Use of Phosphonic Acid as a Generic Hapten in the Production of Broad Specificity Anti-Organophosphate Pesticide Antibody

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Phosphonic acid (*trans*-4-phosphono-2-butenic acid; TPB) was used as a generic hapten in order to generate broad specificity antibodies against a group of organophosphorus pesticides. The polyclonal antiserum showed, in an indirect enzyme-linked immunosorbent assay (ELISA) format, preferential binding toward pesticides containing unsaturated diethyl-phosphate functionalities rather than the equivalent thiophosphate or dimethyl structures. The level of detection in the ELISA using a heterologous system was investigated and showed a 20-fold improvement when a conjugate for which the antibody had lower affinity was immobilized on the plate. Biosensor assays using parathion as a standard indicated that the antibody had a relatively high dissociation rate, and reproducible cycles of regeneration were achieved. The potential for using TPB as a generic hapten is discussed.

Keywords: *Organophosphorus pesticides; phosphonate; broad specificity antibody; ELISA; biosensor; immunoassay*

INTRODUCTION

Organophosphorus pesticides (OPs) are widely used in agricultural and domestic applications (Worthing and Hance, 1991; Racke, 1993). Contaminant residues have been reported in food (U.S. Food and Drug Administration, 1994) and environmental samples (Hallberg, 1989).

As an alternative to the laborious and lengthy chromatographic procedures for OP analysis (Association of Official Analytical Chemists, 1995), several immunoassay methods have been developed both as semiquantitative screening tools and as quantitative methods (Ercegovich et al., 1981; Brimfield et al., 1985; McAdam et al., 1992; Skerritt et al., 1992; Hill et al., 1994; Skerritt and Lee, 1995; Manclus et al., 1996). In general, anti-OP antibodies raised using conventional methodology are highly specific toward a single analyte. Although antibody specificity is an advantageous characteristic for low level detection of trace amounts of individual OPs, there are applications in which broad-specificity antibody recognition of groups of analytes would be desirable, such as for use in cost-effective screening programs, in immunoaffinity columns, and in automated biosensor techniques.

In the past we have successfully developed monoclonal anti-idiotype antibodies that mimic the cutinase active site as one approach to producing a broad-specificity organophosphate antibody (Ward et al., 1999). The anti-idiotype monoclonal antibody (MAb) was able to bind chlorfenvimphos, ethyl paraoxon, tetrachlorvinphos, and demeton-s-methyl in an ELISA format.

Another approach to producing broad-specificity antibodies would be the use of a generic hapten. OPs are nonproteinaceous low molecular weight molecules (<1500 Da), that need to be conjugated to carrier proteins as haptens in order to be made capable of inducing antibodies able to recognize the free hapten. The majority of OPs can be derived from two general structural formulas $(EtO)_2P(X)Y$ and $(MeO)_2P(X)Y$ where X represents O or S and Y represents a range of chemical structures. The functional groups chosen for attachment of the hapten to the carrier protein as well as the length of the spacer between hapten and protein and method of conjugation have all been shown to be fundamental in determining the properties of the resultant antibodies (e.g. Vallejo et al., 1982). Two different strategies have been used to conjugate OPs to the carrier protein: first, attachment to the phosphate ester backbone (McAdam et al., 1992, Skerritt et al., 1992, Manclus et al., 1996) and second, direct attachment to the Y structure, in which the phosphate ester group is left exposed (Ercegovich et al., 1981; Brimfield et al., 1985; Manclus et al., 1996). In both approaches specific antibodies have usually been generated.

Two studies, one by Sudi and Heeschen (1988) and another by Banks et al. (1998) have explored the conserved structure of the majority of OPs and asked whether the use of a common and redundant structure could be used to generate broad specificity antibodies. Banks et al. (1998) have reported the use of a generic thiophosphate hapten linked through a four-carbon spacer to BSA and the production of polyclonal antibodies able to recognize fenitrothion, methacrifos, propetamphos, and dichlorvos. Sudi and Heeschen (1988), in a more comprehensive study, using a variety of haptens and different solid phases, generated antibodies that recognized a great number of OPs, though with reduced assay sensitivity. In particular, the utilization of a short unsaturated linear chain exposing the diethyl phosphono ester group that could either mimic the diethyl phosphate or diethyl phosphothioate groups were attractive approaches.

