level remaining from the two doses at each station: the high significant interaction between the experimental station and the spraying period on the variation of the double/single dose ratio suggests that the amount of pesticide acts on the residue level differently according to the environmental and experimental conditions.

In no case was the residue on the grapes above the Italian legal limit, not even as a consequence of excessive doses.

The experiments carried out appear to have confirmed the general hypothesis that the degradation of pesticides on the plant is a phenomenon that varies with the environment, rather than the dose: the locality and period of spraying are factors that must definitely be taken into account when establishing spraying period and suitable intervals between final spraying and harvest.

Registry No. Vinclozolin, 50471-44-8.

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Mouse Monoclonal Antibodies against Paraoxon: Potential Reagents for Immunoassay with Constant Immunochemical Characteristics

Alan A. Brimfield,* David E. Lenz,[1] Caroline Graham, and Kenneth W. Hunter, Jr.

Mice were immunized with (*p*-aminophenyl)paraoxon conjugated to protein by diazotization. The production and isolation of permanent hybridoma cell lines from the splenic lymphocytes of the immunized animals are described. The specificity of the two resultant paraoxon-specific monoclonal antibodies was tested with paraoxon analogues and seven insecticides. Significant cross reactivity was found with parathion and (*p*-aminophenyl)paraoxon, while no interfering compounds were found among the other insecticides tested. The affinities of the monoclonal antibodies were determined to be 9.4×10^4 and 1.7×10^5 L/mol. Specificity determinations proved the antibodies to be chemically unique. When incorporated into a competitive inhibition enzyme immunoassay, the antibodies provided quantification of paraoxon that compared favorably with gas chromatography on the basis of precision and specificity. The better of the two antibodies produced a response linear from 10 to 100 μg/mL with a minimum detectable concentration of 1 μg/mL in an assay requiring 50 μL of sample. The direction of ongoing work to improve sensitivity is discussed.

INTRODUCTION

Present methods of analysis for insecticides in environmental samples require time-consuming isolation and cleanup procedures, expensive analytical instruments, and highly trained personnel (Association of Official Analytical Chemists, 1980; U.S. EPA, 1980). Radioimmunoassays (Ercegovitch et al., 1981; Langone and Van Vunakis, 1975; Wing and Hammock, 1979) provide the sensitivity and specificity of immunoassay and reduce the need for extensive cleanup steps. However, they also involve expensive equipment and highly trained personnel plus radiation risk and long analysis times.

Competitive inhibition enzyme immunoassay (CIEIA) is a rapid, simple, and inexpensive alternative. Work in our laboratory using heteroantisera has shown the feasibility of this technique applied to paraoxon (Hunter and Lenz, 1982) and soman (Hunter et al., 1983). A similar assay has been successfully applied to parathion residues (Al-Rubae, 1978). An informative review has been published by Hammock and Mumma (1980).

Hapten-specific polyclonal antisera, usually raised in rabbits, form the basis for the immunoassays discussed above. As analytical reagents, heteroantisera posses two substantial drawbacks. They are subject to the variation in immunoglobulin composition and specificity that arise during the maturation of the in vivo immune response (Eisen, 1980). Further, a given heteroantiserum remains available only over the finite lifespan of the immunized animal.

We undertook the present work to supplant the use of heteroantisera in the paraoxon immunoassay procedure. This was accomplished by the development of mouse hybridoma cell lines that produce paraoxon-specific mono-

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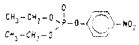
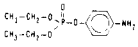
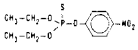
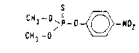
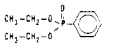
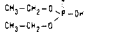
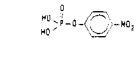
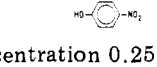
Table I. Specificity of Monoclonal Antibodies toward Paraxon Analogues as Defined by Molar IC_{50}[a]

