Mono- and Polyclonal Antibodies to the Organophosphate Fenitrothion. 1. Approaches to Hapten-Protein Conjugation

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Three different chemical approaches were used to couple the organophosphorus pesticide fenitrothion to carrier proteins for production of polyclonal and monoclonal antibodies. Hapten conjugates coupled through the pesticide phosphate group yielded the most specific and highest affinity antibodies, although antibodies of moderate affinity were obtained by derivatization and coupling through the aromatic nitro group following its reduction and amide formation with an adipic acid spacer arm. Assay formats using either immobilized antibody or immobilized hapten-protein conjugate could be used to determine free fenitrothion. The most sensitive assay could detect 1 ng of fenitrothion.

INTRODUCTION

A major means of protection of wheat and barley from insect infestation during storage is by application of pesticides to the grain at the time of storage. Since pesticides are directly applied to the grain, they must be of low mammalian toxicity. The most popular grain protectants are organophosphates and synthetic pyrethroids. The organophosphate fenitrothion [*O,O*-dimethyl *O*-(3-methyl-4-nitrophenyl) phosphorothioate, FN] has been used as a grain protectant in many countries since the late 1970s. It is a broad spectrum insecticide, toxic to many malathion-resistant species (Kashi, 1972; Ardley and Sticka, 1977; Pearson, 1979), and is especially useful in combination with synthetic pyrethroids, since the lesser grain borer, *Rhizopertha dominica* (F) can then be adequately controlled (Desmarchelier et al., 1981). FN also finds use in horticulture, viticulture, forestry, control of locust plagues, household sprays, and public health applications.

The most commonly used method for analysis of FN residues is gas-liquid chromatography (Sissons and Telling, 1970; Ambrus et al., 1981; Bottomley and Baker, 1984), although HPLC methods have been described (Funch, 1981). While simpler colorimetric tests (based on derivatization or hydrolysis of FN (Kovac and Sohler, 1965; Desmarchelier, 1981) or alternatively thin-layer chromatography/enzyme inhibition assays have been described (Bhaskar, 1982), none of these methods has found widespread use.

Over the past few years, enzyme-linked immunosorbent assay (ELISA) methods have been developed as attractive options for either the identification or quantification of a variety of agrochemicals in water, soil, and, in some cases, agricultural produce (Hammock et al., 1987; Jung et al., 1989). Most work has been done on immunoassays for herbicides, but some work on the organophosphorus pesticides parathion (Ercegovich et al., 1981; Heldman et al., 1985; Ngeh-Ngwainbi et al., 1986) and malathion (Haas and Guardia, 1968; Centeno et al., 1970) and the organophosphorus nerve gas soman (Hunter et al., 1982; Buenafe and Rittenberg, 1987) has been reported. In this paper, the development of specific polyclonal and monoclonal antibodies to FN is reported, with results obtained following three approaches to hapten-protein conjugation.

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MATERIALS AND METHODS

Synthesis of Fenitrothion-Protein Conjugates. Three approaches were used to prepare conjugates of FN and carrier protein for immunization and FN-peroxidase conjugates for use in the immunoassays: (1) reduction of the aromatic nitro group and direct diazo-coupling to protein (Figure 1, scheme A); (2) reduction of the aromatic nitro group and amide formation with an adipic acid spacer arm, which was then coupled to protein (Figure 1, scheme B); and (3) synthesis of a FN derivative in which the phosphate group could be coupled via an amino acid spacer arm to protein (Figure 1, scheme C). FN for synthetic work was obtained from Wellcome Ltd. (Cabarita, Australia) and for use in the ELISAs from Chem Service (West Chester, PA).

1. Diazo Conjugate Method. *O,O*-Dimethyl *O*-(4-amino-3-methylphenyl) phosphorothioate (I) ("reduced FN", 0.25 mmol; Mallet et al., 1978) in 2 mL of 0.5 M HCl was converted to the diazonium salt by treatment with 23 mg of $NaNO_2$ in 1 mL of water. After mixing 10 min at 0 °C, the diazonium salt was reacted at 0 °C with protein (15 mg) in 15 mL 0.2 M potassium phosphate, pH 9.1; 0.1 M NaOH was added to maintain pH.

2. Adipic Acid Spacer Arm Method. To reduced FN (1.0 mmol) in toluene (5 mL, 0 °C) was added 1.2 mmol of ethyladipyl chloride (Morgan and Walton, 1933) in 5 mL of toluene. Sodium carbonate solution (1.0 M) was added to maintain neutral reaction conditions. After 30 min of stirring at 0 °C, the organic phases were washed with brine, dried, and concentrated to give an oil, shown to be homogeneous by both ^1H NMR and thin-layer chromatography. The methyl ester so formed was hydrolyzed in 5 mL of ethanol-2 mL of 1 M KOH (2 h, 20 °C). Following acidification and extraction with ethyl acetate, the acid (340 mg, 91% yield) was purified by chromatography on silica gel (eluted with 70% ethyl acetate-30% petroleum ether) and esterified at 0 °C using *N*-hydroxysuccinimide (NHS, 1.1 mmol) plus dicyclohexylcarbodiimide (DCC, 1.1 mmol) in dichloromethane (10 mL). The product, an oil, was purified by column chromatography on silica gel (eluted with 10% ethyl acetate in chloroform).

