EFFECT OF TWO BIORATIONAL INSECTICIDES, EMAMECTIN BENZOATE AND SPINOSAD, ON ACETYLCHOLINESTERASE AND ESTERASE ACTIVITY AND KINETICS IN THE LARVAL HEADS OF THE COTTON LEAFWORM, *SPODOPTERA LITTORALIS* (BOISD.) (LEPIDOPTERA: NOCTUIDAE)

MOHAMED A. EL-AW

Pest Control and Environmental Protection Department, Faculty of Agriculture (Damanhour), Alexandria University, Egypt

ABSTRACT

The effect of two biorational insecticides, Emamectin benzoate (Proclaim 5% SG) and Spinosad (Tracer 24% SG), on acetylcholinesterase (AChE) and esterase activity and kinetics was evaluated in heads of the sixth larval instar of a laboratory susceptible strain of the cotton leafworm, *Spodoptera littoralis*.

The specific activities of Total-esterases, Cholinesterase (ChE), and Aliesterase (Ali-E) activities in *S. littoralis* larval head (brain) homogenates of untreated larvae (UT-L), Proclaim treated larvae (PT-L), and Spinosad treated larvae (ST-L) of *S. littoralis* were determined. Total-E and ChE reached their peak activity at hr 20 (12 hr post-treatment) in UT-L and at hr 24 (16 hr post-treatment) in both PT-L, and ST-L. Ali-E activity levels exhibited no clear peak. Total-E and ChE activity levels in the larval head homogenates of PT-L and ST-L were significantly higher than that of UT-L. Also, ChE activity levels were clearly higher than Ali-E levels either in UT-L or in both PT-L, and ST-L. However, Spinosad and proclaim increased the ChE activity levels in the *S. littoralis* larval head homogenates while such two insecticides did not significantly affect the activity levels of Ali-E compared with that of untreated larvae.

ChE Km values in UT-L were constant and recorded 1.33 either after 12 hr or 24 hr post-treatment compared with 1.41 at 0 time (hr 8, light on). Such ChE Km values were 1.82 in PT-L either after 12 or 24 hrs. ChE Km values in ST-L were 1.21 and 0.57 after 12 and 24 hr post-treated, respectively, compared with 1.43 at 0 time. Ali-E Km values in PT-L exhibited variability between the tested times indicating photo-and scotophase response. The kinetic criteria indicated that ChE is the predominant esterase in the heads. ChE Vmax values were generally higher than those of Ali-E ones. Vmax values of ChE were mainly higher at 0 time than at those recorded either after 12 or 24 hrs post-treated and vise versa in the case of Ali-E Vmax values.

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The maximal AChE activity levels in UT-L occurs at hr 16 (8 hrs from the start of the photophase), while the minimal activity lies at hr 24 (the end of the photophase). The ratio of the highest/the lowest AChE activity levels of UT-L was 1.504. Such ratios were 3.113- and 3.407-folds of that of the minimal in PT-L and ST-L, respectively. AChE activity levels in the larval head homogenates of PT-L, and ST-L were significantly higher than that of UT-L.

Key Words: Emamectin benzoate, Spinosad, Acetylcholinesterase, esterase, kinetics, *Spodoptera littoralis*.

INTRODUCTION

The economic significance of noctuids, especially the Egyptian cotton leafworm, *S. littoralis*, calls for a detailed examination of the effects exerted by biologically active compounds on these insects. Various natural and synthetic compounds with different chemical structures show very broad spectra of biological activities in insects (Gelbic *et al.*, 2006).

The last decade has seen a far wider variety of new chemicals with new modes of action and enhanced selectivity become available than at any other time since the advent of DDT. Compared with organophosphates, carbamates and pyrethroids, many of these new insect control agents provide improved environmental/mammalian toxicology profiles along with greater opportunities to integrate multiple control tactics (Sparks, 2001). In light of the Egyptian cotton leafworm's long history of resistance development, a true integration of control tactics is essential to the long-term availability of control options for *S. littoralis*.

Abamectin is a fermentation derived insecticide that acts on the insect nervous system functioning to open chloride channels, acting as a gamma-aminobutyric acid (GABA) agonist at binding sites, and/or enhancing the action of GABA at the receptor site or stimulating the presynaptic release of GABA (Fisher, 1993; Miller and Chambers, 1987; Lasota and Dybas, 1991; Turner and Schaeffer, 1989). Emamectin benzoate (Proclaim®) which is an analog of abamectin, produced by the same fermentation system as abamectin (Fisher, 1993), is a second generation avermectin with superior activity against lepidopterans compared with abamectin (Dybas *et al.*, 1989; and Jansson and Dybas, 1997). Given the novel mode of action and high degree of efficacy, emamectin benzoate should be a very useful tool in integrated pest management (IPM) programs for the diamondback moth, *Plutella xylostella* and other lepidopterous pests (Sparks, 2001), such as the Egyptian cotton leafworm (El-Aw, 2003 and 2006).

Spinosad represents a new class of fermentation-derived tetracyclic macrolides (Kirst *et al.* 1992, Sparks *et al.*, 1999). The spinosyns, which act via the insect nervous system, are especially active against a variety of lepidopterous pests (DeAmicis *et al.* 1997; Sparks *et al.*, 1999) and possess very favourable mammalian toxicity and environmental profiles (Sparks *et al.* 1999, Crouse and Sparks 1998). The mode of action appears to involve alteration of nicotinic receptor function as well as the function of GABA gated chloride channels (Salgado *et al.* 1997, Watson 2001). However, Spinosad represents an important choice to be used in IPM where *S. littoralis* is a major pest (Penida *et al.*, 2006).

Esterases in insects have been implicated in reproductive behavior, hormone metabolism, digestion, neurotransmission, and the action of, and resistant to insecticides, particularly organophosphates (OPs) (Parker *et al.*, 1991). Esterases could play a key role in degradation of sex pheromones from Lepidoptera (Merlin *et al.*, 2007).

