Development of an S-Bioallethrin Specific Antibody

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Residue analysis of the pyrethrins and allethrin as well as newer pyrethroid insecticides not containing halogens such as resmethrin and phenothrin is expensive, tedious, and/or of poor sensitivity. The structure of such pyrethroids suggests that they can be analyzed quickly, inexpensively, and at low levels by radioimmunoassay. Reaction of S-bioallethrin (1R,3R,4′S) with carboxymethoxylamine hemihydrochloride led to quantitative conversion to its carbomethoxyoxime derivative. Alternatively, hydroboration–oxidation of S-bioallethrin with disiamylborane led to selective oxidation at the terminal olefin with little loss of specific rotation. Quantitative reaction with succinic anhydride gave S-bioallethrin hemisuccinate which was conjugated via its N-hydroxysuccinimide active ester to tyramine, hemocyanin, or bovine serum albumin (BSA). Rabbits immunized with allethrin–hemocyanin yielded a serum reacting with hemocyanin or allethrin–BSA but not BSA as determined by immunodiffusion. Appearance of the precipitin band to allethrin–BSA could be inhibited by S-bioallethrin, but not by higher levels of permethrin, parathion, carbaryl, DDT, or an inactive allethrin isomer (1S,3R,4′R). The potential of immunochemical methods for residue analysis is discussed.

The analysis of pesticide residues is necessary for insuring environmental quality and human health. Gas-liquid chromatography (GLC) with selective detectors has proven to be the most generally applicable residue analysis technique, but for some groups of compounds GLC analysis has severe limitations. S-Bioallethrin (the 1R-, 3R-, or (±)-*trans*-chrysanthemate of 4′S-allethrelone, compound 1, Figure 1) and the natural pyrethrins (rethrins) have limited but important uses in insect control due to their effectiveness on insects, their very low mammalian toxicity, and their rapid decomposition (Elliott et al., 1978). This low mammalian toxicity has resulted in applications which lead to use on or near human food. The methods for the detection of these compounds at residue levels are, however, expensive, tedious, and/or of poor sensitivity. S-Bioallethrin and other pyrethroids lend themselves to immunochemical methods because they have numerous potential sites for selective antibody recognition. Due to a need for appropriate residue methods for these safe, biodegradable compounds, this study towards the development of immunochemical methods of residue analysis was undertaken.

MATERIALS AND METHODS

Analytical thin-layer chromatography (TLC) was performed in solvent systems described in Table I on 250 μ silica gel F_{254} plates (EM Laboratories), while preparative TLC was done on 2000 μ silica gel plates with fluorescent indicator (Analtech). Compounds were detected by their quenching of gel fluorescence when viewed under short-wave ultraviolet (UV) light (254 nm) or with various reagents previously described (Hammock et al., 1974). High-resolution liquid chromatography (LC) was performed on a prepacked ODS permaphase column (2 × 250 mm, 5 μm, DuPont) by eluting with 40% (allethrin) or 50% (allethrin alcohol, compound 3, Figure 1) aqueous methanol delivered with a Spectra Physics M-3500B pump and monitored at 280 and 254 nm with a Spectra Physics Model 230 UV detector.

Proton magnetic resonance (^1H NMR) spectra were obtained on either a Varian T-60 or EM-390 in dilute deuteriochloroform–tetramethylsilane solutions with or without deuterium oxide. Infrared spectra (IR) were run

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Table I. Relative TLC R_f's of Allethrin and Several Derivatives in Four Solvent Systems

compd	solvent system[a]			
	A	B	C	D
allethrin (1)[b]	0.69	0.70	0.68	0.31
allethrin disiamylborane derivative (2)	0.50[c]	0.52	0.55[c]	
allethrin alcohol (3)	0.19	0.21	0.23	0.08
allethrin hemisuccinate (4)	0.25	0.28	0.03	0.10
allethrin active ester (5)	0.47	0.50	0.03[d]	0.10
allethrin tyramine derivative (6)	0.22	0.22	0.32	0.03
allethrin CMO (7)	0.25	0.28	0.03	0.10

[a] Solvent systems are as follows: (A) benzene-1-propanol, 10:1; (B) benzene-1-propanol-acetic acid, 100:10:1; (C) benzene-1-propanol-ammonium hydroxide, 100:10:1; (D) hexane–ether, 5:1. [b] See Figures 1 and 2 for structures. [c] Partially oxidizes during spotting to the allethrin side chain alcohol. [d] Decomposes entirely to the hemisuccinate and minor products.

as thin films of the respective compounds on silver chloride plates with a Beckman Model 4240, UV spectra in methanol or hexane on a Beckman Model 25, and mass spectra (MS) by the direct insertion probe technique with a Finnigan Model 1015 mass spectrometer interfaced with System Industries Model 150 control system. Fluorometry was performed on an SPF-125 Spectrophotofluorometer (Aminco). Elemental analyses were performed by C. F. Geiger (Ontario, Calif.), while specific rotation was determined from the average of seven measurements of a 2% chloroform solution in a 1-dm cell in a Rudolph Model 1000 Polarimeter using sodium D light.

