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DDT-DEHYDROCHLORINASE IN THE MEXICAN BEAN BEETLE, *EPILACHNA VARIVESTIS* MULS.*

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Abstract—The DDT-dehydrochlorinase level throughout various stages of the beetle was determined and found to increase in proportion to the body weight except in the pupal stage. Here a 35 per cent reduction in enzyme activity was observed; however, following adult feeding the enzyme returned to its previous level. Log dosage–probit lines for topical applications of TDE were determined with adult beetles at various ages. The LD_{50}s, expressed in μg TDE per mg dry wt., showed a close correlation with the enzyme level, expressed in μg TDE dehydrochlorinated per mg dry wt., except for the 3 days following eclosion. The adult tissues were dissected and assayed for enzyme activity. The reproductive organs possess 2·5 times the activity observed in the second most active tissue, the alimentary canal. The other tissues in order of decreasing enzyme activity were: exoskeleton, flight muscles, central nervous system, and fat bodies.

INTRODUCTION

STERNBURG *et al.* (1954) were the first to demonstrate the relationship between insecticide resistance and enzyme activity. The enzyme located in houseflies was designated DDT-dehydrochlorinase (DDT-ase). MOOREFIELD and KEARNS (1957) determined the level of this enzyme throughout the life cycle of the housefly and found it to increase in the larval stages, decline 50 per cent at pupation, and then remain at this level throughout the pupal and adult periods. They concluded that this reduction in enzyme titre at pupation was probably due to the loss of DDT-dehydrochlorination sites in the organized degeneration of tissues characteristic of metamorphosis.

MIYAKE *et al.* (1957) determined the sites of enzyme activity within the female housefly and found that the fat body and brain contained high titres of enzyme. Cuticle, muscle, and haemolymph showed intermediate levels, whereas the ovary and gut contained little or no DDT-ase. From these findings MIYAKE *et al.* postulated that, because of the apparent high level of enzyme in the fat bodies, this organ must play a major role in detoxification.

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Following the initial work on houseflies, SWIFT (1958) and CHATTORAJ and KEARNS (1958) demonstrated that naturally resistant Mexican bean beetle pupae and larvae possessed a comparable enzyme which could detoxify DDT and certain DDT analogues (i.e. TDE and methoxychlor) to their dehydrochlorinated products. The desire to study further this enzyme within the beetle stimulated this investigation. The objectives were to determine if any correlation existed between the enzyme level of various stages of the insect and the LD_{50} of TDE and to determine the relative concentration of DDT-ase of various tissues in the adult beetle.

MATERIALS AND METHODS

Enzyme preparation and measurement

The colony of Mexican bean beetles which furnished all experimental insects was maintained under greenhouse conditions. Acetone powders were prepared by homogenizing the sample for 3 min in 10 vol of cold (−15°C) acetone. The slurry, mixed with an additional 10 vol of acetone, was filtered in a cold Buchner funnel. The filter cake, washed with 3 vol of acetone, was then dried in a vacuum desiccator. The powder was immediately extracted with 10 vol of cold glass-distilled water for 3 hr at 2°C. The extract was centrifuged at 2°C for 15 min at 12,800 *g* and the supernatant immediately assayed for enzyme activity.

The enzyme reaction was conducted in single arm Warburg vessels containing 600 mg of glass beads (15–60 μ in diameter) on which was crystallized 3 mg of TDE, the preferred substrate of the Mexican bean beetle DDT-ase (SWIFT, 1958). 3 mg of reduced glutathione, 1·6 mg of cysteinylglycine, and 1 mg of disodium ethylenediaminetetracetate were added to the vessel's sidearm in 0·5 ml of glass-distilled water. This was followed by the addition of 0·5 ml of 0·09 M sodium bicarbonate and 2 ml of the enzyme solution to the main compartment. The vessels were then attached to Warburg manometers, placed in a 37°C water bath and gassed for 10 min with 95% N_2–5% CO_2 while being shaken at 120 oscillations per minute. The stopcocks and vents were closed and the sidearm contents tipped into the main compartment. This was recorded as zero time and reaction progressed for either 2 or 4 hr.