INHIBITOR	STRUCTURE	MOLAR IC_{50}[b] BD1	CE3
1. PARAOXON[c] [311-45-5]		$7.1(\pm 0.1) \times 10^{-5}$	$2.5(\pm 0.2) \times 10^{-4}$
2. AMINOPARAOXON [14984-58-8]		$5.6(\pm 0.3) \times 10^{-5}$	$7.5(\pm 0.7) \times 10^{-5}$
3. PARATHION [56-38-2]		$2.0(\pm 0.1) \times 10^{-3}$	$2.2(\pm 0.1) \times 10^{-3}$
4. METHYLPARATHION [298-00-0]		$2.4(\pm 0.2) \times 10^{-3}$	$4.7(\pm 0.3) \times 10^{-3}$
5. DIETHYL PHENYLPHOSPHONATE [1754-49-0]		$1.6(\pm 0.2) \times 10^{-3}$	$4.6(\pm 0.6) \times 10^{-3}$
6. DIETHYL PHOSPHATE [598-02-7]		$>10^{-2}$	NI
7. p-NITROPHENYL PHOSPHATE [4264-83-9]		NI	NI
8. p-NITROPHENOL [100-02-7]		NI	NI

[a] Antibody concentration 0.25 μg/mL hapten affinity purified. Conjugate coating concentration 0.5 μg/mL overnight at 4 °C. Antibody plus inhibitor preincubation and all steps in the assay were 30 min at room temperature. [b] Average ±SD of three determinations. [c] CAS nomenclature: (1) phosphoric acid, *O,O*-diethyl *O*-(4-nitrophenyl) ester; (2) phosphoric acid, *O,O*-diethyl *O*-(4-aminophenyl) ester; (3) phosphorothioic acid, *O,O*-diethyl *O*-(4-nitrophenyl) ester; (4) phosphorothioic acid, *O'O*-dimethyl *O*-(4-nitrophenyl) ester; (5) phenylphosphonic acid, *O,O*-diethyl ester; (6) ethylphosphonic acid, *O,O*-diethyl ester; (7) phosphoric acid, *O*-(4-nitrophenyl) ester; (8) 4-nitrophenol.

clonal antibodies. Purification of the immunoglobulin from ascites fluid or cell-free culture fluids by affinity column chromatography yielded reagent antibodies whose characteristics and availability were no longer subject to the vagaries of animal lifespan and immune response maturation. With appropriate monitoring and good culture technique, these cell lines will produce an infinite supply of analytical reagent.

MATERIALS AND METHODS

Reagents. The insecticides used to establish antibody cross reactivity (see Table I) were of primary reference standard grade obtained from the Health Effects Research Laboratory, U.S. EPA, Research Triangle Park, NC. The remaining test compounds were purchased from Alfa Products Ventron Division, Thiokol Corp., Danvers, MA, and were of defined purity. Tritiated paraoxon (New England Nuclear, Boston, MA) had a radiochemical purity of greater than 99% determined by thin-layer chromatography and autoradiography. The specific activity was 41.38 mCi/mmol by scintillation counting and quantification by the spectrophotometric method of Elliot et al. (1960). All other chemicals were of reagent grade or better and were used as received.

Preparation of Paraoxon–Protein Conjugates. The *p*-amino analogue of paraoxon (phosphoric acid, *p*-aminophenyl diethyl triester), 95% pure by elemental analysis, was prepared by Ash Stevens, Inc., Detroit, MI, and was used as supplied. Hemocyanin from giant keyhole limpets (KLH) from Schwartz-Mann, Orangeberg, NY, and crystallized bovine serum albumin (BSA) from Miles Laboratories, Elkhart, IN, were covalently reacted with (*p*-aminophenyl)paraoxon by the method of Nisonoff (1967). Briefly, the paraoxon analogue was converted to a diazonium chloride and then reacted with the desired protein. Unreacted hapten was removed by exhaustive dialysis against phosphate-buffered saline (PBS). Protein concentration was determined by the method of Lowry et al. (1951), and epitope density (moles of hapten/mole of protein) was measured according to the procedure described by Fenton and Singer (1971). The epitope density for the KLH conjugate (KLH-P) was 850 while that for the BSA conjugate (BSA-P) was 14.

