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Development of an Enzyme Immunoassay for Endosulfan and Its Degradation Products

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An enzyme immunoassay (EIA) was developed for the insecticide endosulfan and its degradation products. The EIA was based on antibodies raised against the diol of endosulfan by immunizing rabbits with a keyhole limpet hemocyanin (KLH) endosulfandiol conjugate. With this method, endosulfan was detected in aqueous solutions at a level of 3 ppb without any sample extraction procedure. The measuring range was found to be between 3 and 400 ng/mL. The cross-reactivity of other similar chlorinated hydrocarbon pesticides was tested. Whereas the pesticides lindan, alondan, and aldrin showed only little cross-reactivities, endrin demonstrated a high cross-reactivity of 180%.

Environmental analytical studies of endosulfan (Figure 1) and its degradation product residues have so far relied on conventional analytical techniques, in particular silica gel column chromatography and a subsequent quantitative determination by infrared measurement (Weinmann, 1970) or gas chromatography with a time-consuming cleanup stage (Zweig and Archer, 1960). Therefore, a simple and sensitive method would be a highly desirable aim. Possibly, an immunological procedure, which makes use of a highly specific antibody, could be suitable for a rapid, efficient, and sensitive analytical method.

Recently, immunological methods such as enzyme-linked immunosorbent assay were shown to be applicable to the field of environmental analytical chemistry. The application of immunological methods especially in the analysis of pesticides has been treated in great detail by Hammock and Mumma (1980) and reviewed by Schwalbe-Fehl (1986).

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So far, the present study demonstrates the first development of an EIA method to detect a chlorinated hydrocarbon pesticide. The optimum experimental conditions for the determination and the sensitivity, precision, and specificity of the method are discussed.

MATERIALS AND METHODS

Chemicals. Endosulfan and its degradation products (ether, lactone, diol, sulfate) were provided by Hoechst AG, Frankfurt. The pesticides alodan, aldrin, endrin, and lindan were supplied by Riedel-de Haen, Seelze. Bovine serum albumin, keyhole limpet hemocyanin, peroxidase, o-phenylenediamine dihydrochloride, Tween 20, Freund's adjuvant, dicyclohexylcarbodiimide, and carbonyldiimidazole were obtained from Sigma Chemie, Deisenhofen, and phosgen was purchased from Fluka.

Buffer Solutions. Coating buffer: pH 9.6, 50 mM Na_2CO_3. Phosphate-buffered saline with 0.1% Tween 20 (PBS-t): pH 7.4; prepared by adding NaCl (150 mM),

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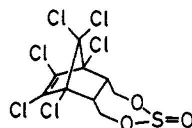

Figure 1. Structure of endosulfan (6,7,8,9,10,10-hexachloro-1,5,5a,6,9,9a-hexahydro-6,9-methano-2,4,6-benzodioxathiepin 3-oxide).

Na_2HPO_4 (50 mM), and 0.1% Tween 20. Citrate–phosphate buffer: 100 mM NaH_2PO_4, adjusted to pH 5.0 with citric acid. Substrate solution: prepared by adding *o*-phenylenediamine (2.5 mM) to citrate–phosphate buffer and 0.5 μL of 30% H_2O_2/mL; solution prepared immediately prior to use.

Antigen Preparation. The endosulfandiol (250 mg) was dissolved in dry pyridine (10 mL), and 750 mg succinic anhydride was added. The resulting solution was heated under reflux for 20 h and the pyridine removed under vacuum (Bauminger et al., 1973). The residue was dissolved in methanol (5 mL) and analyzed by HPLC (RP 18 column). The endosulfan hemisuccinate was obtained in a 90% yield based on the endosulfandiol.

For the synthesis of the active ester (Fitzpatrick and Bundy, 1978; Weygand et al., 1966), 50 mg of the hemisuccinate was dissolved in 1 mL of DMF and 30 mg of dicyclohexylcarbodiimide and 35 mg of *N*-hydroxysuccinimide were added followed by incubation for 50 min at 25 °C. The precipitated dicyclohexylurea was removed by centrifugation. This crude mixture was added dropwise and under stirring to 50 mg of keyhole limpet hemocyanin, dissolved in 2 mL of 0.1 M $NaHCO_3$ and 3.5 mL of DMF. The resulting solution was incubated (18 h, 4 °C), dialyzed against repeated changes of PBS, and stored at −20 °C.

