Journal of Nematology 22(2):182–186. 1990.

Inhibition of Acetylcholinesterases from *Aphelenchus avenae* by Carbofuran and Fenamiphos

D. J. Pree, J. L. Townshend, and K. J. Cole[1]

Abstract: Exposure to carbofuran and fenamiphos for 72 hours reduced the numbers of active *Aphelenchus avenae* in aqueous suspension by >75%. When nematicides were removed, many *A. avenae* exposed to carbofuran resumed normal movement but *A. avenae* treated with fenamiphos did not recover. Acetylcholinesterase (AChE) activity was suppressed by >95% in nematodes treated with carbofuran or fenamiphos. However, 48 hours after treated nematodes had been placed in water, AChE activity in carbofuran treated populations was 98% of the levels in control nematodes. Nematodes that had been treated with fenamiphos showed only slight AChE recovery. The antidotes, atropine sulfate and 2-PAM, were largely ineffective in counteracting the toxic effects of the nematicides.

Key words: acetylcholinesterase, antidote, *Aphelenchus avenae*, carbofuran, fenamiphos, nematicide.

The mode of action of carbamate and organophosphorus nematicides is not clearly understood, but it is generally accepted that acetylcholinesterase (AChE) is inhibited causing both physiological and behavioral effects. Previous studies have shown that these compounds alter reproduction, behavior, and development in several plant-parasitic nematode species (7) and inhibit the movement and dispersion of nematodes in treated soil or sand media (5,9,13–15). Inhibition of acetylcholinesterases (AChE) and behavioral modifications have been reported for nematodes treated with carbamate and organophosphorus nematicides (3,21). Many nonlethal effects of nematicides may be associated with less than complete or reversible interactions between the toxicant and the (physiological) target site.

The objectives of this study were 1) to elucidate the effects of carbofuran (a methyl carbamate) and fenamiphos (an organophosphorous nematicide) on the inhibition of AChE and movement of *A. avenae*, 2) to investigate the reversibility of AChE inhibition by the two nematicides and the resumption of nematode movement, and 3) to determine the effectiveness of selected antidotes in counteracting the nematicidal effects.

Received for publication 28 April 1989.

[1] Insect Toxicologist, Nematode Ecologist (retired), and Research Assistant, Research Station, Agriculture Canada, Vineland Station, Ontario L0R 2E0.

Materials and Methods

Nematode culture: Aphelenchus avenae was reared on the fungus *Botrytis cinerea* Pers. growing on potato dextrose agar in petri dishes (15 × 100 mm) (21). Four-day-old fungus cultures were inoculated with ca. 1,000 nematodes and held in black polyethylene bags at 22–25 C for 1–2 weeks. Nematodes were extracted from fungus cultures in a mistifier and collected at 24-hour intervals for 48–72 hours. *Aphelenchus avenae* cultures with the lids removed were placed in a sealed plastic bag (20 × 20 cm) with a dish of warm (40–50 C) water for 24 hours. This highly humid environment allowed anhydrobiotic nematodes to become active and resulted in lowered mortality in tests. Extracted nematodes were captured on a 38-μm-pore sieve and resuspended in aerated water. Preliminary observation indicated 85–95% of the nematodes were active.

Bioassays: For nematicide toxicity and AChE inhibition tests, 25 ml distilled water containing 10,000–40,000 nematodes/ml (Table 1) was placed in each of six 500-ml Erlenmeyer flasks. Carbofuran or fenamiphos prepared from analytical standards (Chem Services, West Chester, PA) and dissolved in analytical grade acetone was added in 25 μl acetone to produce final concentrations of 0.5 mM carbofuran in each of two flasks and 0.25 mM fenamiphos in each of two flasks. Preliminary assays indicated that these concentrations suppressed *A. avenae* motility. The two re-

maining flasks had 25 μl acetone added and were used as controls. Flasks were loosely plugged with cotton and placed on a Burrell wrist action shaker at low speed for 72 hours at 22–24 C, after which active nematodes in four 1-ml subsamples from each flask were counted. Nematodes in one flask of each treatment were used for AChE inhibition assays. *Aphelenchus avenae* in the other flask from each treatment were concentrated by centrifugation, the supernatant was decanted, and the nematodes were resuspended in 25 ml distilled water; this process was repeated three times. After the third decanting, the *A. avenae* were suspended in 25 ml aerated distilled water in a clean 500-ml Erlenmeyer flask and shaken for 48 hours, after which flasks were resampled and active nematodes counted again. Nematodes in this flask were then used for AChE recovery assays. Nematodes were considered active when visibly flexing. Tests were repeated three times. Data for each test were subjected to an analysis of variance and significant differences were identified using Duncan's multiple-range test ($P = 0.05$).

