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Carbamate and Organophosphorus Nematicides: Acetylcholinesterase Inhibition and Effects on Dispersal

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Abstract: The sensitivities of acetylcholinesterases (ACHE) from the fungus-feeder *Aphelenchus avenae* and the plant-parasitic species *Helicotylenchus dihystera* and *Pratylenchus penetrans* and the housefly, *Musca domestica,* were compared using a radiometric assay which utilized H^3 acetylcholine as a substrate. Nematode ACHE were generally less sensitive to inhibition by organophosphorus and carbamate pesticides than were ACHE from the housefly. ACHE from the plant-parasitic species and *A. avenae* were generally similar in sensitivity. In soil, carbamates were more toxic than the organophosphorus pesticides to *A. avenae.* All pesticides tested affected nematode movement, but fenamiphos was more inhibitory than others. The effects on dispersal of nematodes may be an important mechanism in control by some nematicides.

Key words: acetylcholinesterase, *Aphelenchus avenae,* carbamate, *Helicotylenchus dihystera,* nematicide, organophosphorus, *Pratylenchus penetrans,* radiometric, repellent.

Acetylcholinesterases (ACHE) from the housefly, *Musca domestica* L., have been shown to be more sensitive to inhibition by ACHE-inhibiting organophosphorus and carbamate pesticides than ACHE from the nematode *Aphelenchus avenae* Bastian (13). Pree et al. (13) speculated that mechanisms other than direct inhibition of ACHE might be involved in the nematicidal activity of some pesticides. Their study, however, described investigations with *A. avenae,* but not with any of the plant-parasitic nematode species which are the target of nematicide applications. The spectrophotometric assays used by Pree et al. (13) required large numbers of nematodes precluding tests with plant-parasitic species which are less readily mass cultured than *A. avenae.*

We report here the results of assays using H^3 labeled acetylcholine which compare the sensitivity of ACHE from two species of plant-parasitic nematodes, *Helicotylenchus dihystera* Cobb and *Pratylenchus penetrans* (Cobb), with ACHE from the fungus feeder *A. avenae* and the housefly, *M. domestica.* Also included are studies on the effects of pesticides on the movement of *A. avenae* in soil cores which may be important in explaining the efficacy in the field of some pesticides less effective in inhibiting nematode ACHE.

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Materials and Methods

Rearing: Aphelenchus avenae was isolated from a Vineland silt loam collected from an oak tree nursery at the Agriculture Canada Research Station, Vineland Station, Ontario. The nematode was reared on the fungus *Botrytis cinerea* Pers. which was grown on potato dextrose agar in petri dishes (15 × 100 mm). Four-day-old fungus cultures were inoculated with ca. 1,000 *A. avenae,* enclosed in a black polyethylene bag, and incubated for 2 weeks at 22–25 C; 100,000–150,000 *A. avenae* per plate developed in 2 weeks. The root-lesion nematode, *Pratylenchus penetrans,* which also originated from the Niagara peninsula, was reared initially on celery (*Apium graveolens* Pers. cv. Utah 15) in large plastic tubs (46 × 46 × 27 cm) in a greenhouse and then on sweet corn (*Zea mays* L. cv. Earlivee) in a greenhouse ground bed. The spiral nematode, *Helicotylenchus dihystera,* which originated from New York state, was reared on red table beet (*Beta vulgaris* L.

cv. Detroit Dark Red) in large plastic tubs (46 × 46 × 27 cm) in a greenhouse. The soil used in the nematode cultures was a Vineland silt loam. The greenhouses were maintained at 20–24 C.

Preparation of nematode acetylcholinesterases: Aphelenchus avenae and *P. penetrans* were extracted from fungus cultures and corn roots, respectively, in a mistifier (1) and *H. dihystera* was extracted from soil by the pan method (14). Nematodes extracted by both methods were collected daily, concentrated using a millipore filter, and kept in a shallow layer of water in Erlenmeyer flasks at 5 C before grinding.

