

# Plant-parasitic Nematode Acetylcholinesterase Inhibition by Carbamate and Organophosphate Nematicides<sup>1</sup>

C. H. OPPERMAN AND S. CHANG<sup>2</sup>

**Abstract:** The sensitivity of acetylcholinesterases (AChE) isolated from the plant-parasitic nematodes *Meloidogyne arenaria*, *M. incognita*, and *Heterodera glycines* and the free-living nematode *Caenorhabditis elegans* to carbamate and organophosphate nematicides was examined. The AChE from plant-parasitic nematode species were more sensitive to carbamate inhibitors than was AChE from *C. elegans*, but response to the organophosphates was approximately equivalent. The sulfur-containing phosphate nematicides were poor inhibitors of nematode acetylcholinesterase, but treatment with an oxidizing agent greatly improved inhibition. Behavioral bioassays with living nematodes revealed a poor relationship between enzyme inhibition and expression of symptoms in live nematodes.

**Key words:** acetylcholinesterase, *Caenorhabditis elegans*, carbamate, *Heterodera glycines*, *Meloidogyne arenaria*, *M. incognita*, nematicide, organophosphate.

Nematode locomotion depends upon an array of approximately 90 motor neurons and interneurons that employ the neurotransmitters acetylcholine (excitatory) and gamma-aminobutyric acid (GABA) (inhibitory) (9,16,17). Acetylcholine causes an increase in the rhythmic frequency of muscle cell depolarizations. The action of acetylcholine at the neuromuscular synapse is terminated by acetylcholinesterase (AChE, E.C. 3.1.1.7). The effects of acetylcholine can be potentiated by numerous AChE inhibitors, including carbamate and organophosphate (OP) nematicides (8,16,30).

Genetic mosaic analysis of *Caenorhabditis elegans* has demonstrated that the only vital function of AChE in nematodes is to hydrolyze acetylcholine at the neuromuscular synapse (19). Mutant strains that lack AChE in neurons behave as the wild type, provided AChE is present in the musculature. Those nematodes lacking AChE in the muscles are nonviable. Given the sim-

ilarity in nervous system structure among diverse groups of nematodes (16), this scenario probably holds true for plant-parasitic nematodes as well.

Carbamate and OP nematicide mode-of-action is presumed to be inhibition of AChE (22,26). The presence of AChE in plant-parasitic nematodes has been demonstrated (1,12,20,21,24,27,29,31,32,36), but few investigations concerning its physiological function have been performed. *Aphelenchoides ritzemabosi* cholinesterase did not behave as a true acetylcholinesterase (33) but rather had a broader substrate affinity than mammalian forms of the enzyme. This finding is supported by data generated with *C. elegans* (15).

There are several conflicting reports concerning nematode sensitivity to AChE inhibitors (2,20,24,28). AChE isolated from *Aphelenchus avenae* was sensitive to AChE inhibitors in vitro, but the compounds tested were ineffective at comparable levels on *A. avenae* in bioassays (28). However, *A. avenae* was extremely sensitive to AChE inhibitors at low dosages in a different bioassay (2). Laboratory tests with other nematode species also have shown nematodes to be extremely sensitive to AChE inhibition (5).

We have studied AChE in *Meloidogyne arenaria*, *M. incognita*, and *Heterodera glycines* as part of a project to examine nervous system function in plant-parasitic nematodes. We used *C. elegans* as a model be-

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<sup>2</sup> Assistant Professor and Research Technician, Department of Plant Pathology, North Carolina State University, Raleigh, NC 27695-7616.

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cause its AChE is very well characterized (7,15,18,19,23). In this paper we report the development of methods to analyze plant-nematode enzyme activity, comparative effects of carbamate and OP nematicides on isolated nematode AChE compared with living nematodes, and experiments addressing bioactivation of parent nematicides to their toxic metabolites.

