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MARGARET JANE HARTMAN (Name) for the DOCTOR OF PHILOSOPHY (Degree)

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Title: THE EFFECTS OF FOUR ANTICHOLINESTERASE COMPOUNDS ON THE EMBRYOLOGY OF ACHETA DOMESTICUS (L.) (ORTHOPTERA: GRYLLIDAE)

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P. O. Ritcher

Two organophosphates (Ruelene and Tiguvon) and two carbamates (Sevin and Baygon) were administered topically at various sublethal concentrations to cricket eggs. All the pesticides caused an immediate decrease in the acetylcholinesterase (AChE) activity level in the embryo, but the level usually returned to normal within three days after dosage. The pesticides were found to be competitive inhibitors of AChE. When applied before the AChE was present, the pesticides caused a depression of AChE when it first appeared.

Nymphs were more susceptible than eggs to all the pesticides tested, except Ruelene. Penetration through the chorion was never more than 10% and usually about 3%.

Pretreatment of eggs with carbamates had no effect on the susceptibility of the nymphs to methyl parathion, another anticholinesterase agent. However, pretreatment of eggs with organophosphates

increased the susceptibility of emerging nymphs to an anticholinesterase agent.

When either Sevin or Ruelene was applied to three day old eggs, AChE activity level was observed to be significantly higher at the time of hatching than it was in undosed controls. The higher level of AChE activity led to an increase in tolerance of emerging nymphs to anticholinesterase agents.

The Effects of Four Anticholinesterase Compounds on the Embryology of *Acheta domesticus* (L.) (Orthoptera: Gryllidae)

by

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Chairman of Department of Entomology

Redacted for Privacy

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THE EFFECTS OF FOUR ANTICHOLINESTERASE COMPOUNDS ON THE EMBRYOLOGY OF ACHETA DOMESTICUS (L.) (ORTHOPTERA: GRYLLIDAE)

INTRODUCTION

With the imminent restriction of the chlorinated hydrocarbons and cyclo-diene pesticides, more and more use must be made of the more toxic but less stable organophosphate and carbamate pesticides for insect control. More is known about the mode of action of these anticholinesterase pesticides than is known about the chlorinated hydrocarbons and cyclo-dienes (Smith and Salkeld, 1966). However, studies on the action of the organophosphate and carbamate pesticides have been limited mostly to vertebrates and to larval and adult insects. Little work has been done on the effects of these pesticides on insect embryos, and virtually none on the effects of sublethal doses.

In an actively moving insect, immature or adult, the insect is dependent on its nervous system to respond to its environment. In an insect embryo, although the nervous system is developed early (Wheeler, 1893; Roonwal, 1937; Panov, 1966), it is not used until just prior to hatching, when the insect begins coordinated muscular movements necessary to rupture the chorion. Therefore, an anticholinesterase agent may be present in an embryo for several days before having an observable effect (Mehrotra and Smallman, 1957; Smith and

Wagenknecht, 1956; Mehrotra, 1960).

The embryo presents unique opportunities for study of enzyme systems. The function of an organ is mediated by the enzymes it contains. Thus differentiation of the embryo should be paralleled by changes both in quantity and in distribution of enzymes. If a change in the enzymes can be correlated with a change in an organ, then it is possible to better understand not only the formation of the organ, but also its activation (Salkeld, 1964). This relationship between developing structures and their function might also be investigated in the embryo by use of specific agents affecting enzyme activity. Studies of chemical embryology would aid in understanding both the physiology of the embryo and the physiological significance of the enzymes.

From a practical standpoint, it is important to study the effects of anticholinesterase on embryos in relation to control of insect pests. Different life stages of an insect species may vary in susceptibility to a toxicant. When the egg is the most susceptible stage, or is the stage in which the population is the most concentrated, it seems logical to attempt to control the insect by the use of an ovicide.

Use of ovicides has both advantages and disadvantages. Best results have been obtained when ovicides were used on insect eggs that had been laid in an exposed position on the host plant. Striking examples of this are the peach tree borer (Smith, 1961) and the currant borer (Taschenberg, 1953). In these cases successful control depended

more upon the exposure of the eggs than upon the higher susceptibility of the egg stage to the pesticide (Smith and Salkeld, 1966).

Overwintering eggs can be treated at a season when non-target organisms are least vulnerable. Unfortunately, there are many species whose eggs do not overwinter in an exposed area. Control of these insect eggs poses problems as it is difficult to obtain even pesticide coverage and penetration. Also, use of ovicides as a practical means of insect control is restricted to species which have a large percentage of the population in the egg stage at the same time.

With the common use of granular formulations in soil, the effects of sublethal doses of pesticides on eggs of soil insects should be investigated. There seems to be a belief that resistance is less likely to arise in the egg stage than in later stages (Smith and Salkeld, 1966) but there is no evidence of this.

Resistance to pesticides can be of two types: (1) population resistance, in which genes for resistance are already present in the gene pool. These genes increase in frequency in subsequent generations as susceptible members are selected out and more resistant ones survive to reproduce; (2) individual resistance, in which an individual exposed to a sublethal dose of pesticide builds up an increased tolerance to that group of pesticides. In the former case, the increased resistance is passed on to the next generation; in the latter, the increased tolerance is only for the individual. Although

both types of resistance may occur, it is the first type which has been most often documented and has led to the present problems with resistant insect populations.

The purpose of this study was to compare the effects of two classes of anticholinesterase pesticides, organophosphorus and carbamate compounds, on the embryological development of the cricket, Acheta domesticus (L.). During the course of the study, particular emphasis was given to the following aspects:

(1) the normal morphological development and eclosion of cricket embryos and the effect of the four pesticides, two carbamates (Sevin and Baygon) and two organophosphates (Ruelene and Tiguvon) on these aspects.

(2) the normal course of cholinesterase formation and increase.

(3) the degree of development of the nervous system upon initial appearance of cholinesterase in the embryo.

(4) the differentiation among the different enzymes, contained in the embryo, that hydrolyze acetylcholine.

(5) the effect of the four pesticides on development of cholinesterase.

(6) the effect of embryonic age on the effectiveness of the pesticides.

(7) the rate of penetration of pesticide through the chorion.

(8) the effect of pretreatment of eggs with pesticide on the susceptibility of nymphs to a subsequent application of an anticholinesterase compound.

LITERATURE REVIEW

Morphology and Physiology of the Cricket Egg

The descriptive embryology of the Orthoptera is very well known. Embryogenesis has been studied mostly in grasshoppers and locusts, for example by Wheeler (1893) in Xiphidium ensiferum Scudder, by Slifer (1932a, b; 1937) in Melanoplus differentialis (Thomas) and M. femur-rubrum (DeGeer), by Rooonwal (1936, 1937) in Locusta migratoria migratorioides Reiche and Fairmaire, by Mathée (1951) in Locustana perdalina (Walker), by Bodenheimer and Shulov (1951) in Dociostaurus marocannus (Thumberg), by Pener and Shulov (1960) in Calliptamus palaestinensis Bodenheimer, by Shulov and Pener (1963) in Schistocerca gregaria Forskal.

Among crickets, the embryology of Acheta commodus (Walker) (Brookes, 1952) and Acheta assimilis (Fabricius) (Rakshpal, 1962b) has been studied in great detail, although early workers have referred to the embryology of Gryllidae (summarized by Roonwal, 1936, 1937).

Spermatogenesis in A. domesticus has been studied by Nath and Bhimber (1953) and by Clayton, Deutsch and Jordan-luke (1958). Uptake of labelled thymidine and uridine by spermatogonia and oocytes was studied by Halkka and Heinonen (1966). An earlier study was made of the function of follicle cells in oocyte formation in two other cricket

species, Gryllus abbreviatus Serville and Nemobius fasciatus DeGeer (Murray, 1926).

Panov (1964) found that, in the embryo, the brain ganglia of A. domesticus grew faster than did the nerve cord ganglia, but the reverse was true in the nymph. Further studies indicated that the nervous system of A. domesticus was more developed than that of the mole cricket at hatching because of the greater degree of fusion of the abdominal ganglia (Panov, 1966).