Acetylcholinesterase assays: For AChE assays, *A. avenae* were concentrated by centrifugation in conical tubes and crushed between two ground glass plates in ca. 2 ml cold, 0.05 m sodium phosphate buffer, pH 7.0 (19). Crushed nematodes were flushed with buffer into a 25-ml Erlenmeyer flask to a final volume of ca. 5 ml. Observations with a binocular microscope indicated that 80–100% of the nematodes had been crushed. Protein concentrations were determined by the method of Bradford (2), and the suspension was diluted to approximately 0.14 mg of protein/ml, a concentration which in earlier studies (20) had produced consistent hydrolytic rates for acetylcholine. Crushed nematode preparations were held on ice and used within 4 hours. Acetylcholinesterase assays were with [H^3]-labelled acetylcholine, Specific Activity 2.6 Ci/mmol (Amersham Canada Ltd., Oakville, Ontario). Samples were prepared and stored as described by Pree et al. (21).

TABLE 1. Inhibition and recovery of motility of *Aphelenchus avenae* from treatment with carbofuran or fenamiphos.

Treatment	Active nematodes/ml: 72 hours (Exposure)	Active nematodes/ml: 48 hours (Recovery)
Test 1. Inoculum 17,650 ± 900/ml		
Control	20,000 a	9,700 a
Carbofuran 0.5 mM	5,100 b	3,800 b
Fenamiphos 0.25 mM	3,000 c	1,400 c
Test 2. Inoculum 41,000 ± 6,400/ml		
Control	33,000 a	16,800 a
Carbofuran 0.5 mM	6,600 b	12,100 b
Fenamiphos 0.25 mM	4,500 b	2,640 c
Test 3. Inoculum 10,875 ± 3,700/ml		
Control	11,100 a	18,000 a
Carbofuran 0.5 mM	5,000 b	13,400 b
Fenamiphos 0.25 mM	2,750 c	2,100 c

Numbers followed by the same letter within each test are not significantly different ($P \leq 0.05$) according to Duncan's multiple-range test. Data from each test were analyzed separately.

Nematodes were exposed to pesticide suspension for 72 hours, then allowed to recover 48 hours in distilled water.

The standard assay consisted of 0.5 ml of 0.05 M potassium phosphate buffer (pH 7.0), 0.2 ml of [H^3]-acetylcholine substrate (10 mM), and 0.3 ml of ground nematode preparation to give a final volume of 1.0 ml. The buffer and nematode preparations were premixed and incubated for 15 minutes at 25 ± 1 C on a water bath shaker. The reaction was started by the addition of the acetylcholine substrate. Samples (0.1 ml taken in duplicate) were removed after 0, 10, 20, and 30 minutes and placed in plastic minivials (Beckman Instruments, Mississauga, Ontario). The reaction was terminated in the minivials by adding 200 μl of a solution of 1 M chloroacetic acid, 0.5 N NaOH, and 2 M NaCl in water, and 4.0 ml of scintillation cocktail (0.5% PPO and 0.03% POPOP in toluene containing 10% isoamyl alcohol). Samples were counted on a Beckman LS-6800 scintillation counter programmed to measure dpm at ±2% of the mean.

Effects of antidotes: The antidotal effects of atropine sulfate (Sigma) and pyridine-2-aldoxime methiodide (2-PAM) (Sigma) were tested in bioassays. Twenty milliliters

TABLE 2. Hydrolysis of acetylcholine in *Aphelenchus avenae* treated with nematicides and recovery in distilled water.

