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Studies on the acid phosphatase activity of the diamondback moth, *Plutella xylostella* (L) (Lepidoptera: Plutellidae)

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ABSTRACT Studies on the activity of acid phosphatase in *Plutella xylostella* were carried out using *p*-nitrophenyl phosphate as the substrate. The optimum pH was between 4.75 to 5 and enhancement in phosphatase activity was apparent up to 45°C. Attempts were made to study the effects of malathion, malaoxon, dichlorvos and *p*-hydroxymercuribenzoate on the acid phosphatase activity. Malaoxon and dichlorvos only produced slight inhibition towards acid phosphatase. The specific activity of this enzyme in subsequent developmental stages of the insect was investigated and compared with the changes in nonspecific esterase activity. Isoelectric focusing has also been carried out to separate the enzyme in both the sensitive (S) and resistant (R) strains of *P. xylostella*. Twelve zones for the S-strain and 11 zones for the R-strain were resolved over a pH range of 4.75 to 5.80.

ABSTRAK Kajian ke atas aktiviti asid fosfatase dalam *Plutella xylostella* telah dilakukan menggunakan *p*-nitrofenil fosfat sebagai substrat. pH optimum enzim ini di antara 4.75 hingga 5 dan peningkatan aktiviti adalah jelas sehingga suhu 45°C. Cubaan dibuat untuk mengkaji kesan malathion, malaoxon, dichlorvos (DDVP) dan *p*-hidroksimerkuribenzoat ke atas aktiviti fosfatase. Telah didapati bahawa malaoxon dan DDVP hanya menghasilkan sedikit perencatan kepada asid fosfatase. Aktiviti spesifik enzim ini dalam turutan peringkat perkembangan serangga telah dikaji dan dibandingkan dengan perubahan aktiviti esterase tak-khusus. Pemfokusan isoelektrik juga telah dilakukan untuk memisahkan enzim ini bagi kedua-dua strain *P. xylostella*. Dua belas zon bagi strain-S dan 11 zon bagi strain-R telah diperolehi pada julat pH 4.75 hingga 5.8.

(*P. xylostella*, acid phosphatase, inhibition, organophosphates, resistance)

INTRODUCTION

Most studies of acid phosphatase activity in insect tissues have used cytochemical evidence for lysosomal localization, that is with acid phosphatase as the lysosomal marker. Several investigators reported that the activity of acid phosphatase increases during the larval and pharate development of *Musca domestica* [1], *Stomoxys calcitrans* [2] and *Calliphora erythrocephala* [3]. Some workers reported that the acid phosphatase activity in the fat body decreased during larval development, for example in *Calliphora erythrocephala* [4]. De Loach and Mayer [5] believed that the activity of acid phosphatase might reflect the activity of lysosomes during metamorphosis.

Acid phosphatase is widely distributed in insects and exists in multiple forms known as isozymes. The enzyme is inhibited by compounds which inhibit high-molecular weight-acid phosphatase in eukaryotes, fluoride ions, diisopropyl fluorophosphate (DFP) and anions such as phosphate and molybdate [6,7]. However, very few attempts have been made to study the inhibition of the acid phosphatase activity by organophosphorus compounds, and there is little information concerning its role in metabolizing organophosphorus compounds, especially in insects.

Matsumura and Brown [8] found that on exposure to malathion, the larvae of the resistant strain of *Culex tarsalis*, contained one-third as much malaoxon as normal larvae. This is partly due to a higher phosphatase activity hydrolyzing malaoxon. Krueger and O'Brien [9] established that normal houseflies metabolized malathion mainly by phosphatase enzymes, and in cockroaches the carboxylesterase equalled the phosphatases as hydrolytic agents. According to Price [10], hydrolysis of malathion, for example at the P-S bond, can be oxidative, mediated by mixed-function oxidases or it can be hydrolytic by the action of phosphatases (phosphotriester hydrolases). Darrow and Plapp [11] reported that both the resistant- and susceptible-strains of *Culex tarsalis*, when exposed in water treated with radioactive malathion for 24 hours, converted 5 to 10% of malathion to water-soluble metabolites, of which two-thirds were carboxylesterase products, and one-third phosphatase products.

