INTERNATL MICROBIOL (2000) 3:117–123

RESEARCH ARTICLE

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Esterases in marine dinoflagellates and resistance to the organophosphate insecticide parathion

Received 20 December 1999
Accepted 18 April 2000

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Summary Esterases are involved in the susceptibility or resistance of organisms to organophosphate pesticides. We have examined the action of parathion on the marine dinoflagellates *Crypthecodinium cohnii* and *Prorocentrum micans* by looking at their esterases. One-dimensional gel electrophoresis, immunoblotting and cytochemistry plus image analysis were used to characterize the nature and distribution of the enzymes. Esterases were found in both species, but there appeared to be no particular intracellular localization. The esterase activity of the heterotrophic species *Crypthecodinium cohnii* was 30-fold greater than that of the autotrophic *Prorocentrum micans* and had an antigenic site in common with mosquito esterase. The resistance of *Crypthecodinium cohnii* to parathion was specific and reversible. Less parathion entered the parathion-resistant *Crypthecodinium cohnii* cells than the untreated control cells. Parathion-resistant cell extracts of *Crypthecodinium cohnii* analyzed after immunoblotting also contained an additional band of esterase activity. These results confirm the importance of esterases in toxicological studies of organophosphate insecticides, especially those of marine dinoflagellates.

Key words Dinoflagellates · Esterases · Organophosphates · Parathion · Resistance to pesticides

Introduction

The term “esterases” is used to describe those hydrolases that cleave esters made up of organic acids, mineral acids and alcohols or phenols. Cleavage is often reversible and some esterases can catalyze the synthesis of esters. These enzymes, particularly arylesterases and cholinesterases, interact with organophosphate insecticides in one of two possible ways: insecticides can inhibit them [7, 10, 19, 40, 43], or be degraded by esterases [2, 25, 44] or by other enzymes, the organophosphate acid anhydrases (OPA). These enzymes include A esterase, diisopropyl fluorophosphatase, phosphotriesterases, somanase, parathion hydrolase and paraoxonase [18].

Esterases are widely distributed and several mammalian esterases have been purified and characterized [20]. Ester-cleaving enzymes from eubacteria have also been described [17, 21]. Overproduction of a detoxifying esterase can occur in organophosphate insecticide resistance in mosquitoes [15, 24, N. Pasteur (1977) Ph. D. Thesis Univ Montpellier], the peach-potato aphid *Mysus persicae* [9] and the sheep blowfly *Lucilia cuprina* [26]. The acetylcholinesterase activity of fish extract has been used to monitor pesticide pollution of the marine environment [10].

The Dinoflagellata, or Dinomastigota according to Margulis and Schwartz [22], are a particular phylum of protists. Recent ultrastructural and biochemical investigations of the dinoflagellate nucleus (for a review, see [37, 38]) have demonstrated their uniqueness, and molecular data on their ribosomal RNA sequences indicate that they have a common ancestor with ciliates and Apicomplexa [36]. They are the only eukaryotes whose chromatin is totally devoid of histones and nucleosomes, but is associated with small amounts of basic proteins [11, 35] whose functions are still unclear. They are also distinguished by a permanent nuclear envelope and chromosomes which remain condensed throughout the cell cycle, closed mitosis (dinomitosis) [42], and the high content of the rare base hydroxymethyluracil in their DNA[16] .

There have been few studies of enzymes in dinoflagellates. A type-1 protein phosphatase has been identified in the okadaic acid-producing marine dinoflagellate *Prorocentrum lima* [6]. Moreover, there are periodic acid Schiff (PAS) accumulation

bodies (lysosomes) in the cytoplasm of *Prorocentrum lima* and *Prorocentrum maculosum* [45]. Biochemical and immunocytochemical analyses in *P. lima* have shown okadaic acid in the cytoplasm [33], and okadaic acid and its target, protein phosphatase PP2A, close to the centrosome region [1].

We used dinoflagellates as test organisms in earlier studies on the action of the organophosphate insecticide parathion on the marine environment, because they contain no inhibitable acetylcholinesterase [P. Prévot, 1985, Ph. D. Thesis Univ Paris 6]. Parathion was shown to be toxic for two free-living marine dinoflagellates, the autotrophic *Prorocentrum micans* Ehr. and the heterotrophic *Crypthecodinium cohnii* B. Parathion affects photosystem II of the photosynthetic pathway in *P. micans* [29, 30]. This study demonstrates the presence of esterases in unicellular marine planktonic organisms. The esterase activity in the two dinoflagellate species we studied appears to be different (30-fold greater in *C. cohnii* than in *P. micans*), as does their resistance to organophosphorous compounds. Moreover, the presence of an antigenic site in common with mosquito esterase is demonstrated.

