz, 6 H, $(CH_3O)_2P$]; ^{31}P NMR δ 29.6.

Animals and Preparation of Subcellular Fractions and Incubation Mixtures. Adult male albino Swiss Webster mice (18–20 g) and Sprague Dawley rats (180–200 g) were obtained from Simonsen Laboratories (Gilroy, CA). DEF and PB were administered intraperitoneally (IP) or orally in methoxytriglycol (100 μL for each treatment).

To prepare the microsomal and cytosolic fractions, a homogenate of mouse liver in pH 7.4 100 mM phosphate buffer was centrifugated at 10000g for 15 min and the supernatant thereof at 105000g for 1 h. The microsomal fraction (105000g pellet) was washed with fresh buffer by resuspension and recentrifigation at 105000g for 1 h. The cytosolic fraction was centrifuged again at 105000g for 1 h to remove residual microsomal proteins. All stages of the preparations were carried out at 4 °C, and they were then held at –70 °C for up to 2 weeks until used. Protein

was quantitated according to the procedure of Bradford (1976) with bovine serum albumin as the reference.

Several conditions were standardized throughout the enzyme studies with microsomal and cytosolic fractions as above. Incubations were carried out in pH 7.4 100 mM phosphate buffer for 30 min at 37 °C in a shaking incubator. PB, when present, was added in dimethyl sulfoxide (5 μL) 10 min before addition of DEF in acetone (5 μL). All reaction components are indicated as final concentrations in the incubation mixtures.

Microsomal Oxidative Activation of DEF as an Inhibitor of Electric Eel AChE and Horse Serum BuChE. The coupled AChE or BuChE/MFO system was examined for possible activation of DEF using both the Ellman colorimetric procedure and electrophoresis with esterase staining. For colorimetric studies, incubation mixtures consisted of microsomes (0.1 mg of protein), AChE or BuChE (4 units), NADPH (0 or 67 μM), DEF (0–17 mM), and PB (0 or 100 μM) in 3-mL reaction mixtures. A control experiment established that PB did not inhibit AChE or BuChE activity in the presence or absence of NADPH. Following incubation each reaction was assayed for AChE or BuChE activity by a modification of the method of Ellman et al. (1961). An aliquot (100 μL) was added to a solution (2.9 mL) consisting of acetylthiocholine (ATCh) or butyrylthiocholine (BuTCh) (0.67 mM) and 5,5′-dithiobis(2-nitrobenzoic acid) (10 mM) in pH 7.6 100 mM phosphate buffer with the absorbance change monitored at 412 nm over a period of 10 min at 25 °C. All results are means of two or three experiments.

For electrophoretic assays, the reaction mixtures consisted of AChE (15 units) or BuChE (5 units), microsomes (0.1 mg of protein), NADPH (0 or 1.5 mM), DEF (0–3.7 mM), and PB (0 or 2.2 mM) in buffer (135 μL). After incubation and addition of bromophenol blue (15 μL of a 0.05% aqueous solution) and sucrose (50 μL of a 40% aqueous solution), aliquots (40 μL) were loaded on a native polyacrylamide gel electrophoresis (PAGE) gel (i.e., without sodium dodecyl sulfate) in the Laemmli (1970) buffer system using the Bio-Rad Protean II slab cell. The gels were stained with 6 mM α-naphthyl acetate (αNA) and 2 mg/mL diazonium salt (fast blue RR) in pH 7.0 80 mM Tris-HCl buffer involving shaking for 1 min (Markert and Hunter, 1959). The gels were then destained overnight with 50% aqueous EtOH.

Phosphorylation of Electric Eel AChE and Horse Serum BuChE with [^{3}H]DEF Directly and on Oxidative Bioactivation. [^{3}H]DEF was used to examine phosphorylation of AChE and BuChE directly and on bioactivation with separation of the labeled esterases by native PAGE. In the esterase only (direct) assay AChE (0, 40, 100, or 200 units) or BuChE (0 or 40 units) was incubated with [^{3}H]DEF (1.4 × 10^5 DPM, 5.8 nM) in buffer (400 μL). The bioactivated system also contained microsomes (0.1 mg of protein), NADPH (0 or 5 μM), and PB (0 or 250 μM). Following incubation an aliquot (150 μL) was loaded on the native PAGE gel with development and detection of esterases as above. The AChE or BuChE band was sliced and transferred to a glass scintillation vial containing Solvable gel solubilizer 0.5 M solution (NEN Research Products, Boston, MA) (0.5 mL) and distilled water (0.5 mL). The samples were incubated at 50 °C for 3 h, 10 mL of scintillation cocktail was added, and they were held overnight at room temperature prior to scintillation counting.