3. Coupled Phosphate Group Method. This method required initial synthesis of a bifunctional phosphate intermediate, using a method adapted from Carter et al. (1955), as follows. To β-alanine (90 mmol) was added benzyl chloroformate (100 mmol) and 2 M NaOH (100 mL) with the mixture being stirred at 4 °C for 35 min. Unreacted materials were extracted into toluene, and following ether extraction the aqueous layer was acidified to pH 4 with HCl. The colorless precipitate (*N*-benzyloxycarbonyl-β-alanine) (IV) (69% yield) was practically pure [mp 103-105 °C [lit. 106 °C (Sifferd and Vigneaud, 1935)]].

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Dry dichloromethane (20 mL), IV (200 mmol), and *tert*-butyl alcohol (60 mmol) were mixed and then cooled in an ice bath. (4-Dimethylamino)pyridine (200 mg) and DCC (22 mmol) were added, and the mixture was stirred at 20 °C for 5 h. The mixture was filtered, and the resultant solid was washed twice with dichloromethane (15 mL), with the filtrate and dichloromethane washings being combined. The dichloromethane solution was then washed sequentially with water, dilute acid (1 M HCl), water, 5% sodium bicarbonate solution, water, and brine. The organic layer was dried and filtered, and the solvent was removed to give an oil (5.6 g). The oil was dissolved in ethanol (50 mL)–acetic acid (0.5 mL), and Pd/C (5%, 100 mg) was added. The reaction mixture was shaken for 3 days in a hydrogen atmosphere. The catalyst was removed by filtration, and the filtrate was concentrated to an oil. The oil was dissolved in ether (70 mL), and the amine was extracted by washing twice with 1 M HCl (40 mL); the washings were treated with sodium hydroxide (1 M) to pH >13, and then extracted twice with chloroform (50 mL). The chloroform layers were combined, dried, filtered, and evaporated to give *tert*-butyl-3-aminopropanoate (V), an oil, bp 44–48 °C 1.0 mmHg [lit. 48 °C, 1.5 mmHg (Campbell et al., 1968)].

Synthesis of the spacer arm, *O*-methyl *N*-[2-[(*tert*-butyloxy)carbonyl]ethyl]phosphoramidochloridothioate (VI) was completed as follows. To V (10 mmol) in benzene (40 mL) cooled to 5 °C was added potassium carbonate (20 mmol) and *O*-methyl phosphoroamidochloridothioate (E. J. Tarbor, U.S. Patent 3,005,005). This mixture was stirred overnight at room temperature and then filtered. The filtrate was washed with acid (1 M HCl), the layers were separated, and the organic layer was washed with brine, separated, and dried. Removal of the solvent gave an oil (1.8 g, 67%) which crystallized on standing at −5 °C. The crystals melted below room temperature. Proton NMR was used for identification. Proton NMR (200 MHz): δ 4.35 (b, NH), 3.83 (d, J_{POCH_3} = 14 Hz, OCH_3), 3.28 (dd, $J_{CH_2CH_3}$ = 6.1 Hz, J_{PNCH_2} = 13.5 Hz, NCH_2), 2.50 (t, $COCH_2$), 1.46 [s, $C(CH_3)_3$]. The chloride (VI) was stable at −5 °C under dry conditions for at least 6 months. For developing fenitrothion conjugates, product VI (2.0 mmol) was added to acetonitrile (10 mL) and sodium 3-methyl-4-nitrophenoxide (2.2 mmol). The mixture was refluxed for 1 h and then filtered. The filtrate was concentrated, and the residue was taken up into ethyl acetate (50 mL) and washed sequentially with water, sodium hydroxide (0.1 M), water, acid (1.0 M HCl), water, and brine. The organic layer was dried over $MgSO_4$ and the solvent removed. The residue, an oil, was chromatographed (20 g of silica gel, eluted with 20% ethyl acetate/petroleum ether) to give the product *O*-methyl *O'*-(3-methyl-4-nitrophenyl) *N*-[2-[(*tert*-butyloxy)carbonyl]ethyl]phosphoramidothioate (VII) as an oil (165 mg, 21%). Proton NMR (90 MHz): δ 8.02 (d, $J_{H5',6'}$ = 9.5 Hz, H5), 7.16 (m, H2,6), 3.80 (d, J_{POCH_3} = 14.4 Hz, OCH_3), 3.31 (m, NCH_2), 2.62 (s, Ar CH_3), 2.46 (t, $J_{CH_2CH_2}$ = 6.4 Hz, $COCH_2$), 1.46 [s, $C(CH_3)_3$].

