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Anticholinesterase Effect of Eserine (Physostigmine) in Fish and Crustacean Species

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ABSTRACT

The kinetic characteristic (K_m) of cholinesterase from the crab Chasmagnathus granulata, *the shrimp* Farfantepenaeus paulensis *and the fish Odontesthes bonaeriensis were compared and correlated with the anticholinesterasic effect of eserine (physostigmine). For the crustaceans, the estimated K_m values were about 5-8 times higher than that estimated for the fish (0.04 mM). In the crab and the shrimp, the concentration of eserine which inhibited 50% of cholinesterase activity (IC_{50}) was estimated as $5.33x10^{-4}$ and $4.33x10^{-4}$ mM, respectively. In both cases, it was significantly higher ($P < 0.05$) than that estimated for the fish larvae ($7.43x10^{-5}$ mM). A high K_m could reflect a lower affinity of the cholinesterase for its natural substrate, acetylcholine, or for substrate analogues such as carbamates and organophosphorous pesticides. If we consider the IC_{50} for eserine as an index of enzyme susceptibility to pesticide inhibition, the cholinesterase from the fish larvae may be a better useful tool in assays for pesticide biomonitoring than that from crustacean species.*

Key words: Eserine; physostigmine; acetylcholinesterase; fish; silverside fish; crustacean

INTRODUCTION

Several pesticides, such as carbamates and organophosphorous, are known to be cholinesterase inhibitors. Some experimental work has shown a persistent cholinesterase inhibition after exposure to these agents (Da Silva *et al.*, 1993). Further, relationships between physico-chemical properties of organophosphorous compounds and their acute toxicity to guppies has been established (de Bruijn & Hermens, 1993). However, little is known about the relationship between the kinetic properties of cholinesterase from aquatic organisms and the inhibitory effects of anticholinesterase pollutants. This kind of information may be useful, since it seems a rational criteria to select aquatic organisms to be used as bioindicators. This is an important point to be considered, since some toxicants can differently affect distinct species due to their physiological and/or biochemical differences.

In quantitative structural analysis relationship (QSAR) studies, biological variability is fixed, i.e. an unique species is employed. In this case, the toxicity of several pesticides is correlated with their physico-chemical properties like K_{ow} (de Bruijn & Hermens, 1993). In the present study, we selected a known anticholinesterase agent (eserine) and then correlated its inhibitory effect with a kinetic parameter (K_m) of the cholinesterase from the crustaceans *Chasmagnathus granulata* (Decapoda: Grapsidae) and *Farfantepenaeus paulensis* (Decapoda: Penaeidae) and the fish *Odontesthes bonaeriensis* (Teleostei: Atherinidae). The crab *C. granulata*, a typical salt marsh species from Southern Brazil, has been used in toxicological studies (Rodríguez & Lombardo, 1991). The shrimp *F. paulensis*, and the fishes of

the genus *Odontesthes*, are important fishing resources. They have also been used in bioassays (Wasielesky *et al.*, 1994).

MATERIALS AND METHODS

Adult male crabs *Chasmagnathus granulata* (9.72 ± 0.15 g; N=22) and juvenile shrimps *Farfantepenaeus paulensis* (2.65 ± 0.13 g; N=19) were collected in salt marshes near the city of Rio Grande (Southern Brazil). In the laboratory, they were maintained at 20°C and 20‰ salinity. Three months aged larvae of *Odontesthes bonaeriensis* (0.15 ± 0.02 g; N=20) were obtained as previously described (Bianchini *et al.*, 1997) and maintained at 20°C and 10‰ salinity. The area where all animals were collected is considered to be free of anticholinesterase pollutants (Almeida *et al.*, 1993).

