Bull. Environ. Contam. Toxicol. (1998) 61:569-576

Bulletin of Environmental Contamination and Toxicology

Esterases of *Corbicula fluminea* as Biomarkers of Exposure to Organophosphorus Pesticides

S. B. Basack, M. L. Oneto, J. S. Fuchs, E. J. Wood, E. M. Kesten

Toxicology and Legal Chemistry, Department of Biological Chemistry, Faculty of Exact and Natural Sciences, Buenos Aires University, 4 p. Pab. II- Ciudad Universitaria, 1428 Buenos Aires, Argentina

Received: 15 April 1998/Accepted: 5 October 1998

Organophosphorus pesticides (OPs), well known anticholinesterase agents, are inherently toxic substances that may pose risks to non-target organisms. Their interaction with some esterases led to the classification of these enzymes into three categories: A-esterases, that are able to hydrolyze OPs and are not inhibited by them; B-esterases –cholinesterases (ChEs) and carboxylesterases (CaEs)–, that are typically inhibited by OPs; and C-esterases, that do not interact with this family of pesticides (Aldridge and Reiner 1972; Parkinson 1996).

The inhibition of the activity of ChEs –acetylcholinesterase (AChE, E.C 3.1.1.7) and butyrylcholinesterase (BuChE, E.C 3.1.1.8)– has been frequently used as a biomarker to assess the extent of exposure to OPs, principally in vertebrate species (Walker and Thompson 1991). Bivalve mollusks are often considered as adequate organisms for ecotoxicological monitoring due to their capacity to filter large volumes of water, accumulate a wide range of contaminants, and reflect changes in the pollution status of their environment (Sheehan et al. 1995; Basack et al. 1997). However, until recently there have been few published studies on the use of ChE measurements as exposure biomarkers on aquatic invertebrates, and the activities assessed have been very low (Bocquené et al. 1990; Day and Scott 1990; Escartín and Porte 1997). Evidence shows that ChEs are polimorphic enzymes, which may be soluble or bound to membranes depending on species and tissue. These enzymes have been often studied only in the supernatant fraction obtained from homogenates centrifuged in the range of 10,000–18,000 x g (Day and Scott 1990; Escartín and Porte 1997). However, according to Fairbrother et al. (1991), it is essential to characterize ChE activities before using only the supernatant for enzyme activity determination.

CaEs of bivalve organisms have shown higher sensitivity to OPs than AChE, and so they have been considered good candidates to be employed as biomarkers (Escartín and Porte 1997). Organisms have many esterases, and these enzymes -present in all tissues usually in the cytosol or in the microsomes– show clear evidences of some phylogenetic differences amongst species (Walker and Thompson 1991). Although several CaEs have been found in the mussel *Mytilus galloprovincialis*, there is scarce information on how many esterases may be present in other bivalves (Ozretic and Krajnovic-Ozretic 1992).

Correspondence to: E. M. Kesten

Bivalves of the genus *Corbicula* have been used as biological monitors in freshwater environments and, according to our previous work, may be useful to assess the presence of organic compounds in water, including the less persistent chemicals (Doherty 1990; Basack et al. 1997). In order to evaluate the usefulness of the esterases of *Corbicula fluminea* taken from the Río de la Plata as potential biomarkers of OPs, this study had three different objectives. The first was to determine ChE activities both in the supernatant (ChE-S) and pellet (ChE-P) of whole soft tissue homogenates centrifuged at 10,000 x g, and the activity of CaE in the supernatant fraction. The second was to characterize ChE-S and ChE-P employing *in vitro* assays. The third objective was to assess the inhibition of CaE, ChE-S and ChE-P activities *in vivo*, by exposing the bivalves to different concentrations of the OPs parathion, paraoxon and fenitrothion.

