CHAPTER 2

INHIBITION STUDIES

CHAPTER TWO

2.1 Inhibition studies

2.1.1. Introduction

Specific esterases (eg. cholinesterase and CarE) which are widely distributed in rodent and insect tissues are involved in the mechanisms of insecticide resistance (Sudderuddin , 1972). Detailed studies have been made on the mechanisms and there is evidence that these esterases are involved in the mechanism of insecticide resistance in rodents and insects (Asperen and Oppenoorth, 1960; Motoyama and Dauterman, 1974).

In the overall biochemical defense system for developing resistance in rats and insects, metabolic factors generally do play the most significant role (Brown, 1971; Oppenoorth and Welling, 1976). Metabolic alterations of insecticidal chemicals are clearly recognizable and the enzyme systems responsible for these changes can be identified and characterized.

The mechanism of organophosphate (OP) resistance is mainly detoxification by the enzymes hydrolyzing the insecticides. DDVP-resistant rats are characterized by increased carboxylesterase, which attacks the unique

2.3.2 Determination of I_{50} values

The I_{50} value was obtained by deduction of optical density to get the percentage of inhibition of insecticides (O' Brien , 1960), that is :-

$$\% \text{ Inhibition} = \frac{(\text{optical density of control} - \text{optical density of test}) \times 100}{\text{optical density of control}}$$

From the value of percentage inhibition , the graph of percentage inhibition versus log molar concentration of the insecticide was plotted (Fig. 2.3(a) - 2.3(l)). A linear plot was obtained . Fig 2.4 and Table 2.3, 2.4 and 2.5 depicted the I_{50} values for every exposition to malathion, DDVP and fenitrothion for certain period of time . To get the I_{50} value a line from 50% inhibition was projected from the y - axis to the line plotted in the graph , and the projection showed the value of the x-axis

The I_{50} value denotes the insecticide concentration to reduce 50% of the esterase activity . The result shows that the higher the insecticide concentration the higher the value of percentage inhibition .

α-naphthylacetate + enzyme $\Rightarrow$ α-naphthol + CH_3COOH

[substrate] [acetic acid]

α-naphthol + diazo blue B $\Rightarrow$ α-naphthol diazoate

[diazonium slat] [stable blue dye]

2.2. Materials and methods

2.2.1 Rodents

The effect of inhibitor on esterase enzyme activity in the rodent Rattus rattus diardii was investigated in the present study. Rats were obtained from Pusat Penangkapan Haiwan Bandaraya (DBKL) caught at surrounding areas of Kuala Lumpur. Besides rodent, wild dogs, rabbits and other problem animals were also caught DBKL to avoid more damage to the environment.

Pusat Penangkapan Haiwan Bandaraya is located far away from husing areas to avoid distraction to the residents and spreading of infectious diseases. Some of the animals were used for research on controlling breeding activity and other animals were discarded without causing any torment.

The rodents obtained were kept for 3 – 5 days to acclimatize them to the new environment and food in palette form and water were given daily.

2.2.2. Exposing rodents to the inhibitors

The following inhibitors were used in the experiment:

a)	Malathion	-	57%
b)	Dichlorvos (DDVP)	-	44%
c)	Fenitrothion	-	50%

Rats were exposed to insecticide via intramuscular injection at the hind leg. The weighing procedure for the rats was calculated as follows:-

Rodent weight + catching cloth = W gram

Weight of catching cloth = Y gram

Net weight = (W - Y) gram

= Z gram

Every 1000 grams of rodent weight = 30 mg of inhibitor.

Therefore,

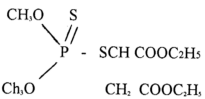
$$\text{Z gram} = \frac{Z}{100} \times 30$$

Amount of inhibitor required = R mg

R grams were diluted in 1 ml of groundnut oil using a syringe and 0.5 ml were injected at the rodent's hind leg. The rodents were exposed to inhibitor for 1, 3, 7, 14, 21 days.

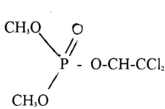
2.2.3. Insecticides used

a) Malathion



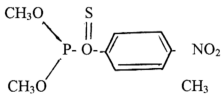
Malathion is the common name approved by BSI and International Organization for Standardization (IOS), for S – 1, 2- di (ethoxycarbonyl) *ethyl O,O*-dimethyl phosphorodithioate. It is soluble in organic solvents and only slightly soluble in water. It is non-systemic insecticides and acaricide of low mamalian toxicity with brief to moderate persistence. In addition to a wide range of agriculatural and horticultural uses, it is used for the control of mosquitoes, flies and other insects.

b) Dichlorvos (DDVP)



Dichlorvos is the common name for 2, 2-dichlorovinyl dimethyl phosphate. This organophosphate is miscible with most organic solvents and aerosol propellants. Dichlorvos is a contact and stomach insecticide with fumigant and penetrant action. It is used as a household and public health fumigant, for protection of stored product and for crop protection. It is non-phytotoxic and non-persistent.