Treatment	Hydrolytic rate (pmol/min/mg protein)†		Recovery‡ (%)
	Treated	Recovered	
Control	2,816.9 ± 487.7	1,443.6 ± 105.2	
Carbofuran 0.5 mM	58.5 ± 56.4 (2.1%)	1,654.0 ± 228.5 (100%)	97.9
Fenamiphos 0.25 mM	71.3 ± 31.9 (2.5%)	121.2 ± 69.0 (8.4%)	5.9

Nematodes were treated with nematicides for 72 hours and allowed to recover in distilled water for 48 hours. Data are means of six separate assays.

† Numbers in parentheses are % activity relative to activity in controls. Data are means of six separate assays.

‡ Calculated as % activity after 48 hours recovery − % activity after 72 hours treatment.

of distilled water containing 2,250 nematodes/ml were placed in each of 12 500-ml Erlenmeyer flasks. Atropine sulfate and (or) 2-PAM (2.5 ml of 2.0mM) were added to each flask. Volumes were adjusted to 25 ml with distilled water. Carbofuran and fenamiphos were added in 25 µl acetone to give final concentrations of 0.5 mM and 0.25 mM, respectively. Flasks were placed on a Burrell wrist action shaker set at low speed at room temperature (21–23 C) for 48 hours. Active nematodes in four 1-ml samples were counted. Data were subjected to an analysis of variance with differences between means separated by Duncan's multiple-range test ($P = 0.05$).

RESULTS

Bioassays: Exposure of *A. avenae* to carbofuran or fenamiphos for 72 hours reduced the numbers of active nematodes by 74.5% and 85%, respectively (Table 1, test 1). In test 1, numbers of active nematodes continued to decline in all treatments, including controls, during the 48-hour recovery period. Less decline occurred during the recovery period with the carbofuran-treated nematodes than with fenamiphos-treated nematodes. Nematodes used in test 1 were 7 days old, whereas in tests 2 and 3 nematodes were 4 days old. In tests 2 and 3, exposure to carbofuran reduced numbers of active nematodes by >50%, but when placed in water and allowed to recover, the numbers of active nematodes increased by approximately 100%. Numbers of active nematodes were reduced by 75–86% by fenamiphos treatment and they continued to decline during the recovery period.

Acetylcholinesterase assays: Nematodes treated with carbofuran or fenamiphos had AChE activity suppressed by 97–98% (Table 2). AChE activity in carbofuran-treated nematodes, after 48 hours recovery in water was similar to that in untreated controls, but activity was only slight (5.9%) in fenamiphos-treated nematodes. AChE activity in control nematodes declined by approximately 50% during the 48-hour recovery period, generally reflecting the decline in numbers of active nematodes during this period in controls (Table 1).

Effects of antidotes: All antidote treatments significantly reduced numbers of active nematodes (Table 3). Both atropine and 2-PAM were toxic when applied alone. Atropine alone reduced the toxicity of carbofuran, but 2-PAM alone or combined with atropine did not affect the toxicity of carbofuran. None of the treatments reduced the toxicity of fenamiphos.

DISCUSSION

The results of this study show a relationship between *A. avenae* poisoning and inhibition of AChE, suggesting that this is a primary mechanism of the nematicidal activity of these compounds. Previous studies (20,21) also had shown that the toxic effects of organophosphorus or carbamate pesticides to *A. avenae* were due, at least in

part, to the inhibition of AChE activity. The large number of nematodes in the samples (necessary for AChE assays) made accurate estimates of numbers difficult to obtain and resulted in a large variance. However, because expected response (i.e., numbers of nematodes affected) in treated samples was >50%, the sampling system used was adequate to indicate these large responses.

Nematicidal effects and AChE inhibition were largely reversible with carbofuran but not with fenamiphos. In a previous study (3), nematodes recovered from paralysis by aldicarb and the recovery also was correlated with changes in AchE inhibition. The irreversible effects shown here with fenamiphos also occurred in their studies with the phosphorodithioate ethoprophos (3). However, Knowles and Casida (10) showed that AChE inhibition by some organophosphorus insecticides (dichlorvos and haloxon), which had low nematicidal activity, was reversible and did not occur with more toxic compounds such as coroxon; they did not test the reversibility of AChE inhibition by carbamates.

