Inhibition of cholinesterase activity by azinphos-methyl in two freshwater invertebrates: Biomphalaria glabrata and Lumbriculus variegatus

Gisela Kristoffa, Noemi Verrengia Guerreroa, Ana María Pechén de D’Angelob, Adriana C. Cochón a,∗

a Departamento de Química Biológica, Facultad de Ciencias Exactas y Naturales, Universidad de Buenos Aires, Ciudad Universitaria, Núcleo, 1428 Buenos Aires, Argentina
b LIBQUMA, Universidad Nacional del Comahue, Buenos Aires 1400, 8300 Neuquén, Argentina

Received 21 November 2005; received in revised form 10 February 2006; accepted 17 February 2006
Available online 4 April 2006

Abstract

In this study, some biochemical features and the extent of inhibition induced by the organophosphorous pesticide azinphos-methyl on the cholinesterase (ChE) activity present in whole soft tissue of two freshwater invertebrate species, the gastropod Biomphalaria glabrata and the oligochaete Lumbriculus variegatus were investigated. Both invertebrate organisms presented marked differences in ChE activity, type of enzymes and subcellular location. Acetylthiocholine was the substrate preferred by B. glabrata ChE. The enzyme activity was located preferentially in the supernatant of 11,000 × g centrifugation and was inhibited by increasing concentrations of substrate but not by iso-OMPA. Results showed that there were progressive inhibitions of the enzyme activity, with values 21%, 59%, 72%, 76%, and 82% lower than the control at levels of 1, 10, 50, 100 and 1000 µM of eserine, respectively. In contrast, L. variegatus ChE activity was distributed both in the supernatant and pellet fractions, with values approximately 6 and 20 times higher than B. glabrata, respectively. Studies with butyrylthiocholine and iso-OMPA suggested that about 72% of the activity corresponded to butyrylcholinesterase. A strong enzyme inhibition (88–94%) was found at low eserine concentrations (1–10 µM). ChE activity from L. variegatus and B. glabrata was inhibited by in vivo exposure to azinphos-methyl suggesting that both species can form the oxon derivative of this pesticide. However, both invertebrate species showed a very different susceptibility to the insecticide. The NOEC and EIC50 values were 500 and 1000 times lower for L. variegatus than for B. glabrata, reflecting that the oligochaetes were much more sensitive organisms. A different pattern was also observed for the recovery of the enzymatic activity when the organisms were transferred to clean water. The recuperation process was faster for the oligochaetes than for the gastropods. Mortality was not observed in either of the experimental conditions assayed, not even at concentrations that induced 90% of ChE inhibition. The differences in substrate specificity, sensitivity to inhibitors, and subcellular location between the ChEs of B. glabrata and L. variegatus could be the main factors contributing to the differential susceptibility to azinphos-methyl ChE inhibition found in the present study.

Keywords: Cholinesterase; Azinphos-methyl; Biomphalaria glabrata; Lumbriculus variegatus

Abbreviations: ChE, cholinesterase; AChE, acetylcholinesterase; AsCh, acetylthiocholine; BChE, butyrylcholinesterase; BsCh, butyrylthiocholine; DTNB, 5,5′-dithio-2-his-nitrobenzate; iso-OMPA, tetraisopropyl pyrophosphoramide

∗ Corresponding author at: Facultad de Ciencias Exactas y Naturales, Universidad de Buenos Aires, Departamento de Química Biológica, Ciudad Universitaria, Pab. II. 4to piso, 1428 Buenos Aires, Argentina. Tel.: +54 11 4576 3342, fax: +54 11 4576 3342.
E-mail address: adcris@qb.fcen.uba.ar (A.C. Cochón).