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10.1021/jf990691m CCC: $19.00
Published on Web 06/02/2000

To set up the basis for a more systematic and comprehensive synthesis program, we report the use of the diethyl *trans*-4-phosphono-2-butenic acid (TPB) as a generic OP hapten. We show that TPB as used by Sudi and Heeschen (1988) can indeed, within certain limits, be used as a generic hapten to generate broad specificity antibodies against OPs. We have characterized the antibodies produced, investigated ways of improving the level of detection in an ELISA format, and also explored their potential use in a biosensor format.

MATERIALS AND METHODS

Chemicals. *trans*-4-Phosphono-2-butenic acid was purchased from Fluka Chemicals, Gillingham, U.K. Parathion-ethyl, parathion-methyl, paraoxon-methyl, and paraoxon-ethyl were purchased from Riedel-de Haën, London, U.K. All the remaining OPs used in the cross-reactivity studies were purchased from Qmx Laboratories Limited, Halstead, U.K. Amino-dextran 40 000 MW (7.4 amines/mole) was purchased from Molecular Probes Europe, Leiden, Holland. The remaining reagents were purchased from Sigma Chemical Co., Poole, U.K.

Preparation of TPB−KLH Conjugate. TPB was coupled to keyhole limpet hemocyanin (KLH) by the carbodiimide method using 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (EDC) as described by Südi and Heeschen (1988). Unreacted hapten was removed by exhaustive dialysis against 5 L of water changed every 12 h at 4 °C for 48 h, and the conjugate was then freeze-dried.

Preparation of Chlorpyrifos−Ovalbumin Conjugate. Chlorpyrifos-propionic acid derivative was synthesized according to the procedures described by Manclús et al. (1996). The derivative was coupled to ovalbumin by the carbodiimide method using EDC as described for TPB−KLH. Unreacted hapten was removed by exhaustive dialysis against 5 L of water changed every 12 h at 4 °C for 48 h, and the conjugate then freeze-dried.

Preparation of OP−Protein and OP−Dextran Conjugates. Parathion-ethyl and paraoxon-ethyl were coupled to bovine serum albumin (BSA) and to amino-dextran by diazotisation, based on the method of Ercegovich et al. (1981). Essentially the nitro group of parathion or paraoxon was reduced by zinc powder and the amino derivative was analyzed by TLC. The amino products run at about half the R_f of the nitro compounds on silica gel-G TLC when a hexane−acetone (7:3) solvent system was employed. The reduced products were then converted to diazonium chloride and reacted with BSA or amino dextran essentially as previously described (Ercegovich et al., 1981). Unreacted hapten was removed by exhaustive dialysis against 5 L of water changed every 12 h at 4 °C for 48 h as before and freeze-dried. The hapten-carrier ratio was determined by measuring the organic phosphate attached to the carrier as described by Garrett et al. (1997).

Immunization Protocol. Two male New Zealand white rabbits were given four subcutaneous injections (250 μL/site) with TPB−KLH conjugate (200 μg/rabbit) suspended in complete Freund's adjuvant−saline (1:1). Booster immunizations were given in a similar manner at monthly intervals using Freund's incomplete adjuvant. Blood was collected in heparinized tubes from the marginal ear vein 10 days after each booster injection. Plasma was separated from the cells by centrifugation and stored at −20 °C.

ELISA. Microtitration plates (Nunc Immunoplate Maxisorp, Gibco Ltd., Uxbridge, U.K.) were coated with parathion or paraoxon−dextran conjugate (1 μg/mL) in phosphate-buffered saline (PBS) for 16 h at 4 °C. Following three washes with distilled water the plates were blocked with 3% nonfat dried milk in PBS plus Tween 20 at 0.05% (PBST) at room temperature for 1 h. Finally the plates were washed three times with PBST, dried, and stored at −20 °C. To perform the assay, polyclonal antisera diluted 1:2000 and the OP standard were added to each well of the coated plate (final volume 100 μL) and left at room temperature for 2 h. The plates were washed five times with PBST and incubated at room tempearture for 2 h with anti-rabbit-HRP (100 μL; Sigma Chemical Co., Poole, U.K), diluted 1:1000 in PBST. The plates were then washed five times in PBST, and detection was achieved by the addition of substrate, 100 μL of 3,3′,5,5′-tetramethyl benzidine solution (Vetoquinol, Bicester, U.K.). After approximately 10 min at room temperature the reaction was stopped by adding sulfuric acid (50 μL, 2 M), and the optical densities of each well were read at 450 nm on a plate reader (Dynatech MR5000, Dynatech Laboratories Ltd., Billingshurst, U.K.). Limits of detection were calculated as the zero value plus 3 standard deviations.