Synthesis. *Allethrin Side Chain Alcohol* (3). Unless otherwise specified, all reactions were carried out under N_2 in subdued light using dry, freshly distilled solvents. S-Bioallethrin (compound 1, Figure 1) was purified by falling film distillation (97.2% chemical purity, $[\alpha]^{25}_D$ [−24.14°], λ_{max} 225 nm, ϵ_{max} 17 800 in hexane). Disiamylborane was prepared by the reaction of diborane with 2-methyl-2-butene (Brown and Moerikofer, 1961), and it was titered by the release of hydrogen gas when exposed to aqueous ethanol. The disiamylborane [10 mmol, 40 mL of tetrahydrofuran (THF)] was added dropwise to the S-bioallethrin (11 mmol) in THF (10 mL, −5 °C). The reaction was allowed to stir (1 h, −5 °C) when NaOH (200 mmol in 10 mL of HOH) was added followed by the careful dropwise addition of hydrogen peroxide (10 mL, 30%). The exothermic reaction was allowed to proceed in an ice bath for 15 min, after which ethanol (30 mL) was added

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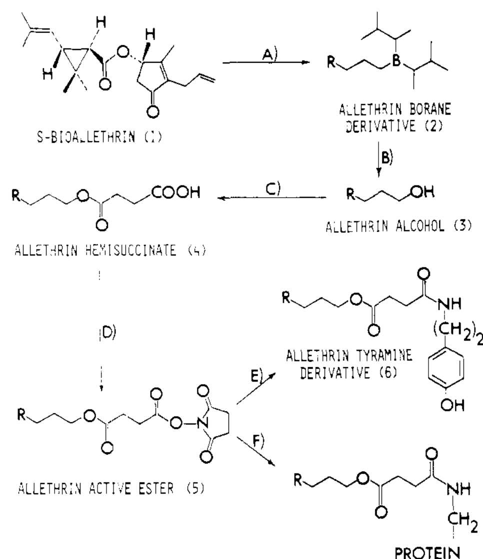

Figure 1. Synthetic route to allethrin protein conjugates and tyramine derivative. Reagents: (a) disiamylborane, (b) basic hydrogen peroxide, (c) succinic anhydride in pyridine, (d) dicyclohexylcarbodiimide and N-hydroxysuccinamide, (e) tyramine, (f) protein (hemocyanin or BSA).

and stirring continued for an equal time at 40 °C. The reaction mixture was poured into saturated brine (100 mL) and extracted with peroxide-free ether (2×, 50 mL). The combined ether extracts were washed sequentially with 1% HCl (100 mL) and distilled water (dHOH) (2×, 100 mL) and dried (anhydrous Na_2SO_4), and the ether was removed under reduced pressure. The residue was chromatographed on Florisil (60–100 mesh) utilizing a hexane–ether step gradient. The desired allethrin alcohol (**3**) was obtained (85% based on the borane utilized) and unreacted allethrin (2 mmol) and three other minor products were also detected. The allethrin alcohol was identified based on its IR, ^{1}H NMR, and MS: IR (thin film) cm^{-1} vs (1728, 1719), s (3420, 2935, 2930, 2880, 1160), and m (1662, 1657, 1428, 1390, 1289, 1243, 1200, 1119); ^{1}H NMR ($CDCl_3$) δ 1.75 (s, 6, $C(CH_3)_2$), 2.05 (s, 3, CCH_3), and 3.58 ppm (t, 2, CH_2OH); loss of 1 proton at 3.15 ppm with D_2O; MS (70 eV, two most intense ions each 14 mass units above 50 and >10% base peak + high mass units) m/e (rel intensity) 55 (46), 56 (25), 67 (25), 69 (24), 83 (91), 85 (97), 93 (21), 95 (17), 107 (28), 117 (18), 123 (100), 124 (22), 135 (21), 136 (17), 153 (19), 154 (26), 168 (48), 303 (0.4), 320 (0.4) (parent ion), 321 (0.4); and $[\alpha]^{25}{}_D$ (−21.26).

