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Article

A Spectrophotometric Assay for Cytochrome P450 Monooxygenase Activity

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An assay for cytochrome P450 monooxygenase activity by determination of the products of organophosphate oxidation via inhibition of acetylcholinesterase was described. Extracts from strains of *Oryzaephilus surinamensis* selected for resistance to chlorpyrifos-methyl (QVOS 102), fenitrothion (VOS F) and malathion (VOS 3), and a standard susceptible strain VOS 48, were incubated with an organophosphate in the presence of a NADPH-generating system and acetylcholinesterase. The degree of inhibition of the acetylchoinesterase activity was converted to monooxygenase activity using standard curves for the inhibition of acetylcholiesterase by chlorpyrifos-methyl-oxon, fenitrooxon and malaoxon. Activity was detectable in VOS 48 and was present at different increased levels with the different organophosphates in the three resistant strains, suggesting that different forms of P450 might be involved in organophosphate oxidation in these insects. The assays were carried out using crude insect homogenates and much smaller samples of insect material than the standard aldrin to dieldrin assay. It should be possible to use the method for determination of monooxygenase activity in single insect.

Key words : *cytochrome P450, monooxygenase activity, aldrin epoxidation activity, organophosphate insecticides.*

Cytochrome P450 monooxygenase systems play an important role in a wide range of metabolic reactions. They are also involved in the metabolism of many xenobiotics by desulfuration, *N*-, *O*-, *S*-dealkylation, and dehydrogenation. Desulfuration of organophosphates results in the compounds more toxic toward AChE, the target enzyme, than the original insecticide.

Early studies of monooxygenase activity with xenobiotics described the epoxidation of organochlorine compounds. In 1953, the epoxidation of heptachlor in dog and rat tissues was measured by the spectrophotometric analysis of the extracted heptachlor epoxide.[1)] The conversion of aldrin to dieldrin in mammalian tissues was first determined in 1955.[2)] The aldrin epoxidation reaction has been widely used as a measure of monooxygenase activity in soil microorganisms,[3)] plant tissues,[4)] animals,[5,6)] and insects.[7-11)]

Organisms may contain more than one form of the cytochrome P450 components of the monooxygenase system and different forms may be related to the metabolism of different substrates. A specific cytochrome P450 component from *Drosophila* was correlated with resistance to DDT, phenylurea and malathion.[12)] Monooxygenase activity with six different substrates was varied according to the level of resistance to pyrethroids in pyrethroid resistant houseflies.[13)] Cytochrome $P450_{lpr}$ present at elevated levels in the insecticide-resistant LPR strain of housefly, has been shown to be responsible for the conversion of chlorpyrifos to chlorpyrifos-oxon.[14)] *In vitro* monooxygenase activity with methyl parathion, determined using [^{14}C]-labelled insecticide, was lower in a strain of *Heliothis virescens* resistant to methyl parathion than in the susceptible strain.[15)] The amount of cytochrome P450 was unchanged in comparison with a susceptible strain, suggesting that the resistant strain contained an altered cytochrome P450 isozyme. Resistance to F(S), in *Anopheles subpicta* was correlated with increased cytochrome P450 levels.[16)] [^{14}C]-F(S) was metabolised by a crude enzyme extract to give a wide range of products, detected by TLC, including substantial amounts of F(O), and 3-hydroxymethyl-fenitrooxon, both of which were more inhibitory to AChE than F(S) itself. Resistance to orgnophosphates in the saw-toothed grain beetle, *O. surinamensis*, has been correlated

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Abbreviations: AChE, acetylcholinesterase; ATCh, acetylthiocholine; ATChI, acetylthiocholine iodide; BAS, bovine serum albumin; CM (O), chlorpyrifos-methyl-o-analogue; CM(S), chlorpyritos-methyl; DTNB, 5,5-dithio-bis-(2-nitrobenzoic acid); GSH, glutathione; F(O), fenitrooxon; F(S), fenitrothion; M(O), malaoxon; M(S), malathion; PVPP, polyvinylpolypyrrolidine.