#### MATERIALS AND METHODS

*Nematode culture:* *Meloidogyne arenaria* and *M. incognita* were maintained on roots of tomato (*Lycopersicon esculentum* cv. Rutgers) in the greenhouse. Egg masses were dissolved with 0.5% sodium hypochlorite (13) and the eggs were collected for hatching. *Heterodera glycines* was cultured on soybean (*Glycine max* cv. Ransom) in the greenhouse. Mature cysts were collected by sieving and gently cracked in a glass homogenizer to release the eggs. The eggs were placed on 25- $\mu$ m-pore screens in water, and hatched second-stage juveniles were collected at 24-hour intervals. The collected nematodes were separated from debris by flotation on 20–40% sucrose and rinsed to remove residual sucrose. The nematodes were pelleted by centrifugation (718 g, 5 minutes) and then resuspended in 1–2 volumes of 100 mM Na borate, 1 mM NaN<sub>3</sub> (as a preservative), 1 mg/ml BSA (to prevent denaturative losses), pH 8.0. Packed nematodes were bulk homogenized in 1-ml fractions and used immediately, or aliquots of this suspension containing approximately 100 nematodes were stored in 1.5-ml tubes at –80 C for microassay.

*Caenorhabditis elegans* was cultured on *Escherichia coli* 'OP50', on nematode growth medium (NGM) (4). Nematode egg suspensions prepared by a hypochlorite method (11) were added to the plate and incubated at 20 C for 4–5 days. Nematodes were harvested by rinsing the plates with borate buffer and were separated from debris and bacteria by flotation on 20–40% sucrose. *Caenorhabditis elegans* suspensions

were aliquoted and stored as described above.

*Nematode preparation:* In initial experiments, fresh suspensions of packed nematodes were 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 with a Virtis 50 ultrasonic cell disrupter (Gardner, NY) at 50% power for three 5-second bursts. The resulting homogenate was used immediately for enzyme assays and protein determinations (3). This method proved to be an inefficient use of tissue; therefore, most experiments utilized a microassay.

Samples (ca. 100 nematodes) were prepared for AChE microassay by subjecting them to six freeze-thaw cycles. After the final cycle, samples were incubated at room temperature overnight. This treatment effectively disrupts nematode cuticle permeability without grossly disrupting the morphology and makes the AChE within the nematode accessible to substrate.

*AChE activity assay:* AChE assays were carried out using a modification of the single vial radiometric method (14). Test compounds were solubilized in acetone such that the solvent concentration in the reaction mixture did not exceed 2.5%. The AChE inhibitors tested included the natural carbamates eserine and neostigmine (Sigma Chemicals, St. Louis, MO); the synthetic carbamates aldicarb, carbofuran, and oxamyl; the thiophosphoryl phosphates parathion, phorate, and terbufos; the phosphorothioate ethoprop; the phosphoramidate fenamiphos; and the phosphoryl phosphate paraoxon (Chem Service, West Chester, PA). Nematode homogenates (40  $\mu$ l) (ca. 1.5–2.0 mg/ml protein for *C. elegans*; ca. 0.5–1.0 mg/ml for all plant-parasitic nematode species) or freeze-thawed nematodes (ca. 100 nematodes per tube) were preincubated for 10 minutes with 2  $\mu$ l inhibitor before addition of substrate. Final inhibitor concentrations in the reaction mixture ranged from 10<sup>–4</sup>

M to  $10^{-8}$  M. Bioactivation of OP inhibitors was performed with meta-chlorperbenzoic acid (mCPBA) (10,34). Inhibitor and mCPBA were preincubated with the nematode preparation for 60 minutes in  $1 \times 10^{-4}$  M concentrations before addition of the substrate,  $22.9 \mu\text{M}$  [ $^3\text{H}$ ]acetylcholine (TRA-244, Amersham, Arlington Heights, IL). After a 20–60-minute incubation period for *C. elegans*, or 60–360 minutes for the plant-parasitic nematode species, the reaction was terminated by addition of 20  $\mu\text{l}$  stop buffer (1 M trichloroacetic acid, 0.5 M NaOH, 2 M NaCl) and then 1.3 ml of a toluene-n-butanol scintillation cocktail (0.5% PPO, 0.03% POPOP in toluene). Substitution of n-butanol for isoamyl alcohol still allows two phasing to occur and is much more economical (15,23). All samples were counted in a Packard Tri-Carb 3255 liquid scintillation spectrometer. Fractional conversion rates to hydrolyzed [ $^3\text{H}$ ]acetate were calculated to correct for substrate depletion during the course of the assay, using the formula:  $\text{fc units} = -\ln(1 - \{(\text{nematode CPM} - \text{blank})/(\text{total CPM} - \text{blank})\})$ . Blank assays contained no nematode preparation. Total substrate hydrolysis was determined by addition of 500 U/ml electric eel AChE (Sigma) at the beginning of the incubation period. There were two to four separate repetitions of each experiment with three replications per treatment.