DEF as an Inhibitor of Erythrocyte AChE and Plasma BuChE in Treated Rats. DEF was administered IP at 100 mg/kg or, in some cases, at this dose 1 h after the rats were treated IP with PB at 200 mg/kg. Blood samples (44.7 μL) were taken from a tail vein using a heparinized micropipet at 0, 0.5, 1, 2, 4, 7, 12, and 24 h after treatment with DEF. The heparinized blood was centrifuged at 2000*g* for 10 min to separate the plasma and erythrocytes. A portion of the plasma (20 μL) was diluted to 1.0 mL with pH 7.6 100 mM phosphate buffer. The erythrocytes were washed twice with 1 mL of fresh buffer, each time by resuspension and centrifugation at 2000*g* for 10 min, then 2% Triton X-100 (100 μL) was added, the tube was shaken, and buffer was added to a final volume of 1 mL. An aliquot (100 μL) of the diluted plasma was used for BuChE assay and of the solubilized erythrocytes for AChE assay as described above. Duplicate analyses were made on samples from each of two animals to obtain the mean activity values.

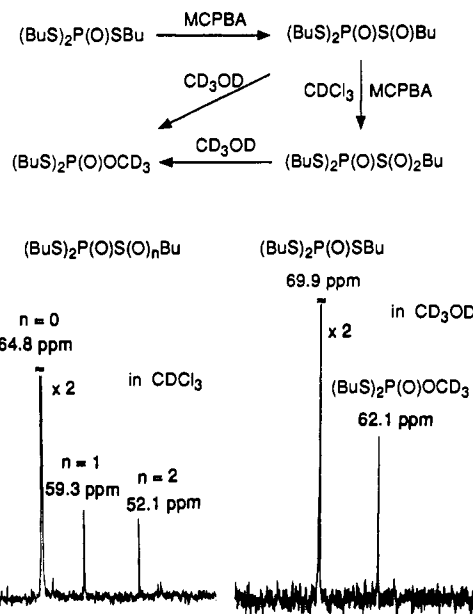

Figure 4. Reactions and ^{31}P NMR spectra of products on oxidation of DEF with 1.5 and 2.0 molar equiv of MCPBA in $CDCl_3$ and CD_3OD, respectively. Additional products in $CDCl_3$, each with a peak height of less than half that of the $(BuS)_2P(O)S(O)Bu$ signal, were at 24.3, 1.0, and –6.9 ppm. The chemical shift of DEF in $CDCl_3$ prior to the addition of MCPBA was 62.4 ppm.

DEF as an in Vitro and in Vivo Inhibitor of Mouse Liver Cytosolic Esterases Analyzed by Electrophoresis with α-Naphthyl Acetate as the Substrate. The in vitro native PAGE and esterase staining assays were made with microsomes, NADPH, DEF, and PB as in the above experiments with AChE and BuChE except that these enzymes were replaced with cytosol (2 mg of protein).

For in vivo studies the mice were treated IP with PB (200 mg/kg) followed 1 h later by DEF at 0, 1, 10, 50, or 100 mg/kg. After an additional 2 h, the liver cytosol was prepared and a 100-μL aliquot (2 mg of protein) was subjected to the electrophoretic esterase assay.

Oxidative Metabolism of [^{3}H]DEF in Mouse Liver Microsome–NADPH System. Following incubation of mixtures consisting of microsomes (0.1 mg of protein), NADPH (0 or 1 mM), and [^{3}H]DEF (50 μM) in buffer (200 μL), an aliquot (20 μL) was spotted directly for TLC in acetonitrile–glacial acetic acid (25:1) (solvent system A) or 1-butanol–1-propanol–0.15 N NH_4OH (2:1:1) (solvent system B). Labeled compounds were detected by radioautography at below 0 °C following spraying with EN^{3}HANCE (NEN) and quantitated by liquid scintillation counting. For cochromatography, the ^{3}H-labeled metabolites from incubation were mixed with standards of unlabeled $(BuS)_2P(O)SH$ and $(BuS)_2P(O)OH$ prior to spotting for TLC. Unlabeled standards were visualized with $Pd(II)Cl_2$ spray reagent (0.5% w/v in dilute aqueous HCl), yielding yellow to brown spots on a white background.

