Development and Evaluation of Antisera for Detection of the *O,O*-Diethyl Phosphorothionate and Phosphorothionothiolate Organophosphorus Pesticides by Immunoassay

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An immunochemical approach for class detection of organophosphorus pesticides was investigated. Synthesis of *O,O*-diethyl phosphorothionate haptens and their use in the generation of polyclonal antisera are described. An indirect inhibition format ELISA coupled with an automated screening procedure was used to characterize the dose−response of the resulting antisera for 17 representative *O,O*-diethyl and *O,O*-dimethyl phosphorothionates/thionothiolates and phosphates.

Keywords: *Organophosphorus pesticides; immunoassay; class-specific detection*

INTRODUCTION

Organophosphorus pesticides (OPs) have proved to be a viable replacement for persistent chlorinated pesticides and, as such, have been used extensively in both agricultural and domestic settings. This success with OPs is in part due to the relatively rapid rate with which they degrade and otherwise disappear from crops or application zones; generally, residues of most OPs are nearly undetectable 4 weeks after application, although weather conditions and the exact identity of the OP will influence this outcome (WHO, 1986). Nevertheless, the acute toxicity of this class of compounds frequently necessitates prudent monitoring of crops. In addition, exposure and risk assessment studies are also called for in society's efforts to balance the risks and benefits associated with OP usage.

Classical methods such as high-performance liquid chromatography (HPLC), (Rengasamy and Parmer, 1988; Thompson et al., 1989) and gas chromatography (GC) [AOAC International, 1990; Cai et al., 1995; U.S. Environmental Protection Agency (EPA), 1992a−d, 1996; U.S. Food and Drug Administration (FDA), 1991; Schenck et al., 1994] have been used successfully, with great sensitivity and reliability, for analysis of OP residues, although the costs and labor requirements associated with these methods may inhibit the scope of monitoring, particularly in field-screening scenarios. Immunochemical techniques, such as immunoassay, offer a number of attractive features, including low cost, high throughput, and portability to field settings lacking extensive laboratory facilities. In addition, the costs associated with purchasing and maintaining instruments such as HPLC or GC can be circumvented in some cases.

Screening assays, such as those based on immunochemical technologies, would permit identification of samples containing OP residues, nearly eliminating the need to expend resources for instrumental analysis of nondetects. As a result of such "triage", the scope of either monitoring or surveying could be enlarged for a given resource base. Alternatively, the requisite surveying/monitoring activity could be carried out at a lower cost. Thus, immunochemical techniques for broad class-specific screening of OP residues can be considered as a complementary tool to GC for OP analysis.

A large number of OPs have been synthesized, and of the 100 000 known OPs, ≈100 are in commercial usage (Hassall, 1982). To be of utility as a screening technique for class-specific detection of OPs, the immunochemical screening method would respond to all OPs and with the same relative response, at least in the "ideal" case. Initial attempts to develop class-specific immunoassays for OPs were reported in the late 1980s (Sudi and Heeschen, 1988). In this work, antibodies were developed against haptens belonging to the *O,O*-diethyl orthophosphate class. Unfortunately, the detection limits were quite high for *O,O*-diethyl phosphorothionates and thionothiolates, which are very commonly used subclasses of the OPs.

Later work described in a patent application (Banks et al., 1994) was based upon antibodies generated against *O,O*-dimethyl phosphorothionate haptens incorporating straight-chain spacer arms. As reported in the application, the system responded well to a limited number of *O,O*-dimethyl phosphorothionates. The reported IC_{50} (the midpoint of the dose−response curve) values ranged from 4.8 to >150 μg/mL for five different OPs (fenitrothion, methacrifos, propetamphos, dichlorvos, and dimethanoate). In addition, seven other OPs were listed and were reported to have poor to no response. This group includes malathion, chlorfenvinphos, etrimfos, chlorpyrifos-methyl, tetrachlorvinphos, pirimiphos-methyl, and glyphosate (the last compound is not a true organophosphate ester). It is not clear, on the basis of the data, whether the immunochemical reagents as described are more broadly applicable.

The goals in the current work are to (1) synthesize haptens incorporating the *O,O*-diethyl phosphorothionate moiety with optimized linker arms, (2) develop polyclonal antisera based upon these haptens, and (3) use a "quick screening" format to characterize the response of the antisera to representative OPs.

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MATERIALS AND METHODS

Chemicals. Neat pesticide standards were obtained from Polysciences (Niles, IL). Analytical stock solutions were prepared in UV−vis grade dimethyl sulfoxide (Fisher Scientific, Fair Lawn, NJ) and stored frozen at 5 °C. Caprolactone, 4-(4-methoxyphenyl)butyric acid, diethylthiophosphoryl chloride, *tert*-butyldimethylsilyl chloride, and *N*-hydroxysuccinimide were obtained from Aldrich Chemical Co. (Milwaukee, WI). Diethyl ether, hexanes, and ethyl acetate, all of ACS reagent grade, were obtained from Fisher Scientific. Goat anti-rabbit/alkaline phosphatase conjugate, *p*-nitrophenyl phosphate substrate tablets, and 1-ethyl-3-[3-(dimethylamino)propyl]carbodiimide hydrochloride were obtained from Sigma Chemical Co. (St. Louis, MO).