Against this background, it is possible that the resistance of *Plutella* towards organophosphorus compounds might be partly due to the activity of acid phosphatase. The present study deals with the inhibition of this enzyme by organophosphorus compounds, the levels of activity of acid phosphatase during the successive developmental stages and between the susceptible and resistant strains of *P. xylostella*, and characterization of the acid phosphatase isozymes.

MATERIALS AND METHODS

Insects

Two strains - susceptible (S) and resistant (R) – of the diamondback moth, *Plutella xylostella* L. were used [12]. Both strains were maintained separately at 25±2°C and 60-80% R.H. The adults were provided with drops of Holloway medium applied on cellophane paper [13]. The larvae were fed daily with fresh *Brassica chinensis* or cabbage leaves.

Preparation of enzyme solution

All operations including centrifugations were carried out at 0 to 4°C. Fifth instar larvae were homogenized in 0.1 M sodium acetate buffer, pH 5 using a hand driven all-glass homogenizer. Triton X-100 (0.1% v/v) was added to the crude homogenate to determine the effect of detergent on enzyme distribution. The homogenate was centrifuged at 1200 g in an MSE, High Speed 18 Centrifuge for 20 minutes. The supernatant was used directly for the acid phosphatase assay.

Acid phosphatase activity

Acid phosphatase activity was measured using *p*-nitrophenyl phosphate (pNPP) as the substrate. The standard assay consisted of 0.1 mL of 3 mM pNPP in 0.1 M sodium acetate buffer, pH 5. One mL of enzyme solution was added to start the reaction and the reaction was stopped by the addition of 1 mL 0.5 M sodium hydroxide after 20 min incubation at 37°C. For inhibition studies, inhibitor or insecticide solution was added instead of sodium phosphate buffer. *p*-Hydroxymercuribenzoate (pHMB) was prepared by dilution with 0.04 M phosphate buffer to give the required concentrations. In the case of malathion, malaoxon and dichlorvos (DDVP), 2% of Triton x-100 were added to obtain a homogeneous emulsion or alternatively, they were dissolved in phosphate buffer solution containing 1% acetone. Assays were performed in triplicates each with a final volume of 3 mL. The constituents were brought to 37°C prior to determination. The yellow colour, due to *p*-nitrophenol being liberated enzymatically, was measured spectrophotometrically at 410 nm. A control sample was incubated similarly, but 1 mL 0.5 M NaOH was added at the beginning of the assay. The control was used to correct for background absorbance.

An extintion coefficient (ϵ) of 18,300 M^{-1} cm^{-1} was used to convert the change in absorbance to mmole [6]. A unit of acid phosphatase activity was defined as mmole of substrate hydrolyzed per hour (units) while the specific activity was expressed as units per milligram protein.

Protein was measured by the method of Lowry *et al.* [14], utilizing bovine serum albumin (BSA) to establish a standard curve. Studies involving inhibitors were carried out using the standard assays with slight modifications.

Electrofocusing procedure

Isoelectric focusing was accomplished in LKB Ampholine PAG plates (LKB 1804-101, pH 4-6.5) using LKB 2117 Multiphor. The procedures used were as described and recommended by LKB. Sample solution (25 µL) was impregnated into a small piece of filter paper which was then laid directly on the surface of the gel. The anode electrode solution was 0.1 M glutamic acid in 0.5 M H_3PO_4, while the cathode electrode solution was 0.1 M β-alanine. A constant voltage source (LKB 2103 Power Supply) was employed. Isoelectric focusing was performed at 4°C by means of a MultiTemp Thermostatic Circulator and the experiments were continued until a steady current was reached. The protein content, ranging from 0.2 mg to 0.6 mg, was used and assayed by the method of Lowry *et al.* [14].

