Journal of Nematology 23(1):20–27. 1991.

Effects of Aldicarb and Fenamiphos on Acetylcholinesterase and Motility of *Caenorhabditis elegans*[1]

C. H. Opperman and S. Chang[2]

Abstract: The ability of *Caenorhabditis elegans* to recover from exposure to high doses of aldicarb and fenamiphos was examined at the organismal and biochemical levels by determination of movement and acetylcholinesterase activity. Nematodes recovered rapidly from a 24-hour exposure to both compounds at concentrations that caused complete paralysis. Acetylcholinesterase regained nearly full activity after a 24-hour exposure to aldicarb but only 10% activity after exposure to fenamiphos. The nematodes were able to move normally, however, on the limited activity that was regained after fenamiphos treatment. Mutant *C. elegans* strains deficient in various molecular forms of acetylcholinesterase were utilized to demonstrate that the mechanism of recovery did not involve new synthesis of enzyme. This result was confirmed by experiments on acetylcholinesterase reactivation from live versus dead nematodes.

Key words: acetylcholinesterase, aldicarb, *Caenorhabditis elegans*, carbamate, carbofuran, fenamiphos, nematicide, organophosphate, oxamyl.

The action of carbamate and organophosphate nematicides is to impair movement or paralyze plant-parasitic nematodes in the soil solution. This action is accomplished by the inhibition of acetylcholinesterase (E.C. 3.1.1.7) (AChE), the enzyme that hydrolyzes the excitatory neurotransmitter acetylcholine at the neuromuscular synapse (15). The various behavioral aberrations, such as twitching, convulsions, and stylet thrusting, reported from nematodes exposed to AChE inhibitors are, for the most part, due to the neuromuscular effects of the compounds. Nonfumigant nematicides are not directly toxic to nematodes (15,24).

Resistance to carbamate and organophosphate pesticides often develops in arthropod pests. This resistance results from either increased metabolism of the compounds (13) or the appearance of AChE forms with reduced sensitivity to the pesticides (19). Resistance to several classes of nematicides has been reported from animal-parasitic nematodes (23), and some studies have indicated that plant-parasitic nematodes under constant exposure to AChE inhibitors can become insensitive or physiologically dependent upon the nematicides (22,25). Some nematodes are capable of metabolizing certain carbamate and phosphate nematicides (1,16), but root-knot and cyst nematodes cannot convert phosphorothioate and dithioate compounds to their toxic oxidized analogues (17). There are few reports of field resistance to nonfumigant nematicides in plant-parasitic nematodes (11,22,26).

The purpose of this investigation was to examine the effects of carbamate and organophosphate nematicides on nematodes at the organismal and cellular levels. We used *Caenorhabditis elegans* as a model to investigate nematode recovery from nematicide exposure relative to the status of the nematode AChE and to examine the role of class C AChE in the recovery process.

Materials and Methods

Nematode culture: Caenorhabditis elegans was cultured according to Brenner (2). *Escherichia coli* 'OP 50', a uracil-requiring strain, was transferred to nematode growth medium (NGM) plates and allowed to grow for 24–48 hours. Mixed-stage nematode suspensions in M9 buffer (2) were added to the plates, and these were incubated at

Received for publication 4 December 1989.

[1] Paper No. 12477 of the North Carolina Agricultural Research Service, Raleigh, NC 27695. Some nematode strains were obtained from the Caenorhabditis Genetics Center, which is supported by Contract N01-AG-9-2113 between the National Institutes of Health and the curators of the University of Missouri.

The use of trade names in this publication does not imply endorsement by the North Carolina Agricultural Research Service of the products named nor criticism of similar ones not mentioned.

[2] Assistant Professor and Research Technician, Department of Plant Pathology, North Carolina State University, Raleigh, NC 27695.