It is important to study the interactions of new insect control agents with some insect biochemical systems to clarify some aspects about the mode of action and the possibility of resistance evolution. Therefore, the purpose of this investigation is to study the probable *in vivo* inhibition of acetylcholinesterase and esterases activities by two selected bio-insecticides, Proclaim and Spinosad and their respective modes of action, as well as to determine esterases kinetics in the larval heads of *S. littoralis*.

MATERIAL AND METHODS

1- Experimental insects

The stock culture of the Egyptian cotton leafworm, *S. littoralis*, was maintained in the laboratory at room conditions for several years. Larvae were reared on castor leaves and adults were fed on 10% sucrose solution. Egg masses were collected from the stock culture and kept, for two generations, under a cyclic alteration between lightness and darkness (LD 16:8) (lightness from 8 a.m., to 12 p.m. and darkness from 12 p.m to 8 a.m., daily), before the conductions of the experiments. Temperature and relative humidity (RH) were 25.0 ± 2.0 ℃ and 70.0 ± 5.0%, respectively. Larvae hatched from eggs were reared in glass jars and provided with the fresh castor leaves until the hatched larvae reached the 6^{th} instar.

2- Tested bio-insecticides

Proclaim 5% SG (Emamectin benzoate, the 4'-deoxy-4'-epi-methyl-amino benzoate salt of avermectin B1) which is, like abamectin, supplied by Syngenta Co., with a recommended rate of 60 gm/ 400 L. water and Tracer 24% SG (the commercial

formulation of Spinosad) is produced by AgroSciences Co., with a recommended rate of 120 gm/ 400 L. water.

3- Procedure

For the determination of the *in vivo* inhibition of the larval head acetylcholinesterase and esterases activity by the two biorational insecticides, Proclaim and Spinosad, castor leaves were dipped for 15 second in each tested insecticide concentration at the recommended rate of each insecticide in 1000 ml tap water and dried in open air. The desired numbers of 6^{th} instar larvae of *S. littoralis* were fed for 24 hrs on the treated castor leaves of each tested insecticide. The experiment was started at 8 a. m., at light on, at which the treated leaves of each tested insecticide were introduced to the larvae. Assays were carried out at 6 different chosen day time intervals since the first assay was carried out immediately after insecticide application (0 time; exactly at the start of photophase at hr 08). Other assays were performed after 4, 8, 12, 16 (at midnight, at light off) and 20 hrs post-treatment. Control larvae were fed for 24 hrs on castor leaves dipped for 15 second in tap water and dried before use.

4- Preparation of head samples for acetylcholinesterase and esterase activity and kinetics

Heads of the 6^{th} larval instar of *S. littoralis* were used for studying the acetylcholinesterase and esterase activity and kinetics in the untreated larvae (UT-L), Proclaim treated larvae (PT-L), and Spinosad treated larvae (ST-L) of *S. littoralis*. The desired number of heads were decapitated on ice, at six different chosen day times after treatment (0, 4, 8, 12, 16, 20 hrs), and the heads were homogenized in ice cold phosphate buffer pH 8 for studying the acetylcholinesterase activity or in ice cold phosphate buffer pH 7.4 for studying the esterase activity and kinetics. An all glass homogenizer in ice was used for the homogenization. Homogenates were centrifuged for 20 min at 6000 g using Universal 32 R Hettich Zentrifugen (Germany) and the supernatants were used for enzyme determinations. Three replicates were secured at each time for each tested insecticide as well as control larvae either for AChE or esterase activity and kinetics.

For the determination of the effect of the two tested biorational insecticides, Proclaim and Spinosad, as well as the day time effect on the larval head esterase kinetics (Km and Vmax). The desired number of heads was decapitated on ice, at three different chosen day times; at 0 time, after 12 and 24 hrs post-treated, and the heads were homogenized in ice cold phosphate buffer pH 7.4. Homogenates were centrifuged for 20 min at 6000 g and the supernatants were used for enzyme determinations of esterase kinetics.

5- Esterase activity and kinetics determination

For the determination of the specific activities of Total-esterase, ChE, and Ali-E, α-NA at a concentration of 2.5 x 10^{-4} M was used as a substrate at pH 7.5 according to the method of Van Asperen (1962) which was slightly modified by Hashem (1971).

For the determination of Michaelis-Menten kinetics, a series of 6 substrate concentrations (0.1, 0.125, 0.25, 0.5, 1.0, and 2.5 x 10^{-4} M) and the enzymatic reactions were allowed for 30 min at 30 ˚C. The protein concentrations in the larval head homogenates were colourimetrically assayed using the method of Lowery *et al.* (1951). Total-esterase, ChE, and Ali-E activities were expressed as μM α-NA hydrolyzed mg protein^{-1} min^{-1} at 30 ˚C. Michaelis-Menten kinetic values were calculated by linear regression of data points on each Lineweaver and Burk (1934) plot.

6- Acetylcholinesterase activity determination

AChE activity in the heads of UT-L, PT-L, and ST-L of *S. littoralis* was determined colouremetrically by the method of Ellman *et al.* (1961). Such method is based on the hydrolysis of acetylthiocholine iodide (ATChI) as substrate by the enzyme AChE to produce thiocholine (TCh) and acetic acid. TCh reacts with 5, 5-dithio-bis (2-nitrobenzoic acid) (DTNB), to produce the yellow anion of 5-thio-2nitro-benzoic acid. The rate of colour production as a function of enzyme activity is measured spectrophotometrically at 412 nm using Jenway 6305 UV/Vis Spectrophotometer. The specific activity expressed as micromoles (μMs) of TCh produced per mg protein per min was calculated using the molecular extension coefficient, 1.36 × 10^4. The protein concentrations in the larval head homogenates were assayed colouremetrically using the method of Lowery *et al.* (1951). The above-mentioned data were statistically analyzed to obtain the analysis of variance (ANOVA) and least significant differences (L.S.Ds) by the method of Steel and Torrie (1984).