Materials and methods

Biological material *Crypthecodinium cohnii* (Seligo) Chatton 1938 (Order Peridiniales) is a heterotrophic species measuring 10 μm in diameter, with a short but complex 9-h reproductive cycle [3], a large number of chromosomes (100) and an average DNA content of 6.9 pg per nucleus [13]. *C. cohnii* cells obtained from the Cambridge Botany School (UK) were cultured in the dark at 27ºC in gelled MLH medium [3], or grown at 27ºC in the dark and without bubbling in 500-ml flasks containing 250 ml of sterile MLH medium.

Prorocentrum micans Ehrenberg 1834 (Order Prorocentrales) cells were obtained from the Cambridge Botany School. This species is autotrophic, measures 30 μm in diameter, and has a rather long life cycle (5.5 days) and a discontinuous, typically eukaryotic interval of DNA synthesis [4], many chromosomes (100) and a large DNA content (42 pg per nucleus) [12]. *P. micans* cells were grown under a 12/12 light/dark cycle (2000 lux, daylight fluorescent tubes, Philips), at 20ºC, without bubbling, in sterile Erd-Schreiber's medium [5]. The cultures were grown in one-liter conical Pyrex glass flasks, each containing 500 ml of medium.

Soluble protein extraction The cells in 1 liter of exponentially growing culture (4–10 × 10^5 cells/ml) were collected by centrifugation (2300× *g* for 15 min), washed 3 times with filtered (0.2 μm filter) sea water and suspended in double-distilled water at 4ºC (10 min). They were collected by centrifugation at 1800× *g* for 10 min and suspended in buffer [Tris HCl pH 7.1 (20 mM), NaCl (50 mM)] and broken by sonication (20 s in ice), followed by treatment in a French press at 14,000 Pascal/inch. The resulting lysate was centrifuged for 1 h at 100,000× *g* at 4ºC and the soluble proteins in the supernatant were saved. Because freezing the cell pellets blocked the esterase activity, we used fresh extracts.

Spectrophotometric assay of the esterase activity Esterase activity was measured by spectrophotometry of the soluble protein extract using a Varian Cary 3E spectrophotometer. The absorbance was read at 505 nm. Esterase activity was revealed by the breakdown of the substrate naphthylacetate (10^{-3} M in acetone) and fast blue RR salt as dye (300 μg in 10 ml H_2O and 3% SDS) in phosphate buffer (5 mM, at pH 6.4). Protein extracts (800 μl) were incubated for 30 min with 300 μl substrate and 300 μl dye. A calibration curve was constructed with purified porcine liver esterase (Sigma).

Enzyme inhibition Enzymes that hydrolyze simple ester substrates such as naphthylacetate are termed nonspecific esterases. They can be subdivided into 3 groups depending on their inhibition by organophosphate: arylesterases (A type), carboxylesterases (B type), and acetyl esterases (C type). This classification coincides with that based on the effects of organophosphate inhibitors. We used the inhibitors eserine and E600 (= paraoxon) at 10^{-5} M for 30 min at 25ºC.

Electrophoresis Esterases were analyzed by non denaturing 10% polyacrylamide gel electrophoresis. Revelation of esterases was done in the presence of the two substrates, 1% α-naphthylacetate and β-naphthylacetate in 0.38 M Tris-HCl buffer in acetone/H_2O (vol/vol). Arylesterases break down the substrates in α- and β-naphthol which is fixed by the diazoic derivative fast blue RR salt (0.1%), giving a red-brown color.

Antibodies and Western blotting *C. cohnii* esterases were revealed by Western blotting using a Millipore apparatus. Proteins were separated by SDS-PAGE and transferred to a nitrocellulose membrane by electroblotting. Western blots were incubated with rabbit anti-mosquito esterase B1 antiserum [23] (a gift from N. Pasteur). Antigen-antibody complexes were revealed by alkaline phosphatase reaction with nitro-blue tetrazolium plus 5-bromo-4-chloro-3 indolyl phosphatase diluted 300-fold in Tris–NaCl–$MgCl_2$.