MATERIALS AND METHODS

Acetylthiocholine iodide (ATCh); butyrylthiocholine iodide (BuTCh); 5,5'-dithiobis-2-dinitrobenzoic acid (DTNB); tetra[monoisopropyl]pyrophosphor-tetramide (iso-OMPA); physostigmine hemisulfate salt (eserine) and paraoxon (O,O-diethyl O-p-nitrophenyl phosphate) approximately 90% were purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A). Technical grade fenitrothion (O,O-dimethyl S-3 methyl-4-nitrophenyl phosphorothioate) was a gift from Sumitomo Chemical Co. (Osaka, Japan) and was greater than 95 % pure. Parathion (O,O-diethyl S-p-nitrophenyl phosphorothioate) 99 % pure was supplied by the Research Center on Pests and Pesticides -CIPEIN-CITEFA-CONICET- Buenos Aires- Argentina. The stock solutions of parathion (2 g/L), paraoxon (0.5 g/L) and fenitrothion (2 g/L) were made in absolute ethanol. Bradford reagent was bought from Bio-Rad Laboratories (Hercules, CA, USA). All other reagents and solvents were analytical grade.

Buenos Aires tap water -filtered through animal carbon, glass wool and filter paper, and later aerated -was used as dilution water for bioassays (total hardness 67 mg/L as $CaCO_3$; alkalinity 29 mg/L as $CaCO_3$; pH 7.0). Bivalves of the species *Corbicula fluminea* were collected on three different occasions between March and August 1997 from the coast of the Rio de la Plata, nearby the Buenos Aires harbor. In the laboratory, they were acclimated to test conditions, according to international standards, in aerated glass aquaria containing dilution water (20±2 °C) with a 16h/8h day/night photoperiod (Basack et al. 1997). During the acclimatization period, the organisms were not fed. The mean length of the bivalves analyzed was 22 ± 2 mm and the mean weight 0.35 ± 0.05 g.

The whole body was excised from the shell, carefully placed on filter paper to drain extra fluids, and weighed. It was immediately frozen at –20 °C. About 15 bivalves were homogenized in a Potter Elvehjem with Tris-HCl buffer 0.1 *MI* 0.25 *M* sucrose pH 7.2) in 1:5 (tissue weight: buffer volume) ratio. The homogenate was centrifuged at 1,000 x g, and the pellet (unhomogenized cells and debris) was discarded. The supernatant fraction was then centrifuged at 10,000 x g. The supernatant and pellet fractions obtained from the latter centrifugation were used for the enzymatic studies. The pellet was washed and

resuspended in Tris buffer pH 7.4 until the protein concentration reached approximately 10 mg/mL. Bradford's method (1976) was used for quantitative determination of proteins in both fractions, employing bovine serum albumin as standard.

ChE activities were assayed by the method of Ellman et al. (1961) with slight modifications. Assay components included: 0.05 *M* Tris buffer (pH=8), DTNB (3.2×10^{-4} *M*, final concentration), ATCh (2×10^{-2} *M* final concentration), and 100 µL of enzyme –pellet or supernatant source, containing 10 mg/mL protein-, in a total assay volume of 2.5 mL. ButCh was used as substrate when BuChE activity was evaluated. The absorbance increase rate (405 nm) was recorded during 3 min at room temperature on a Shimadzu 160A dual-beam spectrophotometer.

CaE activities were determined using DTNB as chromogenic reagent, and phenylthioacetate (PTA) as substrate (Ferrero et al. 1991). The reaction was recorded during 3 min on the spectrophotometer at 412 nm in cuvettes containing 2.0 mL of DTNB/phosphate buffer pH 7.7 (2.5×10^{-4} *MI* 0.1 *M*), 100 µL of PTA (8×10^{-4} *M*, final concentration), and 100 µL of sample (supernatant containing about 1 mg of proteins). PTA was dissolved in absolute ethanol.

In all the cases, the reaction was initiated adding 100 µl of the corresponding substrate in the sample cuvette, and 100 µl of distilled water –for ChEs– or ethanol –for CaEs– in the reference cuvette in order to complete the same final volume. The spontaneous substrate hydrolysis was determined in the same way in the absence of enzyme, and was routinely subtracted. The activities were converted from absorbance units/minute to hydrolyzed substrate nmoles /min x mg protein (or specific activities). Optimum substrate concentrations were determined previously for each subcellular fraction by assaying control samples with a range of different substrate concentrations.

In order to investigate ChE-S and ChE-P behavior, inhibitory studies were performed *in vitro* by preincubating the enzyme preparations from control organisms with eserine (10^{-5} to 10^{-3} *M*, final concentration), or iso-OMPA (10^{-4} and 10^{-3} M, final concentration) in buffer/DTNB medium, 15 min before the substrate was added to start the reaction. Eserine is an inhibitor of ChEs, and iso-OMPA is a selective inhibitor of BuChE. Inhibition by ATCh excess was also assayed (Holmes and Masters 1967; Fairbrother et al. 1991).

