An Immunoassay for Pyrethroids: Detection of Permethrin in Meat

Larry H. Stanker,* Carolyn Bigbee, Jeanette Van Emon,[1] Bruce Watkins, Ronald H. Jensen, Charles Morris, and Martin Vanderlaan

The synthetic pyrethroids are an important class of insecticides because of their widespread use in modern agriculture. Analysis for these compounds has been difficult because of their thermal instability and photoinstability. We report here the development of three monoclonal anti-pyrethroid antibodies. These antibodies differentially recognize a range of synthetic pyrethroids, preferring phenothrin, permethrin, cypermethrin, and deltamethrin. The presence of both the phenoxybenzyl moiety and the cyclopropane ring appears necessary for maximal binding. One of these antibodies, referred to as Py-1, has been incorporated into an immunoassay for permethrin in meats. The assay gives a linear response over a range of 50–500 ppb permethrin in ground beef and, consequently, is sufficiently sensitive to detect permethrin in excess of the current regulatory limit of 150 ppb.

The synthetic pyrethroids represent a large group of insecticides, differing widely in their toxicity, photostability, and chemical structure (Matsumura, 1985). The early pyrethroids, pyrethrin-type insecticides such as allethrin and bioallethrin, were unstable in air and water, qualities that limited their use in agriculture (Smith and Stratton, 1986). Recently developed pyrethroids, however, are more stable in air and to light and have found widespread use in modern agriculture.

In use in the United States, residue limits in meat and fat have been established for permethrin (1), cypermethrin (2), and deltamethrin (3) (Brown, 1987). Conventional methods for detection of these compounds involve multistep sample cleanup procedures followed by gas chromatography and detection by electron capture (GC/EC) or, more recently, analysis by high-pressure liquid chromatography (HPLC) and detection by UV absorbancy (Bottomley and Baker, 1984). Moreover, in a recent review, Papadopoulou-Mourkidou (1983) points out that lack of adequate detection systems has hampered analysis of pyrethroids.

Modern immunoassays, based on monoclonal antibodies, represent potential detection systems for pyrethroids and are an attractive alternative to conventional chemical assays. Immunoassays have already been developed for a variety of small organic molecules including carcinogens, pesticides, toxic chemicals, and DNA adducts [see reviews by Mumma and Brady (1987) and Vanderlaan et al. (1987, and 1988b)]. Recent studies have demonstrated that immunoassays to small organic molecules are highly specific, are often able to distinguish chemical isomers, and have exceedingly high sensitivity (Stanker et al., 1987; Vanderlaan et al., 1988a; Van Emon et al., 1985). In addition, immunoassays of small organic compounds, in many cases, require only simple sample preparation procedures (Monroe, 1984).

Small organic molecules such as permethrin are not, by themselves, immunogenic but can be made immunogenic if they are conjugated with a carrier protein. Molecules with this property are referred to as haptens. The site and chemistry of conjugation influences the specificity of the antibodies produced (Hammock and Mumma, 1980; Vanderlaan et al., 1988a). The general rule, which is supported by the results presented in this paper, is that conjugation should be at a site on the hapten distant from the site where preferential antibody recognition is desired. For example, in the case of the pyrethroids, conjugation as far as possible from the 3-phenoxyphenyl group would be expected to favor the production of antibodies with binding that is sensitive to the presence of the phenoxyphenyl group. Antibodies recognizing this site are desirable since this group is common to several synthetic pyrethroids (Figure 1). Thus, an antibody recognizing this portion of the molecule should recognize a number of different pyrethroids.

We describe the development of several monoclonal anti-pyrethroid antibodies. Monoclonal antibodies (Mabs) were produced since they offer greater specificity than polyclonal antibodies. One of these Mabs forms the basis of a competition enzyme-linked immunosorbent assay (c-ELISA) that is able to detect permethrin in meat. The c-ELISA format was chosen because it is one of the simplest and most sensitive immunoassays (Engvall and Perlman, 1971; Pesce et al., 1981). We also discuss the ability of the assay to detect related pyrethroids, cypermethrin and deltamethrin.

