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SHORT COMMUNICATION

Should the use of inhibition of cholinesterases as a specific biomarker for organophosphate and carbamate pesticides be questioned?

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Recently there has been evidence that contaminants other than organophosphate and carbamate pesticides may inhibit the activity of the enzyme acetylcholinesterase (AChE) both under *in vitro* and *in vivo* conditions. In this study we investigated the *in vitro* effect of three detergents [dodecyl benzyl sulphonate (DBS), sodium dodecyl sulphate (SDS) and a mixture commonly used as domestic detergent (X)] and three metals [molybdenum, barium and chromium (VI)] on AChE activity of *Mytilus galloprovincialis* haemolymph. All the detergents tested significantly inhibited the activity of the enzyme. The lowest observed effect concentrations were equal to 12·5 for DBS and 50 mg l^{-1} for SDS and X. Among the metals, molybdenum and barium had no effect on AChE activity, whereas chromium (VI) significantly depressed the activity of the enzyme at concentrations equal to or higher than 25 mg l^{-1}. These results suggest that the use of AChE as a specific biomarker for organophosphate and carbamate pesticides should be questioned and that the use of this enzyme as a biomarker could be extended.

Keywords: AChE, metals, detergents, biomarker, *Mytilus galloprovincialis*.

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Introduction

Inhibition of cholinesterases has been widely used as a specific biomarker for organophosphate and carbamate pesticides. However, in the last decades it was found that some metals may also inhibit these enzymes, both under *in vivo* and *in vitro* conditions (Herbert *et al.* 1995/96, Guilhermino *et al.* 1996, Labrot *et al.* 1996). Furthermore, some recent studies provided evidence that other environmental contaminants may be anti-cholinesterase agents. For example, Galgani *et al.* (1992) found a variation of AChE activity in tissues of *Limanda limanda* along a pollution gradient in the North Sea. Another recent study performed in Newfoundland (Payne *et al.* 1996) reported a depression in the range of 50 % in muscle tissues of *Salmon trutta* from two urban rivers and in corresponding tissues of *Pleuronectes americanus* from pulp and paper mill areas relative to fishes from reference sites. The nature of AChE-inhibiting substances in the North Sea and in Newfoundland is not known. However, Payne *et al.* (1996) consider it unlikely that the AChE inhibition in fishes observed in their study was due to contamination by pesticides or metals. They suggest that complex mixtures of pollutants may

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of anti-cholinesterase agents in the aquatic environment. Detergents, combustion-type hydrocarbons and pulp mills are potential sources of such agents since they may contain AChE inhibitors (Payne *et al.* 1996).

Detergents are a major class of pollutants. Some of these chemicals contain tertiary and quaternary ammonium compounds which depress the activity of cholinesterases *in vitro* (Witter 1963). In this study, we investigated the effect of three detergents [dodecyl benzyl sulphonate (DBS), sodium dodecyl sulphate (SDS) and a mixture commonly used as domestic detergent (X)] and three metals [molybdenum, barium and chromium (V)] on acetylcholinesterase activity of *Mytilus galloprovincialis* haemolymph in *in vitro* conditions. We used haemolymph as the biological material because it has higher specific activity than other mussel tissues such as gills, adductor muscle, gonads and mantle (Herbert *et al.* 1995/96). All the chemicals tested may be found in the environment as contaminants. Furthermore, chromium, barium and molybdenum (and their compounds) are included in the List II of the European Union dangerous substances directive (CEC 1976) and DBS has been used as reference substance in toxicity studies (Baird *et al.* 1991). As far we know, their potential effects on AChE activity have not been investigated before.

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Methods

Biological material

Mussels were collected on the beach of 'Homem do Leme', near the city of Oporto. They were maintained in the laboratory for 24 h prior to the experiments. Haemolymph was collected with a syringe, diluted (1:4) with phosphate buffer (0·1 M, pH 7·2) and kept on ice until used in the experiments, according to the general procedure described by Herbert *et al.* (1995/96).