Immunization. White female New Zealand rabbits (3 kg) were injected with an emulsion of endosulfan–hemocyanin conjugate (1 mg), dissolved in 0.5 mL of PBS and emulsified with 0.5 mL of Freund's complete adjuvant. Of the emulsion 1 mL was injected subcutaneously into the neck region. After 3 weeks rabbits were boosted with 1 mg of antigen in Freund's incomplete adjuvant. After 3 weeks, the rabbits were again boosted with the same antigen concentration in Freund's incomplete adjuvant. Starting 2 weeks after the last boost, rabbits were bled and the sera stored at −20 °C. Additionally, the sera were examined for antibody production in an ELISA system. Endosulfan coupled to carriers differing from the immunizing antigen and with a different chemical linkage (endosulfandiol was succinylated and coupled to BSA via mixed anhydride (Samokhin and Filimonov, 1985)) was bound to the solid phase of a microtiter plate. After washing, a dilution of serum was added. The fixed rabbit of IgG was detected with an anti-rabbit IgG–antibody peroxidase conjugate. The titer was defined as the serum dilution, showing an extinction of 1.0 in the ELISA system.

Enzyme–Conjugate Preparation. The endosulfan–peroxidase conjugate was prepared with carbonyldiimidazole as activating reagent. A solution of endosulfandiol (100 mg) in 1 mL of DMF was incubated with carbonyldiimidazole (50 mg) in 1 mL of DMF at 37 °C for 1 h. The mixture was further incubated with 2 mL of ethylendiamine at 25 °C for 1 h and then lyophilized.

The oxidation of horseradish peroxidase was carried out according to Nakane and Kawaoi (1974) with slight modifications according to Tijssen and Kurstak (1984). Peroxidase (2 mg) was oxidized, dialyzed against phosphate buffer (200 mM), pH 7.2, and stirred with 0.4 mg of the

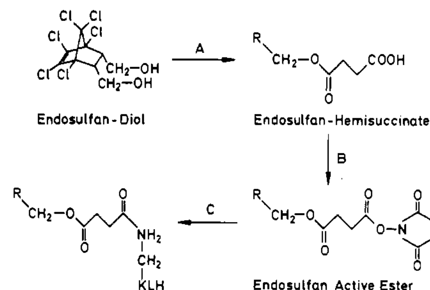

Figure 2. Synthetic route to endosulfan–protein conjugate: (A) succinic anhydride in pyridine; (B) dicyclohexylcarbodiimide and *N*-hydroxysuccinimide; (C) keyhole limpet hemocyanin (KLH).

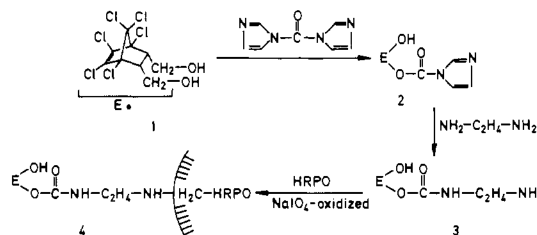

Figure 3. Synthetic route to endosulfan–enzyme conjugate using 1,1-carbonyldiimidazole and ethylenediamine. The endosulfanamine derivative was coupled to $NaIO_4$-oxidized HRPO.

endosulfanamine intermediate, dissolved in DMF/phosphate buffer (200 mM, 1:1), for 3.5 h at room temperature. Subsequently the endosulfan–enzyme conjugate was dialyzed against PBS and 2% DMF. The conjugate was stored at −20 °C in 50% glycerin.

Enzyme activity and immune specificity against endosulfan antibodies were measured.

Enzyme Immunoassay (EIA). Competitive EIA was carried out according to Van Weemen and Schnuurs (1971) and Engvall and Perlmann (1971). All reactions were performed in polystyrene plates (Nunc 96 F). A volume of 200 μL of antibody solution was used in the microtiter plates. The coating antibodies were diluted 1:1000 in coating buffer. The plates were then kept overnight at 4 °C and washed once with PBS.

After addition of PBS containing 1% BSA (200 μL), the plates were incubated for 1 h at 37 °C and stored in a freezer. The coated plates are stable for several months. Before use, the plates were washed three times with PSB-T, and 150 mL of test samples (endosulfan standards in water) together with 50 μL of endosulfan–peroxidase conjugate (150 ng/mL) dissolved in PBS-T/1% BSA were added and incubated for 4 h at 37 °C. The endosulfan standards were made by preparing a stock solution in DMSO (100 μg/mL); further dilutions were made in water. Unbound conjugate was removed by washing three times with PBS-T, and 200 μL of *o*-phenylenediamine substrate solution was added. After 30-min incubation at room temperature the enzyme reaction was terminated by the addition of 50 μL of 60% H_2SO_4 and the optical density of the wells was read at 490 nm on a Titertek Multiscan MC spectrophotometer (Flow Laboratories).

Routinely, standard curves were plotted as shown in Figures 4, 5, 7, and 8. The detection limit of the assay was defined as the lowest concentration of endosulfan showing reduction of the zero standard extinction by double the standard deviation of the zero standard.