Among crickets, diapause in the egg stage may or may not occur. A. domesticus was chosen for this research because the species does not undergo egg diapause (Ghouri and McFarlane, 1958). However, much literature is available on initiation and termination of diapause in related species, A. commodus (Walker) and G. pennsylvanicus Burmeister. In A. commodus, diapause began at the 46 hour stage and was initiated by temperatures below 23°C. The maximum sensitivity to cold occurred immediately prior to the 46 hour stage (Hogan, 1960c). In both A. commodus and G. pennsylvanicus, diapause was terminated by exposure to subzero temperature; the lower the temperature, the shorter period of cold needed to terminate diapause (Hogan, 1960a, b; Rakshpal, 1962a). Ammonium compounds with organic anions, applied to the surface of the egg, also terminated diapause (Hogan, 1962b). Diapausing eggs were still capable of breaking down ammonia and CO_2 (Hogan, 1962a).

Ghouri and McFarlane (1958) found that 35°C was the optimal temperature for development of A. domesticus embryos, although the embryos would also develop at 28°C. The nymphs could develop between 23°C and 41°C, but although time of development decreased as the temperature was raised, mortality increased. Data on preoviposition and oviposition behavior, incubation time, and hatching are also available for Gryllodes sigillatus (Walker) (Khan, 1954) and Gryllus assimilis (Severin, 1930, 1935).

All work on the physiology of A. domesticus embryos has dealt with the properties of the chorion. McFarlane, Ghouri, and Kennard (1959) determined that absorption of water through the chorion occurred up to 96 hours after oviposition. These results were based on weight gain studies (McFarlane et al., 1959) and dye entrance studies (McFarlane, 1960). Water was found to be absorbed over the entire surface, that is, no specialized area of water absorption was found (McFarlane and Kennard, 1960). McFarlane and Furneaux (1964) later discovered a second period of water absorption. The end of the first absorption period was associated with the tanning of the serosa; the beginning of the second period was associated with the liberation of material from the chorion for cuticle formation.

Attempts also have been made to trace the substances involved in membrane tanning and to correlate the timing of tanning with morphological events (McFarlane, 1961, 1962a, b; Furneaux and McFarlane,

1965a, b). The serosal layer tans at blastokinesis, but there is no chitin present at that time (McFarlane, 1961). The catecholamines found in the egg are DOPA (dihydroxyphenylalanine), DOPamine, and N-acetyl DOPamine (Furneaux and McFarlane, 1965a). The histochemical staining of these catecholamines indicated that they are concentrated around the pores in the chorion, the presence of which have been corroborated by electron scan studies of the egg shell (McFarlane, 1965).

Hormoligosis in A. domesticus

Hormoligosis is a phenomenon in which subharmful quantities of a stress agent may be helpful to an organism in a suboptimal environment. Hormoligosis in cricket nymphs has been studied by Luckey (1968). He found that subharmful doses of pesticides increased the growth rates of A. domesticus nymphs at suboptimal (lower) temperatures. Luckey hypothesized that the hormoligant decreases the activating energy required to develop systems in an organism adapting to a suboptimal environment, but he had no data to support this hypothesis.

Mode of Action of Organophosphorus and Carbamate Pesticides

Much evidence indicates that the cholinergic system in insects functions in synaptic transmission as it does in vertebrates, but unlike vertebrates, does not function at the neuromuscular junction

(Treherne, 1966). The cholinergic system consists of three components: (1) acetylcholine (ACh); (2) acetylcholineserase (AChE); (3) choline acetyltransferase (ChA). In vertebrates, acetylcholine causes a decrease in the potential difference at the post synaptic membrane, triggering a nerve impulse in the post synaptic nerve (Eccles, 1965). Acetylcholine is hydrolyzed at the synapse by the enzyme acetylcholinesterase. The hydrolytic process by which acetylcholinesterase splits acetylcholine occurs in two steps. In the first step, an acetylated enzyme is formed and choline is given off. In the second step, the acetylated enzyme reacts with water to form acetate and restored enzyme (Wilson, 1954). Then, in the presence of choline acetyltransferase, the acetate and choline are reformed into acetylcholine.

Studies of insect nervous transmission and tolerance to anticholinesterase compounds have been complicated by a wide variety of related esterases of unknown function. These have been grouped into three classes (O'Brien, 1961):

(1) acetylcholinesterase (AChE): an enzyme specific for acetylcholine, although it hydrolyzes butyrylcholine slowly;

(2) pseudocholinesterase (ChE): a group of enzymes which can hydrolyze acetylcholine, but also hydrolyze other synthetic substrates, such as butyrylcholine. These enzymes are believed to function in the animal to control metabolism of choline esters other than acetylcholine (Augustinsson, 1960);

(3) aliesterases: a group of enzymes which do not hydrolyze acetylcholine, but do hydrolyze such synthetic substrates as phenyl acetate, methyl butyrate, and tributyrin.

Organophosphates act as an alternate substrate for both acetylcholinesterase and pseudocholinesterases. Cholinesterase reacts with the pesticide to form a phosphorylated enzyme and a free radical in the first step. The second reaction, that is to form a restored enzyme and a radical, is almost nonexistent in the case of di-ethyl organophosphates, but does occur to some extent with dimethyl organophosphates (Gage, 1967). Thus in the absence of an enzyme, acetylcholine accumulates, leading to the typical organophosphate poisoning symptoms (O'Brien, 1963).

Carbamates act by forming a carbamylated enzyme. The carbamate reacts with both the anionic and esteratic sites of the enzyme (Koelle, 1965). Carbamylation of cholinesterase is readily reversible, much more so than phosphorylation (Wilson, Hatch and Ginsburg, 1960; Wilson, Harrison and Ginsburg, 1961; O'Brien, 1963; Winteringham and Fowler, 1966).

O'Brien (1961) stated that if organophosphates act on insects by depressing their cholinesterase, then death should occur when the insect cholinesterase activity fell below a certain level. Since carbamates act in much the same manner, death from carbamate poisoning should also occur when insect cholinesterase activity falls below this level. At the present time, there is a controversy as to

whether or not organophosphates do cause death at a certain percent cholinesterase depression. Smith and Goulding (1968, 1970) found that 100% mortality occurred when the cholinesterase activity of the mosquito Aedes aegypti (L.) and of the tick, Dermacentor andersoni Stiles, was depressed 68% by organophosphates, regardless of which one was used. However, Morallo and Sherman (1967) found little correlation between cholinesterase depression and toxicity of organophosphates in house flies. The difference in results may be due to the fact that body cholinesterase seems to be a much better indicator of mortality than head cholinesterase (Bigley, 1966). This has recently been corroborated by histochemical tests (Booth and Metcalf, 1970). Smith and Goulding (1968, 1970) used whole arthropods for their analyses; Morallo and Sherman used only the insect heads. When Culex pipiens pallens Coquillett larvae were poisoned with carbamates, there was no correlation between percent cholinesterase inhibition and mortality (Hsuing, Shieh and Wang, 1963).

Effects of Organophosphorus and Carbamate Pesticides on Insect Eggs

Organophosphates

Organosphosphates as ovicides have been used successfully in the field against the peach-tree borer, codling moth, oriental fruit moth, red-banded leaf roller, grape berry moth, plum curculio,

cherry fruit fly, several species of aphids, and the European red mite (Smith and Salkeld, 1966). In almost all cases, eggs poisoned with organophosphates at any time during their embryonic development continued normal development until just prior to hatching, then died (Salkeld and Potter, 1953; Zschintzsch, O'Brien and Smith, 1965; David, 1959; Smith and Salkeld, 1966). There have been some reports on immediate kill of young embryos by extremely high concentrations of TEPP (tetraethyl pyrophosphate) (Lord and Potter, 1951). In these cases, similar results were obtained by application of carbolic acid derivatives, compounds devoid of anticholinesterase action (Karczmar, 1963). Therefore, it seems likely that such massive dosages may evoke modes of action which do not occur in practical usage (Smith and Salkeld, 1966). It is believed that the normal mode of action of organophosphates is to cause death during the late stages of embryonic development, just prior to hatching.

Two hypotheses attempting to explain this late stage mortality have been presented. The first hypothesis was that embryos died in late stages because the pesticide was trapped in the chorion and only released as the embryo came in contact with the chorion at eclosion (Lord and Potter, 1951; Salkeld and Potter, 1953; Zschintzsch et al., 1965). The second hypothesis was that the pesticides were acting throughout the embryonic life, but that they became lethal when the cholinergic system was first used extensively, that is, near hatching

(Smith and Wagenknecht, 1956; David, 1959; Smith and Salkeld, 1966).

