Inhibition of AChE by single and simultaneous exposure to malathion and its degradation products

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Abstract. *In vitro* inhibition of bovine erythrocytes acetylcholinesterase (AchE) by separate and simultaneous exposure to organophosphorous insecticide malathion and the transformation products, which are generally formed during the storage or natural as well as photochemical degradation pathways of malathion, was investigated.

The increasing concentration of malathion, its oxidation product – malaoxon and isomerisation product – isomalathion inhibited AChE activity in a concentration-dependent manner. The half-maximum inhibitory concentrations (IC_{50} values): $(3.2 \pm 0.1) \times 10^{-5}$ mol/l, $(4.7 \pm 0.8) \times 10^{-7}$ mol/l and $(6.0 \pm 0.5) \times 10^{-7}$mol/l were obtained from the inhibition curves induced by malathion, malaoxon and isomalathion, respectively. However, the products formed due to photoinduced degradation, phosphorodithioic O,O,S-trimethyl phosphorodithioic ester (OOS(S)) and O,O-dimethyl thiophosphate did not noticeably affect the enzyme activity at all investigated concentrations, while diethyl maleate inhibited the AChE activity at concentrations >10 mmol/l. By simultaneous exposure of the enzyme to malaoxon and isomalathion in various concentration combinations the additive effect was achieved by low concentration of inhibitors, while the antagonistic effect was obtained at high concentration ($\geq 3 \times 10^{-7}$ mol/l) of inhibitors.

Inhibitory power of irradiated samples of 1×10^{-5} mol/l malathion can be attributed to the formation of malaoxon and isomalathion, organophosphates about 100 times more toxic than their parent compound, while the presence of non-inhibiting degradation product OOS(S) did not affect the inhibitor efficiency of inhibiting malathion by-products, malaoxon and isomalathion.

Key words: Acetylcholinesterase — Malathion degradation products

Introduction

Organophosphorus compounds (OPs) such as malathion, chlorpyrifos, parathion are commonly used as insecticides for over 50 years. OPs work by attacking the nervous system; they are essentially the nerve poisons. Specifically, these compounds inhibit acetylcholinesterase (AChE, EC 3.1.1.7), enzyme involved in the hydrolysis of the neurotransmitter, acetylcholine at cholinergic synapses in the central and peripheral nervous systems (cholinergic syndrome) (O'Malley 1997; Solbergand Belkin 1997; Musilek et al. 2005).

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OPs are preferred in agriculture because of their relatively low persistence in the environment, but they are indiscriminate pesticides and highly toxic to animals and humans. Besides acute (short-term) toxic effects based on AChE inhibition which leads to accumulation of acetylcholine, chronic low-level exposure has been implicated as a causal factor in a variety of different forms of human ill-health involving the nervous or immune systems (Ray and Richards 2001). Some of OPs show mutagenic or carcinogenic effects (Cantor et al. 1992; Pluth et al. 1996; Blasiak et al. 1999; Cabello et al. 2001; Giri et al. 2002), and they are implicated with vision loss, kidney (Albright et al. 1983) and lung demage (Imamura et al. 1983). The toxicity of some organophosphates, malathion for example, is increased by its break-down products releasing within an organism or in action of sunlight (Borwn et al. 1993).

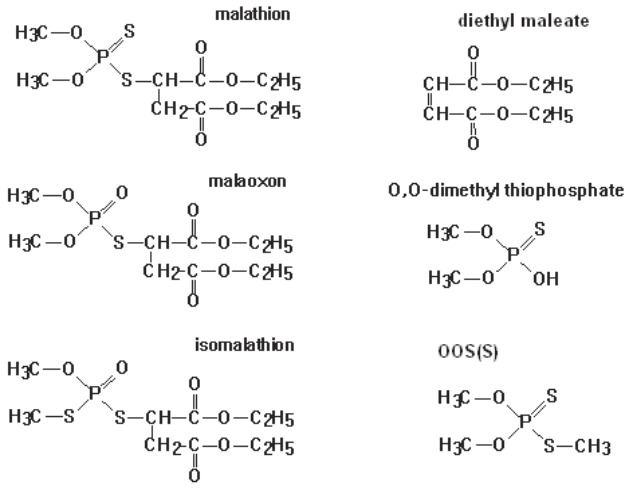

Figure 1. Chemical structures of studied compounds.