Toxicant solutions

The detergents tested were dodecyl benzyl sulphonate (DBS), sodium dodecyl sulphate (SDS) and a mixture commonly used as domestic washing machine detergent (X) containing polycarboxylates (< 5 %), phosphates (15–30 %), anionic, non-ionic and bleaching agents (5–15 %). Metallic compounds were sodium molybdate, barium chloride and sodium dichromate. Test solutions were prepared by dissolving each of the chemicals in double distilled water and making serial dilutions of the original solution.

Incubation procedure

In each experiment, samples of diluted haemolymph (490 µl) were incubated at 20 °C with 10 µl of the test solution, for 30 min. For each substance, a control and four chemical concentrations were used (12·5, 25, 50 and 100 mg l^{-1}, for metallic compounds, these are the concentrations of the metal). At each concentration three samples were randomly selected and for each sample three repeated measurements were made.

Determination of AChE activity

The activity of acetylcholinesterase was determined, in triplicate, by the Ellman method (Ellman *et al.* 1961) adapted to microplates (Herbert *et al.* 1995/96). The protein content of the samples was determined according to the Bradford technique (Bradford 1976) with the modifications described by Herbert *et al.* (1995/96). The activity of AChE in each sample was presented as the mean of the three determinations performed and was expressed in Units (U) per mg of protein (1 U = 1 nmol of substrate hydrolysed per min). A Labsystems Multiskan MS microplate reader was used.

Chemicals

Acetylthiocholine, 5,5′-dithio-bis(γ-nitrobenzoic acid) (DTNB) and γ-bovine globulins were purchased from Sigma (USA), Bradford reagent was from Bio-Rad (UK), DBS was from Unilever (UK) and all the other chemicals were from Merck (Germany). The washing machine detergent was a mixture for domestic use commonly found on the market.

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Data analysis

Data for each tested toxicant solution were analysed using a mixed model analysis of variance, including a fixed effect (concentration) and two random effects (samples and measurements within samples). For the estimation of the 'lowest observed effect concentration' (LOEC) values a series of comparisons was used between the mean AChE activity for each concentration with that of the control (Snedecor and Cochran 1980). The significance level used was 0·05.

Results

All the surfactants tested caused a significant inhibition of AChE activity of mussel haemolymph (table 1 and figure 1). The values of the 'lowest observed effect concentration' (LOEC) were 12·5 mg l^{-1} for DBS ($t = 7{\cdot}14$, df = 4, $p < 0{\cdot}01$), 50 mg l^{-1} for SDS ($t = 9{\cdot}90$, df = 4, $p < 0{\cdot}001$) and X ($t = 3{\cdot}86$, df = 4, $p < 0{\cdot}05$). It is interesting to note that 50 and 100 mg l^{-1} of DBS induced the full inhibition of AChE activity. An inhibition near 100 % was also observed with 100 mg l^{-1} of SDS.

Molybdenum and barium did not significantly inhibit AChE activity of mussel haemolymph, while chromium (VI) significantly depressed the enzyme (table 1 and figure 2), the LOEC being 25 mg l^{-1} ($t = 4{\cdot}70$, df = 4, $p < 0{\cdot}01$). Concentrations of 25, 50 and 100 mg l^{-1} reduced AChE activity by 24 %, 26 % and 38 %, respectively.

Discussion

In this study, acetylthiocholine was the substrate used for the determination of AChE activity and no distinction between AChE and ChE activities was made. However, mussel haemolymph has high specific activity and this activity can be fully inhibited by eserine sulphate (Herbert *et al.* 1995/96) which is a characteristic of AChE (Ellman *et al.* 1961). Results obtained in a previous study indicated that no cross-reaction between pollutants and DTNB or acetylthiocholine occur in our experimental conditions (data not shown).