Nematodes for ACHE studies were crushed between two ground glass plates. The base plate was 20 × 20 cm and the upper plate, which had a wooden cabinet handle glued to the smooth surface, was 15 × 15 cm. The number of nematodes in an aqueous suspension was determined, the nematodes were concentrated by centrifugation in conical tubes, and the supernatant was drawn off by suction. The pellets were transferred to the base plate with a tapered spatula; residual nematodes in the tubes were suspended in 2 ml pH 7.0, 0.05 M sodium phosphate buffer which was poured over the pellet on the base plate, then all nematodes were ground using a circular motion. After grinding, the plates were pried apart and the nematodes on both plates were scraped to the center and reground. This procedure was repeated five times. Finally, the crushed nematodes were scraped from the two plates with a rectangular glass cover slip and flushed with buffer into a 25-ml Erlenmeyer flask to give a final volume of 10–15 ml. The percentage of nematodes crushed was determined under a binocular microscope before the suspension was used for ACHE assays.

Preliminary assays were conducted to determine the amount of ACHE activity per unit of protein for each nematode species. For each inhibition assay, ground nematode preparations were diluted to the appropriate protein concentrations (Table 1). Protein concentrations were determined by the method of Bradford (2). All samples were held on ice until assayed.

Isolation of fly acetylcholinesterases: Adult flies (3–7 days old) from a laboratory colony described previously (13) were used in all experiments. Flies were frozen (−10 C) for 1–2 hours prior to use and decapitated; the heads were homogenized in cold distilled water with a Potter-Elvehjem teflon homogenizer. The homogenates were filtered through glass wool plugs, and the particulate residue was rinsed with additional distilled water. A concentration of 1.5 heads/ml was prepared and diluted to a final protein concentration of 0.0003 mg/ml. Samples were held on ice until assayed.

Preparation of acetylcholine substrate: One millicurie of H^3 acetylcholine chloride (specific activity 2.6 Ci/mmol) (Amersham Canada, Oakville, Ontario) was dissolved in 10 ml ethanol and stored at −20 C. For experimental use, 0.52 ml of this stock solution was removed and treated to remove H^3 acetate background. A method similar to that used by Johnson and Russell (9) was used as follows. The ethanol was driven off with a gentle stream of air, the H^3 acetylcholine was redissolved in 1 ml distilled water, and 5 μl glacial acetic acid per ml was added. Any H^3 acetate formed was removed by extracting three times with 10 volumes of toluene : isoamyl alcohol (10:1). The solution was subsequently extracted three times with 100% toluene to remove any isoamyl alcohol from the aqueous phase. Residual toluene was removed by blowing a gentle stream of air. The solution was diluted to 10 ml with distilled water. This produced a final solution of 2,000 nmol/ml. The solution was stored at −20 C.

Immediately before use, an aliquot of H^3 acetylcholine chloride (2,000 nmol/ml) was removed from the stock solution and diluted with an equal volume of 10 mM unlabeled acetylcholine bromide. Assays were routinely performed at a 2.0-mM concentration of acetylcholine.

Acetylcholinesterase assay: The standard assay mixture contained 0.5 ml of 0.05 M

potassium phosphate buffer (pH 7.0), 0.2 ml of acetylcholine substrate (10 mM), and 0.3 ml enzyme plus H_2O to give a final volume of 1 ml. The assay method was adapted from that used by Johnson and Russell (9).

The buffer, enzyme, and water were premixed and incubated on a water bath-shaker at 25 ± 1 C for 15 minutes. The reaction was started by the addition of substrate (0.2 ml). Samples, 0.1 ml each, were removed after 0, 10, 20, and 30 minutes and placed in plastic minivials (Beckman Instruments, Mississauga, Ontario). The reaction was stopped in the minivials by the addition of 200 μl "stopping mixture" consisting of 1 M chloroacetic acid, 0.5 M NaOH, and 2 M NaCl, made fresh daily, followed immediately by the addition of 4.0 ml scintillation mixture of 0.5% PPO, and 0.03% POPOP (BDH, Toronto, Ontario) in toluene containing 10% isoamyl alcohol.

