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Separation and Characterization of *Heterodera glycines* Acetylcholinesterase Molecular Forms[1]

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Abstract: The composition and biochemical properties of acetylcholinesterases isolated from *Heterodera glycines* were determined. *Heterodera glycines* contains three separable AChE molecular forms that can be grouped into two classes corresponding to classes A and C found in some other nematode species. The apparent lack of class B AChE is unusual and may have significant behavioral ramifications. The class C enzyme isolated from *H. glycines* is similar to that from *Meloidogyne arenaria* and *M. incognita* but is somewhat more sensitive to AChE inhibitors such as eserine. *Heterodera glycines* possesses a larger percentage of its total acetylcholinesterase as class C than other nematodes thus far examined.

Key words: acetylcholinesterase, carbamate, enzyme, *Heterodera glycines*, nematicide, nematode, organophosphate, soybean cyst nematode.

Multiple molecular forms of acetylcholinesterase (AChE) have been described from a number of animal species, including the nematodes *Ascaris lumbricoides, Caenorhabditis elegans, Meloidogyne arenaria, M. incognita,* and *Panagrellus redivivus* (3,6,7,9,10). Biochemical characterization of AChE, however, has been reported for only *C. elegans, M. arenaria,* and *M. incognita* (3,7,8). In these species, AChE molecular forms can be grouped into three discrete classes based on biochemical properties, including substrate affinity, inhibitor sensitivity, detergent sensitivity, and thermolability (3,7). In *C. elegans,* these AChE classes are controlled (and probably encoded) by three unlinked genes (7). Mutational analysis has demonstrated that loss of any one or two classes is not lethal, and the remaining forms are able to compensate, at least partially, for the missing AChE (7). Therefore, it is believed that the nematode AChE forms have overlapping functions.

Of particular interest in the characterization of nematode AChE has been the identification of the class C form. This enzyme possesses a constellation of biochemical properties that are unique among known AChE, both vertebrate and invertebrate. Class C AChE has a remarkably high affinity for the native substrate acetylcholine as it exhibits a K_m in the range of 10 nM (3,8). Additionally, class C AChE is relatively insensitive to carbamate and organophosphate AChE inhibitors when compared to other nematode AChE classes or to vertebrate AChEs (3,8). The functional significance of class C is not known, nor have many cellular localization experiments been performed. Class C AChE probably does occur in or near the CAN cells in *C. elegans,* a pair of neurons associated with the excretory canals (7). In contrast, classes A and B occur primarily at the neuromuscular junctions in the dorsal and ventral cords (6,7). Mutants that lack class C, however, are viable and behave like wild type nematodes (7). Strains that lack both classes A and B are uncoordinated, though developmentally normal, indicating that class C is unable to compensate completely for the loss of the other classes (8). Specific functions for any nematode AChE class have yet to be assigned.

Plant-parasitic nematode response to carbamate and organophosphate nematicides is likely related to the distribution and relative abundance of AChE molecular forms. Besides the classic symptom of paralysis on exposure to AChE inhibitors, plant-parasitic nematodes exhibit an array of sublethal behavioral abnormalities at subparalytic dosages (11,14). Inhibition of

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only the most sensitive AChE forms and incomplete compensation by the uninhibited or partially inhibited forms may cause these behavioral abnormalities. Nematodes may recover from exposure to carbamate and organophosphate nematicides (2,4,12,14). In some cases, recovered nematodes still had approximately 90% of their AChE activity inhibited, even though they appeared to move and behave normally (12). These phenomena are presumably related to relative sensitivity and rates of inactivation or reactivation of the various nematode AChE classes.

