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OXIDATION OF SOME ORGANOPHOSPHATE PESTICIDES WITH ENZYME MYELOPEROXIDASE

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Abstract
Some organophosphorous pesticides (OPs) containing phosphorthioate group were oxidized *in vitro* by enzyme myeloperoxidase (MPO) in the presence of hydrogen peroxide. The products were identified as oxon derivatives (phosphates), where the sulfur atom from thioate group is substituted by an oxygen atom. The oxidation efficiency was determined using acethylcholinesterase (AChE) bioassay and discused in the terms of MPO concentration, OPs concentration and incubation time.

Introduction
The heme enzyme myeloperoxidase (MPO) acts as an oxidant enzyme in the processes of inflammation and atherogenesis [1]. MPO is relatively nonspecific with respect to its reducing substrates and is able to oxidize different substrates.

Organophosphorous insecticides, characterized by one thione moiety (P=S) and three –OR groups are very effective and widely used group of pesticides. OPs and their activated metabolites are potent anti-cholinesterases (anti-ChEs) [2]. They can be activated in the environment to highly reactive oxons (P=O). The oxons are considerably more potent as anti-ChEs than their corresponding phosphorothionates. Therefore, efficient methods for their *in vitro* oxidation are important in their toxicological studies and for the development of methods for their detection [3,4].

In this work the ability of MPO to biocatalyze the transformation of OPs (diazinon and malathion) from thio- to oxo- forms was examined. The aim was to use MPO mediated oxidation of thio OPs to oxo OPs without any preconcentration or extraction step under normal laboratory conditions in order to improve the sensitivity of AChE based bioanalytical assays for their detection.

Material and methods
Myeloperoxidase (MPO) from human neutrophils was obtained from Planta Natural Products, Vienna, Austria. Catalase, from bovine liver, acethylcholinesterase (AChE) from electric eel, acethylthiocholine iodide (ASChI) and 5,5'-dithio-bis-(2-nitrobenzoic acid) (DTNB) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Hydrogen peroxide solutions were prepared daily by diluting a 30% (m/v) stock solution. The pesticides (at least 93% purity) malathion, malaoxon, diazinon and diazoxon were purchased from Pestinal®, Sigma-Aldrich, Denmark. The pesticide working solutions were prepared by dilution of the $1 \cdot 10^{-3}$ M stock solutions in ethanol.

Results and discussion

The desired concentrations of OPs in the range from $1 \cdot 10^{-5}$ - $1 \cdot 10^{-7}$ M were incubated in 50 mM phosphate buffer, pH 6.0, with various concentrations of MPO in a final reaction volume of 0.5 - 1.0 mL. Reaction was started by addition of 50 $\cdot 10^{-6}$ M H_2O_2, and stopped after incubation at various time intervals by adding catalase (100 μg mL^{-1}). The oxidation products were analyzed by UPLC (Ultra Performance Liquid Chromatography) and GC/MS (Gas Chromatography - mass spectrometry) measurements and chromatograms were compared with those of authentic standards. The identification of the oxidation products yielded only one major oxidation product – the oxo-form of pesticides. UPLC chromatograms that were recorded during 60 min, after the oxidation was stopped by catalase, confirmed that there were no other products of oxidation, i.e. the further cleavage of oxo-forms was not observed.

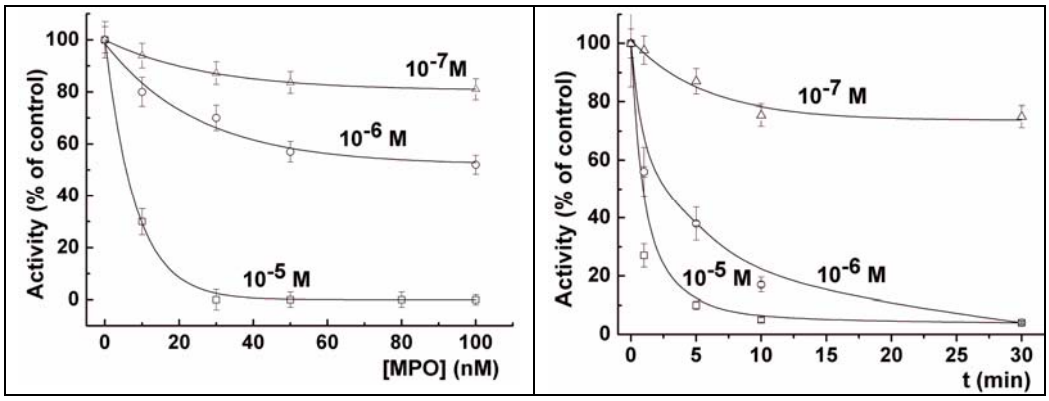

Fig.1. Dependence of AChE inhibition induced by malathion oxidized by MPO during 5 min incubation with OPs as a function of MPO concentration (A) and incubation time of 100 nM MPO with OP (B).

