Journal of Nematology 19(2):188–193. 1987.

Sensitivity of Acetylcholinesterases from *Aphelenchus avenae* to Organophosphorous and Carbamate Pesticides

D. J. Pree, J. L. Townshend, and D. E. Archibald[1]

Abstract: The sensitivities of acetylcholinesterases (ACHE) from the nematode *Aphelenchus avenae* and the house fly *Musca domestica* to various pesticides were compared using a colorimetric assay. ACHE from *A. avenae* were generally less sensitive than ACHE from *M. domestica* to inhibition by organophosphorous and carbamate pesticides. Carbamates were somewhat more inhibiting than organophosphorous pesticides to nematode ACHE. In vivo tests with concentrations of various pesticides up to 500 ppm in sand caused less than 100% mortality of nematodes.

Key words: acetylcholinesterase, *Aphelenchus avenae,* carbamate, nematicide, organophosphate, organophosphorous, pesticide, physiology.

Preplant soil fumigation is considered the most effective method of reducing nematode problems for most crops grown in Ontario (14). However, the adverse environmental attributes associated with several of these fumigants may curtail their continued large-scale application. Alternatives to fumigant nematicides are generally organophosphorous or carbamate pesticides, most of which were initially developed as insecticides. These compounds affect insects primarily through inhibition of the acetyl-cholinesterase system (12,13). Nematodes have also been demonstrated to possess an acetylcholinesterase (ACHE) system (1,7,17). However, the sensitivity of this system in soil-inhibiting nematodes to ACHE-inhibiting pesticides has not been determined. Such data might provide a rational means of selecting compounds for more intensive testing against nematodes. In addition, ACHE-inhibiting pesticides found to be nematicidal (6,8,11) required considerably higher rates of application for nematode control than those effective against soil insects (14). Whether this difference is due to a relatively insensitive nematode ACHE or to other factors has not been well elucidated.

Our objective was to compare the sensitivity of *Aphelenchus avenae* Bastian ACHE with *Musca domestica* L. ACHE to various organophosphorous and carbamate insecticides. In addition, representative insecticides were tested, in sand medium, against *A. avenae* to correlate biological activity with ACHE inhibition.

Materials and Methods

Aphelenchus avenae were extracted by the Baermann pan method (19) from a Vineland silt loam collected from an oak tree nursery at the Agriculture Canada Research Station, Vineland Station. The nematode was propagated on the fungus *Botrytis cinerea* Pers. growing on potato dextrose agar in Petri dishes (15 × 100 mm). Each week 100 four-day-old cultures of *B. cinerea* were inoculated with ca. 1,000 *A. avenae,* enclosed in a black polyethylene bag, and incubated for 2 weeks at 22–25 C. Nematodes were extracted from cultures by placing six nematode-infested fungal mats in a 15-cm-d funnel with the stem inserted into a 450-ml jar in a mist chamber; the funnels were misted for 15 seconds at 10-minute intervals. Fifteen to twenty funnels were set up at one time. Nematodes were collected at 3 and 6 days, concentrated by centrifugation at 1,800 *g* for 10 minutes, and resuspended in 30 ml of 0.1 M sodium phosphate buffer (pH 7.2). The nematodes were ruptured ultrasonically (frequency range 40–90 kHz, 1,000 W) in flasks surrounded with crushed ice;

Received for publication 24 March 1986.

[1] Agriculture Canada Research Station, Vineland Station, Ontario, Canada L0R 2E0.

The authors thank Carol R. Harris, Agriculture Canada, London Research Centre, for some of the pesticide standards and Diane Beaulieu-Aruvee for technical assistance.

95% nematode rupture was achieved. Suspensions of nematodes were sonified for a total of 12 hours (4 hours per day for 3 days) and stored at 2 C until used. Mechanical methods, such as grinding with a mortar and pestle, in fine sand, or with a tissue homogenizer, were ineffective, as was an ultrasonic probe which produced overheating and destruction of the acetylcholinesterase activity. ACHE levels from sonified samples of *A. avenae* were similar to those from small samples of *A. avenae* (200,000–300,000) which had been freshly ground between two ground glass plates (20 × 20 cm). Levels of fly head ACHE sonified similarly for a total of 12 hours were also similar to those in samples freshly prepared in a tissue grinder.

The protein concentration of the sonified solution of *A. avenae* was determined (3) and adjusted just before assays to 0.50 mg/ml.