Staudenmeyer (1953) reasoned that interference with a vital system by a pesticide would cause a change in the gross rate of oxygen consumption. However, he found that no matter when the pesticide was applied to silkworm eggs, there was no change in the respiration until two days before hatching, when the rate decreased.

Other workers have studied the effect of organophosphates on the nervous system enzymes during embryonic development. Mehrotra and Smallman (1957) found that lethal doses of pesticide administered during embryonic development of house flies caused an inhibition of ChE immediately, although morphogenesis continued until just before hatching. The same results were obtained for Pieris brassicae (L.) (David, 1959) and Sanninoidea exitosa (Say) (Smith and Wagenknecht, 1956).

On the basis of this evidence, most workers tend to agree that the pesticide is present in the embryo and remains active but does not exert its lethal effect until the late stages of development.

Carbamates

Carbamates have a mode of action similar to organophosphates in that, regardless of when they are applied to the embryo, no mortality occurs until late embryonic life. Kerr and Brazzel (1960) and Samy (1964) tested the effectiveness of Sevin, a carbamate, against

eggs of several species of Lepidoptera, but obtained poor results. No previous research has been done on the effects of carbamates on the egg cholinergic system.

Recovery of Cholinesterase Activity After Poisoning by Anticholinesterases

In mammals, blood cholinesterase activity level is used as an indicator of the degree of exposure to organophosphates. Workers in the area of animal systemic insecticides have found that, after dosage, the blood ChE activity level remains low for a very long period of time (up to 28 days) although there are no toxic symptoms in the animals (Eddy et al., 1959; Rogoff et al., 1967).

With insects, there is a lack of agreement as to whether the cholinesterase activity of insects will return to normal levels after the initial depression due to a sublethal dose of an anticholinesterase agent. Mengle and Casida (1958) and Mengle and O'Brien (1960) found complete recovery of house fly head cholinesterase activity after poisoning with either organophosphates or carbamates. O'Brien (1956, 1961) used whole house flies and found some recovery of cholinesterase activity after initial poisoning, but the final cholinesterase level after recovery was only 60-70% of the normal level. Stegwee (1960) also found partial, but not complete, recovery of cholinesterase activity in heads and in thoraces of house flies receiving sublethal doses of organophosphates. All work was done with

adult flies and the test time was always 1280 minutes (21 hours, 20 minutes). A longer test period might have resolved these two conflicting results.

MATERIALS AND METHODS

Rearing Crickets

Crickets used in this experiment were obtained from Fluker's Cricket Farm, Baton Rouge, Louisiana. They were identified by Dr. A. B. Gurney[1] as Acheta domesticus (L.). Approximately 250 crickets were placed in a 25 gallon, plastic garbage can which contained about an inch of 30-mesh, white, Monterey sand in the bottom. Four colonies were kept simultaneously. The crickets were fed chicken feed and water was provided from a 100 ml, narrow lipped bottle stoppered with a cotton wick. The lip of the bottle was placed over a plastic petri dish thereby preventing water from dripping onto the sand in the garbage can. The cans were not covered with lids except when oviposition dishes were supplied to the colonies. The cages were cleaned weekly; water and food were replenished as needed. The adult crickets had an egg laying span of two to three weeks. This made it necessary to replace crickets frequently.

Gathering Eggs

To facilitate oviposition, petri dishes filled with moist sand

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[1] Gurney, A. B. 1968. Authority on Orthoptera, Systematic Entomology Laboratory, U. S. D. A., U. S. National Museum. Personal communication. Washington, D. C.

were placed in the cages (Siverly, 1962). Rakshpal (1962b) found that with G. assimilis, the best ratio of sand to water for an oviposition was 3:1. With A. domesticus, however, more eggs were laid when the sand in the dish was less moist. Therefore, dry sand was placed in the petri dish, water in excess was added to moisten all the sand, more sand was added to absorb the excess water, then any excess sand was shaken off. The dish of moistened sand was then placed in the garbage can and the water bottle was removed, so that the only source of moisture was the oviposition dish. After a set interval, usually eight hours, the dishes were removed, covered, and placed in an incubator at $35^{o} \pm 1^{o}C$.

To collect eggs, a portion of the sand from the oviposition dish was put in another petri dish and flooded with distilled water. The eggs fell to the bottom of the dish and were removed either with needle-nose forceps or with a camel's hair brush and placed in specially prepared petri dishes. These dishes had a bottom layer of moist sand, covered with a 9.0 cm B&D filter paper to absorb water. After the required number of eggs was placed on the filter paper, the dish was covered and returned to the $35^{o}C$ incubator. If the sand became dry, the filter paper was removed and the sand remoistened. All data pertaining to date of oviposition, date of application of pesticide, and type and concentration of pesticide used were recorded on both the top and bottom of the petri dishes.

Determination of Stages

Landmark Stages

Each group of crickets was allowed to oviposit for one hour only. The eggs were collected immediately and examined hourly for the beginning of each stage. Determination of eggs in the early stages (II-V) was difficult since the chorion was opaque at this time. It was found that if these eggs were immersed in a mixture of equal parts of glacial acetic acid and clove oil, the chorion was cleared so that the embryonic stage could be identified under a dissecting microscope. However, eggs tested in this manner were destroyed in the process.

Eggs used for determination of landmark stages VI-X could be examined without injury, since the embryo was lying on the surface of the yolk and the chorion was more translucent. The eggs were removed from the petri dish, immersed in water, examined under a dissecting microscope and returned to the petri dish.

Sectioning

To determine the degree of development of the nervous system when the cholinesterase first became active, it was necessary to make serial sections. Two different techniques were used, the rotary microtome method and the cryostat method.

Rotary Microtome Technique. The eggs to be sectioned were

placed in Carnoy's acetic acid-alcohol (Weesner, 1960) for fixation. Much difficulty was experienced because of lack of penetration of fixing and dehydrating agents. To alleviate this, the egg membranes were punctured at the anterior end, in the yolk region. Both the anterior and posterior ends of the egg membranes were then cut off, allowing free flow of dehydrating and embedding agents past the embryo.

The embryos were transferred successively into 70%, 95% and 100% ethanol, a 1:1 mixture of 100% ethanol-xylene, two changes of pure xylene, paraffin saturated xylene (37°C) and then two changes of pure paraffin in a 58°C oven. Ten micron serial sections of the specimens were cut on a rotary microtome (AO-820) and the sections were affixed to microscope slides using Hoyer's affixative. The slides were dried in a 35°C incubator for 24 hours, then stained with acid fuchsin and counterstained with Mallory's triple. Phosphomolybdic acid (2%) was used to destain. The slides were gradually dehydrated and cleared in xylene. They were mounted using Permount.

Cryostat Method. The method used was the one suggested by International Equipment Company (1964). Water was placed on the specimen holder and quick frozen until the surface of the ice was at least 1/4 inch above the surface of the specimen holder. The egg to be sectioned was positioned on the ice, flooded with water, and quick frozen. The sections were cut at 16 microns and placed on cooled slides previously spread with Hoyer's affixative. Best results were

obtained when the slides with affixed sections were stored in the cryostat chamber for 24 hours before staining.

Sections were fixed in absolute methanol on the slide, then quick-stained. A dropper full of hematoxylin (Weesner, 1960) was added to the slide for one minute. The excess hematoxylin was then drained off and the slide rinsed in two changes of acidified tap water. Alcoholic eosin was run over the slide, which was then rinsed in 95%, then in 100% ethanol. The slide was placed in xylene to clear the sections, then covered using Permount.

Treatment of the Eggs

Dosage

Eggs were dosed with one microliter of fluid from a calibrated topical dose applicator (Figure 1), using a 250 µl syringe with a 27 gauge needle. The applicator wheel was notched so that each partial rotation (1/5 of a complete revolution) delivered 0.50 µl/drop of fluid. Therefore, the wheel was turned through two notches, the petri dish containing the eggs was brought up to the tip of the needle and the drop transferred from the needle to an egg. This procedure was repeated with each egg.