In vivo assays (bioassays) were performed one week after collection by exposing the organisms to different sublethal concentrations of OPs during 24 hr. Groups of bivalves were exposed either to solvent (control), to parathion (20, 40 and 80 µg/L), to fenitrothion (10, 50 and 100 µg/L), or to paraoxon (2, 5, 10 and 20 µg/L) test solutions in aerated glass aquaria containing 3 bivalves/L. The solutions for bioassays were prepared adding small amounts of the ethanol solutions to dilution water in order to obtain the desired OP concentrations. The final concentration of ethanol did not exceed 50 µL/L.

All the determinations were performed three times in duplicate, and the results reported are mean values ± standard deviation (SD). Statistical significance was assessed using one-way analysis of variance (ANOVA) followed by Student-Newman-Keuls multiple comparisons test, employing GraphPad InStat software (GraphPad Software, San Diego, USA). Differences with $p<0.05$ were taken as statistically significant. Percent inhibition was calculated relative to mean value for control bivalves assayed.

RESULTS AND DISCUSSION

Table 1 summarizes the specific activities of ChE-P, ChE-S and CaE measured in control organisms of *Corbicula fluminea.* As stated before, CaEs are present in all tissues either in the cytosol or in the microsomes, remaining therefore in the 10,000 x g supernatant. CaE activity was higher and provided more accurate measurements than the activities of both ChE-P and ChE-S. Also, a recent work has reported that CaE activities were higher than ChE activities, measured in the mussel *Mytilus galloprovincialis* (Escartín and Porte 1997).

Table 1. Specific activities of cholinesterases (ChE-P and ChE-S), and carboxylesterase (CaE) from different fractions of soft tissue homogenates of *Corbicula fluminea* control organisms.

ESTERASES	FRACTION *	SPECIFIC ACTIVITY #
ChE-P	Pellet	12.1 ± 1.8
ChE-S	Supernatant	5.5 ± 0.9
CaE	Supernatant	46 ± 1

* After centrifugation at 10,000 x g.
\# All values are mean ± SD (n = 6) in nmol/min x mg protein

CaEs are known to hydrolyze a wide range of exogenous and endogenous esters, but they react only weakly with positively charged carboxylesters, such as acetylcholine (ACh) and butyrylcholine (BuCh) (Maxwell 1992). PTA, one of the many substrates used in the literature to measure CaE activity, was employed in our experiments (as previously done by other authors) because it allows us to use the kinetic method of Ellman et al. (1961), and it is very sensitive to detect CaE activity (Ferrero et al. 1991). Previous results of experiments carried out in our laboratory, indicated that PTA hydrolysis mediated by ChEs was negligible.

Table 2. Characterization of ChE-S and ChE-P from soft tissue homogenates of *Corbicula fluminea* control organisms after centrifugation at 10,000 x g.

	ChE-S	ChE-P
Substrate specificity	ATCh > BuTCh	ATCh > BuTCh
Inhibition with eserine 10^{-5} *M*	35 ± 3 %	91 ± 7 %
10^{-3} *M*	42 ± 5 %	96 ± 5 %
ATCh excess	not inhibited	inhibited
Inhibition with iso-OMPA 10^{-4} *M*	not inhibited	not inhibited