The soybean cyst nematode, *Heterodera glycines* Ichinohe, is an important economic pest of soybeans in the United States and other soybean-growing regions of the world. In general, carbamate and organophosphate nematicides are not economically viable control measures for *H. glycines,* and many growers rely on strategies involving resistant soybean cultivars. In vivo bioassays have suggested that *H. glycines* is less sensitive than *Meloidogyne* to carbamate and organophosphate nematocides (5), and examination of crude nematode AChE response to these compounds supports this finding (11). These results may be related to the composition of *H. glycines* AChE molecular forms and to differential compensation rates on exposure to various nematicide concentrations. In this study, we report the separation and characterization of *H. glycines* AChEs and demonstrate substantial differences in composition and inhibitor sensitivity between these forms and those in *M. arenaria* and *M. incognita.*

Materials and Methods

Nematode culture: Heterodera glycines race 1 (Wilmington, NC) was increased on soybean (*Glycine max* (L.) Merrill cv. Lee 68) in the greenhouse, and second-stage juveniles (J2) were prepared as previously described (11). The nematodes were pelleted by centrifugation (718*g*, 5 minutes), resuspended in 1–2 volumes of 50 mM Tris-HCl (pH 7.5), and stored frozen at −80 C until used.

Acetylcholinesterase preparation: Nematode acetylcholinesterases were prepared and assayed as previously described (3,11). To extract nematode AChE, 0.6 ml of packed J2 in approximately 4 ml of 50 mM Tris-HCl (pH 7.5) were added dropwise to liquid nitrogen and pulverized twice. Extracts were centrifuged (110,000*g*) for 1 hour at 4 C in a Beckman Type 70.1 rotor and the supernatants were saved (3). The pellet was re-extracted with Tris buffer and centrifuged, and the supernatants were pooled to yield the buffer-soluble fraction. The pellet was further extracted twice more with Triton X-100 to yield the detergent-soluble fraction. Protein in each fraction was determined by the Bradford method with bovine serum albumin (BSA) as a standard (1).

Preparative sucrose gradient centrifugation: Nematode AChE forms were separated from buffer-soluble and detergent-soluble supernatants on 5–20% linear sucrose gradients with a Renografin-76 cushion (Squibb Diagnostics, New Brunswick, NJ), as previously described (3). Gradients were centrifuged (90,000*g*) in a Beckman SW28 rotor for 40 hours at 4 C. Approximately 1-ml fractions were collected, and 50 µl was assayed with 1 µM [^{3}H]acetylcholine chloride (1.68 Ci/mM, TRA-277, Amersham, Arlington Heights, IL) or [^{3}H]acetylcholine iodide (73.7 mCi/mM, NET-113, DuPont NEN, Boston, MA) in a total reaction volume of 100 µl. Samples were counted in a Packard Tri-Carb 3255 liquid scintillation counter (3). Total available substrate was determined by complete hydrolysis with electric eel AChE (1 unit), and blanks contained no nematode preparation. Tubes corresponding to enzyme peaks were pooled, dialyzed in 20 mM Tris-HCl (pH 7.5), and stored at −80 C. These experiments were repeated a minimum of 5 times.

Ion-exchange chromatography: To further separate AChE forms, pooled sucrose gradient fractions were loaded onto a 1 × 20 cm Whatman DE-52 (diethylaminoethyl

cellulose) column previously equilibrated with 20 mM Tris-HCl at pH 7.5, (plus 0.1% Triton X-100 for detergent-soluble fractions), washed with equilibration buffer, and eluted with 120 ml of a 0–0.5 M NaCl linear gradient in equilibration buffer at a flow rate of 16 ml/hour. Fractions of 2 ml were collected and assayed with both 1 μM and 10 nM [^{3}H]ACh in microfuge tubes. In order to determine the elution profile, conductivity of some fractions was measured with a Beckman model RC 16B2 conductivity bridge equipped with a YSI 3403 platinum electrode. Tubes corresponding to peaks of enzyme activity were pooled into dialysis tubing with an exclusion limit of 12,000–14,000, concentrated with sucrose powder, dialyzed in 50 mM Tris-HCl (pH 7.5), and stored at −80 C. SM-2 Bio-Beads (Bio-Rad, Richmond, CA) were used to remove Triton X-100 from detergent solubilized samples. These experiments were repeated two to four times.