Urinary Metabolites of DEF in Rats. The rats were treated IP with PB (0 or 200 mg/kg) and 1 h later IP with DEF (100 mg/kg). Alternatively, PB and DEF were administered orally 6 h apart. The 0–24-h urine was filtered (Millex-HV filter unit, 0.45 μm, nonsterilized, Millipore Products Division, Bedford, MA) and lyophilized by SpeedVac concentrator SVC 100 (Savant Instruments, Inc., Farmington, NY), and the viscous liquid obtained was dissolved or resuspended in D_2O (0.5 mL). Phosphorus-containing urinary products were analyzed by ^{31}P NMR, and their ratio was determined by integration of the signals.

RESULTS

Preparation of DEF Sulfoxide and Sulfone and Their Action as Phosphorylating Agents (Figure 4).

Treatment of DEF in $CDCl_3$ with 1.5 molar equiv of MCPBA for 10 min yielded two products with ^{31}P NMR signals at 59.3 and 52.1 ppm, respectively. The ^{31}P

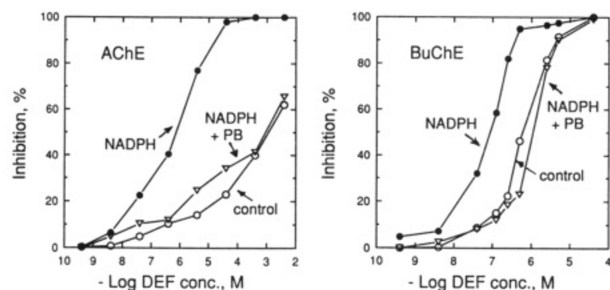

Figure 5. Microsomal oxidative activation of DEF as an inhibitor of electric eel AChE and horse serum BuChE analyzed by the Ellman colorimetric procedure.

chemical shifts of these compounds differed from that of DEF (64.8 ppm in the presence of MCPBA) by 5.5 and 12.7 ppm, which was consistent with the corresponding sulfoxide and sulfone, respectively (Wu et al., 1992). The sulfoxide and sulfone signals disappeared when the reaction mixture was held for several hours at 25 °C. The ratio of sulfone to sulfoxide was greatly increased with 3.0 vs 1.5 molar equiv of MCPBA, indicating their sequential formation, but a more complicated mixture of products was also evident.

A single product was obtained on treatment of DEF in MeOH with 2.0 molar equiv of MCPBA for 20 min, and this product was identified by ^{1}H and ^{31}P NMR as $(BuS)_2P(O)OMe$ (see Materials and Methods). No intermediate was evident in the formation of this methyl phosphate. It was therefore clear that DEF sulfoxide and probably also the sulfone reacted immediately to phosphorylate the MeOH.

Microsomal Oxidative Activation of DEF as an Inhibitor of Electric Eel AChE and Horse Serum BuChE. The potency of DEF as an inhibitor of AChE and BuChE was examined in the presence of mouse liver microsomes (control) or microsomes plus NADPH (oxidase system) or microsomes plus NADPH plus PB using the Ellman colorimetric procedure to assay ATCh and BuTCh hydrolysis, respectively (Figure 5). DEF was a much poorer inhibitor of AChE than of BuChE with concentrations for 50% inhibition (I_{50}s) of 1000 and 1 μM, respectively. The oxidase system increased the potency of DEF by 1000- and 10-fold to I_{50}s of 1 and 0.1 μM for AChE and BuChE, respectively. PB addition to these coupled systems completely blocked the bioactivation (I_{50}s approximating 1000 and 1 μM for AChE and BuChE, respectively). Thus, DEF undergoes oxidase-dependent and PB-sensitive activation as an inhibitor of both AChE and BuChE.