RESULTS AND DISCUSSION

1- In vivo inhibition of larval head esterases

Tables (1 and 2) show the specific activities of Total esterases, ChE, and Ali-E on the basis of μM α-NA hydrolyzed per mg protein per min, in *S. littoralis* larval head (brain) homogenates of UT-L, PT-L, and ST-L. Figure (1) shows that the Total-E and ChE reached its peak activity at hr 8 (12hr post-treatment) in UT-L and at hr 24 (16 hr post-treatment) in both PT-L, and ST-L. Ali-E activity levels exhibited no diurnal rhythmicity or clear peak. Statistical analysis revealed that significant differences in the Total-E and ChE activities in the larval head homogenates were obtained between all of the three treatments; UT-L, PT-L, and ST-L and also between all of the six chosen day times. However, ChE activity levels were clearly higher than Ali-E levels either in UT-L or in both PT-L, and ST-L. Accordingly, the resulted Figure strongly shows that the diurnal

rhythmicity in the esterase activity is attributed to ChE only. It is worthwhile to point to that such phenomenon is in full agreement with that of El-Aw (1997).

The specific activity values of ChE in the larval head homogenates of UT-L ranged from 1.219 to 2.963. Thus, the ratio of the highest/ lowest ChE activity levels was 2.431-fold. Such ratios were 3.250- and 2.565-folds of that of the minimal in PT-L (Table 1) and ST-L (Table 2), respectively. These results give evidence for the occurrence of fluctuations in the head ChE activity levels at intervals of day time. However, the rhythmic dial fluctuations in the larval and moth head ChE activities were revealed in a laboratory strain of *S. littoralis* at 12:12 LD regimen (El-Aw, 1989).

Table (1) shows also ChE activity levels in the larval head homogenates of PT-L. and ST-L of *S. littoralis* in the abovementioned successive chosen day times. The ChE and Ali-E activity values in each point on the curve of UT-L was used as a reference to calculate the ratio of decrease or increase in ChE and Ali-E activities of PT-L and ST-L. The ratio of ChE activity in PT-L and ST-L was related to the ChE activity in the UT-L in each chosen day time (Tables 1 and 2).ChE activity levels in the larval head homogenates of PT-L and ST-L were generally higher than that of UT-L. The ratio of ChE activity of PT-L/UT-L ranged from 1.22- to 1.41-fold (Table 1). Such high levels in ChE activity were more pronounced in the case of ST-L. The ratio of ChE activity of ST-L/UT-L ranged from 1.27- to 1.95-fold (Table 2). However, ChE in both of PT-L and ST-L showed increase in its activity at hr 24 (16 hrs post-treatment). However, spinosad and proclaim increased the ChE activity levels in the *S. littoralis* larval head homogenates but did not affect the diurnal change or fluctuations of such activity levels. The ratio of the highest/the lowest Ali-E activity levels in UT-L was 1.267-fold. Such ratios were 1.297- and 1.489-folds of that of the minimal in PT-L and ST-L, respectively. These results give evidence that there are no fluctuations in the head Ali-E activity levels at intervals of day time either in UT-L or in both of PT-L and ST-L. However, spinosad and proclaim significantly increased the ChE activity levels in the *S. littoralis* larval head homogenates while such two insecticides did not significantly affect the activity levels of Ali-E compared with that of untreated larvae.

ChEs are esterases which under physiological conditions catalyze the hydrolysis of choline esters at a higher rate than that of other esters (Silver, 1974). Carboxylesterases (Car-E or Ali-E) are ubiquitous nonspecific enzymes that hydrolyze a diversity of esters of carboxylic acid. These enzymes are implicated to the following endogenous functions in insects: regulation of juvenile hormone titre, general metabolism in insect muscles, and synthesis and transport of cuticular wax (Sudderuddin and Tan, 1973; Ahmed, 1976). Ali-Es are also implicated in the metabolism of exogenous substrates, *i.e.* insecticides (Ahmed and Forgash, 1976). The hydrolysis of a-NA by homogenates of *S. littoralis* larval heads is

mainly due to the two enzymes, ChE and Ali-E (Hashem, 1971). Accordingly, in the present study, eserine insensitive hydrolytic activities in the larval head homogenates are entirely referred to the Ali-E. Present results demonstrated that spinosad and proclaim increased the ChE activity levels in the *S. littoralis* larval head homogenates but not Ali-E one. However, the esterase inhibitor triphenyl phosphate (TPP) did not act synergistically with abamectin in the NJ-Abm strain of *Bemisia tabaci* (Gennadius) (Wang and Wu, 2007). The authors indicated that enhanced metabolism mediated by P450 monooxygenase and GST is likely to be involved in abamectin resistance and cross-resistance to imidacloprid and emamectin benzoate in the NJ-Abm strain. In contrast, Abo El-Ghar *et al.* (1995) found that abamectin (avermectin B1) caused reduction in the activities of α- and β-esterases in the body homogenates of the fifth instar larvae of *S. littoralis* and that the digestive enzyme activities of amylase, invertase, and trehalase were more affected by abamectin and thuringiensin than non-specific esterases. However, Avermectins block the neurotransmitter GABA at the neuromuscular junction in insects and mites. Visible activity, such as feeding and egg laying, stops shortly after exposure, though death may not occur for several days. Proclaim (emamectin benzoate) acts as Chloride channel activator (Webb and Stansly, 2003; Ware and Whitacre, 2004). Spinosad is a fast-acting, somewhat broad-spectrum material that acts on the insect primarily through ingestion, or by direct contact with a spray droplet or a newly treated surface. It activates the nervous system of the insect, causing loss of muscle control. Continuous activation of motor neurons causes insects to die of exhaustion within 1-2 days (Larson, 1997).

2- Michaelis-Menten kinetic values

The possible effects of the two tested biorational insecticides, Proclaim and Spinosad, as well as the day time effect on the Michaelis-Menten kinetics of the ChE and Ali-E of the sixth larval instar of *S. littoralis* were also investigated in the present study. The kinetics of such enzymes are expressed in terms of the values of their Km, which is the concentration of substrate that gives half maximal rate under experimental condition, and Vmax, which is the maximal velocity at the concentration. Table (3) summarizes the calculated values of Km and Vmax for ChE and Ali-E at 0 time, 12 and 24 hr post-treatment.