**Bioassays:** Behavioral bioassays were conducted by incorporating appropriate concentrations of test compounds into autoclaved NGM (*C. elegans*) or 1.0% water agar (*M. incognita*) at 45 C in a water bath. For assays involving *C. elegans*, *E. coli* OP50 was streaked in the middle of the plates and incubated overnight. For the *M. incognita* bioassay, a newly germinated Rutgers tomato seedling was surface sterilized with sodium hypochlorite, placed at one end of the plate, and allowed to grow for 48 hours. Nematodes were then placed on the plates approximately 5 cm from the root, and the plates were incubated at 25 C in the dark. Root penetration was monitored by staining selected roots with acid fuchsin (6). Be-

havior was observed with a 40 $\times$  dissecting microscope daily for 4–7 days. Controls both with and without solvent were included in each assay to determine baseline responses and to insure lack of solvent effects on nematode behavior. Results are a summation of observations from two to ten separate repetitions of the assays, with six replications per treatment for *C. elegans* and four replications per treatment with *M. incognita*.

## RESULTS

Incubation of nematode homogenates with [ $^3\text{H}$ ]acetylcholine showed a linear increase in substrate hydrolysis between 10 and 60 minutes. Fractional conversion rates of  $0.023 \pm 0.004$  u/minute (*C. elegans*) and  $0.004 \pm 0.001$  (*M. incognita*) were obtained for uninhibited AChE. Conversion rates comparable to these were achieved using 100 freeze-thawed nematodes; therefore, the remainder of these experiments were performed using this method. Comparison of fractional conversions between fresh homogenates and freeze-thaw preparations showed no adverse effect of freeze-thaw cycles on AChE activity. Incubation of freeze-thawed *C. elegans* for 10 minutes provided a conversion rate of 40–50%, but a 3–6-hour incubation was necessary to achieve comparable conversion rates for *M. arenaria*, *M. incognita*, and *H. glycines*. On the basis of saturation curves, preincubation with *C. elegans* homogenate for 10 minutes was chosen for experiments involving inhibitors. Similar responses were observed for plant-parasitic nematodes. Kinetic parameters ( $K_m$ ,  $V_{\text{max}}$ ) were determined to identify optimum substrate concentrations (data not shown).

In general, plant-parasitic nematode AChE was more sensitive to carbamate AChE inhibitors than was *C. elegans* AChE (Table 1). This differential response was particularly evident for *M. arenaria* and *M. incognita* where  $I_{50}$  values were up to 65-fold lower. In all nematodes, eserine and neostigmine were the most potent AChE inhibitors, followed by carbofuran, with aldicarb as the least potent carbamate inhib-

TABLE 1. Concentration of carbamates and organophosphate compounds that reduce nematode acetyl cholinesterase activity by 50% relative to untreated preparations of *Caenorhabditis elegans*, *Heterodera glycines*, *Meloidogyne arenaria*, and *M. incognita*.†