Biosensor Analysis. Antibody properties were examined in a dynamic flow situation using a BIAcore 1000 instrument and CM5 sensor chip both supplied by BIAcore AB (Uppsala, Sweden).

(*i*) *Coupling Reaction for Sensor Surface*. The carboxymethylated dextran matrix was activated by mixing equal volumes of 100 mM *N*-hydroxysuccinimide and 400 mM of EDC and injecting the mixture over the sensor surface for 7 min at a flow rate of 5 μL/min (Lofas and Johnson, 1990). Parathion−BSA (50 μg/mL) was dissolved in 10 mM acetate buffer, pH 4.1, and injected over the surface for 15 min at a flow rate of 2 μL/min. The unreacted sites on the sensor surface were capped by injection of 1 M ethanolamine, pH 8.5, for 7 min.

(*ii*) *Antibody Preparation*. Serum was purified by protein A chromatography and was dialyzed against PBS (pH 7.3, 0.15 M NaCl), and subsequently 2 mg/mL of BSA and 2 mg/mL of carboxymethylated dextran were added. These solutions were preincubated for 1 h at 37 °C. All buffers and solutions used in biosensor were made using ultrapure water, degassed, and sterile filtered.

(*iii*) *Sample Analysis*. Antibody samples prepared as previously described were injected over the sensor surface for 4 min at a flow rate of 5 μL/min.

(*iv*) *Biosensor Immunoassay*. A solution of free parathion (10 mg/mL) was prepared in ethanol. Dilutions were made in PBST in a range between 50 μg/mL and 0.390 μg/mL to provide the assay standards. Each sample was incubated with an equal volume of antibody for 10 min at room temperature and then passed over the BIAcore sensor surface. This procedure was carried out for each standard concentration three times.

(*v*) *Sensor Surface Regeneration*. Regeneration of the sensor surface was carried out by injecting a single 30 s pulse of both 5 mM NaOH and 5 mM HCl.

RESULTS AND DISCUSSION

Although immunoassays for a variety of pesticides have been developed, and some are available commercially, for large residue screening programs immunoassay may suffer from a major practical drawback: high specificity for individual analytes. Several immunoassays would need to be run simultaneously in order to screen an unknown sample. On the other hand, conventional chromatographic techniques, despite being laborious and much less suited to screening programs, can quantify many pesticides individually. Antibodies of broader specificity than those currently available need to be developed.

In the present paper we explore the conserved structure of the majority of OPs and ask whether the use of a common and redundant structure (TPB) could be used to generate broad specificity antibodies.

Antiserum Titer. TPB, a commercially available unsaturated phosphonic acid, was coupled, using conventional carbodiimide coupling chemistry, to KLH and used as immunogen. Figure 1 shows that both rabbits responded well to TPB when the sera were tested by ELISA against immobilized TPB-ovalbumin but did not react with the ethyl-chlorpyrifos−ovalbumin conjugate used as control. When the sera were tested against

