

Kinetics of inhibition of two forms of acetylcholinesterase from *Panagrellus redivivus* by organophosphorus and carbamate compounds

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Summary — Inhibition experiments with paraoxon revealed two types of acetylcholinesterases in *Panagrellus redivivus* i.e. a fast (F-component) and a slow reacting component (S-component). The F-component was thermostable and inactivated by Triton-X-100, while the S-component was thermolabile and insensitive for Triton-X-100. The bimolecular rate constant for inhibition by paraoxon was $25.5 \times 10^3 \text{ M}^{-1}\text{min}^{-1}$ for the F-component and $9.2 \times 10^3 \text{ M}^{-1}\text{min}^{-1}$ for the S-component. Comparable differences in sensitivity between the two components were observed with oxamyl and aldicarb.

Résumé — *Cinétique d'inhibition de deux formes d'acétylcholinestérase par des composés organophosphorés et le carbamate chez Panagrellus redivivus* — Des essais d'inhibition à l'aide du paraoxon ont mis en évidence deux types d'acétylcholinestérase chez *Panagrellus redivivus* : un composant à réaction rapide (composant F) et un composant à réaction lente (composant S). Le composant F est thermostable et peut être inactivé par le Triton-X-100, tandis que le composant S est thermolabile et n'est pas inhibé par le paraoxon. La constante du taux bimoléculaire d'inhibition par le paraoxon est de $25.5 \times 10^3 \text{ M}^{-1}\text{min}^{-1}$ pour le composant F et de $9.2 \times 10^3 \text{ M}^{-1}\text{min}^{-1}$ pour le composant S. Des différences comparables de sensibilité entre les deux composants sont observées en utilisant l'oxamyle et l'aldicarbe.

Key-words : *Panagrellus*, acetylcholinesterase, organophosphorus, Carbamate.

Various reports have described the effects of organophosphorus and carbamate nematicides on the movement, invasion, feeding, development and reproduction of nematodes (Evans, 1973; Bunt, 1975; Wright, 1981). It is thought that these nematicides act principally by inhibition of acetylcholinesterases at cholinergic synapses in the nematode nervous system. Several organophosphorus and carbamate pesticides have proven to be potent inhibitors of cholinesterase activity in crude extracts of various nematode species (Spurr & Chancey, 1967) and some compounds have been shown to inhibit cholinesterase activity in the region of the nematode nerve ring (Wright & Awan, 1976).

Multiple molecular forms of acetylcholinesterases have been reported for various organisms, including nematodes. Detailed studies on the nematode *Caenorhabditis elegans* have established the existence of five multiple molecular forms of acetylcholinesterase in *C. elegans* (Johnson & Russel, 1983). Based on substrate specificity, sensitivity towards various acetylcholinesterase inhibitors, thermostability and inactivation by non-ionic detergents, the five multiple molecular forms were divided in two classes. Analyzing various mutants showed that these two classes are encoded by two different genes (Culotti *et al.*, 1981; Johnson *et al.*, 1981). The kinetic properties of the isoenzymes indicated that both forms are to be referred to as acetylcholinesterases (E.C. 3.1.1.7) rather than cholinesterases (E.C. 3.1.1.8).

Although various aspects of the mode of action of nematicides have been studied, data on isoenzymes or multiple molecular forms and the kinetics of their inhibition are scarce. In a previous report (Mulder & Bakker, 1988) it was shown that two types of acetylcholinesterases from *Panagrellus redivivus* could be differentiated by heating and treatment with Triton-X-100. Kinetic studies revealed distinct differences between the two components with regard to the rate at which they were inactivated by aldicarb and ethoprophos. A suitable method to differentiate multiple molecular forms or isoenzymes of acetylcholinesterase in crude homogenates is to study their inactivation with organophosphorus compounds. In this report we studied the kinetics of inhibition of AChE from *Panagrellus redivivus* by paraoxon. The non-linear decay of the enzyme activity in semi-logarithmic plots revealed the presence of two components. In addition we studied the reactivity of these components with oxamyl and aldicarb.

