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Demonstration of the Excretion by *Dunaliella bioculata* of Esterases Implicated in the Metabolism of Deltamethrin, a Pyrethroid Insecticide

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Deltamethrin (DTM) is a pyrethroid insecticide being derived from the naturally-occurring substance: pyrethrum. This neurotoxic molecule is considered an useful insecticide due to its high insecticidal activity with low mammalian toxicity and limited soil persistence but however with a non-negligible toxicity to the aquatic fauna (Casida et al 1983).

Our previous studies on the uptake and fate of DTM in Dunaliella bioculata, a unicellular marine alga, have revealed that this insecticide was accumulated in cells, particularly in lipid droplets, but was not metabolized (Baeza-Squiban et al 1987,1988). By contrast,a degradation product of DTM was observed in the culture medium, which was linked partially to the spontaneous hydrolysis of the molecule but chiefly to the biological activity. The nature of the metabolites produced and preliminary studies using an esterase inhibitor suggested the role of esterases in this extracellular metabolism. In the present work, our intent was to confirm the excretion of esterases into the culture medium,using two different methods: by studying the inhibition of the DTM metabolism by several known esterase inhibitors, and directly by titrating the esterases in the medium.

MATERIALS AND METHODS

Dunaliella bioculata (Volvocales), a unicellular green alga, was grown on a mineral growth medium (Marano et al 1978) at 24°c, under a 12 hr/12 hr light-dark cycle at 50 W meter^{-2}.

The metabolism of DTM, labeled with C^{14} on either the alcohol moiety or on the acid moiety was studied not only in cells and in their corresponding incubation

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medium but also in a fresh culture medium (i.e., a medium without cells) to determine the spontaneous degradation of DTM, and in conditioned culture medium (i.e., a medium having being used for cell culture with the cells then removed) to determine the influence of the biological activity on DTM degradation. Esterase inhibitors (TEPP: tetraethyl pyrophosphate, DEF: S,S,S tributyl phosphotrithioate and paraoxon: O,O-diethyl para-nitrophenyl phosphate) were used at 0.1 mM in conditioned culture medium.

Metabolites were analysed by TLC on silica gel chromatoplates and further autoradiography and quantification of radioactivity in gel regions scraped from the plates, according to the method already described (Baeza et al 1988).

Before titration of esterases, the conditioned culture medium was concentrated 20- to 30-fold by tangential-flow ultrafiltration (Pellicon System, Millipore, equipped with a 10,000 Da molecular weight cutoff membrane) and desalted by gradual addition of 10 mM Tris-HCl buffer, pH 8. Esterase activity was determined with p-nitrophenyl acetate as substrate. The reaction mixture contained 50 mM Tris-HCl buffer at pH 8, 1.65 mM p-nitrophenyl acetate solution (stock solution: 1% in acetone) and enzyme in a total volume of 3 mL. The absorbance of product formed (p-nitrophenol) was measured, after 5 min incubation at 25°C, at 405 nm using in the reference cuvette a blank containing the enzyme solution boiled 10 min at 120 C. The molar absorption coefficient for p-nitrophenol at pH 8 was 16,500 M^{-1} cm^{-1}. The enzyme activity was calculated as µmol of p-nitrophenol generated/min/L of conditioned culture medium. Protein contents were evaluated using Coomassie Blue (Bradford 1976).

Cytochemical localization of esterases in Dunaliella was performed on algae fixed 1 hr in 1.25% glutaraldehyde. After two rinses with 0.1M sodium phosphate buffer, pH 6.8, cells were incubated overnight at 4°C in a reaction medium containing 500 µL 1% α-naphthyl acetate in acetone as substrate, 25 mg Fast Blue RR salt in 25 mL of 0.1 M sodium phosphate buffer, pH 6.8. The substrate was omitted from control reaction medium.

RESULTS AND DISCUSSION

Analysis of the autoradiochromatogram (Fig. 1,I) did not reveal any degradation products of DTM in cellular extracts. This result suggested that DTM was not metabolizable in Dunaliella either because this alga did not have the necessary enzymatic systems or because the molecule was protected against metabolism through

storage in organelles. In order to elucidate the mechanism implicated, the search of an esterasic activity was undertaken, since esterases are known to metabolize DTM in insects (Jao and Casida 1974) and mammals (Casida et al 1979). As shown by the cytochemical study, the light microscopic examination of algal cells revealed black reaction product in the anterior part of the cell, deposited as cytoplasmic vesicles (Fig. 2,a), but when the substrate was omitted from the reaction mixture, the black reaction product was absent (Fig. 2,b). So, Dunaliella possessed esterases located in vesicles which were probably vacuoles since the vacuoles of plants are now recognized as being a lytic compartment similar to lysosomes in animal cells (Matile 1978). With regard to DTM, given its lipophilic nature, it was accumulated in lipidic structures, as previously shown (Baeza et al 1987). This non-correspondence between DTM storage and the areas with esterasic activity, may explain the preservation of DTM in its native form.