20 C for 5–7 days. Nematodes were harvested by rinsing the plates with borate buffer (100 mM Na borate, 1 mM EDTA, 1mM NaN_3, 1mg/ml BSA, pH 8.0) and were separated from debris and bacteria by flotation on 20–40% sucrose step gradients. After rinsing with borate buffer two times to remove residual sucrose, the collected nematodes were pelleted by centrifugation (718 *g*, 5 minutes) and then resuspended in 1–2 volumes of borate buffer. Strains utilized included N2 (wild type), PR946 (*ace-1 [p1000]*), GG202 (*ace-2 [g72]*), and GG201 (*ace-1 [p1000]*; *ace-2 [g72]*).

Nematode preparation for AChE assay: Fresh nematode suspensions were bulk homogenized by dropwise addition to liquid nitrogen in a chilled mortar, and the liquid nitrogen was allowed to boil away. The remaining frozen beads were crushed to powder with a chilled pestle. The preparation was thawed and then sonicated on ice with a Virtis 50 ultrasonic cell disrupter at 50% power for three 5-second bursts. The resulting homogenate was used for enzyme assay.

AChE activity assay: AChE assays were carried out using a modification (17) of the single vial radiometric technique (5). Aldicarb (a carbamate), carbofuran (carbamate), oxamyl (carbamate), and fenamiphos (organophosphate) were solubilized in acetone. Nematode homogenates (ca. 1.5–2.0 mg/ml protein) were incubated for 20 minutes with 2.0μl nematicide (fc = 1×10^{-4} M) before addition of the substrate. Reaction mixtures were then incubated with 22.9 μM [^{3}H] acetylcholine (TRA-277, Amersham, Arlington Heights, IL) for an additional 20 minutes. The reaction was terminated by adding 20 μl of a stop solution (1 M trichloroacetic acid, 0.5 M NaOH, 2 M NaCl), and 1.3 ml toluene-n-butanol scintillation cocktail was added. The samples were vortexed and counted in a Packard Tri-Carb 3255 liquid scintillation spectrometer. Blank assays contained no nematode preparation, and total substrate available was determined by complete hydrolysis with five units of electric eel AChE (Sigma Chemical Co., St. Louis, MO) per 100 μl. There were three replications per treatment, and the experiments were conducted two times. Class C AChE was assayed by the same method except that [^{3}H]acetylcholine concentration was reduced to 10 nM.

Nematode recovery: Recovery of intact nematodes was assayed in liquid cultures (21). *Escherichia coli* NA 22 was transferred to EC (Difco) broth and allowed to incubate at room temperature for 24 hours. Ten milliliters of this medium were added to 100 ml of M9 buffer containing 5 mg ampicillin (a bacteriostat) and 10,000 units nystatin (a fungicide) and stirred at room temperature for 2 hours (test medium). Compounds to be evaluated were formulated at 1.0 mg/ml in acetone such that the final concentration of solvent in the test medium did not exceed 2.0%. Nematodes were rinsed from NGM plates and concentrated to 1 nematode/μl. One hundred nematodes were added to each well of a 24-well culture plate and 800 μl test medium was added. Finally, 100 μl test compound was added so that the final concentration of nematicide was 100 μg/ml, and the entire contents of the well were mixed thoroughly by pipet. Untreated wells contained either solvent with no nematicide or sterile distilled water as a replacement for nematicide treatment volume. The noncholinergic drug ivermectin and the cholinergic receptor-binding antagonist levamisole were used as drug controls. Nematodes were examined through a dissecting microscope 1 hour and 24 hours after exposure and at 1, 2, 4, 24, and 96 hours after washing. The proportions of active and paralyzed nematodes were recorded. These experiments had six replications per treatment and were conducted twice.

AChE recovery assay: For measurements of AChE recovery, *C. elegans* were washed from 7-day-old NGM plates and concentrated to 100 nematodes/50 μl borate buffer in a 1.5-ml microfuge tube. Nematodes were exposed to 1×10^{-4} M aldicarb, carbofuran, oxamyl, or fenamiphos at room