Mouse Immunization and Cell Fusion. Adult female BALB/C mice (Jackson Laboratories, Bar Harbor, ME) were immunized initially by subcutaneous injection at several sites on the stomach with 100 μg (protein) of KLH-P emulsified in Freund's complete adjuvant (Miles Biochemicals, Elkhart, IN). After 14 days the mice received a booster injection of 100 μg of conjugate in normal saline by the intraperitoneal route. After an additional 3 days the animals were killed by cervical dislocation, their spleens were removed, and splenic lymphocytes were fused with the hypoxanthine–guanine phosphoribosyltransferase deficient mouse plasmacytoma line P3-X63-AG 8.653 (Kearney et al., 1979) as described in Hunter et al. (1983).

Evaluation of Fusion Products. Culture fluid from 14-day-old cultures of fused lymphocyte–plasmacytoma cells (hybridomas) was analyzed for the production of antibodies against paraoxon–protein conjugates by enzyme immunoassay (EIA) (Engvall and Perlman, 1971; Van Weeman and Schurs, 1971). In this procedure the desired paraoxon–protein conjugate was immobilized onto the wells of polystyrene microtiter plates (Dynatech Laboratories, Alexandria, VA) by incubation overnight at 4 °C with 100 μL/well of BSA-P or KLH-P at 0.4 μg/mL in 0.1 M $NaHCO_3$, pH 9.6. The wells were washed with PBS containing 0.05% Tween 20 (Sigma Chemical Co., St. Louis, MO) by five repetitions of filling and emptying of each well.

Aliquots of hybridoma culture medium (50 μL) were added to duplicate BSA-P and KLH-P coated wells and incubated for 30 min at room temperature. Unbound antibody was washed away with PBS–Tween 20, and 50-μL aliquots of rabbit antimouse IgG κ light chain-specific antiserum, 1:300 in PBS–Tween 20 (Miles Biochemicals, Elkhart, IN) were added and incubated at room temperature for 30 min. This reagent binds to virtually all classes of mouse immunoglobulin.

Unbound rabbit antibody was washed away, and 50-μL aliquots of alkaline phosphatase-conjugated goat anti-rabbit IgG antiserum (Sigma Chemical Co., St. Louis, MO), 1:500 in PBS–Tween 20, were added. Following an additional 30-min incubation and a final wash, 100 μL of alkaline phosphatase substrate (*p*-nitrophenyl phosphate, 1 mg/mL in 10% diethanolamine, pH 9.6, Sigma Chemical Co., St. Louis, MO) was added to each well. The enzymatic reaction was allowed to proceed for 30 min at room temperature when *p*-nitrophenol formation was measured spectrophotometrically at 405 nm on a Titertek Multiscan Micro-ELISA reader (Flow Laboratories, Vienna, VA). Duplicate determinations greater than 4 times background were considered to be evidence of hybridomas producing antibody to paraoxon.

To ensure that EIA-positive samples did not contain antibody specific for only the azide linkage, they were further screened for activity to free paraoxon by a modified CIEIA (Hunter et al., 1971). A 50-μL portion of positive culture fluid was incubated for 1 h with 50 μL of distilled water or 50 μL of distilled water containing 2×10^{-3} M paraoxon. A 50-μL portion of this mixture was then tested for antibody binding as described above. In this system, inhibition of color formation to 50% or more of the control (non paraoxon containing) absorbance was considered in-

dicative of antibody reactivity with free paraoxon. Rabbit heteroantiserum against paraoxon (Hunter and Lenz, 1982) was used as a positive control.

Hybridoma Cloning. Hybridoma cultures that tested positive by CIEIA were cloned (0.5–1.0 cell/well) on γ-irradiated mouse macrophage feeder layers as described by Reid (1979). Clones were retested by CIEIA, and positives were recloned to ensure population uniformity.

Immunochemical Characterization. Two clones, designated CE3 and BD1, were chosen for detailed analysis. The cloned hybridomas were established in ascites culture, and antibody was harvested from the pooled collected ascites fluid by 50% ammonium sulfate precipitation. The precipitates were resolubilized, exhaustively dialyzed against potassium phosphate buffer (0.01 M, pH 7.2), and affinity purified on BSA-P bound to glutaraldehyde-activated silica (Boehringer Mannheim Biochemicals, Indianopolis, IN).