Since the oxidized forms of OPs are more potent inhibitors of AChE than the parent compounds, the efficiency of oxons formation was followed using AChE test [2]. Briefly, the free enzyme was exposed to oxidized samples (10 μl) in a final volume of 0.65 mL. The incubation time with AChE in the presence of inhibitors was 20 min, before the reaction was initiated and followed during 8 min. Fig 1A represents the results of the influence of MPO concentration (after 5 min incubation with malathion) on AChE inhibition with oxidised sample. It is obvious, that the increase of MPO concentration above 30 nM resulted in the saturation concentration of the oxo-form. In order to investigate the influence of incubation time between OPs and MPO to achieve the most efficient oxidation, the incubation time between OPs and 100 nM MPO was varied from 1 – 30 min. AChE test was used for monitoring the oxidation efficiency, as described above using 10 fold diluted OPs samples. The results obtained for malathion are presented in Fig 1B. The AChE activity in the presence of the oxidized OPs compared to non-oxidized

samples decreased with increased oxidation times, indicating the increase in the concentration of oxo forms. The saturation level of oxo-forms was reached after 10 min incubation of parent compounds with MPO.

The results for oxidation efficiency after 10 min incubation with 100 nM MPO for diazinon and malathion, obtained using AChE test and UPLC measurements, are given in Table 1. The calibration graph for AChE induced inhibition by oxo forms was constructed for determination of oxo forms concentration formed upon the oxidation. It is obvious that 10 min exposure of $1 \cdot 10^{-4}$ and $1 \cdot 10^{-5}$ M diazinon to 100 nM MPO yielded the formation of about $(3 - 5) \cdot 10^{-6}$ M diazoxon, since $1 \cdot 10^{-6}$ M diazinon was about 50% oxidized. The conclusion can be made that the conversion efficiency increased by lowering diazinon concentration. Similar results were also obtained for malathion.

Table 1. Efficiency of OPs oxidation after 10 min incubation with 100 nM MPO based on UPLC and GC/MS determination of concentration of thio- and oxo- forms.

OPs	Parent OP (M)		Oxo form (M)		[3]Oxidation efficiency (%)	
	Initial concentr. (M)	[1]After incubation (M)	[1]UPLC (M)	[2]AChE test (M)	UPLC	[2]AChE test
diazinon	$1 \cdot 10^{-4}$	$0.95 \cdot 10^{-4}$	$0.05 \cdot 10^{-4}$	$0.02 \cdot 10^{-4}$	5	2
	$1 \cdot 10^{-5}$	$0.73 \cdot 10^{-5}$	$0.27 \cdot 10^{-5}$	$0.04 \cdot 10^{-5}$	27	4
	$1 \cdot 10^{-6}$	$0.85 \cdot 10^{-6}$	$0.50 \cdot 10^{-6}$	$0.10 \cdot 10^{-6}$	50	10
malathion	$1 \cdot 10^{-4}$	$0.93 \cdot 10^{-4}$	-	$>1 \cdot 10^{-5}$	-	>10
	$1 \cdot 10^{-5}$	$0.77 \cdot 10^{-5}$	-	$>1 \cdot 10^{-5}$	-	>25
	$1 \cdot 10^{-6}$	-	-	$>1 \cdot 10^{-7}$	-	>50

[1] Determined by UPLC; [2] Based on AChE inhibition measurements using 10 fold diluted initial solutions and recalculated to initial concentration; [3] [formed oxo form]/[initial OP].

Conclusion

The oxidation of OPs by the enzyme MPO yield only one major oxidation product – the oxo-form of pesticides. The degree of OPs oxidation depends on the OPs and MPO concentrations, as well as on incubation time between OPs and MPO. The 10 min incubation time of OPs in phosphate buffer, pH 6.0, with 50 - 100 nM MPO was found to be applicable for OPs oxidation, to produce oxo-forms with stronger inhibitory power towards AChE.

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