Available online at www.sciencedirect.com
TOXICOLOGY
Toxicol. 222 (2006) 185–194
www.elsevier.com/locate/toxicon

© 2006 Elsevier Ireland Ltd. All rights reserved.
doi:10.1016/j.tox.2006.02.018
1. Introduction

For decades, the activity of the enzyme acetylcholinesterase (AChE, EC 3.1.1.7) has been used as a sensitive biomarker of exposure to organophosphorous and carbamate pesticides (Timbrell, 2000; Walker et al., 2001). Its inhibition is linked with the pesticide mechanism of toxic action, irreversible or reversible binding to the esteratic site of the enzyme and potentiation of cholinergic effects in the nervous system.

As AChE is readily inhibited by organophosphorous compounds it is considered a Type B esterase (Peakall, 1992). This group also includes the butyrylcholinesterase (BuChE: acetylcholine acyl hydrolase, EC 3.1.1.8), neurotoxic esterases, and carboxylesterases (Peakall, 1992). AChE is regarded as a true cholinesterase, since it hydrolyzes preferentially acetylcholine and apart from the nervous system, it is also present in erythrocytes (Huggett et al., 1992). BCME, regarded as a pseudocholinesterase, hydrolyzes preferentially butyrylcholine and it is found in various tissues, e.g. in liver, intestine, serum, heart, and lung (Huggett et al., 1992; Li et al., 2000). In addition, both cholinesterases exhibit different sensitivity towards inhibitors. AChE presents inhibition by an excess of substrate, it is inhibited by eserine but insensitive to iso-OMPA, while BCME is specifically inhibited by iso-OMPA (Hyne and Maher, 2003).

The classification, characteristics and tissue localization given before are generally valid for cholinesterases (ChEs) derived from vertebrates, in particular from mammalian species. In the last case, the ChEs may exhibit a wide variety of substrate specificities. For instances, in the lake trout Salvelinus namaycush predominates AChE, in the crab Uca pugnax it is BCHE, and in ducks it is a ChE propionylcholine specific (Huggett et al., 1992).

On the other hand, ChEs from different sources may present a large variety of molecular forms, different solubility and mode of membrane anchorage (Massoulié et al., 1993).

Although aquatic invertebrates have been widely used as bioindicator organisms in many monitoring programs, the use of ACHE inhibition as a biomarker in these species has been largely neglected (Hyne and Maher, 2003). Inhibition of ChE activity by organophosphorous and carbamate pesticides has been measured in a few aquatic invertebrate species, such as bivalve mollusks (Bocquené et al., 1997; Basack et al., 1998; Mora et al., 1999; Doran et al., 2001; Cooper and Bidwell, 2006), and crustaceans (Day and Scott, 1990; Lundebye et al., 1997; Sturm and Hansen, 1999; Varó et al., 2002). Since the properties of ChE may differ from species to species, it is important to characterize the type of enzyme(s) present in the species to be studied before its use as biomarker. Moreover, more than one ChE may be present and these ChEs may show different sensitivities to anticholinesterase agents. In this sense, Bocquené et al. (1997) described the presence of two ChE in the common oyster, one insensitive to organophosphates and carbamates, anchored to membrane and that hydrolyses AsCh but not BsCh, and the other, hydrophilic and highly sensitive to organophosphates and carbamates that hydrolyses AsCh and to a much lesser extent BsCh. Mora et al. (1999) also described a ChE that was poorly inhibited by organophosphates in marine mussels and in a freshwater bivalve. In addition, Basack et al. (1998) described two ChE activities in Corbicula fluminea, one present in the pellet of 10,000 × g centrifugation sharing all the features of vertebrate ACHE, and another localized in the supernatants of 10,000 × g relatively insensitive to eserine.

In this work two main aspects were investigated. Firstly, a preliminary biochemical characterization of the ChE enzymes present in two freshwater invertebrate species, the gastropod Biomphalaria glabrata and the oligochaete Lumbriculus variegatus, was undertaken. Secondly, a comparative study of the extent of ChE inhibition induced by azinphos-methyl, a widely used pesticide in the Northern Patagonia agricultural areas, was performed.