Characterization of Immunoglobulins. Immunoglobulin class and subclass were determined by Ouchterlony double diffusion (Oudin, 1980) against mouse immunoglobulin class and subclass specific reagents (Kirkigaard and Perry, Inc., Gaithersburg, MD). Aliquots of the cultures were cryopreserved in 15% dimethyl sulfoxide–85% RPMI 1640 culture medium (10% fetal bovine serum) and stored in liquid-nitrogen vapor phase.

Determination of Antibody Specificity. Affinity-purified antibody preparations and a variety of diethyl phosphate esters and analogous compounds were employed in the CIEIA to determine specificity by comparing the affinity of the antibodies for the test compounds relative to their affinity for paraoxon. Details of the CIEIA are supplied in the footnotes to Table I. Briefly, inhibitors were weighed out and dissolved in a volume of methanol that, when diluted to 25 mL with distilled water, yielded a methanol concentration of 25% and a 2.0×10^{-2} M concentration of inhibitor. Inhibitor was diluted serially in the wells of a microtiter plate to give 2×10^{-2} to 2×10^{-7} M concentrations. Diluant at all times was 25% MeOH in distilled water. Moving from low to high concentrations, 50 μL of each inhibitor dilution was transferred to a plate already containing 50 μL of affinity-purified antibody per well at a protein concentration of 0.5 μg/mL. This antibody concentration when diluted 1:2 had been predetermined to be that which gave approximately 50% maximum optical density when the antibody was titrated in the absence of inhibitor.

Antibody plus inhibitor solutions were allowed to react at room temperature for 1 h and then transferred to microtiter plates that had been coated with BSA-P at 0.5 μg/mL (protein) as outlined above. The assay from this point on is exactly as described for the screening of hybridoma culture medium discussed earlier. Each assay plate contained a row (six wells) of uninhibited antibody to provide a maximal response/plate control.

Determination of IC_{50} Values. The mean optical density at each inhibitor concentration was calculated. These means were used to calculate the inhibitor concentration causing a 50% reduction in optical density in the CIEIA (IC_{50}) with the iterative weighted four-parameter computer program of Davis et al. (1980) adapted to Applesoft by A.A.B. This program is available from the Biomedical Computing Technology Information Center, Vanderbilt Medical Center, Department of Radiology, Nashville, TN 37232.

Determination of Affinity. Affinity determinations were performed by equilibrium dialysis using the spectrum equilibrium dialyzer (Preiser Scientific, Beltsville, MD) and [^{3}H]paraoxon. The technique was that described by Karush and Karush (1971) adapted to the instrument. Hydrolysis of paraoxon was controlled by buffering the dialysis system with 0.04 M sodium phosphate, pH 6.8, plus 0.5% NaCl. Thin-layer chromatography and autoradiographic analysis showed less than 1% hydrolysis over the 24-h incubation period using this technique. Radioactivity measurements were made on an LKB 1215 Rackbeta II scintillation counter (LKB Instruments, Inc., Gaithersburg, MD). Efficiency correction was by automatic external standard/channels ratio plot. The equilibrium dialysis results were determined graphically with Sips plots.

Caution! Paraoxon is a potent acetylcholinesterase inhibitor. Special precautions must be taken to prevent inhalation and skin contamination when using this and other organophosphorus compounds.

RESULTS AND DISCUSSION

Culture supernatants from 288 hybridoma cultures were tested for antibody production by EIA with KLH-P (the immunizing conjugate) or BSA-P as the immobilized antigen. All 288 cultures were positive with KLH-P, an indication that hybridomas in each were producing antibody to the protein component of the immunogen. In the assay employing BSA-P, 84 of the 288 cultures were positive. Since no KLH was present in this assay, the 84 positive cultures (29%) were producing antibody specific for either the hapten, some aspect of the azide linking bond, or protein structures common to both BSA and KLH.