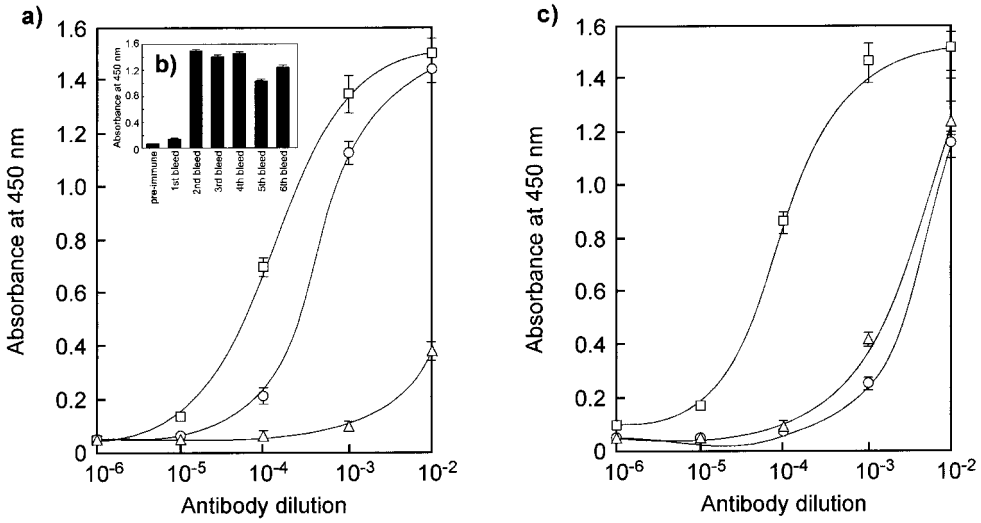


Figure 1. ELISA titration of polyclonal antisera against immobilized OP. Microtitration plates were coated with 1 μg/mL of protein conjugate, and each point represents an average of three replicates (△ = diethyl-chlorpyrifos−ovalbumin control, □ = TPB−ovalbumin, ○ = diethyl-parathion−BSA): (a) antiserum IFRN 559; (b-insert) antiserum IFRN 559 using diethyl-parathion−BSA as solid phase after consecutive bleed; and (c) antiserum IFRN 558.

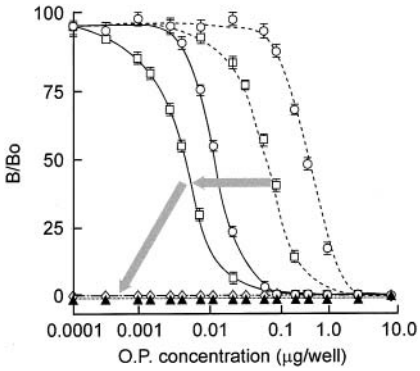


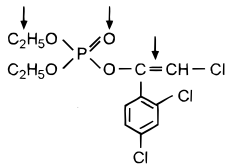
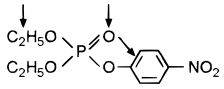
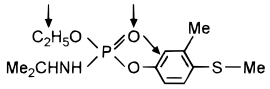
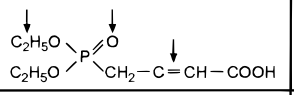
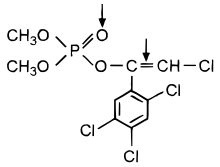
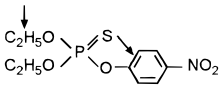
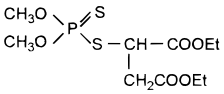
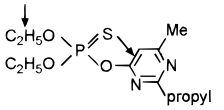
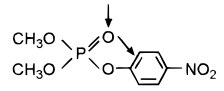
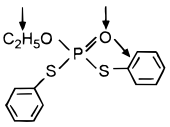
Figure 2. Optimized ELISA using IFRN 559 antiserum. All OP conjugates were synthesized with amino dextran as carrier, and the solid phases were coated using a solution containing 25 ng of parathion-equivalent/well. Each point represents an average of three replicates. Solid phases: (- - -) = diethyl-paraoxon-dextran; (—) = diethyl-parathion-dextran; (···) = dimethyl-parathion and (·-·-) dimethyl-paraoxon-dextran. Two OPs were used with each of these solid phases: □ = diethyl-parathion and ○ = diethyl-paraoxon. Arrow indicates the shift in the assay limit of detection observed from the high affinity solid phase to the lower one.

parathion-BSA, antiserum IFRN 559 showed a higher titer (Figure 1a) than antiserum IFRN 558 (Figure 1c). Furthermore this high titer was clearly induced by consecutive immunizations, as shown in Figure 1b, and was kept constant thereafter.