The microsomal oxidative activation of DEF as an inhibitor of AChE and BuChE is also evident by native PAGE with αNA as the substrate (Figure 6). Inhibition of AChE by DEF at 370 μM in the presence of microsomes was dependent on NADPH and greatly reduced by PB. In other electrophoresis studies not detailed here, the findings with AChE and DEF at 3700 μM were the same as those at 370 μM. In the case of BuChE and microsomes, DEF was a poor direct inhibitor at 3.7 and 37 μM but (although not illustrated) strongly inhibited at 370 and 3700 μM; inhibition at the two lower concentrations was marginally to greatly enhanced by addition of NADPH and retarded by PB. Thus, again, DEF was oxidatively activated as a BuChE inhibitor but to a lesser magnitude than as an AChE inhibitor. The R_f 0.12–0.20 bands designated as microsomal esterases were also very sensitive to bioactivated DEF, i.e., strongly inhibited with microsomes and NADPH and DEF at 3.7 μM but not when PB was also added. It is not known whether these are microsomal esterases released during incubation and analysis or cytosolic esterases adhering to the microsomes during preparation.

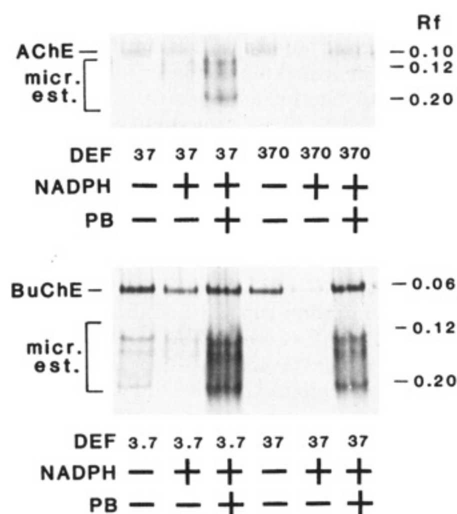

Figure 6. Microsomal oxidative activation of DEF as an inhibitor of electric eel AChE and horse serum BuChE analyzed by electrophoresis with αNA esterase staining. The R_f 0.12–0.20 esterase bands from microsomes are similar to those from cytosol in the same region shown in Figure 8.

Table I. Phosphorylation of Electric Eel AChE and Horse Serum BuChE by [³H]DEF Directly and on Bioactivation

esterase and units	^{3}H bound,[a] dpm		
		esterase + microsomes	
	esterase only	NADPH + PB	NADPH
AChE			
40	63, 32	14, 22	22, 118
100	98, 63	175, 195	419, 537
200	170	199	510
BuChE			
40	2750, 2333	290, 444	−52, 58

[a] Values from individual experiments. Corrected for control values with 0 units of AChE or BuChE as follows (mean ± SE, n = 4): 0 dpm for esterase only; 261 ± 8 dpm for esterase + microsomes + NADPH + PB; 328 ± 31 dpm for esterase + microsomes + NADPH.

Phosphorylation of Electric Eel AChE and Horse Serum BuChE by [³H]DEF Directly and on Oxidative Bioactivation (Table I). Electrophoresis was used to separate labeled AChE and BuChE from other labeled components of the reaction mixtures. The radioactivity electrophoresing in the position of AChE or BuChE was considered to be attributable to $([^3H]BuS)_2P(O)$AChE or $([^3H]BuS)_2P(O)$BuChE. Direct reaction of [³H]DEF with the esterases yielded much more labeling per esterase unit for BuChE than for AChE. Addition of microsomes and NADPH greatly increased the labeling of AChE by a mechanism largely blocked by PB. In contrast, microsomes and NADPH greatly reduced the phosphorylation of BuChE even in the presence of PB.

DEF as an Inhibitor of Erythrocyte AChE and Plasma BuChE in Treated Rats (Figure 7). Rat erythrocyte AChE and plasma BuChE were similarly and progressively inhibited by DEF administered IP at 100 mg/kg with maximum inhibition between 4 and 24 h. PB enhanced the rate at which DEF inhibited erythrocyte AChE and increased the extent of inhibition, but this MFO inhibitor did not alter the effectiveness of DEF in plasma BuChE inhibition.

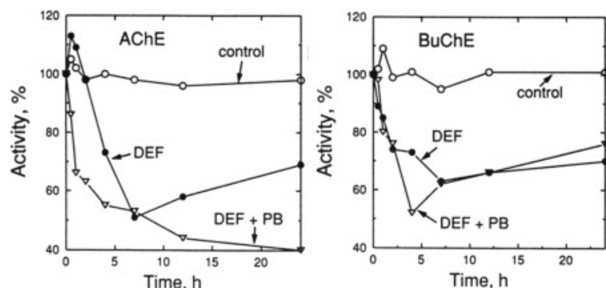

Figure 7. DEF as an inhibitor of erythrocyte AChE and plasma BuChE in treated rats.