with increased levels of monooxygenase activity, determined as aldrin epoxidation and cytochrome P450 concentration.[9] There have been no report of the desulfuration of organophosphates by cell-free preparations from *O. surinamensis.*

Trace amounts of insecticides can be determined by measurement of AChE inhibition.[17] In this method organophosphates were chemically oxidized to the more toxic oxon derivatives. This paper described an assay for the biological oxidation of insecticides by monooxygenase systems, in which the products of the reaction were determined by their inhibition of AChE. This simple spectrophotometric assay has been used to examine the oxidation of chlorpyrifos-methyl, fenitrothion and malathion by strains of *O. surinamensis* selected for resistance to these three insecticides.

Materials and Methods

Insects. The four strains of *Oryzaephilus surinamensis* used in this study were supplied by Dr H. A. Rose, University of Sydney. VOS 48 was an insecticide susceptible reference strain that has been continuously cultured in the laboratory since 1973. VOS 3 was a M(S)-resistant strain collected before the introduction of F(S) and CM (S) selections. VOS F and QVOS 102 were composite Australian field strains, selected with F(S) and CM(S) selection. The strains were reared in our laboratory on a mixture of organic wheatmeal, rolled oats and brewer's yeast (7 : 7 : 1), without any further exposure to insecticide. Resistance factors (RF) relative to VOS 48, determined after culturing without insecticide for 2 years (Lee and Lees, unpublished data), were: VOS 3, RF values CM(S) 4, F(S) 24, M(S) 16; VOS F, RF values CM(S) 5, F(S) 14, M(S) 5; QVOS 102, RF values Cf CM(S), F(S) and M(S) in 2.

Insecticides. CM(S) was supplied by Dowelanco Australia Ltd. CM(O), was purchased from Chem Service Inc. USA. F(S) was supplied by Bayer Australia Ltd.; M (S) and malaoxon, M(O), by Dr. H. A. Rose, University of Sydney. Standard solutions were prepared in 2-methoxyethanol; CM(S), F(S) and M(S) solutions were 100 μg/ml and CM(O). F(O) and M(O) were 10 μg/ml.

Preparation of crude enzyme from *O. surinamensis*. All procedures were carried out at 4°C. Duplicate samples of *O. surinamensis* [0.76 g VOS 48, 0.50 g VOS 3, 0.33 g VOS F and 0.1 g QVOS 102 for the CM(S) assay; 0.76 g VOS 48, 0.50 g VOS 3, 0.33 g VOS F and 0.1 g QVOS 102 for the F(S) assay; and 0.20 g VOS 48, 0.10 g VOS 3, 0.10 g VOS F and 0.1 g QVOS 102 for the M(S) assay], were homogenized in a pestle and mortar with 2.5 ml of 0.1 M potassium phosphate buffer, pH 7.5, containing 1 mM EDTA, 10 mM GSH, and 70 mg BSA and 70 mg PVPP/0.1 g beetles. The homogenates were strained through nylon mesh and centrifuged for 3 min at 12,000 g in a Sorvall RC2B centrifuge. The supernatants were kept on ice as the crude enzyme preparations.