A common solvent was needed to dissolve the pesticides, and to dilute them to the desired strength. Through experimentation it was found that DMSO (dimethyl sulfoxide) was a desirable solvent in that it

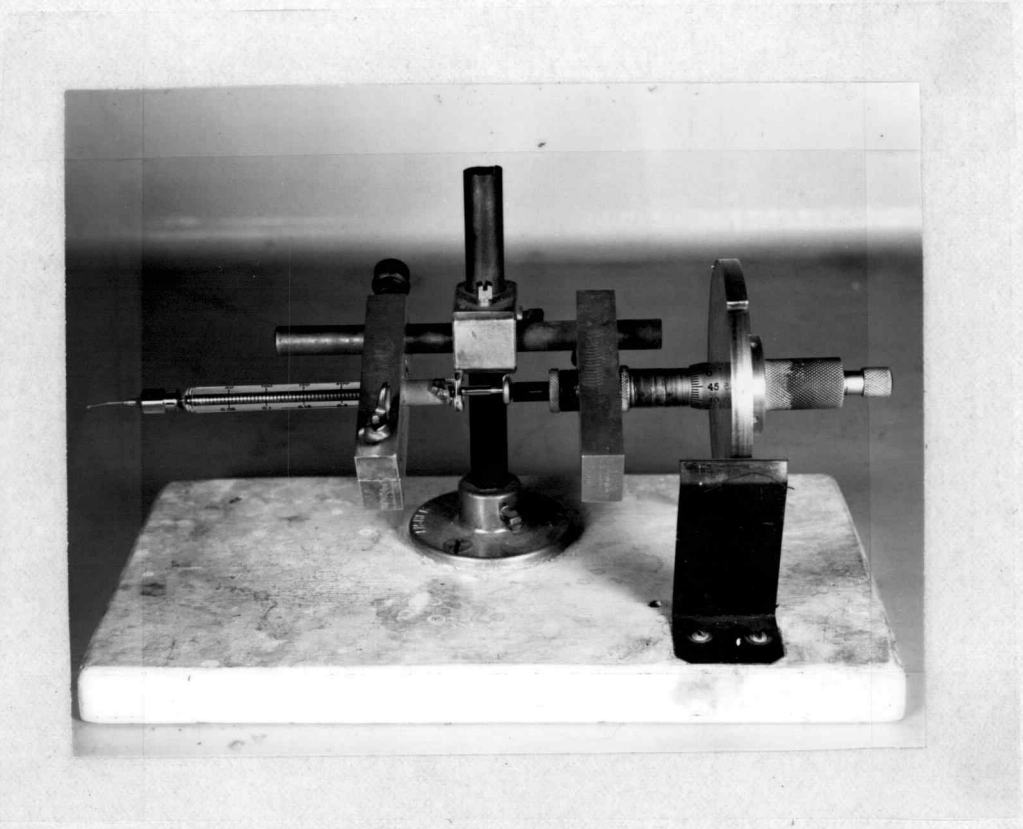

Figure 1. Topical dose applicator.

dissolved all the pesticides and in turn was not a cholinesterase depressant.

Tiguvon[2] was prepared in concentrations of 100, 200 and 300 mg of actual toxicant/liter of DMSO. Concentrations of Ruelene[3] were 500, 800, and 1000 mg actual toxicant/liter of DMSO; concentrations of Sevin[4] and Baygon[5] were each 600, 800, and 1000 mg actual toxicant/liter of DMSO. Different sets of eggs were dosed at 0, 1, 2, 3, 4, 5, 6, 7, and 8 days after oviposition. Any individual was dosed only once in embryonic life, then was moved to a new petri dish containing clean filter paper. Each experiment was repeated three times.

Preserving Eggs and First Instar Nymphs

Samples of 15 eggs were taken daily from the day of dosage until after eclosion (day 9). Samples were rinsed in distilled water, dried, placed in four dram vials, quick frozen, and then stored at -5°C until analysis. Best results were obtained if the eggs were placed in

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[2] Tiguvon, 0, 0-dimethyl 0-(4-(methylthio)-m-tolyl) phosphorothioate, furnished by Chemagro Corporation, Kansas City, Missouri (Appendix I).

[3] Ruelene, 0, -4-tert-butyl-2-chlorophenyl 0-methyl methylphosphoramidate, furnished by Dow Chemical, Midland, Michigan (Appendix I).

[4] Sevin, 1-naphthyl methylcarbamate, furnished by Union Carbide Corp., New York (Appendix I).

[5] Baygon, 0-isopropoxyphenyl methylcarbamate, furnished by Chemagro Corp., Kansas City, Missouri (Appendix I).

absorbant tissue before being frozen, otherwise they tended to adhere to the sides of the vials. Nymphs were aspirated by suction pump, then transferred to four dram vials and frozen. All eggs and nymphs were analyzed for enzyme activity within ten days after freezing.

Controls

Appearance and increase of the enzyme, cholinesterase, was determined by analyzing untreated samples of eggs from the time of oviposition until after eclosion. Twelve samples, consisting of 15 eggs/sample, were analyzed for each day of embryonic life, to establish a mean and confidence limits for normal cholinesterase (ChE) activity. Tests were run to determine the effects of pure DMSO on the mortality and ChE activity of the eggs. DMSO by itself had no ill effects on ChE activity.

Nymphal Dosage

To determine the ability of a cricket which was dosed in the egg stage to withstand a second dose in the nymphal stage, it was necessary to dose the nymphs with a pesticide whose mode of action was also ChE inhibition. It was found that dosing first instar nymphs by topical dose was impractical because the nymphs were so small that even a one μliter drop tended to drown them. Other methods of pesticide application were attempted, including a contact test and a

vapor test. However, best results were obtained when methyl parathion[6] was mixed with the feed and given to the crickets.

One tenth grams of methyl parathion was mixed with 9.9 grams of powdered chicken feed. The mixture was wetted with acetone and placed on a mechanical shaker until the acetone was evaporated. This formed a 10,000 ppm mixture. Further dilutions with chicken feed brought the concentration of pesticide in the feed to the test concentration of 10 ppm. The dilutions always involved the use of acetone to insure that the pesticide was evenly distributed throughout the feed. Controls were fed powdered chicken feed washed with acetone and mixed until dry on a mechanical stirrer.

The tests were run in glass pint jars. The bottom of the jar was covered with sand. Water was provided by an inch long piece of cotton wick, placed on a glass microscope slide to prevent the water from dampening the sand. Food in excess was provided. Approximately 25 first instar nymphs (less than one day old) were placed in the jar. Mortality of crickets in each jar was determined after 24 hours.

Each jar held crickets that had all been treated the same way during embryonic life. This way the mortality of crickets dosed with different pesticides could be compared to each other and to the

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[6]Methyl Parathion, 0,0-dimethyl 0-p-nitrophenyl phosphorothioate, furnished by American Cyanamid, Princeton, New Jersey (Appendix I).

mortality of crickets not dosed during embryonic development.

Cholinesterase Analyses

Radioisotopic Method

A modification of the method of Reed, Goto and Wang (1966) was used for ChE analysis in this experiment. Each sample (10-15 eggs or nymphs/sample) was weighed to the nearest microgram, and placed in a two ml Ten Broeck tissue grinder. Phosphate buffer (Appendix II) was added to give a 1:100 w/v dilution (1 μg per 100 μl) before the eggs were homogenized. Fifty μl of the resulting homogenate were transferred into a 15 ml centrifuge tube, containing 50 μl of the substrate acetyl-1-C^{14} choline (Appendix II). Duplicate runs were made of each sample. The tubes were placed in a 37°C oven and allowed to incubate for ten minutes. During this time, the cholinesterase in the eggs reacted with the substrate, breaking it to acetic acid-C^{14} and choline. After ten minutes, the tubes were removed from the incubator and 300 mg of ion-exchange resin (Appendix II) were added to each tube via a constant delivery device (Smith, Goulding and Priano, 1970). The resin caused the precipitation of the choline and the unhydrolyzed substrate, leaving only the hydrolyzed, labeled acetic acid in solution. Five ml of 100% ethanol were added to each tube and the tubes were shaken vigorously, then centrifuged at 1400 x g for four minutes. Four ml of the supernatant were pipetted into 20 ml counting vials,

containing ten ml of scintillation counting solution (Appendix II). The radioactivity was counted in a Packard Tri-Carb liquid scintillation counter (Model 314EX). Activity was recorded on the machine as counts per ten minutes, which was converted to micromoles of substrate hydrolyzed per minute, as a measure of cholinesterase activity.

Sample blanks were prepared in the same way, except that the homogenate was boiled for 15 minutes before it was added to the substrate. Blank readings provided a correction for background activity and nonenzymatic hydrolysis. Analysis to determine statistical significance of data was based on tests in Mendenhall (1967).