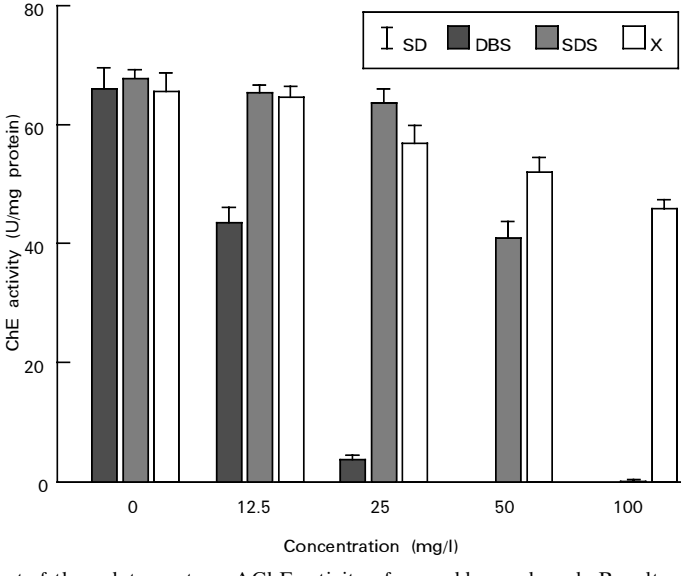

Figure 1. Effect of three detergents on AChE activity of mussel haemolymph. Results are presented as the mean and standard deviation (three measurements within three samples)

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Table 1. Analysis of variance of AChE activity for the tested solutions.

(a) DBS

Source of variation	Sum of squares	df	Mean square	*F*	Prob.
Concentration	33325·4	4	8331·3	562·5	0·000
Samples (within concentration)	148·1	10	14·8	31·7	0·000
Measurements (within samples)	14·0	30	0·47		

(b) SDS

Source of variation	Sum of squares	df	Mean square	*F*	Prob.
Concentration	29443·8	4	7360·9	667·2	0·000
Samples (within concentration)	110·3	10	11·0	12·8	0·000
Measurements (within samples)	25·9	30	0·86		

(c) X

Source of variation	Sum of squares	df	Mean square	*F*	Prob.
Concentration	2516·8	4	629·2	33·9	0·000
Samples (within concentration)	185·4	10	18·5	9·4	0·000
Measurements (within samples)	59·1	30	1·97		

(d) Mo

Source of variation	Sum of squares	df	Mean square	*F*	Prob.
Concentration	403·5	4	100·9	1·7	0·21
Samples (within concentration)	583·3	10	58·3	82·9	0·000
Measurements (within samples)	21·1	30	0·70		

(e) Ba

Source of variation	Sum of squares	df	Mean square	*F*	Prob.
Concentration	108·8	4	27·2	0·5	0·72
Samples (within concentration)	520·6	10	52·1	67·7	0·000
Measurements (within samples)	23·1	30	0·77		

(f) Cr (VI)

Source of variation	Sum of squares	df	Mean square	*F*	Prob.
Concentration	3746·1	4	936·5	56·2	0·000
Samples (within concentration)	166·5	10	16·6	17·0	0·000
Measurements (within samples)	29·3	30	0·97		

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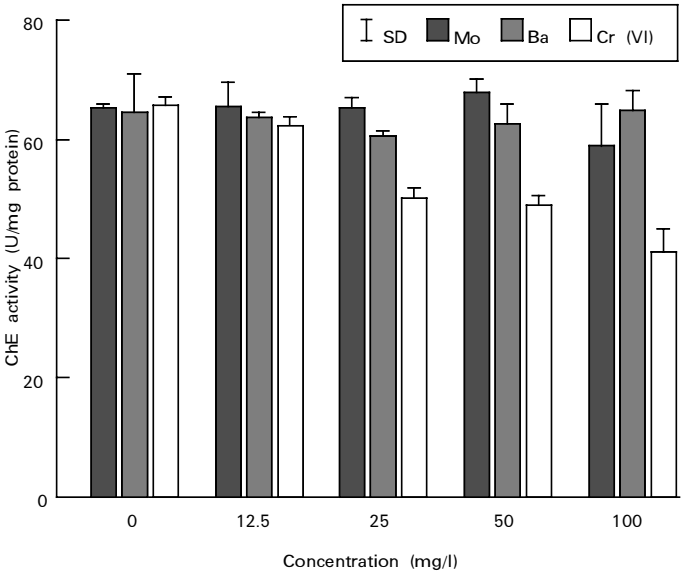

Figure 2. Effect of three metals on AChE activity of mussel haemolymph. Results are presented as the mean and standard deviation (three measurements within three samples).