In our studies, numbers of active nematodes were reduced by up to 86% by nematicide treatment but AChE inhibition was considerably higher, averaging 97.5%. This suggests that some of the AChE activity in nematodes rated as active also was inhibited. Conversely, AChE activity in carbofuran-treated nematodes was restored when nematodes were placed in water to give levels of activity similar to those in untreated controls. Recovery of treated nematodes in these test samples was less than 75% of that in untreated controls. The nematode preparations used for AChE assays included both inactive (dead) and active *A. avenae.* Obviously, some of the AChE measured in recovery assays was from dead or inactive nematodes. Previous assays showed that AChE is a relatively stable enzyme and activity can persist for several days both in vitro and in vivo (20).

Paralysis and reversible inhibition of AChE may explain some of the behavior-modifying effects of carbamates or carbamoyl oximes on nematodes. Reversible inhibition of AChE may be the cause of the nematostatic activity of oxamyl to *Meloidogyne incognita* Chitwood (16). Nelmes (17) also showed the reversible effects of low concentrations of aldicarb (and metabolites) to *Heterodera rostochiensis.* Similar reversible inhibition of AChE also could explain the immobilization and recovery of *Pratylenchus vulnus* observed by Marban-Mendoza and Viglierchio (13,14), who also observed some recovery of fenamiphos-treated nematodes, which was slower and less pronounced as exposure time increased. The reversible inhibition of AChE by carbamates, especially methyl carbamates, and the relatively irreversible effects of organophosphorus pesticides have been well described in both insects and vertebrates (12,19).

Neither of the two synergists tested, atropine and 2-PAM, fully restored AChE activity in *A. avenae.* Atropine, which is an acetylcholine agonist, partially reduced the

TABLE 3. Effects of atropine and 2-PAM on poisoning of *A. avenae* by carbofuran and fenamiphos.

Treatment	Active nematodes/ml	Mortality (%)
Control	1,625	27.8 a
Atropine sulfate 2 mM	1,175	47.8 b
2-PAM 2 mM	665	70.4 c
Atropine sulfate 2 mM + 2-PAM 2 mM	627	72.1 c
Atropine sulfate 2 mM + carbofuran 0.5 mM	380	83.1 d
2-PAM 2 mM + carbofuran 0.5 mM	220	90.2 de
Atropine sulfate 2 mM + 2-PAM 2 mM plus carbofuran 0.5 mM	210	90.7 de
Carbofuran 0.5 mM	115	94.8 e
Atropine sulfate 2 mM + fenamiphos 0.25 mM	110	95.1 e
2-PAM 2 mM + fenamiphos 0.25 mM	100	95.6 e
Atropine sulfate 2 mM + 2-PAM 2 mM plus fenamiphos 0.25 mM	100	95.6 e
Fenamiphos 0.25 mM	85	96.2 e

Means of four samples. Numbers followed by the same letter are not significantly different according to Duncan's multiple-range test ($P = 0.05$).

toxic effects of carbofuran but not fenamiphos. Atropine, which competes with acetylcholine at muscarinic receptor sites, is potentially therapeutic for both carbamate and organophosphorus pesticide poisoning in vertebrates (18). Muscarinic acetylcholine receptors occur in the nematode *Caenorhabditis elegans* (4) but have not been studied in *A. avenae*. The antidote 2-PAM, which acts by dephosphorylating AChE (18) and is useful only against organophosphorus poisoning, had no effect on the toxicity of either carbofuran or fenamiphos to AChE of *A. avenae*. Neither atropine nor 2-PAM are effective for treatment of AChE poisoning in insects (18). Eldefrawi and O'Brien (6) suggested that both of these compounds penetrate poorly into the ganglia of the central nervous system, the probable site of AChE poisoning in insects. A similar difficulty may occur in nematodes. The central nervous system of nematodes consists of a series of ganglia (1,8); physically it is somewhat similar to that of insects. In nematodes, however, AChE distribution may be different from that in insects; Lee and Atkinson (11) report the enzyme(s) is (are) associated with nerve-muscle junctions, the nerve ring, and other organs.