Cytochemistry: Fixation and cryomicrotomy *P. micans* and *C. cohnii* pellets were incubated with polyvinylpyrrolidone (PVP) [41], quick-frozen in liquid nitrogen, and cut into semi thin (1 μm) sections on an ultramicrotome LKB. The sections were placed on coverslips, fixed again with 2% formaldehyde for 10 min, and washed three times for 10 min in 0.1% phosphate-buffered saline (PBS) (0.15 M NaCl, 0.01 M Na_2HPO_4, 0.01 M KH_2PO_4, pH 7.4).

Enzyme detection Non specific esterases were detected with α-naphthylacetate as the substrate and hexazonium pararosaniline as the coupling agent. Slides were incubated for 15 min at 37ºC in α-naphthylacetate, washed three times in double-distilled water, counterstained with 2% methyl green (chloroform-washed), washed in PBS and mounted in glycerol/pyrogallate. Esterase activity appeared reddish-brown, and nuclei were green [28].

Observations and measurements Sections were examined under a Reichert Polyvar light microscope coupled to a computer-

assisted image analyzer consisting of a "Sapphire" (QUANTEL) image processing system, a PC with an SVGA monitor, a LHESA 4015 camera, a Sony RGB control monitor and a Bernouilli box system for image storage. The software was the DIGITHURST image analysis system. Images were processed and averaged, and the background subtracted. A line profile of the grey intensity levels was obtained for a cell diameter. The grey intensity corresponded to the staining intensity of the cell. These profiles were plotted for control cells without substrate and for cells incubated with substrate. Parathion penetration of cells was estimated by gas chromatography [31].

Resistance to parathion A non lethal dose of parathion (5 ppm) slowed cell multiplication. This concentration was used to habituate the cells to parathion. The resistance of *C. cohnii* was determined by comparing the growth of control cells (without parathion) and habituated cells grown in liquid medium containing 5 ppm parathion. Both cultures were then subcultured in medium containing each a lethal dose of parathion (10 and 15 ppm) for three days at 24ºC in the dark. Surviving (motile) cells were counted by using a Coulter Counter (Coultronic, Andilly, France). Cells were grown on solid agar medium containing 20 ppm parathion to isolate resistant clones, which were maintained in 5 ppm parathion.

Specificity and reversibility of parathion resistance The specificity of resistance was assayed by growing cells in paraoxon which is more toxic than parathion in mammals [8]. Sensitive and resistant cells were grown in 10 ppm paraoxon or parathion and cell growth was compared. The reversibility of parathion resistance was assessed by growing resistant cells in medium without parathion for 36 generations, and then placing them in a medium containing 10 ppm parathion. Cell death after 24 h was compared to that of resistant cells (grown in 5 ppm parathion).

Results

Esterase activity in the dinoflagellates The extracts of *C. cohnii* cells contained 30-fold more esterase activity than did those of *P. micans* (Table 1, Fig. 1). No inhibition was detected for either E 600 or eserine. The results suggest the presence of a mixed B type esterase, probably a mixture of A, B and C esterases, with a predominance of type A. As previously shown, there was no acetylcholinesterase [P. Prévot, 1985, Ph. D. Thesis Univ Paris 6].

Table 1 Esterase activity in *Prorocentrum micans* and *Crypthecodinium cohnii*

	Volume of pellet (cm^3)	Absorbance (A_{505})	Equivalent activity of porcine liver esterase	Equivalent activity/Volume of pellet
P. micans	0.1	0.5±0.02	0.001	0.01
C. cohnii	0.5	2.85±0.15	0.185	0.37