2. Materials and methods

2.1. Chemicals

Acetylthiocholine iodide (AsCh), butyrylthiocholine iodide (BuCh), 5,5′-dithio-2-bis-nitrobenzoate (DTNB), physostigmine hemisulfate salt (eserine), and terrasopropyl pyrophosphate (iso-OMPA) were obtained from Sigma (St. Louis, MO). Azinphos-methyl (98.3% pure) was a gift from Bayer, S.A., Argentina. All other chemicals used were of analytical reagent grade.

2.2. Organisms selected

B. glabrata snails were originally obtained from a laboratory culture established at the Invertebrate Laboratory, Department of Biology, Faculty of Exact and Natural Sciences, University of Buenos Aires. The organisms were then cultured in our laboratory under standard conditions in aerated glass aquariums (17–20 L), at a temperature of 22 ± 2°C, and under a 14–10h artificial light-dark photoperiod regime. For the cultures animals were fed lettuce leaves ad libitum (Fried et al., 1992). For all the experiments, adult snails of similar size were used.
A culture of *L. variegatus* was received from the Ecotoxicology Research Group directed by Prof. K. Simkiss, School of Animal and Microbial Sciences, University of Reading, United Kingdom. The cultures were maintained under static conditions in 12 L aquaria, at 22°C, a photoperiod of 14:10 h light/dark. The aquaria were filled with shredded non-bleached paper towels (depth of 3–6 cm) and containing approximately 8 L of dechlorinated tap water, constantly aerated. The overlying water was changed every 7 days. Each culture was fed three times a week with suspensions of finely ground TetraFin fish flakes (0.5 g in 25 ml of dechlorinated water). Adult organisms of 3.5 ± 0.5 cm of length were used.

### 2.3. Bioassays

Acute static bioassays were performed throughout. Triplicate groups of three snails each were placed in small aerated plastic aquaria (3L each, pre-washed with 2% (v/v) nitric acid solution in distilled water), and containing 600 mL of each test solution. In the case of worms, nine groups of 20 organisms were placed in acid washed glass vials containing 20 mL of the test solutions, without aeration. During the treatments animals were not fed. All the tests were performed at a temperature of 22 ± 2°C, and under a photoperiod of 14:10 h light/dark. No mortality was observed either in control animals or in any of the treatments. Carbon filtered dechlorinated tap water was used as the diluent for all tests. The following physico-chemical parameters were recorded: total hardness = 67 ± 3 mg CaCO3 L−1, alkalinity = 29 ± 2 mg CaCO3 L−1, pH = 7.0 ± 0.2 and conductivity = 250 ± 17 μS. Aqueous solutions containing the pesticide azinphos-methyl were prepared by dissolving the pesticide in acetone, and diluted with an appropriate amount of dechlorinated tap water. The concentration of acetone was kept at 0.05% in all pesticide solutions used. Solvent (acetone 0.05%) and solvent-free (dechlorinated tap water only) controls were included. Activities of ChE in *B. glabrata* and *L. variegatus* from the solvent and solvent-free controls did not differ significantly (p>0.05). Therefore, data for ChE activity of both organisms from the solvent and solvent-free controls were combined for statistical analysis.

For the studies with different concentrations of azinphos-methyl, snails were exposed to six nominal pesticide concentrations (0.001, 0.004, 0.005, 0.006, 0.0075, 0.01, 0.05, 0.1, and 0.25 mg L−1). In the case of the worms, the organisms were exposed to nine nominal concentrations (0.006 and 0.1 mg azinphos-methyl L−1 (*L. variegatus*) or 5 mg L−1 (*B. glabrata*), and ChE activity was recorded at 24, 48, 72, and 96 h. In the case of exposure–recovery studies, the organisms were exposed to the above mentioned concentrations of the pesticide for 48 h and then transferred to clean dechlorinated tap water. ChE activity was recorded after 0, 7, 14, and 21 days.