The ester (VII) (0.7 mmol) was added to trifluoroacetic acid (2 mL), and the solution was stirred (1 h). The solution was concentrated and the residue was dissolved in toluene. Removal of the solvent gave an oil which was chromatographed (silica gel, 20 g, eluted with 2% methanol–0.5% acetic acid–97.5% chloroform) to give the acid [*O*-methyl *O'*-(3-methyl-4-nitrophenyl) *N*-(2-carboxyethyl)phosphoramidothioate (VIII)] as an oil (230 mg, 60%). Proton NMR (90 MHz): δ 8.03 (d, $J_{H5,6}$ = 9.8 Hz, H5), 7.18 (m, $H_{2,6}$), 3.81 (d, J_{POCH_3} = 14.0 Hz, OCH_3), 3.34 (m, NCH_2), 2.62 (s, Ar CH_3), 2.62 (t, $J_{CH_2CH_2}$ = 6.5 Hz, $COCH_2$). The acid product (VIII, 230 mg, 0.7 mmol) was dissolved in dichloromethane (0 °C, 5 mL) to which *N*-hydroxysuccinimide (110 mg, 0.9 mmol) followed by DCC (190 mg, 0.9 mmol) and DMAP (10 mg) was added. The mixture was stirred overnight and filtered, and the solvent was removed. Chromatography of the resultant oil on silca gel, eluted with 1% methanol–99% chloroform, gave an ester [*O*-methyl *O'*-(3-methyl-4-nitrophenyl) *N*-[[(succinimidooxy)carbonyl]ethyl]phosphoramidothioate (IX)] as a syrup (195 mg, 62%). Proton NMR (90 MHz): δ 8.04 (d, $J_{H_5,6}$ = 9.5 Hz, H_5), 7.19 (m, $H_{2,6}$), 4.15 (m, NH), 3.81 (d, J_{POCH_3} = 13.0 Hz), 3.54 (m, NCH_2), 2.88 (s, $NCOCH_2CH_2CO$), 2.87 (m, $COCH_2$), 2.61 (s, Ar CH_3).

Coupling of Activated Fenitrothion Succinimide Esters (Methods 2 and 3) to Carrier Proteins and HRP. To either chicken immunoglobulin G (IgY, Sigma, St. Louis, MO) or OA (25 mg) in 15 mL of 0.2 M K_2HPO_4, pH 9.1, at 4 °C was added IX (25 mol equiv) in *N,N*-dimethylformamide. The solutions were mixed and allowed to stand overnight at 4 °C. Uncoupled hapten was removed by passing the solution through Sephadex G25 (Pharmacia, Uppsala, Sweden) using 0.025 M KH_2PO_4–0.15 M NaCl–0.01% NaN_3, pH 7.2 (PBS) as eluant. The concentration of coupled protein in solutions obtained was determined according to the Lowry method (Lowry et al., 1951). The amount of coupling was determined by assuming the principal coupling was with free amines on the proteins. The number of amines coupled was determined by the difference in free amines of coupled protein and untreated proteins, as determined using trinitrobenzenesulfonic acid (Plapp et al., 1971).

Horseradish peroxidase (HRP) and a 40 mole excess of product IX were treated similarly to give the enzyme coupled to the thiophosphate. The eluant was phosphate-buffered saline (as before) using thimerosal (0.01%) as the preservative, as azide inactivates HRP. The concentration of enzyme was determined using E_{403}^{1cm} = 2.25 for a 1 mg/mL solution. The amount of coupling was determined by a modification of the trinitrobenzenesulfonic acid technique above. Thus, to 200 μg of enzyme in 200 μL of PBS was added 0.1 M sodium borate buffer (pH 9.5, 700 μL) and Triton X-100 (100 μL of a 10% solution), and the mixture was heated to 60 °C for 15 min and cooled; then amines were determined according to the method of Plapp et al. (1971). This modification allowed a more reproducible difference between the treated enzyme and untreated enzyme to be determined.

Properties of FN–Protein Conjugates. The protein concentrations of these conjugates were calculated by a modified dye-binding assay (Sharma and Tjhon, 1988) which reduces protein-to-protein variation. Bovine serum albumin (BSA) was used as the assay standard. Substitution of protein with hapten was assessed for the diazo derivative by assuming that tyrosine and histidine residues were the principal reaction sites and use of *N*-acetyl derivatives of these amino acids as model compounds (Fenton and Singer, 1971) for spectrophotometric determination of the degree of substitution.