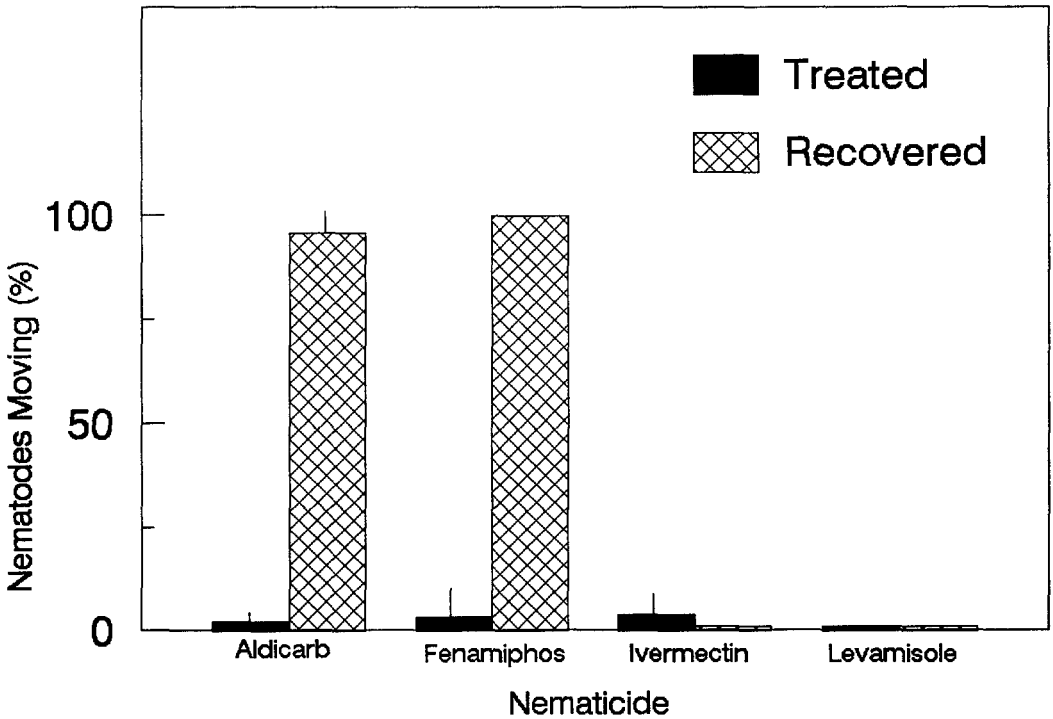

FIG. 1. Recovery of *Caenorhabditis elegans* movement after 24-hour exposure to nematicides (100 μg/ml). Recovery period was 24 hours after washing.

temperature for 24 hours. One half of the nematodes were then washed three times with borate buffer and allowed to recover for 24 hours at room temperature. The other half of the nematodes were exposed to the pesticides, but were not washed or allowed to recover. The nematodes were assayed for AChE activity by freeze-thaw preparation followed by radiometric determination (5,17). Untreated controls were included, and the experiments were repeated two times. Experiments with dead nematodes were conducted in the same manner except that previously frozen and preserved nematodes were used in place of freshly harvested nematodes.

RESULTS

Caenorhabditis elegans recovered after exposure to both aldicarb and fenamiphos for 24 hours (Fig. 1). No recovery was evident in treated nematodes with either levamisole or ivermectin at any of the dosages examined. After washing, some aldicarb-treated and fenamiphos-treated nematodes began to move within an hour, and normal behavior was restored within 24 hours. There were no adverse effects observed from previous exposure to carbamate or organophosphate nematicides with respect to reproduction or development during 5–7 days after recovery over several generations.

Nematode AChE activity was almost completely inhibited in washed and unwashed nematodes by all tested carbamates and phosphates after 24 hours exposure. No inhibition was detected with ivermectin or levamisole (data not shown). AChE activity was restored after removing the carbamate nematicides, but fenamiphos inhibition was nearly as potent after the recovery period as before (Fig. 2). Correlation analysis revealed a significant relationship between enzyme status and nematode movement for aldicarb but not fenamiphos (Table 1). Class C AChE was not completely inhibited by 1×10^{-4} M aldicarb or fenamiphos. The class C I_{50} for

