Hapten Synthesis and Development of ELISAs for Detection of Endosulfan in Water and Soil

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Two enzyme immunoassays, a laboratory assay based on microwell plates and a field test based on the use of small polystyrene tubes, have been developed for the detection of endosulfan (1,4,5,6,7,7-hexachloro-8,9,10-trinorborn-5-en-2,3-ylenedimethyl sulfite) residues in water and soil. To raise antibodies that are sensitive and selective for the toxic forms of endosulfan, three haptens were prepared. One hapten was prepared by derivatization of endosulfan diol [1,4,5,6,7,7-hexachloro-2,3-bis(hydroxymethyl)norborn-5-ene], while the others used derivatives of a rigid five-membered ring adjacent to the bridged hexachlorocyclopentadiene (cyclodiene) ring. Different hapten combinations were used for immunogen and reporter enzyme conjugate in both the microwell and field assays. The optimized assays have detection limits of about 0.2 ppb endosulfan and detect in the range 0.2–10 ppb (0.2–20 ppb for field assay, without sample dilution). Water samples can be analyzed directly without solvent extraction or concentration, while soil samples are simply extracted with 90% methanol. The tests detect endosulfan sulfate (1,4,5,6,7,7-hexachloro-8,9,10-trinorborn-5-en-2,3-ylenedimethyl sulfate) with sensitivity similar to that for endosulfan but are 4–10 times less sensitive to endosulfan diol, and therefore the assays can potentially determine toxic compounds of endosulfan (endosulfan and endosulfan sulfate) from the total endosulfan residues present in the environment.

Keywords: *Immunoassay; endosulfan; organochlorine; insecticide; field test; environmental analysis; soil; water*

INTRODUCTION

Endosulfan is used on a wide range of crops including cereals, coffee, cotton, fruits, oilseeds, tea, and vegetables, yet the most important use of endosulfan in Australia and many other countries is to control *Helicoverpa* species, especially in cotton cropping (Fitt, 1994). It is also used extensively in public health applications in developing countries (Mattiessen *et al.*, 1982). Endosulfan has replaced other organochlorine insecticides such as dieldrin (1*R*,4*S*,5*S*,8*R*)-1,2,3,4,10,-10-hexachloro-1,4,4a,5,6,7,8,8a-octahydro-6,7-epoxy-1,4:5,8-dimethanonaphthalene) and *p,p'*-DDT [1,1,1-trichloro-2,2-bis(4-chlorophenyl)ethylene] because it is less persistent. Several studies have shown that endosulfan may persist in field water samples somewhat longer than in pure water by binding to sediments and soil particles (Chandler and Scott, 1991; Peterson and Batley, 1991; Noegrohati and Hammers, 1992). The effects of the particle-bound endosulfan on organisms are not yet fully understood, but some accumulation of residues in fish has been noted (Mattiessen *et al.*, 1982; Novak and Ahmed, 1989). Problems arising from the environmental studies that have been performed are as follows: (1) an inadequate number of samples is analyzed due to the cost and the time involved in analyzing the samples and (2) often there is a large time gap between the collection and the analysis of the sample.

Endosulfan is generally analyzed by instrumental methods such as gas chromatography with electron capture detection (Quintanilla-Lopez *et al.*, 1992), gas chromatography/mass spectrometry (Wilkes, 1981), or high-performance liquid chromatography (Galeano *et al.*, 1992). Each of these methods needs extraction, cleanup, and concentration of the sample. This is labor-intensive, time-consuming, and expensive, making it the rate-limiting step in environmental studies. Therefore, there is a need for a rapid, simple, and cost-effective method of analysis for endosulfan, such as immunoassay. The development and application of immunoassays in the analysis of environmental pollutants have been reviewed (Hammock *et al.*, 1987; Vanderlaan *et al.*, 1988).

Several immunoassays capable of detecting endosulfan have been described previously in the literature. The structures of endosulfan, its metabolites, and related cyclodiene insecticides are shown in Figure 1. The first assay reported for cyclodienes, some 20 years ago, was the radioimmunoassay of Langone and van Vunakis (1975). Dreher and Podratski (1988) raised antibodies against endosulfan diol conjugated to a carrier protein. Endosulfan diol was conjugated by esterifying a hydroxyl group with succinic anhydride to produce a hemisuccinate linker and then coupled to the carrier protein. This immunoassay was of insufficient sensitivity for analysis of water samples without a need for concentration and also detected the nontoxic metabolite, endosulfan diol, at a sensitivity similar to that of endosulfan. The detection limit of this assay was reported to be 3 ppb of endosulfan. The immunoassay reported by Bushway *et al.* (1988) was developed initially for the cyclodiene pesticide chlordane, but showed a cross-reaction with endosulfan, with a limit of detection of 1.0 ppb. This assay, like the above assay, does not discriminate endosulfan diol. A competitive and inhibition-type immunoassay for the detection of

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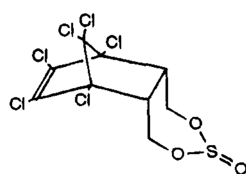
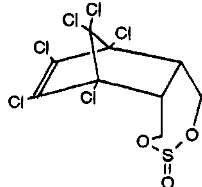
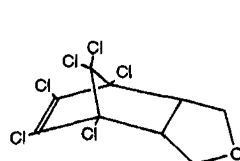
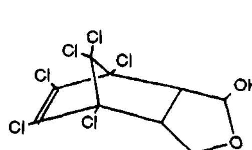
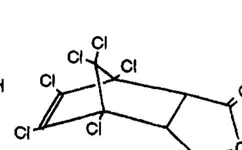
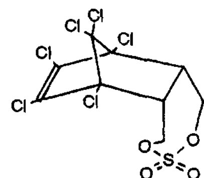


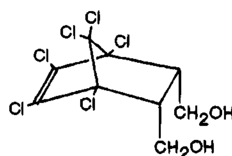


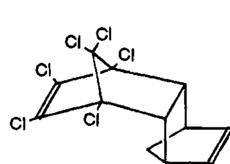


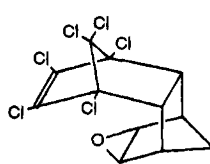


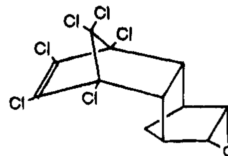


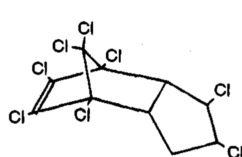












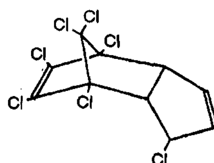


Figure 1. Structures of endosulfan and major metabolites (1), minor metabolites (2), and other organochlorines (related cyclodiene insecticides, 3).

endosulfan was described earlier by Reck and Frevert (1990). Here they employed different spacer structures on endosulfan diol and found that the inhibition-type assay was more sensitive and freer of matrix effects. However, this assay takes longer to complete and involves two incubation steps.

Stanker *et al.* (1991) developed an immunoassay using monoclonal antibodies raised against a chlordene derivative. This assay also was of insufficient sensitivity and shows about 6 times greater sensitivity for β-endosulfan than α-endosulfan. It also cross-reacted strongly with other metabolites of endosulfan. This assay was applied for analysis of cyclodiene residues in fat. Another immunoassay for cyclodienes utilizing monoclonal antibodies produced against the ether analog of aldrin was developed by Karu *et al.* (1990), although it was of rather low sensitivity for endosulfan; this assay also recognized the β isomer better than the α isomer. We have developed two fast and simple immunoassays for the detection of endosulfan residues in water and soil. These assays were designed to selectively detect the derivatives of endosulfan that are most toxic to fish, namely endosulfan and endosulfan sulfate. Three haptens were synthesized to achieve the required sensitivity and specificity.

MATERIALS AND METHODS

Materials. Bovine serum albumin (BSA) and horseradish peroxidase (HRP) were purchased from Boehringer-Mannheim, Germany. Keyhole limpet hemocyanin was purchased from Pierce, Rockville, IL. Fish skin gelatin, Tween 20, and Freund's complete and incomplete adjuvants were obtained from Sigma, St. Louis, MO. Methanol, analytical grade, was obtained from Ajax Chemicals, Clyde, NSW, Australia. Protein A agarose was purchased from Pharmacia, Uppsala, Sweden. Maxisorp polystyrene 96-well strip microwells and tubes were from Nunc, Roskilde, Denmark. Chemicals used in hapten synthesis were obtained from Aldrich, Milwaukee, WI. Silica gel 60 (70−230) mesh was purchased from Merck, Darmstadt, Germany. Standards of endosulfan and metabolites were gifts from Dr. Klaus Stumps, Hoecsht, Frankfurt am Main, Germany. Other pesticides listed in Table 1 were obtained from either Chem Service, Riedel-de-Hahn, Shell, Sandoz, or the Australian Government Curator of Standards, Pymble, NSW.