Supernatant from each of the 84 hybridoma cultures producing BSA-P binding antibodies was tested for binding inhibition in the presence of 10^{-3} M free paraoxon in a CIEIA. Eleven of the 84 were positive by this criterion. These positives were assayed further by titration with decreasing concentrations of paraoxon. Four of the 11 cultures tested in this manner produced antibody that interacted with free paraoxon in a concentration-dependent manner (Figure 1). These four cultures were expanded and frozen. The two that seemed most promising, BD1 and CE3, were cloned and retested.

After cloning, the cultures were adapted to large-scale ascites culture and hapten affinity purified. Testing of the purified antibody preparations in the immunoassay system optimized for immobilized antigen and antibody concentration showed antibody binding in a concentration-dependent manner (Figure 2) and inhibition of antibody binding as a function of free paraoxon concentration (Figure 3). Ouchterlony immunodiffusion testing indicated that both antibodies were of the IgG1 class with κ light chains.

Determination of Affinity. Equilibrium dialysis against [^{3}H]paraoxon yielded affinities of 9.4 ($\pm$14.4) $\times 10^4$ L mol^{-1} for CE3 and 1.7 ($\pm$0.8) $\times 10^5$ L mol^{-1} for BD1 on the basis of the average of three determinations.

Affinity measurements are usually sufficient for the differentiation of one monoclonal antibody from another. In this case, however, the close approximation of the measured values plus the variability of the results with CE3 made it difficult to distinguish these two antibodies on that basis. Comparative specificity determined by CIEIA offered a finely tuned method for resolving this.

The likelihood is small that different antibodies would have the same affinity for the paraoxon–protein conjugate used in the CIEIA and for paraoxon itself. This applied in this case and, indeed, the outcome of a CIEIA using paraoxon as the inhibitor (1, Table I) clarified the equilibrium dialysis results. A higher free paraoxon concen-

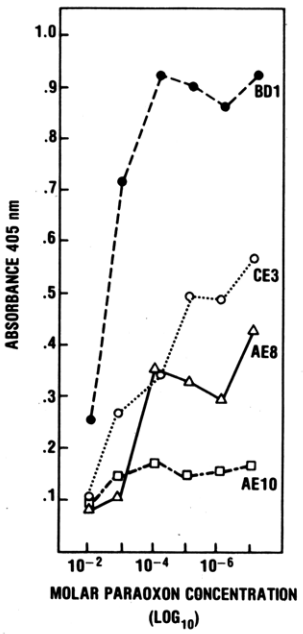


Figure 1. CIEIA of positive fusion culture supernatants with free paraoxon to identify candidates for cloning and further development. Microtiter plates were coated overnight at 4 °C with 2.5 μg/mL BSA-P. Undiluted supernatants (50 μL) were reacted with 50 μL of appropriate inhibitor dilutions in distilled water at room temperature for 2 h. The mixtures were transferred to coated plates, incubated for 1 h, and then assayed as described in Materials and Methods.

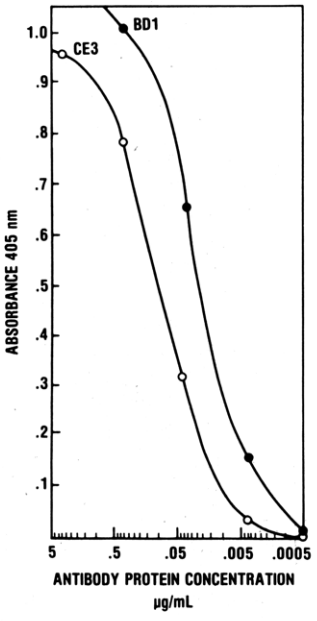


Figure 2. Titration of BD1 (●) and CE3 (○) by enzyme immunoassay after cloning, affinity purification, and protein determination. Assay conditions as described in Materials and Methods.

tration was required to displace 50% of the CE3 from conjugated paraoxon than was necessary to displace an equivalent amount of BD1 (Compare IC_{50} values for 1, Table I). This indicated that BD1 and CE3 were different antibodies having similar affinities for paraoxon.