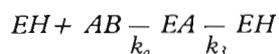
Materials and methods

Panagrellus redivivus (L.) T. Goodey, 1945, was cultured mixtenually at 25 °C on oat meal (Mulder & Bakker, 1988). Nematodes were extracted by soaking 18 to 25 days-old cultures in tap water on a cotton wool filter. After migration through the filter, nematodes were concentrated and homogenized in 50 mM phosphate buffer, pH 7.5 (Mulder & Bakker, 1988). The homo-

genate was stored at -80°C until use. After thawing Triton-X-100 was added to make a final concentration of 1 %. The crude extract was homogenized and centrifuged for 4 min at 1.250 *g*. The supernatant was treated with 3 g Bio-Bead 2 M (BioRad) per ml, by mixing at 4°C for 2 h, to remove the Triton-X-100. This supernatant (S 1) contained two forms of AChE i.e. a fast (F-component) and a slow reacting component (S-component). Heating the S 1 supernatant during 40 min at 45°C and centrifuging for 15 min at 15 000 *g* resulted in a supernatant (S 2) containing solely the activity of the F-component. An extract (S 3) containing predominantly the activity of the S-component was prepared as follows. After thawing, the crude extract was homogenized without Triton-X-100 and centrifuged for 4 min at 1250 *g*. Addition of Triton-X-100 to the supernatant to a final concentration of 1 % revealed a AChE preparation containing the activity of the S-component.

Acetylcholinesterase activity was measured at 25°C using the acetylthiocholine method of Ellmann (Ellmann *et al.*, 1961). Inhibition was measured at 25°C in the absence of substrate by mixing 100 μl inhibitor with 900 μl of a AChE preparation. The remaining activity was measured spectrophotometrically by adding 50 μl portions to assay medium (450 μl) containing acetylthiocholine and 5, 5 di-thio-bis (2-nitrobenzoic acid). Paraoxon and aldicarb were purchased from Serva. Oxamyl was a gift from Shell Nederland BV. Stock solutions of the inhibitors were prepared in 50 mM sodium phosphate, pH 7.5.

When the concentration of the Michaelis complex between enzyme and inhibitor is negligible, the reaction of organophosphorus and carbamate compounds with acetylcholinesterase can be described as :



EH is the active enzyme, AB is the inhibitor and EA is the phosphorylated or carbamylated enzyme. For paraoxon the first order rate constant of spontaneous reactivation (k_j) can be neglected (Clothier *et al.*, 1981; Chemnitius, Haselmeyer & Zech, 1982) and the equation which defines the time course of inactivation is :

$$\log EH_t = \log 100 - 0.43 k_a [AB] t \quad (\text{I})$$

EH_t is the enzyme activity at time t expressed as a percentage of the initial activity. In cases where the enzyme preparation contains one type of AChE, $\log E_t$ is a linear function of the time and the bimolecular rate constant of inhibition (k_a) can be calculated from the slope of the line. Enzyme preparation containing two or more acetylcholinesterases having distinct bimolecular rate constants for paraoxon were characterized by a non linear decay and the bimolecular rate constants were estimated by calculating the partial activities due to each form in the mixture (Chemnitius *et al.*, 1982).

For oxamyl the reactivation was not negligible and the following two equations were used to evaluate the time course of inhibition and the residual enzyme activity at steady state :

$$\ln \frac{EA_{ss}}{EA_{ss} - EA} = (k_a [AB] + k_j) t \quad (\text{II})$$

$$\frac{EH_t}{EA_{ss}} [AB] = \frac{k_j}{k_a} \quad (\text{III})$$

EA_{ss} is the concentration of the inactivated enzyme at steady state expressed as a percentage of the initial enzyme activity. The half-life of the inhibited enzyme is given by :

$$t_{0.5} = 0.69 k_j^{-1} \quad (\text{IV})$$

Results

Inhibition with paraoxon demonstrated that the logarithm of the decay of the acetylcholinesterase activity in the S 1 supernatant is not a linear function of the time (Fig. 1). The enzyme activity is due to two components differing in their reactivity with paraoxon i.e. a fast (F-component) and a slow reacting component (S-component).

Extrapolating the linear part of the inhibition curve to the ordinate shows that approx. 60 % of the total enzyme activity can be contributed to the S-component. The bimolecular rate constant for the S-component is 8.9×10^3 . The decrease in activity of the F-component during incubation with paraoxon was obtained by subtracting the partial activities of the S-component from the total activity. The semi-logarithmic plot, thus obtained was a straight line, showing that the F-component could not be divided in more components. The k_a value of paraoxon for the F-component was 33.5×10^3 .

Testing various extraction procedures demonstrated that the F-component is poorly solubilized in the absence of Triton-X-100. Omitting Triton-X-100 during homogenization and centrifugation resulted in a decrease of the partial activities of the F-component to only 35 %.