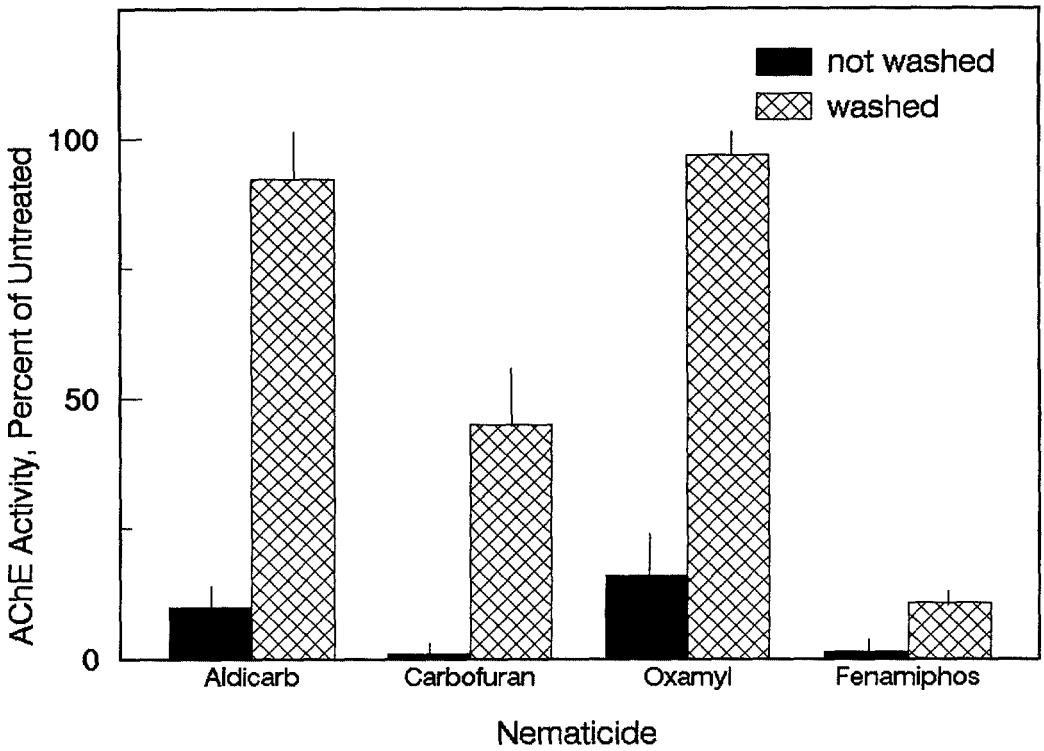

FIG. 2. Recovery of *Caenorhabditis elegans* acetylcholinesterase activity after exposure to carbamate and organophosphate nematicides (1×10^{-4} M). Intact nematodes were washed and allowed to recover for 24 hours prior to acetylcholinesterase assay.

aldicarb was 5×10^{-4} M and for fenamiphos it was 1×10^{-4} M.

Analysis of AChE inhibition in acetylcholinesterase mutant strains by aldicarb showed that there were substantial differences in sensitivity to the inhibitor among the strains (Table 2). Since these differences were reflected in the response of the nematodes behaviorally, a concentration above the sensitivity threshold was chosen for the recovery bioassay (5×10^{-4} M). In the assays comparing the recovery of the wild type strain with three acetylcholinesterase-deficient mutants, no differences were detected among the strains with either aldicarb or fenamiphos (Fig. 3A, B). As was previously observed with the wild type strain, recovery began within several hours of washing, and no differences between the strains were seen. The effects of ivermectin and levamisole on all *ace-** strains were essentially irreversible (data not shown).

Comparison of AChE inhibition and recovery between living and dead nematodes revealed no differences in AChE inhibition between the two groups with aldicarb, carbofuran, fenamiphos, or oxamyl. Howev-

TABLE 1. Relationship of *Caenorhabditis elegans* recovery to acetylcholinesterase inhibition after exposure to aldicarb and fenamiphos.

	Inhibition (%)			
	Aldicarb		Fenamiphos	
Function	Treated†	Recovered‡	Treated†	Recovered‡
Enzyme activity	89	8	99	90
Motility	92	0	88	2
r	0.999***		0.661^{ns}	

Pesticide concentrations for AChE and motility assays were 1×10^{-4} M.