Samples were counted on a Beckman LS-6800 Scintillation counter. Each sample was counted for a period long enough that 95% of the time the results were ± 2% of the mean. Comparisons of this method and the spectrophotometric method used previously (13) indicated similar rates of acetylcholine hydrolysis for both procedures.

Inhibition assays: Pesticides dissolved in analytical grade acetone were added to the reaction vessels before the tests and the solvent allowed to dry. The enzyme, water, and buffer solutions described were added and incubated at 25 ± 1 C for 15 minutes before the addition of substrate. All inhibition assays were conducted with approximately equal rates of acetylcholinesterase activity (Table 1). Pesticides used were all analytical grade standards.

ACHE inhibition assays were at two hydrolytic rates: 146–156 pmol/minute and 71–107 pmol/minute. This was necessary because fly head ACHE did not give consistent results (hydrolytic rates) below 0.0003 mg/ml protein where the rate of acetylcholine hydrolysis was 155 pmol/minute. *P. penetrans* were not available in sufficient numbers to test at hydrolytic rates over 71 pmol/minute. *A. avenae* and *H. dihystera* preparations were tested at both rates.

Effects of pesticides on the dispersal of A. avenae: The mobility of *A. avenae* in response to pesticides was studied in soil cores injected with carbamate and organophosphorus pesticides. Cores of soil (4 cm deep × 1.8 cm d) were prepared in teflon cylinders. Each cylinder consisted of five 1-cm-deep rings held together with masking tape; the bottom ring was closed with fiberglass cloth. A 1-mm-d hole was drilled in the middle of the wall of the bottom ring. A Vineland silt loam was sterilized, air dried, and passed through a 1.7-mm-pore sieve. Prepared soil (14 g) was poured into each cylinder and tapped gently until the soil had settled into the lower four rings. The soil in each cylinder was moistened to field capacity (23%) by placing four cylinders of soil in 13 ml water in a petri dish. After the water was absorbed, the cylinders of moistened soil were placed in a chamber for 48 hours to allow the moisture to equilibrate. The bottom of each cylinder was then sealed with masking tape. Analytical standards of carbamate and organophosphorus pesticides, dissolved in acetone at appropriate concentrations, were injected into the soil in 10 μl acetone through the hole in the bottom ring of the cylinder and the hole was sealed with masking tape. Immediately after the injection of pesticides, 0.5 ml of *A. avenae* aqueous suspension containing 450 nematodes was pipetted onto the soil surface at the opposite end of each cylinder. The inoculated cylinders of soil were placed vertically in a high humidity chamber, and the nematodes were allowed to migrate for 12 days at 24 C. After this time, the four levels of each soil core were separated from the cylinder with a razor blade and the nematodes were extracted from each by the pan method (14). Nematodes were counted with a dissecting microscope. Insecticides were tested at two rates, 0.5 mg and 0.05 mg, in two separate experiments. Two controls were included: one untreated (water only) and a second

that received 10 μl acetone. Each treatment was replicated six times and the experiment was arranged in a randomized block design. Data were subjected to an analysis of variance and means were separated by a Duncan's multiple-range test ($P = 0.05$).

Results

Preparation of nematode acetylcholinesterases: Pellets of *A. avenae* contained from 520,000 to 5,600,000 nematodes of which 74–99% were broken by the grinding process. A lower percentage of *A. avenae* was broken when larger numbers were ground. Pellets of *H. dihystera* contained from 142,000 to 420,000 nematodes; 96–100% of them were successfully crushed. Pellets of *P. penetrans* contained from 325,000 to 800,000 nematodes; 83–100% were crushed.

Preliminary assays to compare the spectrophotometric method used previously with the method described here indicated that the two methods gave similar rates of acetylcholinesterase activity per unit of protein but that the technique using H^3 labeled acetylcholine was 100–500-fold more sensitive and could be used at correspondingly lower protein concentrations.