Instrumentation and Equipment. Proton nuclear magnetic resonance (NMR) spectra were obtained with either a model VXR 300 (300 MHz) or a model EM-360 (60 MHz) NMR spectrometer (Varian, Sunnyvale, CA). High-resolution mass spectrometry (HRMS) was carried out on a VG 70-250SE high-resolution mass spectrometer equipped with a VG 11-250 data system (Micromass, Ltd., Manchester, U.K.). Melting points were determined with a Hoover melting point apparatus (Arthur H. Thomas Co., Philadelphia, PA). Maxisorb II polystyrene microplates were used for all ELISA procedures (Nunc, Roskilde, Denmark). ELISA absorbances were read in the end point mode (450−650 nm) with a Vmax microplate reader (Molecular Devices, Menlo Park, CA). Microplates were washed with a SkanWasher300 (Skatron, Lier, Norway). Microplates were shaken on a Mini-orbital Shaker (Bellco, Inc., Vineland, NJ). Dilutions were performed with a Biomek 1000 robotic workstation (Beckman Instruments, Inc., Palo Alto, CA). Thin-layer chromatography (TLC) was performed on 250 μm silica gel plates with fluorescent indicator (J. T. Baker, Irvine, CA).

Synthesis of Haptens. Compounds **1**−**5** were synthesized for use in the preparation of immunogens and coating antigens.

*4-(4-Hydroxyphenyl)butyric Acid (**1**).* 4-(4-Methoxyphenyl)-butyric acid (5.0 g, 25.7 mmol) was added to a 100-mL round-bottom flask fitted with a condensor, gas inlet, and magnetic stir bar. To this flask was added 50 mL of 48% HBr, and the flask was purged with and maintained under dry argon. The mixture was heated to reflux, at which point the solid completely dissolved. After 4 h, TLC (hexanes/ethyl acetate, 3:1; R_f = 0.07 for product) showed complete consumption of starting material. The reaction mixture was cooled, transferred to a separatory funnel with 100 mL of water, and extracted with diethyl ether (3 × 100 mL). The combined ether extracts were washed with water (3 × 100 mL) and saturated brine (1 × 100 mL), dried over anhydrous $MgSO_4$, filtered, and evaporated to yield a yellow oil, which solidified on standing. The solid was recrystallized from cyclohexane/ethyl acetate to yield tan crystals (2.84 g, 15.76 mmol, 61%), mp 109−111 °C: ^{1}H NMR (60 MHz, acetone-d_6) δ 8.60 (s, br, 2H), 7.11 (d, J = 9.6 Hz, 2H), 6.80 (d, J = 10 Hz, 2H), 2.6 (t, J = 7 Hz, 2H), 2.02 (m, 4H).

*4-(4-Hydroxyphenyl)butyric Acid tert-Butyldimethylsilyl Ester (**2**).* Compound **2** was prepared according to the method of Perich and Johns (1989). To a 100-mL round-bottom flask equipped with a gas inlet and stir bar was added 1.30 g (7.21 mmol) 4-(4-hydroxyphenyl)butyric acid under an atmosphere of dry argon. The acid was dissolved with 25 mL of dried tetrahydrofuran (THF). To this was added 795 μL (731 mg, 7.23 mmol) of dry 4-methylmorpholine, and a solution of *tert*-butyldimethylsilyl chloride (1.088 g, 7.22 mmol, in 5.45 mL of dry THF) was added dropwise. After 30 min, TLC (hexane/ethyl acetate, 3:1; R_f = 0.5 for product) showed consumption of starting acid. The reaction mixture was transferred to a separatory funnel with 75 mL of ether and 50 mL of water. After partitioning, the aqueous layer was further extracted with ether (2 × 50 mL). The combined ether extracts were quickly washed with water (2 × 50 mL) and saturated brine (1 × 50 mL), dried over anhydrous Na_2SO_4, and filtered. The ether was evaporated to yield 2.15 g (101%) of a clear, faintly green mobile oil. This oil was purified by flash chromatography (Still et al., 1978) on silica gel (40 mm column, hexane/ethyl acetate 4:1; R_f = 0.42) to yield 1.52 g (5.16 mmol, 72%) of a colorless oil: ^{1}H NMR (330 MHz, $CDCl_3$) δ 7.03 (d, J = 9 Hz, 2H), 6.75 (d, J = 8 Hz, 2H), 4.99 (s, br, 1H), 2.57 (t, J = 8 Hz, 2H), 2.33 (t, J = 8 Hz, 2H), 1.88 (t, J = 8.2 Hz, 2H), 0.93 (s, 9H), 0.27 (s, 6H); HRMS (resolution 22 000) theoretical 294.1651 Da, found 294.1648 Da.