Compound	I <sub>50</sub> molar‡			
	<i>C. elegans</i>	<i>M. arenaria</i>	<i>M. incognita</i>	<i>H. glycines</i>
<b>Carbamates</b>				
Aldicarb	8.5 × 10 <sup>-5</sup>	5.5 × 10 <sup>-6</sup>	7.7 × 10 <sup>-6</sup>	3.1 × 10 <sup>-5</sup>
Carbofuran	2.3 × 10 <sup>-6</sup>	9.2 × 10 <sup>-8</sup>	7.8 × 10 <sup>-8</sup>	5.1 × 10 <sup>-7</sup>
Oxamyl	4.9 × 10 <sup>-5</sup>	7.3 × 10 <sup>-7</sup>	7.6 × 10 <sup>-7</sup>	2.1 × 10 <sup>-6</sup>
Neostigmine	6.5 × 10 <sup>-8</sup>	2.0 × 10 <sup>-9</sup>	3.0 × 10 <sup>-9</sup>	1.0 × 10 <sup>-8</sup>
Eserine	5.5 × 10 <sup>-8</sup>	1.0 × 10 <sup>-9</sup>	1.0 × 10 <sup>-9</sup>	5.0 × 10 <sup>-8</sup>
<b>Organophosphates</b>				
Fenamiphos	6.9 × 10 <sup>-5</sup>	1.6 × 10 <sup>-5</sup>	1.0 × 10 <sup>-5</sup>	3.5 × 10 <sup>-5</sup>
Ethoprop	> 1.0 × 10 <sup>-4</sup>			
Parathion	> 1.0 × 10 <sup>-4</sup>			
Paraoxon	7.0 × 10 <sup>-7</sup>	7.5 × 10 <sup>-8</sup>	6.0 × 10 <sup>-8</sup>	4.0 × 10 <sup>-7</sup>
Phorate	> 1.0 × 10 <sup>-4</sup>			
Terbufos	> 1.0 × 10 <sup>-4</sup>			

† AChE activity was measured as hydrolysis of [<sup>3</sup>H]acetylcholine by freeze-thawed nematode preparations.

‡ Percentage of inhibition was calculated as 1 - [(treated-blank)/(untreated-blank)] × 100. Approximate conversion rates for untreated nematode preparations were as follows: *C. elegans* 50%, 20-minute incubation; *M. incognita* and *M. arenaria* 35%, 6-hour incubation; *H. glycines* 6%, 6-hour incubation. Total substrate CPM = 20,000.

itor of AChE in vitro for all four nematode species.

With the exception of fenamiphos and paraoxon, the phosphates were very weak inhibitors of nematode AChE (Table 1). The relative potency of the phosphates on AChE from all four nematode species was similar, with paraoxon the most potent. AChE from *M. arenaria* and *M. incognita* were approximately 10,000-fold less sensitive to fenamiphos than they were to the most potent carbamates, and *H. glycines* AChE was approximately 1,000-fold less sensitive. The phosphates with a sulfur moiety in the phosphoryl head (parathion, ethoprop, phorate, and terbufos) exhibited poor activity, whereas the phosphoryl phosphate (paraoxon) and the phosphoramidate (fenamiphos) had activities comparable to the carbamate inhibitors. Dosage-response curves for aldicarb and fenamiphos inhibition of phytonematode AChE were similar (Fig. 1). The AChE from *H. glycines* was less sensitive than AChE from *M. arenaria* and *M. incognita* to both aldicarb and fenamiphos.

*Caenorhabditis elegans* and *M. incognita* behaved similarly in response to both carbamate and OP AChE inhibitors (Table 2). Treatment with active compounds result-

ed in an initial period of hyperactivity followed by contracted paralysis. By 24 hours after treatment, nematode muscle tone was completely lost, resulting in flaccid paralysis. Occasional twitching and pharyngeal pumping occurred in some nematodes. Effects became manifest slightly later in *M. incognita* than in *C. elegans*, but the pattern of symptom expression and the minimum effective concentrations for motility inhibition were virtually identical. Carbamates possessing a quaternary nitrogen group (eserine and neostigmine) were only slightly inhibitory, and thiophosphoryl phosphates had no effect. Behavioral disruption of *M. incognita* without coincident paralysis was observed at much lower dosages than were necessary for motility inhibition (Table 2). Although the nematodes appeared to move normally, they were unable to penetrate host roots.

The poor in vitro activity of OP compounds with sulfur in the phosphate head portion of the molecule led us to perform biological activation experiments with mCPBA, an oxidant. The greatest gain in activity was seen with parathion oxidized to the phosphate form (paraoxon) (Fig. 2). The activation step resulted in a 70% gain in potency on *H. glycines* and smaller in-

creases on *C. elegans*, *M. arenaria*, and *M. incognita* (Fig. 2). A concomitant gain in activity occurred upon pretreatment of phosphate nematicides with mCPBA (Table 3). The dithioates did not gain as much potency by oxidation. Terbufos remained a weak inhibitor on all four nematode AChE, and phorate moderately gained activity on AChE from *M. arenaria*.