This work deals with the *in vitro* investigation of inhibition of bovine erythrocytes AChE (EC 3.1.1.7) by separate and simultaneous exposure to malathion, and its main degradational products which are generally formed during storage or through natural or photochemical irradiation: oxidation product – malaoxon, isomerisation product – isomalathion (Fig. 1). Besides, the inhibitory potencies of O,O,S-trimethyl phosphorodithioic ester (OOS(S)), diethylmaleate and O,O-dimethyl thiophosphate were also investigated, since these compounds are usually formed as a result of cleavage of chemical bonds due to the degradation of the selected organophosphate (Bavcon Kralj et al. 2007a). Malathion, O,O-dimethyl S-1,2-(diethoxycarbamyl) ethyl phosphorodithioate, is a broad-spectrum insecticide and it was chosen as a model compound, because it is one of the most applied OP since 1956.

Materials and Methods

AChE assay

The inhibition of AChE activity was measured using Ellman procedure (Ellman et al. 1961) in the absence (control) and presence of malathion and its related compounds. The experiments were performed by *in vitro* (separate and simultaneous) exposure of 180 μg enzyme to inhibitors in final volume 0.650 ml. The standard medium assays were preincubated for 5 min at 37°C in the absence and presence of desired concentration of investigated compounds. The control tubes contained the corresponding concentration of ethanol without organophosphate. Acetylthiocholine iodide (ASChI) was applied as the enzyme substrate in combination with 5,5'-dithio-bis-2-nitrobenzoic acid (DTNB) as a chromogenic reagent. The product 5-thio-2-nitrobenzoate, formed in the reaction of thiocholine (product of enzymatic reaction) and DTNB, was measured at 412 nm (in buffer solution) by use Perkin Elmer Lambda 35 UV VIS spectrophotometer. All experiments were made in triplicates. Preliminary studies showed that malathion and its by-products or their combinations did not interfere with quantitation of the yellow product 5-thio-2-nitrobenzoate.

Photodegradation procedure

Photodegradation of malathion in aqueous solution was performed by applying previously published method (Bavcon Kralj et al. 2007b). A freshly prepared pesticide solution containing less than 0.5% ethanol was purged with oxygen for 15 min in the dark before irradiation, and was then put into a quartz sample cell (10 × 10 × 40 mm) and placed in front of a xenon light (125 W Cermax Xenon parabolic lamp) at a distance of 45 cm. Individual solution samples were irradiated for different periods of time (10, 15, 20, 30, 45 or 60 min). The samples were analyzed by HPLC (high performance liquid chromatography analysis), ion chromatography and gas chromatography-mass spectrometry (GC-MS) (Bavcon Kralj et al. 2007a,b). According to the results of the analysis, synthetic mixtures of the pure degradation products (commercial chemicals) were composed to investigate the effects on AChE activity of simultaneous exposure to malathion and its photoinduced by-products.

Reagents

All chemicals were of analytical grade. AChE (specific activity 0.28 IU/mg) from bovine erythrocytes, ASChI, and DTNB were purchased from Sigma Chemicals Co. (Taufkirchen, Germany). The materials were used without further purification. The used pesticides were of at least 93% purity. Malathion (97.3%), and malaoxon (93.7%) were from Pestanal (Reidel-de Haën, Seelze, Germany), whereas isomalathion (98.4%) was purchased from Institute of Organic Industrial Chemistry (Poland), diethyl maleate (97%) was from Aldrich Chemical Co. (Gillingham, UK), O,O-dimethyl- thiophosphate and OOS(S) from Sigma Chemical Co. Standard solutions of malathion and its degradation products (0.1 mol/l) were made in ethanol shortly before use.

Statistical analysis

Analysis of variance (one way ANOVA) was used to compare the mathematical sum of inhibitions caused by separate exposure to malaoxon or isomalathion with inhibitions induced by exposure to both organophosphates simultaneously. When a significant continuous probability distribution

(F value) ($p < 0.05$) was obtained, post hoc test Bonferoni was used to determine the differences.

An antagonistic effect was defined as a statistically significant ($p < 0.05$) difference between inhibitions caused by simultaneous exposure and mathematically calculated means of enzyme inhibition for a pair of organophosphates, where the former is lower than the latter.