Instrumentation. Immunoassay microwells were read using a Bio-Rad Model 2550 plate reader (Bio-Rad, Hercules, CA) equipped with a 450-nm filter, while tubes were read using a portable differential photometer (Artel, Windham, ME), also fitted with 450-nm filter. The identities of the chemical intermediates were confirmed by proton and ^{13}C NMR spectroscopy on a 200-MHz (Gemini 200) instrument.

Table 1. Comparison of Assay Sensitivity for Different Immunogen–Enzyme Conjugate Combinations for Haptens I–III in the Microwell Assay

immunogen	enzyme conjugate[a]		
	hapten I	hapten II	hapten III
hapten I–OA[b]	100	8	8
hapten I–KLH[c]	50	7	30
hapten II–OA	12	11	>100
hapten II–KLH	10	6	2.5
Hapten III–OA	>100	7	>100
hapten III–KLH	11	6	100

[a] Data are IC_{50} in ppb for the endosulfan isomer mix, obtained using conjugate dilutions providing a B_0 OD of 0.8–1.2 and using 1 μg of antibody for microwell coating. [b] OA, ovalbumin. [c] KLH, keyhole limpet hemocyanin.

Preparation of Endosulfan Haptens. Three cyclodiene derivative haptens were synthesized for production of endosulfan antibodies and use in the enzyme conjugate. Hapten I (scheme 1, Figure 2) was prepared by esterifying one of the hydroxyl groups of endosulfan diol with succinic anhydride to provide a hemisuccinate spacer arm. The published method (Dreher and Podratski, 1988) used carbonyldiimidazole as an activating reagent, but an enzyme conjugate prepared in this manner (Reck and Frevert, 1990) was reported to be unstable and to contain a large proportion of free enzyme. Thus, the synthetic scheme for the enzyme conjugate has been modified. 1-Hydroxychlordene was prepared according to the methods described by Buchel *et al.* (1966) and Stanker *et al.* (1991) as a starting material for the synthesis of hapten II (scheme 2). For hapten III synthesis (scheme 3), 1,3,4,5,6,7,8,8-octachloro-3a,4,7,7a-tetrahydro-2-hydroxy-4,7-methanoindane was prepared as a starting material using the method described by Velsicol Corp. (1952).

4-Oxobutanoic Acid, 4-(4,5,6,7,8,8-Hexachloro-3a,4,7,7a-tetrahydro-4,7-methano-1H-indenyl-1-oxy) (**1**). To 1-hydroxychlordene (4,5,6,7,8,8-hexachloro-3a,4,7,7a-tetrahydro-4,7-methanoindene) (355 mg, 1.0 mmol) in dry pyridine (2.0 mL) was added succinic anhydride (150 mg, 1.5 mmol) followed by dimethylaminopyridine (DMAP, 20 mg), and then the mixture was stirred overnight. Ethyl acetate (30 mL) was added, and the organic layer was washed with acid (1.0 M HCl), water, and brine (saturated, NaCl) and then dried over $MgSO_4$. The solution was concentrated and the residue chromatographed on silica (methanol/chloroform/acetic acid 3:96.9:0.1) to give the product as a white solid: mp 117–118 °C ($CHCl_3$/petroleum ether); ^1H NMR δ 6.01 (m, H2), 5.93 (m, H3), 5.61 (bs, H1), 3.99 (m, H3a), 3.26 (dd, $J_{H3a,H7a}$ = 7.3 Hz, $J_{H1,H7a}$ = 1.8 Hz), 2.67 (d, $J_{H,H}$ = 8.8 Hz, $COCH_2$), 2.67 (d, $COCH_2$); ^{13}C NMR δ 177.7 (CO_2H), 171.1 (CO), 135.1, 33.6 (C2,3), 131.2 (C5,6), 102.9 (C8), 81.6, 79.3 (C4, 7), 60.5 (C3a), 56.6 (C1,7a), 28.8 (2 CH_2).

4-Oxobutanoic Acid, 4-(4,5,6,7,8,8-Hexachloro-3a,4,7,7a-tetrahydro-4,7-methano-1H-indenyl-1-oxy) Succinimidyl Ester (**2**). To the acid (200 mg, 0.44 mmol) in dichloromethane (10 mL, 0 °C) was added *N*-hydroxysuccinimide (NHS, 115 mg, 0.7 mmol) followed by DMAP (20 mg). The mixture was stirred overnight and then filtered and evaporated, and the residue was chromagraphed on silica (acetone/chloroform 1:9) to give a white solid: recrystallized from dichloromethane/petroleum ether, mp 133–134 °C (found: C, 39.23%; H, 2.16%; H, 2.16%; N, 2.63%; Cl, 38.37%. $C_{18}H_{13}C_{16}NO_6$ requires: C, 39.16%; H, 2.37%; N, 2.54%; Cl, 38.53%); ^1H NMR δ 6.04 (m, H2), 5.97 (m, H3), 5.64 (bs, H1), 4.06 (m, H3a), 3.03 (dd, $J_{H3a,H7a}$ = 7.3 Hz, $J_{H7a,H1}$ = 1.9 Hz, H7a), 2.97 (t, J_{HH} = 6.8, $COCl_2$), 2.85 (s, 2 × $NCOCH_2$), 2.75 (t, $COCH_2$); ^{13}C NMR δ 169.8 (CC), 168.8 (2 × CON), 167.5 (CO), 134.9, 133.7 (C2,3), 131.4, 128.9 (C5,6); 103.8, 102.9 (C8) (isomers at C1), 81.6, 79.6 (C4,7), 60.4 (c3a), 56.3 (c1,7a), 28.6 [C3 (butane)], 26.1 [C2 (butane)], 25.5, 25.4 (2 × CHCON).

4-Oxybutanoic Acid, 4-(1,3,4,5,6,7,8,8-Octachloro-3a,4,7,7a-tetrahydro-4,7-methanoindanyl-2-oxy) (**3**). 1,3,4,5,6,7,8,8-Octachloro-3a,4,7,7a-tetrahydro-2-hydroxy-4,7-methanoindane was treated as for 1-hydroxychlordene previously to give **3** as a white solid: mp 177–181 °C; ^1H NMR δ 5.71 (dd, $J_{H1,H2}$ = 9.6 Hz, H2), 3.85 (m, H1,3), 3.76 (2 × d, $J_{H,H}$ = 2.3 Hz, H3a, 7a), 2.81 [m (11 lines A2B2), 4H, 2 × CH_2]; ^{13}C NMR δ 173.7 (CO_2H), 170.9 (CO), 132.2 (C5,6); 103.6 (C8), 81.7, 79.8 (C4,7), 56.6 (C3a,7a), 55.1 (C1,3), 28.8, 28.7 (2 × CH_2).