Allethrin Hemisuccinate (**4**). The allethrin alcohol (5 mmol) was dissolved in dry pyridine (10 mL) and 10 mmol of succinic anhydride was added. The resulting solution was stirred (24 h, 23 °C) and the pyridine removed under vacuum. The residue was dissolved in ether (50 mL), washed sequentially with aqueous 1% HCl (2x, 50 mL) and dHOH (2×, 50 mL), and the ether layer was dried (Na_2SO_4). The ether was removed under reduced pressure yielding a very viscous light-yellow oil which showed only one spot on TLC in several systems (Table I). The spectral data were identical for the product both before and after preparative TLC in solvent B (Table I). The hemisuccinate was identified on the basis of its IR, ^{1}H NMR, MS, UV, and elemental analysis: IR (thin film) cm^{-1} vs (1740, 1720), s (2960, 2930, 1160), and m (3300, 1659, 1422, 1390, 1285, 1240, 1200, 1115); ^{1}H NMR δ 2.65 (b-s, 4, $(CH_2)_2$) and 4.10 ppm (t, 2, CH_2O); loss of proton at 8.0 ppm with D_2O; MS (70 eV) m/e (rel intensity) 55 (81), 57 (21), 67 (43), 69 (39), 79 (98), 81 (99), 93 (76), 101 (34), 107 (89), 108 (25), 123 (100), 124 (48), 135 (99), 136 (97), 153 (39), 154 (15), 168 (40), 302 (0.6), 303 (0.3), 352 (0.2), 354 (0.9), 355 (0.5), 420 (0.6) (parent ion), 421 (1.0), 422 (0.3); UV λ_{max} 223, ϵ 13900 in hexane; and elemental C, H calcd (found) 65.70 (66.07), 7.67 (8.31).

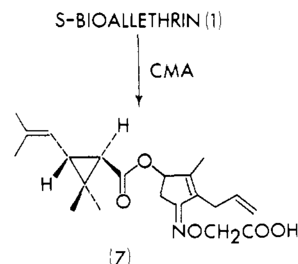

Figure 2. Synthetic route to the carbomethoxyoxime (CMO derivative of allethrin) using carboxymethoxylamine hemihydrochloride (CMA) in pyridine.

Hemisuccinate Active Ester (**5**). The hemisuccinate (2.5 mmol) was dried (5 h, 0.03 torr) and THF containing N-hydroxysuccinimide (5 mL, 2.75 mmol) added followed by cooling (4 °C) and the addition of dicyclohexylcarbodiimide (2.75 mmol). After stirring in the dark (24 h, 4 °C), the mixture was found by TLC to contain only trace amounts of hemisuccinate with the major product being of higher R_f. The precipitated dicyclohexylurea was removed by filtration through Celite followed by washing with THF (Rudinger and Ruegg, 1973). The crude mixture in THF was used for further reactions with tyramine, hemocyanin, and bovine serum albumin (BSA). After TLC in solvent B, IR indicated a loss of the hydroxy group and ^{1}H NMR indicated the presence of succinimide protons at 2.8 ppm.

Allethrin Tyramine Derivative (**6**). To tyramine hydrochloride [p-(2-aminoethyl)phenol·HCl, 4.5 mmol] in dHOH (9 mL) containing sodium bicarbonate (9 mmol) at 4 °C was added cold active ester (1.62 mmol) in THF (15 mL). After stirring 1 h at 4 °C, the solution was extracted with ether (20 mL, 2×), washed with dHOH, then brine (20 mL, 1×) to give 1 g of a viscous oil. After preparative TLC in benzene–propanol (5:1) and extraction with ethyl acetate, the pure allethrin tyramine derivative was obtained in a 71% yield based on the active ester. The derivative was a glassy solid which failed to crystallize. The tyramine derivative was identified based on its IR, ^{1}H NMR, and MS: IR (thin film) cm^{-1} vs (1725, 1710), s (3340, 2910, 1650, 1512, 1150), and m (2825, 1612, 1592, 1550, 1445, 1375, 1360, 1260, 1228, 1189, 1109); ^1H NMR ($CDCl_3$) δ 7.00 (m, 4, aromatic) loss of 1 proton at 3.2 (OH) and a second at 6.6 ppm (NH) with D_2O; MS (70 eV) m/e (rel intensity) 55 (36), 56 (85), 70 (27), 71 (42), 83 (100), 85 (97), 98 (15), 99 (29), 118 (14), 120 (27), 123 (23), 143 (18), 149 (96), 150 (12), 167 (28), 168 (8), 206 (2), 224 (4), 307 (2), 308 (0.4), 447 (0.2) (no parent ion at 538).