Fenitrothion (Sumithion)



The chemical name for fenitrothion is *O,O*-dimethyl O-4-nitro-m tolyl phosphorothioate. It is a brownish-yellow liquid. It is practically insoluble in water but soluble in most organic solvents and of low solubility in aliphatic hydrocarbons. Fenitrothion is a contact insecticide, particularly effective against rice stem borers, and a selective acaricide but of low oricidal activity. It has a low mammalian toxicity.

2.2.4. Evaluation of Specific Enzyme Activity

(i) Enxyme Preparation

In the preparation of enzyme, the rodents were divided into two groups:-

A) Control rodents.

B) Rodents exposed to inhibitor (Malathion - DDVP - Fenitrothion).

Springes were used to collect blood samples and heparin coated Eppendorf tubes were used to store the serum. Blood was drawn direct from the rodent's heart via thorax puncture and transferred into Eppendorf tube. The tubes were then centrifuged at 3000 rpm for 10 minutes. The supernatant was then aspirated using a micropipette and diluted at the ratio of 3 : 5 : 75 with phosphate buffer (0.1 M, pH 7).

The resulting solution were then used as the source of enzyme. Throughout the experiment, crushed ice was used to stop enxyme activity in the sample. The following parameters were considered when the inhibitor effect on enzyme activity was detected:-

- I_{50} (Inhibitor concentration causing 50% inhibition of enzyme activity).

- K_i (The bimolecular rate constant).
- Protein estimation.
- Specific activity of enzyme.
- Electrophoresis.

Experiments were carried out once on treated rats and on the control ones.

(ii) Preparation of reagents

a) Phosphate buffer:

A stock solution of 0.1 M phosphate buffer pH 7 was prepared (15.601 g NaH_2PO_4 + 14.196 g $NaH_2PO_4H_2O$ in 1000 ml of deionized, distilled water). The stock solution was stored at 4 °C. For the required concentration of 0.04 M phosphate buffer for the experiments, 100 ml of 0.1 M PO_4 buffer was diluted with 150 ml deionized distilled water.

a) Substrate (α-naphthyl acetate)

A solution of 3 X 10 °M concentration was prepared by dissolving 0.0056 g substrate in 1 ml acetone and 99 ml 0.04 M phosphate buffer.

b) Diazo-blue solution (DBLS)

5 parts of 5% sodium lauryl sulphate and 2 parts of 1% diazo-blue was prepared with deionized distilled water. To prevent the digradation of the dye, which is very sensitive to light, the container was covered with aluminium foil and kept in a dark place.

d) Inhibittors

Malathion (50%), dichlorvos (DDVP) (44%), and fenitrothion (57% were used as esterase activity inhibitors. All these inhibitors had the concentration value of 10 M prepared through serial dilution. Malathion had to be diluted in 1 ml acetone. These serial dilutions were used for the determination of I_{50} value, K_i for DDVP, malathion and fenitrothion. The dilutions ranged from 2 ML to 10 ML.

(iii) Protein estimation

a) Reagent C :-

For the preparation of reagent C, 100 mL of part A (10 g $Na_2 CO_3$ + 2 g NaOH + 0.8 g Natrium tartarate + 5 g Natrium lauril sulphate dissolved in 50 ml distilled water) diluted with 1 part of B (4 g Cu So_4 . $5H_2O$ dissolved in distilled water).

b) Folin Solution:-

Folin solution was mixed with distilled water at ration 1 : 1 and used in the experiment.

(iv) Experimental design

a) Enzyme activity estimation

The experimental design for enzyme activity estimation was as follows: (Table 2.0).

Reagent	Black C	Black test	Test
Phosphate buffer	1.0 ml	2.5 ml	0.5 ml
Substrate	2.0 ml	------	2.0 ml
Enzyme	--------	0.5 ml	0.5 ml
Mixture were incubated for 15 minutes at 37 °C			
DBLS	0.5 ml distilled water	0.5 ml	0.5 ml

The solution was incubated for 15-30 minutes at 37 °C in a water-bath. Then 0.5 ml DBLS was added to stop the reaction. A blue solution was formed and its optical density (O.D) was measured against the blank at 590 nm. The corrresponding α-naphthol concentration was obtained from the standard curve. The Shimadzu UV-160 A Spectrophotometer was used.

The percentage of inhibition was calculated using the formula:

$$\% \text{ Inhibition} = \frac{\text{O.D. of control - O.D. of test}}{\text{O.D. of control}} \times 100$$

(v) Preparation of standard curve

This was prepared by the addition of different concentration of 2.5 ml α-naphthol and 0.5 ml 0.04 M phosphate buffer to 0.5 ml DBLS. The colour intensities were determined at 590 nm. The O.D. obtained was plotted against the concentration of α-naphthol (Fig. 2.0, Table 2.1).