Staining procedure

Acid phosphatase activity was detected by incubating the gel in 0.05 M sodium acetate buffer adjusted to pH 5 at 37°C, containing 0.1% (w/v) α-naphthyl acid phosphate and 0.1% (w/v) Black K salt. The incubation time varied from 15-30 min and was continued until the phosphatase activity appeared as brown bands against an orange background. The stained gels were fixed in 7% acetic acid.

To determine the actual pH gradient, gels were cut into 12 equal sections of 10 mm with a clean razor blade and equilibrated for 24 hours in 2 mL of distilled water. Then the volume was increased to 5 mL with distilled water and the pH was measured [15].

RESULTS

The activity of acid phosphatase of the 5th instar larvae of *P. xylostella* increased linearly with time for the first 20 minutes of incubation at 37°C, but declined after that period (Fig. 1). The R-strain, however, had lower activity compared to the S-strain.

The enzyme activity rose rapidly with increasing

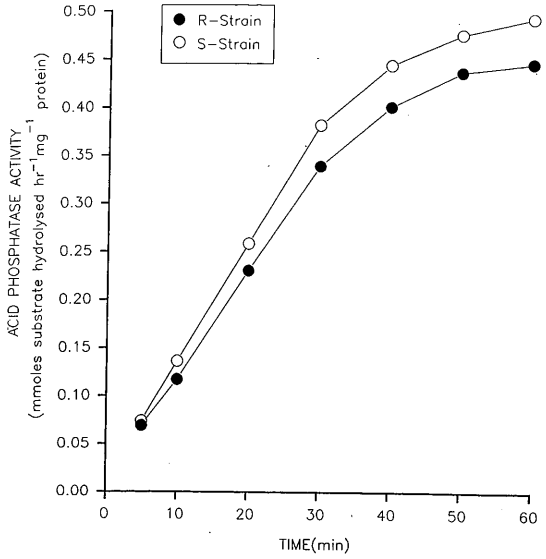

Figure 1. The influence of reaction time on the acid phosphatase activity of 5th instar larvae of *Plutella xylostella* at 37°C and pH 5. Substrate: 0.003 M *p*-nitrophenyl phosphate (pNPP); buffer: 0.1 M sodium acetate buffer.

substrate concentrations of up to 0.25 mM of pNPP after which the rate of activity decreased and leveled off at about 2.8 mM substrate (Fig. 2a).

The K*m* (Michaelis constant) values with pNPP as substrate, as determined by means of the double-reciprocal plot, were 0.188 mM for the S-strain and 0.165 mM for the R-strain (Fig. 2b). The V*max* (maximum reaction velocity) values were 0.58 mmoles hr^{1} mg^{-1} protein for the S-strain and 0.51 mmole hr^{1} mg^{-1} protein for the R-strain.

Optimum pH

The enzyme activity for both the S- and R-strain of *P. xylosrella* was maximal at pH 4.75 to 5.0 (Fig. 3).

Effects of temperature

The ability of the acid phosphatase enzyme to hydrolyze pNPP was studied over a temperature range of 2°C to 66°C (Fig. 4). The enzyme showed maximum activity at 45°C and declined in activity beyond 45°C.

Effects of inhibitors

The effects of malathion and malaoxon on the enzyme activity of acid phosphatase are summarized in Table 1. At concentration of 0.01 M, malathion produced 19.11% inhibition in the S-strain and 15.96% in the R-strain. Malaoxon inhibited very little acid phosphatase activity. PHMB, a sulphydryl inhibitor of arylesterase (ArylE), produced an inhibitory effect on the acid phosphatase activity. The calculated IC_{25} values of PHMB were 1.3 mM for the S-strain and 1.9 mM for the R-strain. Dichlorvos did not inhibit any acid phosphatase activity.