MATERIALS AND METHODS

Reagents. The U.S. Environmental Protection Agency, (Research Triangle Park, NC) provided allethrin [3-allyl-2-methyl-4-oxocyclopent-2-enyl chrysanthemate], cypermethrin [α-cyano-3-phenoxybenzyl 3-(2,2-dichlorovinyl)-2,2-dimethylcyclopropanecarboxylate], fenpropathrin [α-cyano-3-phenoxybenzyl 2,2,3,3-tetramethylcyclopropanecarboxylate], deltamethrin [α-cyano-3-phenoxybenzyl 3-(2,2-dibromovinyl)-2,2-dimethylcyclopropanecarboxylate], fenvalerate [α-cyano-3-phenoxybenzyl 4-chloro-α-(1-methylethyl)benzeneacetate], flucythrinate [α-cyano-3-phenoxybenzyl 4-(difluoromethoxy)-α-(1-methylethyl)-benzeneacetate], permethrin [3-phenoxybenzyl 3-(2,2-dichlorovinyl)-2,2-dimethylcyclopropanecarboxylate], *trans*-permethrin, procyazide, pyrethrins, phenothrin [3-phenoxybenzyl chrysanthemate], and tetramethrin [*N*-(3,4,5,6-tetrahydrophthalimido)-methyl chrysanthemate]. All of the above chemicals were greater than 95% pure for use as analytical standards. The following chemicals were obtained from Sigma Chemical Co. (St. Louis, MO): chrysanthemumcarboxylic acid (mixed isomers) and chrysanthemumcarboxylic acid ethyl ester. The following chemicals were obtained from Aldrich Chemical Co., Inc. (Milwaukee, WI): 3-phenoxybenzyl alcohol, 3-phenoxybenzaldehyde, 3-phenoxybenzoic acid, and phenyl ether. [^{14}C]Permethrin was obtained as a gift from Dr. Bruce Hammock, University of California, Davis.

Instrumentation. Infrared spectra were recorded on a Digilab FTS-20C Fourier transform infrared spectrometer. Proton NMR

Biomedical Sciences Division, Lawrence Livermore National Laboratory, University of California, P.O. Box 5507 L-452, Livermore, California 94550.

[1] Present address: Office of Research and Development, Environmental Monitoring Systems Laboratory—Las Vegas, U.S. EPA, P.O. Box 93478, Las Vegas, NV 89193-3478.

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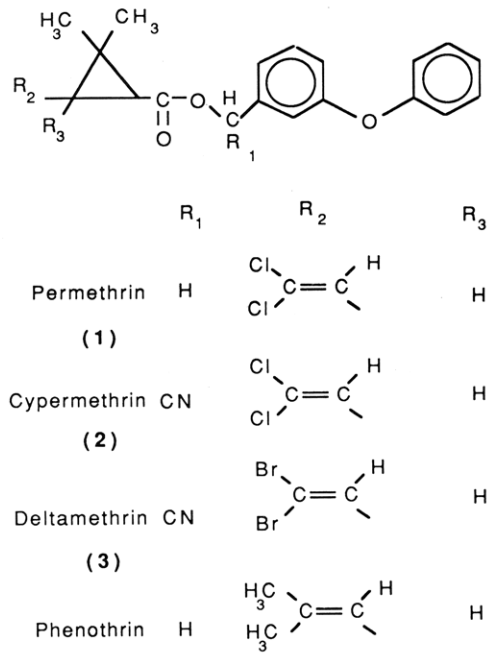

Figure 1. General formula for synthetic pyrethroids as well as the structure for permethrin (**1**), cypermethrin (**2**), and deltamethrin (**3**).

spectra were recorded at 90 MHz on a Varian EM390 spectrometer. Mass spectra were recorded on a Hewlett-Packard 5985A mass spectrometer (low resolution) and a Varian 311-A reverse geometry, Nier-Hohnson double-focussing mass spectrometer (high resolution).

Hapten Synthesis. Hapten synthesis is outlined in Figure 2. Chrysanthemumcarboxylic acid (**4**) (1.28 g, 7.6 mmol, mixed isomer) was suspended in 20 mL of methylene chloride, and oxalyl chloride (740 μL, 8.4 mmol) was added followed by addition of one drop of dimethylformamide. The mixture was stirred for 2 h at room temperature, 3-phenoxybenzyl alcohol (2.00 g, 10 mmol) and pyridine (2 mL) were added, and the mixture was stirred for an additional 12 h. Ether was added, and the mixture was washed with saturated aqueous sodium bicarbonate, followed by water. The organic layer was dried over anhydrous sodium sulfate, evaporated, and purified by column chromatography on neutral alumina (eluted with benzene) to yield 2.2 g of phenothrin (**5**).

A mixture of ozone and oxygen gas (3:97, w/w) (Welsbach Ozone Systems, Sunnyvale, CA; T-408) was passed through a solution containing 1.40 g (4 mmol) of phenothrin (**5**) in 200 mL of ethyl acetate and 10 mL of formic acid at 0 °C for 20 min at a rate of 1.2 L/min. A solution of 30% aqueous hydrogen peroxide (3.0 mL) was then added, and the mixture kept at 4 °C for 12 h. The solution was extracted twice with water and then once with 2 M NaOH. The aqueous layer was acidified with 12 M HCl and then was extracted with methylene chloride. The organic layer was dried over anhydrous sodium sulfate and evaporated. The resulting oil was purified by column chromatography on silica gel (methanol/chloroform, 3:97) to yield 360 mg (26%) of the hapten 3-phenoxybenzyl 2,2-dimethylcyclopropane-1,3-dicarboxylate (**6**) an oil: IR (film) 3066, 1732, 1701 cm^{-1}; ^1H NMR δ ($CDCl_3$) 10.2 (1 H, s, CO_2H), 7.5–6.9 (9 H, m, $C_6H_4OC_6H_5$), 5.16 (2 H, s, CH_2), 2.30 (1.2 H, s, CH), 1.97 (0.8 H, s, CH), 1.38 (1.3 H, s, CH_3), 1.31 (1.7 H, s, CH_3), 1.26, (1.7 H, s, CH_3), 1.22 (1.3 H, s, CH_3); MS, *m/e* (relative intensity) 340 (28, M^+), 264 (6), 200 (40), 183 (100); HRMS for $C_{20}H_{20}O_5$, 340.1313, found 340.1295.