Table 2.1 : Data for the standard curve of α-naphthol.

α-naphthol concentration (μM/ml)	Optical Density at 590nm. 1	2	3	Mean Optical Density.
1.563	0.067	0.070	0.069	0.069
3.125	0.140	0.140	0.140	0.140
6.250	0.245	0.245	0.255	0.248
12.500	0.520	0.500	0.520	0.513
25.000	1.000	1.050	1.050	1.033
50.000	1.800	1.900	1.950	1.883

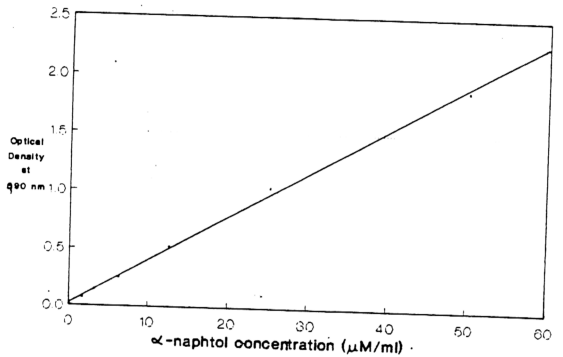


Fig 2.0 : Standard curve for α-naphthol.

(vi) Protein determination

The Folin phenol method used was a combination of those employed by Sutherland *et al.*, (1949) and Lowry *et al.* (1951) as modified by Litwack (1960). The protein content of tissue extracts was determined by comparisons with bovine serumalbumin (BSA). (Fig. 2.2, Table 2.2).

Design of the experiment:

Reagent	Control blank	Test blank	Test
Enzyme	Distill water	0.25 ml	0.25 ml
Reagent C	-------	0.825 ml	0.75 ml
Mixture was incubated for 10 minutes at 25 °C			
Folin	--------	---------	0.075 ml

The mixture was incubated for 45 minutes at 25 °C before the reading of optical density. The O.D. value at 660 nm was obtained using Spectrofotometer UV-1621 and the protein concentration for each individual was determined.

Table 2.2: Data for the standard curve of protein.

protein concentration (µg/ml)	Optical Density at 750nm. 1	2	3	Mean Optical Density.
6.25	0.015	0.005	0.010	0.010
12.50	0.015	0.015	0.020	0.017
25.00	0.455	0.050	0.055	0.050
50.00	0.080	0.095	0.081	0.085
100.00	0.220	0.200	0.200	0.207
200.00	0.400	0.370	0.370	0.380

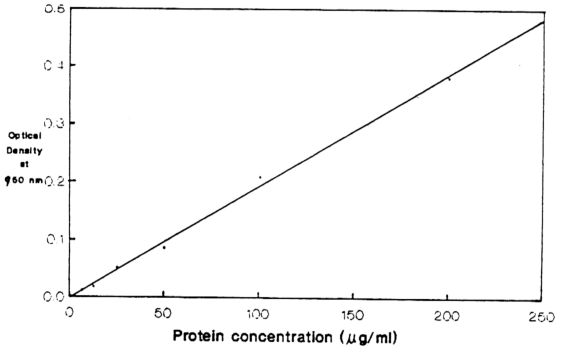
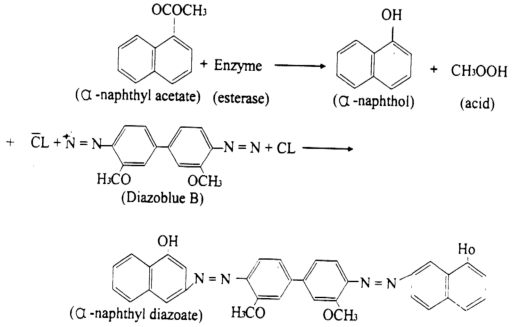


Fig 2.1 : Standard curve for protein concentration.

2.3 Results

2.3.1 Specific activity of esterase

The activity of esterase enzyme can be studied by following their action on substrates , α- naphthyl acetate , which are split into an ester (α-naphthyl) and an acid . The ester many be coupled with a variety of diazonium salt to form highly stable dyes . The activity of esterase can be estimated by measuring these insoluble dyes spectrophotometrically . The susceptibilities and character of these esterases many then be determined by reacting them with specific inhibitors such as an organophosphorus compounds . A summary of these reactions is a follows :-



The specific activity of esterase enzyme was determined based on the amount of α- naphthol produced per time unit for every protein concentration that was used according to the following equation (Asperen , 1962) .

$$\text{specific activity} = \frac{\alpha \text{ - naphthol produced}}{\text{time X protein concentration}} = \text{mM / min / mg protein}$$

α - naphthol concentration yielded was determined by using the standard curve (Fig . 2.0) . As described in the method (2.2.2) , rat was exposed to several types of organophosphate prior to the extraction of their blood . The blood was extracted after 1 , 3 , 7 , 14 and 21 days of exposure and the esterase activities were tested . It was found that the esterase activity increased with time of insecticides exposure (Fig 2.2) . It was shown that the enzyme activities were the highest after 21 days of exposure especially for the fenitrothion . The specific activity of esterase was much more distinct after 21 days of fenitrothion exposure as compared with DDVP and malathion .