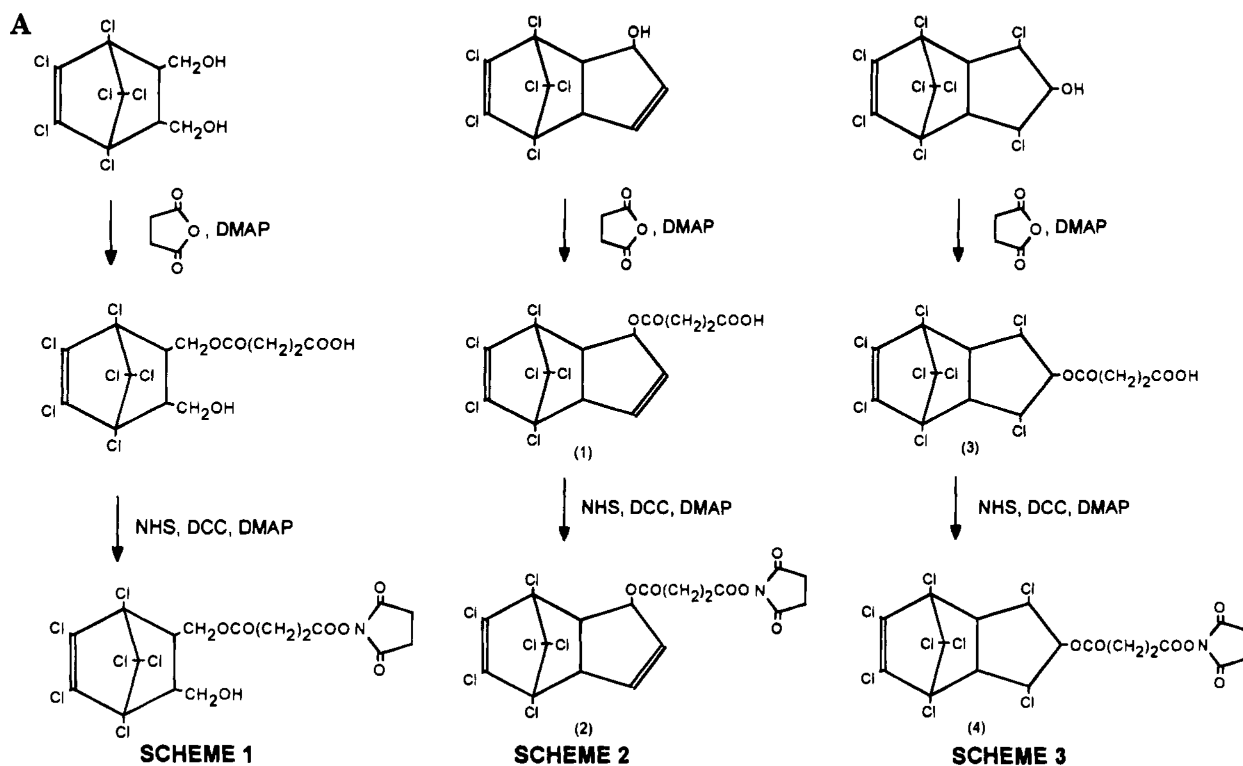
4-Oxybutanoic Acid, 4-(1,3,4,5,6,7,8,8-Octachloro-3a,4,7,7a-tetrahydro-4,7-methanoindanyl-2-oxy) Succinimidyl Ester (**4**). Compound **3** was treated as for **1** to give the succinimidyl ester **4** as a white solid: mp 192–194 °C (chloroform/petroleum ether) (Found: C, 34.78%; H, 1.79%; N, 2.21%; Cl, 45.48%. Requires: C, 34.7%; H, 2.1%; N, 2.25%; Cl, 45.54%; ^1H NMR δ 5.51 (dd, $J_{H1,H2}$ = $J_{H2,H3}$ = 9.8 Hz, H2), 3.69 (m, H1, 3); 3.54 (d, $J_{H,H}$ = 2.4 Hz), 3.53 (d, $J_{H,H}$ = 2.8 Hz, H3a,7a), 3.01 [t, $J_{H2,3}$ = 7.0 Hz, 2H, H2 (butane)], 2.87 [t, 2H, H3(butane)], 2.85 (s, 4H, 2 × CH_2CO); ^{13}C NMR 169.5 [C4 (butane)], 168.8 (2 × CON), 167.2 [C1 (butane)], 132.3 (C5,6), 103.8 (C8), 81.9, 79.8 (C4,7), 56.5 (C2, C4,7), 54.9 (C1,3), 28.4 [C3 (butane)], 26.1 [C2 (butane)], 25.5 (2 × CON).

Mininum Energy Modeling of Haptens. Molecular models of haptens I–III were derived using DTMM molecular modeling software (Oxford University Press, Oxford, U.K.). The stereochemical conformation of the norbornene structure, common to each hapten, was based upon the crystal structures of this moiety in cyclodienes, including endrin and aldrin (DeLacy and Kennard, 1972) and β-endosulfan (Byrn and Siew, 1977), and proton NMR analyses [reviewed by Keith and Alford (1970)]. The software was used to predict the conformation of structures on the hapten that would lie between the norbornene group and the carrier protein or enzyme label, to relate these data to potential cross-reactions of the different assays with endosulfan and metabolites.

Preparation of Protein and Enzyme Conjugates. The *N*-hydroxysuccinimide activated esters of haptens were conjugated to ovalbumin (OA), keyhole limpet hemocyanin (KLH), and horseradish peroxidase (HRP) using the method described earlier (McAdam *et al.*, 1992). Briefly, the active ester dissolved in dry dimethylformamide (DMF) was slowly added to a precooled buffer solution (50 mM K_2HPO_4, pH 9.1) containing the above protein. The mixture was allowed to stand at 4 °C overnight, and then it was desalted using a PD-10 column (Pharmacia), eluting with PBS (50 mM sodium phosphate/0.9% NaCl, pH 7.2) containing either 0.01% merthiolate (for HRP) or 0.05% sodium azide (for OA or KLH) and stored at 4 °C. The protein contents of the conjugates were determined according to the Lowry–Folin procedure (Lowry *et al.*, 1951) and the coupling ratio was measured by determination of the loss of amino groups on the protein using the reaction with trinitrobenzenesulfonate (Plapp *et al.*, 1971). The coupling ratios for protein and enzyme are as follows: (1) for hapten I, 7.5 haptens/mol of OA, 40 haptens/mol of KLH, and 2 haptens/mol of HRP; (2) for hapten II, 8 haptens/mol of OA and 4.5 haptens/mol of HRP (hapten II–KLH precipitated); and (3) for hapten III, 8 haptens/mol of OA, 6 haptens/mol of KLH, and 4 haptens/mol of HRP.

Antibody Preparation. Antibody was raised by intradermal and intramuscular injections of haptens conjugated to keyhole limpet hemocyanin and ovalbumin into New Zealand White rabbits. The immunogens were diluted in 0.9% saline and emulsified in Freund's complete (first immunization) or incomplete adjuvant (subsequent immunizations) to give 1 or 0.5 mg/mL. After three initial fortnightly intervals, booster injections were given monthly. Blood was collected from the marginal ear vein 10 days after each monthly booster injection and the antiserum was stored at −20 °C until purification. Antisera were purified by protein A–agarose affinity chromatography (Goding, 1978).

Preparation of Endosulfan Standards. Endosulfan (isomer mix) (100 ppm) was prepared in methanol as a stock solution. From this stock solution, a 100 ppb standard was prepared by dilution in purified water and then serially diluted to obtain 30, 10, 3, 1, 0.3, and 0.1 ppb in borosilicate tubes for the standard curve. The standard curves for field samples were prepared in the same way using turbid water with turbidities ranging from 27 to 86 nephelometer turbidity units (NTU) using a nephelometer (McVan Instruments, Melbourne,