*O,O-Diethyl, O-[p-(4-Carboxybutyl)phenyl] Phosphorothionate (**3**).* Compound **3** was prepared according to the method of Valerio et al. (1984). Into a dried 100-mL flask equipped with a septum and gas inlet were added 1.502 g (5.10 mmol) of 4-(4-hydroxyphenyl)butyric acid, *tert*-butyldimethylsilyl ester, and 20 mL of dried *p*-dioxane. This solution was cooled to 10−15 °C, and 124 mg (5.16 mmol) of NaH was added in one portion, followed by stirring for 30 min. Diethylthiophosphoryl chloride (1.6 mL, 1.92 g, 10.2 mmol) was added dropwise over 15 min. The mixture was removed from the cooling bath and stirred for 3 h at room temperature. TLC (hexanes/ethyl acetate 3:1; R_f = 0.40 for starting material) showed no remaining starting material. To this mixture was added 10 mL of 3 N HCl, and the resulting solution was stirred at room temperature for 1 h. The dioxane was evaporated, and the remaining solution was transferred to a separatory funnel with 30 mL of water and 25 mL of ether. After partitioning, the aqueous phase was further extracted with ether (2 × 25 mL). The combined ether extracts were washed with water (2 × 25 mL) and saturated brine (1 × 25 mL) and dried over anhydrous $MgSO_4$. The solution was filtered and evaporated to yield 2.59 g of an impure oil. This oil was flash chromatographed on silica gel (40-mm-diameter column, hexane/ethyl acetate/acetic acid, 5:1:1; R_f = 0.41) to yield 701 mg of a slightly impure oil, which still contained diethylthiophosphoryl chloride. This mixture was rechromatographed on a 20-mm-diameter column using the same conditions described above. The fractions containing the desired product were combined in a separatory funnel, diluted with hexane, and washed with water (3 × 50 mL) and saturated brine (1 × 50 mL). The solution was dried over $MgSO_4$, filtered, and evaporated to yield 335 mg (1.01 mmol, 20% overall) of the desired product: ^{1}H NMR (330 MHz, $CDCl_3$) δ 9.97 (s, br, 1H), 7.15 (d, J = 9.6 Hz, 2H), 7.09 (d, J = 9.6 Hz, 2H), 4.27 (m, 4H), 2.64 (t, J = 8.3 Hz, 2H), 2.37 (t, J = 8.3 Hz, 2H), 1.94 (t, J = 7.9 Hz, 2H), 1.36 (t, J = 7.8 Hz, 6H); HRMS (22 000 resolution) theoretical 332.0847 Da, found 332.0855 Da.

*Sodium 6-Hydroxyhexanoate (**4**).* Caprolactone (2.0 mL, 2.06 g, 18.05 mmol) was placed in a 50-mL round-bottom flask along with 20 mL of water, and to this mixture was added NaOH (805 mg, 20.13 mmol). The mixture was heated to reflux for 3 h. The mixture was cooled, and the water was removed under vacuum. The resulting solid was dissolved in hot ethanol and precipitated with diethyl ether to yield 2.08 g (75%) of a white fluffy solid.

*O,O-Diethyl O-(6-Carboxyhexyl) Phosphorothionate (**5**).* Compound **5** was prepared following the method of Schrader and Mühlmann (1956). Into a 50-mL conical flask fitted with a condenser and gas inlet were placed 411 mg (2.66 mmol) of sodium 6-hydroxyhexanoate and 5 mL of dry pyridine. The mixture was stirred vigorously to disperse the salt, and diethylthiophosphoryl chloride (450 μL, 540 mg, 2.86 mmol) was added dropwise over 5 min; the resulting mixture was stirred at 40 °C for 3 h. The mixture was cooled, acidified to pH 3, and transferred to a separatory funnel with 40 mL of water and 50 mL of ether. After partitioning, the aqueous layer was further extracted with ether (2 × 50 mL). The combined ether extracts were washed with water (3 × 50 mL) and saturated brine (1 × 50 mL). The ether solution was dried over anhydrous $MgSO_4$. After filtration, the solution was evaporated to yield 489 mg of crude product. This product was purified by flash chromatography on silica gel (40-mm-diameter column, hexane/ethyl acetate/acetic acid, 5:1:1; R_f = 0.42) to yield 174 mg (0.61 mmol, 23%) of a clear, slightly yellow liquid: ^{1}H NMR (330 MHz, $CDCl_3$) δ 10.2 (s, br, 1H), 4.16 (m, 6H), 2.4 (t, J = 8.2 Hz, 2H), 1.68 (m, 4H), 1.32 (m, 8H).

Preparation of Hapten−Protein Conjugates. Haptens **3** and **5** were conjugated to bovine serum albumin (BSA) and keyhole limpet hemocyanin (KLH). Conjugation was carried out with either 15 or 30 equiv of hapten for each equivalent of carrier protein. The haptens were preactivated as *N*-hydroxysuccinimide esters prior to addition to the carrier protein using a modification of the method of Langone and Vunakis (1975). In a typical procedure, a solution of 6.4 mg (5.56×10^{-2} mmol) of *N*-hydroxysuccinimide and 10.2 mg (6.26×10^{-2} mmol) of 1-ethyl-3-[3-(dimethylamino)propyl]carbodiimide hydrochloride (EDC), in 1.12 mL of dry dimethylformamide (DMF), was added dropwise to a glass vial containing 9.2 mg (2.77×10^{-2} mmol) of *O*,*O*-diethyl *O*-[*p*-(4-carboxybutyl)phenyl] phosphorothionate and a magnetic stir bar. The vial was capped, and the solution was stirred overnight at room temperature. The next day, 20 mg (3.03×10^{-4} mmol) of BSA was dissolved in 4 mL of borate buffer (0.1 M, pH 9.4) and 575 μL of DMF was added to the resulting solution. The activated hapten solution was added to this solution in 25-μL aliquots totaling 185 μL. This corresponds to a mole ratio of 15:1 hapten to BSA and a final DMF concentration of ≈16% v/v. This solution was stirred at room temperature for 2 h and then overnight at 4 °C. The solution was transferred to cellulose dialysis tubing and dialyzed against 2 L of phosphate buffered saline (pH 7.4). The dialysate solution was changed five times over a 3-day period. The final solution was aliquoted into polypropylene microcentrifuge tubes and frozen at −80 °C.

Antisera Production. Antisera production was performed by Lampire Biological Laboratories, Pipersville, PA, following their standard protocol for rabbit polyclonal antibody production. Briefly, pathogen-free New Zealand white rabbits were immunized with an emulsion of 1 mg of immunogen in 1 mL of Freund's complete adjuvant (0.5 mL subcutaneous, 0.5 mL intradermal) at the start of production, followed by two additional equivalent immunizations at the start of weeks 1 and 2. After a rest week, the rabbits were immunized intramuscularly with 1 mL of Freund's incomplete adjuvant containing 1 mg of immunogen. After a second rest week, a test bleed was performed. Booster immunization was again performed after 4 weeks, and terminal production bleeds were performed 2 weeks later.