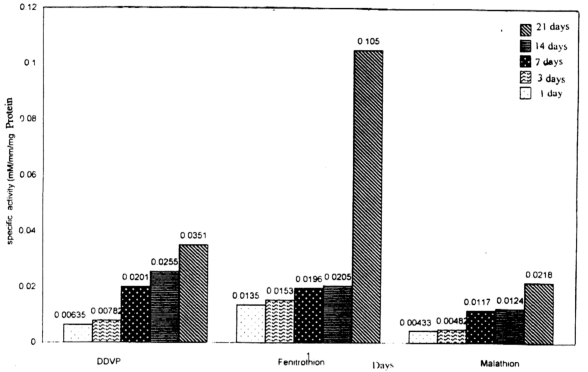


Fig (2.2) Graph for esterase specific activity

2.3.2 Determination of I_{50} values

The I_{50} value was obtained by deduction of optical density to get the percentage of inhibition of insecticides (O' Brien , 1960), that is :-

$$\% \text{ Inhibition} = \frac{(\text{optical density of control} - \text{optical density of test}) \times 100}{\text{optical density of control}}$$

From the value of percentage inhibition , the graph of percentage inhibition versus log molar concentration of the insecticide was plotted (Fig. 2.3(a) - 2.3(l)). A linear plot was obtained . Fig 2.4 and Table 2.3, 2.4 and 2.5 depicted the I_{50} values for every exposition to malathion, DDVP and fenitrothion for certain period of time . To get the I_{50} value a line from 50% inhibition was projected from the y - axis to the line plotted in the graph , and the projection showed the value of the x-axis

The I_{50} value denotes the insecticide concentration to reduce 50% of the esterase activity . The result shows that the higher the insecticide concentration the higher the value of percentage inhibition .

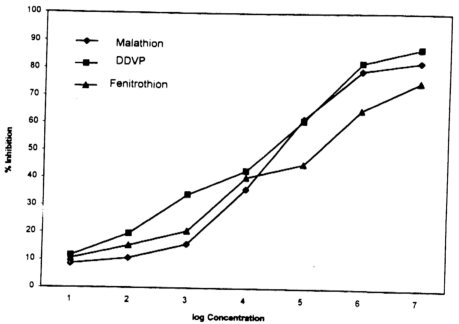


Fig 2.3 (a) Percentage inhibition of esterase of rat by different concentrations of insecticides. The rat has been exposed for 1 day to Malathion prior to the inhibition tests.

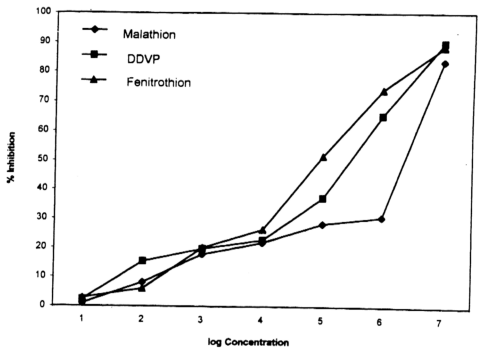


Fig 2.3 (b) Percentage inhibition of esterase of rat by different concentrations of insecticides. The rat has been exposed for 7 days to Malathion prior to the inhibition tests.

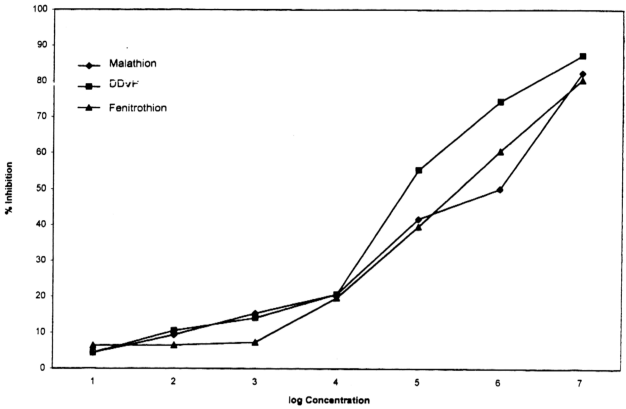


Fig 2.3 (c) Percentage inhibition of esterase of rat by different concentrations of insecticides. The rat has been exposed for 1 day to DDVP prior to the inhibition tests.