### DISCUSSION

Nematode species exhibit considerable diversity in sensitivity of their AChE to carbamate and OP inhibitors (15,22,23,25,28). Our data indicate that, with respect to AChE inhibition, *M. arenaria* and *M. incognita* are among the most carbamate-sensitive nematode species thus far examined. Paradoxically, they appear to be far less sensitive to paraoxon than expected, based on their apparent sensitivity to carbamate AChE inhibitors (26). *Heterodera glycines* showed a similar pattern of relative AChE inhibition by the various inhibitors but was less sensitive than *M. arenaria* and *M. incognita*. Generally, differences among the plant-parasitic nematode species in sensitivity to synthetic AChE inhibitors were small, but considerable variation in in vitro response to the natural carbamates eserine and neostigmine was observed. The interaction of the quaternary nitrogen group present on these compounds with nematode enzyme active sites may indicate differences in AChE conformation among the species examined.

The lack of a clear relationship between in vitro enzyme toxicity and in vivo data may be related to sublethal behavioral effects (21,28,35). Nematode AChE consists of at least two components in nematodes: a behaviorally relevant component occurring at neuromuscular junctions and freely accessible to inhibitors, and the so-called behaviorally irrelevant component, which is not readily accessible to inhibitors (30). Animals treated with sufficient AChE inhibitor doses to induce paralysis still possess substantial in vitro enzyme activity (30). It appears that the inhibitor quickly dis-

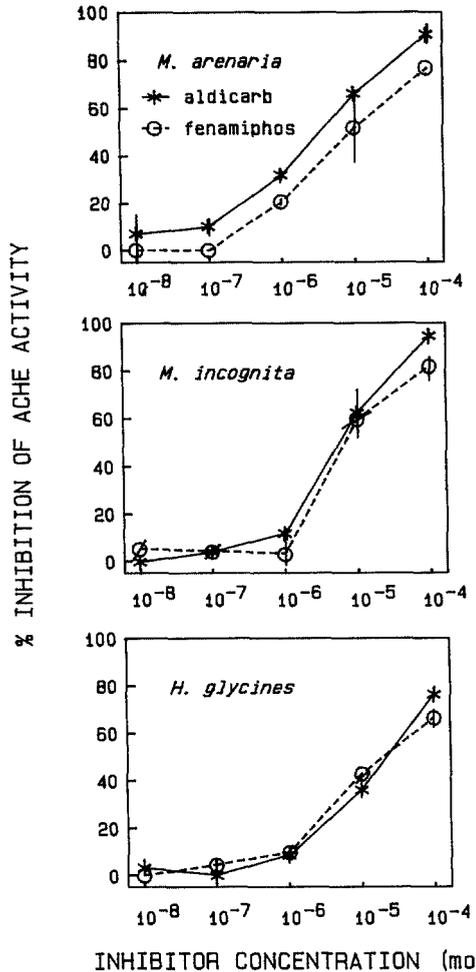


FIG. 1. Effect of various concentrations of aldicarb and fenamiphos on [<sup>3</sup>H]acetylcholine hydrolysis by acetylcholinesterase from *Meloidogyne arenaria*, *M. incognita*, and *Heterodera glycines*. Data are means of at least two experiments using freeze-thaw nematode preparations.

ables the soluble AChE and only slowly inhibits the inaccessible portion of the enzyme. We have observed behavioral disruption by AChE inhibitors at doses 30-fold lower than necessary to inhibit the free portion of the enzyme. These dosages clearly could not be inhibiting the hidden AChE component.

Inhibition of AChE is a stoichiometric reaction; i.e., one molecule of inhibitor binds to a single active site per AChE subunit. Differences in these active sites between AChE species could explain apparent differences within a given nematode

TABLE 2. Behavior of nematodes upon continuous exposure to carbamate and organophosphate inhibitors of acetylcholinesterase.