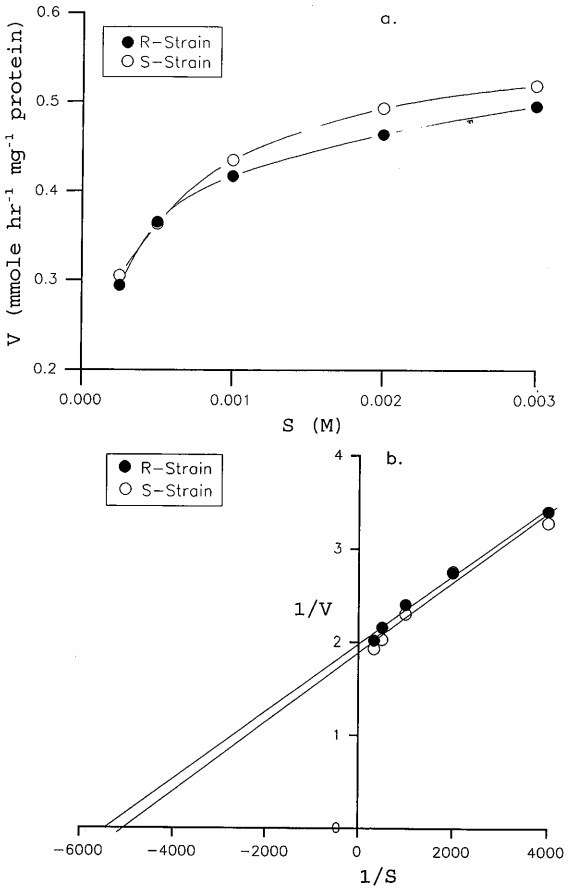

Figure 2. (a) The influence of substrate (S) concentrations on the acid phosphatase activity (V) of S- and R-strains of *P. xylostella*, and (b) the Lineweaver-Burk plot of 1/V versus 1/S. Substrate: 0.003 M pNPP; buffer: 0.1 M sodium acetate buffer.

The inhibitory effect of malathion on acid phosphatase activity in the absence of a substrate is illustrated in Fig. 5. The log percentage of residual activity decreased within 0 to 30 min incubation time of acid phosphatase with 0.04 M malathion. After 30 min of incubation, the acid phosphatase activity recovered slowly to normal. The R-strain recovered within 30 to 60 min whereas the S-strain took longer i.e. up to 150 minutes of incubation.

Developmental changes in acid phosphatase activity

Figure 6 shows the variation in acid phosphatase activity per mg protein in various developmental stages of *P.*

xylostella. The level of acid phosphatase activity was quite high during the earlier instar larvae, decreased as the larvae developed, and then increased again during the late pupal stage. It was highest in the adult. The S-strain consistently showed a higher acid phosphatase activity throughout their developmental stages compared with the R-strain.

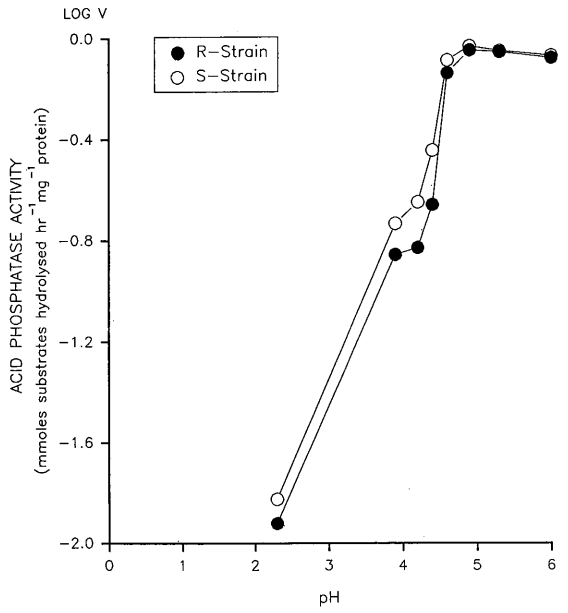

Figure 3. The effect of pH on the activity of acid phosphatase from the S- and R-strains of *P. xylostella*. Buffer : 0.1M sodium acetate buffer; substrate: 0.003 M pNPP; temperature: 37 °C.