Following each reaction quantitative determinations of the substrate, TDE, and its product, dehydrochlorinated TDE, were conducted by the addition of 3 ml of concentrated sulphuric acid to each vessel and extraction with 6 ml of cyclohexane. After 2 hr of continuous shaking an aliquot of 1 ml from each flask was diluted to 25 ml with cyclohexane so that the concentration of the two components fell within the range of standard curves previously prepared. Ultraviolet absorbance at 233 and 260 mμ was measured with a Beckman DU spectrophotometer. Blanks, used for comparison, were prepared similarly by the extraction of the contents of a flask which contained no substrate.

Determination of the ultraviolet absorption spectra of pure *p,p'*-TDE and *p,p'*-dehydrochlorinated TDE dissolved in cyclohexane plus the measurement of

the optical densities of known concentrations of the two compounds enabled the calculation of the amounts of either compound in a two-component system.

Dry weight determination

Determination was made of the dry weight for various stages of the life cycle and also for samples of adult tissue. The material was placed in a previously weighed test tube and retained for 8 hr at 80°C, sufficient to produce a constant tissue weight. Immediately after drying, the weight of the tube was again determined, thus giving the weight of the dried sample.

Aminoid nitrogen of acetone powder

Nitrogen analyses were made on weighed portions of acetone powders which had been assayed for enzyme activity. Each determination was replicated three times, using 3–5 mg of powder for each analysis. The samples were kept in sealed containers under a nitrogen atmosphere at −15°C. The aminoid nitrogen was determined using the micro-Kjeldahl apparatus of STEYERMARK *et al.* (1951) and the methods of NIEDERL and NIEDERL (1942).

LD_{50} of TDE

An apparatus designed to deliver $1{\cdot}00 \pm 0{\cdot}02$ μl was used in applying topically to the beetles known volumes of insecticide solution. The bean beetles possess such great tolerance to the applied TDE that a synergist, DMC (4,4′-dichloro-α-methyl benzhydrol), which possessed no insecticidal activity itself, had to be incorporated to obtain mortality. The two compounds were mixed at a 1 : 1 ratio and all concentrations from 0·005 to 200 mg per ml represent only the concentration of TDE.

The area of insecticidal application was the ventral surface of the thorax, between the coxae of the prothoracic legs. Concentrations of sufficient range to give from 0 to 100 per cent mortality were tested against three replicates of ten insects each. After insecticidal application the insects were retained at room temperature in Petri dishes for sufficient time to obtain 24, 48, and 72 hr mortality counts. All mortality results reported are based, however, on the 72 hr counts.

Tissue dissection

Three replicates of 150 unsexed adults each, between 2 and 4 weeks old, were dissected to furnish sufficient tissue for enzyme determination. The tissues assayed were the entire alimentary canal, reproductive organs, a portion of abdominal body fat, thoracic flight muscles, brain and ventral nerve cord, and the ventral abdominal sclerites of the exoskeleton. These were dissected under saline (STERNBURG *et al.*, 1959) and frozen almost immediately. They were retained in this condition until all 150 insects had been processed, usually within 72 hr after the start of the dissection. The tissues were then assayed for the enzyme in the manner previously described.

RESULTS

Enzyme level and dry weight values for various stages

The average results obtained for the determination of the enzyme level in various stages of the beetle are presented in Fig. 1. The data are plotted as micrograms of product (dehydrochlorinated TDE) formed per insect since a known

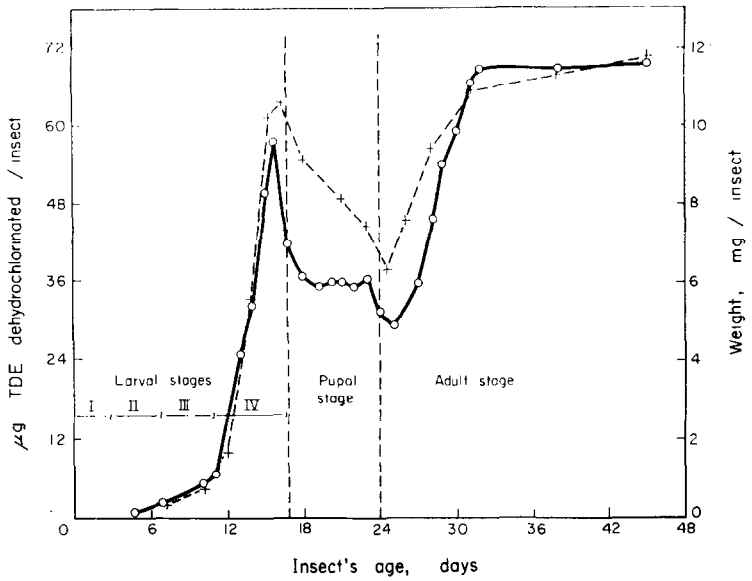

FIG. 1. Level of DDT-dehydrochlorinase and dry weight values throughout various stages of the Mexican bean beetle. μg TDE (O——O); mg weight (+ - - - +).