Allethrin CMO Derivative (**7**). The scheme for the formation of the allethrin carbomethoxyoxime derivative (CMO, **7**) is shown in Figure 2. Small-scale reactions of allethrin (100 μmol) and carboxymethoxylamine hemihydrochloride (200 μmol, 500 μmol, 1 mmol, 2 mmol expressed as the free CMA) in dry pyridine (3 mL) were carried out at 30 °C. The reactions were monitored by TLC in solvent A (Table I) by removing aliquots (100 μL) at various times (1–96 h), the pyridine removed under reduced pressure, before TLC. The reaction with 1 mmol CMA was complete in 18 h, while the reaction with 200 μmol showed mainly starting material. At 96 h the reaction with 200 μmol CMA was also complete. S-Bioallethrin (2 mmol) and CMA (4 mmol) in pyridine (10 mL) were stirred (23 °C, 96 h), then the pyridine was removed under reduced pressure. The glassy residue was dissolved in ether

(25 mL), washed with dHOH (3×, 25 mL), and dried (Na_2SO_4), and the ether was removed under reduced pressure. If the allethrin CMO derivative is washed with 1% HCl or exposed to preparative TLC in a weakly acidic solvent system, a Beckman rearrangement seems to occur with the resulting product showing no acidic protons on NMR. The product also has a similar TLC R_f to the CMO derivative and is weakly toxic to adult house flies. The CMO (**7**) derivative was purified by TLC in hexane–ethyl acetate (4:3) containing a trace of pyridine. The resulting yellow oil was identified on the basis of its IR, ^{1}H NMR, and MS: IR (thin film) cm^{-1} vs (1735), s (2985, 2935, 1163, 1121), m (3150, 3090, 1780, 1430, 1389, 1291, 1245, 1202, 1090, 1031, 1002); ^{1}H NMR δ 1.75 (CCH_3) and 4.55 ppm ($NOCH_2$); loss of one acidic proton at 8.2 ppm with D_2O; and MS (70 eV) m/e (rel intensity) 53 (14), 55 (21), 67 (19), 77 (24), 81 (69), 91 (33), 104 (13), 105 (42), 117 (99), 118 (25), 123 (100), 132 (74), 150 (10), 208 (46), 300 (0.6), 354 (0.2), 375 (2) (parent ion), 376 (4), 377 (1).

1S,3R,4′R-Allethrin. To thionyl chloride (1 g at 5 °C) was added chrysanthemic acid (1 mmol; 96.5% 1*S*, 3*R*, 3.5% 1*R*, 3*S*; and ~0.1% 1*R*, 3*R*, or 1*S*, 3*S* acid) in benzene (0.5 mL). Upon completion of the reaction (1 h), the thionyl chloride was removed under reduced pressure and the resulting yellow residue dissolved in benzene (0.5 mL). To the crude acid chloride (~7 °C) was added allethrolone (1 mmol, 97.6% *R* isomer) in benzene (0.5 mL) and pyridine (1.3 mmol). The resulting reaction mixture was stirred at room temperature overnight. The reaction mixture was diluted in benzene, washed with aqueous sodium bicarbonate (5%, 2×), aqueous HCl (1%, 2×), saturated brine, dried (Na_2SO_4), concentrated under reduced pressure, and purified by preparative TLC in solvent D. The 1*S*,3*R*,4′*R*-allethrin (64% yield) obtained cochromatographed with *S*-bioallethrin in five TLC systems and showed no trans (*R*,*R* or *S*,*S*) isomer on LC. The spectra of the final product were similar to those previously reported (Elliott, 1954; La Forge et al., 1954, 1956; Crombie and Harper, 1958; Bramwell et al., 1969; Elliott et al., 1972; Crombie et al., 1976).

