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BIOCHEMICAL INVESTIGATION OF CHOLINESTERASES AND CARBOXYLESTERASES FROM THE COTTON BOLLWORM *Heliothis armigera*

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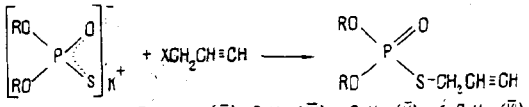
The interaction of the esterases of the cotton bollworm Heliothis armigera with a series of OO-dialkyl S-propargyl phosphorothioates has been studied. In contrast to human and murine ACEs, the hydrophobic regions of the active surfaces of the bollworm ACEs are very feebly expressed. At the same time, the hydrophobic regions in the areas of the esterase points are broader than those of murine CBEs and are adapted for the sorption of normal radicals. These compounds are selective inhibitors of CBEs and can be used as insecticide synergists.

It is known that cholinesterases (CEs) are the target enzymes for the action of many insecticides, while carboxylesterases (CBEs) are enzymes of the insect metabolism. The differences in the properties of these enzymes in mammals and arthropods may serve as a basis for the creation of pesticides and synergists with a selective action [1].

The activity of organophosphorus inhibitors (OPIs) in relation to esterases depends to a considerable degree on the first stage of the interaction of the OPIs with the active centers of the enzymes - the sorption stage. Here a decisive role is played by the degree of correspondnece of te structure of the inhibitor to the structure of the active surface and, in particular, the possibilty of the formation of hydrogen bonds and hydrophobic interactions in this process [2, 3]. Therefore, the study of the inhibiting activity of a series of OPIs with regularly changing structure, which permits the finding of distinguishing features of esterases with different origins and the drawing of conclusions concerning the topography of the active surfacs of enzymes, is not losing its importance.

We have previously [4] shown the peculiarity of the substrate-inhibitor properties of cholinestrases from the cotton bollworm *Heliothis armigera*. In the present paper we report the results of an investigation of the active surfaces of the bollworm acetylcholine-, butyrylcholine- and carboxylesterases with OO-dialkyl S-propargyl phosphorothioates synthesized previously [5] in comparison with the analogous mammalian enzymes. These substances were obtained by the interaction of potassium OO-dialkyl phosphorothioates with propargyl chloride (or bromide) in absolute alcohol:



where R = C_2H_5 (I), C_3H_7 (II), C_4H_8 (III), C_5H_{11} (IV), C_6H_{13} (V), i-C_4H_9 (VI), i-C_5H_{11} (VII); X = Br, Cl.

Mass spectrometry is widely used to establish molecular masses and to identify physiologically active organophosphorus compounds (drugs, pesticides) and their metabolites [6-8]. We have studied the mass-spectrometric fragmentation of OO-dihexyl S-propargyl phosphorothioate. Its mass spectrum was characterized by the presence of an intense signal of an ion with *m/z* 281, due to the splitting out of the propargyl radical from the molecular ion with *m/z* 320. On the breakdown of the molecular ion, a protonated ion of O-hexyl S-propargyl phosphorothioate with *m/z* 237 was also formed.

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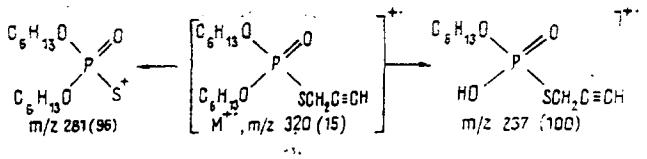
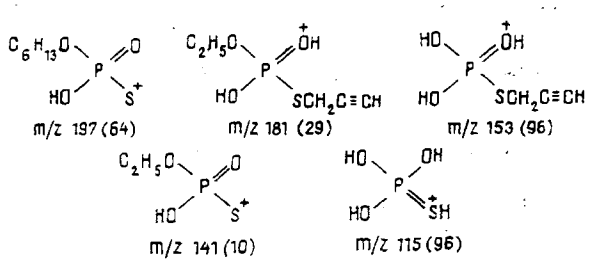


The fragmentation of these ions formed other ions possessing a high intensity (*m/z* 197, 181, 153, 141, 115).



Among the ions arising, the protonated form of phosphorothionic acid (*m/z* 115) is the most important and characteristic fragment in the decomposition of phosphorothioates [9, 10].

The results obtained on the interaction of the OO-dialkyl S-propargyl phosphorothioates with the bollworm ACEs, BuCEs, and CBEs are shown in Tables 1 and 2. Attention is attracted, above all, by the fact that the activities of the inhibitors depended to a large degree on the nature and source of the enzyme. All the substances investigated proved to be selective inhibitors of carboxylesterases. The differences in the constants of the inhibition of the bollworm ACEs and CBEs amounted to 6-7 orders of magnitude, while for mouse ACEs and CBEs it was 2-3 orders of magnitude.