Preparation of fly head acetylcholinesterases: Adult *M. domestica* (3–7 days old) were used in all experiments. The strain used was an ACHE-sensitive strain obtained from F. W. Plapp, Texas A&M University. Flies were frozen for 2–3 hours at −10 C and decapitated. For assays, 12 heads were ground in 5 ml of 0.1 M sodium phosphate buffer (pH 7.2) in a 30-ml Potter-Elvehjem tissue grinder. The homogenate was filtered through glass wool, and the particulate residue was rinsed with additional buffer. A final dilution equivalent to 0.5 heads/ml containing ca. 0.04 mg/ml protein was used; preliminary tests had shown this provided ACHE activity equalling that of *A. avenae* homogenates.

Acetylcholinesterase assays: In a sample assay, 6 ml *A. avenae* homogenate (0.50 mg/ml protein) in a 25-ml Erlenmeyer flask was incubated in a water bath shaker for 15 minutes at 32 C. All reaction mixtures were prepared as described by Robbins et al. (16). The reaction was started by adding an equal volume of 2.5 mM acetylcholine bromide to the incubating enzyme solution, yielding a final protein concentration of 0.25 mg/ml. A 2-ml sample was removed and the reaction stopped immediately by the addition of 2 ml alkaline hydroxylamine prepared as described by Bigley and Plapp (2). The mixture was then shaken on a vortexer for 10 seconds. After 2 minutes, 1 ml of 4 N HCl was added to the sample, followed by 1 ml of 0.37 M ferric chloride in 0.1 N HCl, with shaking after each addition. The sample was filtered through Whatman No. 1 filter paper and its absorbance read at 540 nm in a Bausch and Lomb Spectronic 20 spectrophotometer. A blank for nonspecific color was prepared by reversing the order of addition of the alkaline hydroxylamine and the 4N HCl. Samples were removed and tested at intervals of 0, 10, 20, and 30 minutes. Comparable fly head preparations were assayed at a final protein concentration of 0.02 mg/ml after dilution with acetylcholine bromide solution.

Absorption of experimental samples was compared to a standard curve to determine residual acetylcholine. The rate of disappearance of acetylcholine over time was determined using linear regression analysis. ACHE activity is expressed as the rate of substrate disappearance divided by the protein concentration which is presented in micromoles min^{-1} mg^{-1} protein.

Inhibition assays: Technical or analytical grade inhibitors (Table 1), dissolved in analytical acetone, were added at various concentrations to incubation flasks. For example, where a 10^{-5} M final concentration of inhibitor was desired, 80 μl of 10^{-3} M inhibitor solution was added to the flask using a 50-μl Hamilton syringe and allowed to dry. Four milliliters enzyme solution was incubated for 15 minutes in this flask, and the reaction then was started by adding an equal volume of substrate solution, i.e., a 1:100 inhibitor dilution. Samples were taken for absorbance readings at 0, 10, 20, and 30 minutes. Inhibition of fly head ACHE was tested simultaneously with nematode ACHE. Percentage of inhibition was based on the mean of three separate assays. A control assay containing acetone but no inhibitor was always performed for comparison and to verify activity levels of the ACHE.

TABLE 1. Percentage of inhibition of *Aphelenchus avenae* and *Musca domestica* acetylcholinesterases.

Inhibitor	*A. avenae**		*M. domestica*	
	10^{-5} M	10^{-7} M	10^{-5} M	10^{-7} M
Carbamate insecticides				
Aldicarb	42.8 ± 12.7†	0	75.6 ± 1.8	2.9 ± 5.0
Aldicarb -sulfoxide	89.2 ± 9.8	29.2 ± 14.8		69.6 ± 14.3
Aldicarb -sulfone	80.5 ± 18.6	5.4 ± 9.3		43.4 ± 20.6
Eserine	100.0	32.8 ± 14.6		93.4 ± 10.8
Carbaryl	54.0 ± 11.4	7.8 ± 10.4		54.5 ± 16.5
Carbofuran	95.7 ± 7.4	64.5 ± 19.8		77.5 ± 3.9
Oxamyl	93.0 ± 12.1	28.8 ± 6.4		64.5 ± 4.0
Organophosphorous insecticides				
Azinphosmethyl	69.5 ± 4.9	4.6 ± 4.6	83.7 ± 15.6	52.4 ± 20.2
Azinphosmethyl -oxon	70.7 ± 23.9	8.0 ± 14.0	100.0	100.0
Disulfoton	5.8 ± 8.0		29.3 ± 17.3	
Disulfoton -sulfoxide	0	0	27.2 ± 16.6	
Disulfoton -sulfone	3.2 ± 5.4		92.7 ± 12.6	20.4 ± 10.4
Phorate	10.3 ± 12.1		87.0 ± 4.5	36.2 ± 10.3
Phorate -sulfoxide	31.1 ± 17.5		94.6 ± 9.4	57.0 ± 3.2
Phorate -sulfone	22.4 ± 9.8		100.0	13.9 ± 9.9
Malathion	89.2 ± 18.8	6.6 ± 11.5	79.9 ± 8.1	54.1 ± 10.3
Malaoxon	78.2 ± 10.6	27.4 ± 1.5		
Fensulfothion	8.3 ± 9.7		94.2 ± 10.0	
Fensulfothion -sulfoxide	6.9 ± 8.7		97.3 ± 4.8	10.1 ± 15.6
Fensulfothion -sulfone	0		94.6 ± 9.4	15.8 ± 13.7
Phenamiphos	10.5 ± 4.6		100.0	32.0 ± 15.1
Phenamiphos -sulfoxide	11.1 ± 10.0		100.0	32.7 ± 14.6
Phenamiphos -sulfone	49.1 ± 10.1		100.0	71.8 ± 25.0
Terbufos	0		77.4 ± 23.0	
Terbufos -sulfoxide	4.1 ± 7.1	0	100.0	50.7 ± 2.5
Terbufos -sulfone	36.1 ± 9.4	0	100.0	58.5 ± 8.6
Chlorpyrifos	5.8 ± 9.2			