Antigen Preparation. Keyhole limpet hemocyanin (100 mg A grade, 99.5% Calbiochem) was dissolved in 10 mL of dHOH by brief sonication and the pH adjusted to 8.7 with 0.1 N NaOH. Peroxide free THF (10 mL) was added and the light-purple solution cooled to 4 °C. The active ester (**5**) in THF (300 nmol, 2 mL) was added and the resulting solution stirred (4 °C, 28 h), losing its purple color. The solution was dialyzed against repeated changes of double-distilled water which resulted in precipitation of the protein as the THF was removed. The allethrin–protein conjugate (Figure 1) was then lyophilized in multiple ampules, sealed under N_2, and stored (−30 °C). Dialysis of aliquots of the allethrin–hemocyanin against aqueous ammonium bicarbonate (0.1 M), NaCl (0.15 M), sodium phosphate (0.01 or 0.15 M, pH 7.4), and 50% aqueous ethanol failed to resolubilize the antigen. This procedure was repeated using BSA and tyramine under identical conditions. Proteins used for immunodiffusion studies were additionally precipitated by the addition of cold ethanol (3×, −20 °C) in order to remove hapten molecules which were not covalently bound.

The percent decrease in the free amine groups in the hemocyanin antigen was measured by reacting the antigen, native hemocyanin, and hemocyanin exposed to conjugation conditions in the absence of active ester with fluorescamine using a modification of the method of Böhlen et al. (1973). Measurements were taken by dissolving the protein in 1.5 mL of 0.2 M sodium phosphate buffer titrated from pH 7.8 to 8.5 with sodium hydroxide. To this fluorescamine (0.5 mL of a 30 mg/100 mL solution in dioxane) was added as the sample was vigorously mixed. After 10 min the samples were read at 390 nm excitation, 475 nm emission.

Table II. Quantities of Compounds Required to Inhibit Precipitin Band Formation after Incubation with 100 λ of Undiluted Secondary Serum

compd	micrograms needed to inhibit precipitin band formation (nmol)[a]
S-bioallethrin (**1**)	10 (33.1)
S-bioallethrin side chain alcohol (**3**)	1 (3.14)
S-bioallethrin tyramine derivative (**6**)	1 (1.86)
(−)-*cis*-*R*-allethrin	>100 (>331)
permethrin	>100 (>256)
parathion	>100 (>344)
carbaryl	>100 (>498)
DDT	>50 (>142)

[a] Compounds (varying amounts from 0.01-100 μg) added in ethanol (1 μL) to secondary serum (100 μL) from a rabbit immunized with an allethrin-hemocyanin conjugate. After preincubation (30 min 30 °C, 15 min 4 °C), the serum sample was analyzed by Ouchterlony double diffusion against an allethrin-BSA conjugate.

Injection-Bleeding Schedules. Two injection schedules were examined. On one schedule a white female New Zealand rabbit was injected over a 3-day period with a total of 3 mg of the allethrin–hemocyanin conjugate, suspended in phosphate buffer (pH 7.4, I = 0.2) and emulsified in Freund's Complete Adjuvant (Calbiochem) to a total concentration of 0.5 mG of antigen/mL. Injection was intramuscular, into the back thigh muscles near the popliteal node. After 30 days the rabbit was boosted with a total of 1.5 mg of antigen over a 3-day period in Freund's Incomplete Adjuvant (GIBCO) at a total concentration of 0.5 mg/mL. Starting 10 days after the last boost, the rabbit was again boosted with 1.5 mg of the conjugate in Freund's Incomplete Adjuvant.

On the second schedule a rabbit was injected intravenously in the median ear vein with 3 mg of the allethrin–hemocyanin conjugate in an alum precipitate to reduce metabolism. The precipitate was prepared by suspending the antigen (1 mg) in physiological saline (1 mL), adding 1% $AlCl_3$ (0.5 mL), and titrating to pH 6.5–7.0 with sodium hydroxide (1 M). The boosting schedule was the same as that for the intramuscular injection and both schedules were slight modifications of established procedures (Williams and Chase, 1967).

The rabbits were bled through the median ear vein. Bleedings (20 mL) were taken before injection (control sera), after initial injection (primary sera), and after each boost (secondary sera). The immunodiffusion experiments reported in Table II were carried out using serum taken 9 days after the first boost.

Immunodiffusion. Immunodiffusion experiments were carried out in micro-Ouchterlony double-diffusion systems on glass slides (Ouchterlony, 1958; Williams and Chase, 1971). Agar (1% GIBCO Bacteriological grade in 12.5 mM Tris hydrochloride buffer at pH 7.5 containing 130 mM NaCl and 1 mM EDTA) was heated to a liquid state and 3 mL was applied to a glass slide (25 × 75 mm). After cooling, the agar formed a semi-solid layer ~3 mm thick. Agar plugs were removed by suction to form the desired pattern of wells (Figure 3).