Screening of Antisera with Two-Dimensional Titration. Initial screening of antisera was conducted using an indirect competitive ELISA format (Van Emon et al., 1986). The antibody and coating antigen concentrations were optimized by checkerboard titration (Campbell, 1984).

ELISA-Indirect Inhibition Format. *Day 1.* A solution of coating antigen in carbonate coating buffer (1.59 g of Na_2CO_3, 2.93 g of $NaHCO_3$, and 0.2 g of NaN_3, diluted to 1 L and adjusted to pH 9.6) was added to a microtiter plate (100 μL/well). The coating antigen was prepared with hapten **5** and BSA; the hapten protein loading ratio was 15:1, and coating antigen dilutions typically ranged from 1:1000 to 1:12000 during initial characterization assays. The plate was covered and stored overnight at 4 °C. On the same day, rabbit anti-OP antiserum was serially diluted in phosphate-buffered saline with Tween 20 (PBST), typically from 1:3000 to 1:96000 during the initial characterization stages, and 1 mL of the resulting solution was added to 12 × 75 mm glass tubes, one for each OP standard. To each of these tubes was then added 175 μL of a methanol solution of OP standard. This corresponds to a solution that is 15% (v/v) methanol. The tubes containing these incubation solutions were sealed, and the antibody was allowed to react with the OPs overnight at 21 °C.

Day 2. The antigen-coated plates were washed with PBST (three times). The plates were rotated 180°, such that the wells which had been inserted toward the rear of the washer were now in the front, and re-washed with PBST (3 times). The preincubated solutions from day 1 were added to the antigen-coated microwell plates in triplicate (100 μL/well) and allowed to incubate, with shaking, for 3 h at 21 °C. After the plates were washed again with PBST, 100 μL of a solution of goat anti-rabbit IgG labeled with alkaline phosphatase in PBST (1:1000 dilution) was added to each well. After a 2-h incubation, with shaking, the plates were washed a final time with PBST, and a 1 mg/mL solution of *p*-nitrophenyl phosphate in diethanolamine buffer was added to each well (Voller, 1980). After 30 min, the resultant color was measured at 405−650 nm with a Vmax plate reader (Molecular Devices). A curve fit of optical density versus OP concentration was obtained on the basis of a four-parameter logistic fit using Softmax version 2.33 (Molecular Devices)

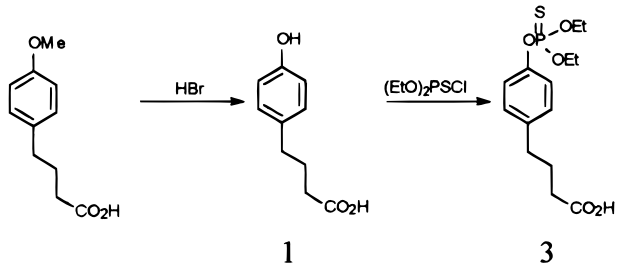


Figure 1. Synthetic scheme for the immunizing hapten.

Screening ELISA Procedure. *Day 1.* The microplates were coated in a manner analogous to the indirect ELISA format discussed above, with CoAg diluted 1:12000 or 1:20000 in carbonate buffer. The OP standard solutions (in DMSO) were added to the first column of a dilution plate and serially diluted robotically 1:5 (60 μL of standard into 240 μL of DMSO). A 1:3000 dilution of antibody was prepared in PBST, and 320 μL of this solution was added to each well of a second microwell plate. The serially diluted standards were then pipetted into the wells containing the antiserum solution (36 μL of diluted standard, 1:10 final dilution, ≈10% DMSO v/v). The plates were covered with acetate plate sealing tape and shaken overnight at room temperature.

Day 2. The antigen-coated plates were washed with PBST (3 times). The plates were rotated 180°, in a manner analogous to the format discussed above, and rewashed with PBST (3 times). The "incubation" plate was removed from the shaker and placed on the robotics unit, and 80 μL of each standard solution was added to antigen-coated plates in triplicate. The plates were removed, covered, and shaken at room temperature for 3 h. The plates were removed and washed as above, and a 1:1500 solution of goat anti-rabbit IgG/alkaline phosphatase conjugate in PBST was added (80 μL/well). The plates were covered and incubated on the shaker for 2 h at room temperature. The plates were then removed and washed as above, and 80 μL/well of a substrate solution in diethanolamine substrate buffer was added. End point optical density was measured at ≈15−30 min as described above, and the IC_{50} values were then calculated on the basis of a four-parameter logistic fit using Softmax version 2.33.

RESULTS AND DISCUSSION

Hapten Synthesis. *Synthesis of Immunization Hapten.* It was hoped that a simple scheme, as shown in Figure 1, would afford the desired hapten **3**.