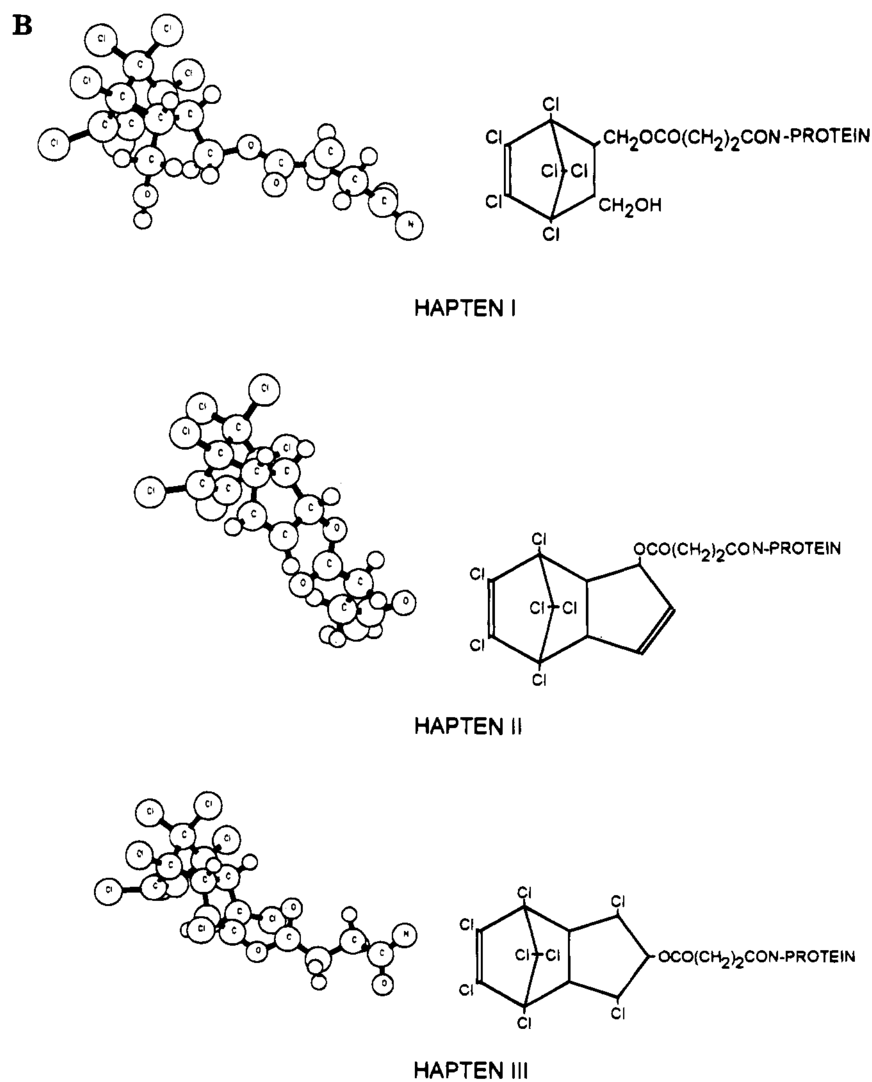


Figure 2. (A) Synthetic schemes for endosulfan haptens. (B) Molecular models showing least energy conformations of endosulfan haptens.

Australia). Standard curves for soil analysis were prepared using an extract of soil (from cotton farms, Narrabri) diluted 1/100 in water. Field water and soil samples for standard curve preparation were prescreened by GC to allow the selection of samples with no endosulfan residues.

Soil Spiking. The soil used for spiking was a black swelling soil, collected from Narrabri (northwest New South Wales, Australia). The soils of this region are alkaline, self-mulching, heavy gray clay, classified Ug 5.25 (Northcote, 1979), and contain predominantly montmorillonite (about 60%) and about 60% of both silt and sand. Ten-gram subsamples of soil, tested by gas–liquid chromatography to be free of endosulfan residues, were distributed into glass jars (with aluminum foil-lined caps) and spiked with 200 μL or less of endosulfan (isomer mix) or endosulfan sulfate. The soil was mixed thoroughly with a stainless steel spatula for 3 min and then allowed to stand at room temperature for 2 days. The soil samples were extracted using 20 mL of 90% methanol, in the original glass jars.

ELISA. *Laboratory (Microwell) Assay.* Antibodies were diluted in 50 mM carbonate buffer, pH 0.6, to 10 μg/mL and were coated at 100 μL per well overnight at 20 °C. The microwells were washed twice with phosphate-buffered saline (50 mM sodium phosphate/0.9% NaCl, pH 7.2) containing 0.05% (v/v) Tween 20 (PBS/T) and then blocked with 150 μL of 1% BSA/PBS [1% bovine serum albumin (Boehringer-Mannheim, Germany) in PBS]. One hundred microliters of endosulfan standard or sample followed by 100 μL of peroxidase conjugate diluted in PBS containing 0.5% (w/v) fish skin gelatin (Sigma) was incubated for 1 h at 20 °C. Peroxidase conjugates were titrated in preliminary experiments to yield an absorbance of 0.9–1.3 units under the conditions used. Hydrogen peroxide substrate/chromogen (3,3′,5,5′-tetramethylbenzidine/hydrogen peroxide in acetate buffer, pH 5.5, 150 μL) was added and incubated 30 min at 20 °C. The color development was stopped by adding 50 μL of 1.25 M sulfuric acid, and the microwell was read at 450 nm. For control and blanks, reverse-osmosis water or field water that is known to be free of organochlorine was used. Blank wells were used to determine the background color, and the absorbances of the wells were calculated by subtraction of the blank wells.

Field (Tube) Assay. Antibodies (12.5 μg/mL) were immobilized at 400 μL per tube overnight at 20 °C. After the tube was washed twice with PBS/T, the unbound sites were blocked by adding 500 μL per tube of 1% BSA/PBS. The reagents were prepared in dropper bottles (Wheaton, Millville, NJ), and the samples and standards were added into the tube using application droppers (Bio-Rad). Four drops (160 μL) of sample and four drops (160 μL) of peroxidase conjugate were added to a tube and incubated for 10 min at 20 °C. The tube was washed with tap water four times and shaken vigorously. Four drops each of substrate (300 μL) and chromogen (150 μL) were added for color development and incubated for 5 min. Finally, four drops (150 μL) of stopping solution (0.625 M sulfuric acid) was added to stop the color development, and absorbance was read with a portable photometer (450 nm).

Soil Extraction, Instrumental and Immunoassay Analysis. For instrumental analysis, 25 g of soil was weighed into a conical flask, and 150 mL of acetone/dichloromethane (3:1 v/v) was added. The flask was shaken for 16 h, and then the solvent was filtered through paper containing 2 g of sodium sulfate. The filtrate (75 mL) was concentrated to 5 mL using a Kuderna-Danish apparatus and then chromatographed on neutral alumina (7 g), containing 7% (w/w) water. The column was eluted using 60 mL of hexane and then 60 mL of acetone/hexane (1:3). The first 10 mL of eluate was discarded and the remainder concentrated to 10 mL. Analysis was performed by gas–liquid chromatography using electron capture detection.

For immunoassay, 10 g of soil was weighed into a 100-mL conical flask, and then 20 mL of 90% methanol was added. The flask was shaken for 1 h and allowed to stand overnight until the particles settled. The supernatant was then diluted 1/100 with water.

RESULTS AND DISCUSSION

Synthesis of Endosulfan Haptens. To develop an immunoassay that is specific to endosulfan, three approaches were initially attempted to synthesize endosulfan derivatives. The simplest was to use the diol metabolite of endosulfan, as in earlier work (Dreher and Podratski, 1988). As would be expected, this gave antibodies which cross-reacted with both sulfone and the diol metabolite (Figure 1). It was proposed that if the sulfone group of endosulfan could be retained in the hapten, the specificity of resulting antibodies for the sulfone and sulfate over the diol would be increased. Other approaches included were to attach the spacer arm at the double bond end and the dichloro bridgehead of endosulfan. The double bond bears two chlorines and was unreactive to a range of nucleophiles such as amines and hydroxide ions. Aminoethanol did react, but instead of a simple addition, simultaneous addition of the amine and alcohol from aminoethanol occurred to produce an oxypiperidine, which was unreactive to attempts to introduce spacer arms. The second approach involved the use of hexachlorocyclopentadiene, formation of the bridged norbornane system, and reactions at the bridgehead, which was a ketone protected with ethylene glycol. Removal of both protecting groups and also dimethyl acetal could not be performed under acidic conditions without dechlorinating the rest of the molecule.

To develop sensitive immunoassays, it is often desirable to use different haptens in immunization and for the enzyme conjugate used in the assay. Haptens II and III consist of a rigid five-membered ring next to the cyclodiene moiety and differ in the number of chlorine atoms and a double bond in the five-membered ring. Hapten II was prepared earlier by Stanker *et al.* (1991). The starting material, 1-hydroxychlordene, was prepared by Diels–Alder addition of hexachlorocyclopentadiene and cyclopentadiene followed by oxidation with selenium dioxide (Buchel *et al.*, 1966). However, Stanker *et al.* (1991) used a carbodiimide reaction for coupling hapten to protein, while active ester formation was employed in the current study. The preparation of 1,3,4,5,6,7,8,8-octachloro-3a,4,7,7a-tetrahydro-2-hydroxy-4,7-methanoindane followed the method described in U.K. patent 714,869 (Velsicol Corp. 1952). Briefly, an addition reaction of 1-chlorochlordene with acetic acid and *tert*-butyl hydrochlorite followed by another addition reaction of the resulting acetate compound with anhydride hydrogen chloride resulted in the chlorohydrin of 1-chlorochlordane. The synthetic schemes are shown in Figure 2A, and molecular models, showing the mininum energy conformations of the haptens, are shown in Figure 2B.