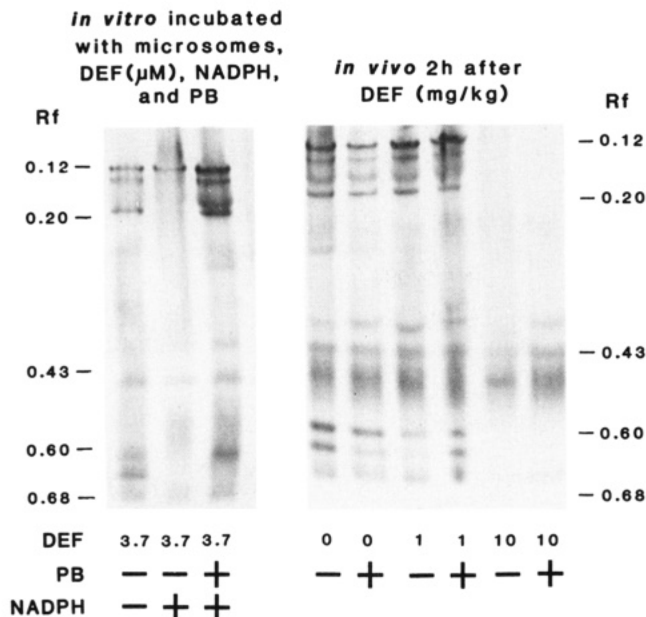

Figure 8. DEF as an in vitro and in vivo inhibitor of mouse liver cytosolic esterases analyzed by electrophoresis with αNA esterase staining. The in vitro assay was the same as that in Figure 6, replacing AChE or BuChE with cytosol. The in vivo assays were made directly on cytosol from treated mice.

DEF as an in Vitro and in Vivo Inhibitor of Mouse Liver Cytosolic Esterases (Figure 8). The cytosolic esterases assayed by electrophoresis with αNA as the substrate were partially inhibited for the R_f 0.12–0.2 bands at 0.37 and 3.7 μM DEF (illustrated for the latter concentration in Figure 8) and completely inhibited at 37 and 370 μM DEF. The R_f 0.43–0.60 bands were less sensitive to DEF than the R_f 0.60–0.68 bands, which retained their activity even at 370 μM DEF. Cytosolic esterase inhibition by DEF in the presence of microsomes was sometimes but not always enhanced by NADPH, and PB was an inhibitor of this oxidative activation.

The cytosolic esterases were inhibited little if at all by DEF at 1 mg/kg, but at 10 mg/kg most of the esterase bands were strongly inhibited and the four to six refractory bands in the R_f 0.43–0.60 region were also insensitive at 50 and 100 mg/kg. PB did not affect this inhibition pattern at any level of DEF.

Oxidative Metabolism of [^{3}H]DEF in the Mouse Liver Microsome–NADPH System (Table II). [^{3}H]-DEF underwent relatively little metabolism on direct incubation with microsomes, but when NADPH was also added, it gave three major metabolites plus a region of minor metabolites of intermediate R_f values and material at the origin evident by TLC following direct spotting of the metabolite mixtures. Two of the metabolites were identified as $(BuS)_2P(O)SH$ and $(BuS)_2P(O)OH$ by cochromatography with authentic standards in solvent systems A and B. The major unidentified metabolite(s) was (were) more polar than these diesters as appropriate for oxidative modifications within a butyl substituent of a diester or conversion to a monoester of phosphorothioic or phosphoric acid. Thus, the metabolism by mouse microsomes was primarily oxidative in nature and was initiated by cleavage at both the S–C and P–S linkages.

Urinary Metabolites of DEF in Rats (Figure 9). The urine 0–24 h after administration of DEF IP at 100 mg/kg contained at least four major new ^{31}P NMR signals at 78.8, 37.2, 15.5, and 8.8 ppm in a peak area ratio of 2.6:1.0:4.4:1.7, respectively. The identity and ratio of these peaks were not changed by oral administration of DEF and by pretreatment of the rats with PB.