The thoracic and the sub-esophageal ganglia from the crab and shrimp respectively, were isolated and used as enzyme source. The fish was frozen and then decapitated, the head being employed as enzyme source. Tissues were kept frozen (-20°C) not longer than one week. All samples were weighed and homogenized (crustaceans: 2% w/v; fish: 5% w/v) employing cold phosphate buffer (crustaceans: 0.25 M, pH 7.40; fish: 0.05 M, pH 7.40) (Habig *et al.*, 1988). Homogenates were then centrifuged at 8,100 g for 20 min at 4°C. The supernatant was used as cholinesterase source. Cholinesterase activity was determined using a colorimetric method (Ellman *et al.*, 1961). Acetylthiocholine iodide (AcSCh, Fluka) was used as substrate. Rates of AcSCh hydrolysis (25°C, pH 7.40) were determined in duplicate, using substrate concentrations ranging from 0.24 to 9.24 mM (crab), 0.20 to 7.84 mM (shrimp), and 0.08 to 3.63 mM (fish). K_m and V_{max} values were estimated after Eadie-Hofstee transformation. Homogenate protein content was determined using a commercial kit (Microprote®, Doles Ltda, Brazil), which is based on the method described by Bradford (1976).

The concentrations used for *in vitro* estimation of the eserine (Sigma) concentration that inhibited 50% of cholinesterase activity (IC_{50}) ranged from $2x10^{-5}$ to $7x10^{-3}$ mM for the crustacean species (in triplicate) and from 10^{-6} to $1.12x10^{-2}$ mM for the fish species (in duplicate). The enzyme was exposed to the inhibitor during 5 min (25°C, pH 7.40) before measurements of cholinesterase activity.

The IC_{50} for the three species were estimated using probit analysis (Finney, 1971) and then compared, considering the higher IC_{50}/lower IC_{50} ratio (α = 0.05) as previously described (Rodríguez & Lombardo, 1991). The IC_{50} values obtained in this experiment and other data from literature were correlated with their respective K_m values, using regression analysis after logarithmic transformation of both variables.

RESULTS AND DISCUSSION

In the crustacean species, the K_m values estimated for cholinesterase were about 5-8 times higher than that estimated for the fish species. The IC_{50} for eserine in *C. granulata* and *F. paulensis* cholinesterases were estimated as $5.33x10^{-4}$ and $4.33x10^{-4}$ mM, respectively. These values were significantly higher than that estimated in *O. bonaeriensis* ($7.43x10^{-5}$ mM) (Table 1).

For the three species tested, high concentrations of the substrate (AcSCh) induced an inhibition of the cholinesterase activity. Considering V_{max} as 100%, inhibition was of 14.1 and 24.5% when 4.62 and 9.24 mM ATCh, respectively, were employed in *C. granulata*. In *F. paulensis* and *O. bonaeriensis*, the inhibition was of 13.0 and 47.8%, employing 7.63 and 3.63 mM AcSCh, respectively (Fig. 1).

The specific cholinesterase activity was higher for the crustacean species than for *O. bonaeriensis*. However, slight differences were observed when V_{max} of each species was related to their respective K_m value. It must be noted that the enzyme source for *O. bonaeriensis* was the whole head, while for *C. granulata* and *F. paulensis* were the thoracic and subesophageal ganglia, respectively. Probably, proteins other than cholinesterase were more abundant in *O. bonaeriensis* than in crustaceans homogenates, resulting in a lower specific enzyme activity for the fish species. On the other hand, the inhibition registered at high substrate concentration suggest the possibility that the cholinesterases of the three species were acetylcholinesterases (Habig *et al.*, 1988).

Using the K_m and IC_{50} values estimated in the present study, and those estimated by other authors for different species, a positive and significant relationship between the logarithmic values of K_m and IC_{50} was observed (r=0.96; N=10; $P < 0.05$; Fig. 2).

Despite of the fact that the literature not always mentions the inhibition time used, a positive and significant relationship between the logarithmic values of K_m and IC_{50} was observed. When estimating this relationship, we assumed that the threshold inhibition level was reached in all cases. Although this assumption could not be checked, a very good correlation between the two variables was observed (r=0.96).