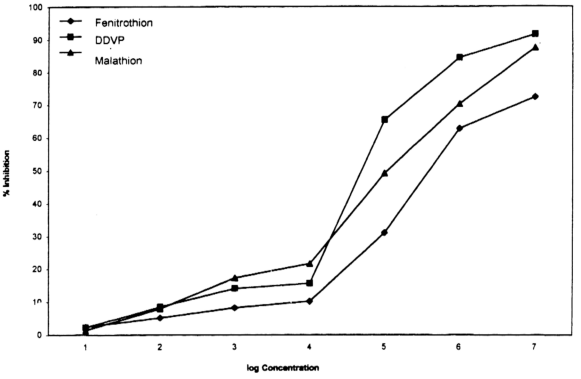


Fig 2.3 (d) Percentage inhibition of esterase of rat by different concentrations of insecticides. The rat has been exposed for 3 days to DDVP prior to the inhibition tests.

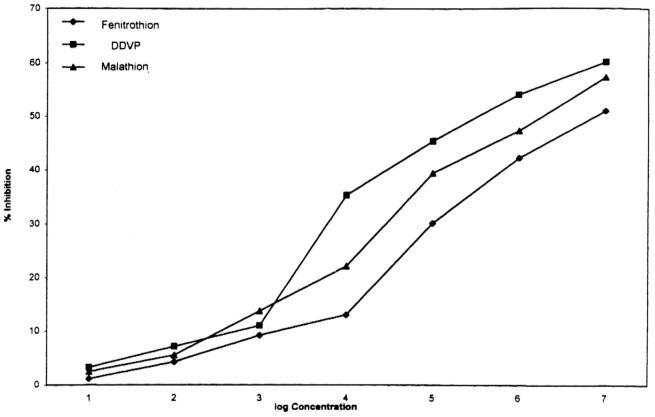


Fig 2.3 (e) Percentage inhibition of esterase of rat by different concentrations of insecticides. The rat has been exposed for 7 days to DDVP prior to the inhibition tests.

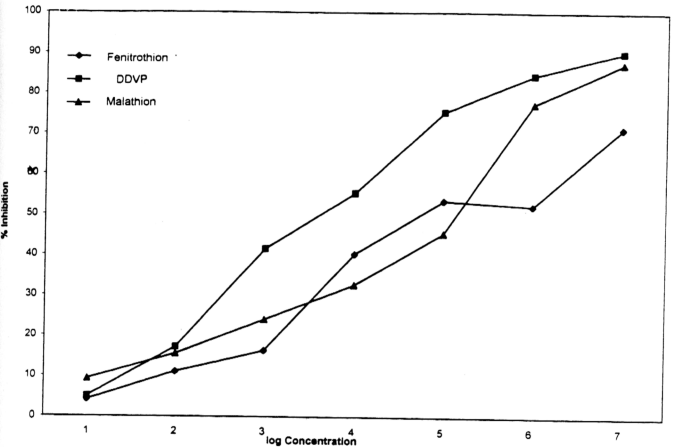


Fig 2.3 (f) Percentage inhibition of esterase of rat by different concentrations of insecticides. The rat has been exposed for 14 days to DDVP prior to the inhibition tests.

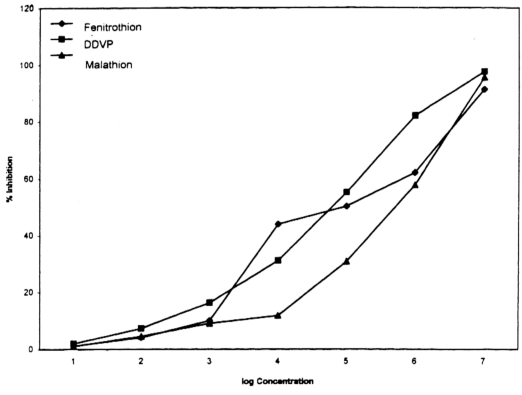


Fig 2.3 (g) Percentage inhibition of esterase of rat by different concentrations of insecticides. The rat has been exposed for 21 days to DDVP prior to the inhibition tests.

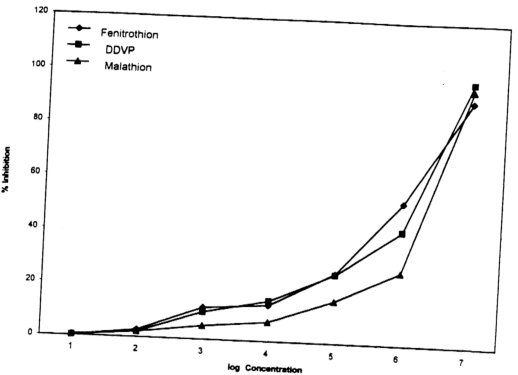


Fig 2.3 (h) Percentage inhibition of esterase of rat by different concentrations of insecticides. The rat has been exposed for 1 day to Fenitrothion prior to the inhibition tests.