* Hydrolytic rate adjusted to 22.6 ± 4.6 μmol acetylcholine bromide min^{-1} for *M. domestica* ACHE and to 18.8 ± 7.9 μmol min^{-1} for *A. avenae*.

† Percentage of inhibition ± SE.

In vivo tests: To compare the toxicity of selected pesticides against *A. avenae* in vivo, known numbers of nematodes were added to sterile sand containing various concentrations of the pesticides. Pesticides included those most inhibitory in vitro to *A. avenae* ACHE, poor *A. avenae* ACHE inhibitors, and some effective in the field against plant-parasitic nematodes (e.g., phenamiphos). Rates selected in initial assays, 5–15 ppm, approximated the concentrations at which soil pesticides are applied in band treatments. Weight calculations were based on an acre furrow slice of 6 inches weighing 2.5 million pounds (4). Each pesticide was added in two 2-ml aliquots of analytical grade acetone to 50 g of sand in a 454-ml jar. After each addition of pesticide solution, the jar was sealed and shaken for 2 minutes on a Fisher-Kendall mixer. Sand mixes were air evaporated in a fume hood for 2–2.5 hours at 22 C. Controls received 4 ml of acetone only. Jars were then covered with standard Mason lids and held at

−5 C until used. Ten milliliters of a suspension containing 10,000–20,000 *A. avenae* was deposited with a syringe onto the sand surface in each jar. Additional water (1–3 ml) was added to moisten the sand completely. Jars were arranged in a randomized block design, and treatments were replicated four times. After 3 days exposure at ca. 22 C, the nematodes were extracted by Baermann pans and counted. Data were subjected to analysis of variance, and differences between means were analyzed by Duncan's multiple-range test ($P = 0.05$).

Results

Approximately 150,000 *A. avenae* developed in a single *B. cinerea* plate culture, and 8–11 million nematodes were extracted and sonified each week. Ninety-five percent of the nematodes in suspension were ruptured in the ultrasonic cleaner. *A. avenae–B. cinerea* cultures more than 2 weeks old could not be used because many of the *A. avenae* were in an anhydrobiotic state and died when moistened suddenly in the mist chamber.

The rate of hydrolysis of acetylcholine bromide was 226.0 ± 46.1 μmoles min^{-1} mg^{-1} protein for *M. domestica* head ACHE and 40.9 ± 17.1 μmol min^{-1} mg^{-1} protein for *A. avenae* ACHE. This difference was not unexpected, since only *M. domestica* heads, which contain large amounts of nervous tissue, were used. Homogenates of both were adjusted to approximately equal rates of ACHE activity for inhibition assays (Table 1). These rates were linear over the 30-minute assay period at the substrate concentration used. Activity of nematode ACHE preparation declined after storage at 2 C for more than 7 days. All assays were conducted with preparations less than 5 days old.