Titrametric Method

Since the radioisotopic method is at the present time limited to use with one substrate, acetylcholine, the titrametric method was used to further characterize the enzyme. The analysis was based on the method described by Nachmansohn and Wilson (1955). Substrates used were acetylcholine, acetyl-methyl-choline, and benzoyl choline.

For the test, the automatic titrator (TTT1) was first standardized. Then 0.6 ml of sodium barbitol (0.02M, pH 7.4), 0.6 ml of magnesium chloride (0.2M) and 1.4 ml of distilled water were added to the titration chamber. One tenth ml of enzyme was added, then the reaction was adjusted to pH 7.4. The automatic titrator was set to a

pH of 7.4. At time zero, substrate was added to the titration chamber, and as the enzyme reacted with the substrate, liberating acetic acid, the titrator added 0.02N NaOH to hold the pH in the reaction vessel constant at 7.4. The amount of base added/unit time was automatically recorded on a chart, and from this the μmoles of substrate hydrolyzed was calculated by the formula:

$$\frac{\mu M \text{ substrate hydrolyzed}}{\text{minute}} = \frac{\mu l \text{ of NaOH added}}{\text{minute}} \times 0.02 \ \mu equiv/\mu l$$

Kinetics studies were run on both pure enzymes and cricket egg homogenates to determine the effect of enzyme and substrate concentrations. Cricket egg homogenates were made by diluting 1/100 w/v with distilled water. Based on the activity of the pure enzymes, the amount of enzyme in the eggs giving the same activity could be determined. Blanks were determined by using enzyme or cricket homogenate which had been placed in an 81°C water bath for 20 minutes to inactivate the enzyme.

Determination of Percent Penetration of Pesticides

To determine the percent pesticide which penetrated through the chorion, a bioassay was run to determine the amount of pesticide present inside dosed eggs. Eggs were dosed as before and samples of 100 eggs were collected 0, 15, 30, 60, and 120 minutes after dosage. Fifty eggs from each sample were washed in distilled water, blotted dry, and homogenized in 0.5 ml of distilled water; the other 50 eggs

were dechorionated in 5.25% sodium hypochlorite, blotted dry, and homogenized in 0.5 ml of distilled water. It was found that there was no significant difference between washed and dechorionated eggs. Two replicates were run of each experiment.

The egg homogenates were placed in an 81°C water bath for 20 minutes to inactivate the enzyme. A bioassay to determine the amount of pesticide present in the eggs was run using the titrametric method (see above). The enzyme used was 0.5 μg pure acetylcholinesterase/assay; the substrate was 1.1 μM acetylcholine/assay. Egg homogenate (0.2 ml) was added to the reaction mixture before the addition of acetylcholine, and the resulting inhibition of reaction was read against a standard curve to determine the total pesticide present in 0.2 ml homogenate. This total pesticide was divided by 20 to determine the average amount of pesticide present in each egg. Results were expressed as percent of the pesticide penetrating through the chorion. Percent penetration was determined for each pesticide for days 3 through 8 of embryonic life.

Inhibitor Constants

Inhibition constants (K_i) were determined for each pesticide on egg homogenate by the method of Dixon (Dixon and Webb, 1959). The enzyme and substrate concentrations were held constant, and the amount of inhibitor added was varied. A similar series of tests was

run at a higher substrate concentration and the same enzyme concentration and the inverse of the velocity was plotted against inhibitor concentration for both substrate concentrations. The intersection of the two lines gave $-K_i$ directly, K_i is the reciprocal of the affinity of the enzyme for the inhibitor.

RESULTS AND DISCUSSION

Normal Morphological Development

Embryonic Development

For convenience in determining the effect of pesticides on the length of development, ten embryonic stages were selected, similar to the developmental stages of Gryllus assimilis, described by Rakshpal (1962b).

Stage I. Within 20 hours, the embryo lies as a small band of cells at the posterior pole of the egg. This is visible in sectioned eggs under a compound microscope (745X), but not in intact eggs.

Stage II. The embryo has moved to the dorsal surface of the yolk. It is visible in cleared eggs and consists of two parts: an anterior, wide, shorter protocephalic region and a posterior, longer, more narrow, protocormic region (Figure 2).

Stage III. The embryo has moved from the dorsal surface and lies in a U shaped position at the posterior pole. It is longer than Stage II and the protocephalic region has begun to develop lateral lobes (Figure 3).

Stage IV. In this stage, the embryo occupies the ventral surface of the egg. The protocephalic region is now facing caudad. Primary segmentation has begun (Figure 4).

Stage V. At this point the embryo has sunk into the yolk and is

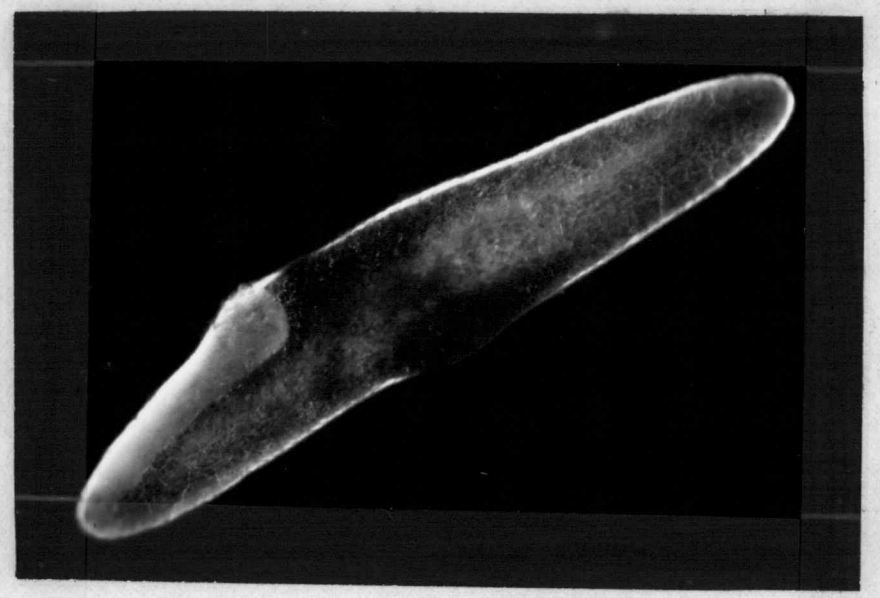


Figure 2. Stage II embryo (cleared).

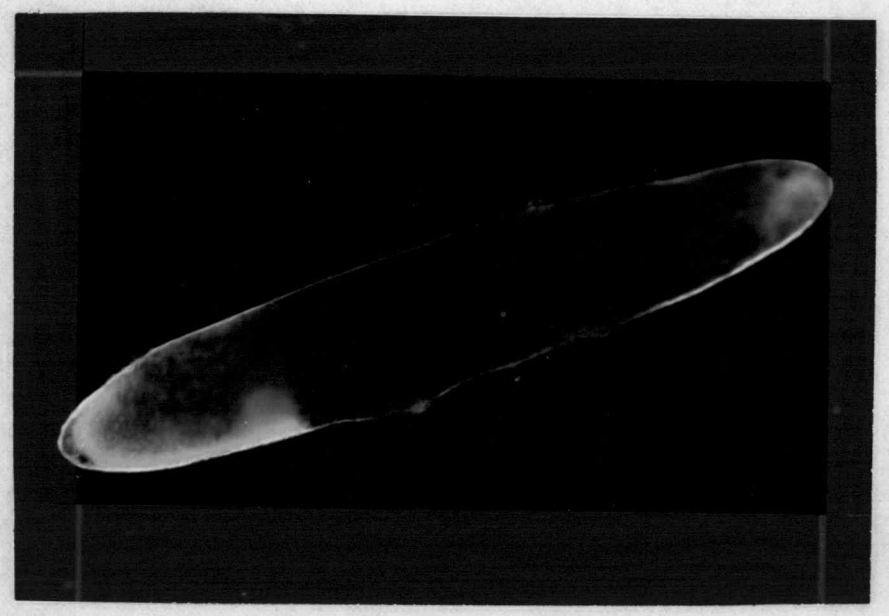


Figure 3. Stage III embryo (cleared).

not visible. During this stage, blastokinesis occurs as well as an increase in segmentation and the formation of appendages.

Stage VI. In this stage, the embryo has emerged from the yolk. The embryo faces caudad and lies on the dorsal surface near the posterior end of the egg. It begins to rotate until it lies on the ventral surface with its cephalic end toward the anterior pole (Figure 5).