The chemical shifts of the 78.8 and 37.2 ppm signals were appropriate for compounds with three and two P–S linkages, respectively, and their ^{31}P–^{1}H coupled spectra each had five peaks consistent with two P–S–Bu substituents. These findings suggested that the 78.8 and 37.2 ppm signals were attributable to $(BuS)_2P(O)SH$ and $(BuS)_2P(O)OH$, respectively, a proposal confirmed by adding the authentic standards to urine and obtaining an enhanced single signal in the appropriate position in each case.

Two metabolite ^{31}P signals appeared at 15.5 and 8.8 ppm, suggesting the possibility that one or more of these compounds might contain one P–S linkage. Neither of these signals was due to $BuSP(O)(OH)_2$, since when the standard was added to urine, its signal (14.1 ppm) appeared between those of these two metabolites; this monoester standard decomposed on holding in urine for 12 h at 25 °C, so it could conceivably have been a metabolic intermediate that was lost due to instability. The 15.5 ppm region probably contained compounds with only one P–S (possibly $PSCH_2$) linkage. The ^{31}P–^{1}H coupled spectra showed at least seven peaks, and the decoupled spectra showed two or three partially overlapping peaks rather than a singlet, indicating a mixture of closely related metabolites. The unknown at 8.8 ppm gave a singlet in the decoupled spectrum and a doublet in the ^{31}P–^{1}H coupled spectrum ($^3J_{PH}$ = 24 Hz), indicating a possible $(HO)_2P(O)SCH$ functionality. $(NaO)_2P(S)ONa$ gave a chemical shift of 35.2 ppm on addition to the urinary metabolite mixture, so this monothio acid was not a metabolite.

A minor metabolite signal at −2.0 ppm appeared on the shoulder of the major endogenous urinary ^{31}P compound at −2.4 ppm. This −2.0 ppm compound gave a ^{31}P–^{1}H coupled spectrum appropriate for $HP(O)(OH)_2$ (d, $^1J_{PH}$ = ~600 Hz) with confirmation by spiking with the authentic standard. The major signal in urine from both the treated rats and the controls was attributable to $HOP(O)(OH)_2$. When a mixture of the hydrolysis products of DEF in aqueous alkali [i.e., $(BuS)_2P(O)OH$, $BuSP(O)(OH)_2$, $HP(O)(OH)_2$, and $HOP(O)(OH)_2$] was added to the urine, the only unique ^{31}P signal from the hydrolysate compared with those in the urine was due to $BuSP(O)(OH)_2$, providing further evidence that this monoester was not a metabolite (or decomposed as previously noted) and that $HOP(O)(OH)_2$ was the major urinary phosphorus compound. A ^{31}P signal sometimes appeared at −14.4 ppm, as appropriate for a pyrophosphate functionality, in the urine of both control and DEF-treated rats.

DISCUSSION

Many phosphorothiolates are propesticides which are converted to potent AChE inhibitors on biooxidation (Wing et al., 1983, 1984). The activated intermediates are considered to be phosphorothiolate sulfoxides on analogy with findings in model chemical systems, usually MCPBA

Table II. Mouse Microsomal Metabolites of [^{3}H]DEF

^{3}H compd or fraction	TLC R_f in indicated solvent system[a]		% ^{3}H recovery[b] ± NADPH in indicated solvent system[a]			
			alone		+NADPH	
	A	B	A	B	A	B
$(BuS)_2P(O)SBu$	0.78	0.72	85.0 ± 0.2	83.1 ± 1.1	3.6 ± 0.5	3.7 ± 1.3
$(BuS)_2P(O)OH$	0.19	0.54	2.7 ± 0.3	2.9 ± 0.4	20.7 ± 0.4	15.5 ± 1.7
$(BuS)_2P(O)SH$	0.64	0.62		6.3 ± 1.4		28.0 ± 1.5
unknowns	0.09	0.46	10.5 ± 0.5[c]	4.0 ± 0.1	57.9 ± 1.6[c]	23.1 ± 1.3
	0.25–0.60	0.05–0.40		3.1 ± 0.4		15.6 ± 0.7
	0.00	0.00	1.8 ± 0.1	0.6 ± 0.0	14.7 ± 0.6	7.8 ± 0.4
recovery			100.0	100.0	96.9	93.7

[a] A, acetonitrile–glacial acetic acid 25:1. B, 1-butanol–1-propanol–0.15 N NH_4OH 2:1:1. [b] Mean ± SE (n = 3). [c] The ratio of $(BuS)_2P(O)SH$ and the R_f 0.09 and 0.25–0.60 unknowns in solvent system A (tabulated collectively) differs from that in B (tabulated individually), suggesting possible decomposition in one of the solvent systems, probably A.

