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Acetylcholinesterase Activity in Juveniles of *Daphnia magna* Straus

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In the last few decades there has been an increase in public concern over the environmental contamination by pesticides. In response, the agrochemical industries developed more selective and less persistent compounds. Organophosphate insecticides have been used since the 1930s. Their success was mainly due to two characteristics: they are degraded quite rapidly in the environment and apparently they do not accumulate in food chains. Unfortunately, many are very toxic to non-target organisms (Eto 1974), including fishes and aquatic invertebrates. Furthermore, some organophosphates appear to be much more persistent than previously believed (Eto 1974) and, in some cases, the products of degradation are even more pernicious than the parent compound.

The main toxic effect of organophosphates is the inhibition of the enzyme acetylcholinesterase (AChE) (Gallo and Lawryk 1991) leading to the disruption of the nervous system. The activity of AChE has been used as an indicator of organophosphate toxicity in terrestrial (Brown and Bryson 1992), aquatic (Day and Scott 1990) and marine organisms (Galgani and Bocquené 1990; Martínez-Tabche et al. 1992).

In this work we optimized the conditions for the use of AChE activity in toxicity tests with Daphnia magna juveniles and we evaluated the effect of some experimental procedures on the enzyme activity. These organisms were selected because they are sensitive to a broad spectrum of toxicants and they are among the most favourable test animals in aquatic toxicology (Soares et al. 1992)

MATERIALS AND METHODS

Daphnids used in experiments were individually cultured in 100 mL of ASTM hard water (ASTM 1980) with organic additive (Baird et al. 1989b), at 20°±1°C under a 16L:8D regime and fed daily with Chlorella vulgaris

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(Carbon ration 0.322 mg per 100 mL of medium). All experiments were carried out with animals from the clone A (*sensu* Baird et al. 1989a) and initiated with 3rd to 5th brood neonates. Daphnids less than 24 hr old were transferred in groups of five to 100 mL of ASTM and not fed during the test period.Temperature and photoperiod were as described above. After 24 hr, homogenates for enzymatic determinations were prepared.

Homogenates were prepared in phosphate buffer (0.1 M, pH = 7.2) using a Ystral GmbH d-7801 Dottingen homogenizer and kept on ice during homogenization. In experiments with juveniles, 30 animals per 0.8 mL of buffer were used. The activity of AChE was determined immediately, except in the experiment where the effect of freezing the samples was investigated. AChE activity in homogenate was assayed at room temperature by Ellman method (Ellman et al. 1961) adapted to microplate (A. Herbert, personal communication). In a typical assay, 0.250 mL of the reaction solution [30 mL of phosphate buffer, 1 mL of reagent dithiobisnitrobenzoate 10 mM (DTNB) (acid dithiobisnitobenzoate and sodium hydrogen carbonate in phosphate buffer) and 0.200 mL of acetylthiocholine iodide 0.075 M] were added to 0.1 mL of *D. magna* homogenate. Enzyme activities, in quadruplicate, were performed after an incubation period of 10 min in a Biomerieux microreader Axia at 405 nm during 5 min. The reaction solution (buffer + DTNB + substrate) was used as blank. All chemicals were from Sigma (USA). The enzyme activity was expressed in Units/mg protein; a Unit is a nmol of substrate hydrolysed per minute. The concentration of protein in the samples was determined in triplicate by the Bradford method (Bradford 1976), using bovine globulin's (Sigma, USA) as standard.

To test the effect of the number of animals used per sample on AChE activity, homogenates of 10, 20, 30, and 40 animals were prepared in triplicate from isolated juveniles (< 24 hr) and cultured for 24 hr in the conditions described above. The range of AChE activity in non treated animals was determined by measuring the activity of the enzyme in 19 samples prepared from juveniles produced by different females (30 juveniles per sample). The enzymatic determinations were performed in quadruplicate on different days using freshly prepared solutions. In order to determine the effect of freezing the samples, we determined the AChE activity in fresh homogenates and in samples stored for 40 d at -30°C or -70°C. To study the variation of the AChE activity in samples kept at 20°C we used homogenates prepared as described above and kept at 20°C for 12 hr; determinations of AChE activity were done after 0, 3, 6, and 12 hr from the time of preparation. The effect of feeding the animals on AChE activity in tests of short duration (24 or 48 hr) was investigated by measuring the AChE activity in homogenates prepared from juveniles cultured in absence or presence of food, during 24 and 48 hr. At the start of the-test, all animals were less than 24 hr old. Neonates (360) were pooled in groups of five per 100 mL of medium. From these, 36 groups were fed at

the start of the test with 0.322 mg Carbon of C. vulgaris; from these 18 groups were feed again with the same ration after 24 hr. The remaining 36 groups were not fed. After 24 and 48 hr, homogenates of daphnids fed and not fed were prepared (30 animals per 0.8 mL of buffer). The AChE as a function of animal age was assayed in homogenates prepared with animals of different ages. Samples prepared from adults were centrifuged in order to obtain a clean homogenate. Before studying the variation of AChE activity with age, we investigated the effects of centrifugation on enzyme activity. For this purpose, we compared the level of AChE in six samples prepared from juveniles cultured in the conditions described above. Three of these samples were centrifuged at 2500 g for 5 min in a Eppendorf centrifuge whereas the remaining samples were not centrifuged. To study the AChE variation with age, groups of three daphnids were cultured in 200 mL ASTM (described above) with organic additive and fed daily with 0.644 mg C. vulgaris. Then, 24 hr after the start of the test, 3 replicates of 30 animals were used to prepare homogenates for enzymatic determinations. After 7, 14, and 21 days, 3 replicates of 3 to 5 animals were used to measure AChE activity. Homogenates were centrifuged at 2500 g for 5 min and the supernatant was used to measure enzyme activity.