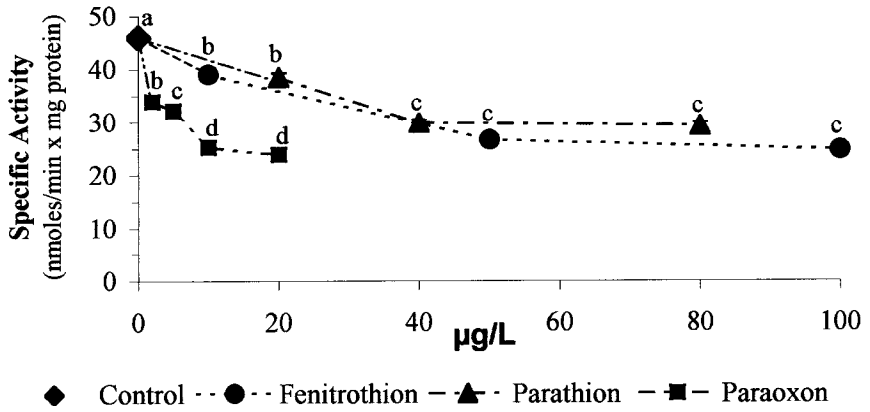

Figure 1: Effect of the organophosphorus pesticides (OPs) on the carboxylesterases (CaEs) of *Corbicula fluminea.* Each point represents the mean of three independent experiments. SD are lower than the size of symbols used (n=6). Within each treatment group, those points that do not share the same lowercase letter are significantly different ($p < 0.05$).

Some reports pointed out undetectable or very low levels of ChE activities in the supernatant fraction of mussel tissue homogenates centrifuged in the range of 10,00–18,000 x g (Bocquené et al. 1990; Ozretic and Krajnovic-Ozretic 1992; Escartín and Porte 1997). Since ChEs of some species are polimorphic enzymes, and they may have different subcellular location and association to membranes, we measured ChE activities both in pellet and supernatant fractions (Fairbrother et al. 1991). We found that ChE-P and ChE-S showed low specific activities, and that ChE-P activity was twice that of ChE-S. These results led us to investigate ChE-P and ChE-S behavior, Although it is known that ChEs of invertebrates and vertebrates may differ, their characterization is typically done using assays which are well established in vertebrates (Fairbrother et al. 1991; Bocquené et al. 1997; Escartín and Porte 1997).

The results of the *in vitro* assays, performed to characterize *Corbicula fluminea* ChEs, are summarized in Table 2. ChE-P shared all the features of vertebrate AChE, such as preferential hydrolysis of ATCh over BuTCh as substrate, nearly complete inhibition by eserine $10^{-3}M$ and $10^{-5}M$, and inhibition by ATCh excess displaying a bell shaped curve. Although the role of ChE-P in *Corbicula fluminea* must be clarified, this result may be significant since AChE is assumed to be the target enzyme for OPs toxicity in most species (Boone and Chambers 1996). ChE-S was more active against ATCh than against BuTCh, it was not inhibited by substrate excess, and showed a lack of sensitivity to iso-OMPA $10^{-5}M$ or $10^{-4}M$. Consequently, it could not be considered a BuChE. On the other hand, ChE-S had a significant residual activity even in the presence of $10^{-3}M$ eserine (near 40% inhibition), which at $10^{-5}M$ concentration totally inhibits ChEs in most species (Holmes and Masters 1967). It might be the case of an ChE less sensitive to eserine. In relation to these results, it should be mentioned that, employing invertebrate species, other authors have characterized ChEs that differ in substrate specificity and susceptibility to inhibitors from those of vertebrates (Talesa et al.