Kinetic experiments: Acetylcholinesterase forms separated by density centrifugation and ion-exchange chromatography (25–37.5 μg protein) were assayed at substrate concentrations ranging from 1 nM to 200 μM in 100-μl reaction volumes with two replications per substrate level (3). Incubations were carried out at ca. 24 C in 4.5-ml scintillation vials, and the experiments were performed a minimum of two times.

Calculation of I_{50}: Aldicarb, fenamiphos, eserine, and neostigmine were formulated in acetone and stored at −20 C. Enzyme (25–37.5 μg protein), purified by ion-exchange chromatography, was preincubated with 2 μl inhibitor ranging from 3×10^{-3} to 3×10^{-11} M (final reaction concentration) for 20 minutes prior to introduction of 10 nM or 1 μM [^{3}H]ACh in a total reaction volume of 100 μl (3,11). There were three replications per inhibitor concentration. Incubations were performed at room temperature in 4.5-ml scintillation vials. These experiments were repeated a minimum of four times.

Thermal inactivation: Enzyme purified by ion-exchange chromatography was diluted sixfold with a solution of 1 mg/ml BSA in 50 mM Tris-HCl (pH 7.5) preequilibrated to 45 C. At predetermined time intervals from 0 to 1 hour, two 80-μl samples of the enzyme mixture were transferred to microfuge tubes on ice and kept there until all samples had been removed. Samples were allowed to warm to room temperature before assay with 20 μl of 10 nM or 1 μM [^{3}H]ACh. Controls consisted of enzyme samples similarly diluted but equilibrated at room temperature. These experiments were performed one to two times.

Results

Velocity sedimentation of the buffer-soluble fractions resulted in one well-defined activity peak at approximately 12S and an amorphous region sedimenting higher in the gradient (Fig. 1A). The detergent-soluble fractions yielded two discrete activity peaks that sedimented at 12S and 7S (Fig. 1B). Analysis of the separated AChEs on ion-exchange media revealed that the buffer-soluble 12S fraction had only one activity peak eluting at 100 mM NaCl, whereas activity from the amorphous region was not recoverable despite repeated attempts (data not shown). Chromatography of the 12S detergent-soluble fraction also resulted in isolation of a single activity peak eluting at 100 mM NaCl (data not shown). Analysis of the detergent-soluble 7S fraction, however, revealed the presence of two distinct AChE forms (Fig. 1C), respectively, eluted with ca. 100 mM and 250 mM NaCl. Further examination of these forms by lowering the substrate concentration from 1 μM to 10 nM showed a large increase in activity in the later eluting peak, whereas activity of the early eluting peak remained approximately the same, and extremely low, at both substrate levels (Fig. 1D). This enhanced activity at low substrate levels was not observed in any of the other fractions.

Kinetic experiments with the separated fractions demonstrated that the early eluting 7S peak had no repeatably detectable activity. The three isolated forms fell into