Table 1 - Cholinesterase kinetic parameters (K_m and V_{max}) and concentrations of eserine which inhibit 50% of enzyme activity (IC_{50}) in *Chasmagnathus granulata*, *Farfantepenaeus paulensis*, and *Odontesthes bonaeriensis*. Equal letters represent means not significantly different ($P > 0.05$) for each parameter analyzed. K_m and IC_{50} were expressed in mM. V_{max} was expressed in µmoles. mg protein^{-1}.min^{-1}.

Species	K_m (± 1 SE)	V_{max} (± 1 SE)	IC_{50}
C. granulata	0.28 ± 0.03 (**A**)	1.76 ± 0.05 (**A**)	$5.33.10^{-4}$ (**A**)
F. paulensis	0.19 ± 0.02 (**B**)	1.41 ± 0.04 (**B**)	$4.33.10^{-4}$ (**A**)
O. bonaeriensis	0.04 ± 0.01 (**C**)	0.26 ± 0.01 (**C**)	$7.43.10^{-5}$ (**B**)

Some authors have reported that insect resistance to carbamate or organophosphorous pesticides could be related to high K_m values (Fournier & Muero, 1994). A high K_m could reflect a lower affinity of the cholinesterase protein for its natural substrate, acetylcholine, or for substrate analogues such as carbamate and organophosphorus pesticides. Considering IC_{50} for eserine as an index of enzyme sensitivity to pesticide inhibition, a positive relationship with K_m values was expected, as indeed observed. According to our data, *O. bonaeriensis* could be a better *in vitro* bioindicator, since its acetylcholinesterase presents a lower K_m, and a correspondingly lower IC_{50} than *C. granulata* and *F. paulensis* acetylcholinesterases.

When considering the advantages of *in vitro* assays, fastness and simplicity are obvious. However, one should consider if the *in vitro* results reflect or not the *in vivo* processes. It

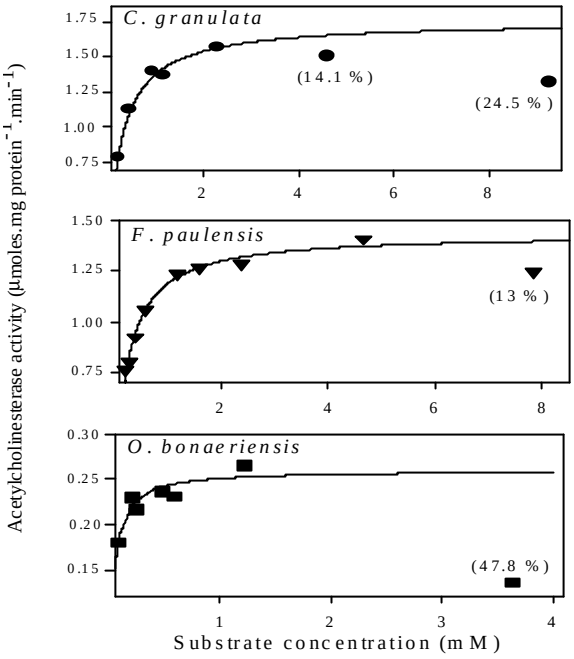

Figure 1 - Substrate concentration and acetylcholinesterase activity relationship for *Chasmagnathus granulata*, *Farfantepenaeus paulensis* and *Odontesthes bonaeriensis*. Values into brackets represent enzyme inhibition registered at high substrate concentration.