The following degrees of substitution were obtained for antigens and immunogens: (1) FN–diazo proteins, 18, 112, and 0.5–2.5 mol of FN/mol of OA(ODF), KLH (KDF), and HRP; (2) FN–C6 proteins, 9.2, 27.8, and 2.8 mol of FN/mol of OA (ONF), IgY (YNF), and HRP; (3) FN–P proteins, 5.8, 15.0, and 1.0 mol of FN/mol of OA (OPF), IgY (YPF), and HRP. Lower degrees of substitution for each FN–protein complex were less immunogenic.

Polyclonal Antibody Production. For each FN–protein route of coupling, either or both of the OA and IgY conjugates (or KLH for diazo proteins) were used as immunogens in rabbits and mice and as coating antigens in the ELISA. For immunizations, 1.0 mg of FN–protein, dissolved in 0.5 mL of 0.9% NaCl (saline), was emulsified with an equal volume of Freund's complete adjuvant and half the dose was injected subcutaneously and half intramuscularly into New Zealand white rabbits. Subsequent booster injections (0.5 mg of conjugate in Freund's incomplete adjuvant–saline) were performed 2, 4, 8, 16, 20, 24, 28, 32, 36, and 40 weeks later. Blood was collected 8–9 days following each immunization. IgG was purified from the serum fraction using affinity chromatography on protein A–Sepharose (Pharmacia) according to the method of Goding (1978). Up to four rabbits were immunized with each FN–protein conjugate, but results shown here were obtained from individual rabbits and individual bleeds (after the fourth injection).

Monoclonal Antibody (MAb) Production. Mice were immunized as for rabbits, except that 0.4 mg of protein–FN conjugate in 0.4 mL was used and doses were divided intraperitoneally and subcutaneously. Following two boosters at fortnightly intervals, mice were bled 8 days later. Those animals with a combination of high titer and good displacement by free FN were rested 6–10 weeks and boosted intraperitoneally with 0.4 mg of protein–FN conjugate; their spleens were removed for fusion of spleen cells with Sp 2/0 myelomas. Methods used for monoclonal antibody production in our laboratory have been described elsewhere (Skerritt and Underwood, 1986). Following recloning, appropriate hybridomas were grown as ascites tumors

in mice. IgG antibody from peritoneal fluids in these mice was purified by protein G–Sepharose chromatography (Akerstrom et al., 1985).

Immunoassays. *1. ELISA Using Solid-Phase Bound Fenitrothion–Protein Conjugates.* A checkerboard assay, in which sera was titrated against varying amounts of a conjugate of FN and the protein not used as the immunogen, was used to optimize antigen coating and antibody concentrations. The lowest antigen concentration (usually 150 ng/well) yielding an absorbance of 1.0 or more with serum dilutions greater than 1/5000 was chosen. The ELISA assays using solid-phase bound FN–protein conjugates were performed as follows: Antigen in 100 μL of 50 mM sodium carbonate–bicarbonate buffer, pH 9.6, was coated (16 h, 20 °C) onto each well of 96-well Nunc Maxisorp plates (Roskilde, Denmark). Plates were then washed twice in phosphate-buffered saline (PBS, 50 mM sodium phosphate–0.9% NaCl, pH 7.2)–0.05% Tween 20 (Sigma), and unbound active sites were blocked with 1% BSA in PBS for 1 h at 20 °C. All assay steps were performed at room temperature (20–24 °C).

FN standards in methanol or methanol wheat extracts were diluted at least 1/5 in PBS–1% BSA–0.05% Tween 20, and 50 μL was added to appropriate wells. Fifty microliters of horseradish peroxidase-labeled antibody, diluted in PBS–1% BSA–Tween 20, was immediately added, and microwell contents were mixed by gentle agitation. [In preliminary antibody characterization experiments, unlabeled FN-binding antibodies were used, and following a washing step, antibody binding was assessed by use of HRP-labeled swine-anti-rabbit or rabbit-anti-mouse antibodies (Dako, Glostrup, Denmark).] Following four washes with PBS–Tween, peroxidase substrate–chromogen [2,2′-azinobis(3-ethylbenzthiazoline-6-sulfonic acid)], 4.5 mM in 100 mM sodium citrate buffer, pH 4.5, containing 0.003% (v/v) hydrogen peroxide (total volume 150 μL), was added and incubated for 15 min. Color development was terminated by addition of 50 μL of 3% oxalic acid, and the absorbance was read at 414 nm (Skerritt and Hill, 1991).

2. ELISA Using Solid-Phase Bound Antibody. These assays were performed as per the ELISA with solid-phase bound antibody but with the following modification: Purified IgG antibody in 100 μL of 50 mM sodium carbonate buffer, pH 9.6, was coated for 60 min, and then immediately after addition of FN standard or test sample, 50 μL of HRP-labeled fenitrothion diluted in PBS–BSA–Tween was added to each well. After a 30-min incubation, plates were washed and peroxidase substrate–chromogen was added.