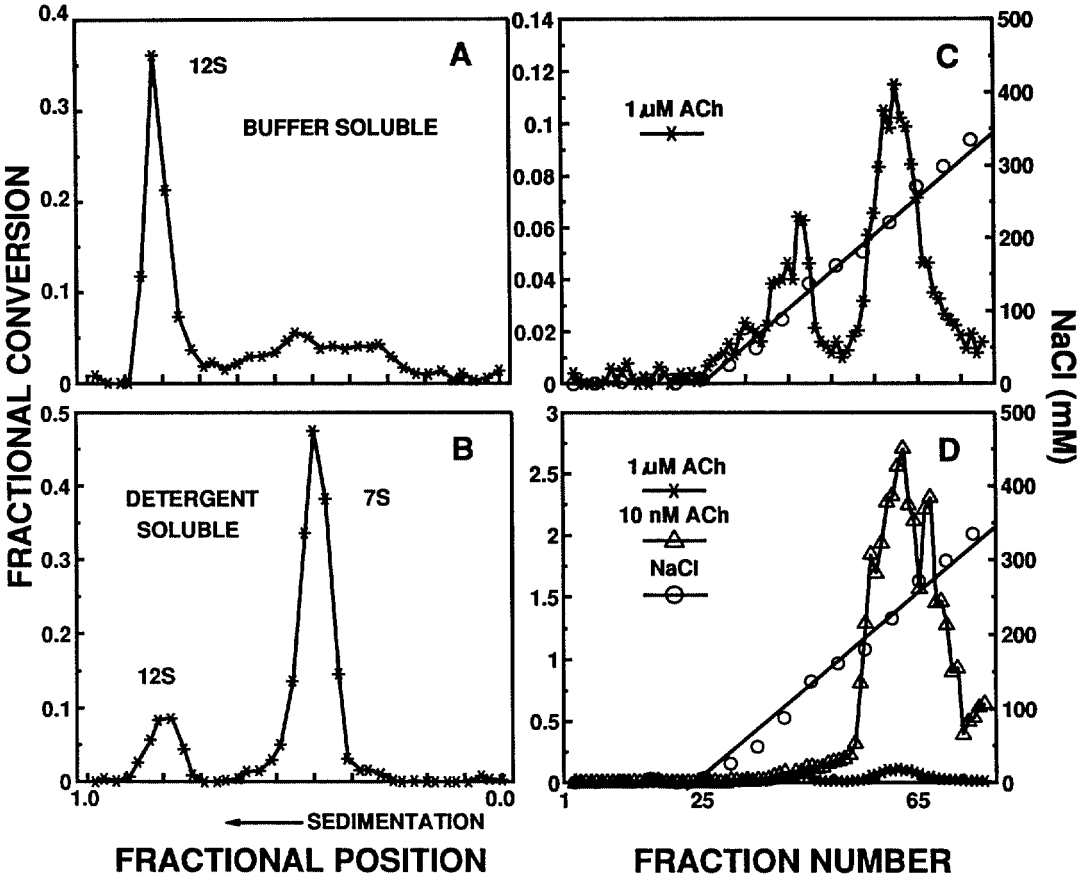

FIG. 1. Separation of *Heterodera glycines* acetylcholinesterase molecular forms by sucrose gradient sedimentation of buffer-soluble and detergent-soluble fractions and ion-exchange chromatography. The AChE activity is plotted as fractional conversion of substrate, i.e., $-\ln(1 - [(\text{assay CPM} - \text{blank})/(\text{total CPM} - \text{blank})])$. Differences in the numbers of fractions collected were compensated by plotting fractions at their fractional positions from the gradient top. Approximate sedimentation coefficients were determined by parallel gradients with previously characterized *Caenorhabditis elegans* AChE molecular forms (5). A) Buffer-soluble fraction. B) Detergent-soluble fraction. C,D) Ion-exchange chromatography of *Heterodera glycines* 7S AChE forms assayed at 1 μM ACh (C) or 10 nM ACh (D).

two discrete groups based on substrate affinity (K_m) (Table 1). The buffer-soluble 12S and detergent-soluble 12S forms had K_ms of 4.7×10^{-5} and 12.0×10^{-5} M, respectively, whereas the late eluting 7S peak had a K_m of 10.3×10^{-9} M (Table 1, Fig. 2). Other tests further delineated the forms into two groups, with the two 12S forms differing from the 7S (Table 1). The 7S form was not sensitive to detergent treatment, unlike the 12S forms. The 12S forms were also more stable to heat, remaining active for prolonged periods of incubation at 45 C. The 7S form exhibited remarkable thermolability, as it lost over 50% activity in less than 10 minutes (Fig. 3).