Assay Optimization. Titers of all antibodies were checked with all enzyme conjugates, and optimized conditions for all of the combinations were established by titration of serum dilutions against various concentrations of the immunizing hapten coupled to ovalbumin (in the case of antibodies raised to KLH conjugates) or KLH (in the case of antibodies raised to OA conjugates). A high-titer response was produced to each immunogen conjugate; for example, color development of approximately 1.0 optical density (OD) unit was obtained at the following serum dilutions: antisera to hapten I–OA, 1/500000; to hapten I–KLH, 1/300000; to hapten II–OA, 1/60000; to hapten II–KLH, 1/30000; to hapten III–OA, 1/300000; and to hapten III–KLH, 1/15000. The IC_{50} (calculated as the concentration of endosulfan

Table 2. Cross-Reactions of Three Optimized Assays with Endosulfan Metabolites, Using the Microwell Assay

endosulfan isomer		cross-reaction[a] (%)		
		assay 1	assay 2	assay 3
	imunogen:	I	II	III
	conjugate:	II	III	II
isomers				
isomer mix		100	100	100
α or I isomer		90	80	20
β or II isomer		120	120	360
major metbolites				
sulfate		65	90	80
diol		20	9	8
minor metabolites				
lactone		10	10	10
hydroxy ether		20	30	10
ether		40	45	4

[a] Data shown are relative to the IC_{50} of the isomer mixes, calculated as IC_{50} (endosulfan)/IC_{50} (test compound) × 100.

giving 50% inhibition of the color development for each combination) is shown in Table 1. In general, assays using the same hapten for immunogen and conjugate showed much lower sensitivity, except one with hapten H−OA antibodies and hapten II−enzyme conjugate. The combinations that gave highest sensitivity were (1) hapten I−KLH antibody with hapten II−enzyme conjugate, (2) hapten II−KLH antibody with hapten III−enzyme conjugate, and (3) hapten III−OA or −KLH antibody with hapten II−enzyme conjugate.

The choice of the most suitable antibody for the final assay was determined not just on overall assay sensitivity but by the specificity of the antibody for endosulfan metabolites. The most desirable assay would detect endosulfan and endosulfan sulfate at similar sensitivities but have a lower sensitivity for detection of the diol metabolite (Figure 1). The third antibody−conjugate combination recognizes β-endosulfan much more sensitively than α-endosulfan or endosulfan sulfate and thus would give erroneous results for "total endosulfan" in environmental samples, as the rates of dissipation of α- and β-endosulfan differ considerably (Stewart and Cairns, 1974; Singh *et al.*, 1991). Therefore, the second combination was chosen for use in the microwell plate format, since the detection of endosulfan diol was 10 times less sensitive than that of the parent forms and the sulfate (Table 2).

Different hapten−conjugate combinations were needed in the tube format. A comparison of the combinations used in microwell assays 1−3 (Table 2) showed that only assay 1 (the hapten I−KLH antibody/hapten II−enzyme conjugate combination) obtained sufficient color and sensitivity in the tube format. Using 2.5 μg of coating antibody, optimal sensitivities and OD values were obtained as follows: assay 1, OD = 0.94, IC_{50} = 1.4 ppb; assay 2, OD = 0.47, IC_{50} = 3 ppb; assay 3, OD = 0.70, IC_{50} 3 ppb. Higher OD values could be obtained in assays 2 and 3 by use of greater conjugate concentrations, but the sensitivities of the assays were reduced considerably.

Standard Curves. A mean standard curve representing data from eight curves obtained on four different days is shown in Figure 3. The standard curve in purified water showed a limit of detection (mean endosulfan concentration providing 15% inhibition of color development) of about 0.2 ppb. The IC_{50} was 1.6 ± 0.5 ppb, and a 10-fold difference in endosulfan concentration over the most linear part of the curve (between 1 and 10 ppb) gave a 43% difference in color development, suggesting that the slope of the standard curve was steep enough for quantitative analysis. Potential matrix effects for water and soil extract were examined by comparing the standard curve generated from purified water with those produced from the turbid water (Figure 2) and soil extract (Figure 4). The standard curves were superimposed, suggesting there were no significant matrix effects from the soil extract.

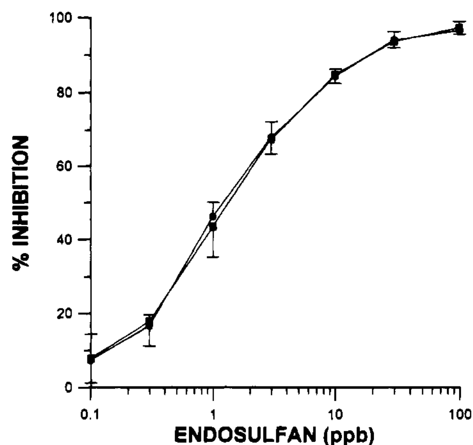


Figure 3. Microwell assay standard curve for endosulfan using antibody to II−KLH (with III−HRP conjugate): standards in purified water (●) vs turbid field water (▲, 27−86 NTU).

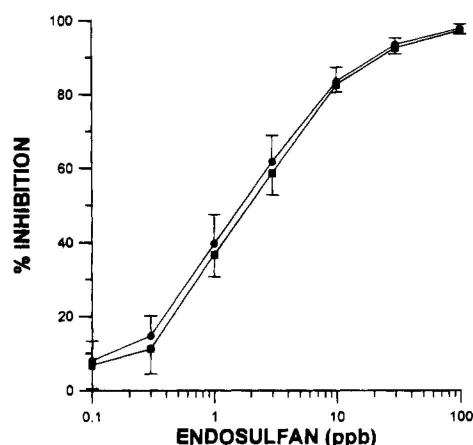


Figure 4. Microwell assay standard curve for endosulfan using antibody to II−KLH (with III−HRP conjugate): standards in purified water (●) vs soil extract (■, black swelling soil).

A comparison of the standard curves for the tube assay and the microwell assay of the same antibodies is shown in Figure 5. The tube assay had a detection limit similar to that of the microwell assay, but as the standard curve for the tube assay was less steep [a 36% difference in OD over the linear part of the standard curve (2−20 ppb endosulfan)], determination of endosulfan was less quantitative than the microwell assay.

Assay Specificity. Endosulfan sulfate, one of the major metabolites, has a toxicity to fish similar to that of the parent compound of endosulfan, whereas the hydrolytic product in water, endosulfan diol, is much less toxic (Novak and Ahmad, 1989; Chapman and Sunderam, 1992; Sunderam *et al.*, 1992). The specificity of the assay was manipulated by using enzyme conjugate prepared by different hapten structures. The assay developed by raising antibodies against hapten III and using hapten II conjugated to horseradish peroxidase as the enzyme conjugate was 10 times less sensitive for endosulfan diol (calculated as the IC_{50}) than for endosulfan (both isomers) and endosulfan sulfate. On the

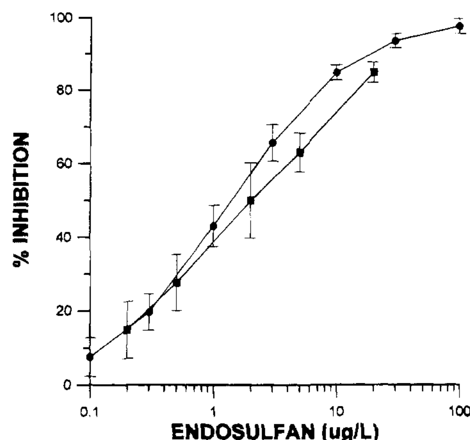


Figure 5. Comparison of standard curves for tube (●) and microwell (■) assays. The microwell assay used antibody to II–KLH (with III–HRP conjugate), and the tube assay used antibody to I–KLH and II–HRP conjugate.

other hand, the assay using antibodies raised against hapten I–protein and enzyme–conjugate using hapten III–HRP detected endosulfan and two metabolites, endosulfan sulfate and endosulfan diol, at similar sensitivities. Other minor metabolites, endosulfan lactone, endosulfan ether, and endosulfan hydroxy ether (Figure 1), were detected at lower sensitivity on both assays. The residues of metabolites other than endosulfan sulfate and endosulfan diol in environmental matrices occur at insignificant levels and therefore are not expected to interfere in the analysis of the toxic compounds, namely endosulfan and endosulfan sulfate. Comparison of the cross-reactions of the three different antibodies with endosulfan metabolites is shown in Table 2.