In contrast, DTM was degraded in the incubation culture medium of algae (Fig. 1,II), chiefly by hydrolysis of the ester bond since the metabolites were in different location on the chromatogram according to the label used. The observation of the pattern of degradation of DTM in the fresh culture medium (Fig. 1,III) revealed the absence of some of the metabolites compared to the pattern in the incubation medium, and particularly the metabolite Rf:0.8 (labeled on the alcohol moiety) which by contrast was present in the conditioned culture medium (Fig. 1,IV)

These observations led us to consider this metabolite Rf: 0.8 as a marker of a biological activity in the conditioned culture medium, especially since it disappeared after the boiling of the conditioned culture medium (Fig. 3,C_b). Since the release of this product was linked to the hydrolysis of DTM, the existence of an esterase excreted by cells into the culture medium was suspected. In order to confirm this hypothesis, various esterase inhibitors were tested in the conditioned culture medium. At 0.1 mM these inhibitors inhibited the release of the product Rf: 0.8 with decreasing potencies as follows: DEF> TEPP> paraoxon.

Indirectly, by studying of the metabolism of DTM, we demonstrated an extracellular esterase activity. Now, it remained to prove the existence of esterases in the culture medium by titration of this enzyme.

The conditioned culture medium contained few proteins

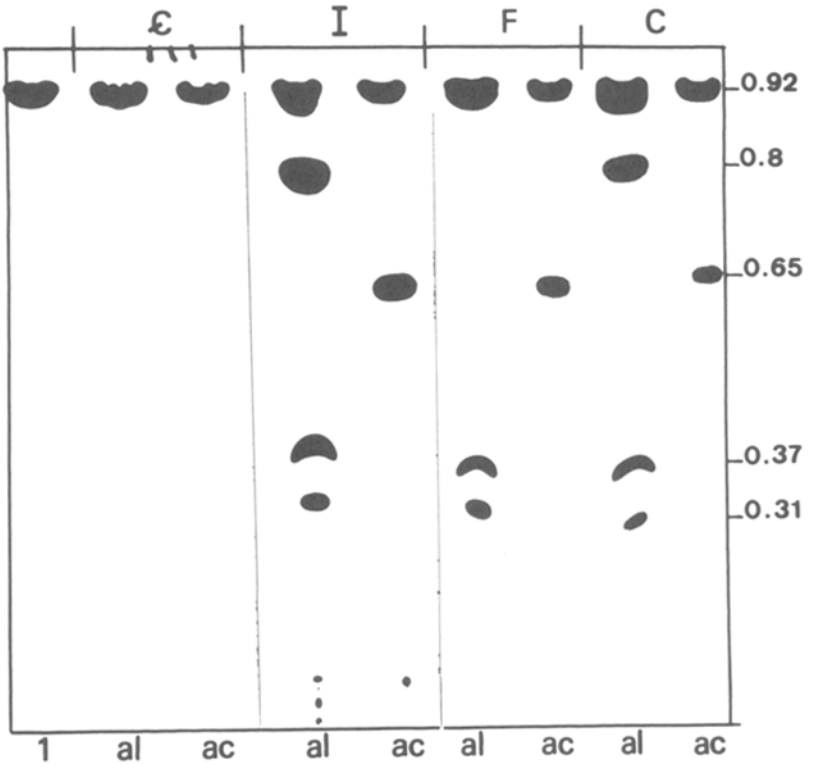

Figure 1. Autoradiochromatogram of organic extracts from 1- control DTM, I: cells (C), II: the incubation medium (I), III: the fresh culture medium (F), IV: the conditioned culture medium (C)
In each case, preparations were treated with DTM labeled with C^{14} either on the acid moiety (ac) or on the alcohol moiety (al)

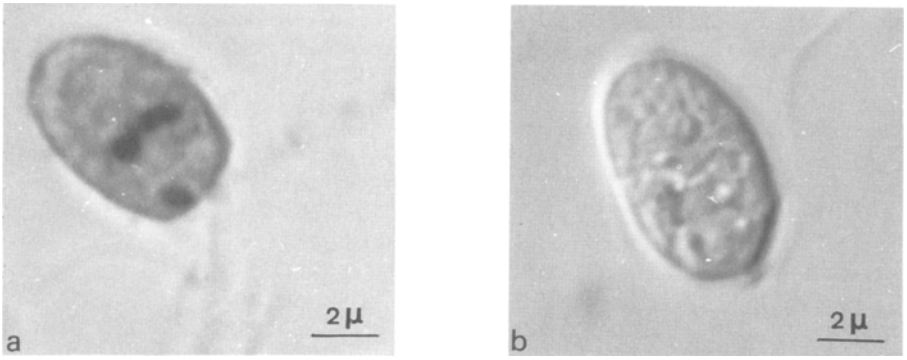

Figure 2. a)Cytochemical demonstration of esterases in Dunaliella incubated with α-naphthyl acetate pH 6.8 at 4°C for 16 hr and Fast Blue RR (x 5500).
b)Dunaliella incubated without substrate (x 5500)