RESULTS

Antibody Response to Different FN–Protein Conjugates. In initial studies, the FN nitro group was reduced and the resulting amino group was coupled either to protein by direct diazotization (Figure 1) or to protein amino groups through a 6-carbon spacer arm based on the acyl chloride of ethyl adipate. Polyclonal rabbit antibodies produced to the diazo conjugates of both FN–keyhole limpet hemocyanin (KLH) (conjugate is termed KDF) and FN–OA (termed ODF) recognized fenitrothion when coupled to protein. However, such binding was inhibited by free fenitrothion in the case of the KDF antibody only, and inhibition (50% at 3 μg/mL FN, Figure 2A) was very weak. Mice immunized with ODF yielded 11 hybridomas (from 384 wells) reactive with KDF antigen, and mice immunized with KDF yielded 9 hybridomas (from 384 wells). However, inhibition of antibody–antigen binding with 10 μg/mL FN was very weak (<20%) in all cases. Attempts to develop an immobilized antibody-based assay for the antibodies to FN–diazo proteins were not successful, since enzymic activity of the marker enzyme used, horseradish peroxidase, was destroyed during diazotization with the fenitrothion amine over a wide range of hapten–enzyme ratios.

More sensitive detection of FN was possible when the 6-carbon spacer arm was used with the FN amine. Polyclonal antisera to FN–C6–IgY (YNF) was of especially

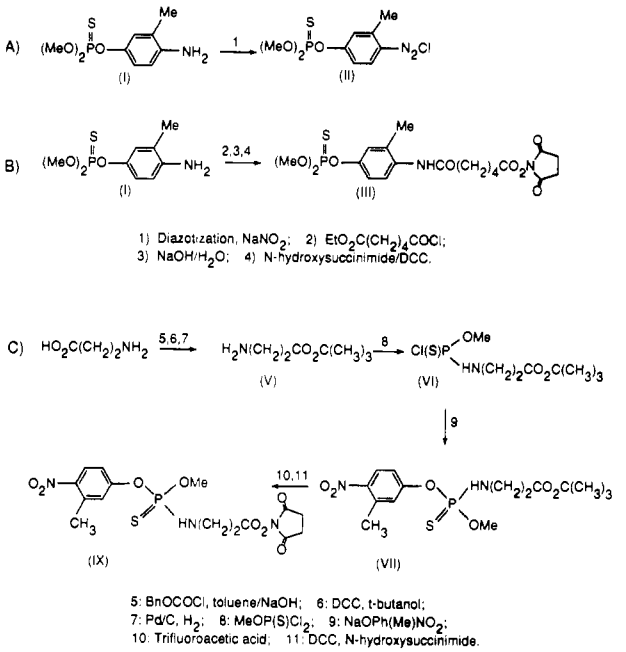

Figure 1. Synthetic scheme for the production of various fenitrothion–protein conjugates: (A) direct diazotization (ODF, KDF); (B) linking through reduced nitro group using a 6-carbon spacer arm (ONF, YNF); (C) linking through the fenitrothion phosphate group (OPF, YPF). See text for further detail. Reaction 5 yielded intermediate IV, *N*-benzyloxycarboxy-β-alanine.

high titer. Appreciable inhibition by free FN was seen in the immobilized antibody form but not in the immobilized antigen (FN–C6–OA) format. In contrast, antibodies to ONF only performed in the immobilized antigen format (Figure 2B). IgY was used in place of KLH for this and subsequent conjugates due to the low solubility of KLH. Specific monoclonal antibodies were obtained to FN–C6–IgY (YNF). The yield of hybridomas recognizing both FN–C6–OA (ONF) and free FN (over 30% inhibition at 10 μg/mL) was 4 of 192.

Direct diazo coupling of the reduced amine form of the aromatic nitro-organophosphate pesticide has been used with moderate success in the case of parathion, which is structurally related to FN (Ercegovich et al., 1981). Subsequent work (Vallejo et al., 1982) used a variety of spacer arms, including two linked through the reduced nitro group. The glutarimine spacer arm used by these workers is similar to the adipic acid spacer used in the present study. However, these authors obtained poor inhibition with antibodies to this derivative with free parathion. Diazotized aminophenyl spacer arms worked well in generations of soman antibodies (Buenafe and Rittenburg, 1987).