Results

The in vitro influence of malathion and its related compounds on AChE activity

The separate influence of malathion and its degradation products, usually formed due to the chemical conversion or photochemical treatment of the selected organophosphate, on AChE activity was investigated in the concentration range 1×10^{-9} to 1×10^{-1} mol/l.

The obtained results show (Fig. 2) that malathion, malaoxon, isomalathion and diethylmaleate inhibited AChE in a concentration-dependent manner but with various inhibition potencies. Dependence of the AChE activity, expressed as the percent of control value (obtained without inhibitor), on the inhibitor concentration fitted a sigmoidal function in all cases (Fig. 2). The inhibition parameters, half-maximum inhibitory concentration (IC_{50}) values and Hill coefficient n_H determined using the Hill method (Fig. 3) by linear regression analysis of log [% activity/(100 – % activity)] *vs.* log [concentration of inhibitor plots], are given in Table 1. The differences between values of IC_{50} values obtained using the Hill

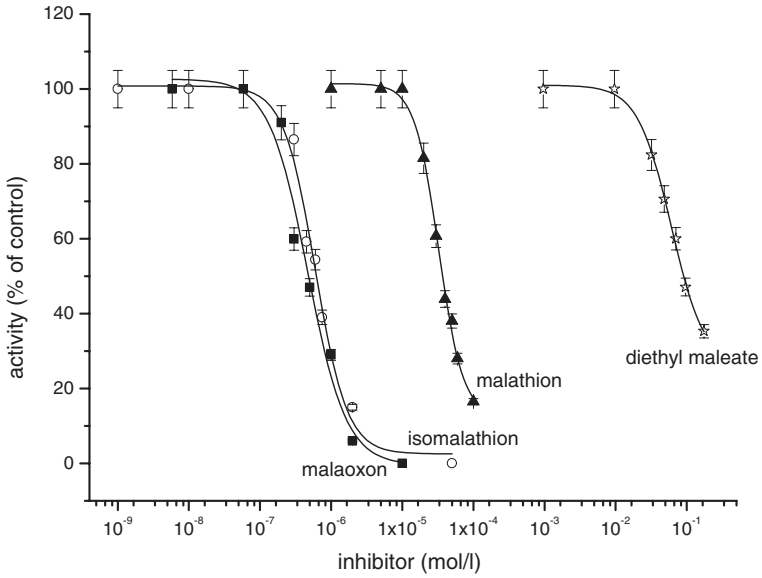

Figure 2. The concentration-dependent inhibition of bovine erythrocytes AChE by malathion (triangle), malaoxon (square), isomalathion (circle) and diethyl maleate (asterisk). The values are expressed as mean ± S.E.M.

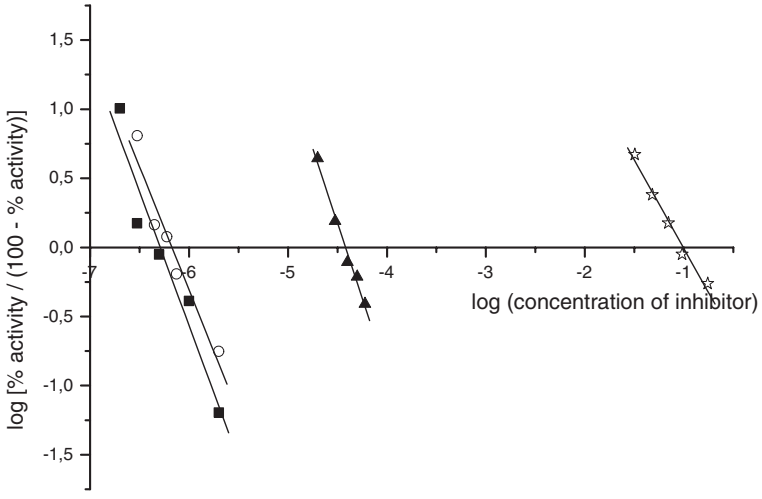

Figure 3. Hill analysis of inhibition of bovine erythrocytes acethylcholinesterase activity induced by malathion (triangle), malaoxon (square), isomalathion (circle) and diethyl maleate (asterisk).