Initial attempts to prepare **3** from the dianion of **1** with *O*,*O*-diethylthiophosphoryl chloride in ethanol (Fletcher et al., 1948) failed. In an effort to emulate thiophosphorylation of a singly charged phenoxide anion, it was decided that masking the carboxyl group should be attempted. A protecting group that could be removed without destroying the thiophosphoryl moiety was desired. A number of protecting groups were tried, including benzyl and phenacyl. Using these protecting groups, the phenolic hydroxyl group was successfully thiophosphorylated with thiophosphoryl chloride using Fletcher's conditions (hydroxide ion in ethanol). Subsequent reactions aimed at removal of the protective group, unfortunately, required conditions vigorous enough to cleave the newly formed O−P bond. This led to efforts at using the dimethyl *tert*-butyl silyl ester, which

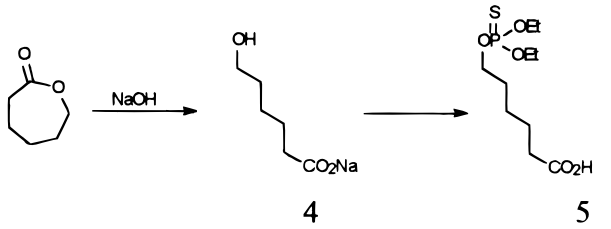


Figure 2. Synthetic scheme for the CoAg hapten.

was prepared with *tert*-butyldimethyl silyl chloride and *N*-methylmorpholine in THF (Perich and Johns, 1989). Unfortunately, this silyl ester was not stable under Fletcher's conditions. Apparently, hydrolysis of the silyl ester was very rapid. As an alternative, the protected silyl ester was treated with a suspension of NaH in dry dioxane, followed by addition of 2 equiv of thiophosphoryl chloride (Valerio et al., 1984). The resulting *O*,*O*-diethyl thiophosphorylated silyl ester was relatively labile, and because of this it was convenient to cleave the silyl protecting group without isolation of the protected compound, to produce the desired hapten **3**. The phosphorothionate moiety proved to be robust under the acidic conditions required for silyl ester cleavage.

Synthesis of Coating Antigen Hapten. Preparation of **5** proceeded from caprolactone. The two-step synthesis is depicted in Figure 2. Simple aqueous hydrolysis with sodium hydroxide afforded the sodium salt of 6-hydroxyhexanoic acid. Initial attempts at protecting the carboxyl group in a fashion analogous to the phenyl compound **2** proved to be fruitless, due to the difficulty of working with an ionic compound under Perich's conditions (anhydrous THF). Attempts were not made at isolating the protonated form of 6-hydroxyhexanoic acid, due to obvious problems of spontaneous lactonization. The sodium salt was suspended in dry pyridine and treated with diethylthiophosphoryl chloride, which afforded the desired compound **5** in adequate yield.

Preliminary Screening of Prospective Antisera. Initial antisera generation encompassed immunization of five rabbits, designated 4781−4785, with immunogens derived from hapten **3**. After identification of approximate reagent concentrations as outlined above, the sera were screened using the direct inhibition format. Solutions of each candidate antiserum were pipetted in triplicate to the incubation tubes. Methanol only was added to one of the triplicate tubes; this tube provided the B_0 response. A methanol solution of ethyl parathion was added to the second tube. This solution served as the "prototype" *O*,*O*-diethyl, *O*-phenyl compound. A methanol solution of disulfoton was added to the third tube. This compound was used as a "representative" *O*,*O*-diethyl, *O*-alkyl compound. Immunoassay was performed on these solutions, and the results are depicted in Figure 3.

Assuming that ethyl parathion and disulfoton are truly representative of ring-containing OPs and nonring-containing OPs, respectively, then the idealized response for class-specific purposes would be a strong and equal inhibition for both the ethyl parathion-containing and disulfoton-containing tubes. It can be observed that antisera 4783 and 4784 exhibited this behavior, whereas antisera 4785 and 4781 exhibited a significant response differential, being much less responsive to the straight-chain OP. Antiserum 4782 did not exhibit differential response; however, it did exhibit

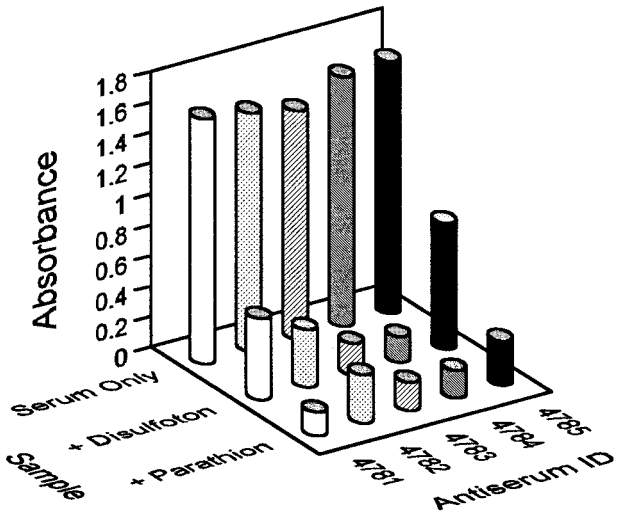


Figure 3. Response for antisera derived from hapten **3**.

Table 1. Structure of Phosphorus Esters Found in Predominant Organophosphorus Pesticides

phosphorus ester	structure
O,*O*-dimethyl phosphate	—O–P(=O)(OMe)OMe
O,*O*-diethyl phosphate	—O–P(=O)(OEt)OEt
O,*O*-dimethyl phosphorothioate	—O–P(=S)(OMe)OMe
O,*O*-diethyl phosphorothioate	—O–P(=S)(OEt)OEt
O,*O*-dimethyl phosphorothionothiolate	—S–P(=S)(OMe)OMe
O,*O*-diethyl phosphorothionothiolate	—S–P(=S)(OEt)OEt

a reduced response relative to antisera 4783 and 4784. On the basis of these data, antisera 4783 and 4784 were selected for the remainder of the work described here. These antisera were generated with immunogens prepared with KLH as the carrier protein, and hapten/protein ratios (preparative) were 15:1 and 30:1, respectively.