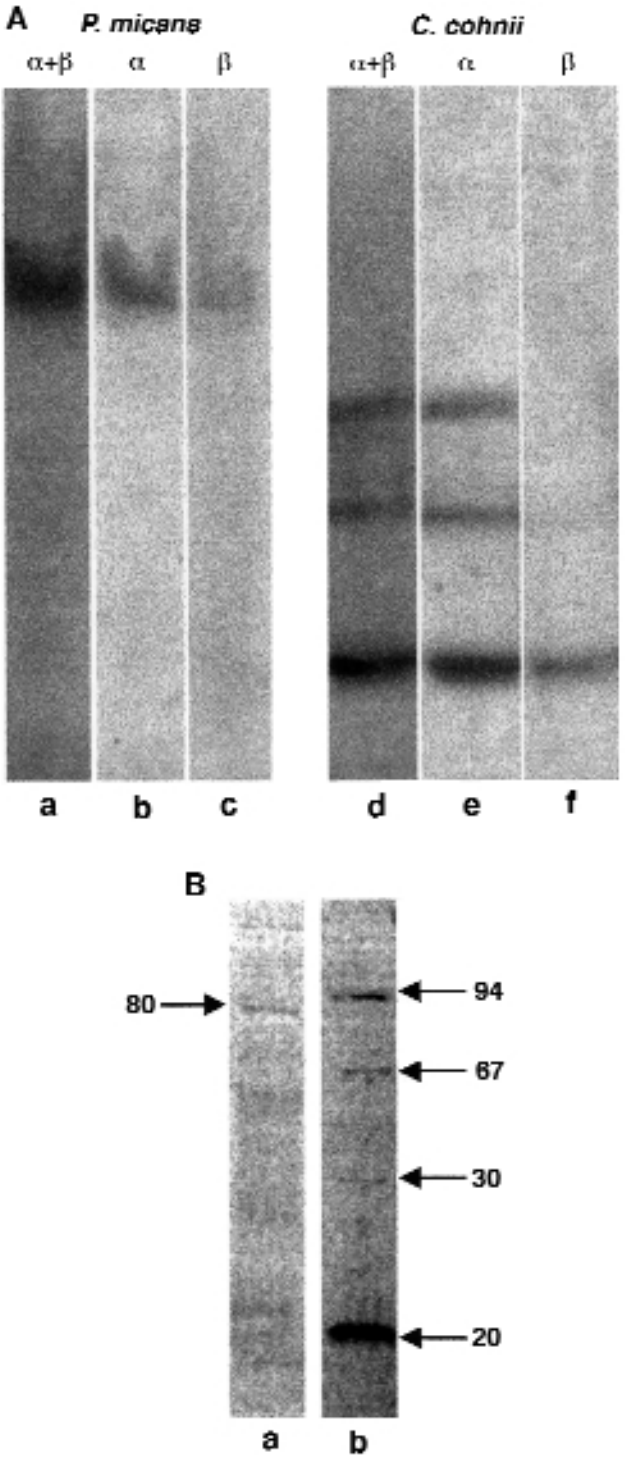

Fig. 1 Esterase activity in two dinoflagellates. (A) Non-denaturing polyacrylamide gel electrophoresis of soluble proteins extracted from *Prorocentrum micans* and *Crypthecodinium cohnii*. **a**, **d**: α+β-Naphthylacetate; **b**, **e**: α-naphthylacetate; **c**, **f**: β-naphthylacetate (B) Western blot of *C. cohnii* extracts after reaction with rabbit anti-esterase B1 antiserum (numbers indicate kDa). **a**: Protein extracts treated with anti-esterase B1antibody; **b**: molecular weight markers

Esterase activity after electrophoresis Proteins extracted from exponentially growing cultures (Fig. 1A) of *P. micans* (lane **a**) and *C. cohnii* (lane **d**), separated by non-denaturing gel electrophoresis and stained with α+β naphthylacetates, showed esterase activity in both species. The *P. micans* esterase activity was different from the *C. cohnii* activity. The *P. micans* extract contained fewer bands than the *C. cohnii* extract, and their positions were different (Table 1, Fig. 1A). The electrophoretic patterns of cell extracts incubated with the esterase inhibitor tributyl phosphorothionate (10 ppm in acetone) yielded no bands (data not shown), indicating that the activity measured was that of a true esterase. The esterase activity in *C. cohnii* (Fig. 1A, lanes **e**, **f**) and *P. micans* (Fig. 1A, lanes **b**, **c**) extracts was revealed by α- and β-naphthylacetate alone. In both species more esterase activity was revealed with α-naphthyl than with β-naphthyl substrates. The rabbit anti-*Culex pipiens* esterase B1 immune serum revealed an 80 kDa band in the *C. cohnii* cell extract (Fig. 1B).

Esterase revealed by cytochemistry There was esterase activity in cryosectioned cells of *P. micans* and *C. cohnii*, as shown by the reddish-brown color after the specific cytochemical reaction (Fig. 2). The staining was not very intense and not restricted to a specific compartment of the cell. Nevertheless, the semiquantitative study revealed the presence of esterases in both organisms. The staining was clearly more intense with naphthylacetate (Fig. 2A, C) than in the controls without substrate (Fig. 2B, D). There was more esterase activity in *C. cohnii* than in *P. micans*.