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Some studies published recently indicate that environmental contaminants other than organophosphate and carbamate pesticides may depress the activity of the enzyme AChE. For example, AChE inhibitor effects of chemicals such as metals and undetermined components of complex mixtures of pollutants both *in vivo* and *in vitro* conditions have been reported (Schmidt and Ibrahim 1994, Labrot *et al.* 1996, Payne *et al.* 1996). Here, we demonstrated that Cr(VI) and some detergents depress the activity of the enzyme in *in vitro* conditions. The concentrations of contaminants used in this study (range of mg per litre) are higher than those commonly found in the sea and in freshwater systems which are in general at the μg per litre level (Martin and Whitfield 1983, Mance 1987). However, concentrations of metals at mg per litre range may be found in domestic, mine and industrial effluents or receiving streams (Connell and Miller 1984, Mance 1987, Whiting *et al.* 1994). Furthermore, metals may accumulate in the organisms. Thus, the concentration of metals in tissues of some organisms may be considerably higher than those found in the water where they live (Mance 1987, Depledge *et al.* 1994). Modern detergents tend to be rapidly degraded in surface waters and their concentrations in the aquatic environment are in general at the range of μg per litre (Malcolm *et al.* 1995). However, higher concentrations have been measured in river sediments (<1–10 mg l^{-1}) and at sites near point sources (Malcolm *et al.* 1995) and also probably occur in some polluted industrial effluents.

Detergents may inhibit AChE by causing its denaturation. For example, SDS is known to denature several enzymes at concentrations near 1 %. The concentrations of detergents used here are considerably lower than 1 % (for example, the highest SDS concentration is about 0·03 %). Thus, it seems unlikely that the AChE inhibition observed was due to the denaturation of the enzyme caused by a high detergent concentration. Detergents may induce alterations in surface charges of proteins, changing their behaviour in solution (Scopes 1982). May this mechanism be responsible for the inhibitory effects observed? Our data do not answer this question.

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Several recent studies indicate inhibition of AChE by metals at relatively high concentrations (mg l^{-1} range). For example, cadmium, copper, mercury, lead and uranium have been shown to depress AChE activity of several species at concentrations in the range of mg l^{-1}, both *in vitro* and *in vivo* (Schmidt and Ibrahim 1994, Guilhermino *et al.* 1996, Herbert *et al.* 1995/96, Labrot *et al.* 1996). As far as we know, the mechanism of AChE inhibition by metals is unknown. It is generally accepted that metals may inactivate enzymes by binding to specific groups of these proteins (Viarengo 1989, Simkiss *et al.* 1993). An hypothesis is that metal cations may bind to the anionic site of acetylcholinesterase molecules. The first step in the formation of the acetylcholine–AChE complex seems to be the establishment of an electrostatic linkage between the positive charge of the quaternary nitrogen of the choline part of acetylcholine and the anionic site of AChE. This linkage seems to be a prerequisite to the linkage of the acetate part of acetylcholine to the esteratic site of the enzyme (Gallo and Lawryk 1991). Thus, if the anionic site of the enzyme is occupied by the metal, acetylcholine cannot bind properly to the enzyme and it is not degraded. According to this hypothesis, differences in the potential to inhibit AChE among metals may be explained by properties such as ionic size, capacity of complex formation, electronegativity and reduction potential. Another hypothesis is the inhibition of the enzyme by a mechanism of salting-out (precipitation of proteins at high concentrations of salts).

In summary, results obtained in this study and literature data indicate that several chemicals, including detergents, metals and components of complex mixtures may inhibit AChE activity. Labrot *et al.* (1996) questioned the use of this enzyme as a specific biomarker for organophosphate and carbamate pesticides. We support this viewpoint and we suggest a more general use for this biomarker.

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