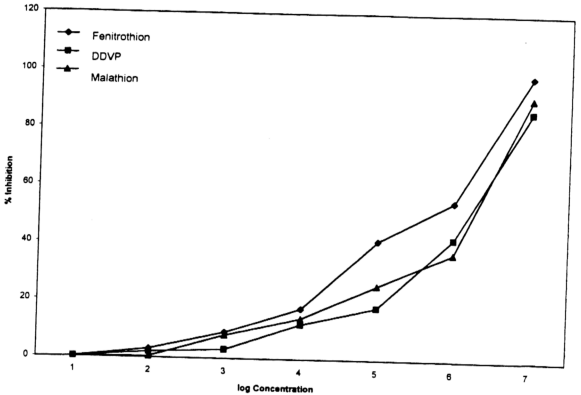


Fig 2.3 (i) Percentage inhibition of esterase of rat by different concentrations of insecticides. The rat has been exposed for 3 days to Fenitrothion prior to the inhibition tests.

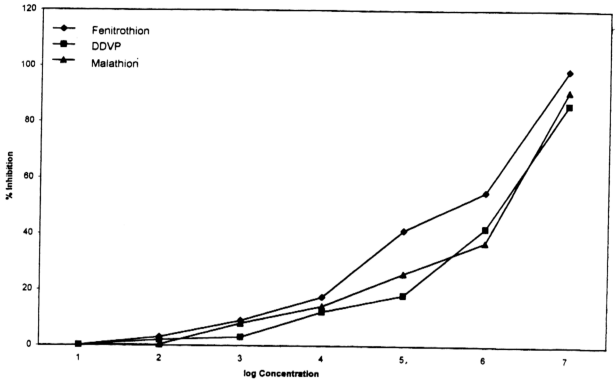


Fig 2.3 (j) Percentage inhibition of esterase of rat by different concentrations of insecticides. The rat has been exposed for 7 days to Fenitrothion prior to the inhibition tests.

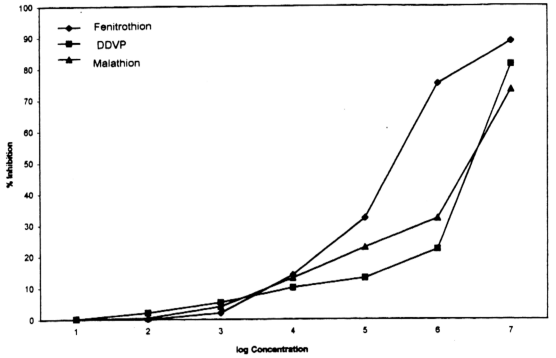


Fig 2.3 (k) Percentage inhibition of esterase of rat by different concentrations of insecticides. The rat has been exposed for 14 days to Fenitrothion prior to the inhibition tests.

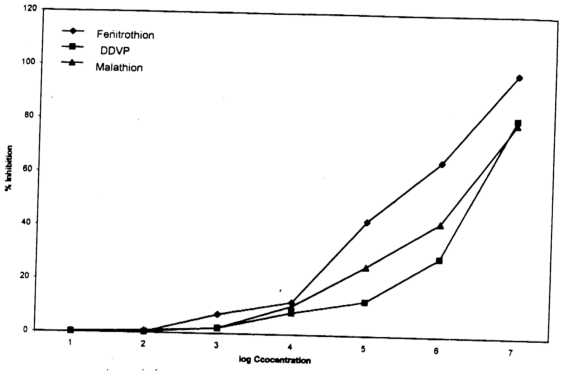


Fig 2.3(1) Percentage inhibition of esterase of rat by different concentrations of insecticides. The rat has been exposed for 21 days to Fenitrothion prior to the inhibition tests.

(i) Dichlorvos (DDVP) exposure

The blood was extracted after 1 , 3 , 7 , 14 and 21 days of exposure with DDVP and the inhibition of malathion , fenitrothion and DDVP on the esterase activities were tested . The I_{50} values obtained are shown in Table 2.3 and Fig 2.4 and the values were increased to 37 times as compared to day 1 exposure when tested with DDVP . Increase in I_{50} value was not obvious for the fenitrothion but it was distinct for malathion where it reaches 59 times after 21 days exposure . The I_{50} values for DDVP and malathion were dropped after 7 days exposure . But this did not happen when fenitrothion were used as the inhibitor .