Assay of monooxygenase activity in crude extracts of strains of *O. surinamensis* by determination of the inhibition of AChE by the oxidation products of chropyrifos-methyl, fenitrothion and malathion. The duplicate crude extracts prepared from the four strains of insects were assayed in duplicate. Insecticide samples, 10 μg/ml solutions of CM(S), F(S) and M(S) in 2-methoxyethanol were placed in test tubes and the solvent was evaporated by a stream of nitrogen. Potassium phosphate buffer, 0.1 M, pH 7.5 (0.12 to 0.31 ml), phosphate buffer containing 1 mM EDTA and 2.6% BSA (0.09 ml), and 50 μl of an NADPH generating system containing 1.1 μmol NADP, 12.1 μmol glucose 6-phosphate and 2.8 units of glucose 6-phosphate dehydrogenase (D-glucose-6-phosphate : NADP 1-oxidoreductase, EC 1.1.1.49, from yeast) were added to the test tubes. AChE, (acetylcholine acetylhydrolase, EC 3.1.1.7) about 0.1 mg, (from *Electrophorus electricus*) was dissolved in 1 ml of 0.1 M phosphate buffer, pH 7.5, to give a solution containing 100 units of enzyme/ml and 40 μl (4 units) of this solution was added to the reaction mixture. The reaction was initiated by the addition of 10 to 200 μl of the crude enzyme to give a total volume of 0.5 ml. Control reaction mixtures were set up without an NADPH generating system, and containing 0.17 to 0.36 ml of phosphate buffer. Reaction mixtures were incubated at 37°C and 10 μl aliquots were removed for AChE assay at t0 and times up to 70 min.

AChE activity was assayed by Ellman's method.[18] The 10 μl sample was added to a solution containing ATChI, (0.5 mM) and DTNB (3.0 mM) in 0.1 M potassium phosphate buffer, pH 8.0, in a total volume of 3 ml, incubated at 37°C and the A412 was monitored using a Perkin-Elmer Lambda 1 spectrophotometer linked to a Perkin-Elmer 561 Recorder. The activity of the AChE was calculated as ΔA412 per min and converted to nmol ATChI hydrolysed/min using a standard curve in the range from 0 to 150 nmol ATCh. Units of AChE were calculated as μmole ATChI hydrolysed/min for the 0.5 ml reaction mixtures. The unit of AChE inhibited as a result of monooxygenase activity in the reaction mixtures was calculated as the difference between units of AChE inhibited in the presence and absence of an NADPH generating system.

Standard curves for the inhibition of AChE by CM(O), F(O) and M(O) were prepared, Standard CM(O), F(O) and M(O) solutions were diluted to give a range of concentrations; 10 μl aliquots were transferred to test tubes and the 2-methoxyethanol was evaporated by a stream of nitrogen gas. Potassium phosphate buffer, 0.1 M, pH 7.5, (0.36 ml) and phosphate buffer containing 1 mM EDTA and 2.8% BSA (0.1 ml) were added to the test tubes. Diluted AChE solution (40 μl, 4 units) was added to the

test tubes at t_0 to give a total reaction mixture of 0.5 ml, and the reaction mixture was incubated at 37°C. Samples (10 μl) were withdrawn from the reaction mixture at t_0 and t_{60} and assayed for AChE activity. Units of AChE (μmol ATChI hydrolysed/min) inhibited in a 0.5 ml reaction mixture were plotted against the amount of CM(O), F(O) or M(O) in the reaction mixture.

Using these standard curves, the units of AChE inhibited in the reaction mixtures containing the monooxygenase activity and organophosphates were converted to ng of CM(O), F(O) or M(O) produced in the reaction mixtures. Activity was calculated as pmol of CM(O), F (O) or M(O) produced/min/g insect.

Results and Disussion

The oxidation of chropyrifos-methyl, fenitrothion and malathion by cytochrome P450 monooxygenase activity in *Oryzaephilus surinamensis*. Standard curves for the inhibition of AChE by CM(O), F(O) and M(O) were shown in Fig. 1. Incubation of AChE with the insecticides for 60 min gave maximum inhibition for the amount of inhibitor used. The standard curves were linear up to 1 ng for CM(O), 6 ng for F(O) and 2 ng for M(O), and showed that AChE was less susceptible to inhibition by F (O) than by CM(O) and M(O).