Table 1. The inhibition parameters (IC_{50} values and Hill coefficients n_H) of malathion and its related compounds for AChE obtained by Hill analysis and fitting the experimental inhibition curves by sigmoidal function

Compound	Sigmoidal fitting	Hill analysis	
	IC_{50} (mol/l)	IC_{50} (mol/l)	n_H
Malathion	$(3.2 \pm 0.1) \times 10^{-5}$	3.8×10^{-5}	2.2 ± 0.1
Malaoxon	$(4.7 \pm 0.8) \times 10^{-7}$	5.1×10^{-7}	1.9 ± 0.3
Isomalathion	$(6.0 \pm 0.5) \times 10^{-7}$	6.7×10^{-7}	1.8 ± 0.2
Diethyl maleate	$(6.0 \pm 0.5) \times 10^{-2}$	9.8×10^{-2}	1.3 ± 0.1

method and by fitting the sigmoidal inhibition curves were in the range of experimental error.

Although the investigated organophosphates: malathion, malaoxon and isomalathion have similar structure (moreover, malathion and isomalathion are isomers), it is obvious that their inhibitor efficiency is quite different. At the concentration of 1×10^{-5} mol/l malaoxon as well as isomalathion completely inhibited AChE activity, while the effect of the same concentration of malathion on the enzyme activity was negligible. The IC_{50} of the enzyme activity was achieved at $(3.2 \pm 0.1) \times 10^{-5}$ mol/l of malathion, while the same effect was observed when the concentrations of malaoxon $(4.7 \pm 0.8) \times 10^{-7}$ mol/l and isomalathion $(6.0 \pm 0.5) \times 10^{-7}$ mol/l have been two orders of magnitude lower than concentration of malathion.

On the contrary, diethyl-maleate up to concentrations of 10 mmol/l did not cause a marked reduction of enzyme activity (Fig. 2), while O,O-dimethylthiophosphate as well as OOS(S) did not noticeably affect the bovine erythrocytes AChE activity at all investigated concentrations (1×10^{-9} to 1×10^{-1} mol/l).

Table 2. Inhibition of AChE by single and simultaneous exposure to different combinations of malathion and/or its photoinduced by-products; The values in parenthesis represent concentration (mol/l) of OPs identified by GC-MS chromatography after different time of 1×10^{-5} mol/l malathion photodegradation and used in inhibition experiments

Time of irradiation (min)		0	10	15	20	30	45	60
		Inhibition (%) [Concentration of inhibitor (mol/l)]						
Single exposure	Malathion	0 [1×10^{-5}]	0 [9×10^{-6}]	0 [5×10^{-6}]	0 [2×10^{-6}]	/	/	/
	Malaoxon	/	71 [1×10^{-6}]	94 [2×10^{-6}]	94 [2×10^{-6}]	94 [2×10^{-6}]	/	/
	Isomalathion	/	/	80 [3×10^{-6}]	80 [3×10^{-6}]	80 [3×10^{-6}]	80 [3×10^{-6}]	67 [1×10^{-6}]
	OOS(S)	/	/	/	0 [3×10^{-6}]	0 [5×10^{-6}]	0 [7×10^{-6}]	0 [9×10^{-6}]
Simultaneous exposure *	Malathion + malaoxon	/	74.0	90.4	92.7	/	/	/
	Malathion + isomalathion	/	/	78.6	76.4	/	/	/
	Malaoxon + OOS(S)	/	/	/	93.2	92.1	/	/
	Isomalathion + OOS(S)	/	/	/	80.5	79.3	82.5	67
	Malathion + malaoxon + isomalathion	/	/	96.0	98.5	/	/	/
	Malathion + malaoxon + isomalathion + OOS(S)	/	/	/	95.1	/	/	/

* concentrations of mixture components used in simultaneous exposure experiments (lower part of table) are the same as given (as values in parenthesis) in the data for single exposure (upper part of table); OOS(S), O,O,S-trimethyl phosphorodithioic ester.

Inhibition of AChE activity by simultaneous exposure to malathion and its by-products

Different synthetic mixtures of malathion and its by-products are prepared on the basis of literature data (Bavcon Kralj et al. 2007b) indicating the composition of malathion solution after photochemical treatment. In many cases, the inhibition of AChE by mixture of these compounds was not shown to be a sum of individual inhibition capacities but expressed synergistic/antagonistic effects.