ELISA Cross-Reactions. To further characterize the nature of the antibody recognition, cross-reactions were determined. A selection of 29 OPs, over a wide range of concentrations, was tested in an ELISA using antiserum IFRN 559 and parathion-BSA as the assay solid phase. The curves obtained with the 29 OPs (data not shown) showed a low background, similar to those shown in Figure 2; the I_{50} and limit of detection are displayed in Tables 1 and 2. One of the main features of the recognition of serum IFRN 559 was its broad specificity toward a large number of OPs. The results suggest that the recognition is specific for the phosphate group as the antiserum did not bind to organophosphate breakdown products such as trichloro-2-pyridinol (Table 2). Antiserum IFRN 559 recognized OPs that possess the phosphate group (such as ethyl-paraoxon) better than those with the thiophosphate group (such as ethyl-parathion) and diethyl ester derivatives (such as ethyl-paraoxon) rather than the dimethyl ester derivatives (such as methyl-paraoxon). In contrast to the results described by Südi and Heeschen (1988), in which OPs with aromatic side chain were preferentially recognized by an antiserum raised against the same TPB hapten, linear side chain OPs were also recognized by serum IFRN 559. Interestingly, the recognition was higher than for the TPB for at least three of the OPs tested: chlorfenvimphos, diethyl-paraoxon, and fenamiphos. These three OPs possess an unsaturation adjacent to the phosphate group besides the phosphate group and the ethyl ester. The importance of the unsaturation is further stressed as a common structure present in most of the 10 pesticides with high binding as displayed in Table 1 and in the binding of tetrachlorvimphos and monocrotophos where the dimethyl esters were used. In general, the cross-reactivity results were in agreement with what might have been predicted by using TPB as immunogen and suggest that at least three parts of the structure, the phosphate group, the diethyl ester, and the unsaturated bond adjacent to the phosphate group (highlighted in Table 1), make up the epitope.

ELISA Limit of Detection. The unusual recognition of the parathion derivative by IFRN 559 led us to investigate the effect of the immobilized OP conjugate on ELISA limit of detection. Based on the cross-reactivity results, several parathion derivatives were synthesized and conjugated to the inert carrier amino dextran in order to provide a range of materials with different affinities for the antibody. ELISA plates were coated with equal densities of methyl-parathion, ethyl-parathion, ethyl-paraoxon, and methyl paraoxon on a dextran carrier, and an inhibition assay was set up using either ethyl-parathion or ethyl-paraoxon standards (Figure 2). As shown in Figure 2, a 20-fold improvement in the limit of detection was observed when an OP of lower affinity, in this case the thiophosphate ethyl rather than a phosphate group, was used as solid phase. The use of conjugates with lower affinity as solid phase in order to improve the limit of detection can only operate within a certain range of affinities, as

Table 1. ELISA Cross-Reactions Using IFRN 559 Antiserum[a]

Rank	Structure	Name	I_{50} ng/ml	LOD ng/well
1		Chlorfenvinphos	24	0.3
2		Paraoxon-ethyl	37	0.6
3		Fenamiphos	100	1.2
4		TPB	225	3.8
5		Tetrachlorvinphos	450	5.4
6		Parathion-ethyl	600	12
7		Malathion	700	10
8		Diazinon	900	12
9		Paraoxon-methyl	1400	13
10		Edifenphos	1500	23

[a] All reactions were performed in triplicate on microtitration plates coated with diethyl-parathion−BSA at 1 μg/mL as solid phase. Only the structure of the 10 highest cross-reactions are shown, the remaining OP tested are shown in Table 2. LOD = limit of detection.

antibodies that were weakly bound would be removed during the washing steps as exemplified in Figure 2 by the methyl ester derivatives. When TPB conjugate was used in the assay, the limit of detection was between values given by the ethyl-parathion and ethyl paraoxon (data not shown). The improvement in the limit of detection of the assay obtained by using compounds with different affinities compared to the analyte is a well described phenomenon, and our results corroborate the use of heterologous assays for improved sensitivity (Marco et al., 1995).