Percentage of inhibition was calculated using the value for the untreated controls as 100% active (0% inhibited).

r = coefficient of linear correlation between enzyme status and nematode motility. *** $P < 0.001$, ns = not significant at $P < 0.05$.

† Treatment period = 24 hours.

‡ Recovery period = 24 hours.

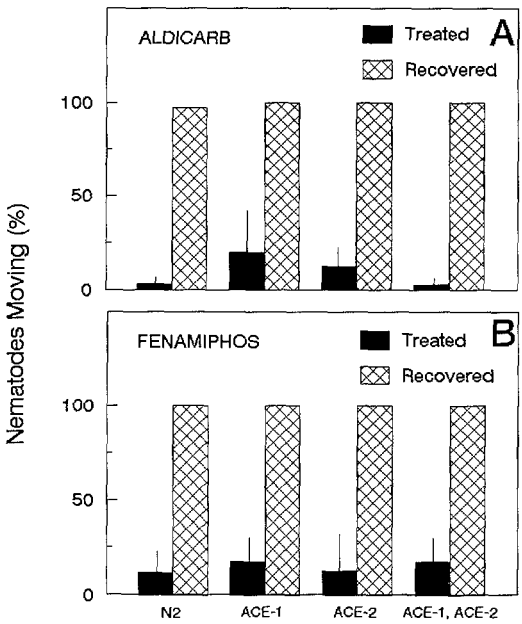

FIG. 3. Recovery of *Caenorhabditis elegans* N2 (wild type) and acetylcholinesterase mutant strains from exposure to nematicides (100 μg/ml). A) Aldicarb. B) Fenamiphos.

er, AChE recovery after washing in dead nematodes was less than recovery observed in live nematodes (Fig. 4). As was observed in previous experiments, AChE activity recovery from exposure to carbamate nematicides was substantial, but only slight recovery was observed for the organophosphate, fenamiphos.

DISCUSSION

These data demonstrate that *C. elegans* can recover from short exposure to both aldicarb and fenamiphos. Our experiments with other carbamate and organophosphate nematicides have revealed the same trend. *Meloidogyne incognita* has also demonstrated recovery from carbamate and organophosphate nematicides (Opperman, unpubl.). It is significant to note that the effects of both ivermectin and levamisole were apparently irreversible in these experiments. These drugs do not interact with AChE (7,10).

The apparent lack of relationship between AChE recovery and the ability to move observed with fenamiphos indicates

TABLE 2. Effect of aldicarb on acetylcholinesterase activity in wild type and mutant *Caenorhabditis elegans* strains.

Strain	I_{50}, M†	Immobilized‡
N2 (wild type)	8.3×10^{-5}	95
ace-1	1.5×10^{-4}	50
ace-2	4.8×10^{-5}	70
ace-1, ace-2	$> 1.0 \times 10^{-4}$	95

† Concentration of aldicarb necessary to inhibit AChE activity by 50% relative to untreated control.

‡ Percentage of nematodes immobilized at the I_{50} concentration in bioassay.

that the nematode is able to move normally with less than 15% of normal AChE activity. This observation is supported by the AChE deficient mutant analysis. The *ace-1, ace-2* strain used in this study completely lacks two of the major classes of AChE, and therefore possesses only ca. 5% of the AChE found in the wild type (6). The untreated *ace-1, ace-2* nematodes move, feed, and reproduce with only mild uncoordination. The readiness with which AChE activity is restored from carbamate effects and the lack of substantial reactivation from the organophosphate inhibitor is similar to other systems (4,8,18). Recovery of nematode AChE from the methylcarbamate carbofuran was slower than from the oxime carbamates aldicarb and oxamyl. Carbofuran is also a more potent AChE inhibitor in vitro than either aldicarb or oxamyl (17). However, in this case only a small amount of recovery was necessary to allow normal behavior.