RESULTS AND DISCUSSION

The structures of antigen and enzyme conjugate are shown in Figures 2 and 3. The synthesis of the endosulfan–peroxidase conjugate turned out to be extremely difficult. Because of the strongly hydrophobic character

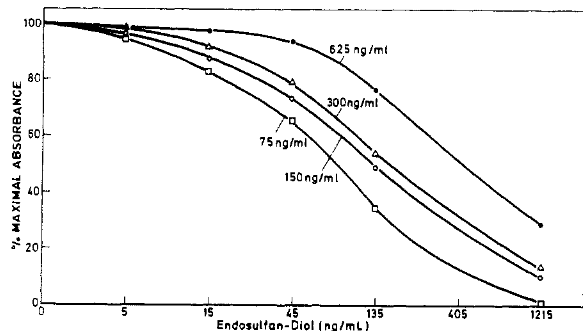

Figure 4. Effect of varying the endosulfan–enzyme conjugate concentration on the sensitivity of competitive EIA for endosulfan.

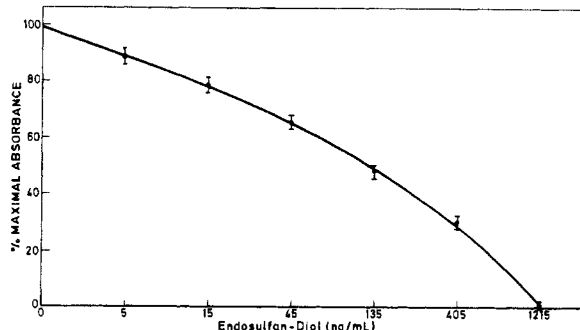

Figure 5. Typical standard curve for endosulfan. The curve represents the mean and standard deviation of an 11-fold determination.

of endosulfan, the conjugates of endosulfan and carrier protein or enzyme, respectively, proved to be water insoluble in most cases. This caused the precipitation or inactivation of the peroxidase. In contrast there was no problem in synthesis of the antigen, since insoluble antigens injected as suspension prove to be very effective immunogens, which was also demonstrated by the high titers to endosulfan (1:72 000).

The problem of the inactivation of peroxidase was solved by the synthesis of an amine derivative of endosulfandiol (Figure 3). This strategy reduced the hydrophobic character of endosulfandiol, and the coupling of the amine derivative to peroxidase via a periodate oxidation of peroxidase resulted in coupling of only one endosulfan molecule per molecule of peroxidase. However, this method led to a distinctly large amount of uncoupled peroxidase. This became evident by the effect of enzyme–conjugate concentrations on the sensitivity (Figure 4).

The highest sensitivities under the used conditions were obtained with a conjugate concentration of 75 ng/mL, but the absolute value of the absorbance was too low (100% maximal absorbance was 0.35 absorbance unit). For that reason, a conjugate concentration of 150 ng/mL was necessary for getting a sufficient absorbance (100% maximal absorbance was 0.8 absorbance unit) and simultaneously a good sensitivity.

Since one test required 50 μL of conjugate (approximately 7.5 ng of peroxidase) to achieve sufficiently high extinction values, it is estimated that only 20–30% of the peroxidase molecules were really coupled to endosulfanamine.

Figure 5 shows the standard curve for endosulfan in the competitive EIA system. The curve represents the mean and standard deviation of an 11-fold determination, and it is apparent that the sensitivity approached 3 ng mL^{-1} or 3 ppb.

Since the EIA was developed for residue analysis, it was necessary to be able to detect the most common degra-

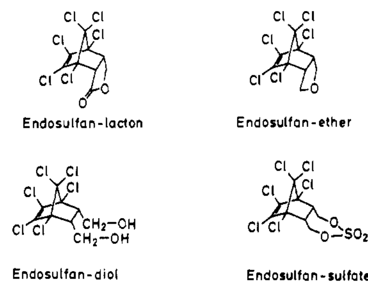

Figure 6. Structures of endosulfan degradation products: (a) endosulfan lactone (4,5,6,7,8,8-hexachloro-1,3,3a,4,7,7a-hexahydro-4,7-methanoisobenzofuran-1-one); (b) endosulfan ether (4,5,6,7,8,8-hexachloro-1,3,3a,4,7,7a-hexahydro-4,7-methanoisobenzofuran); (c) endosulfandiol (1,4,5,6,7,7-hexachlorobicyclo-[2.2.1]hept-5-ene-2,3-dimethanol); (d) endosulfan sulfate (6,7,8,9,10,10-hexachloro-1,5,5a,6,9,9a-hexahydro-6,9-methano-2,4,3-benzodioxathiepin 3,3-dioxide).