Monoclonal Antibody Production. The pyrethroid hapten **6** was conjugated to keyhole limpet hemocyanin (KLH) (py-KLH), and to bovine serum albumin (BSA) (py-BSA) by the mixed-anhydride method (Erlanger et al., 1959). Six-month-old BALB/cBkl mice (Bantin and Kingman Laboratories, Fremont, CA) were injected intraperitoneally (ip) with 100 μg of py-KLH conjugate mixed 1:1 with complete Freund's adjuvant. Mice received a single ip injection every other week for a total of three injections. Four days prior to fusion, each mouse was given an intrasplenic injection of 100 μg of py-KLH conjugate in sterile

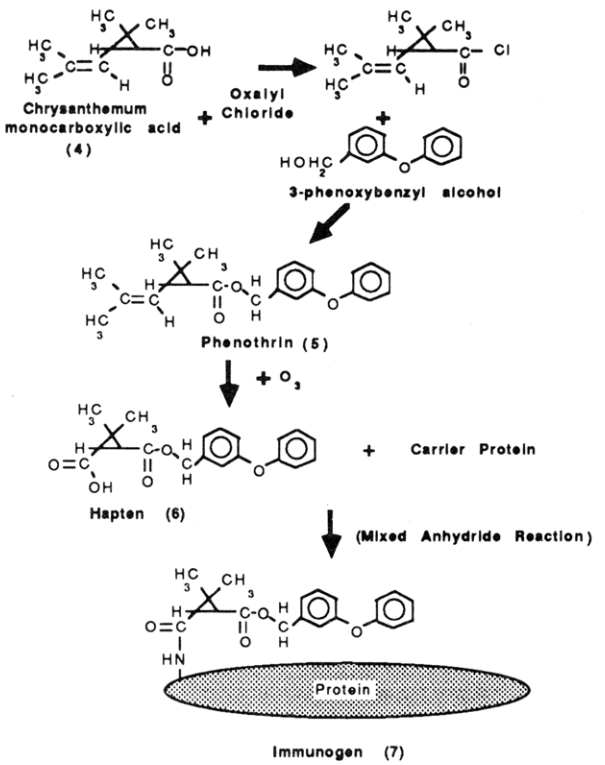

Figure 2. Synthetic pathway for production of the immunogen. Briefly, chrysanthemumcarboxylic acid (**4**) and 3-phenoxybenzyl alcohol were reacted to form phenothrin (**5**), which was acidified to form the hapten (**6**). The immunogen was formed by linking compound **6** to the carrier protein KLH and is depicted as compound **7**.

saline. The spleen was removed, and the splenocytes were fused with SP2/0 myeloma cells and grown under conditions described by Stanker et al. (1986).

A direct-binding ELISA, described by Stanker et al. (1986) and modified as described below, was used to screen culture fluid from the growing hybridomas for antibodies to pyrethroids. In this procedure, 96-well Immulon-II microtiter plates (Dynatech Laboratories, Alexandria, VA) were coated with py-BSA (0.2 μg/well) in carbonate–bicarbonate buffer (pH 9) for 18 h at 4 °C. Nonreacted sites on the plastic microtiter plates were blocked for 1 h at room temperature with a 3% solution of ovalbumin and then incubated for 1 h at 37 °C with the hybridoma supernatants. The plates were carefully washed with a solution of 0.05% Tween-20 in water, and peroxidase-conjugated goat anti-mouse antiserum (United States Biochemicals, Cleveland OH) diluted 1:500 in assay buffer (0.005 M sodium phosphate, 0.075 N NaCl, 0.001% Tween-20, pH 7.2) was added to each well. Following a second 1-h incubation at 37 °C, the plates were washed again and the substrate, 2,2′-azinobis(3-ethylbenzothiazoline-6-sulfonic acid (ABTS), added. Absorbance measurements at 405 nm were taken as a function of time, and the resulting data were transferred to a Macintosh computer and subsequently analyzed with the Cyberdoma ELISA software described by Slezak et al. (1983).

Hybridoma cells from wells showing a positive response in the ELISA screen were expanded and subcloned twice by limiting dilution to ensure their monoclonal origin. Ascites fluid was prepared in irradiated mice (Stanker et al., 1986), and the monoclonal antibodies were purified from the ascites by hydroxylapatite chromatography (Stanker et al., 1985). Isotype determination was done by ELISA using mouse heavy- and light-chain-specific antisera (Southern Biotechnology Associates, Birmingham, AL).