Our data indicate that inhibition of AChE in nematodes is an important aspect in the control of nematodes by organophosphorus and carbamate pesticides. The antidotes atropine and 2-PAM did not block the toxic action of carbofuran or fenamiphos.

Literature Cited

1. Bird, A. F. 1971. The structure of nematodes. New York: Academic Press.

2. 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.

3. Cuany, A., J-B. Bergé, J-M. Bride, and S. Hamadène-Sellami. 1984. Inhibition et réactivation des acétylcholinestérase amphidiales de *Meloidogyne javanica* par l'éthoprophos et l'aldicarbe: Tentative de correlation avec une réaction compartmentale. Revue de Nematologie 7:173–176.

4. Culotti, J. G., and W. L. Klein. 1983. Occurrence of muscarinic acetylcholine receptors in wild type and cholinergic mutants of *Caenorhabditis elegans*. Journal of Neuroscience 3:359–368.

5. DiSanzo, C. P. 1973. Nematode response to carbofuran. Journal of Nematology 5:22–27.

6. Eldefrawi, M. E., and R. D. O'Brien. 1967. Permeability of the abdominal nerve cord of the American cockroach, *Periplaneta americana* to quaternary ammonium salts. Journal of Experimental Biology 46: 1–12.

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

8. Hirschmann, H. 1971. Comparative morphology and anatomy. Pp. 11–63 *in* B. M. Zuckerman, M. F. Mai, and R. A. Rohde, eds. Plant parasitic nematodes, vol. 1. Morphology, anatomy, taxonomy and ecology. New York: Academic Press.

9. Hough, A., and I. J. Thomason. 1975. Effects of aldicarb on the behavior of *Heterodora schachtii* and *Meloidogyne javanica*. Journal of Nematology 7:221–229.

10. 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.

11. Lee, D. L., and H. J. Atkinson. 1976. Physiology of nematodes, 2nd ed. New York: MacMillan.

12. Main, A. R. 1980. Toxicant–receptor interactions: Fundamental principles. Pp. 180–192 *in* E. Hodgson and F. E. Guthrie, eds. Introduction to biochemical toxicology. New York: Elsevier.

13. Marban-Mendoza, N., and D. R. Viglierchio. 1980. Behavioral effects of carbofuran and fenamiphos on *Pratylenchus vulnus* I. Mobility and dispersion. Journal of Nematology 12:102–114.

14. Marban-Mendoza, N., and D. R. Viglierchio. 1980. Behavioral effects of carbofuran and fenamiphos on *Pratylenchus vulnus* II. Attraction to bean roots. Journal of Nematology 12:114–118.

15. Marban-Mendoza, N., and D. R. Viglierchio. 1980. Behavioral effects of carbofuran and fenamiphos on *Pratylenchus vulnus* III. Penetration and development. Journal of Nematology 12:119–129.

16. McGarvey, B. D., J. W. Potter, and M. Chiba. 1984. Nematostatic activity of oxamyl and N,N-dimethyl-l-cyanoformamide (DMCF) on *Meloidogyne incognita* juveniles. Journal of Nematology 16:328–332.

17. Nelmes, A. J. 1970. Behavioral response of *Heterodera rostochiensis* larvae to aldicarb and its sulfoxide and sulfone. Journal of Nematology 2:223–227.

18. O'Brien, R. D. 1967. Insecticides: Action and metabolism. New York: Academic Press.

19. O'Brien, R. D. 1976. Acetylcholinesterase and its inhibition. Pp. 271–296 *in* C. F. Wilkinson, ed. Insecticide biochemistry and physiology. New York: Plenum Press.

20. Pree, D. J., J. L. Townshend, and D. E. Archibald. 1987. Sensitivity of acetylcholinesterases from *Aphelenchus avenae* to organophosphorus and carbamate pesticides. Journal of Nematology 19:188–193.

21. Pree, D. J., J. L. Townshend, and D. E. Archibald. 1989. Carbamate and organophosphorus nematicides: Acetylcholinesterase inhibition and effects on dispersal. Journal of Nematology, 21:483–489.