### 2.4. Cholinesterase activity

Following pesticide exposure, animals were placed on ice for 6–8 min. In the case of the snails, the shells were carefully removed and the soft tissue isolated at 0°C. The whole body tissues were washed in distilled water, placed on filter paper to drain extra fluids, and weighed. Pools of 10 worms each were treated similarly. Tissues were homogenized in 20 mM Tris/HCl buffer, pH 7.5, plus 0.5 mM EDTA. Homogenates were centrifuged at 11,000 × g for 20 min at 4°C. ChE activity was measured, in duplicate, in 100 mM phosphate buffer, pH 8.0, 0.2 mM DTNB, AsCh 0.75 mM according to the method of Ellman et al. (1961). Activity was recorded continuously at 412 nm. Rates were corrected for spontaneous hydrolysis of the substrate and non-specific reduction of the chromogen by tissue extracts. The enzymatic activity was expressed as μmol min−1 mg protein−1.

To assay the in vitro effects of eserine (1, 10, 50, 100, and 1000 μM) and iso-GMPS (1 mM) on the enzyme activity the compounds were preincubated with the enzyme for 15 min at 20°C before substrate addition. Eserine and iso-GMPS dilutions were prepared in distilled water. Controls with distilled water were also included. Four independent replicates of each inhibitor concentration were performed.

Substrate preference in supernatants and pellets was assayed using AcCh (0.75 mM) and BsCh (1.5 mM) as substrates. The effects of increased substrate concentration on supernatant ChE activity were determined with concentrations of AsCh and BsCh ranging from 0.7 to 33.6 mM. At least three independent replicates of each substrate were performed.

Protein content was determined according to the method of Lowry et al. (1951), using bovine serum albumin as standard.

### 2.5. Data analysis

Results were expressed as mean ± S.D. Data were subjected to one-way ANOVA. When overall F statistic was significant, pair-wise comparisons among treatment groups were performed by Tukey–Kramer test (Sokal and Rohlf, 1997). Values of effect concentration 50 and 95% confidence intervals were used to determine the level of azinphos-methyl exposure that produces 50% of inhibition on ChE activity (IC50). They were calculated using the US EPA Probit Analysis Program, version 1.5 (US EPA, 1992). Computations performed by the program are based on Finney (1971). The ChE inhibition no observed effect concentration (NOEC) was experimentally...
determined as the maximum concentration causing an inhibition not significantly different from control values, tested by ANOVA. In all cases, results were considered to be significant at $p < 0.05$.

3. Results

3.1. Characterization of ChE

Measurements of ChE activity from the whole body soft tissue of $B. glabrata$ and $L. variegatus$ were performed using two substrates: AcCh and BsCh. The use of AsCh allows the determination of the total ChE activity, while the use of BsCh reflects more closely the activity of BChE only. The activity was measured either in the supernatant or in the pellet obtained after centrifuging at 11,000 $\times g$ for 20 min. The results are shown in Table 1.

As expected the highest enzymatic activity was observed when using AsCh either in the supernatant or in the pellet from both organisms. In $B. glabrata$ almost all the activity was found when using AsCh as the substrate and it was principally located in the supernatant fraction. Instead very low levels of activity were detected when BsCh was used, both in the supernatant and the pellet. A different pattern was found for $L. variegatus$. By far, the oligochaetes presented higher values of enzymatic activity than $B. glabrata$ organisms regardless of the substrate assayed. In addition, comparable values of activity were detected with the two substrates both in the supernatant fraction and in the pellet. According to these results, all other experiments were performed using AsCh as the substrate and analyzing the supernatant fraction, where the highest enzymatic activity was observed for both organisms.

The extent of enzyme inhibition caused by iso-OMPA and eserine is presented in Fig. 1A and B for $B. glabrata$ and $L. variegatus$ supernatants, respectively. ChE from the supernatants of $B. glabrata$ was not significantly inhibited by iso-OMPA, confirming the low value of activity observed when it was assayed with BsCh as substrate (Table 1). On the other hand, iso-OMPA induced a significant inhibition of ChE activity in the supernatants of $L. variegatus$. It is worth noting that

![Graph showing ChE activity inhibition by iso-OMPA and eserine](image)

**Fig. 1.** Effect of iso-OMPA and eserine on ChE activity of $B. glabrata$ and $L. variegatus$. ChE inhibition by (A) iso-OMPA and (B) eserine was determined by pre-incubating $B. glabrata$ and $L. variegatus$ supernatants with various concentrations of drug for 15 min and then assaying for enzyme activity in the presence of AsCh. Each bar represents the mean ± S.D. of four determinations. The asterisk indicates significant differences from controls at $p < 0.05$.