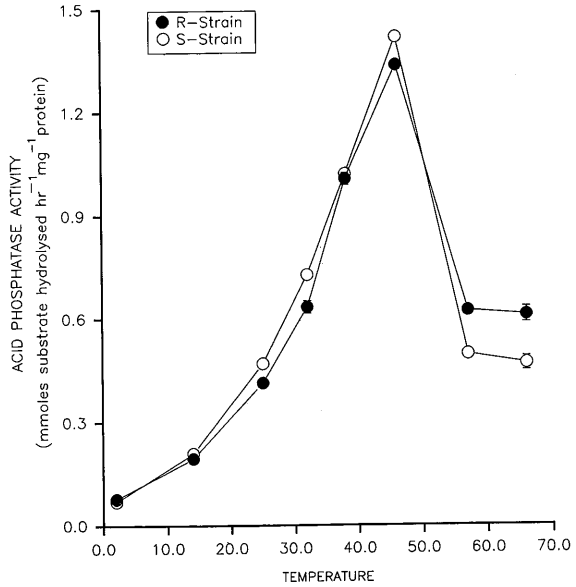

Figure 4. The effect of reaction temperature on the acid phosphatase activity of the S- and R-strains of *P. xylostella*. Enzyme activity was determined with 0.003 M pNPP, 0.1 M acetate buffer pH 5 for 20 min incubation.

Isoenzyme patterns

The acid phosphatase isozyme patterns in the S- and the R-strains of *P. xylostella* are illustrated in Fig. 7. The pI of each band of acid phosphatase is given in Table 2. There were 12 zones in the S-strain and 11 in the R-strain. Compared with the R-strain, the S-strain lacked bands P11 and P12; on the other hand the P4, P7, and P9 bands were absent in the R-strain.

DISCUSSION

The homogenates of *Plutella xylostella* larvae contain a relatively active acid phosphatase. Although *p*-nitrophenyl phosphate is not the normal substrate of acid phosphatase, the fact that it exhibits a low K*m* value and a high V*max* value indicates that it is an excellent substrate to estimate the activity of this enzyme. The S-strain showed higher activity of acid phosphatase compared to the R-strain. Matsumura and Brown [8] also observed that the S-strain of *Culex tarsalis* showed higher acid phosphatase activity than the resistant mosquito.

The release of *p*-nitrophenyl was linearly related to the substrate concentration. The enzyme acid phosphatase was temperature dependent and showed an optimum activity at about 45°C. In *Drosophila virilis*, the optimum temperature for this enzyme activity was

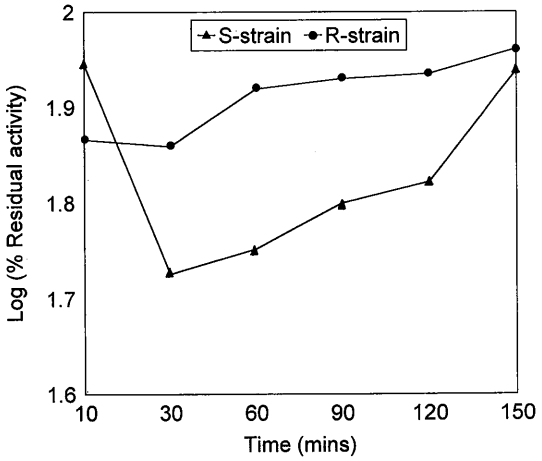

Figure 5. *In vitro* inhibition of the acid phosphatase activity in the S- and R-strains of *P. xylostella* by 0.04 M malathion in the absence of substrate.

Table 1. The influence of various inhibitors on the acid phosphatase activity in the S- and R-strains of *P. xylostella*. Substrate: 3 x 10^{-3} M pNPP; buffer: 0.1 M acetate buffer pH 5; temperature: 37°C.