Competition Enzyme-Linked Immunosorbent Assay. A competition enzyme-linked immunosorbent assay was developed to quantify the amount of permethrin in solution and to evaluate the ability of the antibodies to distinguish among various natural and synthetic pyrethroids. Preliminary work to optimize the sensitivity of the assay showed greatly improved sensitivity if the

py-BSA coating antigen was replaced with 3-phenoxybenzoic acid (3-pba) conjugated to BSA (data not shown). The commercial availability of 3-pba is an additional advantage of use of 3-pba-BSA versus the py-BSA conjugate as the coating antigen. The coating antigen (BSA linked to 3-phenxoybenzoic acid) was synthesized as follows. A 50-mg portion of BSA dissolved in 5 mL of distilled water was added to 50 mg of 3-phenoxybenzoic acid dissolved in 5 mL of water. The pH was then adjusted to 7.0 by addition of 1 N sodium hydroxide. Then, 50 mg of 1-ethyl-3-[3-(dimethylamino)propyl]carbodiimide (EDC) (Pierce Chemical Co., Rockford, IL) was added, and the mixture was stirred overnight at ambient temperature and dialyzed for 48 h against four changes of PBS (0.01 M sodium phosphate, 0.15 M NaCl, pH 7.2). Microtiter plates were coated with 0.5 μg/well 3-pba-BSA (100 μL/well of a 5 μg/mL solution of 3-pba-BSA in distilled water was allowed to evaporate (at 37 °C) onto the bottoms of the wells) and blocked with ovalbumin as described above. Competitors (dissolved in acetonitrile) were added to the assay buffer such that the resulting solution was 6% acetonitrile. An aliquot (200 μL) of this competitor assay buffer solution was added to an antigen-coated well. Then, the amount of competitor was serially diluted down the microtiter plate, so each well contained 100 μL of competitor in a 6% acetonitrile assay buffer solution. Next an equal volume of assay buffer containing a predetermined amount of Mabs (for Mabs Py-1, 100 μL of a 0.2 μg/mL solution) was added to each well. Thus, each well contained 200 μL of a 3% acetonitrile solution in assay buffer containing antibody and competitor. The antibodies described here can tolerate up to 5% acetonitrile with little loss of activity. Plates were incubated for 1 h at 37 °C and then processed as described above except alkaline phosphatase conjugated goat anti-mouse (Sigma) was used as the secondary antibody and *p*-nitrophenyl phosphate (Sigma) was used as the substrate (Van Emon et al., 1985).

In each experiment microtiter wells containing all components except competitor were prepared, and the activity in these wells was taken to represent 100% activity. The test wells, each containing different amounts of competitor, were normalized to the 100% activity wells. Percent inhibition then was calculated by subtracting the normalized percent activity from 100.

Extraction of Permethrin from Meat. The extraction procedure described below is a modification of the method described by Braun and Stanek (1982). A 5-g sample of ground beef (purchased from local supermarkets) was mixed with 50 mL of an acetonitrile/water (85:15) solution and homogenized with a Polytron (Brinkman Instruments, Westbury, NY) at setting 8 for 2 min. The resulting slurry was centrifuged at 100*g* for 2 min in a counter-top centrifuge to sediment the particulate. The liquid phase was recovered and set in a freezer (−20 °C) for several hours to allow the fats to separate. Then 8.5 mL of the upper acetonitrile phase (one-fifth of the sample) was shaken by hand against 10 mL of hexane in a separatory funnel for 30 s. Next, 40 mL of 2% NaCl in distilled water was added, and the mixture shaken for 1 min and allowed to stand for 1 min. The aqueous fraction was discarded and the hexane fraction reextracted with 5 mL of water and gentle shaking for 30 s. The hexane fraction was recovered, and the separatory funnel was rinsed with an additional 5 mL of hexane, and both hexane fractions were pooled and dried by passing over Na_2SO_4. The pooled hexane fraction (15 mL) was next passed over a 2-cm alumina column prepared in a disposable Pasture pipet with a 1-cm layer of Na_2SO_4. The column was then rinsed with 10 mL of hexane. Permethrin was eluted with 10 mL of benzene. The benzene fraction was dried under a gentle stream of nitrogen and the sample resuspended in 60 μL of acetonitrile followed by the addition of 940 μL of assay buffer. The sample was then used in the c-ELISA described above. The alumina used to prepare the above columns (Bio-Rad acid alumna, AG4, 100–200 mesh, Bio-Rad Laboratories) was first washed with methylene chloride for 24 h in a Soxhlet apparatus, dried at 130 °C for 24 h, and stored at 110 °C until used.