The activities of the F and S-component could also be measured separately by subjecting the homogenates to a heat treatment or adding Triton-X-100 (Fig. 2). The S-component could be removed from the S 1 supernatant by heating for 40 min at 45°C and centrifugation. Inhibition of the enzyme activity of the heat treated homogenate (S 2) with paraoxon resulted in a linear decay of the enzyme activity and showed only the activity of the F-component. Inhibition experiments carried out with the S 3 supernatant containing 1 % Triton-X-100 showed that the F-component could be inactivated by Triton-X-100, and that the activity of the F-component was completely restored after removal of the Triton-X-100. Heating and addition of Tri-

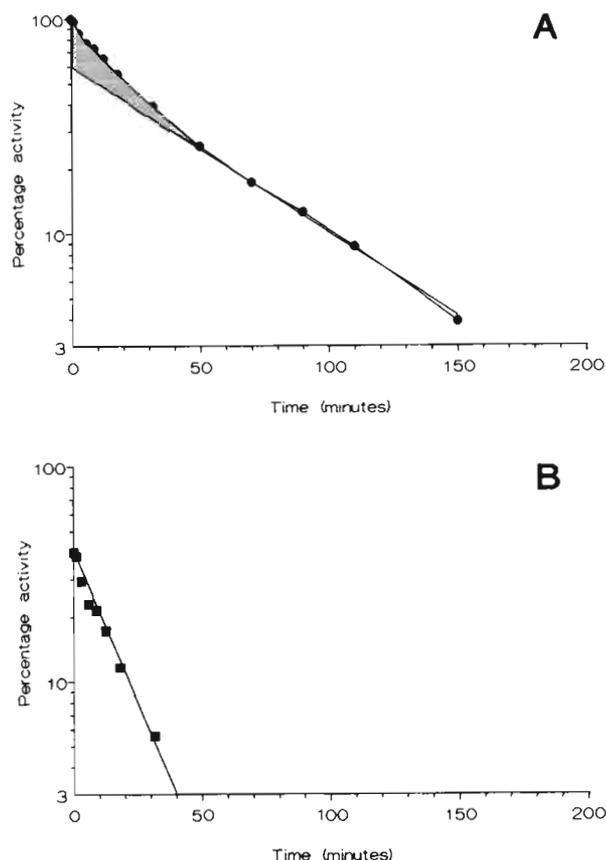


Fig. 1. Identification of two forms of acetylcholinesterase of *Panagrellus redivivus* differing in their reactivity with paraoxon. A : Time course of inhibition of total enzyme activity in a crude homogenate by paraoxon (2 μ M). The enzyme activity of the slow reacting component (S-component) is extrapolated to $t = 0$ and the activity of the fast reacting component (F-component) is marked by hatching. B : Time course of inhibition of the activity of the F-component obtained by subtracting the activity of the S-component from the total activity shown in A.

ton-X-100 did not adversely affect the kinetics of inhibition. The estimates of the bimolecular rate constants of inhibition by paraoxon were similar to those obtained with the S 1 supernatant i.e. 25.5×10^3 and 9.2×10^3 for the F- and S-component, respectively.

As shown in Fig. 3 the enzyme activities in the S 2 and S 3 supernatant are reversibly inhibited by oxamyl and aldicarb. After approximately 40 minutes the inhibition reaches a steady state i.e. the rate of inhibition equals the rate of reactivation. At short inhibition times reactivation is negligible (Clothier *et al.*, 1981) and the bimolecular rate constants were estimated from the initial slopes of the lines by utilizing equation I. The bimolecular rate constants of inhibition by oxamyl and aldicarb differed

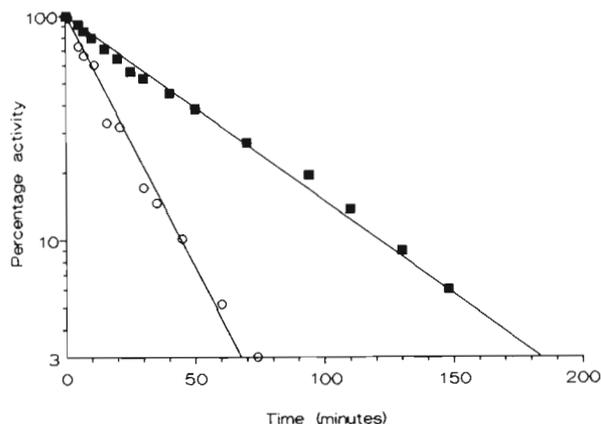


Fig. 2. Time course of inhibition of the F-component (○---○) and the S-component (■---■) of acetylcholinesterase from *Panagrellus redivivus* by paraoxon (2 μ M) — The F- and S-component were differentiated by heating and treatment with Triton X-100. Further details : see text.