Inhibitor	Percentage Inhibitors pI (-log molar concentration)					
	7	6	5	4	3	2
PHMB						
S-strain	ND	3.48	14.83	16.90	23.95	-
R-strain	1.59	2.89	14.20	18.09	22.72	-
DDVP						
S-strain	-	-	-	-	ND	-
R-strain	-	-	-	-	ND	-
Malathion						
S-strain	ND	ND	ND	1.74	-	19.11
R-strain	ND	ND	ND	1.62	-	15.96
Malaoxon						
S-strain	-	-	-	-	ND	1.81
R-strain	-	-	-	-	ND	1.55

ND - not detected

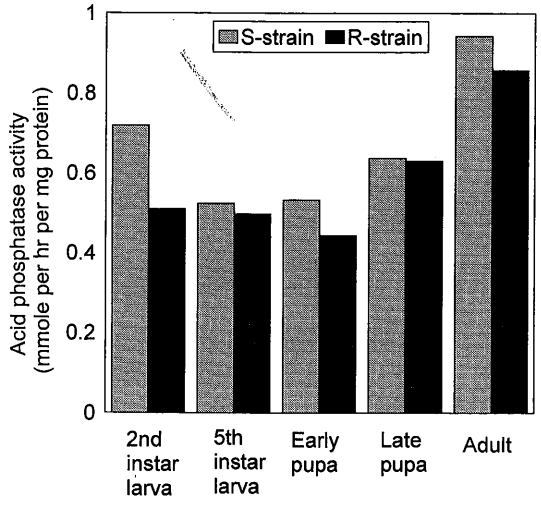

Figure 6. Acid phosphatase activity during the successive developmental stages of the S- and R-strains of *P. xylostella*. Substrate: 0.003 M pNPP; buffer: 0.1 M acetate buffer pH 5; temperature: 37 °C; incubation time: 20 min.

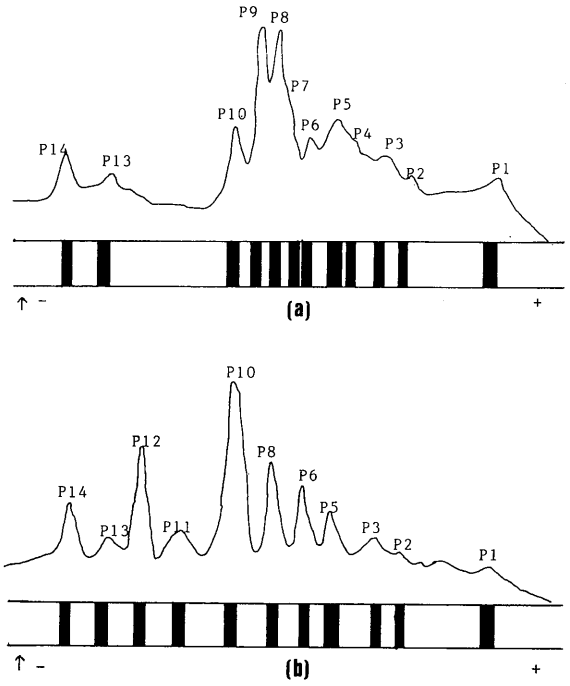

Figure 7. Zymogram and densitometric scan of whole larval acid phosphatase of (a) the S-strain, and (b) the R-strain. Arrow indicates the origin of the sample.

Table 2. The acid phosphatase bands or zones and corresponding pH (pI) for the S- and R-strains of *P. xylostella*.

Band (acid phosphatase)		pH
S-strain	R-strain	
P1	P1	4.75
P2	P2	5.07
P3	P3	5.12
P4	-	5.20
P5	P5	5.23
P6	P6	5.26
P7	-	5.30
P8	P8	5.35
P9	-	5.38
P10	P10	5.40
-	P11	5.55
-	P12	5.65
P13	P13	5.70
P14	P14	5.80

53°C and it was relatively heat stable [7]. In some other insects the optimum temperature was lower than 45°C. In the giant silkmoth, for example, it was found to be 38°C [16].