Analysis of Molecular Immunodeterminants. In light of the similar measured affinities for paraoxon, the difference in IC_{50} values indicated that some aspect of the BSA–paraoxon conjugate not present in free paraoxon was contributing to the binding of CE3 but not BD1. While this is not entirely unusual when making antibodies to

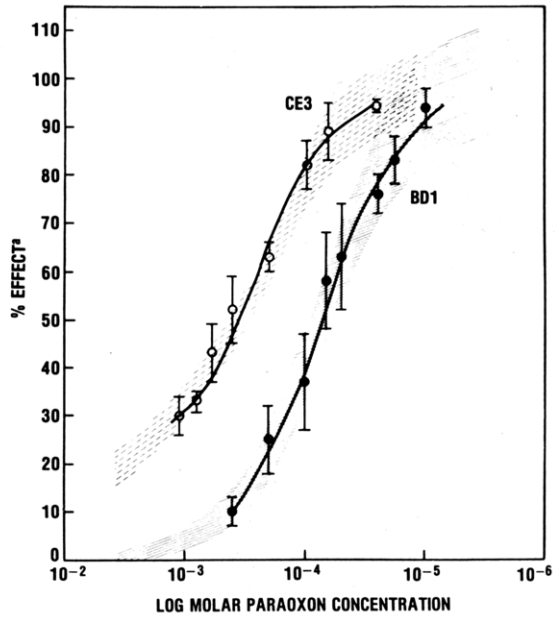


Figure 3. Standard curve for paraoxon in phosphate buffer measured with CE3 and BD1. Ordinate is the percent of optical density at 405 nm with no inhibitor present. Bars indicate the average ±SD (n = 3) of actual points generated in the CIEIA. The shaded area indicates the 95% confidence interval generated by the four-parameter logistic curve-fitting method of Davis et al. (1980) for comparison. Conditions are as outlined in Figure 1.

protein-bound haptens (Eisen, 1980), it was unlikely that CE3 was recognizing the protein portion of the immobilized conjugate because those sources of interaction had been eliminated during the selection process. Other than constituents of BSA itself, the only sites on BSA–paraoxon not present on the free material were the phenylamine group participating in the azo linkage and the azo group itself.

(*p*-Aminophenyl)paraoxon (2, Table I) was approximately 3-fold better as an inhibitor of CE3 than paraoxon (P = 0.001, two-tailed t-test, 4 df), results consistant with our hypothesizing. The *p*-aminophenyl analogue displayed only modestly increased inhibitory power toward BD1 (Compare IC_{50}'s of 1 and 2, Table I). The phosphonate analogue with the nitrogen removed (5, Table I) showed reduced inhibitory power toward both antibodies. The more closely analogous diethyl phenyl phosphate was not available so this effect could not be completely differentiated from the loss of the ester linkage.

Loss of the phenyl substituent altogether in diethyl phosphate (6, Table I) extinguished all inhibitory potential toward CE3 but still produced weak interaction with BD1. This provided additional indication of an involvement of the phenyl end of the molecule in binding to CE3 and indicated that the specificity of BD1 may be partially directed toward the alkyl substituents. This lack of reactivity is fortuitous, since diethyl phosphate is one of the hydrolysis products of paraoxon. Such low sensitivity toward it on the part of the antibodies means that diethyl phosphate is unlikely to interfere in paraoxon analysis.

Testing of the second product of paraoxon hydrolysis, *p*-nitrophenyl (8, Table I), produced no measurable inhibition of either antibody. This result, together with the effect of diethyl phosphate discussed above, was not entirely surprising. In previous work with anti-phosphonate antibodies (Brimfield et al., 1985) the hydrolysis products were tested with similar results. *p*-Nitrophenyl phosphate (7, Table I), a paraoxon analogue from which the alkyl

groups are removed, was also tested against BD1 and CE3 and produced no inhibition.

Substitution of parathion (3, Table I) for paraoxon allowed us to evaluate antibody–hapten interaction through the phosphoryl oxygen. In our previous work with monoclonal antibodies toward pinacolyl methylphosphonofluoridic acid this was a key molecular immunodeterminant (Brimfield et al., 1985); substitution of sulfur for the phosphoryl oxygen completely abolished interaction with the antibodies. We postulated in that case that the effect was due to a reduction in hydrogen-bonding potential that occurs upon the conversion of phosphonates to phosphonothioates (Gramstadt and Fuglevik, 1962).