Data in Table (3) show that the Km values for ChE were not changed from 12 to 24 hrs post-treated in UT-L. The Km values for ChE were increased from in PT-L 1.409 at 0 time to 1.818 either after 12 or 24 hrs post-treated. While in ST-L, the Km values for ChE were decreased from 1.429 at 0 time to 1.209 and 0.571 after 12 and 24 hrs post-treatment, respectively. The Km values for Ali-E in UT-L exhibited changes between the tested time periods of the day such as it increased from 0.714 at 0 time to 1.333 either after 12 or 24

hrs post-treatment. The same trend was observed in the case of PT-L and ST-L. The Km which represents the α-NA concentration at which 50 % velocity of the reaction is attained as an important tool to indicate the affinities of enzymes to their substrates. The changes in Km values of ChE and Ali-E from 0 time to 12 or 24 hrs post-treatment within the day indicate changes in the affinity of such two esterases to α-NA. However, such changes are mainly present in ST-L. The larvae treated with Proclaim had high Km values or low affinities to α-NA within the day compared with those of UT-L. While the larvae treated with Spinosad had lower ChE Km values or higher affinities to α-NA within the day. This indicates that the esterase affinity to α-NA may vary in response to the insecticide treatment and day time at which the enzyme is obtained from the animal, for assaying its activity and determination of its Km and Vmax, which characterize the enzyme. The affinity was mainly decreased in the scotophase.

The results likewise indicate that the Km values for Ali-E in ST-L were mainly higher than those for ChE, while the Vmax values were generally lower. This shows that Ali-E enzyme has lower affinity than ChE towards α-NA and that ChE is highly active and is predominant in the larval head tissue. Such results is not held true all through the different treatments, as in a few cases Ali-E has equal, or even slightly higher, affinity to α-NA. However, such conclusion is further supported by the higher Vmax values for ChE than those for Ali-E, approximately up to 3-folds, in some treatments. These points to the higher substrate turnover, which may reflect the physiological importance of the ChE in the function of the nervous tissue of the head (brain). Hashem (1971) demonstrated that the hydrolytic reactions of esterases to α- and ß-NAs proved that Ali-E had lower affinity than ChE to both substrates in the whole larval body homogenates of the 3^{rd} and 6^{th} instars of *S. littoralis*. His results showed also that there was only one Ali-E in such homogenates splitting α-NA, whereas there were more than one ChE in the 6^{th} instar and only one ChE in the 3^{rd} instar hydrolyzing ß-NA. However, these aspects of the kinetics of *S. littoralis* larval head esterases are of interest since the enzymes of defined physiological roles generally exhibit a low Km and a high Vmax (Kapin and Ahmed, 1980).

The Vmax values for ChE in the larval head homogenates are generally higher than those of Ali-E at all of the three tested day times. The data also indicate that the Vmax values of Ali-E in the head homogenates of UT-L, PT-L, and ST-L are generally higher after 24 hr post-treatment than at 0 time. Vmax values of Ali-E after 12 hr were generally moderate. This trend is not the same in the ChE as they are lower after 12 hr than at 0 time or after 24 hr post-treatment. When the mean values of each insecticide were considered the Vmax for ChE was found to increase in the ascending order; ST-L (0.392), PT-L

(0.446), and UT-L (0.465). Similarly, the Vmax for Ali-E was found to increase in the ascending order; UT-L (0.242), PT-L (0.276), and ST-L (0.304 μ mole α-NA).

It may be obvious that there were no differences in ChE Km values between 12 hr and 24 hr post-treatment neither in UT-L nor in PT-L. The ChE Vmax value was mainly higher at 0 time and after 24 hr than after 12 hr post-treatment. ChE Vmax values of PT-L and ST-L were about 88 and 72 % of that of control ones after 24 hr post-treatment. The Km value for ChE of UT-L, after 24 hr post-treatment, was about 2.3-fold of those of ST-L. The data show that the Vmax values of such enzyme were affected by both insecticides and assaying time. The two kinetic criteria, Km and Vmax, for Ali-E were mostly affected by both factors. The Ali-E Km values were lower in Proclaim and Spinosad treated larvae than control one. Such lower Michaelis constant may indicate the presence of Ali-E of higher affinity to a-NA in the heads of the larvae treated with Proclaim and Spinosad.

It is interesting to observe that the Michaelis constants for ChE remain unaltered, in UT-L and PT-L. While in ST-L, ChE Km values were decreased after 24 hr post-treatment (the end of the scotophase) The Vmax values changed from UT-L to PT-L and ST-L of *S. littoralis* and from 12 to 24 hrs post-treatment in all treatments. The Ali-E Vmax values were mainly higher after 24 hr post-treatment. While The ChE Vmax values were mainly higher at 0 time (the start of the photophase). This fact indicates that the number of active sites on the ChE of the larval head homogenates increased at 0 time (hr 08). Such changes may follow daily rhythmical fluctuations which are altered by the photoperiodism. Kerkut *et al.* (1972) found a decreased in the activity of the major ChE iso-enzymes in the km of the enzyme from the controls to the trained animals. The ChE and Ali-E of *S. littoralis*, in the present work, perhaps undergo reversible conformational changes, under different insecticides and day times, produced by another change in endogenous factor(s). No data concerning the effects of such the two tested bioinsecticides, Proclaim and Spinosad, or the day time on the kinetic parameters Km and Vmax of enzymes are, unfortunately so far available. However, Hashem (1989) found that *S. littoralis* larval head esterase affinity to α-NA may vary in response to day time at which the enzyme is obtained from the animal, for determination of its Km and Vmax, which characterize the enzyme. The affinity was mainly decreased in the scotophase. Therefore, the interpretation of mechanism of changes of such parameters becomes difficult and the subject is still under investigation.

