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Cholinesterase activity during embryonic development in the blood-feeding bug *Triatoma patagonica*

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Abstract. *Triatoma patagonica* Del Ponte (Hemiptera: Reduviidae), a vector of Chagas' disease, is widely distributed in Argentina and is found in sylvatic and peridomiciliary ecotopes, as well as occasionally in human dwellings after the chemical control of *Triatoma infestans*. Anti-cholinesteratic products can be applied in peridomiciliary areas and thus knowledge of cholinesterase activity during embryonic development in this species might contribute further information relevant to effective chemical control. Cholinesterase activity was characterized by reactions to eserine 10^{-5} M, to increasing concentrations of substrate and to varying centrifugal speeds. Acetylcholinesterase activity was detected on day 4 and was significant from day 5. A reduction in cholinesterase activity towards acetylthiocholine (ATC) was observed on days 9 and 10 of development. Cholinesterase activity towards ATC and butyrylthiocholine (BTC) in homogenates of eggs was inhibited by eserine 10^{-5} M. The shape of the curve indicating levels of inhibition at different concentrations of ATC was typical of acetylcholinesterase. Activity towards BTC did not appear to be inhibited by excess substrate, which parallels the behaviour of butyrylcholinesterases. Cholinesterase activity towards ATC was reduced in supernatant centrifuged at 15 000 **g** compared with supernatant centrifuged at 1100 **g**. The cholinesterase system that hydrolyzes mainly ATC seems to belong to the nervous system, as indicated by its behaviour towards the substrates assayed, its greater insolubility and the fact that it evolves parallel to the development of the nervous system. Knowledge of biochemical changes associated with the development and maturation of the nervous system during embryonic development would contribute to the better understanding of anti-cholinesteratic compounds with ovicidal action that might be used in control campaigns against vectors of Chagas' disease.

Key words. *Triatoma patagonica*, cholinesterase activity, eggs, embryonic development, nervous system, vector control of Chagas' disease.

Introduction

In Argentina, the domestic species *Triatoma infestans* is the principal vector of *Trypanosoma cruzi* (Kinetoplastida: Trypanosomatidae), the aetiological agent of Chagas' disease. *Triatoma patagonica* is found in sylvatic and peridomiciliary ecotopes, and occasionally in human dwellings after the chemical control of *T. infestans* (Mazza, 1937; Lent & Wygodzinsky, 1979; Ferrero *et al*., 1999).

Given that the difficulties in controlling vectors of Chagas' disease are caused in part by the lack of ovicidal effect of insecticides (Wright, 1971) and that *T. patagonica*

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(a peridomiciliary species) may be present in human dwellings after the chemical control of *T. infestans* (Ferrero *et al*., 1999), the study of cholinesterase activity in *T. patagonica* proved to be of particular interest.

For several years it has been argued that longterm control of domestic triatomines requires the development of highly residual insecticide formulations [World Health Organization (WHO), 2002]. Anti-cholinesteratic products can be applied in peridomiciliary areas (WHO Expert Committee, 1991) and thus learning about cholinesterase activity during embryonic development in this species may contribute additional information to further the efficacy of chemical control.

Cholinesterases, which are ubiquitous in the animal kingdom, are enzymes that hydrolyze esters of choline faster than other esters and are inhibited by low concentrations ($\leq 10^{-5}$ M) of eserine (Augustinsson, 1957). According to their substrate specificity, these enzymes are classified as acetylcholinesterase (AChE), proprionylcholinesterase (PChE) or butyrylcholinesterase (BuChE) (Principato *et al.*, 1989). AChE (EC 3.1.1.7) differs from BuChE (EC 3.1.1.8) in that it hydrolyzes acetylcholine and its analogue acetylthiocholine (ATC) more effectively and faster than it does other substrates. AChE is inhibited by high concentrations of substrate (ATC) and also by BW284C51 or eserine (Zhu & Clark, 1994). BuChE hydrolyzes butyrylthiocholine (BTC) and is not inhibited by high concentrations of substrate (Augustinsson, 1957).

In insects, as in other animals, the cholinergic system is associated with the nervous system tissue. It is present in the central nervous system of insects (Eto, 1974), as well as in insect eggs (Smallman & Mansingh, 1969), and plays the same role in synaptic transmission in insects as it does in vertebrates.

The basic differences between embryonic and post-embryonic insect stages make it necessary to study each stage independently. The eggs are characterized by the developmental stage of nervous system tissues, which are targeted by the majority of insecticides, and AChE represents the molecular target of organophosphate and carbamate insecticides (O'Brien, 1967). Thus, it is necessary to conduct biochemical studies to investigate enzymological changes in developing embryos in order to understand the toxic response of insect eggs to insecticides.