Further experiments with AChE inhibitors demonstrated a marked difference in sensitivity between the late-eluting 7S form and the others (Table 1). The late-eluting 7S form was insensitive to carbamate and organophosphate inhibitors, whereas the 12S forms were very sensitive. Although this difference was evident with aldicarb and fenamiphos, it was much more substantial with the aromatic carbamates eserine and neostigmine (Fig. 4).

TABLE 1. Properties of separated *Heterodera glycines* acetylcholinesterase molecular forms.

	12S†	12S	7S
Class	A	A	C
K_m (μM)	47.5	120.0	0.01
NaCl (mM)‡	100	100	250
Thermal sensitivity§	low	low	high
Triton sensitivity‖	+	+/−	−
pI_{50}¶			
aldicarb	6.4	5.5	4.7
eserine	9.9	8.9	5.6
neostigmine	9.7	9.3	4.4
fenamiphos	5.5	3.9	3.6

† Approximate sedimentation position in a 5–20% linear sucrose gradient based on comparison to standard AChE from *Caenorhabditis elegans*.

‡ Concentration of NaCl necessary to dissociate AChE from a Whatman DE-52 ion-exchange column.

§ Sensitivity to thermal inactivation (T_{50} < 10 minutes).

‖ + = >50% inhibition by 0.05% Triton X-100, +/− = ca. 50% inhibition, − = <50% inhibition.

¶ −log (inhibitor concentration necessary to decrease enzyme activity by 50% compared to untreated enzyme).

DISCUSSION

Heterodera glycines contain three easily separable forms of acetylcholinesterase. The two 12S forms have similar, though not identical, properties, and may represent differentially soluble species of the same form. Based on a set of biochemical characteristics, including inhibitor sensitivity, substrate affinity, and thermolability, these forms can be grouped into two classes. These classes roughly correspond to classes A and C previously reported from *C. elegans, M. arenaria,* and *M. incognita* (3,7). Substrate affinity is very similar among the respective classes from each nematode species. Also, characteristics such as detergent sensitivity, thermolability, and class A sensitivity to AChE inhibitors are consistent across *C. elegans, H. glycines, M. arenaria,* and *M. incognita.* There are, however, some substantial differences between the AChE forms isolated from *H. glycines* and those from *Meloidogyne* species. The class C form in *Meloidogyne* is less sensitive to inhibitors than that in *H. glycines* (3). Additionally, the NaCl elution profiles from ion-exchange columns are quite different, with *H. glycines* class C AChE requiring higher salt concentrations to dissociate from the DE52 column. Finally, only three separable forms were found in *H. glycines,* compared with five from *Meloidogyne* (3).