Ground beef samples were spiked with permethrin standards, in acetonitrile, by delivering between 100 and 500 μL of standard to the meat sample at three or four locations. Neat acetonitrile was added to the unspiked meat samples. In some cases (indicated in individual experiments in the results section) samples were spiked as above after extraction with the acetonitrile/H_2O solution. In these cases the spike was delivered to the recovered acetonitrile after the fat was separated.

RESULTS

Hapten Synthesis. Permethrin is a small organic molecule, and thus, it must be conjugated to a carrier protein in order to render it immunogenic. Since permethrin does not have any functional groups through which it could be conjugated to a carrier protein, it was necessary to synthesize an analogue, the 3-phenoxybenzyl monoester of cyclopropanedicarboxylic acid (**6**). The permethrin analogue (hapten) was synthesized, as illustrated in Figure 2 and described in Materials and Methods. Its properties were similar to those reported for the optically pure materials (Nambu et al., 1980). The hapten was then conjugated to KLH and the resulting immunogen (**7**) (see Figure 2) used to immunize mice.

Hybridoma Production. Spleen cells from a BALB/c mouse immunized with py-KLH were fused with SP 2/0 myeloma cells, and the resulting hybridomas were cultured in 30 96-well microculture dishes. Growing hybridomas were observed in greater than 90% of the wells 10 days after fusion. The supernatant from each well was screened for antibody activity against py-BSA in an ELISA (BSA represents an extraneous protein because the immunogen was py-KLH, therefore, anti-KLH antibodies will not be detected). Approximately 500 wells were observed to be secreting antibody that recognized the py-BSA conjugate but not BSA itself. The cells from those wells showing the strongest response and specificity (approximately 250 wells) were expanded and tested against py-BSA, py-KLH, BSA, and KLH. Antibody that recognized both hapten conjugates, but did not bind either of the unconjugated carrier proteins, was observed in 29 wells. Of these, 13 were subcloned, while the remaining 16 were frozen for possible future use. Next, the monoclonal antibodies were evaluated for their ability to recognize unconjugated permethrin and cypermethrin in a competition ELISA. Only 3 of the 13 hybridomas produced antibodies that recognized the nonconjugated compounds. These antibodies are named Py-1, Py-3, and Py-4 and were determined (using an isotype-specific ELISA) to be IgG2a antibodies with κ light chains.

Assay Development. Py-1 was titrated against immobilized antigen (3-phenoxybenzoic acid–BSA) (0.5 μg/well) in a direct-binding ELISA. A concentration of 0.02 μg of antibody/well resulted in 40% of the plateau activity. This concentration of Py-1 was used in the c-ELISA. Py-3 and Py-4 were used as unpurified culture fluids at a final dilution of 1:200 in the assay wells (these concentrations also were near 50% of the plateau activity). The concentration of detergent in the sample buffer was found to greatly influence the behavior of the assay. Py-1 binding to immobilized antigen was inhibited in the presence of detergent (Tween-20) at concentrations greater that 0.01% (data not shown). Other components of the assay buffer, i.e., salt concentration from 0.15 to 0.018 M, had little effect on antibody–antigen binding. We routinely used 0.001% detergent in all buffers.

Antibody Characterization. Typical c-ELISA results for Py-1, Py-3, and Py-4 using permethrin as competitor are shown in Figure 3. The concentration of permethrin that resulted in a 50% inhibition (I_{50}) of the control activity (i.e., wells with no competitor present) is in the low nanograms/well range for all three antibodies. Repeated c-ELISA's using Py-1 and permethrin as competitor (49 samples run over a 5-month inverval) indicate that the average I_{50} for permethrin is 1.55 ng with a standard deviation of ±0.6 ng/well. Similar variations were observed

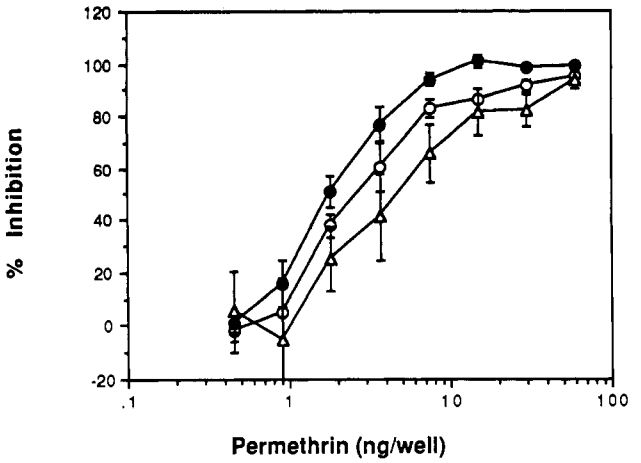

Figure 3. Average c-ELISA data for Mabs Py-1, (open circles), Py-3 (closed circles), and Py-4 (open triangles), using permethrin as competitor. Bars equal ± 1 standard deviation. The competitor solution (100 μL) containing the amount of competitor indicated was added to an antigen-coated well followed by 100 μL of antibody solution. Thus, nanograms/well are converted to nanograms per milliliter or parts per billion simply by multiplying by 10.