The wells were filled with either rabbit serum (12 λ) or various protein solutions in phosphate buffer (pH 7.4, I

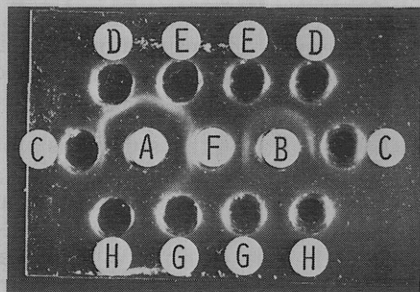

Figure 3. Ouchterlony double diffusion of BSA–allethrin against undiluted secondary serum: (a) 0.6% BSA–allethrin, (b) 0.06% BSA–allethrin, (c) untreated serum; remaining wells contain serum incubated with (d) EtOH, (e) 0.01 μg of *S*-bioallethrin, (f) 0.1 μg of *S*-bioallethrin, (g) 1 μg of *S*-bioallethrin, (h) 10 μg of *S*-bioallethrin.

= 0.2, 12 λ) and allowed to diffuse toward each other in individual moisture chambers for 20 h at room temperature or 36 h at 4 °C. They were then examined for precipitation band formation over a light box.

For band inhibition studies undiluted secondary serum was incubated (30 min, 30 °C in a shaking incubator followed by 15 min, 4 °C) in glass microfuge tubes (Kimble Products, 6 × 50 mm) with potential inhibitors added in ethanol (1 λ). The resulting serum sample was then used for Ouchterlony double diffusion as described above.

RESULTS

Synthesis. In order to raise antibodies specific for a small nonimmunogenic molecule (hapten), it is usually necessary to conjugate it to a protein. Therefore, a functionality must be present on the molecule of interest which will react with a protein. Based on spectral data, its specific rotation, and the appearance of only one peak on LC (in a system which separates allethrin into four major peaks), the *S*-bioallethrin (**1**) used in antigen formation was considered to be highly pure. As expected from published data (Brown and Moerikofer, 1961, 1962; Brown, 1972), hydroboration–oxidation occurred very selectively to give the anti-Markovnikov alcohol (**2**) on the allyl side chain of *S*-bioallethrin. A single UV dense peak was observed for the alcohol on LC and the >5000 theoretical plate column is likely to have separated alcohols formed at the other possible positions. The high specific rotation of the allethrin side chain alcohol indicates, but does not prove, that racemization was negligible. This synthetic method should be directly applicable to haptens for pyrethrins I and II and rotenone and should give usable mixtures of haptens for the cinerins and jasmolins, as well as provide a route to possible metabolites of these compounds.

The *S*-bioallethrin CMO (**7**) derivative was formed successfully and the carboxylic acid of the CMO derivative may be useful in the future to provide a hapten bound at the center of the molecule (possibly in conjunction with hapten handles on the *gem*-dimethyl or isobutenyl group of the chrysanthemic acid moiety) to develop different antibody populations for enhanced specificity through multiple antibody technology. The apparent instability of the CMO derivative of *S*-bioallethrin, however, made it inappropriate for initial studies.

Other attempts to synthesize a hapten handle on the allyl side chain of allethrin were less successful. No ninhydrin sensitive material was obtained from the reaction of hydroxylamine-*O*-sulfonic acid (Brown et al., 1964; Rathke et al., 1966) with the disiamylborane derivative of *S*-bioallethrin (**2**) in an attempt to place an amine on the allyl side chain. Attempted reaction of the disiamylborane derivative of *S*-bioallethrin with ethyl bromoacetate to yield a carboxylic acid on the allyl side chain (Brown et al., 1968a, 1968b) failed due to proton abstraction from the allethrolone moiety in the strongly basic conditions used, followed by polymerization and extensive ester cleavage (La Forge et al., 1952).

Synthesis of the hemisuccinate (**4**) of *S*-bioallethrin alcohol was essentially quantitative as was the formation of the active ester (**5**) and tyramine (**6**) derivatives. The active ester can be purified and is moderately stable under slightly acidic conditions (Rudinger and Ruegg, 1973; Bolton and Hunter, 1973), but better results can be obtained by using the active ester immediately after its formation. The tyramine derivative of *S*-bioallethrin is a convenient moiety to radiolabel (^{3}H, ^{14}C, ^{125}I) for radioimmunoassay, and it additionally provides a convenient method for monitoring antigen formation. It is difficult to monitor the reaction of the hemisuccinate active ester with protein, so simultaneously a reaction was carried out under identical conditions with tyramine and monitored by TLC in solvent C (Table I).