Stage VII. This stage is characterized by the appearance of the red eye spots.

Stage VIII. In this stage, the embryo occupies the full length of the egg (Figure 6). The yolk is confined to a small area dorsad to the embryo; it is absorbed at the end of this stage.

Stage IX. This stage is characterized by the appearance of a pair of black lines, marking the lateral margins of the labrum.

Stage X. In this stage, the body becomes covered with rows of bristles.

Table 1 gives the time of formation of each stage at a constant temperature of 35°C. These eggs were all laid within one hour and examined hourly for changes in stages.

Eclosion

The chorion splits down the dorsal surface. There does not seem to be any special structure to aid in eclosion. The split runs from approximately the anterior region of the thorax through the

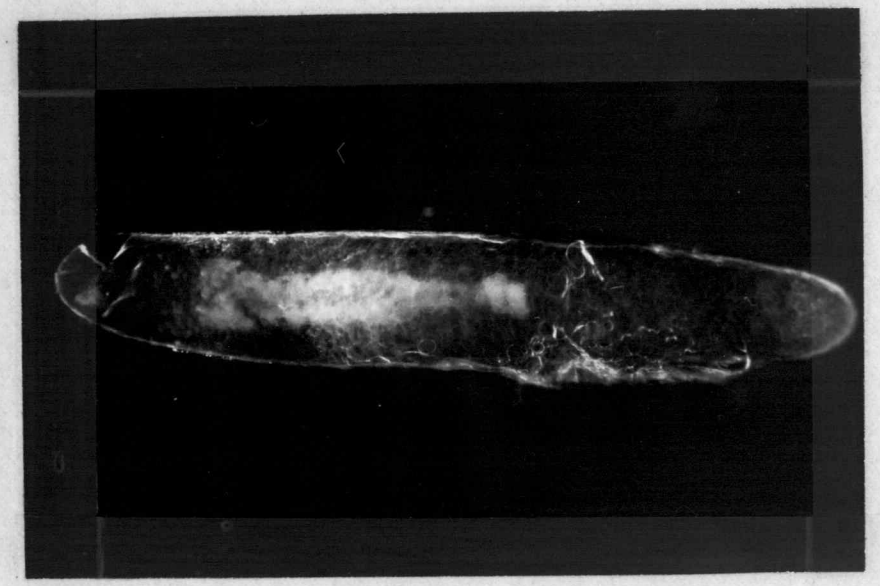


Figure 4. Stage IV embryo (cleared).

Figure 5. Stage VI embryo.

Table 1. Time of development of Acheta domesticus embryos at a constant temperature (35°C).

Stage	Mean time in hours	95% C. L.
II	30	±0.63
III	36.2	±0.62
IV	39	±0.63
V	42	±1.75
VI	84.5	±1.19
VII	101.4	±0.96
VIII	115.2	±2.18
IX	138.6	±1.10
X	180.0	±1.01
hatching	191.1	±4.26

anterior abdominal segments. The animal forces itself out of the chorion by a series of muscular contractions.

Once out of the chorion, the nymph is still encased in a sheath, called the embryonic membrane (Slifer, 1937; Brookes, 1952). The head is in an opisthognathous position (Figure 7).

With ventral side down, the larva goes through a series of dorso-ventral movements of the abdomen, which frees the cerci from their obtect condition. Balancing on its elongated head and its cerci, the nymph starts a series of caudal to cephalic contractions. The dorso-caudal section of the head is pulled back toward the prothorax, causing the head to assume its typical hypognathous shape. The nymph then sheds the embryonic membranes, freeing the legs and head

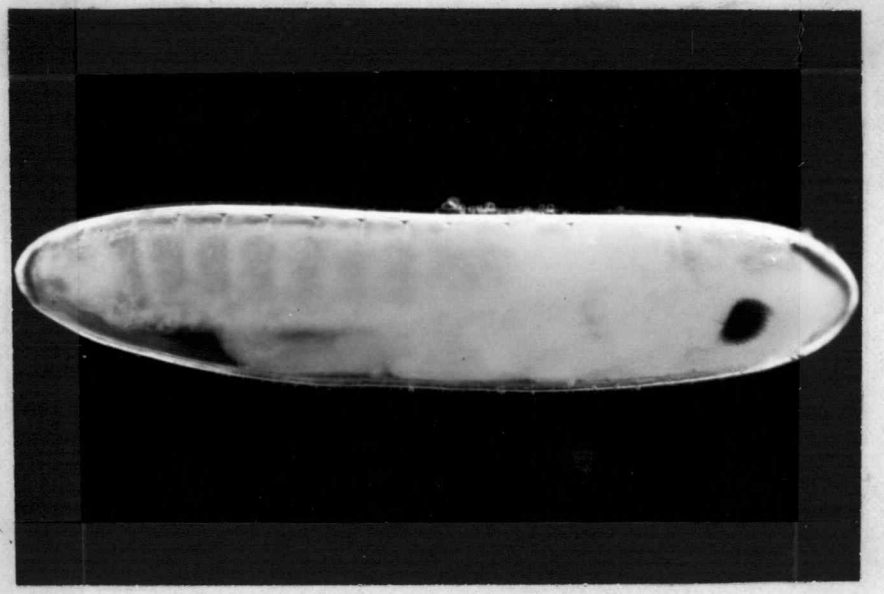


Figure 6. Stage VIII embryo.

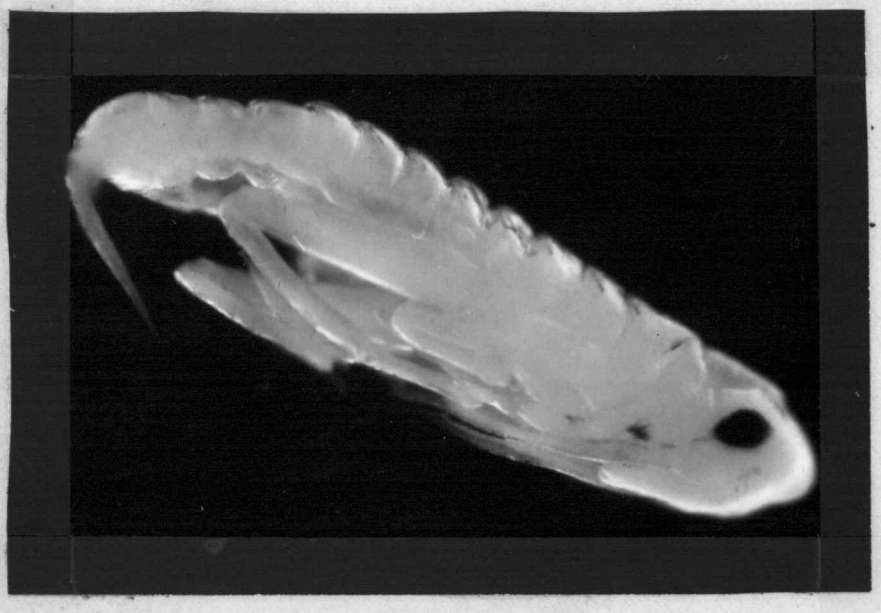


Figure 7. Nymph encased in embryonic membrane.

appendages first, and finally each individual cercus. The nymph can immediately walk and jump.

Effect of Anticholinesterase on Development

Embryonic Development

The four pesticides were each applied to eggs that were 3, 4, 5, 6, 7, or 8 days old. This included eggs in stages V-X. In all cases, development proceeded normally through Stage X. In no case was the development of the embryos (based on stages) significantly different from the controls. This agreed with most literature published on the effect of anticholinesterases on insect embryos (Smith and Salkeld, 1966).

Eggs were also dosed at days 0, 1, and 2 of embryonic life. There was 100% mortality by day 3 in all cases. However, eggs dosed with pure DMSO at these times also had early mortality.

This phenomenon has been previously reported with eggs of Diataraxia oleracea L. and Ephestia kuhniella Zell by Lord and Potter (1951), when they dosed eggs with high concentrations of parathion. Lord and Potter (1951) postulated that anticholinesterase activity was not necessarily the only way in which organophosphates could exert their toxic action on insect embryos. Similar early mortality of insect eggs was also obtained with compounds (carbolic acid derivatives) with no anticholinesterase activity (Karczmar, 1963).

Therefore, the early mortality of cricket eggs could be attributed to noncholinergic activity of DMSO or the noncholinergic activity of both pesticide and DMSO.