While the antibodies raised to spacer-arm-derivatized proteins (coupled through the reduced nitro group) had reasonably high specificities (Hill et al., 1992), it was proposed that antibodies of greater specificity and possibly higher affinity may be produced by conjugation of fenitrothion through the thiophosphate group, thus exposing the functional groups unique to fenitrothion (Erlanger, 1981). In fact, antibodies to such conjugates (OPF and YPF) were higher in affinity (Figure 2B) than those raised by other means. Specific monoclonal antibodies were also obtained: for mice immunized with OPF, 5 hybridomas/192 wells recognized YPF antigen, 3 showing >30% inhibition at 10 μg/mL FN; YPF mice, 7/192 recognized OPF antigen, 5 showed significant inhibition. With these hybridomas, detection of FN in the immobilized antibody

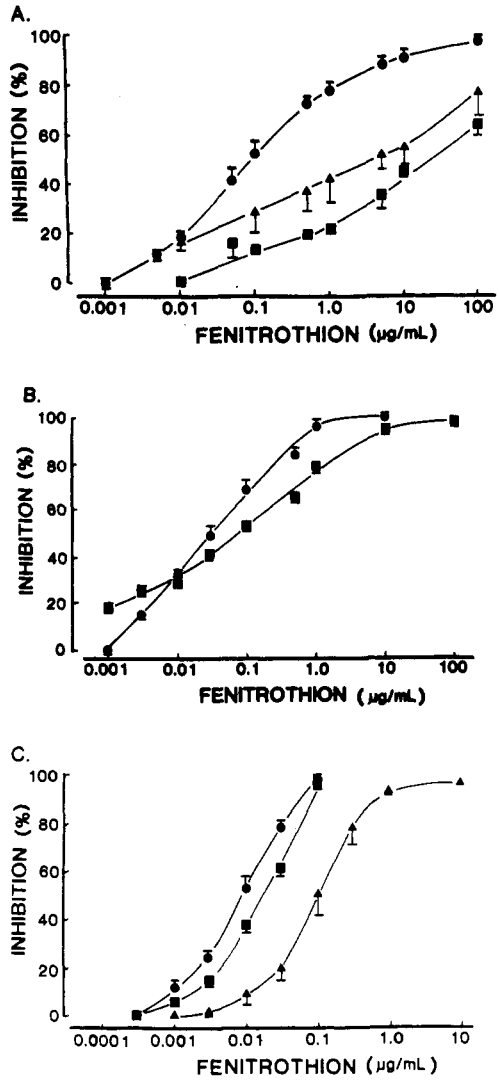

Figure 2. Standard curves for different fenitrothion antibodies and assay formats. (A) Polyclonal antibodies to fenitrothion–protein conjugates coupled through the nitro group. Antibodies: PAb YNF on solid phase (●); PAb ONF in solution (■); PAb KDF in solution (▲). (B) Polyclonal antibodies to FN conjugates coupled through the phosphate group. Antibodies: PAb YPF solid phase (●); PAb YPF in solution (■). (C) Monoclonal antibodies to FN conjugates coupled through the phosphate group. Antibodies: MAb YPF 10/20 on solid phase (●); MAb YPF 10/20 in solution (■); MAb OPF 8/24 in solution (▲). Data shown are means ± SD of 2–10 determinations.

format was especially sensitive, with a limit of detection (10% inhibition) at 2 ng for the polyclonal antibody (Figure 2B) and 3 ng for the monoclonal antibody (Figure 2C; Table I).

Dynamic Response to Fenitrothion. The FN concentration–absorbance plots for the more sensitive assays were sigmoidal, with a linear portion between 25 and 75% of maximal absorbance (Figure 2B,C). The absorbance change per change in FN concentration could be calculated from the slope of the linear part of the plot. The most dynamic antibody was using monoclonal antibody PF10/20 (raised to OPF) on the solid phase. A 5-fold change in FN concentration caused a 35% change in absorbance in this format. However, the assay with the corresponding polyclonal antibody was rather similar. A steep standard curve is important to enable quantitative analysis of pesticide in the sample.

Table I. Comparison of Sensitivity and Dynamic Response of Different Fenitrothion Assays

assay format/antibody	sensitivity: limit of detection[a]	sensitivity: I_{50}[b]	dynamic response[c]
solution-phase antibody			
1. Anti P-FN			
PAb-YPF (2)	0.001	0.09	30 ● 1
MAb-YPF 10/20 (13)	0.004	0.07	30 ● 2
solid-phase antibody			
1. Anti C6-FN			
PAb-YNF (2)	0.004	0.100	14 ± 1
2. Anti P-FN			
PAb-YPF (11)	0.002	0.023	26 ± 2
MAb-YPF 10/20 (18)	0.003	0.028	35 ± 6

[a] Concentration (μg/mL) providing [a]10% inhibition, [b]50% inhibition, [c]difference in % inhibition for a 5-fold difference in concentration, at center of concentration–response curve. Number of determinations is shown in parentheses.

GENERAL DISCUSSION

Several antibodies and formats were developed which could detect FN sensitively; however, the most suitable of these appeared to be the test based on a monoclonal antibody (prepared using an FN immunogen coupled through the phosphate group to carrier proteins) immobilized on the microwell. This assay was quite sensitive and rather dynamic. Monoclonal antibodies also offer the advantages of potential scaleup of production of a well-defined antibody with constant properties. The corresponding polyclonal antibody prepared the same way and used in the same assay format was only slightly less sensitive but had a flatter concentration–response curve. In general, assays with solid-phase bound antibody were more sensitive (to free FN) than those using solid-phase FN–protein derivatives. Most likely, this results from mass action considerations; e.g., both immobilized antibody and enzyme-labeled FN contain two hapten binding sites and up to three haptens respectively, while FN–protein derivates are more highly substituted.