The confusing picture that emerges when behavior of intact nematodes is compared to the status of AChE may also be related to the enzyme distribution within the nematode neuroanatomy. Acetylcholinesterase occurs in two states in nematodes: a soluble fraction operating at the neuromuscular synapse and thought to be the immediate target of inhibitors; and a relatively insoluble fraction with unknown primary function (20). Our nematicide formulations should have enabled the inhibitor to reach both the soluble and insoluble fractions. One possible function of the insoluble portion of AChE may be to act as

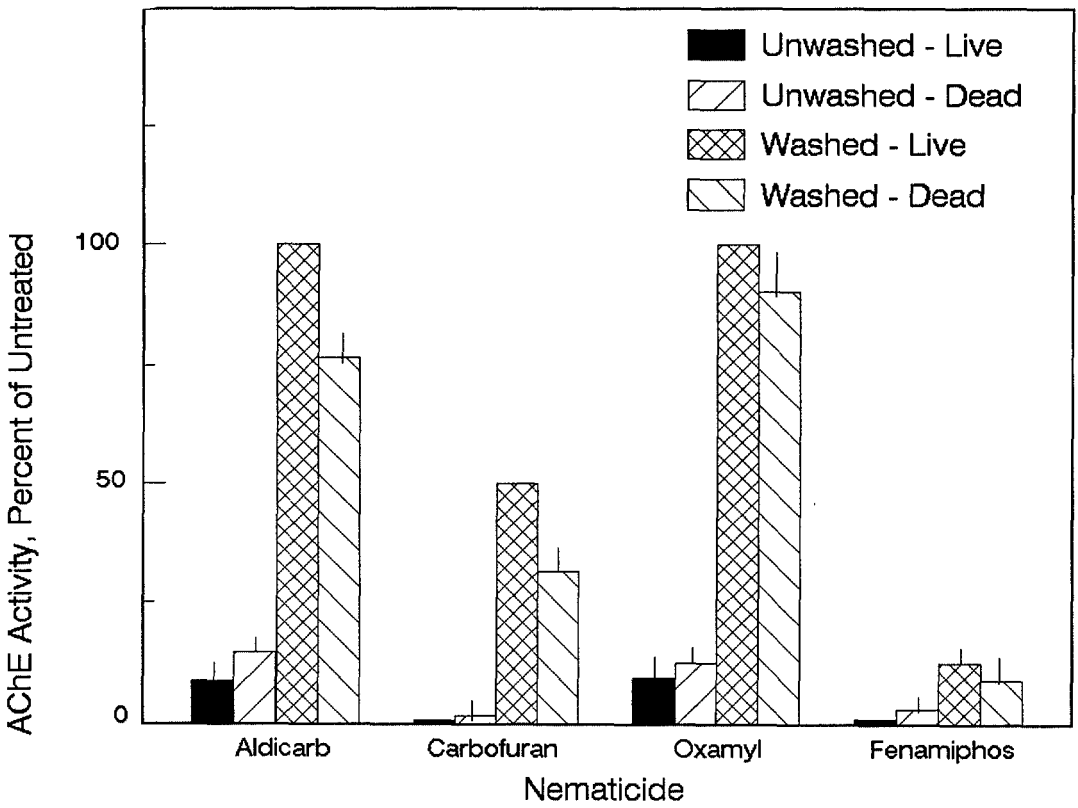

FIG. 4. Comparison of *Caenorhabditis elegans* acetylcholinesterase recovery from exposure to nematicides (1×10^{-4} M) between live and dead nematodes.

a backup system for the soluble fraction (20). At least some of the insoluble fraction is composed of class C AChE, a molecular form of AChE that is, so far, unique to nematodes (9). Class C AChE is relatively insensitive to inhibitors compared to the more prevalent AChE classes A and B, and its affinity for acetylcholine is extremely high compared to classes A and B (A $\simeq$ 13 μM, B $\simeq$ 73 μM, C $\simeq$ 16 nM) (9). At the substrate concentrations of these experiments, and those of other investigators, class C AChE activity would be masked by the other classes. Our experiments demonstrate that class C AChE would be the first form reactivated during a recovery period. In addition, the fact that it is an insoluble form may make it less accessible to inhibitors, resulting in a longer period necessary for nematicide action.