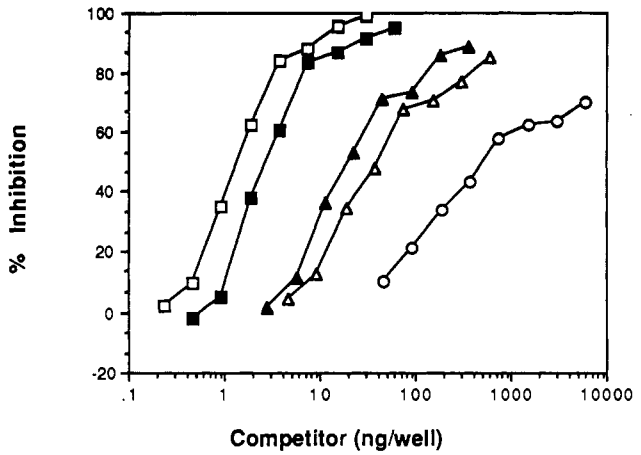

Figure 4. Typical c-ELISA showing the ability of Mab Py-1 to react with phenothrin (open squares), permethrin (closed squares), deltamethrin (closed triangles), cypermethrin (open triangles), and fenvalerate (open circles).

with other competitors. The Py-1 antibody used in these experiments, and all subsequent experiments, was purified from ascites fluid, while Py-3 and Py-4 were used as unpurified culture media.

Next, the antibodies were characterized as to their ability to recognize related pyrethroid insecticides. Representative inhibition curves for Py-1, with permethrin, cypermethrin, deltamethrin, phenothrin, and fenvalerate as competitors are shown in Figure 4. These data clearly illustrate the relative ability of Py-1 to distinguish between related pyrethroids. Similar c-ELISA data for Py-1, Py-3, and Py-4 were collected with 13 different competitors and are summarized in Table I. The average I_{50} values for permethrin shown in Table I were 1.5, 1.7, and 12 ng for Py-1, Py-3, and Py-4, respectively, with standard deviations of 0.6, 0.3, and 7.0 ng. These averages are based on 12 independent assays on six separate microtiter plates for Py-3 and Py-4 and 49 assays using Py-1. Py-1 and Py-3 appear to have similar specificities and sensitivities, whereas Py-4 has a lower sensitive for most of the compounds tested.

Immunoassay of Permethrin in Meats. An immunoassay capable of detecting permethrin in meat was developed with Py-1. Briefly, a sample was extracted in an acetonitrile/water solution, partitioned against hexane, partially purified on an alumina column, and analyzed with the c-ELISA described above. In one experiment, samples from unspiked beef were tested for assay interference at several stages in the purification. Aliquots were saved (i) after extraction, (ii) after partitioning into hexane, and (iii) after elution from the alumina column. The resulting competition curves were compared with the competition curve obtained when permethrin was analyzed in the standard c-ELISA assay buffer. In companion experiments, the acetonitrile extract was spiked (300 ng of permethrin/8.5 mL of extract) and then partitioned into hexane followed by chromatography on alumina. Again, aliquots after each step were saved and assayed in the c-ELISA. Samples of the acetonitrile extract and of the hexane fraction from the unspiked samples resulted in 40–50% inhibition (five trials) as compared to the no-permethrin standard (i.e., the no-permethrin samples run in assay buffer). Additional competition, above this background level, was generally not observed in the spiked acetonitrile fractions (data not shown). Thus, interfering compounds that compromise the c-ELISA appear to be present in the crude meat extracts. In contrast, little or no inhibition (usually between 5 and 10%) was observed in the benzene fraction recovered from the nonspiked meat samples. Furthermore, spiking the acetonitrile fraction or the benzene eluant from the alumina column resulted in competition curves that were similar to those observed with permethrin standards. Thus, it is clear that components that occur in the early extracts interfere with the assay and are effectively removed by alumina chromatography.

Table I. Average[a] 50% Inhibition Values (I_{50}) in Nanograms of Competitors Listed for Py-1, Py-3, and Py-4

compound	monoclonal antibodies		
	Py-1	Py-3	Py-4
phenothrin	1.5	0.5	6
permethrin (mixed isomers)	1.5	1.7	12
permethrin trans	1.7	nd[b]	nd
hapten	2	0.3	0.6
deltamethrin	15	10	10
fenpropathrin	17	10	25
3-phenoxybenzaldehyde	22	nd	nd
cypermethrin	30	20	22
flucythrinate	120	45	275
3-phenoxybenzoic acid	200	140	330
fenvalerate	350	160	4000
tetramethrin	>600[c]	nd	nd
chrysanthemic acid	>600	nd	nd

[a] Averages calculated from at least 12 assays. [b] nd = not done. [c] 50% inhibition was not observed at the highest concentration of competitor.