Biosensor Analysis. If affinity is a critical parameter in an assay format such as ELISA, in applications that rely on dynamic equilibrium at solid surfaces, affinity assumes even more importance due to the need for solid-phase regeneration. To characterize IFRN 559 in a dynamic flow situation, BIAcore analyses, using parathion−BSA as immobilized conjugate, were set up. The coupling of the parathion−BSA conjugate to the dextran surface was carried out using conventional carbodiimide coupling chemistry (Lofas and Johnson, 1990). The pI of the conjugate was found to be 4.1 which suggested a high parathion−BSA ratio. Immobilization of the conjugate resulted in approximately 10 000 RU of parathion−BSA bound to the surface. A response of 10 000 RU in the BIAcore indicates a protein concentration of approximately 10 ng/mm^2. Therefore, immobilization of parathion−BSA was successful, linking a large quantity of conjugate to the surface. Preincubation of all samples with BSA and dextran resulted in any nonspecific binding being eliminated. Antiserum IFRN 559 gave a good binding response to parathion. To achieve significant binding of antibody to the surface of the sensor chip, undiluted antiserum was found to

Table 2. ELISA Cross-Reactions Using IFRN 559 Antiserum[a]

rank	name	I_{50} ng/mL	LOD ng/well
11	vanidothion	1900	30
12	propetamphos	1900	17
13	pyrimiphos-ethyl	2500	40
14	mevinphos	3700	52
15	dicrotophos	5200	50
16	fenitrothion	5600	110
17	monocrotophos	6100	66
18	parathion-methyl	10 500	160
19	carbofenothion	18 800	49
20	propetamphos	19 000	140
21	azamethiphos	25 000	240
22	etrimphos	27 000	730
23	cyanofenphos	63 000	120
24	chlorpyrifos-ethyl	67 000	530
25	methacrifos	70 000	400
26	pyrimiphos-methyl	Nx	
27	methamidophos	Nx	
28	chlorpyrifos-methyl	Nx	
29	trichloropyridinol	Nx	

[a] All reactions were performed in triplicate on microtitration plates coated with diethyl-parathion-BSA as solid phase at 1 μg/mL as solid phase. LOD = limit of detection. Nx = do not cross react, I_{50} higher than 100 μg/mL.

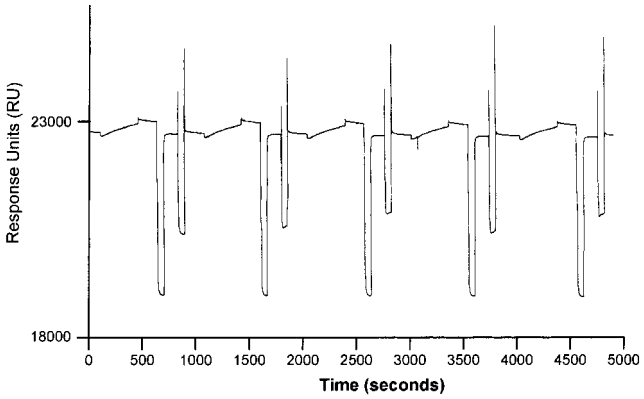


Figure 3. Sensorgram showing five antibody binding and regeneration cycles. Each cycle consisted of a pulse of antibody at 5 μL/min for 4 min followed by injection of a single 30 s pulse of both 20 mmol/L NaOH and 20 mmol/L HCl.

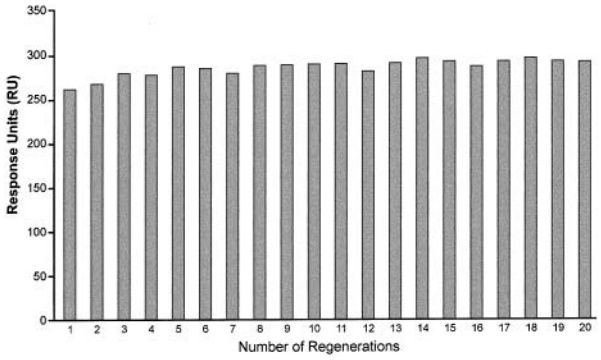


Figure 4. Graph showing the reproducibility of regeneration of the sensor chip. Twenty regenerations of the sensor surface to which parathion−BSA was immobilized. No damage to the solid phase is apparent. The mean ± SD of response units for the 20 regenerations was 286.77 ± 8.4025.