number of insects, usually 200, was used for each analysis. The dry weight values for similar periods in the life cycle of the beetle are also shown. This figure clearly illustrates that the enzyme level, as well as the dry weight, increases markedly in the fourth instar. Both reach a maximum approximately 16 hr prior to the insect's attachment as a pupa to the leaf surface.

The pupal period, divided into successive stages based on pigmentation, experiences an early, rapid decline in enzyme concentration but soon levels off, whereas the loss in weight over the same period is more gradual and proportionally not as great. Soon after feeding is initiated in the newly emerged adult, both the enzyme and dry weight curves increase gradually over the next 6–7 days after which they remain relatively constant.

To determine if the enzyme concentration varies directly with the body weight, a plot of enzyme activity per unit of dry weight was made (Fig. 2). The values are for alternating days beginning with the sixth following hatching and continuing to the fourteenth day of the adult stage. There appears to be, throughout the insect's life cycle, a constant ratio of enzyme to dry weight, except for a reduction of one-third from the eighteenth to the twenty-sixth day.

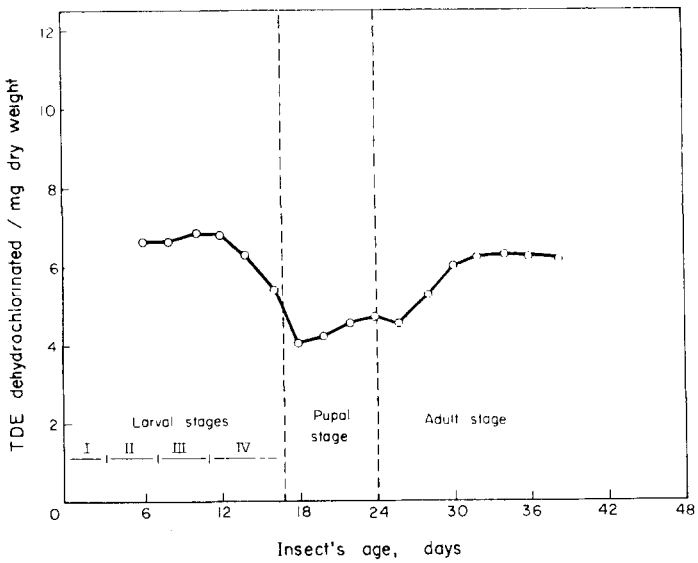

FIG. 2. Ratio of micrograms of TDE dehydrochlorinated per milligram of dry weight for various stages of the Mexican bean beetle.

LD_{50} levels of TDE for the adult Mexican bean beetle

Topical application of 1 μl per insect of an insecticide solution of known concentration was the method employed for these determinations. The LD_{50}s of TDE, both as μg TDE per insect and per mg dry wt., for the different-aged adults are shown in Table 1. A column showing the amount of TDE dehydrochlorinated per mg dry wt. for each age (from data in Fig. 2) is presented for rapid comparison.

TABLE 1—LD_{50} VALUES OF TDE FOR THE MEXICAN BEAN BEETLE LARVAE AND ADULTS

Age of adults (days)	LD_{50} of TDE		TDE dehydrochlorinated per mg dry wt. (μg)
	per insect (μg)	per mg dry wt. (μg)	
0·5	2·7	0·42	4·65
1·5	1·6	0·22	4·58
3·0	6·4	0·76	4·90
4·0	18·9	2·01	5·29
5·0	23·5	2·36	5·65
6·0	29·4	2·80	6·00
7·0	30·8	2·81	6·10
14·0	26·7	2·37	6·16
28·0	22·3	2·16	6·15

Aminoid nitrogen determination

The per cent protein of the various stages of the beetles was determined by nitrogen analyses of acetone powders. The values as shown in Table 2 illustrate that there is a slightly higher range in the pupal and adult stages than in the larval periods.