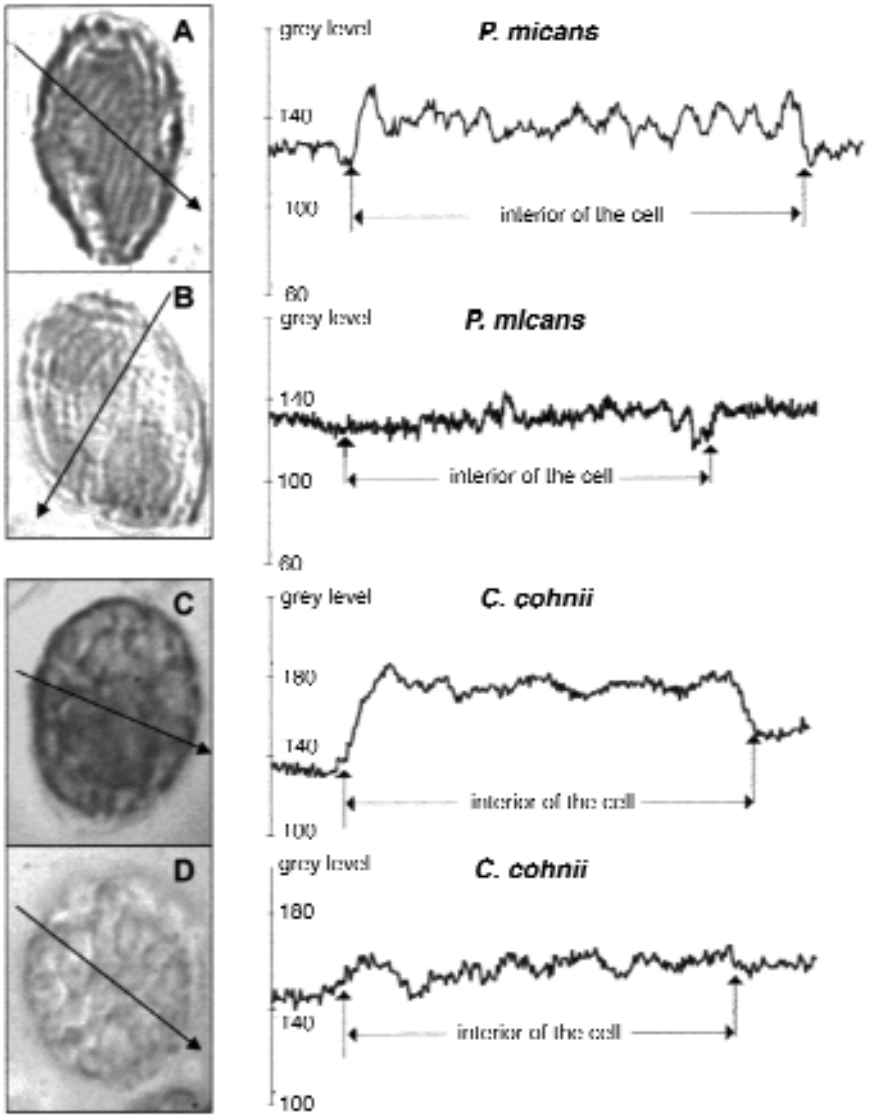

Fig. 2 Video microscope images of cryosectioned cells and line profile of the grey intensities of a cell diameter revealing esterase activity. Photographs A and C: cells with substrate (α-naphthylacetate). Photographs B and D: cells without substrate

Resistance to parathion The sizes of the control and treated cells of *C. cohnii* were compared. The size varied when cells were grown in medium containing 10, 15 or 20 ppm parathion. The rate of division was reduced in cells grown in 5 ppm parathion. This concentration was used to habituate cells to parathion.

Cells grown in 5 ppm parathion for 80 generations (2.5 divisions per day) became resistant. Non-treated (control) and treated (as just described) cells reacted differently when grown in 10 ppm parathion (lethal concentration). The survivals of those two types of cells (treated and non-treated) were different regardless of the incubation time in lethal concentration. After incubation at 10 ppm for three or four days, the survival of control cells was only 1.5%, whereas it was 30% for treated cells. A clone of resistant cells was isolated on solid agar medium. We monitored the behavior of these cells in lethal medium for three days. Fifty percent of control cells were dead after 24 h incubation, but surviving cells started to grow again slowly after 48 h. The resistant cells were not killed, but the growth rate decreased somewhat.