The general aim of this study was to determine cholinesterase activity in *T. patagonica* eggs during embryonic development. The specific objective was to characterize the cholinesterase system according to its reaction to eserine 10^{-5} M, to increasing concentrations of substrate and to varying rates of centrifugal speed.

Materials and methods

Insects

Triatoma patagonica eggs were obtained from our laboratory colony. This colony is maintained at 28 ± 1 °C, at a relative humidity (RH) of 60–70%, in complete darkness and is fed weekly on pigeons. The protocol for feeding on pigeons was approved by the ad hoc committee of the Centro de Investigaciones en Plagas e Insecticidas [CONICET-CITEFA (Pest and Insecticide Research Centre), Buenos Aires, Argentina] and is archived in our laboratory. Eggs representing each day of development (days 1, 2, 3... until hatching) were selected according to their morphological characteristics (Visciarelli *et al*., 2001).

Chemicals

The chemicals used were: 5,5′-dithiobis-(2-nitrobenzoic acid) (DTNB) (Sigma Aldrich de Argentina SA, Buenos Aires, Argentina); eserine sulphate 10^{-5} M (physostigmine) (Sigma Aldrich de Argentina SA); phosphate buffer 0.2 M ($NaH_2PO_4H_2O$, pH 7.2); physiological solution (NaCl 0.9%); acetylthiocholine iodide (ATC) (Sigma Aldrich de Argentina SA), and butyrylthiocholine iodide (BTC) (Sigma Aldrich de Argentina SA). Substrate concentration (0.545 mM) was the optimal reported (de Villar *et al*., 1980).

Enzyme preparation

Groups of 30 eggs representing each day of development were homogenized in 1 mL of NaCl 0.9% using a Teflon glass homogenizer (Glas-Col LLC, Terre-Haute, IN, U.S.A.). The homogenate was filtered (through glass wool) and centrifuged at 1100 **g** at 4 °C for 10 min. The supernatant was used as the enzyme source for assays of cholinesterase activity.

Cholinesterase activity assays

Cholinesterase activity was determined in eggs representing each day of development using the method described by Ellman *et al.* (1961). The assay mixture contained 2.7 mL of DTNB and 0.3 mL of enzyme preparation. Each assay was initiated by the addition of 0.3 mL of substrate (ATC or BTC).

Enzyme activity was expressed in enzymatic units (EU), which represent the enzyme level that hydrolyzes 1 nmol of substrate/min at 22 °C and pH 7.2. This activity was monitored for 10 min at 412 nm using a Shimadzu UV-1203 photometer with the Kinetics-2-Program Pack P/N (206-62029-10; Shimadzu Corp., Kyoto, Japan). Absorbance was converted to nmol of hydrolyzed substrate/min/egg using a molar extinction coefficient of 13 600 mM^{-1}.

Inhibition by eserine 10^{-5} m

Cholinesterase activity inhibition by eserine was determined in 6- and 15-day-old eggs. The assay mixture contained 2.7 mL of DTNB, 0.3 mL of enzyme preparation and 0.3 mL of 10^{-4} M eserine sulphate. It was incubated for 15 min at 22 °C. The reaction was initiated by the addition of 0.3 mL of substrate (ATC or BTC).

Cholinesterase activity in increasing concentrations of substrate

Cholinesterase activity was determined in 6-day-old eggs using ATC as substrate and in 15-day-old eggs using BTC as substrate. These ages were considered optimal to avoid cross influences of different activities.

Acetylthiocholine concentrations of 5×10^{-5} M, 5×10^{-4} M, 10^{-3} M, 10^{-2} M and 10^{-1} M were used.

Butyrylthiocholine concentrations of 10^{-4} M, 5×10^{-4} M, 10^{-3} M, 5×10^{-3} M and 10^{-2} M were used.

Cholinesterase activity after different rates of centrifugation

The effects of different rates of centrifugation were determined in 15-day-old eggs using ATC and BTC as substrates after centrifugation at 1100 **g** and 15 000 **g** at 4 °C for 10 min.

Statistical analysis

The significance of differences in cholinesterase activity between days was analysed by analysis of variance (ANOVA) and mean comparisons were made using the Bonferroni test (Zar, 1999). Each assay was replicated three times.