Compounds	<i>C. elegans</i>				<i>M. incognita</i>				
	1 hr	24 hr	96 hr	MEC	1 hr	24 hr	96 hr	MEC†	PEN‡
Carbamates									
Aldicarb	P+	P++	P++	5 $\mu$ M	SE	P++	P++	5 $\mu$ M	0.26 $\mu$ M
Carbofuran	P+	P++	P++	20 $\mu$ M	SE	P++	P++	20 $\mu$ M	10.00 $\mu$ M
Eserine	NE	NE	P	ND	NE	NE	NM, RP, G	ND	ND
Neostigmine	NE	NE	P	ND	NE	NE	NM, RP	ND	ND
Oxamyl	P+	P+	P+	20 $\mu$ M	SE	P++	P++	20 $\mu$ M	2.28 $\mu$ M
Untreated	NE	NE	NE		NE	NE	NE		
Organophosphates									
Ethoprop	P+	P++	P++	10 $\mu$ M	NE	P++	P++	10 $\mu$ M	2.89 $\mu$ M
Paraoxon	P++	P++ <sup>H</sup>	P++	3 $\mu$ M	NE	P++ <sup>H</sup>	P++	3 $\mu$ M	ND
Parathion	NE	NE	NE	ND	NE	SE	NM, RP	ND	ND
Phenamiphos	P+	P++	P++	3 $\mu$ M	NE	P++	P++	3 $\mu$ M	0.10 $\mu$ M
Phorate	NE	SE	P	ND	NE	SE	NM, RP, G	ND	ND
Terbufos	NE	NE	NE	ND	NE	SE	NM, RP, G	ND	ND
Untreated	NE	NE	NE		NE	NE	NE		

Nematodes were placed on inhibitor-amended agar in the presence of a food source (*Escherichia coli* for *Caenorhabditis elegans*, and tomato root for *Meloidogyne incognita*) to elicit a behavioral response.

Behavioral responses: P = some slowing; P+ = marked slowing and contraction; P++ = complete paralysis; NE = no effect; ND = not determined; SE = slight effects on movement; NM = normal movement; RP = root penetration; G = galls developing on tomato roots infected with *M. incognita*.

† Minimum effective concentration necessary for symptom expression.

‡ Minimum effective concentration necessary for 50% inhibition of penetration of tomato roots by *M. incognita*.

species in relative power of inhibitors, but the organismal data do not support this conclusion. Although AChE inhibition data revealed 10–1,000-fold differences among inhibitors within and between nematode species, inhibitors functioned at similar concentrations on the intact nematodes.

The nematode species in our experiments apparently lack the appropriate cellular machinery to rapidly oxidize sulfur-containing phosphates. This inability suggests that oxidation of carbamate and organophosphate nematicides does not alter their efficacy. *Ascaris lumbricoides* does not metabolize organophosphate AChE inhibitors either (22). Treatment of inhibitors with an oxidizing agent demonstrated that their oxidation products are indeed toxic to nematodes. The relatively small increases in AChE inhibition for the phosphorothioates phorate and terbufos are similar to corresponding increases due to the synthetically prepared metabolites in *Aphelenchus avenae* (28). The activation of parathion to paraoxon and the sulfoxidation of fenamiphos and ethoprop also yield

results consistent with those obtained with the synthetic analogs.

The natural carbamates eserine and neostigmine possess a quaternary nitrogen group. Their difficulty in penetrating the living nematode cuticle could result in the lack of biological activity observed. In general, the carbamates were powerful inhibitors of nematode soluble AChE, as expected because they were designed to mimic the natural substrate, acetylcholine. The major function of AChE inhibitors in plant-

TABLE 3. Effect of meta-chlorperbenzoic acid on activity of parathion against *Caenorhabditis elegans*.

Treatment	Motile nematodes† (%)	AChE inhibition‡ (%)
Control	100	0.00
Parathion	100	13.61
Parathion plus m-CPBA	0	100.00
Paraoxon§	0	97.00
m-CPBA	100	0.14

† Motile at 20 minutes at  $3 \times 10^{-4}$  M.

‡ Assayed at  $5 \times 10^{-4}$  M.

§ Chemically synthesized analog of parathion + m-CPBA reaction product.