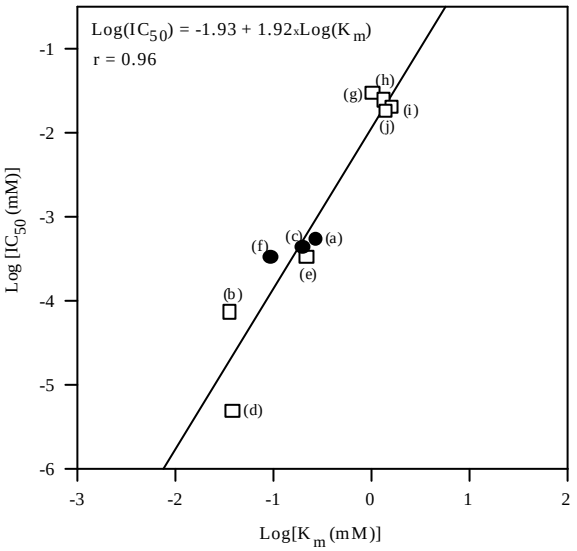

Figure 2 - Relationship between IC_{50} for eserine and cholinesterase K_m values for different species of (•) crustacean and ( ) fish species. (a), (b) and (c) are data from the present study; (d) is from Magnotti *et al*. (1994); (e) and (f) are from Habig *et al*. (1988); (g), (h), (i) and (j) are from Kabeer Ahmmad Sahib & Ramana Rao (1980).

must be noted that animal sensitivity to organophosphorous compounds is related to the dynamics of activation and deactivation of these compounds. Differences in the rates of these processes, for example, can explain the differences in toxicity of these compounds to rats of different sex and age (Murphy *et al.*, 1968). More recent work (Thompson *et al.*, 1995) showed that the LD_{50} of different organophosphorous compounds for avian species were well correlated with *in vitro* IC_{50} for brain cholinesterase (r=0.88), suggesting that a variable which integrated *in vivo* toxicological processes could be predicted by an *in vitro* measure of toxicity. Thus, it could be reasonable to consider *in vitro* enzyme sensitivity as a good criterion for selection of species to be employed as monitors of anticholinesterase compounds.

Another aspect to be considered is if the sensitivity to one compound, such as eserine, implies in general sensitivity. We re-analyzed some previous results (Wang & Murphy, 1982) in order to correlate in vitro IC_{50} for different species and distinct anticholinesterase compounds. There was a strong relationship between pairs of IC_{50} of different organophosphrous compounds (Table 2), suggesting that sensitivity to one specific compound could indicate a general sensitivity. Also, IC_{50} values present a high and significant ($P < 0.05$) correlation with a more specific inhibitor measure, such as the bimolecular inhibition constant (K_i), which quantifies the proportion between inhibited enzyme and the complex inhibitor-enzyme (Fig. 3). At this point, it can be hypothesized that general sensitivity of cholinesterase to organophosphorous and carbamates compounds can be estimated using kinetic parameters, like K_m.

There are several theories to explain differences in enzyme sensitivity to anticholinesterasic compounds. Some authors determined the cholinesterase activity of resistant and non-resistant arthropods strains to organophosphorous (Zahavi *et al.*, 1971). The non-resistant strains showed a greater enzyme activity than the resistant strain, when butirylthiocholine iodide (BuSCh) was employed as substrate, and lower when acetylthiocholine iodide (AcSCh) was used.

Table 2 - Correlation between IC_{50} (expressed in M) of several organophosphorous pesticides for cholinesterases of different animal species (*Rana sp.*, *Gallus domesticus*, *Cavia porcellus*, *Macaca fascicularis*, *Rattus rattus* and *Ictalurus sp.*). Values into brackets represents the p-value for the correlation test. The analysis was made using data reported by Wang & Murphy (1982).

	DFP	MP	P	G	EG
DFP	-	0.98 (0.001)	0.96 (0.002)	0.96 (0.003)	0.99 (0.000)
Methyl paraoxon (MP)	0.98 (0.001)	-	0.98 (0.001)	0.90 (0.013)	0.95 (0.003)
Paraoxon (P)	0.96 (0.002)	0.98 (0.001)	-	0.91 (0.013)	0.95 (0.003)
Gutoxon (G)	0.96 (0.003)	0.90 (0.013)	0.91 (0.013)	-	0.99 (0.000)
Ethyl gutoxon (EG)	0.99 (0.000)	0.95 (0.003)	0.95 (0.003)	0.99 (0.000)	-