Approach. Most OPs can be classified as phosphoric acid derivatives. There are 12 subclasses of OP pesticides known, with the bulk of commercially used OPs coming from the phosphorothionate, phosphorthionothiolate, and phosphate classes. The phosphorothionothiolate, phosphoramide (phosphoramides, phosphorodiamidates, phophoramidothionates, and phosphoramidothiolates), and phosphonate (phosphonates, phosphonothionates, and phosphonothionothiolates) subclasses are very small in terms of number of insecticides in use (Chambers, 1992).

A survey of the structures of the phosphorothionates, phosphorthionothiolates, and phosphates suggests that the phosphate ester moiety in the bulk of the pesticides in these classes can be emulated by one of the six groups shown in Table 1. The *O*,*O*-diethyl phosphorothionate class was selected for the initial attempts, along an *O*-phenyl substitution. It was hoped that the larger size of the ethyl group (as opposed to methyl) along with the phenyl group would help increase antigenicity. It was also hoped that the sulfur atom, which is larger than

oxygen, would also increase antigenicity. Luckily, sulfur, rather than oxygen, is used in the bulk of commercially important OPs.

The structure of the immunizing hapten is by design very "generic", and it was hoped that the cross-reactivity of the resulting antisera would be such that members from the six groups would be detectable. The results of the current study indicate that the cross-reactivity is not broad enough to detect members from each of the six groups but, rather, that performance was very good for the subclass of compounds related to the immunizing hapten. This result suggests that an immunochemical approach for detection of OPs as a broad class may be achievable using a multiantibody approach, perhaps utilizing mixed monoclonal antibodies.

Dose−Response. Dose−response characteristics were determined for PBST buffered antisera solutions to which standard solutions of OPs dissolved in DMSO were added. Due to high viscosity, DMSO is often not an ideal solvent for ELISA procedures. The primary objective in this phase of the study was to grossly characterize multiple antisera with respect to their dose−response for multiple OPs. This was facilitated by use of robotics. The robotics system used, however, imposed its own requirements. Dilution of standards in microplate wells took ≈12 min from start to finish. Use of more volatile solvents such as methanol, ethanol, or 2-propanol was impossible due to evaporative errors, whereas DMSO did not suffer from this limitation.

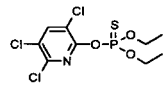
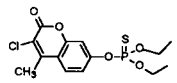
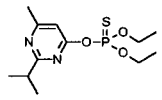
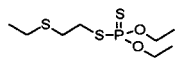
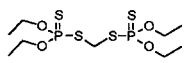
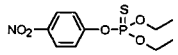
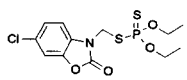
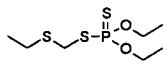
Another consideration which entered into the selection of DMSO rather than alcohols was that of solvolysis (alcoholysis). Solvolytic decomposition by nucleophilic species such as water or alcohols is a general phenomenon with OPs. Analytical schemes with specific OP compounds often resort to immediate preparation of analytical standards just prior to analysis to avoid problems. This potential problem was avoided by using DMSO.

Dose−response was investigated for 17 OPs. These compounds were selected on the basis of a number of criteria, including Agency interest and overlap with the previous work described in the Introduction. In addition, the selected compounds are among the most heavily applied OPs in the United States and thus would be expected to be frequent screening targets. Dose−response curves were constructed for each of the compounds using the "screening ELISA" procedure. Each compound was run three or more times. Twelve standards were run for each compound, spanning 8 orders of magnitude in concentration, ensuring that in most cases both the upper and lower asymptotes were well defined. Meeting this requirement is a necessary condition for valid calculation of IC_{50} values.

Results for IC_{50} calculations are shown in Tables 2−5. The compounds are classified according to the alkyl groups on the phosphorus ester and whether they contain oxo or thio esters. The third column reports the ratio of the literature IC_{50} values (IC_{50}, lit., taken from Sudi and Heeschen or Banks et al.) to the IC_{50} values determined in the current study (IC_{50} current). Thus, a value >1 indicates that the immunoassay system described in the current work has a lower IC_{50} value than previously described in the literature.

A number of observations are apparent from Table 2. First, the current system responds to both ring-containing OPs and non-ring-containing OPs, although not equally to all of the compounds surveyed. Not surprisingly, the current system responded most strongly to ethyl parathion, which emulates the immunizing hapten most closely; the calculated IC_{50} in this case is ≈5 ng/mL. In almost all cases, the current system exhibits a greater response than previously reported in the literature.

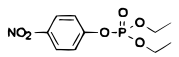
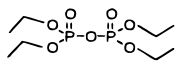
Table 2. Fifty Percent Inhibition Level for *O,O*-Diethyl Phosphorothionates and Phosphorothionothiolates[a]

Compound	IC_{50}, μg/mL	IC_{50}, Literature/ IC_{50} Current
 chlorpyrifos	1	56
 coumaphos	0.01	no data
 diazinon	0.037	273
 disulfoton	0.11	29
 ethion	28	0.11
 ethyl parathion	4.8×10^{-3} 9.4×10^{-4}	210 1064 Ab 4783
 phosalone (zolone)	3	no data
 phorate	0.09	35

[a] Literature values taken from Sudi and Heeschen (1988).