(ii) Fenitrothion exposure

Rats were exposed to fenitrothion for 1 , 3 , 7 , 14 and 21 days . Then they were extracted and tested with malathion , fenitrothion and DDVP . Results obtained for I_{50} value are shown in Table 2.4 and Fig 2.4 . It was found that the I_{50} values increases with increasing time of exposure . It is clear that the I_{50} value for fenitrothion increased 23 times as compared with the day 1 exposure . The I_{50} values for DDVP and malathion also increases with increasing time of exposure but for malathion the I_{50} values was less than half when the rat exposed to DDVP.

Table 2. 3 I_{50} value of fenitrothion, malathion and DDVP after the rat has been exposed to DDVP at different period of time.

Insecticides	I_{50} value (m)									
	day 1	ratio	day 3	ratio	day 7	ratio	day 14	ratio	day 21	ratio
DDVP	5.18×10^{-5}	1	5.57×10^{-4}	10.75	2.02×10^{-4}	3.90	1.21×10^{-3}	23.36	1.93×10^{-3}	37.26
Fenitrothion	1.18×10^{-4}	1	2.8×10^{-4}	2.37	2.98×10^{-4}	2.53	3.42×10^{-4}	2.90	3.83×10^{-4}	3.25
Malathion	2.04×10^{-5}	1	2.14×10^{-4}	10.49	4.11×10^{-5}	2.01	3.07×10^{-4}	15.04	1.21×10^{-3}	59.31

Table 2.4 I_{50} value of fenitrothion, malathion and DDVP after the rat has been exposed to fenitrothion at different period of time.

Insecticides	I_{50} value (m)									
	day 1	ratio	day 3	ratio	day 7	ratio	day 14	ratio	day 21	ratio
DDVP	$1.12X10^{-4}$	5.11	$1.19X10^{-4}$	5.43	$3.03X10^{-4}$	4.33	$1.2\ X10^{-3}$	17.45	$4.25X10^{-3}$	60.81
Fenitrothion	$2.19X10^{-5}$	1	$4.18X\ 10^{-5}$	1.91	$2.05X10^{-4}$	2.93	$3.81X10^{-4}$	5.45	$5.11X10^{-4}$	7.31
Malathion	$5.59X10^{-5}$	2.55	$2.29X10^{-4}$	10.46	$6.99X10^{-5}$	1	$9.34X10^{-4}$	13.36	$1.32X10^{-3}$	18.81

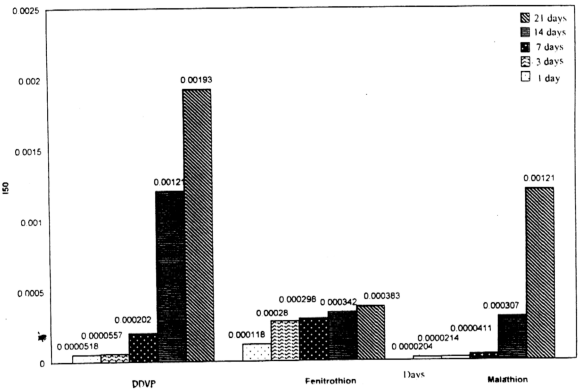


Fig (2.4 Graph for Is of DDVP, Fenitrothion and Malathion after the rat has been exposed to DDVP

(iii) Malathion exposure

In another experiments rat were exposed with malathion for 1 , 3 , 7 , 14 and 21 days . Their blood were extracted and tested with malathion , fenitrothion and DDVP . Increase in I_{50} values for DDVP and fenitrothion were not obvious but for malathion the values increased from 2.33 times , 80.09 times and 132.9 times after 1 , 3 , 7 , 14 and 21 days of exposure respectively (Table 2.5 and Fig 2.5) .

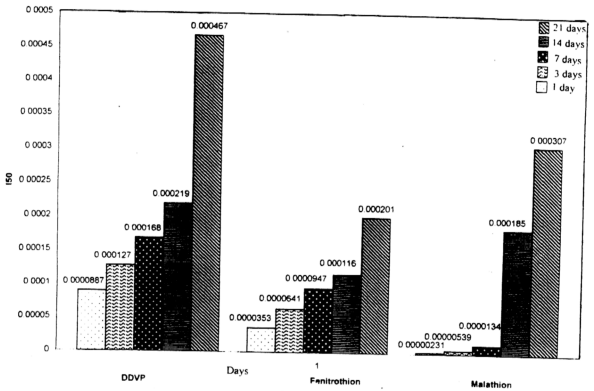


Fig (2.5) Graph for I_{50} of Malathion , Fenitrothion and DDVP , after the rat has been exposed to Malathion

Table 2.5 I_{50} value of fenitrothion, malathion and DDVP after the rat has been exposed malathion at different period of time.