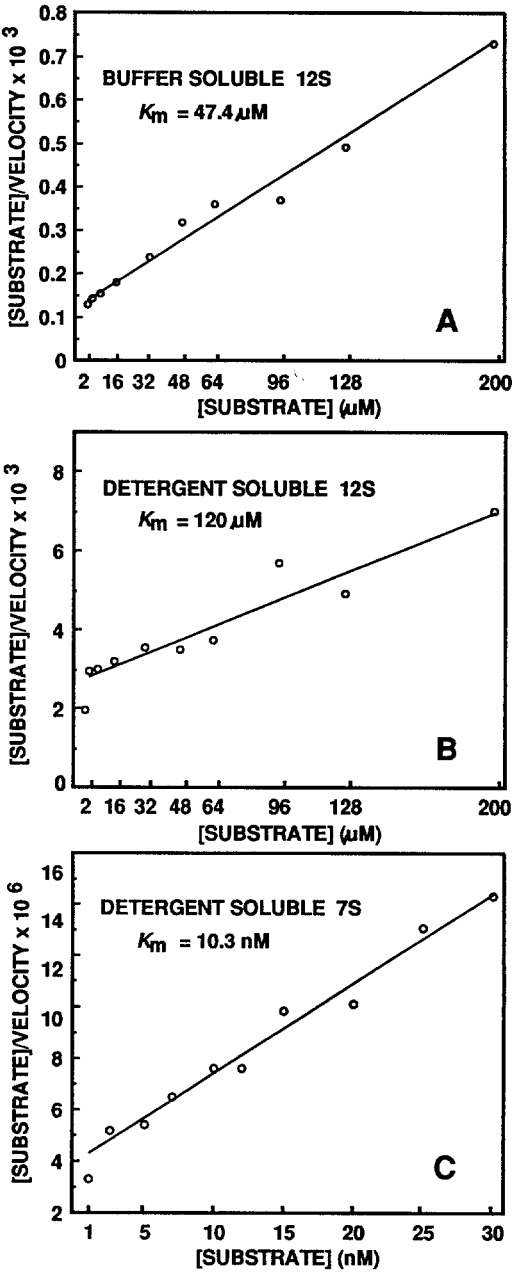

FIG. 2. Hanes-Woolfe plots of substrate affinities of separated AChE forms from *Heterodera glycines.* Substrate concentrations ranged from 0.5–200 μM ACh (A,B) and 1–30 nM (C). Incubation times were adjusted to yield between 2–30% conversion rates. The ratio of substrate concentration to velocity was plotted against substrate concentration, and the K_m was determined by linear regression.

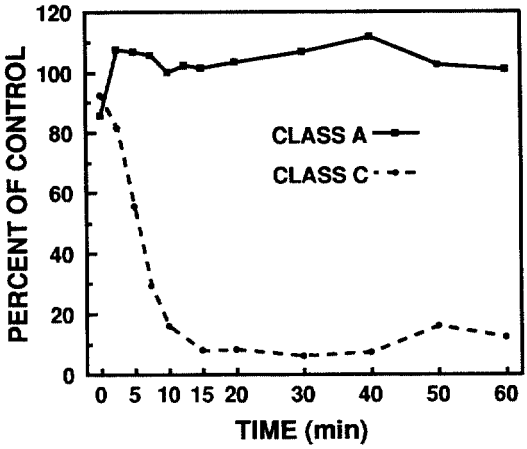

FIG. 3. Thermal inactivation of class A and C AChE from *Heterodera glycines*. Class A (buffer-soluble 12S form) was assayed at 1 μM ACh, and class C (late-eluting 7S form) was assayed at 10 nM. Activity is shown as percentage of parallel untreated controls.

Somewhat surprisingly, forms corresponding to class B type AChE were not recoverable in *H. glycines*. Class B AChE in *M. arenaria* and *M. incognita* is a detergent-soluble form that co-sediments with class C in sucrose gradients. These classes can be separated by ion-exchange chromatography and by their very different substrate affinities. Although class B exhibits similar substrate affinity to class A, it is not detergent-sensitive and is 10–100 times less sensitive to carbamate and organophosphate inhibitors than is class A. Repeated efforts were made to characterize the early eluting 7S minor peak that was separated from the class C-like AChE by ion-exchange chromatography, but the early peak was present in very small quantities and consistent activity was not obtained. This peak was possibly a degradation product from one of the other forms because its appearance was variable. If not a degradation product, it must represent a very minor form of AChE. The fact that its activity was not enhanced by low substrate levels indicates that it was not related to the later eluting, more abundant class C peak.

From a behavioral standpoint, the lack of class B AChE activity may affect immediate sensitivity to low doses of nematicides. Class A type AChE is the most sensitive class to inhibitors and is thought to be most likely to occur extracellularly at the neuromuscular synapse, whereas class B and C type AChE forms are likely to be intracellular and membrane bound (6,13). Because classes A and B occur at the neuromuscular junctions, species that have class B type AChE, such as *Meloidogyne*, may compensate for class A inhibition more rapidly under low dosage stress. Mutational analysis in *C. elegans* indicates that class C AChE is unable to completely compensate for the loss of the other AChE classes (7). Because *H. glycines* has only classes A and C AChE, sublethal behavioral effects may occur at lower inhibitor doses during the initial exposure period than in nematodes with classes A, B, and C. Compensation by class C may either take longer to be implemented or is not complete.