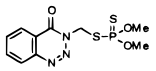
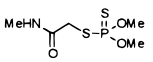
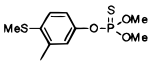
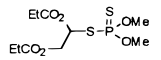
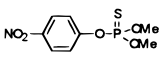
The data in Table 3 demonstrate that the system reported here is less responsive to *O,O*-diethyl phosphates than previous work, which is not surprising given that the immunizing hapten in previous work emulates ethyl paraoxon (Sudi and Heeschen, 1988). In one sense, this is inconsequential given that thionates and thionothiolates represent the bulk of applied OPs and generally are more toxic than the phosphates (Chambers, 1992). Interestingly, in the current work there is a smaller relative performance differential between thionate/thionothiolate and phosphate OPs than there is for the oxo-derived antisera based system reported by Sudi and Heeschen (1988). This result may be conceptualized in terms of the antibody binding site. For the previously reported phosphoro-derived antisera,

Table 3. Fifty Percent Inhibition Level for *O,O*-Diethyl Phosphates[a]

Compound	IC_{50}, μg/mL	IC_{50}, Literature/IC_{50} Current
 Ethyl Paraoxon	0.1	0.32
 TEPP	80	0.13

[a] Literature values taken from Sudi and Heeschen (1988).

Table 4. Fifty Percent Inhibition Level for *O,O*-Dimethyl Phosphorothionates and Phosphorothionothiolates[a]

Compound	IC_{50}, μg/mL	IC_{50}, Literature/IC_{50} Current
 azinphos-methyl (guthion)	4	79
 dimethoate	$>10^3$	no data
 fenthion	0.14	no data
 malathion	6.6	11.5
 methyl parathion	0.2	900

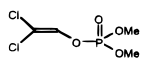
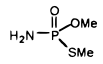
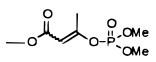
[a] Literature values taken from Sudi and Heeschen (1988).

one can hypothesize that a subpopulation of the antibodies have a binding site which allows "docking" of the O=P group. Clearly, the size of this site may be such that it is too small to accommodate the S=P group. In the case of the thionate-derived antisera reported in the current work, however, the analogous docking site would be large with respect to the O=P group, allowing it to readily "fit" loosely, while the remainder of the molecule interacts with epitopic features found in both classes of molecules.

In the case of *O,O*-dimethyl thionates, thionothiolates, and phophates (Tables 4 and 5), the current system provides mixed results. In cases for which comparative data exist, the present system has a gain of 1 to ~3 orders of magnitude increased response. Understandably, fenthion and methyl parathion, which closely resemble the immunizing hapten, gave the best response. For dimethoate and methamidophos, however, the present system exhibits poor response.

Further understanding of the behavior of the system can be gained by examining the dose−response data for these compounds. Figures 4, 5, and 6 depict dose−response curves for the *O,O*-diethyl thionates (and thionothiolates), *O,O*-dimethyl thionates (and thionothiolates), and the *O,O*-diethyl and *O,O*-dimethyl phosphates, respectively. All optical densities were normalized to a yield a maximum value of 1. Although the size of the data set is small, it is nevertheless quite interesting to note that in the case of the *O,O*-diethyl thionates (Figure 4), the data for all of the pesticides plotted cluster tightly about one of two four-parameter curve fits, labeled "A" and "B" in Figure 4. This is suggestive of a bimodal response. These data clearly show that for some of the OPs, the current system responds in a uniform and sensitive sigmoidal manner (in fact, the r^2 value for curve fit A was >0.93).

Table 5. Fifty Percent Inhibition Level for *O,O*-Dimethyl Phosphates[a]

Compound	IC_{50}, μg/mL	IC_{50}, Literature/IC_{50} Current
 DDVP	0.95	1.89, 95
 methamidophos (monitor)	$>10^3$	no data
 mevinphos	>0.1	no data

[a] Literature value taken from Banks et al. (1994) and Sudi and Heeschen (1988).

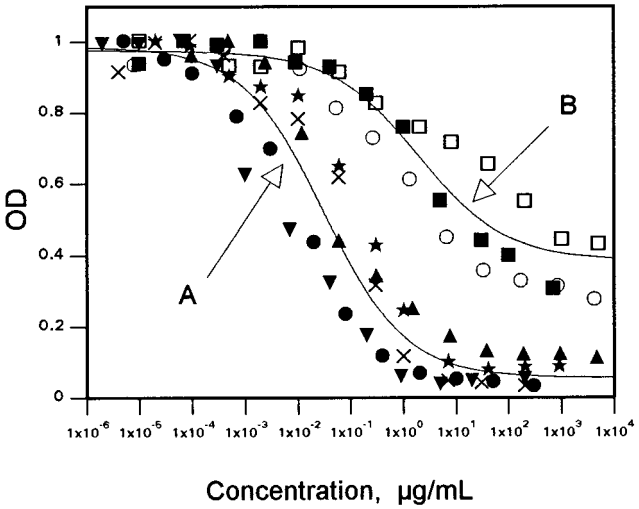


Figure 4. Dose−response for *O,O*-diethyl thionates and thionothiolates: chlorpyrifos (○); coumaphos (●); diazinon (▲); disulfoton (×); ethion (□); ethyl parathion (▼); phorate (★); zolone (■).

Detection limits for the *O,O*-diethyl thionates and thionothiolates were approximated as 3 standard deviations above the zero standard, calculated on the basis of the four-parameter curve fit for each of the compounds. These data are presented in Table 6. The current system would function well as a screen for the compounds clustered about curve fit A or any additional compounds that are found to respond in a similar manner upon further assay characterization. The three compounds clustered about curve fit B exhibit a shallower dose−response curve, which may be adequate for detection of these compounds, particularly if the assay is optimized further for sensitivity enhancement.