The recovery of permethrin from beef samples was evaluated with use of [^{14}C]permethrin. Ground beef samples were spiked at 500 ng/g with nonradiolabeled permethrin in the presence of a trace amount of ^{14}C-labeled permethrin. Our results (from six experiments) were that 72 ± 1.7% of the radiolabeled permethrin was recovered in the acetonitrile extract (after freezing-out the fat), 65.5 ± 3.5% of the original spiked radioactivity was recovered in the hexane fraction, and 62 ± 2.9% of the original radioactivity was recovered in the final benzene fraction.

Finally, ground beef samples were spiked with various amounts of permethrin, extracted, purified, and analyzed with the c-ELISA. In one experiment, shown in Figure 5, meat samples were spiked with permethrin at 500 and 100 ppb. In this particular experiment, 50% inhibition was observed following a 16- and a 3-fold dilution of the

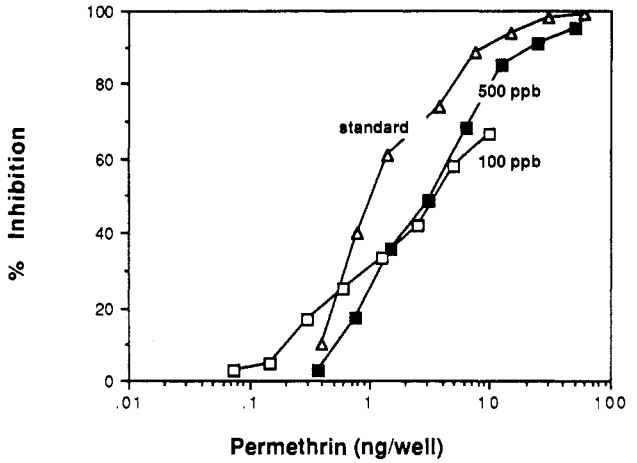

Figure 5. c-ELISA showing the ability of Mab Py-1 to detect permethrin in meat. Samples were spiked with permethrin at 500 ppb (closed squares) and at 100 ppb (open squares). In each case the extract from 0.1 g of meat was used as the starting material to generate the inhibition curves shown (the permethrin value plotted on the x-axis represents the amount of spike present in 0.1 g of sample if 100% was recovered). Permethrin standard is also presented (open triangles).

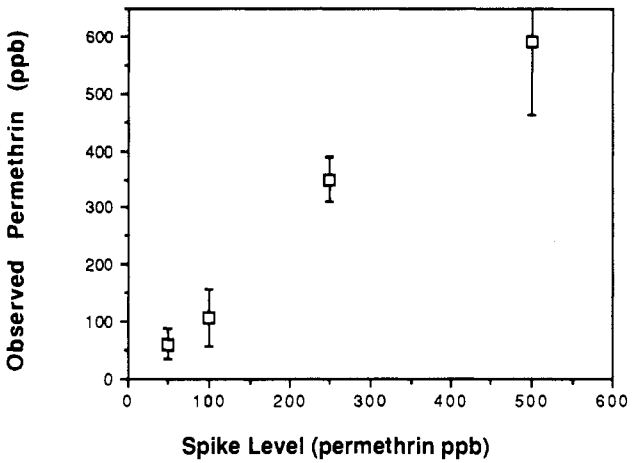

Figure 6. c-ELISA used to determine the level of permethrin contamination in beef samples spiked at 500, 250, 100, and 50 ppb (at least six replicates for each point). Permethrin levels were calculated from the competition curves as described in the text with a recovery of 62%. Error bars represent 1 standard deviation.

starting 500 and 100 ppb extracts, respectively. Since 1.5 ng (±0.6 ng) of permethrin results in 50% inhibition of the standard curve, and assuming a 62% recovery from the sample, permethrin levels of 400 and 69 ppb, respectively, were calculated for these samples. The results from similar fortification experiments are summarized in Figure 6 in which the amount of permethrin determined by the c-ELISA (assuming a 62% recovery) is plotted versus the level of spike added (bars represent ±1 standard deviation). Good correlations between the expected levels and the measured levels of permethrin were observed in all cases when samples were spiked at levels greater that 50 ppb.

DISCUSSION

The synthetic pyrethroids represent a major class of insecticides. Their desirability is in part due to their high toxicity to a wide spectrum of insects and relatively low toxicity to mammals (Elliott, 1976). Residue limits for many pyrethroids have been set by the World Health Organization (WHO, 1977, 1980) as well as the U.S. Department of Agriculture (Brown, 1987). Pyrethroid monitoring in food and environmental samples, however, has been hampered by the lack of convenient methods for their detection (Papadopoulou-Mourkidou, 1983).