In deriving least energy molecular models of the haptens, we investigated the role of flexibility of the spacer arm and of the substituted cyclopentane ring distal to the spacer. The polychlorinated norbornene structure, furthest from the point of coupling, was assumed to adopt a similar conformation in each hapten. This assumption was borne out by the cross-reaction of the final assays (Table 3) and of the other antibody–conjugate combinations (Lee and Skerritt, unpublished results) with cyclodienes such as dieldrin and endrin (Figure 1). Haptens II and III consist of a five-membered ring next to a norbornane structure and thus retain the basic ring structure of endosulfan. Thus, the assays that used combinations of haptens II and III as immunogens and conjugates recognized endosulfan and the sulfate selectively, over the diol, which did not have a five-membered ring. Substituents on the five-membered ring, such as lactone or hydroxy ether, lowered recognition. The greater cross-reaction of assays using hapten I with endosulfan diol is not unexpected since this hapten is a derivative of endosulfan diol. The somewhat greater recognition of β-endosulfan over the α-form by antibodies to hapten III was explicable from the molecular models, as hapten III adopted an exo-like conformation, similar to that of β-endosulfan.

Since antibodies were generated by haptens retaining the polychlorinated norbornene moiety of the cyclodiene insecticides, the cross-reactivity for common cyclodienes was investigated (Table 3). Among the cyclodienes tested, dieldrin and endrin showed cross-reactions of 100% or higher. Aldrin and heptachlor also showed significant cross-reactions. Pesticides other than cyclodienes did not show any cross-reaction at 1000 ppb. The cotton industry in many countries has a history of heavy usage of *p,p'*-DDT, which has been replaced by endosulfan (Fitt, 1994). *p,p'*-DDT and its metabolites are still detected in soil at significant levels; the predominant one is 1,1-dichloro-2,2-bis(4-chlorophenyl)ethane (DDE) (Poels *et al.*, 1991; Szeto and Price, 1991). DDE residues may interfere with gas chromatographic analysis of endosulfan in soil, and so the soil extracts routinely undergo extensive cleanup to remove such residues before this analysis can be performed. The immunoassay did not show cross-reaction with *p,p'*-DDT, DDE, or pesticides other than cyclodienes at 1000 ppb. Therefore, an added advantage of ELISA is that there is no need for cleanup of soil extracts to remove possible DDE interference.

Effects of Enzyme Conjugate Diluents, Salt Ions, and Solvent on the Assay. Five different enzyme conjugate diluents were tested for their effects on the color and the sensitivity of the assays. They were PBS, PBS/T, 1% BSA/PBS, 1% BSA/PBS/T, and 0.5% FG/PBS. The sensitivity was reduced by factor of 10 wherever BSA and Tween 20 were used, and the effects were more pronounced with Tween 20 (Figure 6). PBS alone increased the sensitivity; however, the color development in the assay tended to fluctuate between assays, perhaps due to nonspecific stickiness of the conjugate. The use of fish gelatin/PBS gave reproducible color development and high sensitivity.

Major ions commonly found in soil and field water are Ca^{2+}, Mg^{2+}, Na^{+}, Cl^{-}, SO_4^{2-}, and HCO_3^{-}, with small amounts of K^{+} and NO_3^{-} (White, 1987). The following concentrations of salt ions were tested for their effects on performance of two microwell assays and also on the tube assay: up to 1000 mM NaCl, up to 500 mM $NaHCO_3$, 1 mM $FeSO_4$, up to 5 mM $MgSO_4$, up to 5 mM $CaCl_2$, and up to 5 mM KNO_3. There were only slight reductions in the color of assay using hapten II antibodies (with hapten III–enzyme conjugate) for most of the ions tested, except Fe^{2+}. The color of the assay was reduced by 43% in the case of Fe^{2+}, but there was no significant effect on the sensitivity of the assay. The tube assay and the microwell assay using hapten I antibodies were more tolerant of the salt ions as no color reduction was observed for ions except for Fe^{2+}. The color was reduced by 35% in this case. Fe^{2+} is rarely found in field water, at least in Australian cotton farms, and so the performance of the immunoassays would not be affected for the analysis of water and soil.

The extraction of soil endosulfan residues is generally done by a number of solvents having a range of polarity (Goebel *et al.*, 1982). The common ones are hexane, acetonitrile, acetone, dichlomethane, methanol, or a mixture of these solvents. Immunoassay requires water-miscible solvent for direct analysis. Methanol has been reported to be one of the most effective solvents for extraction of endosulfan residues in soil (Goebel *et al.*, 1982). The effects of methanol were determined by comparing the standard curve prepared in methanol with purified water. Methanol at 10% or less did not alter the sensitivity of the assay. Fifty percent inhibition of color development occurred at 2 ppb (for 0, 5, and 10% methanol), while at 20 and 40% methanol the IC_{50} value was 3 ppb. Using a final methanol concentration of 0.5%, the curves obtained with solvent and with solvent extract of black swelling soil (diluted 1/100) were superimposable with the standard curve prepared using standards diluted in water (Figure 4).

Table 3. Specificity of Microwell and Tube Endosulfan Assays[a] for Endosulfan Metabolites and Other Organochlorines

	microwell assay			tube assay		
	cross-reaction (%) based on relative			cross-reaction (%) based on relative		
compound	IC_{50} value	IC_{15} value	IC_{15} (ppb)	IC_{50} value	IC_{15} value	IC_{15} (ppb)
endosulfan						
isomer mix	100	100	0.2	100	100	0.15
α-isomer	80	70	0.3	100	100	0.15
β-isomer	120	130	0.15	100	100	0.15
sulfate	90	100	0.2	25	75	0.2
diol	9	6	3.5	15	35	0.4
ether	45	100	0.2	100	100	0.2
hydroxy ether	30	35	0.6	20	29	0.7
lactone	10	6	3.5	21	100	0.2
other organochlorines						
aldrin	10			35		
dieldrin	100			250		
chlordane	10			700		
heptachlor	50			30		
endrin	250			100		
lindane	0.1			1		
DDT	not detected at 1000 ppb					
methoxychlor	not detected at 100 ppb					

[a] Microwell assay using antibody to II−KLH (with III−HRP conjugate) and tube assay using antibody to I−KLH and II−HRP conjugate. Cross-reaction data are relative to the IC_{50} (or IC_{15} value, as stated) of the isomer mix, calculated as IC_{50} (endosulfan)/IC_{50} (test compound) × 100.

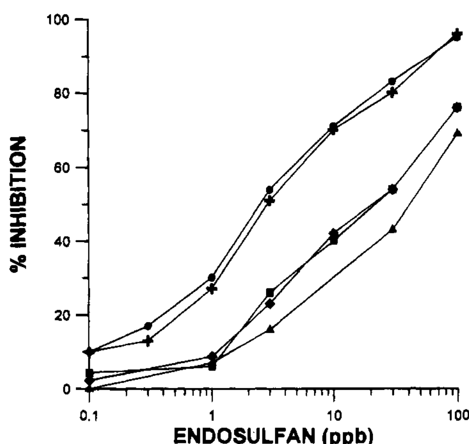


Figure 6. Comparison of conjugate and sample assay diluents: PBS (●), 1% BSA/PBS (■), PBS/0.05% Tween (◆), 1% BSA/PBS/0.05% Tween (▲), and 0.5% FG/PBS (+) in the microwell assay using antibody to II−KLH (with III−HRP conjugate).

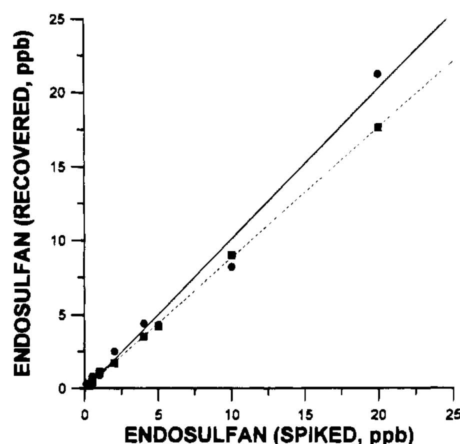


Figure 7. Spike and recovery of endosulfan standards in water, using the microwell (●, solid lines) and tube (■, dashed lines) assays. The microwell assay used antibody to II−KLH (with III−HRP conjugate), and the tube assay used antibody to I−KLH and II−HRP conjugate.