Acetylcholinesterases from house fly heads were generally more sensitive than nematode preparations to inhibition by the compounds tested. Fly ACHE varied from 2–3 times more sensitive with aldicarb sulfoxide or eserine to at least 100 times more sensitive with the oxygen analog of azinphosmethyl (oxon). Only carbofuran was equally inhibitory to ACHE from both organisms. Carbamates, including the carbamoyl oximes, generally were more effective than organophosphorous compounds in inhibiting nematode ACHE. Phenamiphos at 10^{-5} M inhibited only about 10% of the nematode ACHE activity, whereas fly ACHE activity was completely inhibited. As expected, the oxygen analogs (oxons) of azinphosmethyl and malathion inhibited fly ACHE much more effectively than the phosphorodithioate precursors. Similar data were obtained with oxidative metabolites of aldicarb, disulfoton, phorate, fensulfothion, phenamiphos, and terbufos. Both aldicarb sulfoxide and sulfone were ca. twofold more effective than aldicarb in inhibiting nematode ACHE. The sulfoxides and sulfones derived from disulfoton, phorate, fensulfothion, and terbufos were poor inhibitors of nematode ACHE at concentrations as high as 10^{-5} M. Phenamiphos sulfone inhibited nematode ACHE ca. 50% at 10^{-5} M. Terbufos at 10^{-5} M effectively inhibited *M. domestica* ACHE but not *A. avenae* ACHE.

Inhibitor concentrations of 5–15 ppm reduced nematode population densities in the in vivo sand tests, but the high mortalities (>90%) normally associated with pest control were not achieved (Table 2). As pesticide concentrations were increased, there were corresponding increases in the numbers of nematodes killed, but even at 500 ppm, nematode mortality was only 50–71% (Table 2, test 3).

Discussion

Nematode ACHE were less sensitive than fly ACHE to inhibition by pesticides. This result is not surprising, since most pesticides tested were developed and marketed as insecticides. ACHE from the nematode *Caenorhabditis elegans* were less sensitive than ACHE from the electric eel to inhibition by aldicarb or eserine (7). Knowles and Casida (9) tested some 56 compounds, including many organophosphorous insecticides, against ACHE from *Ascaris lumbri-*

TABLE 2. In vivo pesticide assays with *Aphelenchus avenae* in treated sand.

Treatment	Inhibitor concentrations (ppm)	Nematodes recovered (no.)	Reduction from control (%)
Test 1. Inoculum 20,900/jar; recovery from control 71%			
Aldicarb	5	12,450 abc	16
Aldicarb	15	9,250 a	38
Carbofuran	15	10,500 ab	29
Disulfoton	15	12,550 abc	16
Malathion	15	13,000 bc	13
Phenamiphos	10	10,050 ab	32
Phenamiphos	15	10,750 ab	28
Control		14,850 c	
Test 2. Inoculum 10,930/jar; recovery from control 76%			
Carbofuran	15	6,580 ab	21
Carbofuran	30	4,930 b	41
Carbofuran	50	5,230 b	37
Carbofuran	150	3,000 b	64
Control		8,300 a	
Test 3. Inoculum 14,000/jar; recovery from control 54%			
Aldicarb	500	2,580 ab	67
Carbofuran	500	2,710 ab	65
Disulfoton	500	3,910 c	50
Malathion	500	3,440 bc	56
Phenamiphos	500	2,290 a	71
Control		7,760 d	

Means followed by the same letter are not significantly different ($P = 0.05$) according to Duncan's multiple-range test. Each test was analyzed separately.

coides and found inhibition, for the most toxic materials, occurred at concentrations of ca. 10^{-4} M. They also reported that eserine concentrations inhibitory to ACHE from *Ascaris* were higher than the concentration necessary to inhibit ACHE from mammals or insects. Only six of the compounds we tested were also tested by Knowles and Casida. All six—phorate, disulfoton, malathion, malaoxon, azinophosmethyl, and eserine—appeared more toxic to ACHE from *A. avenae* than from *Ascaris*.

In ACHE inhibition tests, the carbamates or carbamoyl oximes seemed more effective than the organophosphorous pesticides; the former also tended to be more water soluble than the latter (20). However, phenamiphos at 500 ppm, despite appearing to be a relatively poor inhibitor of ACHE in vitro, was as effective as aldicarb or carbofuran in vivo. Spurr (18) reported that aldicarb was nematicidal at 10^{-3} M and inhibited ACHE of the bacteria feeder *Panagrellas redivivus* at ca. 10^{-6} M in water contact tests.

Although nematode ACHE are less sensitive than fly head ACHE, pesticides at concentrations of 500 ppm were expected to cause higher mortalities than was observed with some materials (e.g., aldicarb). Nematode control usually requires pesticide application rates higher than those effective for control of soil insects (6,14). Generally, concentrations of 15 ppm or less can cause 100% mortality of soil insects (15). This suggests that other factors (e.g., failure to penetrate the nematode integument, reduced exposure, etc.) may also be important. Nematodes are much less mobile than insects, and because movement results in increased contact with pesticides, the exposure of nematodes in soil should be considerably less than that of insects. As with insects, the cuticle can function as a formidable barrier to many compounds. Only bacteria-feeding nematodes ingest particulate materials, whereas the fungus feeder *A. avenae* feeds only through its stylet on hyphae.