Insecticides	I_{50} value (m)									
	day 1	ratio	day 3	ratio	day 7	ratio	day 14	ratio	day 21	ratio
DDVP	$8.87X10^{-5}$	1	$1.27X10^{-4}$	1.43	$1.68X10^{-4}$	1.89	$2.19X10^{-4}$	2.47	$4.67X10^{-4}$	5.26
Fenitrothion	$3.35 X10^{-4}$	5.23	$6.41X10^{-5}$	1	$9.47X10^{-5}$	1.48	$1.16 X10^{-4}$	1.81	$2.01X10^{-4}$	3.14
Malathion	$2.31 X 10^{-6}$	1	$5.39 X 10^{-6}$	2.33	$1.34 X 10^{-5}$	5.8	$1.85 X 10^{-4}$	80.09	$3.07 X 10^{-4}$	132.9

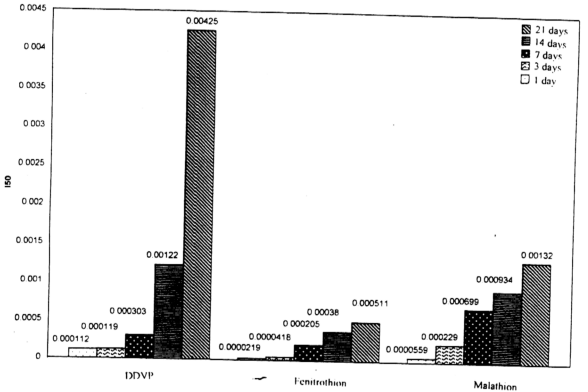


Fig (2.6) Graph for I50 of Fenitrothion, Malathion, and DDVP, after the rat has been exposed to Fenitrothion

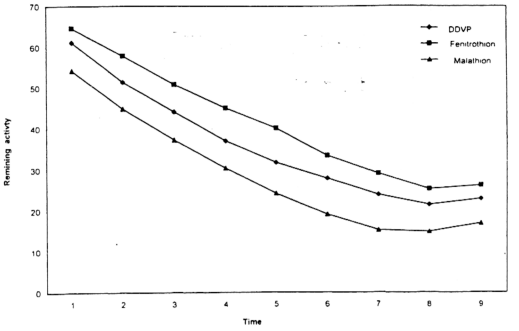


Fig 2.71(a) In vitro inhibition of esterase of rat by different concentrations of insecticides. The graph for K_i of DDVP and the rat has been exposed to DDVP after 1 day prior to inhibition tests.

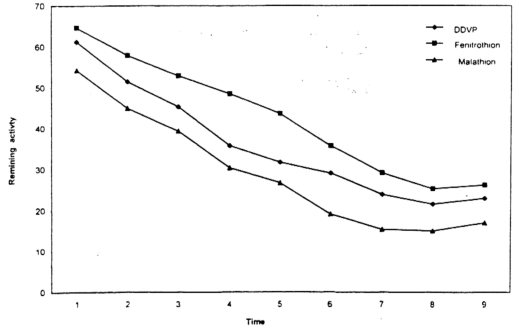


Fig 2.71(b) In vitro inhibition of esterase of rat by different concentrations of insecticides. The graph for K_i of DDVP and the rat has been exposed to DDVP after 3 days prior to inhibition tests.

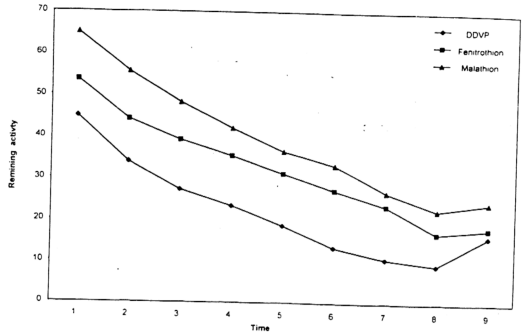


Fig 2.71(c) In vitro inhibition of esterase of rat by different concentrations of insecticides. The graph for K_i of DDVP and the rat has been exposed to DDVP after 7 days prior to inhibition tests.

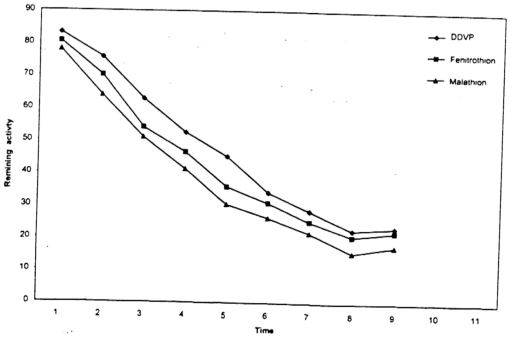


Fig 2.71(d) In vitro inhibition of esterase of rat by different concentrations of insecticides. The graph for K_i of DDVP and the rat has been exposed to DDVP after 14 days prior to inhibition tests.