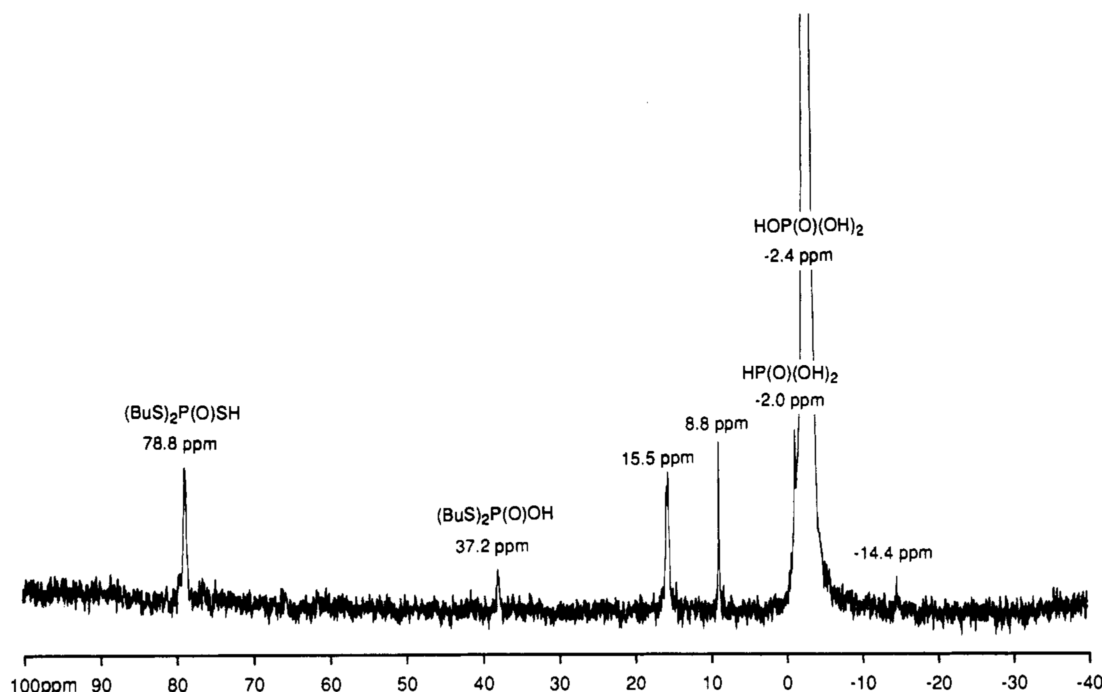

Figure 9. ^{31}P NMR spectrum of rat urinary metabolites of DEF. $HOP(O)(OH)_2$ is an endogenous urinary constituent, and little if any of this signal is attributable to a DEF metabolite.

oxidation in various aprotic and protic solvents (Segall and Casida, 1983). The low yields of DEF sulfoxide and sulfone and the formation of many byproducts in aprotic solvents are probably due to the multiple sulfurs available for oxidation (Wu et al., 1992). On the other hand, DEF sulfoxide is probably the principal and possibly the only intermediate in the conversion of DEF to $(BuS)_2P(O)$-OMe on oxidation with MCPBA in MeOH.

Several aspects of the toxicology of DEF appear to be associated with its bioactivation as an esterase inhibitor. In this case the AChE or BuChE serine hydroxyl substituent, like the MeOH hydroxyl in the chemical model system, serves to trap DEF sulfoxide by undergoing a phosphorylation reaction. The acute toxic effects of DEF possibly involve the cholinergic system, yet it is a very poor AChE inhibitor (Murphy and DuBois, 1959). However, it is moderately active as an in vivo AChE inhibitor and can undergo 1000-fold activation on in vitro MFO metabolism. In an apparent anomaly, PB blocked the microsomal oxidative activation of DEF as an AChE inhibitor in vitro but increased its rate of erythrocyte AChE inhibition in vivo, possibly reflecting the dual role of MFO-catalyzed sulfoxidation in both activation and detoxification. The delayed neurotoxicity and NTE inhibition are proposed to result from DEF sulfoxide (Chow et al., 1986; Lapadula et al., 1984). The action of DEF as a BuChE inhibitor and insecticide synergist may also be