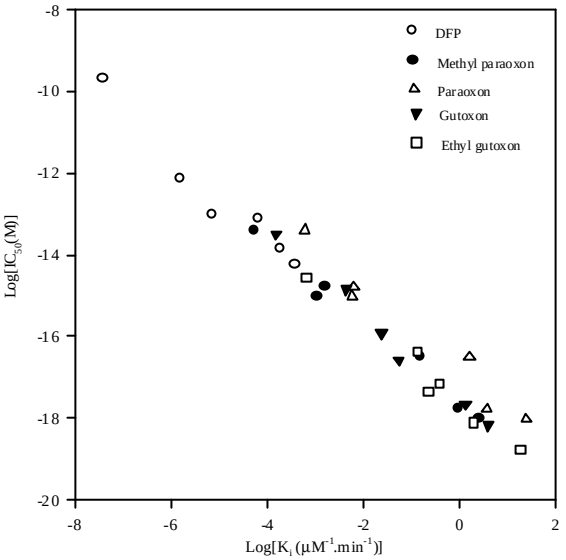

Figure 3 - Correlation between *in vitro* IC_{50} of different organophosphorous and bimolecular inhibition constant (K_i) for cholinesterases of different animal species (*Rana sp.*, *Gallus domesticus*, *Cavia porcellus*, *Macaca fascicularis*, *Rattus rattus* e *Ictalurus sp.*). The analysis was made using data reported by Wang & Murphy (1982).

The activity quotient BuSCh/AcSCh was estimated as 0.16 and 0.05 for non-resistant and resistant strains, respectively. This data suggested that the esteratic site of the cholinesterase of non-resistant species was greater than that of resistant ones. This in turn could lead to higher probability of inhibitor-enzime complex formation in non-resistant species.

For *C. granulata* cholinesterase, we registered an activity quotient higher than that reported for the sensitive cholinesterases from the arthropod *Tetranychus cinnabarinus* (Zahavi *et al.*, 1971). It was estimated as 0.25, since the activity registered was 0.34 and 1.37 μmoles.mg protein^{-1}.min^{-1} when 1.2 mM of AcSCh and 1.11 mM of BuSCh was employed, respectively. It remains to be determined the BuSCh/AcSCh quotient for a more sensitive species to eserine, such as *O. bonaeriensis*. Finally, it must be noted that in the available literature only one work has reported *in vitro* sensitivity of acetylcholinesterases to organophosphorous compounds to be lower for marine invertebrates (*Palaemon serratus* and *Mytilus edulis*) than for fishes (Galgani & Bocquéné, 1990).

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RESUMO

As caraterísticas cinéticas (K_m) de colinesterases do caranguejo *Chasmagnathus granulata*, o camarão *Farfantepenaeus paulensis* e o peixe *Odontesthes bonaeriensis* foram comparadas e correlacionadas com os efeitos anticolinesterásicos da eserina (fisostigmina). Nos crustáceos, o valores estimados de K_m foram aproximadamente 5-8 vezes maiores do que aquele estimado para a espécie de peixe (0.04 mM). No caranguejo e camarão, a concentração de eserina que inibiu 50% da atividade colinesterásica (CI_{50}) foi estimada em $5.33x10^{-4}$ e $4.33x10^{-4}$ mM, respectivamente. Estes valores foram significativamente maiores ($P < 0.05$) que aquele estimado para as larvas de peixes ($7.43x10^{-5}$ mM). Um valor de K_m mais elevado poderia refletir uma menor afinidade da colinesterase pelo seu substrato natural, acetilcolina, ou análogos tais como inseticidas carbamatos e fosforados. Se a CI_{50} para eserina é considerada como um índice da susceptibilidade da enzima a inibição por inseticidas, logo a colinesterase de larvas de peixes poderiam ser uma ferramenta mais útil no monitoramento de inseticidas do que aquelas das espécies de crustáceos.

REFERENCES

Almeida, M. T.; Baumgarten, M. G.; Silva Rodrigues, R. M. (1993), *Identificação de possíveis fontes de contaminação das águas que margeiam a cidade do Rio Grande.* Documentos Técnicos - Oceanografia, Rio Grande .