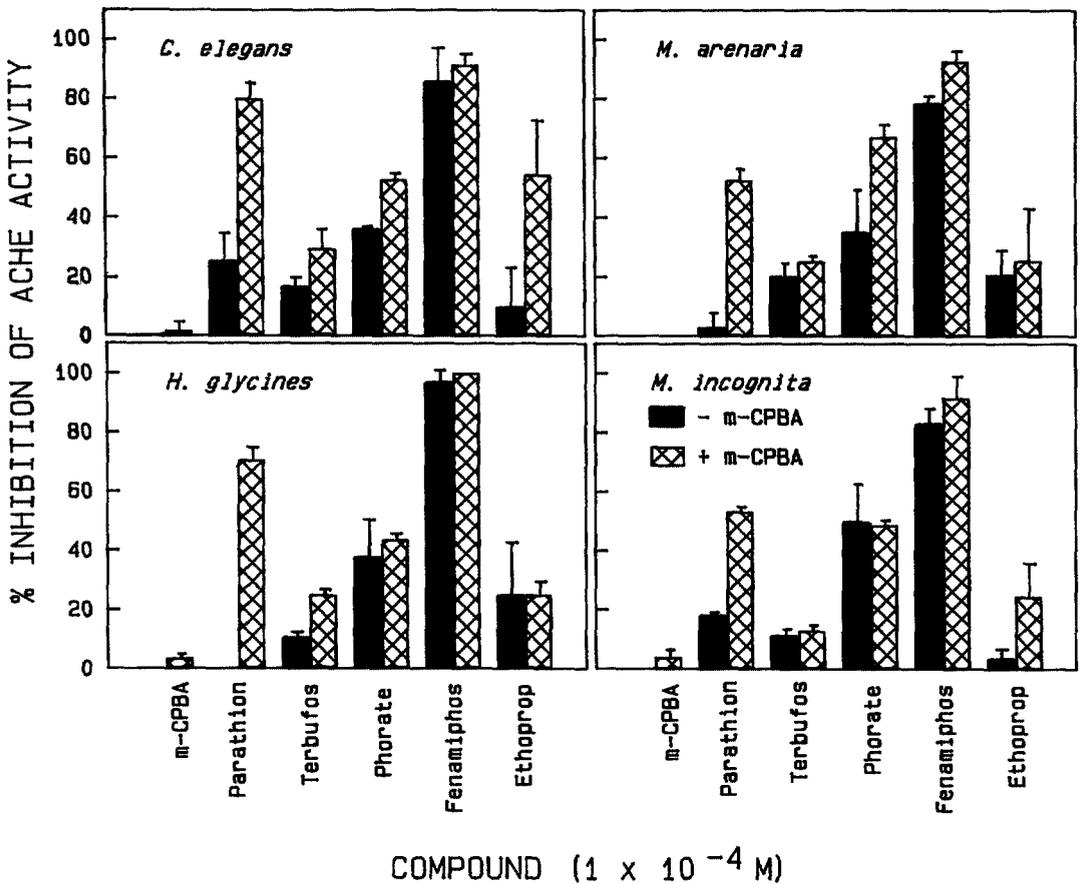


FIG. 2. Inhibition of nematode acetylcholinesterase activity before and after bioactivation of organophosphates by meta-chlorperbenzoic acid. The inhibitor and the mCPBA were incubated for 1 hour before addition of  $11.9 \mu\text{M}$  [ $^3\text{H}$ ]acetylcholine. Percentage of inhibition was calculated as  $1 - ((\text{treated} - \text{blank}) / (\text{untreated} - \text{blank})) \times 100$ , as measured by hydrolysis of [ $^3\text{H}$ ] substrate. Paraoxon was the positive control in these experiments. Inhibition by  $2 \times 10^{-4}$  M paraoxon alone was 99% for *Meloidogyne arenaria* and 100% for *Caenorhabditis elegans*, *Heterodera glycines*, and *M. incognita*.

parasitic nematodes is neuromuscular paralysis. Our data demonstrate that differences between *C. elegans* and plant-parasitic species are relatively minor. This is to be expected because of the evolutionary conservation of nervous system development seen in many animal species.

Multiple molecular forms of AChE have been isolated from *C. elegans*, *A. lumbricoides*, and *Stephanurus denatus* (15,23). These classes of AChE differ from each other in their biochemical properties, including sensitivity to inhibitors. The difference between species in sensitivity of total nematode AChE to inhibitors, which we have reported here, seem likely to be related to differential distribution of AChE classes between these species.

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