The concentrations of malathion and its by-products in the mixture were chosen using two criteria: the concentrations of mixture components were similar to the concentrations identified by GC-MS after photodegradation of 1×10^{-5} mol/l malathion in the time interval of 0 to 60 min (Bavcon Kralj et al. 2007b) (1) and concentrations of malathion inhibiting by-products, malaoxon and isomalathion, were in the concentration range around or lower than the IC_{50} values (2).

1. Inhibition of AChE activity obtained after 5 min of preincubation of the enzyme with desired combinations of malathion and/or its by-products is presented in Table 2 and compared to the inhibition achieved in the presence of same concentration of single compound (upper part of Table 2). It is obvious that conversion of 10% of 1×10^{-5} mol/l malathion to malaoxon caused more than 70% of AChE inhibition. The amount of malathion (9×10^{-6} mol/l) remaining unconverted after 10 min of photodegradation did not affect the inhibitor capacity of malaoxon at concentration 1×10^{-6} mol/l, since malaoxon by separate exposure inhibited the enzyme 71% of initial AChE activity (Table 2). Combinations of malathion and its by-products formed during 15–30 min of photodegradation (Bavcon Kralj et al. 2007b) completely inhibited the activity of AChE. The obtained results were in accordance with our beforehand expectations because malaoxon at the concentration of 2×10^{-6} mol/l (by separate exposure as well as in the presence of investigated concentrations of malathion) inhibited AChE up to 90%, while the separate exposure of the enzyme to 3×10^{-6} mol/l isomalathion induced inhibition of 80%. The presence of malathion (at concentrations which did not affect AChE activity by single exposure) in combinations malathion/malaoxon or malathion/isomalathion did not affect the inhibition caused by single exposure to malaoxon or isomalathion. OOS(S) at all investigated combinations with malaoxon or isomalathion did not alter their inhibitor capacity.

2. Synergism/antagonism between malaoxon and isomalathion inhibition power was studied by 5 min preincubation of the enzyme with the mixtures of malaoxon and isomalathion at concentrations which produced by individual exposure around 50% of inhibition or less by single exposure. Table 3 shows means of experimental values and mathematical data calculated as the sum of organophosphate-induced inhibitions measured separately.

Table 3. Inhibition of AChE activity induced by simultaneous exposure to malaoxon and isomalathion in mixtures. Values in parenthesis represent organophosphate-induced inhibition measured separately (by single exposure)

Inhibition (%)				
Isomalathion (mol/l) / Malaoxon (mol/l)	2×10^{-7} (10%)	3×10^{-7} (13.5%)	4.5×10^{-7} (35.8%)	6×10^{-7} (45.6%)
2×10^{-7} (9%)	21.3%	25.0%	42.0%	39.7%
3×10^{-7} (41%)	39.5%	40.3%	63.1%	51.5%
4×10^{-7} (57.1%)	53.5%	56.9%	57.9%	67.5%

As can bee seen from Table 3, during the simultaneous exposure of enzyme to malaoxon and isomalathion in various concentration ratios, the additive inhibition effects at lower concentration of inhibitors is observed, while the statistically significant antagonistic inhibition (i.e. lower than the sum of OPs-induced inhibitions assayed separately) was obtained at higher concentration of inhibitors ($\geq 3 \times 10^{-7}$ mol/l).

Discussion

Results of our study showed that malathion, its oxidation product-malaoxon and isomerisation product – isomalathion, as previously reported (Bajgar 2004, 2005; Bavcon Kralj et al. 2006) inhibited AChE activity with varying potencies (Table 1). The obtained IC_{50} values for malaoxon (4.7 ± 0.8) $\times 10^{-7}$ mol/l and isomalathion (6.0 ± 0.5) $\times 10^{-7}$ mol/l are two orders of magnitude lower than the IC_{50} value of their parent compound, malathion (3.2 ± 0.1) $\times 10^{-5}$ mol/l. Obtained results are in accordance with previously reported findings that malathion toxicity increased by its break-down products (Brenner 1992; Marrs 1993; Smulders et al. 2004; Bajgar 2005). As malathion reacts and breaks down within an organism or sunlight, one of the chemicals released is malaoxon, organophosphate about 100 times more toxic than its parent compound – malathion (Borwn et al. 1993). Obtained Hill coefficients $n_H > 1$ for malathion and its inhibiting by-products are in accordance with previously reported n_H values for another organophosphates (Smulders et al. 2004) as well as allosteric effects between the top and the bottom of the gorge (Boublik et al. 2002).