The percent decrease in free amine groups in hemocyanin was measured by fluorometry after conjugation with the allethrin active ester. A linear standard curve was generated relating amount of unreacted hemocyanin to percent relative intensity; subsequent measurement of a known quantity of allethrin–hemocyanin indicated that 38% of the free amine groups had become unavailable for reaction with fluorescamine. There was additionally a 6% increase in hemocyanin free amines available for reaction after exposure to conjugation conditions in the absence of active ester.

Immunodiffusion. Antibodies specific to an antigen will comprise a heterogeneous population differing in the region of recognition and in binding affinity. When serum containing this population and the specific antigen are allowed to diffuse radially toward each other in a gel matrix, they will form a visible precipitin band at their optimal relative concentrations or equivalence point (Ouchterlony, 1958). There were no antibodies detectable by immunodiffusion from the alum-injected rabbit after three boosts, while serum from the rabbit injected intramuscularly showed an allethrin-specific antibody titer when bled 9–12 days after the first boost. This undiluted secondary serum displayed a precipitin band when allowed to diffuse against a 0.6% solution of a BSA–allethrin conjugate or native hemocyanin, but no band formed with unreacted BSA. This serum was then used for band inhibition studies as shown in Table II.

The antibody population of this serum shows a dose-dependent inhibition of precipitin band formation between 1 and 10 μg of *S*-bioallethrin/100 μL of serum probably due to saturation of the allethrin-specific immunoglobulins making them unavailable for cross reaction with the BSA–allethrin conjugate (Figure 3). The allethrin alcohol and tyramine derivative were found to inhibit band formation at lower levels. The greater sensitivity of the antibody to these derivatives relative to the parent allethrin may be due to their greater resemblance to the actual hapten injected and/or superior solubility in the incubation system used. The formation of the precipitin band was not inhibited by the 1*S*,3*R*-chrysanthemate of 4′*R*-allethrolone even at 100 μg/100 μL of serum, indicating the configurational and/or geometrical specificity of the antibody population. These results imply that stereochemical purity should be considered in immunochemical studies. The formation of the precipitin band was not inhibited by permethrin, parathion, or carbaryl (100 μg/

100 μL of serum) or DDT (50 μg/100 μL of serum; greater amounts of DDT were insoluble in ethanol).

DISCUSSION

Although widely used in the health and some biological sciences, immunological techniques have received limited use in environmental chemistry (see review by Ercegovich, 1971; Lukens et al., 1977; Chae et al., 1977; Kado and Wei, 1978). This limited utilization is due in part to the success of other techniques for residue analysis such as GLC with selective detectors. Some environmental contaminants including some insect growth regulators and pyrethroids do not lend themselves to such analysis. Electron-capture GLC of the rethrins demonstrates poor sensitivity and requires extensive workup (Moore, 1973). The detection of some other pyrethroids such as resmethrin and phenothrin is even more difficult (Ueda, 1977). Saponification methods necessitate extensive workup (George et al., 1977), and while GLC–mass spectral analysis is sensitive, it requires the dedication of very expensive equipment for residue analysis (Pattenden et al., 1973; Crobie et al., 1976; Holmstead and Soderlund, 1977).

For compounds such as allethrin, immunological techniques offer a wide range of possible assay methods. Radial immunodiffusion (Williams and Chase, 1971) and similar techniques can provide moderate sensitivity in a rapid procedure requiring no sophisticated equipment. Such a procedure may be very useful for rapid measurements under field conditions and may be the appropriate technology for estimation of pyrethrum content of *Chrysanthemum cinerariaefolium* during plant breeding or for residue studies in some developing countries. Immunochemical techniques are easily interfaced with LC to essentially provide a sensitive, selective detector, and antibodies can be additionally used in the actual cleanup of a residue sample.

As shown by this and many previous studies, such as Landsteiner and van der Scheer (1928) and Karush (1956), immunochemical methods can be selective or specific for absolute configuration, while this selectivity is beyond the capability of most classical methods. As with any biologically active material, the mammalian and insect toxicity of insecticides is often related to their configuration. The different isomers may additionally show selective toxicity, differing rates of biodegradation, and unique chronic effects (Sagar et al., 1972; Elliott et al., 1974; Ohkawa et al., 1977; Casida and Soderlund, 1977; Wickham, 1977; Henrick et al., 1978; for earlier references, see Wustner, 1971). It will become increasingly important for industry and regulatory agencies to monitor the configuration of some products. Immunochemical methods will allow quality control of optical purity without extensive cleanup, they will facilitate monitoring the configurational shelf-life of compounds, and they will allow the investigation of changes in configurational composition during metabolism or in the composition of residues under field conditions. From a more basic standpoint, immunochemical tools may additionally be useful for the elucidation of the conformation of biologically important molecules in aqueous solutions (Pressman et al., 1948) as appears currently important to direct the synthesis of new pyrethroids (Elliott et al., 1974; Elliott and Janes, 1977).