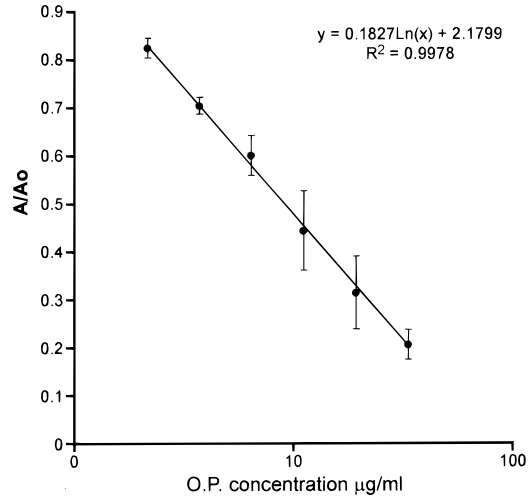


Figure 5. Biosensor interassay variation. Parathion−BSA conjugate was coated on the surface of the sensor chip at a concentration of 50 μg/mL, and the antibody was used undiluted. Mean values were taken from three assays. A linear range of detection was found between 1.562 and 50 μg/mL of parathion.

be required. Approximately 300 RU bound to the surface at each binding pulse. Five regeneration cycles of antibody binding to the parathion−BSA surface of the sensor chip are shown in Figure 3. The interaction displayed a moderate dissociation between antibody and antigen as regeneration of the surface was achieved easily with 20 mM NaOH and 20 mM HCl. These solutions removed the bound antibody from the surface with minimal effect on the conjugate-coated surface. Thus, it was possible to carry out a number of regenerations without damage occurring to the surface of the chip. Figure 5 is a typical sensorgram of five binding-regeneration cycles. Regeneration of the surface was carried out 20 times under these regeneration conditions (Figure 4).

Biosensor Assay. A competitive assay was then set up using a parathion-BSA immobilized surface. Standards of parathion were prepared and incubated with an equal volume of antibody and then passed over the surface of the chip. The binding of antibody to the surface of the chip was inversely proportional to the amount of free parathion in solution. The linear range of detection was found to be between 1.562 μg/mL and 50 μg/mL. Interassay accuracy and precision were then determined. Response unit values of the standards (A_1) were divided by the response unit values for 0% inhibition (A_0), and the mean and standard deviation values were plotted against concentration of parathion. This indicated a high accuracy level (Figure 5).

The pesticide assay data presented in this paper were generated from samples prepared in buffer. The next stage of this work will be to perform an extensive study applying this assay, and others under development in our laboratory, to contaminated agricultural and food samples. The aim of this second study will be 2-fold: (i) to demonstrate that the assay sensitivity is sufficient for screening trace levels of relevant pesticides and (ii) to investigate potential sample matrix effects and, if any are found, methods of eliminating them. The work is part of an EU-funded project, and suitable test substrates (e.g. wheat, maize, rice, bread), containing up to six relevant pesticides, are under preparation by other partners.

The results suggest that there is considerable merit in using TPB hapten−protein conjugates as immunogens for obtaining broad specificity antibodies against organophosphate pesticides. Also, it has stressed the importance of the unsaturation within the immunogen linear chain, the typical between-animal variation in antibody response, and the role of antibody affinity for

different immobilized competitors in determining assay sensitivity. The interaction between antibody and parathion was found to have a relatively high dissociation rate so that regeneration of the biosensor surface was successful. This characteristic is favorable as it allows sequential analysis of a single surface without significant damage. In this case 20 regeneration cycles were completed with minimal alteration to the surface during each binding-regeneration cycle. The linear range of detection was found to be between 1.562 and 50 μg/mL when parathion−BSA was immobilized onto the surface of the chip. Interassay accuracy and precision indicated a high accuracy level.

ACKNOWLEDGMENT

This work was supported by the Commission of the European Communities, Agriculture and Fisheries (FAIR) CT96-1181. C. Doyen was supported by a Lavoisier grant from the Ministére des Affaires Etrangéres.

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Received for review June 25, 1999. Revised manuscript received March 28, 2000. Accepted April 2, 2000.

JF990691M