Results

Cholinesterase activity

Cholinesterase reactions to ATC and BTC in the homogenates of *T. patagonica* eggs of each day of development showed different patterns of evolution ($P \ll 0.01$). Enzymatic hydrolysis [enzymatic reaction towards both substrates (ATC and BTC)] was apparent on day 4 of embryonic development. Activity with ATC as substrate was significantly higher on day 5, increased to day 8 and subsequently declined until day 10. From day 11, a continuous increase in activity until the end of development was observed (Bonferroni $\alpha = 0.05$, d.f. $= 26$; $P \ll 0.05$) (Fig. 1). When BTC was used as the substrate, activity in *T. patagonica* embryos was measurable from day 6 and showed a continuous increase until hatching (Bonferroni $\alpha = 0.05$, d.f. $= 26$; $P \ll 0.05$) (Fig. 2).

Cholinesterase reaction to eserine 10^{-5} m

Cholinesterase activity towards ATC and BTC in homogenates of *T. patagonica* eggs was totally inhibited by eserine 10^{-5} M.

Cholinesterase reaction to increasing concentrations of substrate

Cholinesterase activity towards ATC was inhibited when the substrate concentration was increased and a concentration of 10^{-3} M was found to be optimal (Fig. 3). Activity measured at different concentrations of BTC showed increasing activity at concentrations up to 5×10^{-3} M and no further inhibition up to the maximum concentration of 10^{-2} M (Fig. 4).

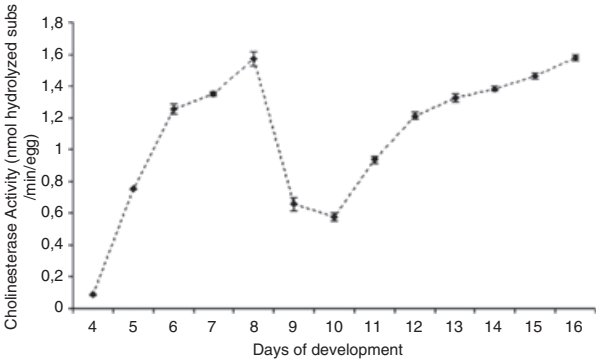

Fig. 1. Cholinesterase activity towards acetylthiocholine (ATC) in *Triatoma patagonica* eggs during embryonic development expressed in nmol of hydrolyzed substrate/min/egg.

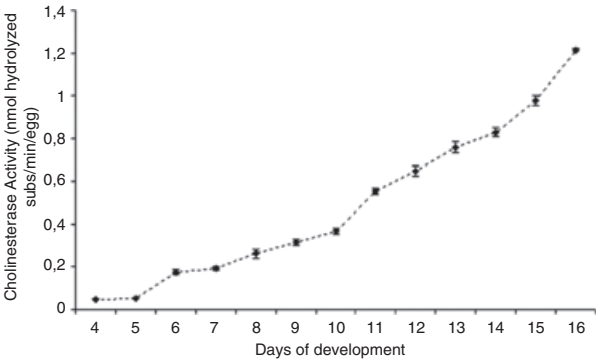

Fig. 2. Cholinesterase activity towards butyrylthiocholine (BTC) in *Triatoma patagonica* eggs during embryonic development expressed in nmol of hydrolyzed substrate/min/egg.

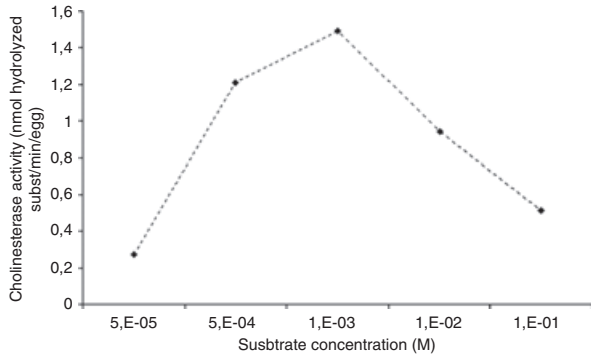

Fig. 3. Cholinesterase activity in 6-day-old eggs of *Triatoma patagonia* towards different concentrations of acetylthiocholine (ATC) expressed in nmol of hydrolyzed substrate/min/egg.

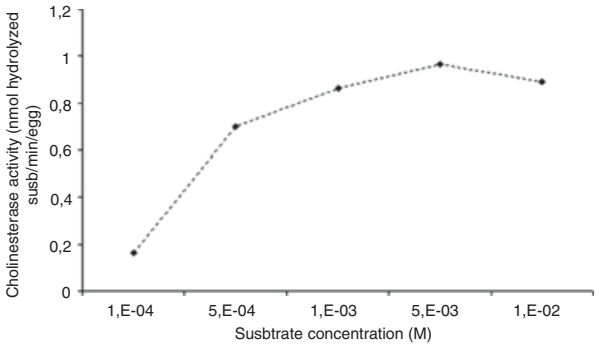

Fig. 4. Cholinesterase activity in 15-day-old eggs of *Triatoma patagonia* towards different concentrations of butyrylthiocholine (BTC) expressed in nmol of hydrolyzed substrate/min/egg.