Eclosion

The data on the mortality due to Ruelene, Tiguvon, Sevin, and Baygon are presented in Tables 2-5, respectively. The mortality is based on samples of 50 eggs. Tests were run in triplicate. Controls had an average percent hatch of 95.7%. The 95% confidence limits ranges from 88.8 - 100+%.

At the dosages tested, only Tiguvon caused mortality significantly greater than in the control eggs. The earliest and latest stages seemed to be more susceptible than the middle stages. On the whole, the embryonic stages of house crickets did not appear to be very susceptible to poisoning by the anticholinesterases used in this study.

Development of Cholinesterase Activity in the Embryo

Characterization of the Enzyme

The action of the enzyme found in the embryo was compared to the action of pure acetylcholinesterase (AChE) isolated from the electric eel and the action of butyryl cholinesterase (BuChE) isolated

Table 2. Mortality of eggs treated with Ruelene.

Dosage Rate	Day Treated	Percent Mortality
0.5 µg/egg	3	3.2
	4	2.8
	5	0
	6	0
	7	3.7
	8	3.6
0.8 µg/egg	3	5.6
	4	0
	5	5.8
	6	0
	7	3.3
	8	3.7
1.0 µg/egg	3	2.7
	4	0
	5	6.4
	6	0
	7	0
	8	3.7

Table 3. Mortality of eggs treated with Tiguvon.

Dosage Rate	Day Treated	Percent Mortality
0.1 µg/egg	3	0
	4	10.7
	5	15*
	6	3.6
	7	0
	8	6.5
0.2 µg/egg	3	14.0*
	4	13.7*
	5	10.5
	6	0
	7	4.2
	8	34.3*
0.3 µg/egg	3	26.3*
	4	16.3*
	5	7.3
	6	3.6
	7	46.4*
	8	31.1*

*Significantly (95% confidence limit) different from the control.

Table 4. Mortality of eggs treated with Sevin.

Dosage Rate	Day Treated	Percent Mortality
0.6 µg/egg	3	6.8
	4	9.0
	5	6.9
	6	0
	7	4.2
	8	3.6
0.8 µg/egg	3	5.6
	4	6.2
	5	0
	6	0
	7	7.8
	8	0
1.0 µg/egg	3	7.3
	4	0
	5	8.4
	6	0
	7	4.7
	8	0

Table 5. Mortality of eggs treated with Baygon.

Dosage Rate	Day Treated	Percent Mortality
0.6 µg/egg	3	0
	4	0
	5	7.7
	6	4.2
	7	7.8
	8	0
0.8 µg/egg	3	10.5
	4	8.8
	5	0
	6	10.0
	7	0
	8	11.1
1.0 µg/egg	3	0
	4	10.3
	5	6.3
	6	7.7
	7	3.6
	8	10.5

from horse serum.[7]

Figure 8 is a graphical representation of the action of AChE on two substrates, acetylcholine (ACh), the nonspecific substrate for cholinesterases, and on acetyl-methyl-choline (AmCh), the substrate specific for AChE (Koelle, 1965). Tests were run to determine the action of AChE on benzoyl choline (BeCh), but there was no reaction, as was expected (Koelle, 1965).

Figures 8A and 8B illustrate the velocity of reaction (v) as the enzyme concentration was varied with each substrate. The pure enzyme was more active with ACh than with AmCh, which agreed with earlier research (Nachmansohn and Wilson, 1955).

Various techniques have been used to graph the effects of varying the substrate concentration on the initial velocity. According to the Linweaver-Burke representation (Dixon and Webb, 1959), 1/s is plotted against 1/v. The y-intercept of the line obtained by the plot of the points is the reciprocal of the maximum velocity (V_{max}) of the reaction. The x-intercept of the line is $-1/K_m$, where K_m is the dissociation constant of the enzyme-substrate complex (the reciprocal of the enzyme-substrate affinity).

Figures 8C and 8D illustrate the effect of varying the concentration of ACh and AmCh respectively. V_{max} of ACh was 0.833 μmoles ACh/minute at an enzyme concentration of 0.2 μg. V_{max} for AmCh

[7] Worthington Biochemical Corporation, Freehold, New Jersey.

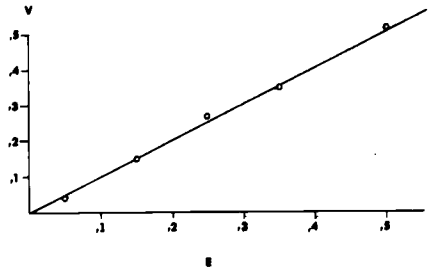


Fig 8A

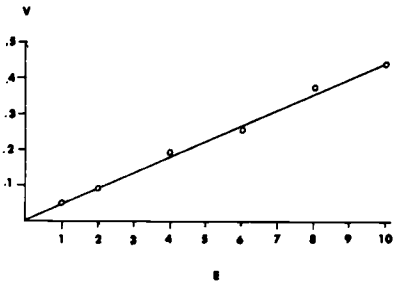


Fig 8B

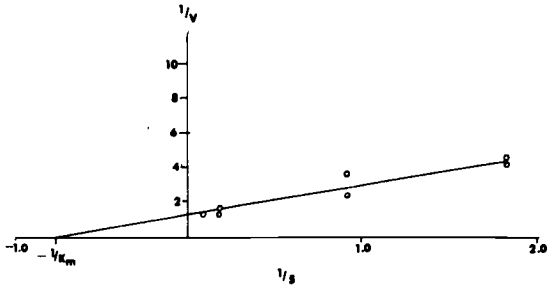


Fig 8C

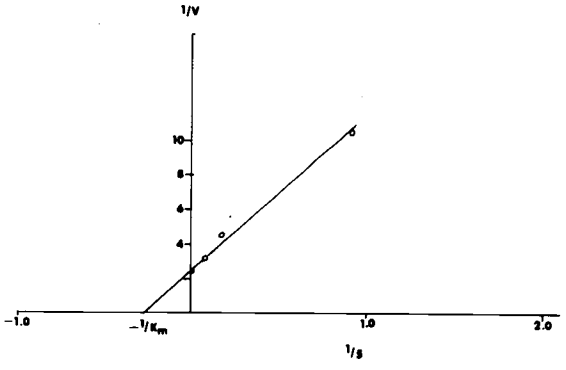


Fig 8D

was 0.417 μmoles AmCh/minute/0.2 μg enzyme. K_m for AmCh (2.78) was larger than the K_m of ACh (1.33) when the enzyme was AChE. Thus AChE had a greater affinity for ACh, than for its specific substrate, AmCh. This agreed with data of Wilson (1960). However, ACh is the naturally occurring substrate for AChE, and AmCh is an artificial substrate.

Figure 9 illustrates the action of butyrylcholinesterase (BuChE), a pseudocholinesterase, on two substrates, ACh and BeCh (benzoyl choline), its specific substrate (Koelle, 1965). Tests were also run to determine the action of BuChE on AmCh, but there was no reaction, as was expected from the literature (Koelle, 1965).

Figures 9A and 9B represent the increase in the velocity of the reaction of BuChE with each substrate as the enzyme concentration was varied. BuChE can be seen to be approximately twice as active with its specific substrate than with ACh. This agreed with the discussion by Augustinsson (1960).

Figures 9C and 9D are Linweaver-Burke plots of reaction velocity as the substrate was varied (1/v against 1/s). With ACh, V_{max} was 0.2 μmoles ACh/minute at an enzyme concentration of 20 μg and the K_m was 6.67. With BeCh, V_{max} was 0.4 μmoles BeCh/minute and the K_m was 4.08.