The effect was similar with CE3 and BD1 when the phosphorothioate analogue was substituted for the phosphate but varied in degree. Rather than an extinction of binding, the result was only a reduction: approximately 30-fold for BD1 and 10-fold for CE3 (compare 1 and 3, Table I). Clearly the P=O group is a strong source of interaction, but not so critical to binding as in the earlier work. The reduction in binding seen with parathion may also represent an alteration in hydrogen-bonding potential, but a simple steric effect cannot be ruled out without further experimentation.

Methylparathion (4, Table I) was included as an alternative means for evaluating the importance of the alkyl groups as molecular immunodeterminants. Methylparaoxon, a better analogue for this purpose, was unavailable. However, it was felt that the comparison of parathion vs. methylparathion would provide an indication of ethoxy group contribution to antibody binding.

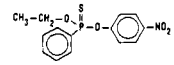
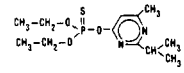
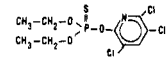
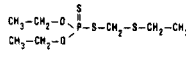
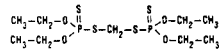
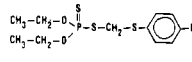
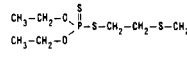
Interestingly, methylparathion interacted with BD1 with the same strength as parathion while the magnitude of the effect on binding to CE3 was somewhat greater (Compare CE3, 3 and 4, Table I) but still slight. A greater reduction in binding to BD1 had been expected, especially considering the minor effect on affinity brought about by changes in the phenyl end of the molecule. Perhaps the presence of the more bulky sulfur in place of the phosphoryl oxygen reduced the overall fit to a point where the structure of the alkyl groups was of little importence to BD1.

Insecticide Cross Reactivity. The cross reactivity of the antibodies with various insecticidal organophosphorus compounds similar in structure to paraoxon was also tested with the CIEIA (Table II). The resultant IC_{50} values give an indication of the potential of each compound for interference in a measurement of paraoxon concentration using the competitive assay. The data indicate that none of these compounds would seriously interfere with antibody–paraoxon interaction.

In the majority of the tests there was no cross reactivity at all in the concentration range used (0.001–10 mM). BD1 was slightly inhibited in the presence of high concentrations of EPN and ethion (1 and 2, Table II). A similar effect was seen with CE3 in the presence of high concentrations of chlorpyrifos and phorate (3 and 4, Table II). In each case the IC_{50} fell beyond the concentration range tested, however, and probably exceeded the aqueous solubility of the inhibitors. BD1 was inhibited by chlorpyrifos to an extent that made the generation of an IC_{50} feasible. However, the quality of the data was such that we have only been able to report it as a range (3, Table II). On the basis of the results shown in Table I, we can speculate that the P=O analogues of these compounds would be correspondingly better inhibitors.

Quantification of Paraoxon with BD1 and CE3. Having identified the molecular determinants and, to a degree, the insecticide cross reactivity of BD1 and CE3,

Table II. Cross Reactivity of Monoclonal Antibodies with Selected Pesticidal Compounds as Defined by Molar IC_{50}[a]

INHIBITOR	STRUCTURE	MOLAR IC_{50}[b] BD1	CE3
1. EPN[c] [2104-64-5]		$>10^{-2}$	NI
2. DIAZANON [333-4]		NI	NI
3. CHLORPYRIFOS [2921-88-2]		$2 \times 10^{-3} < IC_{50} < 10^{-2}$	$>10^{-2}$
4. PHORATE [298-02-2]		NI	$>10^{-2}$
5. ETHION [563-12-2]		$>10^{-2}$	NI
6. CARBOPHENTHION [786-19-6]		NI	NI
7. DISULFOTON [298-04-4]		NI	NI