We have isolated three monoclonal antibodies that recognize selected synthetic pyrethroids, including permethrin, cypermethrin, and deltamethrin. One of these antibodies, Py-1, was used to generate an immunoassay that is able to detect permethrin in meat. The ability of these antibodies to recognize different pyrethroids was analyzed by a c-ELISA. While the results of these studies indicate that some generalizations can be made concerning the nature of the epitope recognized by these antibodies, each recognized the immunogen in a slightly different orientation. For example, the I_{50} values obtained with permethrin, phenothrin, and hapten **6** were almost identical for Py-1, indicating that the antibody recognized these compounds equally. Furthermore, the I_{50} values for these compounds were the lowest values obtained for all competitors, indicating that Py-1 recognizes these compounds more effectively than the other compounds tested. Thus, the antibody does not distinguish between the presence of a dichloro or dimethylvinyl group on the molecule. In fact, the ability of Py-1 to recognize 3-phenoxybenzaldehyde at I_{50} = 22 ng/well and 3-pba at I_{50} = 200 ng/well indicates that a dichloro or dimethylvinyl group is not necessary for antibody binding nor does its presence interfere with binding if the cyclopropane and/or phenoxybenzyl portions of the molecule are present. These results suggest that the vinyl region of the molecule is probably not intimately associated with the epitope and support the generalization that those regions of a hapten furthest from the linkage position will be most specifically recognized by the antibody (Hammock and Mumma, 1980; Vanderlaan, 1988a). Elimination of the cyclopropane ring causes approximately a 100-fold reduction in binding (e.g., binding to flucythrinate, 3-pba, or fenvalerate is greatly reduced as compared to permethrin), suggesting that this portion of the molecule is involved with antibody binding. However, chrysanthmic acid was not recognized, suggesting that the cyclopropane ring itself is not sufficient for binding. Furthermore, the ability of the antibody to recognize 3-phenoxybenzaldehyde, I_{50} = 22 ng, suggests that strong binding can occur in the absence of the cyclopropane ring and suggests that the electron distribution at the α-carbon of the benzyl moiety also is important for antibody binding. The results observed for binding of deltamethrin and cypermethrin indicate that antibody binding is reduced by approximately 1 order of magnitude if a cyano group is substituted for a hydrogen on the α-carbon of the benzyl moiety. These observations taken together suggest that Py-1 recognizes both the phenoxybenzyl and the cyclopropane portion of the molecule and is also sensitive to the substitutions on the α-carbon.

Similar conclusions can be made for Py-3 and Py-4. However, Py-3 recognized phenothrin and flucythrinate approximately 3-fold better that did Py-1, suggesting that the two antibodies recognize slightly different epitopes. Likewise the details of Py-4 binding are slightly different from those of Py-1 and Py-3, and the absolute affinity of Py-4 for most pyrethroids tested appears to be lower than that observed with Py-1 and Py-3.

A c-ELISA using Py-1 capable of detecting permethrin in spiked meat samples was developed. Permethrin was extracted from fortified samples in an acetonitrile/water solution and partitioned against hexane followed by cleanup on an alumina column. This assay can easily detect permethrin contamination in the range 50–500 ppb starting with only 5 g of material and with minimal sample

cleanup. Substance(s) that interfere with the c-ELISA appeared to be present in the acetonitrile extract and in the hexane-partitioned material but were effectively removed by alumina chromatography. The nature of these substance(s) is unknown. Radiotracer experiments indicate that 62% of the sample is repeatedly recovered with this method. The current regulatory limit for permethrin in meats is 150 ppb (Brown, 1987). Consequently, this immunoassay can readily be used to detect contamination in excess of the regulatory limit. However, the specificity of Py-1 suggests that, in addition to permethrin, other pyrethroids, such as cypermethrin and deltamethrin, also will be detected (albeit with lower sensitivities). Thus, a positive response in the immunoassay is not absolute proof of the presence of permethrin in the sample. Instead, it represents the total response to those pyrethroids that the antibody can detect. Moreover, the relative affinity that the antibody has for a given pyrethroid will govern the sensitivity of the assay to that pyrethroid. For example, a 50% reduction in the ELISA activity caused by undiluted extract (i.e., the equivalent of 0.1 g of sample) suggests the presence of permethrin at 24 ppb in the sample, cypermethrin at 484 ppb, or fenvalerate at 5645 ppb, or some combination of these pesticides at proportional concentrations (assuming the "cleaned-up" extract from 0.1 g of meat is analyzed with Py-1 and a 62% recovery is valid for all pyrethroids). This degree of uncertainty about the exact composition of the contamination serves to illustrate that even with monoclonal antibodies some cross-reactivity with related chemicals occurs. However, monoclonal antibodies are invariant reagents that need be extensively characterized only once to determine the range of compounds detected and their relative sensitivities. In contrast, polyclonal antibodies need to be characterized, and the assay optimized following production of each lot of antisera.

We anticipate that the immunoassay we described here will be most useful as a screening tool, allowing for a rapid, inexpensive, easily automatable test for pyrethroid contamination. A positive response can then be confirmed by GC/EC or an equivalent analytical method if the exact nature of the contamination must to be ascertained.

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