3- *In vivo* inhibition of larval head acetylcholinesterase

Table (4) shows the specific activity of AChE in the larval head homogenates of UT-L, PT-L, and ST-L of *S. littoralis*. Statistical analysis revealed that significant differences in the AChE activities in the larval head homogenates were obtained between

all of the three treatments; UT-L, PT-L, and ST-L and also between all of the six chosen day times.

Fig. (2) shows the changes in AChE activity levels in the larval head homogenates of UT-L, PT-L, and ST-L of *S. littoralis* in the abovementioned successive chosen day times. In the case of UT-L, the maximal AChE activity levels occurs at hr 16 (8 hrs from the start of the photophase), while the minimal activity lies at hr 24 (the end of the photophase). The specific activity values of AChE in the larval head homogenates of UT-L ranged from 3.576 to 5.381. Thus, the ratio of the highest/the lowest AChE activity levels was 1.504. Such ratios were 3.112- and 5.065-folds of that of the minimal in PT-L and ST-L, respectively. These results give evidence for the occurrence of fluctuations in the head AChE activity levels at intervals of day time. However, determination of the head AChE activity at different intervals of day time showed distinct fluctuations in male and female moths of *S. littoralis* (El-Aw and Hashem, 2000) as well as in larval head AChE activities of both laboratory and field strains of *S. littoralis* at 16:8 LD regime (El-Aw, 1997). Also, AChE activity in the central nervous system extract of the cockroach kept in LD 12:12, showed its maximal level at 12 hr (noon) (Vijayalakshmi *et al.*, 1977). However, AChEs appeared to be mainly localized in the central nervous system of the German cockroach, *Blattella germanica* (Kim *et al.*, 2006).

Table (4) shows also AChE activity levels in the larval head homogenates of PT-L, and ST-L of *S. littoralis* in the abovementioned successive chosen day times. AChE activity levels in the larval head homogenates of PT-L, and ST-L were generally higher than that of UT-L. Such high levels in AChE activity were more pronounced in the case of ST-L. However, AChE in PT-L showed increase in its activity at hr 20 p.m. (12 hrs post-treatment). While AChE in ST-L showed increase in its activity at hr 12 a.m. (4 hrs post-treatment). However, spinosad and proclaim increased the AChE activity levels in the *S. littoralis* larval head homogenates but did not affect the diurnal change or fluctuations of such activity levels. The AChE activity in each point on the curve of UT-L was used as a reference to calculate the ratio of decrease or increase in AChE activity of PT-L and ST-L. That the ratio of AChE activity in PT-L and ST-L was related to the AChE activity in the UT-L in each chosen day time (Table 4). Such ratios were 1.2 and 2.9 folds for PT-L and ST-L, respectively, 4 hrs after the application of the two insecticides. The ratio were 1.99 and 1.66 folds for PT-L and ST-L , respectively, after 12 hrs post-treatment and decreased less than 1.0 after 20 hr post-treatment to be 0.51 and 0.87 folds for PT-L and ST-L , respectively. However, results of the first part of the present study proved also the occurrence of rhythmicity in esterases activity which is attributed to the ChE. So far, very

little data are available about the diurnal fluctuation in the AChE in normal and/or Proclaim- and Spinosad-treated insects.

The *in vivo* studies by proclaim and spinosad on the AChE activity of *S. littoralis* showed that spinosad increases significantly the larval head AChE activity. Present results are in a good agreement and support the findings of Khedr (2005) who found that spinosad increases the larval head AChE activity of *S. littoralis* both *in vivo* and *in vitro*. Choe *et al.* (2003) reported that ATP acts via $P2Y_1$ receptors (localized at the junction, proposed by Choe *et al.* (2003), to mediate a trophic role for synaptic ATP) to stimulate AChE expression. Khedr (2005) found also that spinosad inhibit total ATPases and H^+/K^--ATPase. The author concluded that this will lead to accumulation of ATP which may lead to stimulation of AChE expression, according to its function. The *in vivo* effect of spinosad on the Glutathione S-transferases (GST) from mid-gut of 4^{th} instar larvae of *S. littorals* showed that spinosad inhibit the GST activity and the inhibition is concentration dependent. The author concluded that GST in lepidopterous larvae may be a target to spinosad. On the other hand, the activity *in vitro* of microsomal-*O*-demethylase and GST in the resistant strain was 5.2- and 1.0-fold of the susceptible strain, respectively. The results implied that microsomal-*O*-demethylase might be important in conferring spinosad resistance in the *S. exigua* population (Wang *et al.*, 2006).

It is concluded from the present results that the enzyme AChE in the larval heads of *S. littoralis* is not inhibited by proclaim or spinosad. However, AChE is an important regulatory enzyme responsible for controlling the transmission of nerve impulses across cholinergic synapses where it acts to hydrolyze the excitatory neurotransmitter, acetylcholine. AChE in insects has important toxicological significance because it is readily inhibited by OP and carbamate insecticides commonly used as chemical control agents. A major threat to the continued efficacy of OP and carbamate insecticides is the development of resistance via modified AChEs. Such modification of AChE has been implicated in the resistance mechanisms of a number of important medical, veterinary, and agriculture insects and acarine pest species for which OP and carbamate insecticides are commonly used for control (Siegfried, 1990).

Insect GABA receptors are known to be affected by various insecticidal compound classes, including cyclodiens, polychlorocycloalkanes, fiprols and avermectins (vonKeyserlingk and Willis, 1991; El-defrawi and El-defrawi, 1988; Lummis, 1990; and Gant, 1998). Emamectin is a member of the chemical class of avermectins, macrocyclic lactones, produced by fermentation of the soil actinomycete, *Streptomyces avermitilis*. Chemical modification of this fermentation product has yielded hundreds of analogues (Fisher and Mrozik, 1984) including ivermectin, abamectin, moxidectin and doramectin

which are widely used for control of animal and human parasites as well as insects and mites on crops (Fisher, 1997). The mechanism of avermectin killing is disruption of chloride ion movement in nerves and thus neurotransmission through competitive binding to glutamate-gated chloride channels of invertebrate nerves. This mode of action differs from that of organophosphates that inhibit neurotransmission by disruption of cholinesterase activity and of insect growth regulators that inhibit chitin synthesis to disrupt cuticle formation. This unique mode of action should reduce the potential for cross-resistance with other approved products (Arena *et al.*, 1995) used for *S. littoralis* control. However, the present results indicated that there was slight and insignificant increase in head AChE activity in *S. littoralis* larvae treated with proclaim. In other words, no inhibition was observed in head AChE activity in *S. littoralis* larvae treated with proclaim. It may indicates that the enzyme AChE is not a target for the action of emamectin benzoate (proclaim) in *S. littoralis* larval heads (brains).