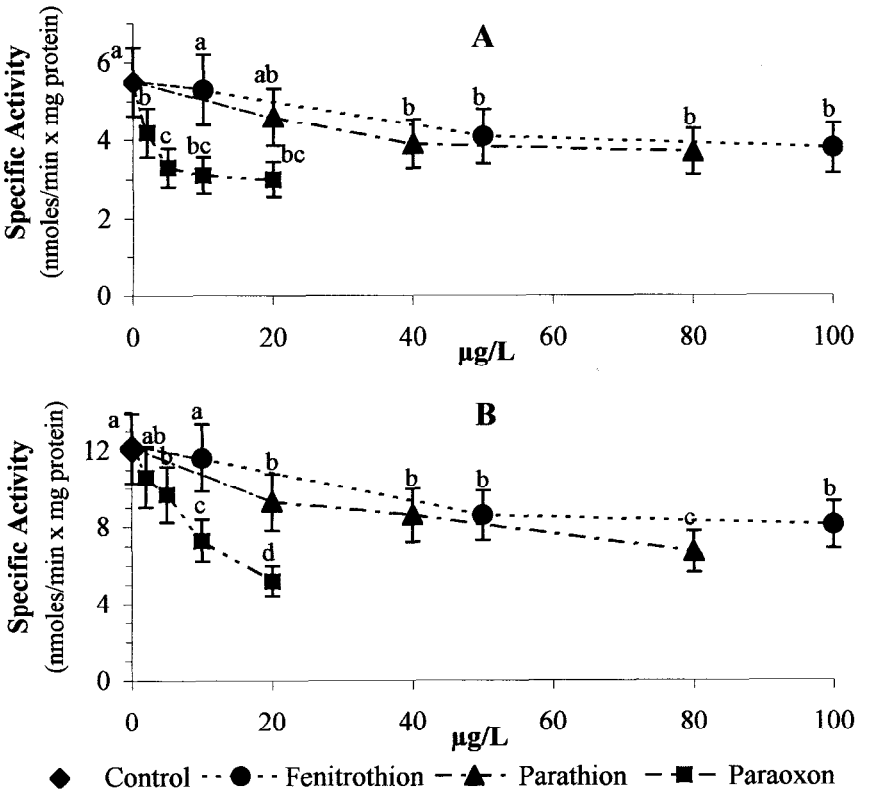

Figure 2: Effect of the organophosphorus pesticides (OPs) on the cholinesterases (ChEs) of *Corbicula fluminea.* **A.** ChE-S **B.** ChE-P. Each point represents the mean of three independent experiments.The error bars indicate SD values (n=6). Within each treatment group, those points that do not share the same lowercase letter are significantly different ($p < 0.05$).

1996). Within this context, Bocquené et al. (1997) identified different ChEs in the marine oyster *Crassosteca gigas,* one of which was insensitive to OPs. Further research is necessary to characterize *Corbicula fluminea* ChE-S better.

Regarding bioassay results, after a 24 hr exposure period to sublethal concentrations of paraoxon, parathion and fenitrothion, no mortality occurred in controls and exposed bivalves. As it can be observed in Figure 1, significant CaE inhibitions with respect to the controls were obtained at all the concentrations tested on the organisms exposed to the three pesticides. Inhibitions from 15 to 46%, 16 to 36%, and 26 to 48% were reached at the fenitrothion, parathion and paraoxon concentration ranges, respectively. However, CaEs proved to be saturated at the highest pesticide concentrations, and this agrees with the data reported by other authors (Maxwell 1992; Boone and Chambers 1996). The physiological role of CaEs is uncertain, but they are generally thought to play an important role in the detoxication of some OPs, and can afford protection against poisoning in different species (Parkinson 1996).

In Figure 2 quite different patterns of ChE-S and ChE-P inhibition by paraoxon were obtained. ChE-S appeared to be sensitive to the lowest concentration of paraoxon (2 µg/L) but it was not able to discriminate between the higher concentrations. On the other hand, although ChE-P did not show a significant inhibition at 2 µg/L paraoxon compared to the controls, its inhibition pattern seemed to be dose related, since there were statistical differences between test samples at 5, 10 and 20 µg/L paraoxon respect to the controls, as well as among the test samples themselves (Figure 2-B).

Parathion and fenitrothion produced a similar inhibition pattern for both ChE-S and ChE-P (Figure 2 A and B). The inhibitions reached suggest the possible formation of the respective oxon active metabolites. Although it is accepted that xenobiotic biotransformation reactions in bivalves are very limited, the assessment of parent compounds and their respective oxons in *Corbicula fluminea* tissues could help to explain the extent of the bioactivation (Livingstone et al. 1989; Basack et al. 1997).

According to Figures 1 and 2, paraoxon was the most inhibitory to the three enzymes studied. For example, when the organisms were exposed to paraoxon 20 µg/L, ChE-P, ChE-S, and CaE were inhibited from 2.5 to 3 times more than when they were exposed to the same concentration of parathion. This result agrees with the classical mechanism of type B-esterases inhibition by OPs in other species, where the "oxon" form is the active one (Boone and Chambers 1996).

These results represent a preliminary approach in the search for potential biomarkers for monitoring aquatic contamination by OPs. *Corbicula fluminea* esterases may be of interest since we could measure different B-esterase activities, –ChE-S, ChE-P and CaE– that were inhibited *in vivo* by the three OPs assayed. However, in designing a toxicity test we would suggest that two esterases, CaE and ChE-P, should be included: the first for its higher sensitivity, and the second because it has all the characteristics of the AChE of other species. In this work we achieved significant results with enzyme inhibitions from 25 % upwards, compared to controls. Further research should be addressed to extrapolate the importance of the reduction in enzyme activities, obtained in the laboratory, to aquatic ecosystems polluted with OPs.