Characteristics of resistant cells Gas chromatography showed decreasing penetration of parathion into resistant cells. The sensitive cells contained an average of 0.25 pg parathion, whereas resistant cells contained 0.7 to 1 pg parathion. The cells grown in medium containing 5 ppm parathion had a higher total protein content. Resistant and revertant cells contained an extra esterase band (supplementary esterase, E_{sup}) after degradation of β-naphthylacetate (Fig. 3, arrow).

Specificity of the resistance to parathion Paraoxon is the oxidized form of parathion; its neurotoxic and anticholinesterasic effect in metazoa is much stronger than that of parathion. It was very toxic for dinoflagellates, but less toxic than parathion (Fig. 4A), in contrast to findings in mammals. Because resistant *C. cohnii* cells were as sensitive to paraoxon as control cells (Fig. 4B), the resistance was assumed to be specific to parathion.

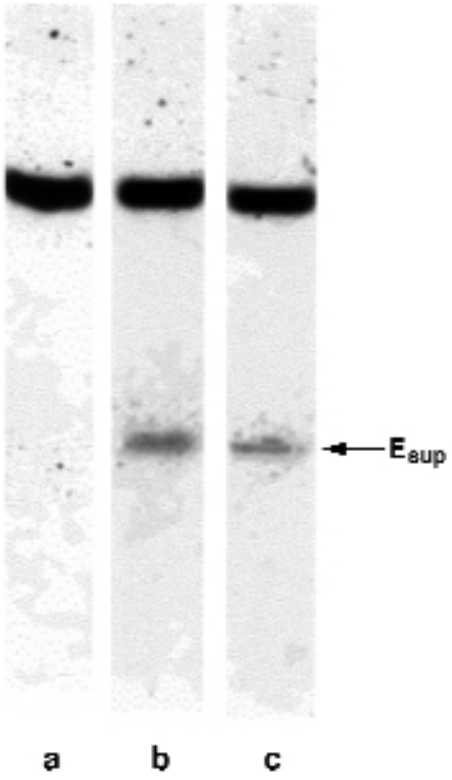

Fig. 3 Evidence for a supplementary esterase band (E_{sup}, arrow) in *Crypthecodinium cohnii*, after non-denaturing polyacrylamide electrophoresis of soluble proteins revealed by β-naphthylacetate. **a**: Sensitive (control); **b**: resistant; **c**: revertant

Discussion

Esterases in dinoflagellates We have demonstrated the presence of esterase activity (arylesterase) in two dinoflagellates, the autotrophic *P. micans* and the heterotrophic *C. cohnii*. There is less esterase activity in *P. micans* than in *C. cohnii* (30-fold) and there is no specific localization of esterases in these cells. The numbers of esterase bands after non-denaturing electrophoresis of *C. cohnii* and *P. micans* cells differ; there are more bands in *C. cohnii* than in *P. micans* protein lysates. Conversely, it has been shown that the esterases in *P. micans* are more sensitive to parathion than those in *C.cohnii* [P. Prévot, 1985, Ph. D. Thesis Univ Paris 6].

The difference in the sensitivities of *P. micans* and *C. cohnii* esterases to parathion cannot be due to differences in esterase activity alone. Parathion acts on photosystem II in *P. micans* (which is photosynthetic), by blocking it and producing plastoglobuli [28], but this is not the case in *C. cohnii*, which is not a photosynthetic organism. On the contrary, parathion increases intracellular ATP concentration in *C. cohnii* and increases its mitochondrial content, whereas in *P. micans* considerable alterations in mitochondria appear [32]. In the rat liver, the respiratory control ratio decreases in the presence of pesticides [39], and some authors have suggested that organophosphorous compounds act as inhibitors of the mitochondrial electron-transport system by modifying the permeability of the inner mitochondrial membrane. In contrast, other authors observed in vitro alterations of calmoduline-active conformation (known to regulate Ca^{2+} transport), and inhibition of its biological activity [27]. Esterases are present in many organisms, from protists and invertebrates to vertebrates, and parathion seems to act differently depending on the specialization of the cells. Thus it is not unreasonable to think that organophosphorous compound sensitivity and resistance are managed by separate systems dependent on different cell types.