It can be seen from Table 1 that a lengthening of the radicals from ethyl to amyl increased the inhibitory activity of the compounds for the bollworm intestinal CBE 700-fold, for the bollworm fatty body CBE 400-fold, and for mouse liver CBE 225-fold. On passing to hexyl, the activities in relation to the bollworm CBEs rose still further, but in relation to the mouse CBE it fell somewhat. Thus, the hexyl derivative proved to be the most powerful inhibitor of the bollworm CBE. The compounds with isoamyl radicals were 2 to 10 times weaker than their analogues with *n*-alkyl radicals. An increase in the length of the branched radicals from isobutyl to isoamyl likewise led to a rise in inhibiting activity in the cases of all three carboxylesterases.

Consequently, the active surfaces of the carboxylesterases from these sources possess pronounced hydrophobic regions. It is possible that such a region is smaller in the case of mouse liver CBE, as is indicated by the somewhat lower inhibiting activity of the dihexyl than of the diamyl derivative. The large role of hydrophobic interactions was also shown in the investigation of [the inhibition of (?)] locust carboxylesterase by a series of alkyl methylphosphonates [11]. It is interesting to trace the influence of the branching of a radical on anticarboxylesterase activity. At an overall length of 3 carbon atoms (propyl and isobutyl derivatives) branching led to a pronounced enhancement of the effect in relation to the bollworm carboxylesterases but scarcely affected the mouse enzyme. The branching of longer radicals (compare the butyl and isoamyl derivatives) exerted practically no influence, and this indicates a definite limited nature of the hydrophobic regions of the bollworm CBEs and their relative closeness to the esterase centers.

The results of an investigation of the inhibiting activities of a number of the compounds studied in relation to the bollworm and mammalian cholinesterases (Table 2) showed a pronounced resistance of the bollworm ACEs to these inhibitors. The inhibition constants of human and mouse ACEs and also of the bollworm BuCE were 2-4 orders of magnitude higher than for the bollworm ACEs.

Considerable differences were detected between the cholinesterases investigated in the nature of the dependence of the inhibiting activity on the length of the alkyl radicals. In the case of the bollworm ACEs a lengthening of the radicals, on the whole, lowered the activity of the OPIs, while in the case of the bollworm BuCE the dependence of the activity on the length of the radical revealed a pronounced maximum for the dibutyl derivative.

It is known from the literature [12-16] that a maximum sensitivity to the dibutyl or diamyl homologues of various series of OO-dialkyl phosphorothioates is characteristic for human and bovine ACEs, while for the cholinesterases of arthropods (flies, aphids, the rice weevil, the mealybug, the red spider mite) in the majority of cases a maximum sensitivity to the diethyl and, more rarely, the dipropyl homologues is characteristic. This agrees with the results that we have obtained on the sensitivity

TABLE 1. Anticarboxylesterase Activities (k_2, $M^{-1}\cdot min^{-1}$) of OO-Dialkyl S-Propargyl Phosphorothioates

Compound	Cotton bollworm		mouse liver
	intestine	fatty body	
I	$(9.6+0.1)\times10^4$	$(2.2+0.2)\times10^5$	$(2.0+0.2)\times10^5$
II	$(5.9+0.3)\times10^5$	$(9.4+1.5)10^5$	$(1.2+0.1)\times10^6$
III	$(3.1+0.3)\times10^7$	$(5.4+0.7)\times10^7$	$(2.1+0.1)\times10^7$
IV	$(6.8+0.5)\times10^7$	$(8.5+0.8)\times10^7$	$(5.5+0.4)\times10^7$
V	$(3.1+0.3)\times10^8$	$(2.9+0.3)\times10^8$	$(4.0+0.4)\times10^7$
VI	$(2.6+0.2)\times10^6$	$(4.5+0.5)\times10^6$	$(2.1+0.2)\times10^6$
VII	$(3.8+0.3)\times10^7$	$(4.0+0.5)\times10^7$	$(2.3+0.2)\times10^7$

TABLE 2. Anticholinesterase Activities (k_2, $M^{-1}\cdot min^{-1}$) of OO-Dialkyl S-Propargyl Phosphorothioates

Compound	Cotton bollworm		Human erythrocyte ACE	Mouse brain ACE
	nerve cord ACE	whole-body homogenate BuCE		
I	$(4.9+0.5)\times10^2$	$(3.7+0.4)\times10^4$	$1.9\times10^{4*}$	$7.0\times10^{3*}$
II	$(1.1+0.1)\times10^3$	$(1.5+0.2)\times10^4$	$1.6\times10^{5*}$	$2.0\times10^{4*}$
III	$(3.1+0.3)\times10^1$	$(8.5+0.2)\times10^6$	$1.9\times10^{5*}$	$1.4\times10^{5*}$
IV	$(1.2+0.1)\times10^2$	$(6.3+0.5)\times10^5$	$(3.5+0.4)\times10^4$	$(1.6+0.2)\times10^5$
V	$(2.5+0.4)\times10^1$	$(6.3+0.6)\times10^5$	$(1.2+0.1)\times10^5$	$(8.0+0.9)\times10^3$
VI	$(3.4+0.5)\times10^1$	$(2.2+0.2)\times10^5$	$2.1\times10^{5*}$	$3.7\times10^{5*}$
VII	$(1.7+0.3)\times10^3$	$(3.4+0.3)\times10^4$	$(3.4+0.3)\times10^4$	$(1.0+0.1)\times10^6$

of the bollworm ACEs and the ACEs of mammals to small alkyl radicals and indicates a smaller extent of the hydrophobic sections in the regions of the esterase points of arthropd ACEs.