The cytochrome P450 monooxygenase activities of four strains of *O. surinamensis* assayed using CM(S), F(S) and M(S) as substrates were shown in Table 1. Activity has been calculated as pmol CM(O), F(O) and M(O) produced [or pmol CM(S), F(S) and M(S) oxidised]/min/g insect. Activity with the three organophosphates could be detected in the susceptible strain VOS 48. Higher activity was measured in the strains selected for resistance to the insecticides.

The results presented here showed that monooxygenase activity determined as the rate of oxidation of a particular organophosphate differed with the organophosphate and the strain of insect examined. In using CM(S) as substrate, only the QVOS 102 strain had high activity. When the four strains were assayed with M(S), there

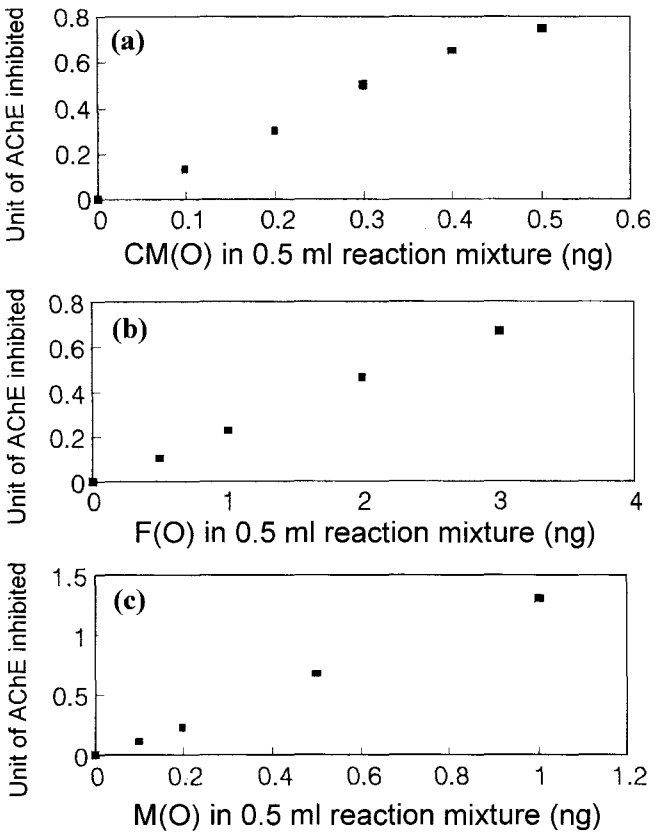

Fig. 1. Standard curves for the inhibition of AChE by CM(O), F(O) and M(O). Reaction mixtures containing the insecticides and AChE in a total volume of 0.5 ml were incubated at 37°C and AChE in a total volume of 0.5 ml was incubated at 37°C and AChE activity assayed at t_0 and t_{60} min; (a) Δ, CM(O); (b) F (O); (c) M(O).

was high activity in both VOS 3 and QVOS 102 strains. Substantial monooxygenase activity with F(S) was detected only in QVOS 102 and was not observed in VOS F [cultured without F(S) for two years] to F(S), CM(S) and M(S) with published data[9] showed that resistance to F(S) in the VOS F strain was very low. VOS 3, the M(S) resistant strain isolated before the use of F(S) and CM(S) as grain protectants, had high activity only with M(S) as substrate, whereas QVOS 102 was active with all three insecticides. QVOS 102 was developed by selection with

Table 1. Cytochrome P450 monooxygenase activity of four strains of *O. surinamensis* assayed with CM(S), F(S) and M(S) as substrates.