The chemicals used in this study were purchased from Merck location except acetylthiocholine iodide, DTNB, and bovine globulin's (purchased from Sigma location) and the reagent for protein determination (purchased from Bio-Rad location).

Results were analysed by ANOVA followed by Tukey test. The significance level was $P = 0.05$.

RESULTS AND DISCUSSION

The number of animals used per sample significantly affected the measurement of AChE activity (one-way ANOVA, $F = 58.406$, $P < 0.05$). The enzyme activity increased with the number of daphnids per sample up to 30 animals. Differences between homogenates prepared from 30 daphnids and samples prepared from 40 animals were not statistically significant. This result should not be a consequence of substrate limitation since the activity of AChE calculated from the difference between 15 and 20 min after the start of the reaction remained constant. Other factors seem to be responsible for the differences in AChE activity detected among samples prepared with different number of animals. It is very important to find the number of animals per sample that gives the accurate measurement of the enzyme activity which will consequently allow to investigate test compound effects in future experiments. This number should be a compromise between the sensitivity of enzymatic determinations and costs (in money, time, and human effort) needed to culture the daphnids required to run the experiment. Consequently, all further tests were done with samples prepared with 30 juveniles per 0.8 mL

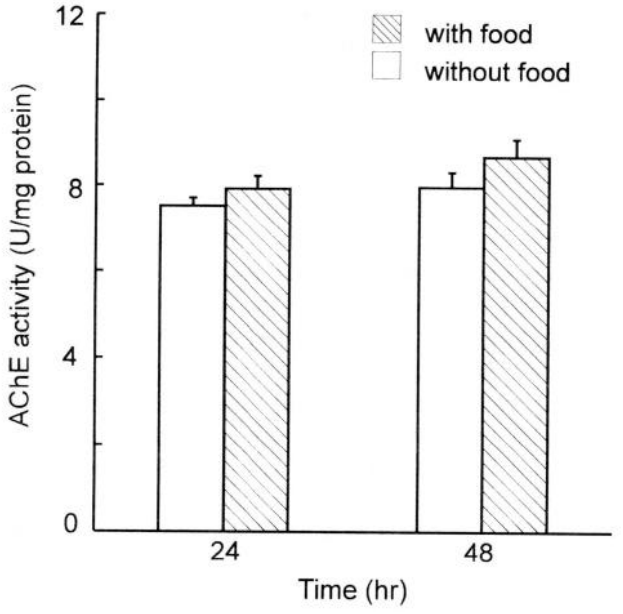

Figure 1. AChE in homogenates from juvenile (<24 h old) D. magna cultured for 24 and 48 hr in the absence or presence of food. Results are expressed as the mean±SE of three samples (4 determinations per sample).

of buffer.

The mean activity of 19 samples prepared from D. magna juveniles (30 per sample) was 8.20±0.995 SD Units. This level of enzyme activity is lower than the activity reported by Day and Scott (1990) with D. magna.

The procedure of freezing the samples at -30°C or at -70°C for 40 d did not affect the enzyme activity (one-way ANOVA, $F = 0.507$, $P > 0.05$). Sample freezing is a very useful procedure that allows the measurement of enzyme activity on samples obtained in different days or weeks; several other authors used frozen samples to measure AChE activity (Day and Scott, 1990; Bocquené et al., 1990; Bocquené et al., 1995). Bocquené et al. (1990) reported a slight reduction of AChE activity in homogenates of marine animals stored at -20°C.

There was no significant loss of AChE activity in samples kept at 20°C for 3, 6 and 12 hr (one-way ANOVA, $F = 0.712$, $P > 0.05$). This result may indicate that enzymatic determinations could be done several hours after the preparation of the homogenates, with no need for special preservation techniques.