Nevertheless, *H. glycines* possesses a reserve of class C AChE. Previous studies have revealed that class C is relatively inaccessible to inhibitors (13). Experiments in our laboratory (Opperman, unpubl.) have demonstrated that *H. glycines* possesses significantly more class C AChE activity per mg protein than do either *C. elegans* or *Meloidogyne* spp. Thus, *H. glycines* probably tolerates higher doses of carbamate and organophosphate nematicides than species with lesser amounts of this enzyme type, such as *Meloidogyne* spp. For example, in *M. arenaria* and *M. incognita*, the 7S detergent-soluble peak is composed of both classes B and C AChE, whereas in *H. glycines* it is almost entirely class C. This distribution accounts for the lower overall sensitivity of *H. glycines* crude AChE preparations and the lower in vivo sensitivity to conventional dosages of carbamate and organophosphate nematicides than respective properties of *Meloidogyne* species. Additionally, once an *H. glycines* female has initiated a feeding site and has lost the ability to move, it may utilize class C AChE exclusively for the pharyngeal musculature. If this is the case, then systemic nematicides may not have much effect on nema-

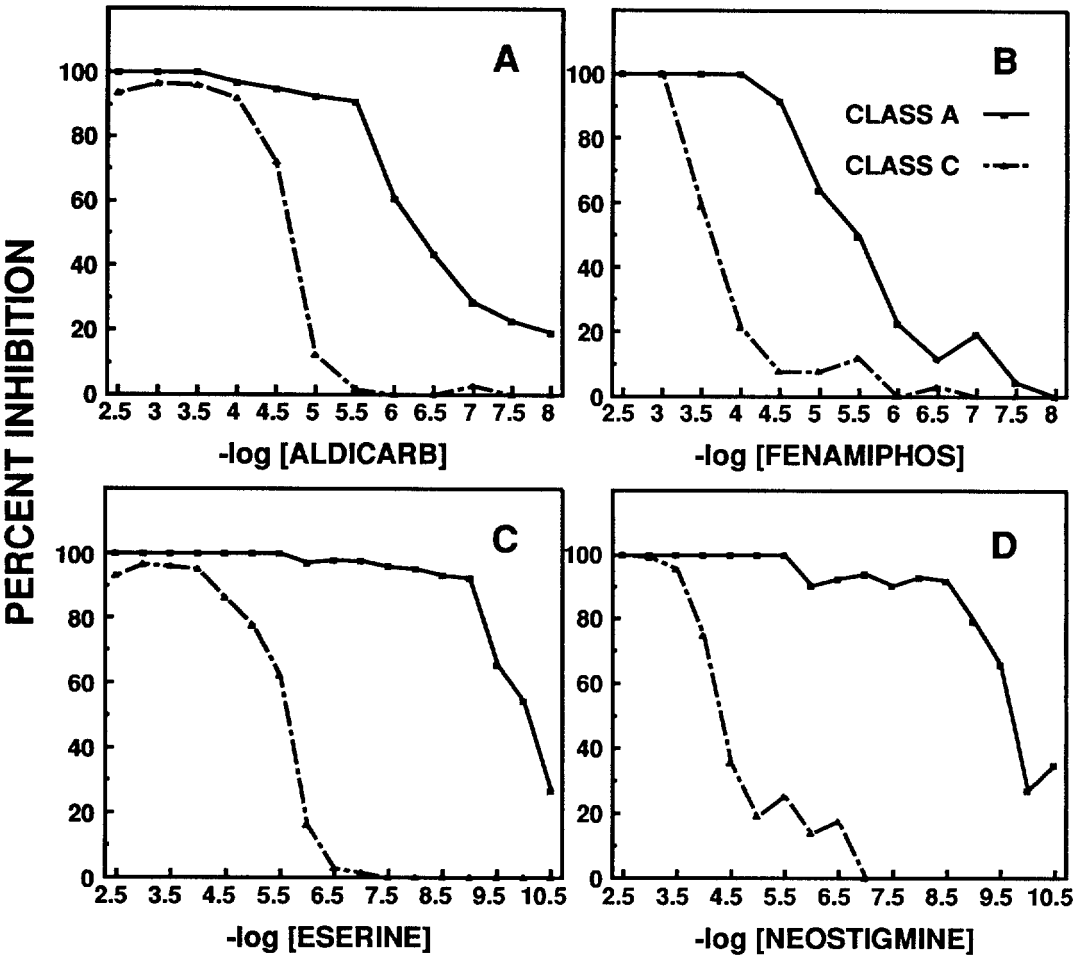

FIG. 4. Effects of inhibitors on classes A (solid line) and C (broken line) AChE from *Heterodera glycines*. Activities are plotted as 1 − [(treated CPM − blank)/(untreated CPM − blank)] × 100. Class A (buffer-soluble 12S form) was assayed at 1 μM ACh, and class C (late eluting, detergent-soluble 7S form) was assayed at 10 nM ACh. Inhibitor concentrations ranged from 3×10^{-3} M to 3×10^{-11} M. A) Aldicarb. B) Fenamiphos. C) Eserine. D) Neostigmine.

todes already within roots. Although these scenarios have yet to be fully examined, they indicate the critical need to understand the developmental biochemistry of target pests when nematicides are applied. Further analysis will be necessary to understand the relative roles of AChE molecular forms in plant-parasitic nematode behavior.

LITERATURE CITED

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