Inhibition assays: ACHE from housefly heads was more sensitive to inhibition by both carbamate and organophosphorus compounds than were any of the nematode species (Table 1). This was especially apparent at the lower (10^{-7} M) concentration. Aldicarb sulfoxide, eserine, and carbofuran inhibited nematode ACHE more than did fenamiphos sulfone. Malaoxon was as inhibitory as the carbamates. Differences in sensitivity between fly head ACHE and nematode ACHE to malaoxon were also less than to fenamiphos sulfone. In general, ACHE from the plant-parasitic nematode species *H. dihystera* and *P. penetrans* were similar in sensitivity to inhibition to that from *A. avenae* (Table 1). Oxygen analogs of malathion and fenamiphos were much more inhibitory to ACHE than their precursors. Malaoxon was more inhibitory than fenamiphos sulfone to nematode ACHE. Fenamiphos sulfone had no effect on ACHE from *P. penetrans.* Aldicarb sulfoxide was more active than aldicarb against *H. dihystera* ACHE but both were about equally inhibitory to *M. domestica* and *A. avenae* ACHE at 10^{-5} M. Aldicarb sulfoxide at 10^{-7} M was less inhibitory to ACHE from the plant-parasitic species than that from *A. avenae.* Carbofuran was about equally inhibitory to ACHE from all nematode species.

Effects on dispersal of A. avenae: Based on the distribution of nematodes in the different soil cores (Table 2), all compounds except aldicarb at the 0.5-mg rate reduced the movement of *A. avenae.* Nematodes were not always randomly distributed in the four soil core levels in controls treated with water or with acetone (Table 2). When the percentages of nematodes in each soil core were compared (Table 3), however, only fenamiphos and carbofuran treatments had significant effects on nematode dispersal. At the 0.5-mg treatment level, fenamiphos had the greatest effect; a higher percentage of the nematodes remained in soil core level 1 than for other treatments.

Carbofuran and aldicarb were more toxic than either fenamiphos or malathion at both the 0.5-mg and 0.05-mg rates. Mortality caused by both compounds was reduced at the 0.05-mg rate but these compounds were still more toxic than either fenamiphos or malathion. In both tests, aldicarb was more toxic than carbofuran. Nematode numbers in malathion-treated cores were not significantly reduced relative to untreated or acetone-treated controls. Fenamiphos, while more toxic than malathion, killed significantly fewer nematodes than aldicarb or carbofuran.

Discussion

The data presented here generally support those described earlier for comparisons between *M. domestica* and *A. avenae* by Pree et al. (13). Nematode ACHE, including those from the plant-parasitic species, *H. dihystera* and *P. penetrans,* were less susceptible to inhibition by organophospho-

TABLE 1. Inhibition (%) of the hydrolytic activity of acetylcholinesterases from houseflies and nematodes by organophosphorus and carbamate pesticides.