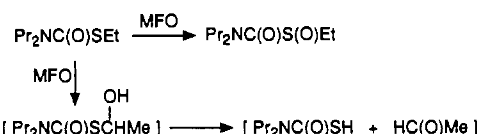

Figure 10. Microsomal oxidative metabolism of the thiocarbamate herbicide EPTC.

related in part to the sulfoxide as the activation product. The mechanism of defoliant action of DEF and its effect on bone and cartilage in fish (Cleveland and Hamilton, 1983) remain unknown, although the target in each case is probably phosphorylated by DEF sulfoxide. These proposals will remain as only working hypotheses until conditions are established to directly observe DEF sulfoxide in a metabolic system.

Oxidative metabolism of thiocarbamate herbicides serves as a possible model for phosphorothiolate pesticides. EPTC, as an example, undergoes MFO-catalyzed sulfoxidation and hydroxylation at each carbon with preference for the *S*-methylene functionality (Figure 10). EPTC sulfoxide is the major product in MFO systems, whereas the α-hydroxyethyl intermediate decomposes to acetaldehyde and the thiocarbamic acid which in turn yields dipropylamine and COS (Chen et al., 1978). DEF activation is probably analogous in mechanism and the enzymes involved, but the products are considerably different in

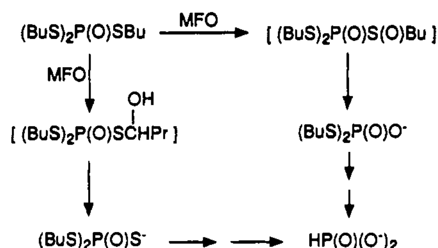

Figure 11. Metabolite pathways for DEF in rats.

stability than those derived from EPTC. DEF sulfoxide is detected here as $(BuS)_2P(O)OH$ from its hydrolysis, and $(BuS)_2P(O)SCH(OH)Pr$ is analyzed as $(BuS)_2P(O)SH$. More generally, cleavage of the P–S bond of phosphorothiolates may involve hydrolysis or sulfoxidation and then hydrolysis, whereas cleavage of the S–C bond probably involves a PSC(OH) intermediate. BuS(O)H liberated on hydrolysis of DEF sulfoxide probably reverts in the most part to BuSH, but a portion may be oxidized to $BuS(O)_2OH$. An unexpected metabolite observed from DEF is $HP(O)(OH)_2$, possibly formed via $BuSP(O)(OH)_2$. Figure 11 summarizes the proposed metabolic pathways for DEF in rats and the mouse liver MFO system.

ABBREVIATIONS USED

AChE, acetylcholinesterase; ATCh, acetylthiocholine; Bu, *n*-butyl; BuChE, butyrylcholinesterase; BuTCh, butyrylthiocholine; DEF, *S,S,S*-tributyl phosphorotrithioate; Et, ethyl; I_{50}, inhibitor concentration for 50% inhibition; IP, intraperitoneal; MCPBA, *m*-chloroperoxybenzoic acid; Me, methyl; MFO, mixed-function oxidase; αNA, α-naphthyl acetate; NTE, neuropathy target esterase; PAGE, polyacrylamide gel electrophoresis; PB, piperonyl butoxide; Pr, *n*-propyl; TLC, thin-layer chromatography.

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Registry No. DEF, 78-48-8; DEF sulfoxide, 142635-81-2; DEF sulfone, 141931-23-9; MFO, 9040-60-2; AChE, 9000-81-1; BuChE, 9001-08-5; $BuSP(O)(OH)_2$, 44843-57-4; $(BuS)_2P(O)OH$, 72284-33-4; $(BuS)_2P(O)SH$, 142635-82-3; P_2S_5, 1314-80-3; MeOH, 67-56-1; $(Ks)_2P(S)OMe$, 20132-37-0; $(BuS)_2P(S)OMe$, 22082-31-1; $(BuS)_2P(O)S^-$, 142635-80-1; $BrCH_2CH_2CH{=}CH_2$, 5162-44-7; $(BuS)_2P(O)SCH_2CH_2CH{=}CH_2$, 142635-84-5; $(BuS)_2P(O)SCH_2CH_2CHTCH_2T$, 142635-83-4; $(BuS)_2P(O)OMe$, 13194-47-3.