This paper describes a simple and sensitive ELISA assay for the organophosphate fenitrothion. While this compound is used in a number of agricultural and domestic applications, our special interest is in the analysis of residues on grain. In the accompanying paper (Hill et al., 1992) we assess the specificity of antibodies and their performance in quantitation of FN residues in grain.

ABBREVIATIONS USED

FN, fenitrothion; NHS, *N*-hydroxysuccinimide; DCC, dicyclohexylcarbodimide; OA, ovalbumin; KLH, keyhole limpet hemocyamin; HRP, horseradish peroxidase; IgY, chicken immunoglobulin G; BSA, bovine serum albumin; PBS, phosphate-buffered saline; Y/ODF, IgY or albumin coupled to fenitrothion by diazotization; KY/ONF, KLH, IgY, or albumin coupled to fenitrothion by 6-carbon spacer arm; Y/OPF, spacer arm coupled to fenitrothion through the phosphate group.

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LITERATURE CITED

Akerstrom, B.; Brodin, T.; Reis, K. J.; Lawman, M. J. P. Protein G: A powerful tool for binding and detecting monoclonal and polyclonal antibodies. *J. Immunol.* **1985**, *135*, 2589–2592.

Ambrus, A.; Lantos, J.; Visi, E.; Csatlos, I.; Sarvari, L. General method for determination of pesticide residues in samples of plant origin, soil and water. I. Extraction and cleanup. *J. Assoc. Off. Anal. Chem.* **1981**, *64*, 733–742.

Ardley, J. H.; Sticka, R. The effectiveness of fenitrothion and malathion as grain protectants under bulk storage conditions in New South Wales, Australia. *J. Stored Prod. Res.* **1977**, *13*, 159–168.

Bhaskar, S. V. An enzymatic method for TLC detection and determination of fenitrothion in water. *Talanta* **1982**, *29*, 133–138.

Bottomley, D.; Baker, P. G. Multiresidue determination of organochlorine, organophosphate and synthetic pyrethroid pesticides in grain by gas-liquid and high performance liquid chromatography. *Analyst* **1984**, *109*, 85–90.

Buenafe, A. C.; Rittenburg, M. B. Combining site specificity of monoclonal antibodies to the organophosphate hapten, soman. *Mol. Immunol.* **1987**, *24*, 402–407.

Campbell, R.; Purkayastha, R.; Young, G. T. Amino acids and peptides. XIX. The use of S-benzylthiomethyl L-cysteine in peptide synthesis: Synthesis of glutathione and homoglutathione. *J. Chem. Soc. C* **1968**, 1219–1224.

Carter, H. E.; Frank, R. L.; Johnston, H. W. Carbobenzoxy chloride and derivatives. *Organic Syntheses*; Wiley: New York, 1955; Collect. Vol. III, pp 167–169.

Centero, E. R.; Johnson, W. J.; Sehon, A. H. Antibodies to two common pesticides, DDT and malathion. *Int. Arch. Allergy Appl. Immunol.* **1970**, *37*, 1–13.

Desmarchelier, J. M. A rapid, colourimetric procedure for determination of fenitrothion. *J. Environ. Sci. Health* **1981**, *B16*, 419–426.

Desmarchelier, J.; Bengston, M.; Connell, M.; Henning, R.; Ridley, E.; Ripp, E.; Sierakowski, C.; Sticka, R.; Snelson, J.; Wilson, A. Extensive pilot use of the grain protectant combinations, fenitrothion plus bioresmethrin and pirimiphos-methyl plus bioresmethrin. *Pestic. Sci.* **1981**, *12*, 365–374.

Ercegovich, C. D.; Vallejo, R. P.; Gettig, R. P.; Woods, L.; Bogus, E. R.; Mumma, R. O. Development of a radioimmunoassay for parathion. *J. Agric. Food Chem.* **1981**, *29*, 559–568.

Erlanger, B. F. Principles and methods for the preparation of drug-protein conjugates for immunological studies. *Pharm. Rev.* **1981**, *25*, 271–280.

Fenton, J. W.; Singer, S. J. Affinity labeling of antibodies to the p-azophenyltrimethylammonium hapten, and a comparison of affinity-labeled antibodies of two different specifities. *Biochemistry* **1971**, *10*, 1429–1437.

Funch, F. H. Analysis of residues of seven pesticides in some fruit and vegetables by high-pressure liquid chromatography. *Z. Lebensm. Unters. Forsch.* **1981**, *173*, 95–98.