Analysis with the mutant strains demonstrates that recovery is based on the inherent instability of the enzyme-inhibitor covalent linkage, and not on synthesis of new enzyme. If new synthesis were occurring, there would have been a time differential in recovery among the mutant strains and wild type, and there was no differential. Although the concentrations of nematicide were well above the threshold for movement inhibition, the strain with only 5% normal AChE recovered as rapidly as the wild type. The experiments with live versus dead nematodes further support this data. Substantial AChE recovery occurred in dead nematodes from exposure to the carbamate nematicides, indicating that recovery is a mostly passive process. The differences in amount of recovered AChE activity between live and dead nematodes may reflect a partial role for active metabolism of the carbamate nematicides (16). The organophosphate fenamiphos, however, be-

haved similarly on both live and dead nematode AChE.

A recent study reported that *Aphelenchus avenae* recovered from exposure to carbofuran, but not fenamiphos, after a 72-hour exposure period (18). Nematode recovery from exposure to carbamate nematicides has been documented in several other instances as well (3,4,12,14). Recovery from organophosphate exposure has been reported for *Ascaris* AChE (8), but the effects of ethoprop on *M. javanica* amphidial AChE were apparently irreversible (4). In a different study, the effects of fenamiphos on *Pratylenchus vulnus* were reported to be reversible, but the amount of recovery decreased as exposure time increased (12). In combination with the results reported in our study, the following picture emerges. Nematode AChE can recover rapidly from exposure to carbamate nematicides under most circumstances and after relatively long exposure periods. Recovery from exposure to organophosphates is conditional, slower, and less pronounced than recovery from carbamates. The structural features of the toxic moiety of phosphate compounds may also impact the recovery period. The rapidity with which behavior was restored after fenamiphos exposure in our experiments is probably related to the short exposure time, resulting in incomplete inhibition of class C AChE. The relative insensitivity of class C AChE to both carbamates and organophosphates, combined with its less accessible location, suggests that it plays a significant role in the recovery process (9,20).

Our results support previous studies on nematode recovery from carbamate nematicide exposure, and we also have shown that recovery from short exposures (< 24 hours) to organophosphates is rapid. Recovery is a result of instability of the inhibitor-enzyme complex. The speed and amount of recovery is dependent upon both the duration and intensity of nematicide exposure combined with the distribution and relative sensitivity of the AChE molecular classes within the nematode neuroanatomy.

Literature Cited

1. Batterby, S., G. N. J. Le Patourel, and D. J. Wright. 1977. Accumulation and metabolism of aldicarb by the free-living nematodes *Aphelenchus avenae* and *Panagrellus redivivus*. Annals of Applied Biology 86:69–76.

2. Brenner, S. 1974. The genetics of *Caenorhabditis elegans*. Genetics 77:71–94.

3. Bunt, J. A. 1975. Effect and mode of action of some systemic nematicides. Mededeling Landbouwhogesschool Wageningen 75–10:1–127.

4. Cuany, A., J.-B. Berge, J.-M. Bride, and S. Hamadene-Sellami. 1984. Inhibition et reactivation des acetylcholinesterases amphidiales de *Meloidogyne javanica* par l'ethoprophos et l'aldicarb: Tentative de correlation avec une reaction compartmentale. Revue de Nématologie 7:173–176.

5. Johnson, C. D., and R. L. Russell. 1975. A rapid, simple radiometric assay for cholinesterase, suitable for multiple determinations. Analytical Biochemistry 64:229–238.

6. Johnson, C. D., J. B. Rand, R. K. Herman, B. D. Stern, and R. L. Russell. 1988. The acetylcholinesterase genes of *C. elegans*: Identification of a third gene (*ace-3*) and mosaic mapping of a synthetic lethal phenotype. Neuron 1:165 –173.

7. Kass, I. S., C. C. Wang, J. P. Walrond, and A. O. W. Stretton. 1980. Avermectin B_1a, a paralyzing anthelminthic that affects interneurons and inhibitory motoneurons in *Ascaris*. Proceeding of the National Academy of Sciences USA 77:6211–6215.

8. Knowles, C. O., and J. E. Casida. 1966. Mode of action of organophosphate anthelminthics. Cholinesterase inhibition in *Ascaris lumbricoides*. Journal of Agricultural and Food Chemistry 14:566–572.