Acknowledgments. We thank the Buenos Aires University for grant 191 (1994/1997) to EM Kesten and for the research fellowship given to SB Basack. This study is part of SB Basack's PhD thesis. We also thank Ms. Laura Gutiérrez for advice given regarding the English for the manuscript.

REFERENCES

Aldridge WN, Reiner E (1972) Enzyme inhibitors as substrates. North-Holland Publishing Company Amsterdam

Basack SB, Oneto ML, Verrengia Guerrero NR, Kesten EM (1997) Accumulation and elimination of pentachlorophenol in the freshwater bivalve *Corbicula fluminea*. Bull Environ Contam Toxicol 58: 497-503

Bocquené G, Galgani F, Truquet P (1990) Characterization and assay conditions for use of AChE activity from several marine species in pollution monitoring. Mar Environ Res 30: 75-89

Bocquené G, Roig A, Fournier D (1997) Cholinesterases from the common oyster (*Crassosteca gigas*). FEBS Letters 407: 261-266

Boone JS, Chambers JE (1996) Time course of inhibition of cholinesterase and aliesterase activities, and non protein sulfhydryl levels following exposure to organophosphorus insecticides in mosquitofish (*Gambusia afinis*). Fundam Appl Toxicol 29: 202-207

Bradford MM (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem 72: 248-254

Day KE, Scott IM (1990) Use of acetylcholinesterase activity to detect sublethal toxicity in stream invertebrates exposed to low concentrations of organophosphate insecticides. Aquat Toxicol 18: 101-114

Doherty FG (1990) The asiatic clam, *Corbicula* spp. as a biological monitor in fieshwater environments. Environ Monit Assess 15:143-181

Ellman GL, Courtney KD, Andres V Jr., Featherstone RM (1961) A new and rapid calorimetric determination of acetylcholinesterase activity. Biochem Pharmacol 7 88-95

Escartín E, Porte C (1997) The use of cholinesterase and carboxylesterase activities from *Mytilus galloprovincialis* in pollution monitoring. Environ Toxicol Chem 16: 2090-2095

Fairbrother A, Marden BT, Benett JK, Hooper MJ (1991) Methods used in the determination of cholinesterase activity. In: Mineau P (ed) Cholinesterase-inhibiting insecticides. Elsevier, Amsterdam, p 35

Ferrero A, Wood EJ, Pícollo de Villar MI, Reale C, Zerba E (1991) Relación entre la actividad de esterasas y la resistencia a malatión en una cepa de *Tribolium castaneum.* Acta Bioquím Clín Latinoam 25: 435-442

Holmes RS, Masters CJ (1967) The developmental multiplicity and isoenzyme status of cavian esterases. Biochim Biophys Acta 132: 379-399

Livingstone DR, Kirching M, Wiseman A (1989) Cytochrome P-450 and oxidative metabolism in molluscs. Xenobiotics 19: 1041-1062

Maxwell DM (1992) Detoxication of organophosphorus compounds by carboxylesterases. In: Chambers JE and Levi PE (eds) Organophosphates: Chemistry, fate and effects. Academic Press, San Diego, California, p 183

Ozretic B, Krajnovic-Ozretic M (1992) Esterase heterogenity in mussel *Mytilus galloprovincialis:* Effects of organophosphate and carbamate pesticides *in vitro.* Comp Biochem Physiol C 103:221-225

Parkinson A (1996) Biotransformation of xenobiotics. In: Klaassen CD (ed) Casarett and Doull's Toxicology. The basic science of poisons. 5th edition. New York, McGraw-Hill, p 115

Sheehan D, McIntosh J, Power A, Fitzpatrick PJ (1995) Drug metabolizing enzymes of mussels as bioindicators of chemical pollution. Biochem Soc Trans 23 : 419-422

Talesa V, Romani R, Rosi G, Giovannini E (1996) Acetylcholinesterase in *Dendrobaena veneta* (Oligochaeta: Opisthopora) is present with forms sensitive and insensitive to phosphatidylinositol phospholipase C. Eur J Biochem 238: 538-

Walker CH, Thompson HM (1991) Phylogenetic distribution of cholinesterases and related esterases. In: Mineau P (ed) Cholinesterase-inhibiting insecticides. Elsevier, Amsterdam, p 1