Extensive studies conducted over a period of several years have found that spinosad had no remarkable toxicologically significant effects in assays for many insecticide and drug target sites, suggesting that it has a novel mode of action. The present study is one of those studies that could contribute to the limited published data (Slagado *et al.*, 1997 and 1998; and Watson, 2001, Khedr, 2005). It has shown that spinosyns can activate a cation that is blocked by the nicotinic acetylcholine receptor (nAChR) antagonist α-bungarotoxin (Slagado *et al.*, 1997). Watson (2001) found that spinosyns have potent effects on the function of the GABA receptors of small-diameter cockroach neurons and can elicit small amplitude Cl^-. These findings may have implications toward the insecticidal mechanism of action of spinosyns. The present study on the interaction of both compounds, proclaim and spinosad, with the enzymes esterases or AChE, which regulate the cholinergic neurotransmitter, ACh, in the nervous system, revealed remarkable activation on the function of these enzymes. However, further investigation is needed to study the cholinergic drug profile in attempts to explore the compound interactions with the nicotinic-receptor-enzyme regulating functions.

Spinosad is a fermentation metabolite of the actinomycete, *Saccharopolyspora spinosa*, a soil-inhabiting microorganism. It has both contact and stomach activity against lepidopteran larvae, leaf miners, thrips, and termites, with long residual activity (Thompson *et al.*, 1999; Dow AgroSciences Co., 2001). Electrophysiological studies have shown that spinosyn A acts on the insect nervous system to increase the spontaneous activity, leading to involuntary muscle contraction and tremors. This increase in excitation appears to result from the persistent activation of nAChRs and prolongation of acetylcholine responses, in a manner that is distinct from other nicotinic active molecules. In addition, spinosyns can

also alter the function of GABA-gated chloride channels (Salgado, 1997 and 1998, Salgado *et al.* 1997). This unique mode of action contribute in that spinosad has little or no cross-resistance in laboratory strain of *Holicoverpa virescence* previously selected for high levels of resistance to insecticides (Sparks *et al.*, 1996), or known to possess specific target site-or metabolism-based mechanisms. Also, there was no cross-resistance in the beet armyworm, *Spodoptera exigua* between spinosad and fenvalerate, phoxim, methomyl, abamectin, and cyfluthrin (Wang *et al.*, 2006). Given its favorable safety profile, a high degree of initial and residual activity comparable with methomyl and lack of cross-resistance to other chemistries, spinosad bait may be a valuable component of house fly control programs to help control or delay the emergence of resistant populations (White *et al.*, 2007).

It is concluded from the present results that esterase and AChE activity levels are significantly variable according to the day time at which the enzyme is assayed. However, the results of the present study are of experimental importance as they draw attention to standardize the conditions under which the subjected animal is kept. It should be stressed that the time of the day at which the enzyme is obtained from the animal, for assaying its activity and determination of its Km and Vmax, which characterize the enzyme, must be defined. To realize more accuracy the replication of such experiments must also be performed at the same time of the day (Hashem, 1989; El-Aw and Hashem, 2001).

Insects can become resistant to any insecticide if it is used repeatedly. This also applies to alternating insecticides with similar modes of action. To complicate matters, some insecticides in the same class have different modes of action and some unrelated chemicals have the same mode of action. In general, pesticides with the same mode of action should be used no more than twice in any crop cycle if residual activity is short and only once if residual activity is long. In addition to alternating insecticides with different modes of action, integrating other non-chemical control measures in a pest management program should help to delay resistance (Webb and Stansly, 2003). However, with new compounds which have new modes of action, such as Proclaim and Spinosad, as alternatives to the recommended OP and carbamate insecticides currently in use, could be successfully included in the management program of *S. littoralis*.

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TABLE (I)

Larval head esterases activity following feeding of the sixth larval instar of cotton leafworm, *Spodoptera littoralis*, on castor oil leaves treated with the recommended fieled concentration of the biorational insecticide, Proclaim.

Time Intervals in hrs	Spesific activity (SA*)								
	Total-E		PT-L/UT-L ratio	ChE		PT-L/UT-L ratio	*Ali-E*		PT-L/UT-L ratio
	UT-L	PT-L		UT-L	PT-L		UT-L	PT-L	
0	2.406 ±0.223 ^{c}	2.406 ±0.223 ^{f}	1.000	1.244 ±0.124 ^{d}	1.244 ±0.124 ^{c}	1.000	1.162 ±0.104 ^{b}	1.162 ±0.104 ^{b}	1.000
4	3.561 ±0.258 ^{b}	4.308 ±0.242 ^{c}	1.363	2.212 ±0.298 ^{b}	2.884 ±0.210 ^{b}	1.304	1.349 ±0.125^{ab}	1.424 ±0.034 ^{ab}	1.056
8	3.214 ±0.033 ^{b}	3.724 ±0.535 ^{d}	1.159	1.743 ±0.269 ^{c}	2.128 ±0.250 ^{c}	1.221	1.471 ±0.236 ^{a}	1.596 ±0.287 ^{a}	1.085
12	4.411 ±0.320 ^{a}	5.004 ±0.048 ^{b}	1.134	2.963 ±0.195 ^{a}	3.667 ±0.051 ^{a}	1.229	1.449 ±0.127 ^{a}	1.337 ±0.098 ^{ab}	0.936
16	4.362 ±0.0.217 ^{a}	5.550 ±0.252 ^{a}	1.260	2.865 ±0.026 ^{a}	4.043 ±0.307 ^{a}	1.411	1.497 ±0.128 ^{a}	1.507 ±0.058 ^{a}	1.007
20	2.347 ±0.190 ^{c}	3.018 ±0.258 ^{e}	1.286	1.219 ±0.271 ^{d}	1.713 ±0.225 ^{d}	1.405	1.128 ±0.081 ^{b}	1.305 ±0.184 ^{ab}	1.157
L.S.D.$_{0.05}$	0.388	0.527		0.395	0.377		0.249	0.282	

SA*; expressed as μ Mol. α-NA/mg protein/min.