2. Bunt, J. A. 1975. Effect and mode of action of some systemic nematicides. Mededeling Landbowhogeschool Wageningen 75-10:1–125.

3. Chang, S., and C. H. Opperman. 1991. Characterization of the molecular forms of acetylcholinesterase of the root-knot nematode, *Meloidogyne*. Molecular and Biochemical Parasitology 49:205–214.

4. Cuany, A., J.-B. Bergé, J.-M. Bride, and S. Hamadène-Sellami. 1984. Inhibition et réactivation des acétylcholinestérases amphidiales de *Meloidogyne javanica* par l'éthoprophos et l'aldicarb: Tentative de corrélation avec une réaction comportementale. Revue de Nématologie 7:173–176.

5. Gourd, T. R. 1990. Differential activity of selected nematicides on *Heterodera glycines* races and *Meloidogyne* species. Ph.D. Thesis, North Carolina State University, Raleigh, NC.

6. Johnson, C. D., and A. O. W. Stretton. 1980.

Neural control of locomotion in *Ascaris:* Anatomy, electrophysiology, and biochemistry. Pp. 159–195 *in* B. M. Zuckerman, ed. Nematodes as biological models, vol. 1. New York: Academic Press.

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

8. 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.

9. Mulder, J. G., and J. Bakker. 1988. Selective inhibition of acetylcholinesterase isozymes from *Panagrellus redivivus* by aldicarb and ethoprop. Mededelingen Faculteit Landbouwwetenschappen Rijksuniversiteit Gent 53/2b:911–918.

10. Nordmeyer, D., and D. W. Dickson. 1990. Multiple molecular forms of cholinesterase in the plant-parasitic nematodes *Meloidogyne incognita* and *Radopholus similis.* Revue de Nématologie 13:311–316.

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

12. Opperman, C. H., and S. Chang. 1991. Effects of aldicarb and fenamiphos on acetylcholinesterase and motility of *Caenorhabditis elegans.* Journal of Nematology 23:20–27.

13. 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.

14. Wright, D. J. 1981. Nematicides: Mode of action and new approaches to chemical control. Pp. 421–449 *in* B. M. Zuckerman and R. A. Rohde, eds. Plant parasitic nematodes, vol. 3. New York: Academic Press.