The enzyme activity was maximally active at pH 4.85. This optimal pH is very similar to that reported for *Drosophila virilis* (pH 4.75) [7], but a higher pH optimum was reported for the giant silkmoth i.e. pH 5.6 [16]. On the other hand, in *Drosophila melanogaster*, the optimum pH is relatively low, that is pH 4, [6].

The acid phosphatase was inhibited by PHMB and malathion, only slightly by malaoxon (10^{-5} M) and almost uninhibited by DDVP. The PHMB (10^{-3} M) which is a sulfhydryl inhibitor of arylesterase, produced approximately 23% inhibition on the acid phosphatase activity of both the strains of *Plutella*. Dinan *et al.* [6], reported that the sulfhydryl reagents did not affect the acid phosphatase activity and Narise [7] showed that *p*-chloro-mercuribenzoate produced 3.9% inhibition at 0.1 mM concentration towards the acid phosphatase activity. There seem to be a difference between the acid phosphatase from *Plutella* and *Drosophila*, eventhough both insects have enzymes with a similar optimum pH. However, the optimum temperature for their acid phosphatase activity was different (*Plutella* at 45°C and *Drosophila* at 53°C).

The present results show that malathion inhibited the acid phosphatase activity in both strains of *Plutella*, with the R-strain being slightly less susceptible than the S-strain. Malaoxon, at 10^{-5} M inhibited approximately 1.5% of acid phosphatase activity in both the strains. The fact that both strains of *Plutella* recovered well after the inhibition by malathion (Fig. 5) indicates that malathion was probably degraded by an acid phosphatase which attacked the P-S-C bonds [8,9,17]. According to Matsumura and Brown [8], when the larvae of either strain were exposed to equal concentration of malathion, the normal strain contained about two-thirds as much malaoxon as the resistant strain. This variance was thought to be caused by the greater enzyme activity of hydrolyzing malaoxon. When the larvae were exposed to malaoxon, the malaoxon was eliminated by the resistant strain but not by the susceptible strain. The rate of degradation of malathion is probably faster in the R-strain of *Plutella* since this strain showed a faster rate of recovery (Fig. 4) compared to the S-strain.

The acid phosphatase activity in the whole body of *Plutella* decreased throughout the larval stages, then slowly increased during the late pupal stages reaching a maximum in the adult stage. This result is in agreement with that obtained by Price [4], in which the acid phosphatase activity in *Calliphora* fat body was maximal from 5-day larvae thereafter falling slightly over the remainder of the instar. However, several investigators have reported that the opposite effect can occur. In other insects the acid phosphatase activity increases steadily during the larval stages such as in *Musca domestica* [1], *Drosophila melanogaster* [18], *Stomoxys calcitrans* [19] and *Calliphora erythrocephala* [20]. Price [4] suggested that the decrease in acid phosphatase activity of the fat body of *Calliphora* was due to the synthesis of this enzyme during the early period of the third instar which was the period of maximum overall protein synthesis by the fat body. On the contrary, Pant and Lacy [21] suggested that the high level of acid phosphatase activity during pupal development could be related to an active glycogen mobilization with dephosphorylation at the acid side of the pH range.

With regards to the acid phosphatase activity, the S-strain showed higher activity than the R-strain in which 12 zones of acid phosphatase in the S-strain and 11 zones in the R-strain have been resolved.

In summary, acid phosphatase does not appear to play a role in the resistance mechanism towards malaoxon and DDVP, as these inhibitors produce almost no inhibitory effect on this enzyme. This indicates that both malaoxon and DDVP are not the substrates for acid phosphatase. On the other hand, acid phosphatase plays a role in the metabolism of malathion. Acid phosphatase recovered well after 140 minutes inhibition by malathion. This means that malathion is a substrate for acid phosphatase. Dauterman [22] believed that the metabolism of paraoxon by the R-strain flies was due to the activity of acid phosphatase. However, previous attempts by Welling *et al.* [23] failed to demonstrate the presence of a phosphatase that was capable of degrading parathion. The contribution of acid phosphatase to organophosphates resistance was therefore of minor importance because the level of acid phosphatase was not proportional to the level of resistance.

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