Australian cotton cropping areas have slightly alkaline soils, with typical pH values of about 8 (Ward *et al.*, 1988), although there is some variation between regions; surface water is of a similar pH. The effect of pH on the endosulfan immunoassays was investigated by comparing standard curves prepared in water of pH 3, 5, 7, 9, and 11, by adjustment of the pH of purified water accordingly with either acetic acid or NaOH. Color development in both the tube and microwell assays was not affected by pH, and the sensitivity of the assay was only reduced at pH 11, probably due to hydrolysis of endosulfan to endosulfan diol. At acidic pH values (3 and 5) the sensitivity of the assay increased by 50−100%.

Spike and Recovery Studies. Twenty-six water samples of various tubidities (27−86 NTU) were spiked with endosulfan at eight levels (0.05−20 ppb), and recovery was assessed using both microwell and tube assays. Data shown in Figure 7 represent recovery means for each spike level for each assay format. The equations for the lines of best fit for 26 spiked samples were, for the microwell assay, endosulfan (recovered) = 1.02 × endosulfan (spiked) − 0.09 (r = 0.99), and for the field assay, endosulfan (recovered) = 0.88 × endosulfan (spiked) + 0.01 (r = 0.99). These data demonstrate that good recoveries were observed for water samples using either assay.

The cross-reaction studies showed the microwell assay was selective to endosulfan and endosulfan sulfate. As endosulfan diol is the major metabolite in water, we examined the performance of the assay (microwell format) by spiking endosulfan, endosulfan sulfate, and endosulfan diol at different concentrations in water (Figure 8). Both sets of samples with and without endosulfan diol gave approximately 100% recovery of "toxic endosulfans" (i.e. sum of α- and β-endosulfan and sulfate). This shows the ELISA was capable of selectively detecting the more toxic endosulfan isomers and metabolites.

Figure 9 presents the result of spike and recovery studies in soil. Ten soil samples were spiked with endosulfan (Figure 9A) and endosulfan sulfate (Figure 9B) in methanol at different concentrations ranging between 0.05 and 10 ppm and analyzed in both microwell and tube assays. The average recovery rate was 91% from soil. The soil samples shown to be free of

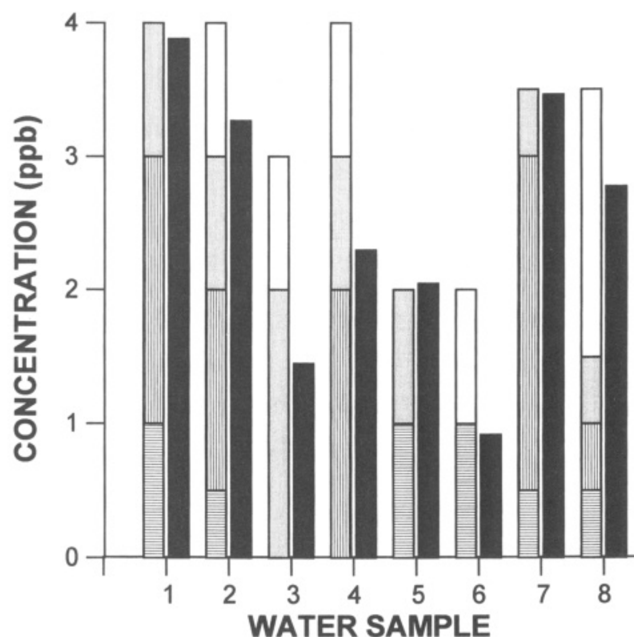


Figure 8. Immunoassay results (right-hand bar) obtained using eight purified water samples spiked (left-hand bar) with different combinations of α-endosulfan (horizontally striped bar), β-endosulfan (vertically striped bar), endosulfan sulfate (dotted bar), and endosulfan diol (empty bar). Microwell assay using antibody to II–KLH (with III–HRP conjugate).

endosulfan residues by gas–liquid chromatography also gave negative results with the ELISA.

Precision of the Immunoassays. The precision of the assays was studied by determining the intra-assay repeatability and between-assay reproducibility. Ten soil samples were spiked with endosulfan at concentrations of 0.05, 0.075, 0.1, 0.5, 1, 2, 3, 4, 6, and 10 ppm and extracted as described under Materials and Methods. Analysis of the same samples on two days gave deviations of the data from means of 7, 13, 0, 7, 0, 34, 20, 18, 5, and 0% for each of the respective endosulfan concentrations using the microwell assay and of 116, 108, 76, 3, 21, 31, 0, 6, 22, 19%, respectively, using the tube assay. The high imprecision of the tube assay for analyses of soils containing 0.01–0.1 ppm would suggest that at these concentrations the microwell assay should be used for quantitative analysis. For four replicates of water samples spiked with 0.5, 2, and 4 ppb of endosulfan (isomer mix) assayed on the same day, the coefficients of variation about the mean result were 13, 19, and 36%, respectively, using the microwell assay, and 22, 35, and 20%, respectively, using the tube assay.

Conclusion. Two enzyme immunoassays, a laboratory assay using microwell plates and a field assay using polystyrene tubes, have been developed for detection of endosulfan residues in water and soil. These assays are sufficiently sensitive to detect endosulfan at the ecotoxicological level in water without need for extraction, cleanup, and concentration. The soil samples need only a simple extraction with 90% methanol and dilution 1/100 with water for immunoassay. The assays are specific for endosulfan and cyclodiene pesticides; however, the latter compounds are not typically applied together with endosulfan. Therefore, they are not a concern for the immunoassay in these matrices and samples. The cross-reactivity and the recovery studies with endosulfan analogs and metabolites suggest that in environmental matrices the assays would detect predominantly endosulfan and endosulfan sulfate. As the presence of particulates and dissolved organic compounds in water and soil extracts does not interfere with the assay, water with endosulfan concentrations between 0.2 and 10 ppb can be analyzed directly. These immunoassays may be a very useful tool for regular monitoring of water and soil samples for possible contamination of endosulfan, and they can also be used as a research tool for environmental studies that may require many samples that need to be analyzed in a short period of time.

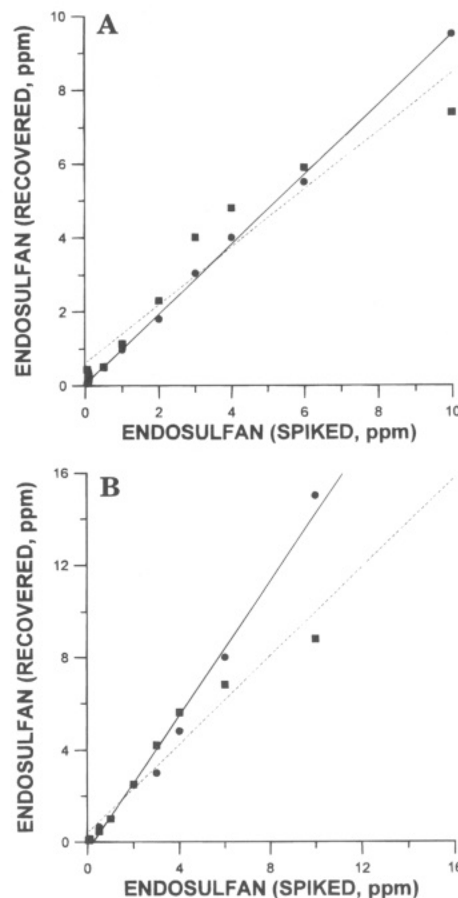


Figure 9. Spike (x axis) and recovery (y axis) of (A) endosulfan (mixed isomers) and (B) endosulfan sulfate in soil, using the microwell assay (●, solid lines) or tube assay (■, dashed lines). The microwell assay used antibody to II–KLH (with III–HRP conjugate), and the tube assay used antibody to I–KLH and II–HRP conjugate. (A) Microwell assay: $y = 0.94x + 0.05$, $r = 0.998$. Tube assay: $y = 0.79x + 0.6$, $r = 0.964$. (B) Microwell assay: $y = 1.44x + 0.13$, $r = 0.993$. Tube assay: $y = 0.96x + 0.4$, $r = 0.969$.

LITERATURE CITED

Buchel, K. H.; Ginsberg, A. E.; Fischer, R. Synthesis and structure of heptachloromethanotetrahydroindanes. *Chem. Ber.* **1966**, *99*, 405–415.