Strain	Substrate					
	CM(S)		F(S)		M(S)	
VOS 48	0.16±0.03	(1)[a]	0.91±0.29	(1)[a]	0.63±0.17	(1)[a]
VOS 3	6.30±2.30	(39)[b]	6.20±2.00	(7)[b]	245.00±31.80	(390)[b]
VOS F	5.50±1.10	(34)[c]	4.20±1.20	(5)[c]	3.24±1.22	(5)[c]
QVOS 102	210.00±18.00	(1300)[d]	366.00±53.00	(400)[d]	388.00±68.60	(620)[d]

All cytochrome P450 monooxygenase acticity was calculated as nmol 'oxon' derivative produced $min^{-1}g$ $beetles^{-1}$. The figure in brackets (*) indicates the ratio of monooxygenase activity with an organophsphates substrate in a resistant insect to monooxygenase activity with that substrate in VOS 48, the standard susceptible strain. The data (mean±SE values) were determined from duplicate assays. Means in the column followed by the same letter were not significantly different (P>0.05) as determined by paired t tests assuming unequal variances.

CM(S) of field strains which had not been exposed to M (S) for many years. These data suggested that monooxygenase activity which contributed to the development of resistance to M(S) has been retained in the insects while resistance to other organophosphates has developed. Activity with M(S) was low in the VOS F strain which was also originally selected from insects resistant to M(S).

In an earlier report of monooxygenase activity in VOS 48, VOS 3, VOS F and VOS CM, the cytochrome P450 content and aldrin epoxidation activity were elevated in the three resistant strains but levels were similar in the three beetles.[9] The results obtained using the AChE assay showed that the assay of monooxygenase activity by aldrin epoxidation and cytochrome P450 determination gave only limited information about the activity and role of monooxygenases in organophosphate metabolism in these insects. The differences in monooxygenase activity between the QVOS 102 and VOS 3 strains in particular, suggested that there might be different forms of cytochrome P450 which acted in the oxidation of different organophosphates.

In these experiments, using CM(S), F(S) and M(S) as substrates, monooxygenase activity has been determined as the CM(O), F(O) and M(O) produced by oxidation of the organophosphate. It might be that other oxidation products which were more inhibitory to AChE than the original insecticide, have also been produced. It has been shown that F(S) was metabolized into two good inhibitors of AChE, F(O) and 3-hydroxymethyl-fenitrooxonin in *A. subpicta*.[16] Such metabolism could occur in other species. Nevertheless, this method did give a good measure of the capacity of the monooxygenase activity of a strain of insect to produce toxic metabolites of a particular insecticide. Isolation and characterization of the cytochrome P450 species of the insects, and reconstitution experiments using specific organophosphates should allow a better understanding of the role of monooxygenases in resistance to these insecticides.

This assay allowed to determine monooxygenase activity with much smaller samples of insect than usually used for the aldrin epoxidation method. In the standard method described for assay in *O. surinamensis*,[8,9] a microsomal preparation was used and the monooxygenase reaction mixture contained the equivalent of 225 insects. Only a small fraction of the hexane-extracted aldrin and dieldrin mixture was used for GLC analysis. It was possible to reduce the scale of this assay to use the equivalent of about 25 insects per assay, but limitations of hexane extraction procedures made it difficult to scale the procedure further down. In the assay described here, crude extracts of insect homogenates have been assayed, and for resistant insects monooxygenase activity has been determined in samples containing less than the equivalent of 1 insect in a reaction mixture of 0.5 ml, from which 10 µl samples have been taken for AChE assay. The inclusion of EDTA and BSA in the reaction mixture was essential to prevent degradation of the AChE by the crude insect homogenate. For homogenates containing high levels of endogenous esterase activity, which could also be inhibited by the oxon forms of organophosphates, it was necessary to ensure that the amount of AChE added to the reaction mixture was substantially greater than the level of endogenous esterase activity present. It should be possible to increase the sensitivity of this assay to determine monooxgenase activity in a single *O. surinamensis* insect by determining the relative amounts of insect extract and AChE used, the insecticide concentration in the assay, and the size of the sample taken for AChE assay. Single insect assay of esterase activity can be readily carried out, enabling examination of the distribution of this activity in populations of insects.[19] Analysis of monooxygenase activity in this way has not been possible so far, but further development of this method should enable such assays to be carried out.

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