Bianchini, A.; Monserrat, J. M.; Phonlor, G.; Oneto, M. L. (1997), Anticholinesterase effects of methyl parathion to juveniles of *Odontesthes bonaeriensis* (Teleostei: Atherinidae). *Arq. Biol. Tecnol.*, **40**, 225-231.

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

Da Silva, H. C.; Guilherme Medina, H. S.; Fanta, E.; Bacila, M. (1993), Sub-lethal effects of the organophosphate FOLIDOL 600 (Methyl parathion) on *Callichthys callichthys* (Pisces: Teleostei). *Comp. Biochem. Physiol.*, **105C**, 197-201.

De Bruijin, J.; Hermens, J. (1993), Inhibition of acetylcholinesterase and acute toxicity of organophosphorus compounds to fish: a preliminary structure-activity analysis. *Aquat. Toxicol.*, **24**, 257-274.

Ellman G. L.; Courtney D.; Andres Jr. V.; Featherstone, R. M. (1961), A new and rapid colorimetric determination of acetylcholinesterase activity. *Biochem. Pharmacol.*, **7**, 88-96.

Finney, D. J. (1971), *Probit Analysis*. Cambridge University Press, Cambridge.

Fournier, D.; Muero, A. (1994), Modification of acetylcholinesterase as a mechanism of resistance to insecticides. *Comp. Biochem. Physiol.*, **108C**, 19-31.

Galgani, F.; Bocquéné, G. (1990), *In vitro* inhibition of acetylcholinesterase from four marine species by organophosphates and carbamates. *Bull. Environ. Contam. Toxicol.*, **45**, 243-249.

Habig, C.; Di Giulio, R. T.; Abou-Donia, M. B. (1988), Comparative properties of channel catfish (*Ictalurus punctatus*) and blue crab (*Callinectes sapidus*) acetylcholinesterases. *Comp. Biochem. Physiol.*, **91C**, 293-300.

Kabeer Ahammad Sahib, I.; Ramana Rao, K. V. (1980), Correlation between subacute toxicity of malathion and acetylcholinesterase inhibition in the tissues of the teleost *Tilapia mossambica*. *Bull. Environ. Contam. Toxicol.*, **24**, 711-718.

Magnotti Jr., R. A.; Zaino, J. P.; McConnell, R. S. (1994), Pesticide-sensitive fish muscle cholinesterases. *Comp. Biochem. Physiol*., **108C**, 187-194.

Murphy, S. D.; Lauwerys, R. R.; Cheever, K. L. (1968), Comparative anticholinesterase action of organophosphorus insecticides in vertebrates. *Toxicol. Appl. Pharmacol.*, **12**, 22-35.

Rodríguez, E. M.; Lombardo, R. J. (1991), Acute toxicity of parathion and 2,4 D to estuarine adult crabs. *Bull. Environ. Contam. Toxicol.*, **46**, 576-582.

Thompson, H. M.; Langton, S. D.; Hart, A. D. M. (1995), Prediction of interespecific differences in the toxicity of organophosphorus pesticides to wildlife - a biochemical approach. *Comp. Biochem. Physiol*., **111C**, 1-12.

Wang, C.; Murphy, S. D. (1982), Kinetic analysis of species difference in acetylcholinesterase sensitivity to organophosphate insecticides. *Toxicol. Appl. Pharmacol*., **66**, 409-419.

Wasielesky, W. J.; Marchiori, M. A.; Santos, M. H. S. (1994), Efeito da amônia no crescimento de pós-larvas do camarão rosa, *Penaeus paulensis*, Perez-Farfante, 1967 (Decapoda: Penaeidae). *Nauplius*, **2**, 99-105.

Zahavi, M.; Tahori, A. S.; Klimer, F. (1971), Insensitivity of acetylcholinesterase to organophosphorus compounds as related to size of esteratic site. *Molec. Pharmacol.*, **7**, 611-619.

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