Bushway, R. J.; Pask, W. M.; King, J.; Perkins, B.; Ferguson, B. S. Determination of chlordane in soil by enzyme immunoassay. *Field Screening Methods for Hazardous Waste Site Investigations*, Proceedings of the First International Symposium; U.S. Environmental Protection Agency: Las Vegas, NV, 1988; pp 433–437.

Byrn, S. R.; Siew, P. Y. Structure and conformation of β-thiodan in the solid state and in solution. Application of the infrared-X-ray method. *J. Chem. Soc., Perkin Trans. 2* **1977**, 144–149.

Chandler, G. T.; Scott, G. I. Effects of sediment-bound endosulfan on survival, reproduction and larval settlement of meiobenthic polychaetes and copepods. *Environ. Toxicol. Chem.* **1991**, *10*, 375–382.

Chapman, J.; Sunderam, R. Endosulfan and the aquatic ecosystem. *Aust. Cottongrower* **1992**, *13*, 51–58.

DeLacy, T. P.; Kennard, C. H. L. Insecticides. Part III. Crystal structures of endrin (1,2,3,4,10,10-hexachloro-6,7-epoxy-1,4,4a,5,6,7,8,8a-octahydro-*endo*-1,4-*endo*-5,8-dimethanonaphthalene) and aldrin (1,2,3,4,10,10-hexachloro-1,4,4a,5,8,8a-hexahydro-*endo*-1,4-*exo*-5,8-dimethanonaphthalene). *J. Chem. Soc., Perkin Trans. 2* **1972**, 2153–2158.

Dreher, R. M.; Podratzki, B. Development of an enzyme immunoassay for endosulfan and its degradation products. *J. Agric. Food Chem.* **1988**, *36*, 1072–1075.

Fitt, G. P. Cotton pest management. 3. An Australian perspective. *Annu. Rev. Entomol.* **1994**, *39*, 543–562.

Galeano, T.; Guiberteani, A.; Salinas, F. Rapid determination of alpha-endosulfan and beta-endosulfan in formulations and potatoes by high-performance liquid chromatography. *Anal. Lett.* **1992**, *25*, 1797–1804.

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

Goebel, H.; Gorbach, S.; Knauf, W.; Rimpau, R. H.; Muttenbach, H. Properties, effects, residues and analytics of the insecticide endosulfan. *Residue Rev.* **1982**, *83*, 1–174.

Hammock, B. D.; Gee, S. J.; Cheung, P. Y. K.; Miyamoto, T.; Goodrow, M. H. VanEmon, J.; Seiber, J. N. In *Pesticide Science and Biotechnology*; Greenhalgh, R., Roberts, T. R., Eds.; Blackwell: Oxford, U.K. 1987.

Karu, A. E.; Liliental, J. E.; Schmidt, D. J.; Lim, A. K.; Carlson, R. E.; Swanson, T. A.; Buirge, A. W.; Chamerlik, M. Monoclonal antibody-based immunoassay of cyclodienes. *Proceedings, 6th Annual EPA Waste Testing and Quality Assurance Symposium*; U.S. GPO: Washington, DC, 1990; pp I-237–I-245.

Keith, L. H.; Alford, A. L. Review of the application of nuclear magnetic resonance spectroscopy in pesticide analysis. *J. Assoc. Off. Anal. Chem.* **1970**, *53*, 1018–1035.

Langone, J. J.; Van Vunakis, H. Radioimmunoassay for dieldrin and aldrin. *Res. Commun. Chem. Pathol. Pharmacol.* **1975**, *10*, 163–171.

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.

Mattiessen, P.; Fox, P. J.; Wood, A. B. Accumulation of endosulfan residues in fish and their predators after aerial spraying for the tsetse fly in Botswana. *Pestic. Sci.* **1982**, *13*, 39–48.

McAdam, D. P.; Hill, A. S.; Beasley, H. L.; Skerritt, J. H. Mono- and polyclonal antibodies to the organophosphate fenitrothion. I. Approaches to hapten-protein conjugation. *J. Agric. Food Chem.* **1992**, *40*, 1466–1470.

Noegrohati, S.; Hammers, W. E. Sorption-desorption kinetics of some organochlorine insecticides in silt-water suspensions. *Toxicol. Environ. Chem.* **1992**, *34*, 187–206.

Northcote, K. H. *A Factual Key for the Recognition of Australian Soils*, 3rd ed.; Rellim Technical Publications: Glenside, South Australia, 1979.

Novak, B.; Ahmad, N. Residues in fish exposed to sublethal doses of endosulfan and fish collected from cotton growing areas. *J. Environ. Sci. Health* **1989**, *B24*, 97–109.

Peterson, S. M.; Batley, G. E. Fate and transport of endosulfan and diuron in aquatic ecosystems. Investigation Report CET/LH/IRO 13; Centre for Advanced Analytical Chemistry, CSIRO Division of Coal and Energy Technology, Menai, Australia, 1991; 105 pp.

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.

Poels, C. L. M.; Gruntz, U.; Isnard, P.; Riley, D.; Spitler, M.; ten Berge, W.; Veerkamp, W.; Bontinck, W. J. Hazard assessment of chemical contaminants in soil. *Chemosphere* **1991**, *23*, 3–24.

Quintanilla-López, J. E.; Lebrón-Aguilar, R.; Polo-Díez, L. M. Comparative study of clean-up and fractionation methods for the determination of organochlorine pesticides in lipids by gas chromatography. *J. Chromatogr.* **1992**, *591*, 303–311.

Reck, B.; Frevert, J. Competitive- and inhibition-type immunoassay for determination of endosulfan. In *Immunochemical Methods for Environmental Analysis*; van Emon, J. V., Mumma, R. O., Eds.; ACS Symposium Series 442; American Chemical Society: Washington, DC, 1990; pp 193–198.

Singh, N. C.; Dasgupta, T. P.; Roberts, E. V.; Mansingh, A. Dynamics of pesticides in tropical conditions. 1. Kinetic studies of volatilization, hydrolysis, and photolysis of dieldrin and α- and β-endosulfan. *J. Agric. Food Chem.* **1991**, *39*, 575–579.

Stanker, L. H.; Watkins, B.; Vanderlaan, M.; Ellis, R.; Rajan, J. Analysis of heptachlor and related cyclodiene insecticides in food products. In *Immunoassays for Trace Chemical Analysis*; Vanderlaan, M., Stanker, L. H., Watkins, B. E., Roberts, D. W., Eds.; ACS Symposium Series 451; American Chemical Society: Washington, DC, 1991; pp 108–123.

Stewart, D. K. R.; Cairns, K. G. Endosulfan persistence in soil and uptake by potato tubers. *J. Agric. Food Chem.* **1974**, *22*, 984–986.

Sunderam, R. I. M.; Cheng, D. M. H.; Thompson, G. B. Toxicity of endosulfan to native and introduced fish in Australia. *Environ. Toxicol. Chem.* **1992**, *11*, 1469–1476.

Szeto, S. Y.; Price, P. M. Persistence of pesticide residues in mineral and organic soils in the Fraser valley of British Columbia. *J. Agric. Food Chem.* **1991**, *39*, 1679–1684.

Vanderlaan, M.; Watkins, B. E.; Stanker, L. H. Environmental monitoring by immunoassay. *Environ. Sci. Technol.* **1988**, *22*, 247–254.

Velsicol Corp. Improvements in or relating to halogenated dicyclopentadiene epoxides. Br. Pat. 714,869, 1952.

Ward, W. T.; McTainsh, G. H.; McGarry, D. The influence of parent rocks and sediments on soil variation in the Narrabri district. *Proceedings of the Fourth Australian Cotton Conference*; Australian Cotton growers' Research Association: Wee Waa, NSW, Australia, 1988; pp 367–371.

White, R. E. *An Introduction to the Principles and Practice of Soil Science*; Blackwell Scientific Publishers: London, 1987.

Wilkes, P. S. Gas-chromatographic-mass spectrometric confirmation of endosulfan and endosulfan sulfate in apples and carrots. *J. Assoc. Off. Anal. Chem.* **1981**, *64*, 1208–1210.

Received for review October 19, 1994. Revised manuscript received March 27, 1995. Accepted April 17, 1995.[⊗] We acknowledge the Cotton Research and Development Corp. of Australia and Millipore Australia Pty Ltd for partial financial support of this project.

JF940589J

[⊗] Abstract published in *Advance ACS Abstracts,* June 1, 1995.