<table>
<thead>
<tr>
<th>Substrate</th>
<th>$B. glabrata$</th>
<th>$L. variegatus$</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Supernatant</td>
<td>Pellet</td>
</tr>
<tr>
<td>AsCh</td>
<td>0.046 ± 0.006</td>
<td>0.013 ± 0.006</td>
</tr>
<tr>
<td>BsCh</td>
<td>0.003 ± 0.001</td>
<td>0.005 ± 0.001</td>
</tr>
<tr>
<td>AsCh/BsCh ratio</td>
<td>15.33</td>
<td>2.60</td>
</tr>
</tbody>
</table>

The enzyme activity was determined using acetylcholine (AsCh), and butyrylcholine (BsCh) as substrates. Results are given as mean ± S.D. of four determinations.
a similar inhibition (71%) by iso-OMPA was also found in the pellet fraction of the oligochaetes (data not shown).

Eserine induced a significant inhibition in the supernatant ChE activity of both organisms. A high inhibition was observed in L. variegatus assayed at all eserine levels assayed. However, at the lower levels of eserine, ChE activity in B. glabrata was not so strongly inhibited. Nevertheless, the remaining activities were significantly lower than the value observed for control snails.

Finally, the effect of increasing substrate concentrations on ChE activity was investigated in the supernatant fractions. The results are presented in Fig. 2. Increased concentrations of ACh induced a progressive decrease in the ChE activity for both organisms, reaching values higher than 80% of inhibition at 33.6 mM of substrate. A very similar pattern was observed for increased concentrations of BsCh in L. variegatus but the level of 80% of inhibition was found at 11.2 mM of substrate.

3.2. Enzyme inhibition by azinphos-methyl

The values of ChE activity in B. glabrata and L. variegatus treated for 48 h with different nominal azinphos-methyl concentrations are shown in Fig. 3A and B, respectively. Concentration-dependent enzyme inhibition could be detected in both species. However, L. variegatus was much more sensitive than B. glabrata to the pesticide-induced ChE inhibition. A significant enzyme inhibition could be detected in L. variegatus at a level as low as 0.004 mg azinphos-methyl L⁻¹. Instead, a comparable ChE inhibition could be detected in B. glabrata at a level 625 fold higher (2.5 mg L⁻¹). The highest inhibition achieved in B. glabrata and L. variegatus was 66% at 15 mg azinphos-methyl L⁻¹ and 99% at 0.25 mg azinphos-methyl L⁻¹, respectively.

Table 2 shows the concentrations of azinphos-methyl required to cause 50% inhibition of enzyme activity (EIC50) and the no observed effect concentration (NOEC) for the two organisms. Both parameters were
Table 2

Values of azinphos-methyl concentration where it was found the 50% of inhibition (EIC50) and the non-observed effect concentration (NOEC) on ChE activity.

<table>
<thead>
<tr>
<th></th>
<th>B. glabrata</th>
<th>L. variegatus</th>
</tr>
</thead>
<tbody>
<tr>
<td>EIC50</td>
<td>5.960 (2.670–8.410)</td>
<td>0.006 (0.005–0.010)</td>
</tr>
<tr>
<td>NOEC</td>
<td>0.500</td>
<td>0.001</td>
</tr>
</tbody>
</table>

*a 95% confidence intervals.

*b Experimentally determined as the maximum concentration eliciting a ChE activity not significantly different from control values, tested by analyses of variance.

much higher for B. glabrata than for L. variegatus (three and four orders of magnitude, respectively).