The obvious direction for further immunochemical research on pyrethroids and insect growth regulators is towards the development of radioimmunoassay techniques as have been recently reported for some cyclodienes (Langone and Van Vunakis, 1975) and parathion (Ercegovich et al., 1977). Radioimmunoassay promises to greatly enhance the sensitivity, reproducibility, and speed of residue analysis for compounds such as allethrin, while at least providing alternate or confirmatory techniques for pesticides currently assayed by classical methods.

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Extraction Efficiencies for Pesticides in Crops. 1. [^{14}C]Carbaryl Extraction from Mustard Greens and Radishes

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^{14}C-labeled carbaryl (1-naphthyl *N*-methylcarbamate) suspended in a commercial carbaryl formulation was sprayed on mustard greens and radishes. At three intervals, postapplication, the crops were extracted using methanol, acetonitrile, or acetone. Crops were either blended and leached or repetitively blended followed by Soxhlet extraction. Essentially all of the extractable radioactivity was removed by blending. The ^{14}C was more difficult to extract from radishes than from mustard greens and with increasing time after application. For mustard greens 92, 83, and 77% of the ^{14}C at harvest was extractable at 3, 7, and 14 days, respectively; for radishes 91, 76, and 58% was extractable at the same intervals. Methanol was generally the best solvent and the blend–Soxhlet process was superior to the blend–leach process. Thin-layer chromatography of the organic soluble extracts indicated that the majority of ^{14}C was carbaryl. Acid hydrolysis of the extracted tissues released 40–50% of the residual ^{14}C.

Quantitive data for many pesticide residue analytical extraction methods consist primarily of determinations made on representative sample types fortified in the laboratory with the compounds of interest. Such studies provide data on recovery of the pesticide through the various manipulations of the method, but fail to provide the equally essential information of the ability of the extraction step of a method to remove "field-incurred" residues from the sample. This problem is well understood by pesticide analysts but relatively little work has been reported to provide an estimate of the magnitude of the problem or solutions to it.

One of the earliest reports was that by Klein et al. (1959) who reported a nonextractable residue of radioactivity remaining in spinach after it was sprayed with labeled methoxychlor [2,2-bis(*p*-methoxyphenyl)-1,1,1-trichloroethane].

Mumma et al. (1966), Wheeler et al. (1967), and Wheeler and Frear (1966) reported that root absorbed and translocated [^{14}C]dieldrin [1,2,3,4,10,10-hexachloro-*exo*-6,7-epoxy-1,4,4a,5,6,7,8,8a-octahydro-1,4-*endo*,*exo*-5,8-dimethanonaphthalene] was not efficiently extracted by blending with *n*-hexane–isopropyl alcohol (2:1, v/v). A subsequent exhaustive extraction using chloroform–methanol (1:1, v/v) in a Soxhlet extractor recovered 20 to 40% of the total dieldrin residue. Several subsequent reports (Burke and Porter, 1966; Burke et al., 1971; and Caro, 1971) compared various extraction systems to the exhaustive extraction procedure of Mumma et al. (1966).

Bowman et al. (1968) evaluated nine procedures for the extraction of six organophosphate insecticides and their metabolites from field-treated crops. They detected the highest total residues when the samples were extracted by using 10% methanol in chloroform in a Soxhlet extractor.

Very little work has been reported on the evaluation of the efficiency of extraction of carbamate insecticides. Watts (1971) applied [^{14}C]carbaryl [1-naphthyl *N*-methylcarbamate] to bean larves and was able to extract 100% of the applied radioactivity 48 h later.

Van Middelem and Peplow (1973) studied the extraction of [^{14}C]carbofuran [2,3-dihydro-2,2-dimethyl-7-benzofuranyl methylcarbamate] from cabbage after soil application of the pesticide. At postapplication intervals up to 35 days, 90% of the ^{14}C was extractable by acid digestion, Soxhlet extraction using methanol, or by blending in methanol.

Although a number of investigators agree that one of the most effective means to evaluate extraction efficiency is

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