9. Kolson, D. L., and R. L. Russell. 1985. A novel class of acetylcholinesterase, revealed by mutations, in the nematode *Caenorhabditis elegans*. Journal of Neurogenetics 2:93–110.

10. Lewis, J. A., J. S. Elmer, J. Skimming, S. McLafferty, J. Fleming, and T. McGee. 1987. Cholinergic receptor mutants of the nematode *Caenorhabditis elegans*. Journal of Neuroscience 7:3059–3071.

11. MacDonald, D. H. 1976. Effects of continued application of aldicarb to greenhouse rose beds infested with *Pratylenchus hamatus*. Journal of Nematology 8:293–294.

12. Marban-Mendoza, N., and D. R. Viglierchio. 1980. Behavioral effects of carbofuran and phenamiphos on *Pratylenchus vulnus*. 1. Motility and dispersion. Journal of Nematology 12:102–114.

13. Mouches, C., N. Pasteur, J.-B. Berge, D. Hyrien, M. Raymond, B. P. De Saint Vincent, M. De Silvestri, and G. P. Georghiou. 1986. Amplification of an esterase gene is responsible for insecticide resistance in a California *Culex* mosquito. Science 233: 778–780.

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

15. Nelmes, A. J., D. L. Trudgill, and D. C. M. Corbett. 1973. Chemotherapy in the study of plant

parasitic nematodes. Pp. 95–112 *in* A. E. R. Taylor and R. Muller, eds. Chemotherapeutic agents in the study of parasites. London: Blackwell Press.

16. Nordmeyer, D., D. W. Dickson, L. T. Ou, and H. L. Cromroy. 1989. Uptake, accumulation, and metabolism of carbofuran and fenamiphos by the phytoparasitic nematodes *Meloidogyne javanica* and *M. incognita*. Pesticide Biochemistry and Physiology 34: 179–184.

17. Opperman, C. H., and S. Chang. 1990. Plant-parasitic nematode acetylcholinesterase inhibition by carbamate and organophosphate nematicides. Journal of Nematology 22:481–488.

18. Pree, D. J., J. L. Townshend, and K. J. Cole. 1990. Inhibition of acetylcholinesterases from *Aphelenchus avenae* by carbofuran and fenamiphos. Journal of Nematology 22:182–186.

19. Price, N. R. 1988. Insecticide-insensitive acetyl cholinesterase from a laboratory selected and a field strain of housefly (*Musca domestica* L.). Comparative Biochemistry and Physiology 90C:221–224.

20. Russell, R. L., and R. H. Burns. 1987. Nematode responses to anti-AChE anthelminthics: Genetic analysis in *C. elegans*. Pp. 407–420 *in* A. MacInnis, ed. Molecular paradigms for eradicating helminthic parasites. New York: Alan R. Liss.

21. Simpkin, K. G., and G. C. Coles. 1981. The use of *Caenorhabditis elegans* for anthelminthic screening. Journal of Chemical Technology and Biotechnology 31:66–69.

22. Smolik, J. D. 1978. Influence of previous insecticidal use on ability of carbofuran to control nematode populations in corn and effect on corn yield. Plant Disease Reporter 62:95–99.

23. Waller, P. J., R. J. Dobson, D. L. Obendorf, and R. J. Gillham. 1986. Resistance of *Trichostrongylus colubriformis* to levamisole and morantel: Differences in relation to selection history. Veterinary Parasitology 21:255–263.

24. Wright, D. J. 1981. Nematicides: Mode of action and new approaches to chemical control. Pp. 421–434 *in* B. M. Zuckerman and R. A. Rohde, eds. Plant parasitic nematodes, vol. 3. New York: Academic Press.

25. Yamashita, T. T., D. R. Viglierchio, and R. V. Schmitt. 1986. Responses of nematodes to nematicidal applications following extended exposures to subnematicidal stress. Revue de Nématologie 9:49–61.

26. Yamashita, T. T., and D. R. Viglierchio. 1987. Field resistance to nonfumigant nematicides in *Xiphinema index* and *Meloidogyne incognita*. Revue de Nématologie 10:327–332.