Moreover, locomotive disorders were observed in L. variegatus exposed to NOEC and higher concentrations. At the NOEC level (0.001 mg azinphos-methyl L⁻¹), the oligochaetes presented uncoordinated movements and curling of the tail. At the EIC50 level (0.006 mg azinphos-methyl L⁻¹), only the anterior segments of the oligochaetes conserved mobility. At 0.1 mg azinphos-methyl L⁻¹ (90% ChE inhibition) individuals showed total lack of movement, contraction and curling of the whole body. The 0.006 and 0.1 mg L⁻¹ concentrations are documented in Fig. 4.

Fig. 4. Gross morphological appearance of L. variegatus after exposure for 48 h to azinphos-methyl. (a) Control, (b) azinphos-methyl 0.006 mg L⁻¹ and (c) azinphos-methyl 0.1 mg L⁻¹.
Fig. 5. Time course of ChE inhibition and recovery following exposure to azinphos-methyl. *B. glabrata* (A) and *L. variegatus* (B) were exposed to the insecticide for 48 h (days: −2 to 0) and then transferred to clean water for 21 days (days: 0–21). The arrow indicates the time at which the organisms were transferred. ChE activity was assayed with AsCh as substrate. Each point represents the mean ± S.D. of nine determinations. Means not followed by the same uppercase letter are significantly different at *p* < 0.05.

the other hand, no locomotive disorders were observed in *B. glabrata* organisms exposed to azinphos-methyl (0.05–15 mg L$^{-1}$) for 48 h.

The profiles illustrating time-course exposure-recovery for ChE activities in the two species can be seen in Fig. 5A and B. The organisms were exposed to the respective EIC$_{50}$ for 48 h and then transferred to clean water for 21 days. In addition, the recovery process in *L. variegatus* was also analyzed using the concentration that elicits a ChE inhibition higher than 90% (0.1 mg azinphos-methyl L$^{-1}$). In the time-course exposure study, statistically significant inhibition of ChE activity was observed after 24 h of treatment with the insecticide in both species (Fig. 5A and B). In the depuration study, *B. glabrata* snails showed a partial recovery of the enzyme activity at day 7. No further recovery was observed until day 21. At this time point, ChE activity was still 72% of the control activity (Fig. 5A). In contrast, *L. variegatus* worms exposed to their EIC$_{50}$ for 48 h showed nearly complete recuperation of ChE activity at day 21 (95% of the control value). On the other hand, the appearance of these worms resembled the relaxed appearance of control worms. Also they did not show impaired mobility. When the recovery process was analyzed after exposure to 0.1 mg azinphos-methyl L$^{-1}$ for 48 h, no significant recovery of the activity could be observed after 21 days of depuration (Fig. 5B). However, the worms showed more motility and a more relaxed appearance than before the recovery process.

4. Discussion

In the present study we performed a partial characterization of ChE activity in homogenates from the whole body soft tissue of the freshwater invertebrates *B. glabrata* and *L. variegatus*. Although both invertebrates have been recommended for water toxicity tests, to our best knowledge the ChEs present in these species have not yet been investigated. The use of snail whole body soft tissue was required to allow direct comparisons with *L. variegatus* organisms, where anatomical regions are not easily distinguished due to the small size of these oligochaetes.

According to our results, both invertebrate organisms present marked differences in ChE activity, type of enzymes and subcellular location. When using AsCh as substrate, almost all the activity was confined to the supernatant fraction of the whole body homogenates of *B. glabrata* snails. In addition, the enzyme activity was inhibited by increasing concentrations of substrate, reaching 91% of inhibition at 33.6 mM of AsCh. When using BsCh as substrate, very low levels of activity were detected which were very similar both from the pellet and the supernatant fraction. In vitro studies showed that there were progressive inhibitions of the enzyme activity, with values 21%, 59%, 72%, 76%, and 82% lower than the control at levels of 1, 10, 50, 100 and 1000 µM of eserine, respectively. In addition, *iso*-OMPA induced a slight decrease in total ChE activity. These results suggest that a very low proportion of the enzyme activity could be due to BChE and that about 28–18% of activity might be the contribution of esterases other than ChE since 50–1000 µM of eserine inhibited only 72–82% of the activity. A comparable percent of activity that was not inhibited at concentrations of eserine from 50 to 800 µM were found in *Artemia salina* and *A. parthenogenetica* (Varó et al., 2002). In contrast, Basack et al. (1998) reported that about 58% of activity was not inhibited at 1000 µM of eserine in the supernatant fraction of *C. fluminea*.