Table 6. Detection Limits for *O,O*-Diethyl Thionates and Thionothiolates

compound	detection limit, μg/mL	compound	detection limit, μg/mL
chlorpyrifos	1.6×10^{-3}	ethion	1.8×10^{-3}
coumaphos	1.5×10^{-4}	ethyl parathion	1.1×10^{-4}
diazinon	2.4×10^{-3}	phorate	1.4×10^{-3}
disulfoton	1.5×10^{-3}	zolone	3.1×10^{-2}

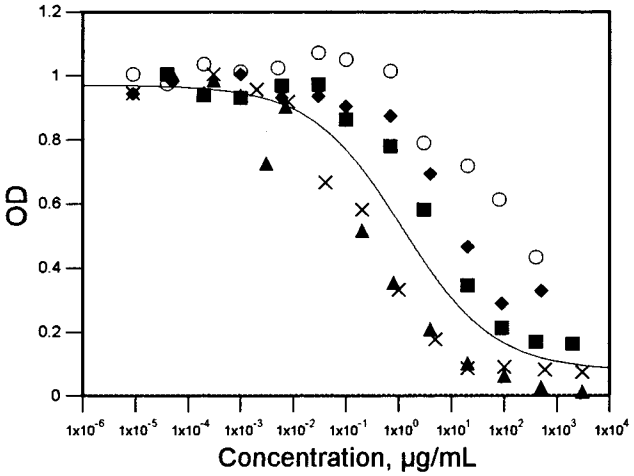


Figure 5. Dose−response for *O,O*-dimethyl thionates and thionothiolates: azinphos-methyl (■); dimethoate (○); fenthion (▲); malathion (◆); methyl parathion (×).

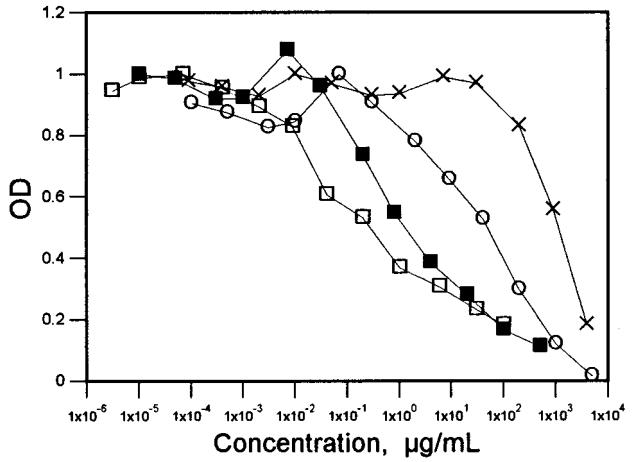


Figure 6. Dose−response for *O,O*-diethyl and *O,O*-dimethyl phosphates: DDVP (■); ethyl paraoxon (□); tetraethyl pyrophosphate (○); mevinphos (×).

In the case of the *O,O*-dimethyl thionates (Figure 5), the current system exhibits fairly smooth and uniform sigmoidal response, with the exception of dimethoate. The overall response for dimethoate may not be sigmoidal, although it cannot be determined from the present data because the lower asymptote was not defined by the standards used. Nevertheless, the dose−response for this particular group of compounds varies over a wide range and has a reasonably steep slope, allowing detection of at least these particular *O,O*-dimethyl thionates. With further optimization, "detection limits", which appear to be sub-parts per million currently, could be improved.

The *O,O*-diethyl and *O,O*-dimethyl phosphates exhibited more widely varied behavior (Figure 6). In the case of ethyl paraoxon, which very closely resembles the immunizing hapten, sigmoidal behavior is apparently exhibited, although the lower asymptote is poorly defined by the standards used. Detection of ethyl paraoxon, at fairly low levels (sub-parts per million) was readily possible. In the case of the other three compounds, the response is either irregular at the "detection limit" or sensitivity is not low enough. Nevertheless, the current system shows approximate sigmoidal response to all the analytes screened, although with irregularities or low sensitivity.

Conclusion. The present work, though carried out on a somewhat limited set of organophosphorus pesticides, demonstrates the feasibility of using immunochemical approaches for detection of multiple OP residues. It is clear that the currently reported system does not meet the "ideal" criteria for uniform response to all organophosphoro moiety-containing compounds. It does, however, work well for the *O,O*-diethyl thionates/thionothiolates reported here. In the case of the *O,O*-dimethyl thionates/thionothiolates, the results are mixed, whereas for the phosphates, the data show unacceptable performance, except for perhaps ethyl paraoxon, which closely resembles the immunizing hapten.

These results suggest that antibodies could be developed for different subclasses of OPs and, furthermore, that these antibodies could be combined to form a mixed system that would respond to a broad number of OPs. For example, one could envision the production of antibodies for the *O,O*-dimethyl thionates/thionothiolates, as well as antibodies for the phosphates. These immunochemical reagents could be used in an ELISA or in a time-resolved fluoroimmunoassay utilizing multiple lanthanide chelate labels.

It is important to bear in mind that optimization of assay parameters was not carried out extensively in the manner with which one might optimize a single-analyte immunoassay. The assay as presented, however, does allow for demonstration of a useful "cross-reactivity" toward multiple OPs belonging to the *O,O*-diethyl thionate/thionothiolate group. Keeping in mind the above caveats, it is clear that the current system represents significant progress toward the capability of broad-spectrum detection of OPs. Development of antibodies for the *O,O*-dimethyl thionates/thionothiolates and the phosphates represents a next logical step, as does investigation of sensitivity-enhancing formats and broadening the list of characterized OPs.

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