Inhibitor	Concentration	*Musca domestica*	*Aphelenchus avenae*		*Helicotylenchus dihystera*		*Pratylenchus penetrans*
		155.6 ± 54.4† pmol/min	154.3 ± 24.1 pmol/min	107.3 ± 24.4 pmol/min	145.8 ± 5.2 pmol/min	80.3 ± 16.4 pmol/min	70.6 ± 48.6 pmol/min
Carbamate pesticides							
Aldicarb	10^{-5} M	100‡	78.2 ± 30.9	77.1‡		20.1‡	
Aldicarb	10^{-5}	95.5 ± 5.3	85.1 ± 12.7	91.8 ± 14.3	64.2 ± 13.1	69.3 ± 26.6	83.9 ± 14.2
Sulfoxide	10^{-7}	91.5 ± 7.6	40.5 ± 9.7	42.8 ± 24.2	16.8 ± 13.8		14.2 ± 12.9
Eserine	10^{-5}		97.7 ± 3.9	100‡	83.1 ± 16.6	100‡	94.6 ± 9.3
	10^{-7}	96.8 ± 5.5	75.4 ± 7.0	61.8 ± 23.0	24.0 ± 11.1	18.5 ± 15.7	28.5 ± 24.7
Carbofuran	10^{-5}	90.3 ± 4.0	96.1 ± 4.2	99.7 ± 0.6	79.3 ± 8.3	96.0 ± 6.5	86.3 ± 23.8
	10^{-7}	94.0 ± 9.0	54.9 ± 3.5	55.8 ± 15.1	51.6 ± 8.1	54.6 ± 8.8	53.6 ± 26.5
Organophosphorous pesticides							
Malathion	10^{-5}	0	5.5 ± 7.8		0		
Malaoxon	10^{-5}	99.1 ± 1.6	92.5 ± 4.4	97.4 ± 4.5	88.2 ± 20.4	97.4 ± 4.6	97.4 ± 4.5
	10^{-7}	96.3 ± 5.9	31.5 ± 13.8	40.1 ± 4.9	9.4 ± 9.1	10.9 ± 18.9	40.1 ± 4.9
Fenamiphos	10^{-5}	91.2 ± 6.0	1.0 ± 1.3		0		0
Fenamiphos	10^{-5}	99.1 ± 1.6	25.9 ± 8.9	47.8 ± 2.4	13.6 ± 14.5	73.6 ± 37.3	0
Sulfone	10^{-7}	89.6 ± 0.8	0	6.2 ± 10.7	7.7 ± 9.7	38.8 ± 33.7	0
Protein concentration (mg/ml)		0.0003	0.006	0.003	0.025	0.013	0.025

Data are means of three separate assays ± SD.
† Hydrolytic rate.
‡ Data based on single assay.

TABLE 2. Dispersal of *Aphelenchus avenae* in pesticide-treated soil.

Treatment	Number of nematodes†				Total living nematodes‡	Mortality (%)
	Level 1	Level 2	Level 3	Level 4		
			Test 1			
Water	126.2 a	96.2 a	122.5 a	110.0 a	454.9 v	0.0
Acetone (10 μl)	125.0 a	120.0 a	116.0 ab	47.5 b	408.5 w	9.2
Malathion (0.5 mg)	157.5 a	98.7 b	80.0 b	61.2 b	397.4 w	11.7
Fenamiphos (0.5 mg)	158.7 a	23.7 b	16.2 b	6.3 b	204.9 x	54.5
Carbofuran (0.5 mg)	82.5 a	38.7 b	42.5 b	2.5 c	166.2 y	63.1
Aldicarb (0.5 mg)	12.5 a	10.0 a	6.3 a	10.0 a	38.8 z	91.4
			Test 2			
Water	157.5 a	80.0 a	118.8 ab	72.5 b	428.8 v	4.7
Acetone (10 μl)	163.7 a	125.0 b	85.0 c	40.0 d	413.7 v	8.1
Malathion (0.05 mg)	195.0 a	117.5 b	60.0 b	57.5 b	430.0 v	4.4
Fenamiphos (0.05 mg)	227.5 a	102.5 b	20.0 c	21.5 c	371.5 w	17.4
Carbofuran (0.05 mg)	182.5 a	55.0 b	31.3 bc	12.5 c	281.3 x	37.5
Aldicarb (0.05 mg)	67.5 a	25.0 b	27.5 b	7.5 b	127.5 y	71.7

† Nematodes (450) applied at soil level 1. Insecticide applied in 10 μl acetone to soil core level 4. Distribution determined after 12 days exposure. Numbers in same rows (levels 1–4) followed by same letter are not significantly different ($P = 0.05$) by Duncan's multiple-range test. Data are means of six replications.

‡ Numbers followed by same letter in this column are not significantly different ($P = 0.05$) by Duncan's multiple-range test. Comparisons are within each test. Data are means of six replications.

rus and carbamate pesticides than were ACHE from the housefly. ACHE from plant-parasitic nematode species *H. dihystera* and *P. penetrans* and the fungal feeding nematode species *A. avenae* were generally similar in sensitivity. This suggests that physiological and toxicological assays with *A. avenae* may be extrapolated to plant-parasitic nematode species.