UT-L; untreated larvae, PT-L; Proclaim treated larvae.

TABLE (II)

Larval head esterases activity following feeding of the sixth larval instar of cotton leafworm, *Spodoptera littoralis*, on castor oil leaves treated with the recommended fieled concentrations of the biorational insecticide, Spinosad.

Time intervals In hrs	Spesific activity (SA*)								
	Total-E		ST-L / UT-L ratio	ChE		ST-L / UT-L ratio	*Ali-E*		ST-L / UT-L ratio
	UT-L	ST-L		UT-L	ST-L		UT-L	ST-L	
0	3.072 ±0.164 ^{c}	3.072 ±0.164 ^{c}	1.000	2.041 ±0.142 ^{c}	2.041 ±0.142 ^{c}	1.000	1.031 ±0.151 ^{b}	1.031 ±0.151 ^{b}	1.000
4	2.641 ±0.289 ^{cd}	4.537 ±0.495 ^{b}	1.718	1.725 ±0.260 ^{c}	3.328 ±0.357 ^{b}	1.929	0.916 ±0.097 ^{b}	1.009 ±0.169 ^{b}	1.102
8	4.281 ±0.201 ^{b}	6.294 ±0.337 ^{a}	1.470	2.909 ±0.116 ^{b}	4.863 ±0.178 ^{a}	1.672	1.372 ±0.132 ^{a}	1.431 ±0.216 ^{ab}	1.043
12	5.293 ±0.362 ^{a}	6.187 ±0.296 ^{a}	1.169	3.835 ±0.445 ^{a}	4.852 ±0.360 ^{a}	1.265	1.458 ±0.263 ^{a}	1.535 ±0.379 ^{a}	1.053
16	4.654 ±0.685 ^{ab}	6.703 ±0.808 ^{a}	1.440	3.231 ±0.504 ^{b}	5.235 ±0.503 ^{a}	1.620	1.423 ±0.185 ^{a}	1.468 ±0.311 ^{ab}	1.032
20	1.971 ±0.335 ^{d}	3.284 ±0.474 ^{c}	1.666	1.063 ±0.167 ^{d}	2.071 ±0.288 ^{c}	1.948	0.908 ±0.167 ^{b}	1.213 ±0.189 ^{ab}	1.336
L.S.D.$_{0.05}$	**0.673**	**0.843**		**0.553**	**0.584**		**0.305**	**0.444**	

SA*; expressed as μ Mol. α-NA/mg protein/min.

UT-L; untreated larvae, ST-L; Spinosad treated larvae.

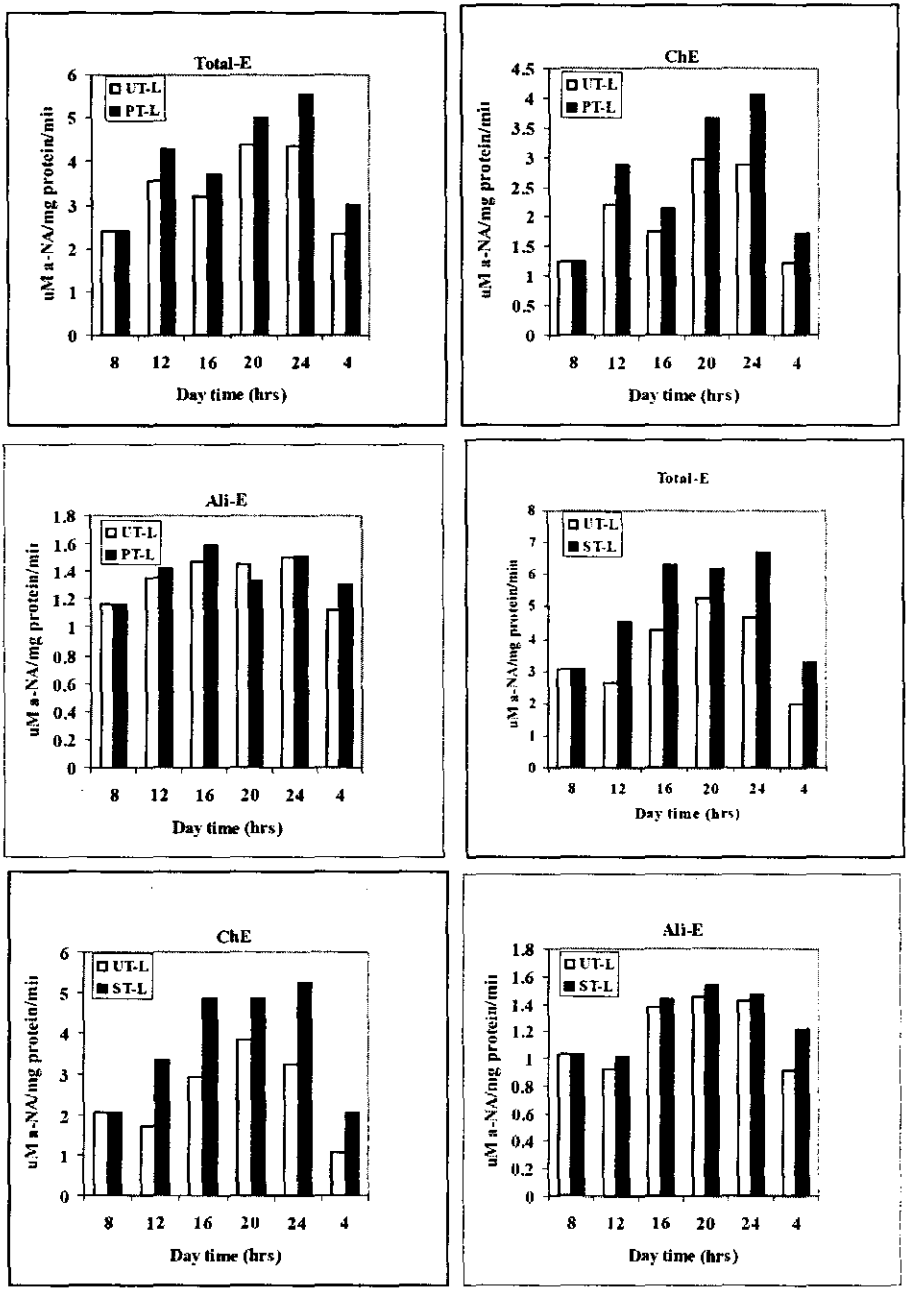
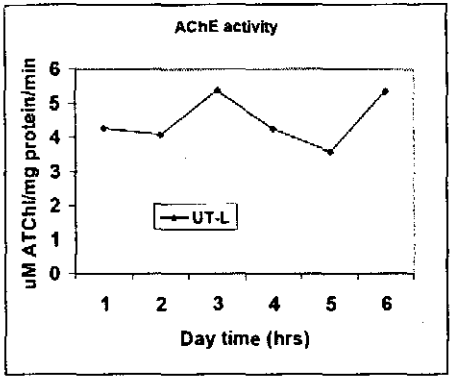
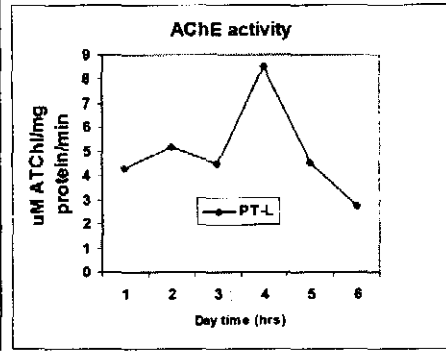