The spatial extent of this section can be estimated indirectly by studying series with α-branched alkyl radicals. It has been shown in [13-16] that, as a rule, activity in relation to mammalian ACEs remains approximately the same or increases on passing from dipropyl to diisobutyl and from dibutyl to diisoamyl derivatives. In the case of arthropod ACEs, however, the replacement of propyl by isobutyl radicals and, in some cases, also of butyl by isoamyl radicals sharply lowers inhibiting activity, this apparently being explained by the appearance of steric hindrance in the sorption of branched radicals. A comparison of the activities of the normal and iso radicals of the series of compounds investigated (see Table 2) in general confirms this tendency in relation to the bollworm ACEs and mammalian ACEs. The bollworm ACEs revealed some peculiarity in the structure of their active surfaces; the diisobutyl homologue proved to be 15 times more active than the dipropyl derivative, and the isoamyl homologue 5 times weaker than the butyl derivatiuve.

Thus, we have shown that the same enzymes with different origins differ appreciably from one another in their sensitivity to inhibitors. This can most probably be explained by differences in the structures of the hydrophobic environments of their active centers. Even more profound differences have been found between different esterases. As already mentioned, the OPIs studied proved to be selective inhibitors of carboxylesterases. Since these enzymes participate directly in the metabolic detoxication of the OPIs, potential insecticide synergists may be found among their inhibitors.

EXPERIMENTAL

In our experiments we used cotton bollworm caterpillars of the 5th instar grown in the laboratory on a synthetic medium [17]. The source of ACEs was a homogenate of isolated caterpillar nerve cords (2 cords/ml), and the source of BuCE

a homogenate of whole caterpillars (100 mg/ml) in which the ACEs were inhibited by preincubation for 10 min with the inhibitor Gd-42 (the methyl sulfomethylate of O-ethyl S-(ethylthioethyl) methylphosphonothioate) with, for comparison, a partially purified preparation of human erythrocyte acetylcholinesterase (produced by the Perm Scientific-Research Institute of Vaccines and Sera; specific activity 2 U/mg) and a mouse brain homogenate (10 mg/ml).

The homogenates were prepared in a glass homogenizer cooled with water and ice in 0. 05 M Na phosphate buffer, pH 7.5, and were then centrifuged at 10,000 rpm for 3 min, and, after the deposit had been discarded, the CE activity in the supernatant was determined by Ellman's method [18], using as substrate acetylthiocholine or butyrylthiocholine (for the bollworm BuCE) in a concentration of 1×10^{-3} M.

As the source of carboxylesterases we used the supernatant fraction of a homogenate the intestines (20 mg/ml) and fatty bodies (10 mg/ml) of bollworm caterpillars and of mouse liver (100 mg/ml) obtained by homogenizing the tissue in a glass homogenizer in Na phosphate buffer, pH 7 (M/15) and centrifuging for 15 min at 2500 *g* (bollworm CBEs) or 10,000 *g* (mouse CBEs). The activities of the carboxylesterases were determined calorimetrically from the rate of hydrolysis of *p*-nitrophenyl acetate in a concentration of 1×10^{-3} M at 30°C in Na phosphate buffer, pH 8. In view of the fact that the homogenates contained other hydrolases hydrolyzing *p*-nitrophenyl acetate besides CBEs [19], supplementary control experiments were performed. To exclude the possibility of the hydrolysis of the substrate by the CEs present in the sample, proserine (neostigmine) was added, to a final concentration of 1×10^{-5} M . Hydrolysis of the substrate by other hydrolysases was taken into account in control experiments in which the homogenates were incubated beforehand with paraoxon (diethyl *p*-nitrophenyl phosphate) in a concentration of 5×10^{-5} M for 20 min [20].

The inhibiting activities of the compounds were evaluated by means of the bimolecular rate constant of their interaction with the enzyme, calculated from the formula

$$k_2 = \frac{1}{[I]t} lg \frac{V_0}{V_t} \; M^{-1} \cdot \min^{-1},$$

where V_0 and V_t are the rates of enzymatic transformation of the substrates in the absence of the inhibitor and after t (1-5) minutes' incubation of the enzyme with the inhibitor in various concentrations [1].

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