about a three-fold between the F- and S-component. The rate constants of spontaneous reactivation (k_j) were derived from the k_a values and the concentration of the inhibited enzyme at the steady state by using equation III. No significant differences between the two forms were observed for the rate constants of decarbamylation (Table 1). The half-life of the carbamylated enzyme was about 40 min. The accuracy of the estimates of the k_a and k_j values was evaluated by using equation III. As shown in Fig. 4 the calculated values and experimentally determined values of the residual enzyme activities were very similar.

Table 1. Bimolecular rate constants of inhibition (k_a) and rate constants of spontaneous reactivation (k_j) of two different types of acetylcholinesterase in *Panagrellus redivivus*.

	S-component		F-component	
	$k_a \cdot 10^{-3}$	$k_j \cdot 10^2$	$k_a \cdot 10^{-3}$	$k_j \cdot 10^3$
Paraoxon (homogenate)	8.9	—	33.5	—
Paraoxon (separated)	9.2	—	25.5	—
Aldicarb	0.8	1.9	2.4	1.8
Oxamyl	12.0	1.5	35.0	1.9

Data are means of at least two experiments. Individual values deviated usually less than 10 % from the mean values.

The estimated kinetic parameters, by using different inhibitor concentrations, showed that k_a values were not significantly affected by the concentration of the inhibitor used, indicating that Michaelis complex formation is negligible.

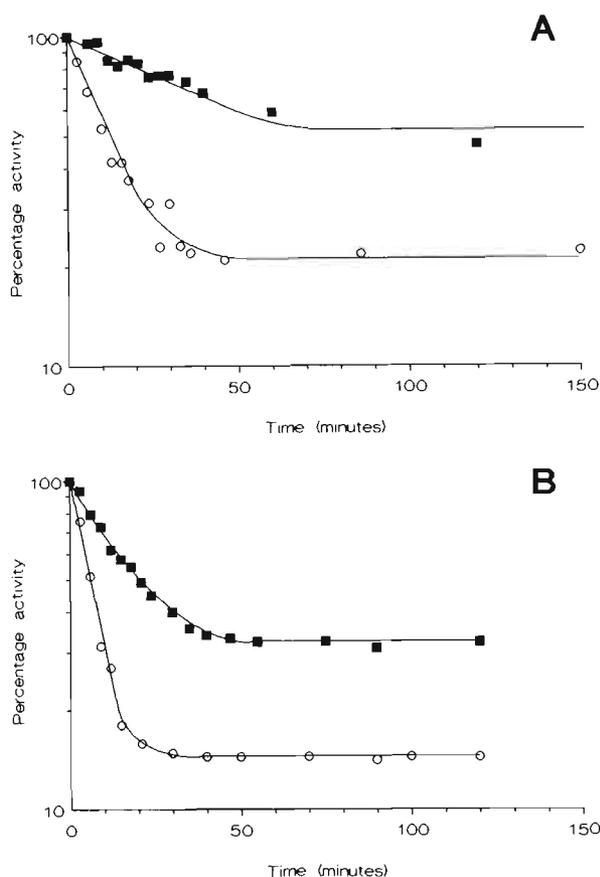


Fig. 3. Time course of inhibition of the F-component (○---○) and the S-component (■---■) of acetylcholinesterase from *Panagrellus redivivus*. A : Inhibition by oxamyl (2 μM); B : Inhibition by aldicarb (0,5 μM). The F- and S-component were differentiated by heating and treatment with Triton-X-100. Further details see text.

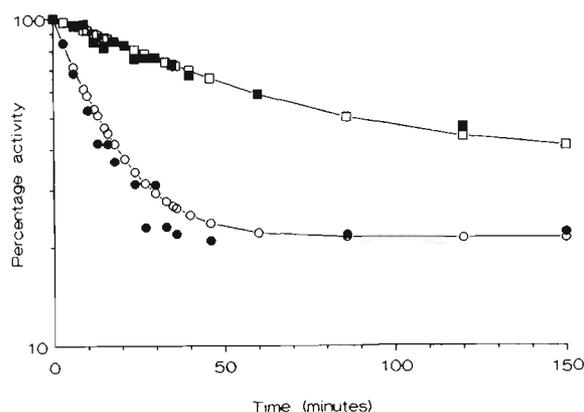


Fig. 4. Time course of inhibition of the F-component (○---○) and the S-component (■---■) of acetylcholinesterase of *Panagrellus redivivus*. Inhibition by oxamyl (2 μM) (● and ■ measured, ○ and □ calculated).