Fig. (1): Esterases activity in heads of the sixth larval instar of cotton leafworm, *Spodoptera littoralis*, fed on castor oil leaves treated with the recommended fieled concentrations of the two biorationalb insecticides, Proclaim and , Spinosad. UT-L; untreated larvae, PT-L; Proclaim treated larvae, ST-L; Spinosad treated larvae.





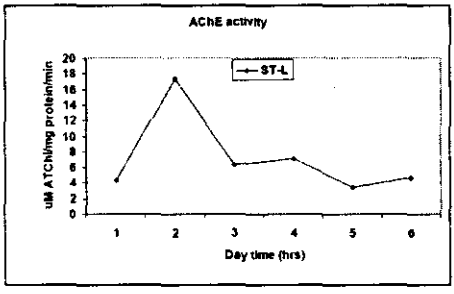


Fig. (2): Acetylcholinesterase activity in heads of sixth larval instar of cotton leafworm, *Spodoptera littoralis*, fed on castor oil leaves treated with the recommended fieled concentrations of the two biorational insecticides, Proclaim and Spinosad. UT-L; untreated larvae, PT-L; Proclaim treated larvae, ST-L; Spinosad treated larvae.

TABLE (III)

Michaelis-Menten kinetics for α-NA hydrolyzed by sixth larval head esterases of *S. littoralis* fed on castor oil leaves treated with the recommended fieled concentrations of the two biorational insecticides, Proclaim and Spinosad.

Insecticide	Treatment	Time post-treatment (hrs)	Km		Vmax	
			ChE	Ali-E	ChE	Ali-E
Proclaim	UT-L	0	1.409	0.714	0.513	0.163
		12	1.333	1.333	0.348	0.242
		24	1.333	1.333	0.533	0.320
	PT-L	0	1.409	0.714	0.518	0.163
		12	1.818	1.053	0.348	0.301
		24	1.818	1.333	0.471	0.364
Spinosad	UT-L	0	1.429	0.952	0.476	0.235
		12	1.333	1.667	0.353	0.308
		24	1.333	1.667	0.444	0.408
	ST-L	0	1.429	0.952	0.476	0.235
		12	1.209	1.563	0.381	0.296
		24	0.571	1.563	0.320	0.381

Km, $\times 10^{-4}$ M; Vmax, μ Mol. α-NA hydrolyzed/mg protein/min.

UT-L; untreated larvae, PT-L; Proclaim treated larvae, ST-L; Spinosad treated larvae.

TABLE (IV)

Larval head acetylcholinesterase activity following feeding of the sixth larval instar of cotton leafworm, *Spodoptera littoralis*, on castor oil leaves treated with the recommended fieled concentrations of the two biorational insecticides, Proclaim and Spinosad.

Time Intervals in hrs	Spesific activity (SA*)				
	UT-L	PT-L	ST-L	PT-L / UT-L ratio	ST-L / UT-L ratio
0	4.304 ± 0.084	4.304 ± 0.084	4.304 ± 0.084	1.000	1.000
4	4.068 ± 0.115	5.056 ± 0.176	11.658 ± 0.996	1.243	2.866
8	5.381 ± 0.140	4.482 ± 0.073	6.418 ± 0.015	0.833	1.174
12	4.272 ± 0.043	8.497 ± 0.282	7.087 ± 0.159	1.989	1.659
16	3.576 ± 0.148	4.527 ± 0.012	3.442 ± 0.101	1.266	0.963
20	5.251 ± 0.096	2.696 ± 0.01	4.670 ± 0.022	0.510	0.873
$L.S.D._{0.05}$	**0.377**	**0.512**	**0.823**	-	-

SA*; expressed as μMol. ATChI/mg protein/min.
UT-L; untreated larvae, PT-L; Proclaim treated larvae, ST-L; Spinosad treated larvae.
Values are the mean ± SE of three determinations (each with triplicate incubations).

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