TABLE 2—AMINO NITROGEN AND EQUIVALENT PROTEIN CONTENT OF THE MEXICAN BEAN BEETLE

Age of insect (days)	Stage of development	Average amino nitrogen (%)	Equivalent protein (%)
6	Larval	8·9	55·6
8		8·8	55·0
10		8·3 ± 0·2	51·9
12		8·1 ± 0·5	50·1
14		8·8 ± 0·6	55·0
16	Pupal	9·2 ± 0·9	57·5
18		9·3 ± 0·7	58·1
20		9·7 ± 0·5	60·6
22		9·8 ± 0·6	61·3
24		10·5 ± 0·8	65·6
26	Adult	10·6 ± 0·3	66·3
28		9·7 ± 0·4	60·6
30		10·1 ± 0·4	63·1
34		10·6 ± 0·6	66·3
38		11·1 ± 1·0	69·4

TABLE 3—AVERAGE VALUES FOR TDE DEHYDROCHLORINATED, DRY WEIGHT, AND RATIO OF TDE DEHYDROCHLORINATED PER MILLIGRAM OF DRY WEIGHT FOR THE SIX BODY TISSUES OF THE ADULT MEXICAN BEAN BEETLE

Tissue sample	TDE dehydrochlorinated per insect (μg)	Dry weight per insect (μg)	TDE dehydrochlorinated per mg dry wt. (μg)
Alimentary canal	6·68 ± 2·40	1·12 ± 0·11	6·0
Reproductive organs	46·30 ± 6·40	2·10 ± 0·05	22·0
Flight muscles	4·80 ± 1·00	2·77 ± 0·52	1·7
Fat bodies	0·14 ± 0·06	0·50 ± 0·10	0·3
Central nervous system	0·39 ± 0·11	0·35 ± 0·00	1·1
Exoskeleton	3·30 ± 1·10	0·74 ± 0·30	4·5

Enzyme activity in tissues of the adult Mexican bean beetle

The analyses of six tissues of the adult beetle, which entailed careful dissection, were conducted to determine the possible sites of enzyme location. The data resulting from these tests, including the tissue dry weight values and a ratio of TDE dehydrochlorinated per mg dry wt., are shown in Table 3. The reproductive organs possessed the greatest amount of enzyme, the activity being more than 3·5 times that of the alimentary canal, which was the second most active tissue.

DISCUSSION

The DDT-ase activity (μg TDE dehydrochlorinated/mg dry wt.) of the Mexican bean beetle was found to be relatively constant over most of the larval and adult stages. There was a 35 per cent decrease in enzyme activity when the insects entered the pupal period and a similar increase early in the adult period. Thus, during the transformation from the larva to the adult, the level of detectable enzyme underwent a definite change.

Since analyses show the presence of enzyme in various tissues, and it is true that tissues undergo reorganization during pupation, it is a good assumption that the enzyme is lost in this process. A logical conclusion, and one proposed by MOOREFIELD and KEARNS (1957), is that the enzyme is hydrolysed by proteolytic enzymes. Also, the rate of enzyme synthesis is probably concurrently reduced, returning to the normal level only following adult feeding. The question of whether the enzyme is indiscriminately utilized, as are cell membranes, or whether the enzyme is a labile protein and is preferentially acted upon is debatable.

A correlation was anticipated, and found to be present, between the tolerance to TDE and the *in vitro* TDE dehydrochlorinating ability of the adult beetle (Table 1). During the first 7 days of adulthood, the LD_{50}s increase from 0·42 to 2·81 μg/mg and there is a corresponding change in the enzyme activity over the same period. The ratio of LD_{50} to enzyme activity is not constant, however. During the first 3 days the LD_{50} value is less than for older adults, but this may be due to the fact that the exoskeleton is more permeable, thus permitting the absorption of a greater percentage of the applied dose (RICHARDS, 1953).

Analyses of the specific adult tissues for enzyme activity revealed that the enzyme is widely distributed, being present in all tissues examined. The site of the greatest concentration is the reproductive organs, followed by the alimentary canal. The low concentration of enzyme within the fat bodies and central nervous system of the bean beetle may seem surprising, since MIYAKE *et al.* (1957) found these two organs to be the predominant sites of the enzyme in resistant female houseflies. They also reported that the reproductive organs contained the lowest level of all tissues investigated. Thus, the distribution of enzyme among the tissues differs markedly between the female housefly and the Mexican bean beetle. There is, however, no reason to expect the enzyme distribution to be identical in the two species; one insect is naturally tolerant to DDT whereas the other has become resistant through DDT selection pressure.

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