It is generally considered that organophosphate pesticides are substrate analogues of acetylcholine. Like the natural substrate, OPs enter the active site, which is a 20 Å deep gorge with a catalytic triad (Ser 200, Glu 327 and His 440)

at the bottom (Sussman et al. 1991). As in acetylation, the organophosphate is split and hydroxyl group of Ser 200 i.e the enzyme is phosphorylated. The difference in substrate behavior lies in the next step; while the acyl enzyme quickly hydrolyzed to regenerate the enzyme, dephosphorylation is very slow. As phosphorylated enzyme cannot hydrolyses neurotransmitter, the post-synaptic membrane remains depolarized, synaptic transmission does not work and organophosphates are often considered as irreversible inhibitors (Boublik et al. 2002).

Although the investigated organophosphates: malathion, malaoxon and isomalathion have similar structure, it is clear that combination of the substituents at the central phosphorous atom is responsible for their different inhibition power. This finding can be ascribed to the polarity of the chemical bond that binds phosphorous to sulfur atom. It seems that the strong covalent character of malathion P-S complicates the nucleofilic attack of serine –OH group from AChE. The consequence is the decrease of the phosphorylation, i.e. relative high IC_{50} value. In the case of malaoxon and isomalathion, P-S bond is more polar and phosphorous atom is more suitable for nucleophilic attack of serine –OH group from AChE compared to malathion. Moreover, double bonded oxygen atom in both cases increases the electropositivity of the central phosphorous atom, and facilitates the phosphorylation of the enzyme. The consequence is the higher inhibitory potency of malathion inhibiting by-products, malaoxon and isomalathion.

The absence of inhibitory effect of O,O-dimethyl thiophosphate on AChE activity is undoubtedly a consequence of absence of the 1,2-(diethoxycarbamyl) ethyl group which is present in molecules of investigated organophosphate inhibitors. It seems that this group is necessary for interactions between the organophosphate and anionic subsite of AChE active site, which are responsible for appropriate position of substrate in the enzyme active site (Harel et al. 1993). Moreover, these findings are in agreement with obtained inhibitory effect of diethiyl-maleate at concentrations ≥10 mmol/l.

The absence of inhibitory effect of OOS(S) obtained at all investigated concentrations is in agreement with previously reported findings (Talcott et al. 1979). In fact, OOS(S) at all investigated combinations with malathion inhibiting by-products (Table 3) did not affect their inhibition power. The compound requires metabolic activation, probably through oxidation to O,O,S-trimethyl phosphorothioate (OOS(O)) or isomerisation to O,S,S-trimethyl phosphorodithioate (OSS(O)). The toxicity of these compounds (OOS(O) and OSS(O)) as anticholinesterases comes from their ability of rapid aging of the enzyme (Clothier et al. 1981).

The additive inhibition of AChE activity obtained by simultaneous exposure to low concentrations of malaoxon and isomalathion (Table 3) confirms that both inhibitors bind to the same binding site and that there is an excess of inhibitor binding sites. The antagonistic inhibition of bovine erythrocytes AChE in the presence of mixtures of inhibitors at higher concentration ($\geq 3 \times 10^{-7}$mol/l) can be explained by competition between inhibitors for a limited number of inhibitor binding sites on the enzyme.

The complete inhibition of the enzyme activity by simultaneous exposure to combinations of selected compounds formed in the period from 15^{th} untill 60^{th} min of irradiation of 1×10^{-5} mol/l malathion (Table 2) confirms that inhibitory power of malathion-irradiated samples can be attributed to the formation of malaoxon and isomalathion during the malathion photodegradation. It could be concluded that compounds (malaoxon and isomalathion) which are generally formed during storage or through natural or photochemical degradation of malathion are about 100 times more toxic than their parent compound. However, photoinduced malahion by-product formed as a result of P-S-C bond cleavage (diethiyl-maleate, O,O-dimethyl thiophosphate and OOS(S)) did not significantly affect AChE activity as well as the inhibitor efficiency of malathion inhibiting by-products, malaoxon and isomalathion.

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