Our study and others (7) have shown that ACHE in nematodes are different from those in other animals and are less sensitive to inhibition by pesticides. In *Ascaris lumbricoides,* ACHE are located in the nerve ring and not in ganglion cells (10). Although such limited distribution of ACHE may not be the case in all species of nematodes, the effectiveness of ACHE-inhibiting pesticides in vivo may be reduced by inaccessibility of the enzymes. Further studies should be undertaken to determine whether *A. avenae* ACHE are typical of free-living and (or) plant-parasitic nematodes. The techniques used here probably are not sensitive enough for such comparisons, given the difficulties of producing the numbers of nematodes necessary to conduct such studies. It should not be assumed, however, that ACHE inhibition is necessarily the primary mode of action of these

compounds. Nematode control may be achieved through effects on reproduction, repellency, interference with movement, and (or) ovicidal action (5). Certainly the results of our study suggest that mechanisms other than ACHE inhibition may be involved where nematode control occurs in the field.

Literature Cited

1. Benton, A. W., and R. F. Myers. 1967. Esterases, phosphatases, and protein patterns of *Ditylenchus triformis* and *Panagrellus redivivus.* Nematologica 12: 495–500.

2. Bigley, W. S., and F. W. Plapp, Jr. 1960. Cholinesterase and ali-esterase activity in organophosphorous susceptible and resistant house flies. Annals of the Entomological Society of America 53:360–364.

3. Bradford, M. 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Analytical Biochemistry 72:248–254.

4. Brady, N. C. 1974. The nature and properties of soils, 8th ed. New York: MacMillan.

5. Hague, N. G. M., M. Damadzadeh, S. K. Garabedian, and K. H. Radwan. 1983. Methods of assessing the efficacy of non-volatile nematicides. Pesticide Science 14:587–595.

6. Herne, D. H. C., C. M. Simpson, and J. L. Townshend. Aldicarb: A systemic soil pesticide useful for establishing apple plantings. Proceedings of the Entomological Society of Ontario 103:27–31.

7. Johnson, C. D., and R. L. Russell. 1983. Multiple molecular forms of acetylcholinesterase in the nematode *Caenorhabditis elegans.* Journal of Neurochemistry 41:30–46.

8. Johnson, P. W., J. A. Dirks, D. J. Pree, and C. F. Marks. 1982. Systemic nematicides for control of *Pratylenchus penetrans* during apple orchard establishment in Ontario. Plant Disease 66:299–301.

9. Knowles, C. O., and J. E. Casida. 1966. Mode of action of organophosphate anthelmintics. Cholinesterase inhibition in *Ascaris lumbricoides.* Journal of Agricultural and Food Chemistry 14:566–573.

10. Lee, D. L. 1962. A histochemical study of esterase enzymes in the nervous system of *Ascaris lumbricoides.* Journal of Parasitology 48:26 (Abstr.).

11. Marks, C. F., and J. M. Elliot. 1975. Control of the root-lesion nematode (*Pratylenchus penetrans*) in flue-cured tobacco in Ontario. Canadian Journal of Plant Science 55:309–314.

12. Matsumura, F. 1974. Toxicology of insecticides. New York: Plenum Press.

13. O'Brien, R. D. 1960. Toxic phosphorous esters. New York: Academic Press.

14. Ontario Ministry of Agriculture and Food. 1985. Vegetable production recommendations. Publication No. 363, p. 28.

15. Read, D. C. 1983. Bioassay of toxicity of insecticides in soil using eggs and larvae of the cabbage maggot, *Hylemya brassicae.* Canadian Entomologist 115: 573–576.

16. Robbins, W. E., T. L. Hopkins, and A. R. Roth. 1958. Application of the colorimetric whole-blood method to the measurement of bovine red-blood-cell cholinesterase activity. Journal of Economic Entomology 51:326–329.

17. Rohde, R. A. 1960. Acetylcholinesterase in plant parasitic nematodes and anticholinesterase from asparagus. Proceedings of the Helminthological Society of Washington 27:121–123.

18. Spurr, H. W., Jr. 1966. Nematode cholinesterases and their inhibition by nematocidal carbamates. Phytopathology 56:902–903.

19. Townshend, J. L. 1963. A modification and evaluation of the apparatus for the Oostenbrink direct cottonwool filter extraction method. Nematologica 9:106–110.

20. Worthing, C. R., and S. B. Walker, editors. 1983. The pesticide manual, 7th ed. Croydon, England: The British Crop Protection Council.