In soil, the carbamates were more toxic to *A. avenae* than were the organophosphorus pesticides. Poor control was not unexpected in the case of malathion which is readily hydrolyzed in soil and not persistent (12). Fenamiphos, which is an effective soil nematicide (6), was less toxic to *A. avenae* than was carbofuran or aldicarb.

In our tests to measure effects on dispersal, nematodes were not always randomly distributed through the various soil levels, even after 12 days. Studies of nematode movement patterns, reviewed by Croll (3), have indicated that nematode movement in the absence of a stimulant does not constitute a good dispersal system. Croll and Blair (4) ascribed the asymmetric movement patterns they saw to corresponding asymmetries in the nervous system. Hence, in the absence of any behavioral stimulation, a completely random distribution of *A. avenae* may not be expected. Other studies (8,10,11) that have assayed for repellent effects or effects on dispersal have shown that both carbamate and organophosphorus pesticides affect nematode dispersion. Marban-Mendoza

TABLE 3. Distribution (%) of *Aphelenchus avenae* in four levels of pesticide-treated soil cores.

Treatment	Level 1	Level 2	Level 3	Level 4
		Test 1 (0.5 mg)		
Control				
Water	27.7 d	21.1 ab	27.0 a	24.2 a
Acetone	30.6 cd	29.5 a	28.3 a	11.6 ab
Malathion	39.5 c	24.8 ab	20.1 ab	15.6 a
Fenamiphos	77.4 a	11.6 b	7.9 b	3.1 b
Carbofuran	49.6 b	23.3 ab	25.6 a	1.5 b
Aldicarb	32.2 cd	25.8 ab	16.3 ab	25.8 a
		Test 2 (0.05 mg)		
Control				
Water	36.7 b	18.7 a	27.7 a	16.9 a
Acetone	39.6 b	30.2 a	20.5 ab	9.7 abc
Malathion	45.3 b	27.3 a	14.0 bc	13.4 ab
Fenamiphos	61.2 a	27.6 a	5.4 c	5.8 d
Carbofuran	64.8 a	19.6 a	11.1 c	4.5 cd
Aldicarb	52.9 ab	19.6 a	21.6 ab	5.9 bcd

Nematodes (450) applied to level 1. Insecticide applied to level 4 in 10 μl acetone. Distribution of nematodes determined after 12 days. Numbers in each column followed by same letter are not significantly different ($P = 0.05$) by Duncan's multiple-range test. Data are means of six replications.

and Viglierchio (10,11) showed that low concentrations of carbofuran and fenamiphos suppressed dispersion of *Pratylenchus vulnus* Allen and Jensen, even in the presence of a gradient of an attractant from bean roots. Di Sanzo (5) showed that carbofuran inhibited movement of *Tylenchorhynchus claytoni* Steiner toward corn (*Zea mays* L.) seedlings. In our study, all compounds had some effect on *A. avenae* but fenamiphos, and to a lesser extent carbofuran, inhibited movement of *A. avenae* through the soil. Hough and Thomason (8) observed that migration in sand columns of *Meloidogyne javanica* and *Heterodera schachtii* Schmidt juveniles was inhibited by aldicarb. Aldicarb reduced dispersion in our studies but was also highly toxic at the concentrations tested.

The data suggest that reduced movement or dispersion, rather than direct mortality, may be an important part of controls obtained from fenamiphos treatments. On the other hand, direct mortality appears to occur more readily with aldicarb or carbofuran treatment. The ACHE inhibition studies here and in previous studies with *A. avenae* (13) support the possibility that different nematicides may be providing controls based on different primary modes of action. Carbamate and carbamoyl oximes may act primarily as ACHE inhibitors (i.e., killing the nematodes by ACHE inhibition), whereas fenamiphos may be effective primarily by reducing dispersive movement. This does not preclude the possibility that ACHE inhibition has a major role in reducing dispersal. Certainly, other modes of action may also be involved: e.g., effects on reproduction, ovicidal action, or reduced movement as suggested by Hough and Thomason (8) and Hague et al. (7).

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