On the other hand, and when using AsCh as substrate, *L. variegatus* oligochaetes presented values approxi-
G. Kroon et al. / Toxicology 222 (2006) 185–194

approximately 6 and 20 times higher ChE activity than *B. glabrata* snails, either in the supernatant fraction or in the pellet, respectively. As in *B. glabrata*, ChE activity in the oligochaetes was inhibited by increasing concentrations of substrate, reaching 86% of inhibition at 33.6 mM of AcCh. A strong enzyme inhibition (88–94%) was found in the range of 1–10 μM of eserine, confirming that the activity measured was due to ChE and not to other types of esterases. When using BoCh as substrate, significant values of enzyme activity were detected that represented about 72% of the total ChE activity from both the supernatant and the pellet. In agreement with this, the activity measured in the presence of *iso*-OMPA was 28% and 29% of that measured in the absence of the inhibitor, both in supernatant and pellet, respectively. These results suggest that approximately 72% of the enzyme activity could be attributed to a BoChE activity in *L. variegatus*. However, this activity did not resemble vertebrate BoCHe since it showed inhibition by excess of BoCh. In agreement with our results, Pezzennenti et al. (2003) described the presence of a BoChE that hydrolyses and shows substrate inhibition with both AoCh and BoCh in the amphipod *Branchiostoma floridanus*.

The present study shows that ChE activity from *L. variegatus* and *B. glabrata* was inhibited by in vivo exposure to azinphos-methyl. However, both invertebrate species showed a very different susceptibility to the insecticide. The NOEC and EIC50 values were 500 and 1000 times lower for *L. variegatus* than for *B. glabrata*, reflecting that the oligochaetes were much more sensitive organisms. Mean ChE activity was significantly reduced in both organisms within 24 h of exposure, with higher reductions in activity at 48 h. In agreement with studies in other species (Ferrari et al., 2004b), exposure of both organisms to azinphos-methyl for 72 and 96 h, either in static assays or with renewal of the media, resulted in a ChE inhibition not significantly different from that observed at 48 h (data not shown). It is worth noting that we did not observed mortality in any of the experimental conditions assayed, even at concentrations that induced 90% ChE inhibition. The correlation between ChE inhibition and mortality has not been fully established. Thus, while some species tolerate high levels of ChE inhibition without mortality some others die (Chambers and Carr, 1995; Fulton and Key, 2001). In agreement with our results, Ferrari et al. (2004a) described that after exposing goldfish for 96 h to 0.1 mg azinphos-methyl L−1 a ChE inhibition of 90% was induced without lethality. In fact, the lethal concentration 50 (LC50) was 70-fold higher. Similar results have been reported for other fish species and crustaceans where sublethal concentrations of organophosphates induced more than 70–90% of ChE inhibition (Gruber and Munn, 1998; Varó et al., 2002; Varó et al., 2003). Moreover, the correlation between ChE inhibition and the onset of symptoms of intoxication is complex and variable. In this study we observed locomotive disorders in *L. variegatus* even at the lowest concentration of azinphos-methyl assayed. On the contrary, no symptoms of locomotive disorders were observed in *B. glabrata* despite ChE inhibition reached 66%. Similarly, Ferrari et al. (2004a) have not observed signs of intoxication in goldfish exposed to azinphos-methyl even at inhibitions of 90% of ChE activity. At this respect, Pope (1999) postulated that although the initial step in organophosphates pesticides mechanism of toxicity is the interaction between the toxicant and the target enzyme ChE, it is not sufficient by itself to lead to cholinergic toxicity. Thus, modulation of any of the neurochemical processes involved in acetylcholine synthesis and release, cholinergic receptor binding, or signal transduction, concurrent with the pesticide exposure, could influence the progression of events from AChE inhibition to the expression of neurotoxicity.