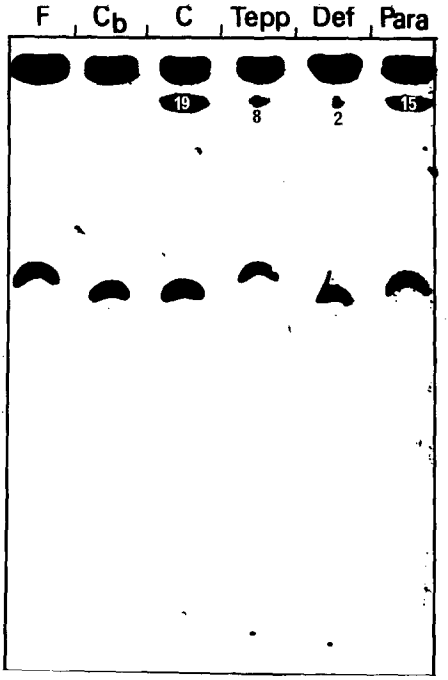

Figure 3. Autoradiochromatogram of organic extracts
from F: fresh culture medium
C_b:conditioned medium boiled 10 min. at 120°C
C: conditioned medium
Tepp: conditioned medium treated with 0.1 mM TEPP
Def: conditioned medium treated with 0.1 mM DEF
Para: conditioned medium treated with 0.1 mM paraoxon
1- control deltamethrin
Preparations were only treated with DTM labeled on the alcohol moiety
Numbers on the spots represent the rate of the compounds isolated by TLC (results expressed in % of radioactivity on TLC plates of the different compounds in each extract).

(about 0.1 µg/L of conditioned medium) but many salts (0.4 M NaCl) capable of interfering with esterase activity (Mastropaolo and Yourno 1981), thus the concentration and desalting of the medium was necessary to allow the titration. The hydrolytic activity of the extracellular esterase toward a non-specific substrate of esterase: p-nitrophenyl acetate, was evaluated. The esterase activity measured in the conditioned culture medium was: 0.45 $\pm$ 0.17 µmol p-nitrophenol generated per minute and per liter of conditioned culture medium.

Thus, this study revealed that algae released esterases into their culture medium. These enzymes kept their

activity during 3 days in this extracellular medium and were able to metabolize DTM. This excretion mechanism that may be continuous or induced by a chemical stress is an original cellular feature little described in literature except by Wink (1984,1985) on various suspension-cultured plant cells.

So, we are now thoroughly studying this phenomenon, evaluating the effect of various factors such as the growth phase, the period of the cell cycle or even the possible induction of this excretion by xenobiotics. Indeed, it will be interesting to determine if it is possible to optimize the excretion of enzymes by cells in view of using them for the cleaning of contaminated medium.

REFERENCES

Baeza-Squiban A, Marano F, Ronot X, Adolphe M, Puiseux-Dao S (1987) Effects of deltamethrin and its commercial formulation Decis on different cell types in vitro: cytotoxicity, cellular binding and intracellular localization. Pestic Biochem Physiol 28: 103-113

Baeza-Squiban A, Meinard C, Marano F (1988) Metabolism of deltamethrin in two cell types in vitro. Pestic Biochem Physiol 32:253-261

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

Casida JE, Gaughan LC, Ruzo LO (1979) Comparative metabolism of pyrethroids derived from 3-phenoxy benzyl and α-cyano-3 phenoxybenzyl alcohols. In H Geissbuehler (ed) Advances in pesticide science" Pergamon- Oxford, pp182-189

Casida JE, Gammon DW, Glickman AH, Lawrence LJ (1983) Mechanisms of selective action of pyrethroid insecticides. Ann Rev Pharmacol Toxicol 23:413-438

Jao LT, Casida JE (1974) Insect pyrethroid-hydrolysing esterases. Pestic Biochem Physiol 4:465-472

Marano F, Amancio S, Durrand AM (1978) Synchronous growth and synthesis of macromolecules in a naturally wall-less volvocale, Dunaliella bioculata. Protoplasma 95:135-144

Mastropaolo W and Yourno Y (1981) An ultraviolet spectrophotometric assay for naphtyl butyrate esterases.Anal Biochem 115:188-193

Matile P (1978) Biochemistry and function of vacuoles. Ann Rev Plant Physiol 29:193-213

Wink M (1984) Evidence of an extracellular lytic compartment of plant cell suspension cultures: the culture medium. Naturwissenschaften 71:635-637

Wink M (1985) Composition of the spent cell culture medium. I:time course of ethanol formation and the excretion af hydrolytic enzymes into the medium of suspension cultured cells of Lupinus polyphyllus. J Plant Physiol 121:287-293

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