Table 1. Rates of cholinesterase activity towards acetylthiocholine (ATC) and butyrylthiocholine (BTC) in 15-day-old eggs of *Triatoma patagonica* in supernatant centrifuged at 1100 **g** and 15 000 **g**.

	1100 **g**	15 000 **g**
Activity towards ATC	100%	25%
Activity towards BTC	100%	70%

Cholinesterase activity at different rates of centrifugation

Cholinesterase activity at different rates of centrifugation is shown in Table 1. Activity towards ATC in supernatant centrifuged at 15 000 **g** was remarkably reduced in comparison with activity in supernatant centrifuged at 1100 **g**. The decrease in cholinesterase reaction towards BTC was less significant.

Discussion

The determination of cholinesterase activity in *T. patagonica* eggs was useful in establishing the initiation and evolution of the nervous system in these triatomines. Acetylcholinesterase activity was detected on day 4 and was measurable from day 5. Enzyme activity associated with the cholinergic system is related to the differentiation of neuroblasts and the developing nervous system (Poulson & Boell, 1946; Chino & Yushima, 1954; Potter *et al.*, 1957). In enzymatic and histological studies in *T. infestans* eggs, Picollo de Villar (1979) demonstrated that AChE activity coincided with the histologically observable development of nervous system cells. Thus, our results may indicate that the detection of AChE activity may be related to neuroblastogenesis and to the growth and maturation of the nervous system, as shown by Picollo de Villar (1979) in *T. infestans* embryos.

A reduction in cholinesterase activity towards ATC was observed at 9 and 10 days of development. This could be attributed to the inhibiting action of the ocular pigment over AChE. Wood *et al.* (1979) demonstrated the presence of an endogenous inhibitor in the *T. infestans* head, with the characteristics of a selective inhibitor of AChE in insects, probably associated with the compound eyes of insects.

The sustained increase in cholinesterase activity in the last stage of embryonic development would indicate the presence of an already mature nervous system.

Cholinesterase activity towards ATC and BTC in homogenates of *T. patagonica* eggs was inhibited by eserine 10^{-5} M. This indicated that these enzymes are cholinesterases. Esterases that are not inhibited by eserine 10^{-5} M should not be referred to as cholinesterases (Augustinsson, 1957).

The shape of the curve indicating levels of inhibition at different concentrations of ATC was typical of AChE. Activity against BTC did not appear to be inhibited by an excess of substrate until the maximum concentration was assayed, in line with the behaviour of BuChE.

The results of assays of cholinesterase activity at different centrifugation rates presumably indicate that the cholinesterase reaction to ATC implies the presence of an insoluble enzyme, probably a membrane enzyme (Picollo de Villar, 1979; de Villar *et al.*, 1980). Activity towards BTC seems to indicate the presence of a soluble enzyme because high-speed centrifugation reduced activity towards ATC to a far greater extent.

Thus, the cholinesterase system in *T. patagonica* eggs may be made up of two enzymes. The enzyme that hydrolyzes acetylcholine is inhibited by excess substrate and pertains to the nervous system. It is called acetylcholinesterase, and is true or specific cholinesterase and would correspond to Group I of Augustinsson (1957). The other enzyme hydrolyzes mainly butyrylcholine and is not inhibited by excess substrate. It is called butyrylcholinesterase, pseudocholinesterase or non-specific cholinesterase and would correspond to Group II of Augustinsson (1957).

The cholinesterase system that hydrolyzes mainly ATC seems to belong to the nervous system according to its behaviour towards the substrates assayed, its greater insolubility and the fact that its evolution parallels the development of the nervous system; consequently, it may be presumed to be the molecular target of organophosphate compounds in the eggs studied.

The results of our findings in *T. patagonica* eggs are similar to those reported by Picollo de Villar (1979) in *T. infestans*. It is likely that insecticides with anti-cholinesterase activity that proved effective against *T. infestans* will also prove effective against *T. patagonica.* As expected, increasing levels of cholinesterase activity were observed in the second half of embryonic development, during which the embryo may be considered to be more susceptible to insecticides. In most cases, eggs showed significant AChE activity during the last stage of embryonic development, which is associated with the increased susceptibility to toxics reported in more mature eggs (Michaelides & Wright, 1997).

Knowledge of the biochemical changes associated with the development and maturation of the nervous system during embryonic development in *T. patagonica* will contribute to better understanding of anti-cholinesteratic compounds with ovicidal action that can be used in control campaigns against vectors of Chagas' disease.

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