A different pattern was also observed for the recovery of the enzymatic activity when the organisms were exposed to their respective EIC50 values for 48 h and then transferred to clean water. After 21 days of depuration, the snails showed a slight increase in ChE activity but they were unable to reach the control value. Instead, after 14 days *L. variegatus* reached more than 80% and after 21 days approximately 95% of the control value, indicating that the recuperation process was faster for the oligochaetes. It is worth noting that when ChE inhibition reached 90%, no significant recovery of the activity was observed in *L. variegatus* after 21 days of depuration. Similarly, Ferrari et al. (2004a) reported that 35 days of depuration were needed to almost reach the value of control activity in goldfish exposed to a level of azinphos-methyl that had induced a 90% of ChE inhibition.

Azinphos-methyl is an organophosphate insecticide containing a phosphorothioate group. Thus, the parent compound needs to form the oxon derivative to actually inhibit the ChE enzyme. In vertebrates this reaction is mediated by the mixed function monoxygenase system (MFO), located in microsomes, which contains the cytochrome P450, a flavoprotein and the reduced form of either β-nicotinamide adenine dinucleotide phosphate (NADPH) or β-nicotinamide adenosine dinucleotide (NADH) as cofactor (Timbrell, 2000). Interspecies variations of metabolic activities, either quantitative or qualitative, may account for differences in the toxicity of the organophosphate insecticides, such as the extent of cholinesterase inhibition (Livingstone, 1998; Timbrell, 2000). Previous studies have demonstrated that the activ-
ity of the MPO system is maximal in mammals, followed by birds and finally fish and other invertebrate species (Walker, 1980; Di Giulio et al., 1995; Livingstone, 1998). However, for most aquatic invertebrates the metabolic pathways are still not completely elucidated so that it is difficult to establish general trends among them. Components of the cytochrome P450-dependent monoxygenase system have been characterized in various gastropods (Solé and Livingstone, 2005) and the isolation and characterization of the full-length cDNA encoding a member of the cytochrome P450 superfamily from B. glabrata has recently been reported (Lockyer et al., 2005). On the other hand, L. variegatus ability to metabolize xenobiotics is uncertain (A eskley and Collyard, 1995; Verrengia Guerrero et al., 2002). However, our data showing in vivo ChE inhibition by azinphos-methyl in B. glabrata and L. variegatus suggest that both species can form the oxon derivative of this pesticide.

Besides toxicokinetic processes, such as absorption, metabolism and excretion, toxicoelastic processes, such as differences in the sensitivity of the target(s) enzyme(s), can contribute to the differential susceptibility to ChE inhibition between species. Several works support this hypothesis. Studies performed in Gasterosteus aculeatus revealed that BCHE was 1000-fold more sensitive to paraoxon than AChE (Wogram et al., 2001). On the other hand, the presence of ChEs relatively insensitive to organophosphates in some aquatic invertebrates has been reported (Bocquené et al., 1997; Mora et al., 1999). In addition, a correlation between the size of the steratic site of the enzyme and insensitivity to organophosphorous compounds has been observed. Thus, the steratic site of the ChE of non-resistant arthropods strains was greater than that of resistant ones (Zahavi et al., 1971). Thus, the differences in substrate specificity, sensitivity to inhibitors, and subcellular localization between the ChEs of B. glabrata and L. variegatus could be the main factors contributing to the differential susceptibility to azinphos-methyl ChE inhibition found in the present study.

Acknowledgments

This work was supported by grants from the ANPCYT (PICTR 2002-02023) and from the University of Buenos Aires. G. Kristoff is recipient of an ANPCYT fellowship. We thank Carmen Aldonatti for technical assistance, Dr. Silvina Gazzaniga for photographic assistance, Valot, S.A., and the Unit of Toxicology and Legal Chemistry for providing some materials.

References