Because the anti-*Culex pipiens* esterase antibody reacted with a *C. cohnii* esterase site, we surmise that the antigenic site

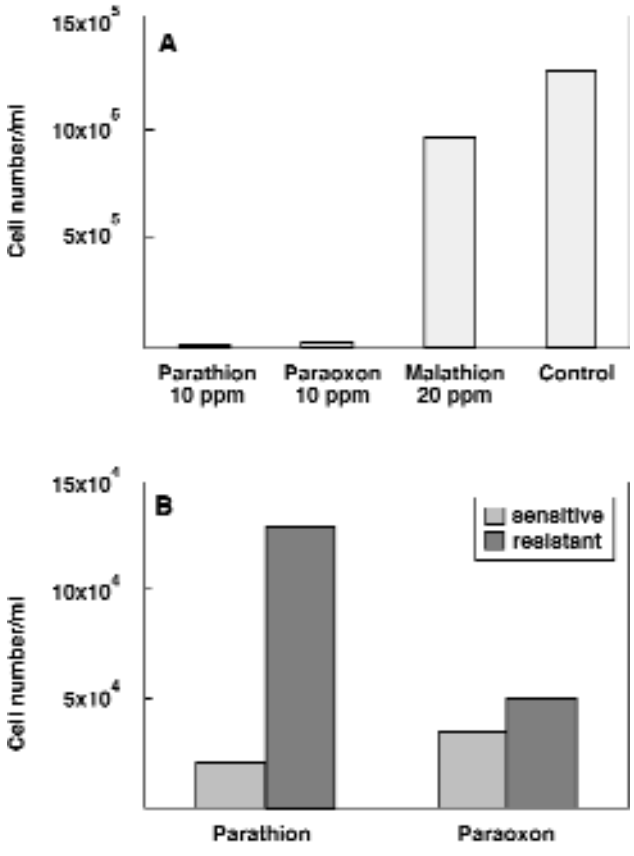

Fig. 4 Specificity and reversibility of the resistance of *Crypthecodinium cohnii* cells to organophosphate. (A) Toxicities of parathion, paraoxon and malathion in *C. cohnii* cells. (B) Parathion and paraoxon toxicities for resistant and sensitive *C. cohnii* cells

on the *C. pipiens* esterase was similar to that of a dinoflagellate esterase (*P. micans* was not treated). It is noteworthy that esterases from species as different as mosquitoes and dinoflagellates have a common antigenic site, indicating a high degree of conservation of this enzyme. Esterases may play a part in the resistance of dinoflagellates to parathion, as they do in mosquitoes and other animals [15, 23, 24, 26, 32, N. Pasteur, 1977, Ph. D. Thesis Univ Montpellier].

Resistance and reversibility We selected for resistance to parathion in *C.cohnii* and obtained cells that were resistant to a lethal dose of parathion and grew more slowly than untreated cells. This might have been due to a genetically transmitted resistance *sensu stricto*, or habituation in which cells adapt to an unfavorable environment, and which is lost when the toxic agent is removed. This is the case for parathion-habituated *C. cohnii* cells, as they recovered their initial characteristics. The demonstration of genetic mutations will require amplification of the selection pressure by cultivating resistant cells for more than one thousand generations.

C. cohnii contains an extra esterase (E_{sup}) which may play a part in resistance by increasing parathion breakdown in the cells. However, this esterase is also present in revertant cells. Similar qualitative esterase differences were found in *Anopheles stephensis* and *Anopheles arabiensis*, which are resistant to malathion [14].

Thus, resistance of dinoflagellates to organophosphorous compounds such as parathion is probably due to multiple causes. The contributions of esterases to this phenomenon should become clearer once these enzymes have been purified and their corresponding genes cloned, and their structure and regulation studied. Esterases may be important indicators of the action of organophosphate insecticides on marine phytoplankton, and may hence be essential for ecotoxicological studies, especially those of both photosynthetic and non-photosynthetic dinoflagellates.

Acknowledgments We thank Marie Albert and Danièle Saint-Hilaire for technical assistance and Dr. Nicole Pasteur for donating the rabbit anti-esterase B1 antiserum. This work was supported by the Direction des Recherches et Études Techniques (DRET), Délégation Ministérielle pour l'Armement, contract number 90/143 and by CNRS UMR 7628 for M-O. S-G. and P.P.

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