[a] Conditions as described in Table I. [b] Average ±SD of three determinations. [c] CAS nomenclature: (1) phenyl phosphonothioic acid, *O*-ethyl *O*-(4-nitrophenyl) ester; (2) phosphorothioic acid *O,O*-diethyl *O*-[6-methyl-2-(1-methylethyl)4-pyrimidinyl] ester; (3) phosphorothioic acid, *O,O*-diethyl *O*-(3,5,6-trichloro-2-pyridinyl) ester; (4) phosphorodithioic acid, *O,O*-diethyl *S*-[(ethylthio)methyl] ester; (5) bis(phosphorodithioic acid), *S,S'*-methylene *O,O,O',O'*-tetraethyl ester; (6) phosphorodithioic acid, *O,O*-diethyl *S*-[[(4-chlorophenyl)thio]methyl] ester; (7) phosphorodithioic acid, *O,O*-diethyl *S*-[2-(ethylthio)ethyl] ester.

the final step in the initial characterization of these new reagent antibodies was to run standard curves to define the sensitivity and range of a potential assay. The standard concentrations were adjusted from those used for IC_{50} generation to provide a greater number of data points in the critical linear region of the curves. Paraoxon standard solutions were prepared in the same manner as those described in Materials and Methods for CIEIA. Separate preliminary EIAs defined antibody concentrations giving 50% maximum optical density at 405 nm after 30 min of incubation and the optimal concentration of solid phase antigen, 0.5 μg/mL for both.

The results of two such determinations with the limits defined about the curves by 1 standard deviation unit are shown in Figure 3. BD1 provided the more sensitive test of paraoxon concentration as one would predict from its slightly higher affinity for free paraoxon and its apparent lower affinity for BSA-P. The heteroscadastic variability is characteristic of immunoassays generally. It translates to an 8–14% variability depending on standard concentration, values that compare favorably with gas chromatographic determinations. The linear range for the BD1 curve spans approximately 1.5 orders of magnitude corresponding to a concentration range of approximately 10–100 μg/mL. The minimum detectable concentration, defined as the lowest concentration giving a response 2 standard deviation units away from the zero dose asymptote (Wilson and Miles, 1977), was 1 μg/mL for BD1 and 8 μg/mL for CE3 under these assay conditions. IC_{50} values for paraoxon read from these curves were 7.0×10^{-5} and 3.6×10^{-4} M for BD1 and CE3, respectively, completely consistant with the values generated on different days (Table I) shown in the results of the specificity determinations.

It is interesting to note that rabbit heteroantisera (Hunter and Lenz, 1982) gave slightly lower IC_{50} values with paraoxon than the mouse monoclonal antibodies [2×10^{-6} vs. 7×10^{-5} M, respectively]. That is, the polyclonal antiserum based immunoassay was more sensitive. However, when cross reactivity was examined, the monoclonals were clearly superior reagents. The rabbit polyclonal antiserum interacted with *p*-nitrophenol ($IC_{50} = 5 \times 10^{-3}$) as well as with diethyl phosphate ($IC_{50} = 3 \times 10^{-4}$) while the monoclonal antibody did not (Lenz et al., 1983). Since these are the primary products of paraoxon hydrolysis, reactivity toward these compounds renders the polyclonal antiserum of little value in paraoxon quanitification since samples will almost always contain some of these hydrolysis products.

Summary and Conclusions. We have shown that hybridoma technology can provide reagent antibodies with potential as immunoassay reagents for organophosphate insecticide residues. While the ranges of sensitivity of the assays based on BD1 and CE3 were somewhat disappointing, the specificity was very good. We are now in the process of identifying assay conditions that will enhance the sensitivity and useful range of the CIEIA with BD1 by employing solid-phase antigens for which the antibody has a lower affinity. This work, and a rigorous examination of precision and accuracy, will be published in a later paper. An evaluation of extraction methods for dislodgable residues on actual plant material is under way.

Registry No. EPN, 2104-64-5; paraoxon, 311-45-5; aminoparaoxon, 14984-58-8; parathion, 56-38-2; methylparathion, 298-00-0; diethyl phenylphosphonate, 1754-49-0; diethyl phosphate, 598-02-7; *p*-nitrophenyl phosphate, 330-13-2; *p*-nitrophenol, 100-02-7; diazinon, 333-41-5; chlorpyrifos, 2921-88-2; phorate, 298-02-2; ethion, 563-12-2; carbophenothion, 786-19-6; disulfoton, 298-04-4.

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