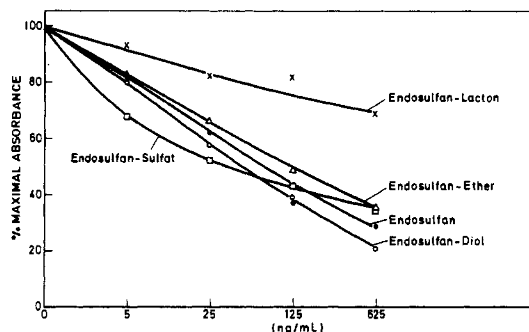

Figure 7. Standard curves for endosulfan and its degradation products, endosulfandiol and endosulfan ether, sulfate, and lactone. See Figure 6 for structures and IUPAC designation.

Table I. Sensitivity of EIA for Endosulfan Degradation Products with Regard to Endosulfan[a]

degradation product	sensitivity, %
endosulfan	100
endosulfandiol	158
endosulfan sulfate	203
endosulfan ether	57
endosulfan lactone	0.02

[a] Sensitivity is expressed as that concentration of endosulfan causing a 50% inhibition of binding × 100, divided by the concentration of the endosulfan degradation product that caused a 50% inhibition of binding.

Table II. Cross-Reaction of Other Chlorinated Hydrocarbon Pesticides in Competitive EIA with Regard to Endosulfan

pesticide	cross-reactn, %	pesticide	cross-reactn, %
endosulfan	100	lindan	3
aldrin	16	endrin	180
alodan	7		

dation products, such as endosulfandiol and endosulfan ether, sulfate, and lactone (Figure 6). Figure 7 shows that all endosulfan degradation products are detected in the same concentration range as endosulfan, except the lactone derivative.

The sensitivities with respect to the degradation products are shown in Table I. In addition, EIA studies were also performed on other chlorinated hydrocarbon pesticides. The results are shown in Figure 8 and Table II. Aldrin, alodan, and lindan showed rather small cross-reactivities of 16%, 7%, and 3%, respectively, whereas endrin exhibited cross-reactivity of 180%, meaning that its limit of detection is lower than that for endosulfan.

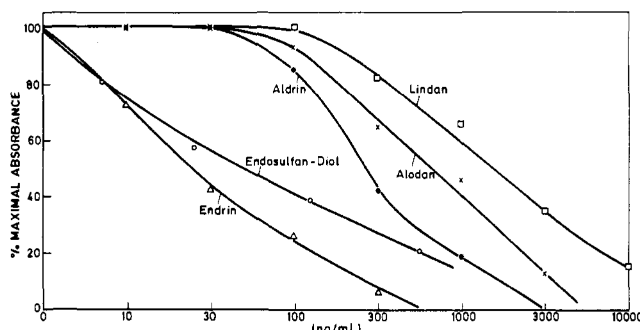

Figure 8. EIA curves for other chlorinated hydrocarbon pesticides endrin, aldrin, alodan, and lindan in comparison with endosulfan.

This high cross-reactivity with endrin cannot be explained solely on the basis of the common hexachloro structure, since this structure is also found in the other chlorinated hydrocarbon compounds that were examined, with lindan as a hexachlorohexane showing the lowest cross-reactivity.

CONCLUSIONS

This study presents, for the first time, a competitive EIA for a pesticide with a chlorinated hydrocarbon structure. The endosulfan EIA provides an accurate method for the determination of residues in water as well as the most important degradation products. The assay shows a detection limit of 3 ng mL^{-1} and allows a rapid throughput of samples, making the method particularly useful for the simultaneous analysis of a large number of samples. The method is currently adapted to soil residue analysis. The assay possesses a low cross-reactivity against the chlorinated hydrocarbons aldrin, alodan, and lindan and a high cross-reactivity against endrin. These cross-reactivities can probably only be abolished by use of monoclonal antibodies angainst endosulfan.

In spite of the great problem concerning the solubility and activity of the peroxidase conjugate during the synthesis of the conjugate, the possiblity was demonstrated to develop an EIA for a hydrophobic substance such as endosulfan. The sensitivity of the assay (3 ppb) is in comparison with other developed ELISAs for pesticides in a similar range (Wie and Hammock, 1982; Newsome, 1986) or better (Huber and Hock, 1985; Niewola et al., 1986).

The EIA method for endosulfan determination should prove to be a powerful analytical tool in environmental studies, and there is a clear scope for the development of assays for other chlorinated hydrocarbon pesticides.

Registry No. Endosulfan, 115459-37-5; endosulfan ether, 13447-74-0; endosulfan lactone, 13447-76-2; endosulfandiol, 17378-23-3; endosulfan sulfate, 13383-10-3; endosulfan hemisuccinate, 115384-49-1; endosulfan active ester, 115384-50-4.

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