Discussion

Our data show that studying the kinetics of inhibition of two distinct forms of acetylcholinesterase from *P. redivivus* can be achieved without the application of arduous separation techniques, which may modify the kinetic properties of the enzymes. Evidently, it can not be excluded that more components are present in the crude homogenates of *P. redivivus*. Components present in extreme low quantities or components having slightly different kinetic properties are not resolved by performing inhibition experiments with paraoxon.

In vitro inhibition of acetylcholinesterase is for most pesticides usually indicative for the mode of action *in vivo*. In spite of the widespread use of I_{50} values in literature, i.e. the concentration at which 50 % of the enzyme is inhibited after a fixed period of time, the bimolecular rate constant (k_a) is the parameter best suited to describe the inhibition. Most active pesticides have bimolecular rate constants within the range of 10^3 to $10^6 \text{ M}^{-1}\text{min}^{-1}$. Lower k_a values usually indicate that activation is required to exert *in vivo* acetylcholinesterase inhibition. Although the bimolecular rate constants of inhibition by paraoxon of the S-component ($K_a = 9.2 \cdot 10^3 \text{ M}^{-1}\text{min}^{-1}$) and F-component ($K_a = 25.5 \cdot 10^3$) are within this range, they are rather insensitive for paraoxon when compared with acetylcholinesterases from other organisms. The bimolecular rate constants of acetylcholinesterase from bovine erythrocytes ($9.0 \cdot 10^5$; Chemnitz *et al.*, 1982), house fly ($5.8 \cdot 10^5$; Devonshire & Moores, 1984), mites ($1.5 \cdot 10^7$; Smitsaert *et al.*, 1970) and house sparrow ($9.9 \cdot 10^6$; Yaweth *et al.*, 1979) are much higher. A proper evaluation of the relative sensitivity of the acetylcholinesterases of *P. redivivus* to aldicarb and oxamyl is not possible, because data on the bimolecular rate constants of these compounds for acetylcholinesterases from other organisms are scarce.

Decarbamylation rate constants are usually estimated by measuring the rate of reactivation after removal of the inhibitor (Aldridge & Reiner, 1972). In this report we did not remove the inhibitor but estimated the reactivation constants by utilizing equation III, which allows the calculation of k_3 when k_a and the residual activity at steady state are known (Clothier *et al.*, 1981). Application of equation II demonstrates that the k_3 values listed in Table 1, but also the k_2 values, describe the time course of inhibition fairly well. The maximum difference between the calculated and experimentally determined values of residual enzyme activities was usually less than 10 %. The observation that the rate constants of spontaneous reactivation for aldicarb and oxamyl are not significantly different for the S- or F-component is concordant with the fact that both compounds are N-monomethyl carbamates, which form identical carbamylated enzymes. The result showing that the reactivation rate constants of the S- and F-component are

similar is consistent with the finding that the decarbamylation rate constants of N-monomethyl carbamates show little variation (Hastings *et al.*, 1970). Studies on a range of organisms have shown that the reactivation rate constants usually range from $0.6 \cdot 10^{-2}$ to $3.4 \cdot 10^{-2}$, corresponding with half-lives of 20 to 120 min.

Whether the F- and S-component are isoenzymes, i.e. encoded by genes at distinct loci, can obviously not be inferred from our data. Circumstantial evidence that the F- and S-components may be isoenzymes comes from studies on *C. elegans* (Johnson & Russel, 1983). Similar to the F- and S- component, the two isoenzymes in *C. elegans* can also be distinguished by heating, treatment with Triton-X-100 and inhibition with pesticides. It has been demonstrated that the two isoenzymes in *C. elegans* have overlapping functions and can substitute for one another functionally. Histochemical studies showed that the two forms were located in the same areas and mutants lacking either of the two acetylcholinesterases displayed no distorted functional behavior (Culotti *et al.*, 1981; Johnson *et al.*, 1981). These findings indicate that if the occurrence of such isoenzymes is a wide spread feature in the phylum Nematoda, the effectiveness of organophosphorus and carbamate compounds is predominantly determined by the inhibition of the most insensitive acetylcholinesterase isoenzyme.

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