Goding, J. W. Use of Staphyloccal protein A as an immunological reagent. *J. Immunol. Methods* **1978**, *13*, 215–226.

Haas, J. H.; Guardia, E. J. Production of antibodies against insecticide-protein conjugates. *Proc. Soc. Exp. Biol. Med.* **1968**, *129*, 546–551.

Hammock, B. D.; Gee, S. J.; Cheung, P. Y. H.; Miyamoto, T.; Goodrow, M. H.; Van Emon, J.; Seiber, J. N. *Utility of immunoassay in biotechnology*; Greenhalgh, R., Roberts, T. R., Eds.; Blackwell Scientific: Ottawa, 1987; pp 309–316.

Heldman, E.; Balan, A.; Horowitz, O.; Ben-Zion, S.; Torten, M. A novel immunoassay with direct relevance to protection against organophosphate poisoning. *FEBS Lett.* **1985**, *180*, 243–248.

Hill, A. S.; Beasley, H. L.; McAdam, D. P.; Skerritt, J. H. Mono- and polyclonal antibodies to the organophosphate, fenitrothion. 2. Antibody specificity and assay performance. *J. Agric. Food Chem.* **1992**, following paper in this issue.

Hunter, K. W.; Lenz, D. E.; Grimfield, A. A.; Naylor, J. A. Quantification of the organophosphorus nerve agent soman by competitive inhibition enzyme immunoassay using monoclonal antibody. *FEBS Lett.* **1982**, *149*, 147–151.

Jung, F.; Gee, S. J.; Harrison, R. O.; Goodman, M. H.; Karu, A. E.; Braun, A. L.; Li, Q. X.; Hammock, B. D. Use of immunochemical techniques for the analysis of pesticides. *Pestic. Sci.* **1989**, *26*, 303–317.

Kashi, K. P. An appraisal of fenitrothion as a promising grain protectant. *Int. Pest Control* **1972**, 20–22.

Kovac, J.; Sohler, E. Determination of O,O-dimethyl(O-3-methyl-4-nitrophenyl)thiophosphate residues in fruits and vegetables following the separation of coextracted dyes by thin-layer chromatrography. *Fresnius' Z. Anal. Chem.* **1965**, *208*, 201–204.

Lowry, O. H.; Roseborough, N. J.; Farr, A. L.; Randall, R. J. Protein measurement with the Folin phenol reagent. *J. Biol. Chem.* **1951**, *193*, 265–275.

Mallet, V. N.; Brun, G. L.; MacDonald, R. N.; Berkane, K. A comparative study of the use of XAD-2 resin and the conventional serial solvent extraction procedure for the analysis of fentrothion and some derivatives in water-preservation techniques. *J. Chromatogr.* **1978**, *160*, 81–88.

Morgan, G. T.; Walton, E. New derivatives of p-arsanilic acid. IV. p-Arsanoadipanilic acid and related compounds. *J. Chem. Soc.* **1933**, *91*, 91–93.

Ngeh-Ngwainbi, J.; Foley, P. H.; Huan, S. S.; Guilbault, G. Parathion antibodies on piezoelectric crystals. *J. Am. Chem. Soc.* **1986**, *108*, 5444–5447.

Pearson, R. Effectiveness of liquid fenitrothion against pests in stored grain. *Milling Feed Fert.* **1979**, *162*, 30–32.

Plapp, B. V.; Moore, S.; Stein. W. M. Activity of bovine pancreatic deoxyribonuclease A with modified amino groups. *J. Biol. Chem.* **1971**, *246*, 939–945.

Sharma, H. K.; Tjhon, C. Determination of avidin and steptavidin by a modified Bradford method. *Anal. Biochem.* **1988**, *170*, 135–139.

Sifferd, R. M.; du Vigneaud, V. A new synthesis of carnosine with some observations on the splitting of the benzyl group from carbo-benzoxy derivatives and from benzyl thioethers. *J. Biol. Chem.* **1935**, *108*, 753–761.

Sissons, D. J.; Telling, G. M. Rapid procedure or the routine determination of organophosphate insecticide residues in vegetables. I. Determination of hexane-soluble insecticides by gas-liquid chromatography and total phosphorous procedures. *J. Chromatogr.* **1970**, *47*, 328–340.

Skerritt, J. H.; Hill, A. S. Enzyme-immunoassay for determination of gluten in foods: collaborative study. *J. Assoc. Off. Anal. Chem.* **1991**, *74*, 257–264.

Skerritt, J. H.; Underwood, P. A. Specificity characteristics of monoclonal antibodies to wheat grain storage proteins. *Biochim. Biophys. Acta* **1986**, *874*, 245–254.

Vallejo, R. P.; Bogus, E. R.; Mumma, R. O. Effects of hapten structure and bridging groups on antisera specificity in parathion immunoassay development. *J. Agric. Food Chem.* **1982**, *30*, 572–580.

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