Figure 1 shows the level of AChE in homogenates of animals cultured in the

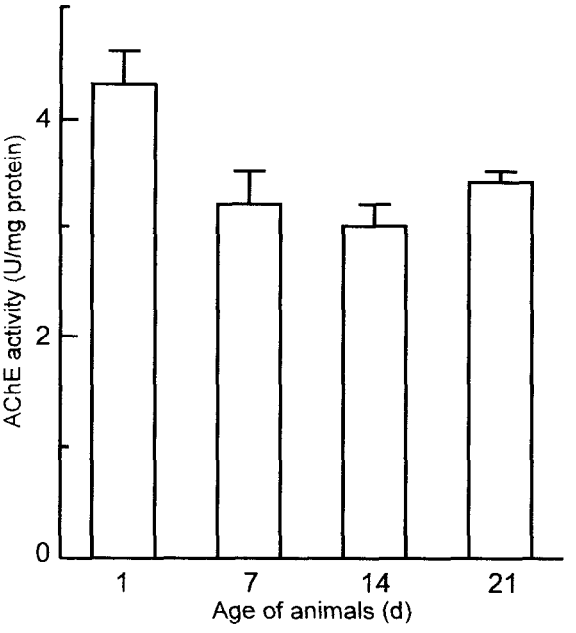

Figure 2. AChE activity as a function of the animal age. Results are expressed as a mean±SE of three samples (4 determinations per sample).

presence and absence of food during 24 and 48 hr. The activity of AChE does not differ significantly in homogenates prepared from animals 24 and 48 hr old (two-way ANOVA, $F = 3.373$, $P > 0.05$) or in animals fed and not fed (two-way ANOVA, $F= 2.886$, $P > 0.05$). In addition, interaction between the two factors was not statistically significant (two-way ANOVA, $F = 0.234$, $P > 0.05$). This may indicate that (i) 24 or 48 hr without food were not sufficient time periods to induce alterations on enzyme activity due to fasting or that (ii) the activity of this enzyme is not affected by the nutritional condition of the animals. Furthermore, our results seems to indicate that the AChE activity remains constant on the first 3 d of daphnids life.

The AChE activity determined in sample supernatants from juveniles centrifuged is lower than the activity measured in samples not centrifuged (one-way ANOVA, $F = 143.968$, $P < 0.05$). This may be due to the partial removal of the enzyme from the supernatant that goes in the pellet linked to the intact cells and large fragments. Our results indicate that juveniles have higher levels of AChE than adults (Figure 2). The differences among homogenates from animals 1, 7, 14, and 21 d were statistically significant (one-way ANOVA, $F = 5.522$, $p < 0.05$). The decrease in AChE activity seems to start before the seventh day of life. This may be related to the start of the period of reproduction that usually occurs at this time.

In summary, our results indicate that i) homogenates prepared with 30 juveniles per 0.8 mL of buffer are suitable for AChE determinations, ii) AChE is stable at 20°C up to 12 hr and iii) enzyme activity is not altered by freezing the samples. Furthermore, AChE activity seems to vary with animal age and the presence or absence of food during the incubation period of the juveniles appears to have no effect on AChE activity. The system described in this paper has been tested for sensitivity and selectivity in our laboratory. The results obtained in these experiments led us to propose the inibition of AChE as an effect criterion in acute toxicity tests with juvenile D. magna (Guilhermino et al., 1996).

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REFERENCES

Adema DMM (1978) Daphnia magna as a test animal in acute and chronic toxicity tests. Hydrobiologia 59: 125-134

ASTM (1980) Standard Practice for Conducting Acute Toxicity Tests with Fishes, Macroinvertebrates and Amphibians. (Unpublished Rep. E-729-90). American Standards for Testing and Materials. Philadelphia

Baird DJ, Barber I, Bradley M, Calow P, Soares AMVM (1989a) The Daphnia bioassay: a critique. Hydrobiologia 188/189: 403-406

Baird DJ, Soares AMVM, Girling A, Barber I, Bradley MC, Calow P (1989b) The long-term maintenance of Daphnia magna Straus for use in ecotoxicity tests: problems and prospects. In: Lokke H, Tyle H, Bro-Rasmussen F (ed) Proceedings First European Conference on Ecotoxicology, Lyngby, pp 144-148

Bocquené G, Bellanger C, Cadiou Y, Galgani F (1995) Joint action of combinations of pollutants on the acetylcholinesterase activity of several marine species. Ecotoxicology 4: 266-279

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

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

Brown TM, Bryson PK (1992) Selective inhibitors of methyl parathion-resistant acetylcholinesterase from Heliothis virescens. Pest. Biochem Physiol 44: 155-164

Day K, 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

Eto, M (1974) Organophosphorous pesticides: organic and biological chemistry. CRC Press, Cleveland, Ohio

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

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

Gallo MA, Lawryk NJ (1991) Organic phosphorous pesticides. In: Hayes, WJ, Jr, Laws, ER Jr (eds) Handbook of pesticide toxicology, vol 2. Academic Press, San Diego, pp 917-1123

Guilhermino L, Lopes MC, Carvalho AP, Soares AMVM (1996) Inhibition of acetylcholinesterase activity as effect criterion in acute tests with juvenile Daphnia magna. Chemosphere 32: 727-738

Martínez-Tabche L, Estrada BM, Galar I (1992) Parathion and salinity effects on gills and mesonephros carbonic anhydrase activity of the fish Oreochromis hornorum. Bull Environ Contam Toxicol 49: 929-934

Soares AMVM, Baird DJ, Calow P (1992) Interclonal variation in the performance of Daphnia magna Straus in chronic bioassays. Environ Toxicol and Chem 11: 1477-1483