Graphs in Figure 10 illustrate the action of cricket homogenate on ACh and on AmCh. Figure 10A and 10B illustrate the velocity of

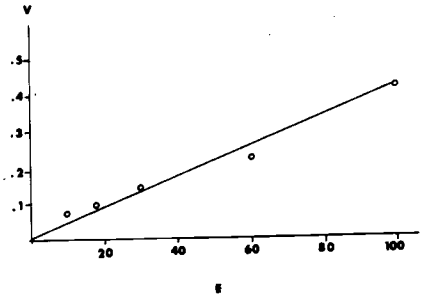


Fig 9A

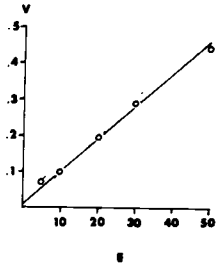


Fig 9B

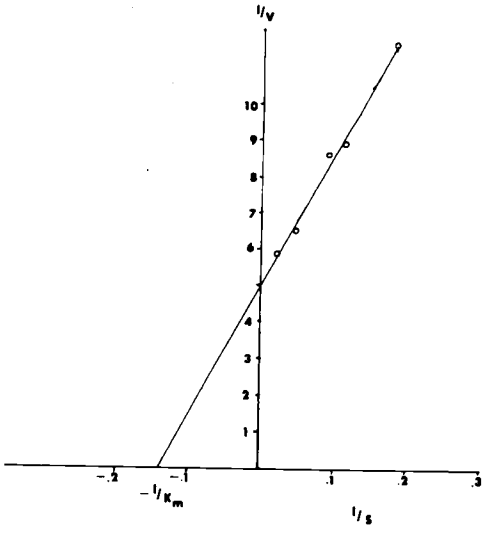


Fig 9C

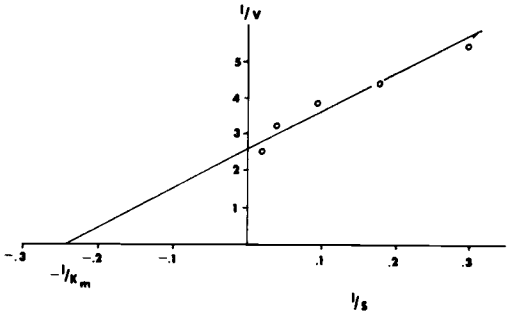


Fig 9D

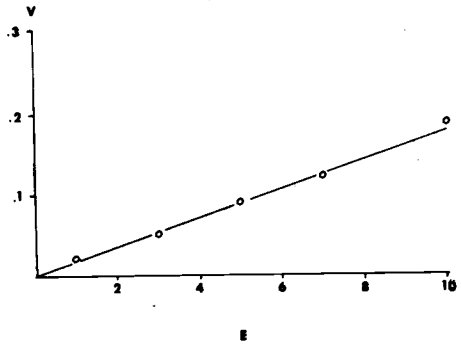


Fig 10A

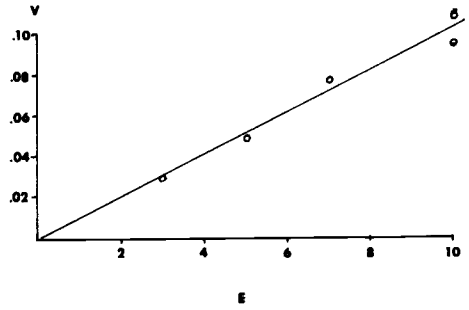


Fig 10B

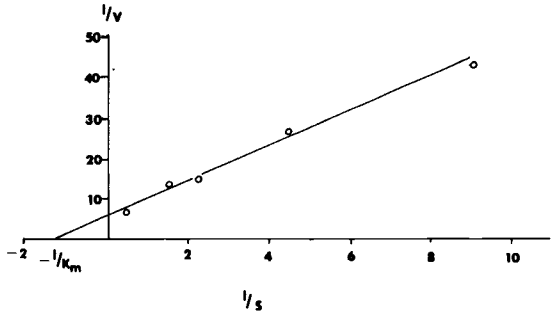


Fig 10C

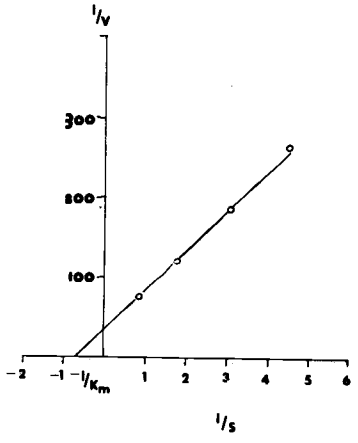


Fig 10D

the reaction as the enzyme concentration is varied with ACh and AmCh. The cricket homogenate was approximately twice as active with ACh as with AmCh. There was no reaction when cricket homogenate was combined with BeCh, indicating that the only enzyme in the cricket which was hydrolyzing ACh was true cholinesterase (AChE).

Figures 10C and 10D are graphs representing effects of substrate concentration. With ACh (Figure 10C), the maximum velocity was 0.2 μmoles ACh/minute and the K_m was 0.833. With AmCh (Figure 10D), the maximum velocity was also 0.2 μmoles AmCh/minute and the K_m was 1.43.

The K_m values for cricket homogenate were not the same as the K_m values for the pure enzyme, when the substrate is either ACh or AmCh. This was to be expected, because source and purity of the enzyme affect the K_m value (Dixon and Webb, 1959).

The effect of pH on velocity of reaction for pure AChE and cricket homogenate is illustrated in Figures 11 and 12, respectively. With either enzyme, there was no reaction below pH 4.8. The reaction reached its maximum velocity at about pH 7, and remained at this level. There was no optimum pH. This agreed with data for pure AChE cited in Dixon and Webb (1959).

Development of Acetylcholinesterase in the Cricket Embryo

The normal AChE activity of the embryos was determined by

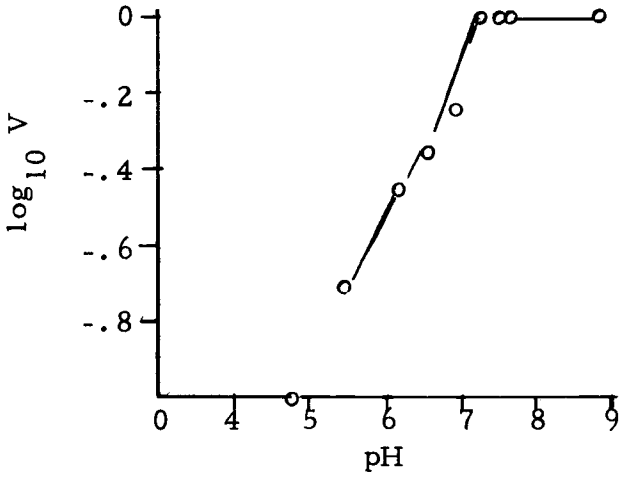


Figure 11. Effect of pH on velocity of reaction of pure acetylcholinesterase acting on acetylcholine.

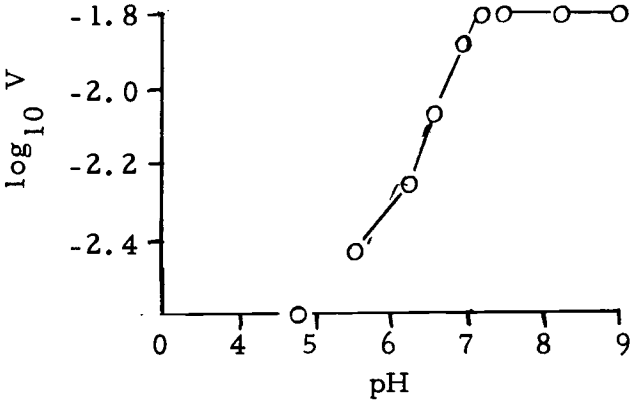


Figure 12. Effect of pH on velocity of reaction of cricket homogenate acting on acetylcholine.

analysing samples of embryos at each day of development. Both the radioisotopic method and the titrametric method were used.

Figure 13 is a graph of the development of the AChE based on one mg of cricket homogenate, analyzed by the radioisotopic method. The enzyme was first present at day 4 and increased thereafter. After day 7, AChE activity leveled off and increased only slightly at the time of hatching (day 9).

Figures 14 and 15 represent the development of AChE activity against ACh and AmCh respectively, using the titrametric method. Cricket enzyme was more active with ACh than with AmCh, but reactions with both substrates showed the same general curve of increase.

An attempt was made to determine the amount of enzyme present in the cricket embryo at various days of development. On the basis of the titrametric method, one mg of day 5 embryo had an AChE activity equal to 2.8×10^{-3} µg of pure AChE; one mg of day 6 embryo had an AChE activity equal to 15.2×10^{-3} µg of pure AChE; one mg of day 7 embryo had an AChE activity equal to 19.0×10^{-3} µg of AChE; one mg of day 8 cricket embryo had an AChE activity equal to 19.6×10^{-3} µg of pure AChE; one mg of first instar cricket nymph had an AChE activity equal to 21.0×10^{-3} µg of pure AChE.

The radioisotopic method was used for analysis of the pesticidal effect on AChE activity of embryos as, through experimentation, it was

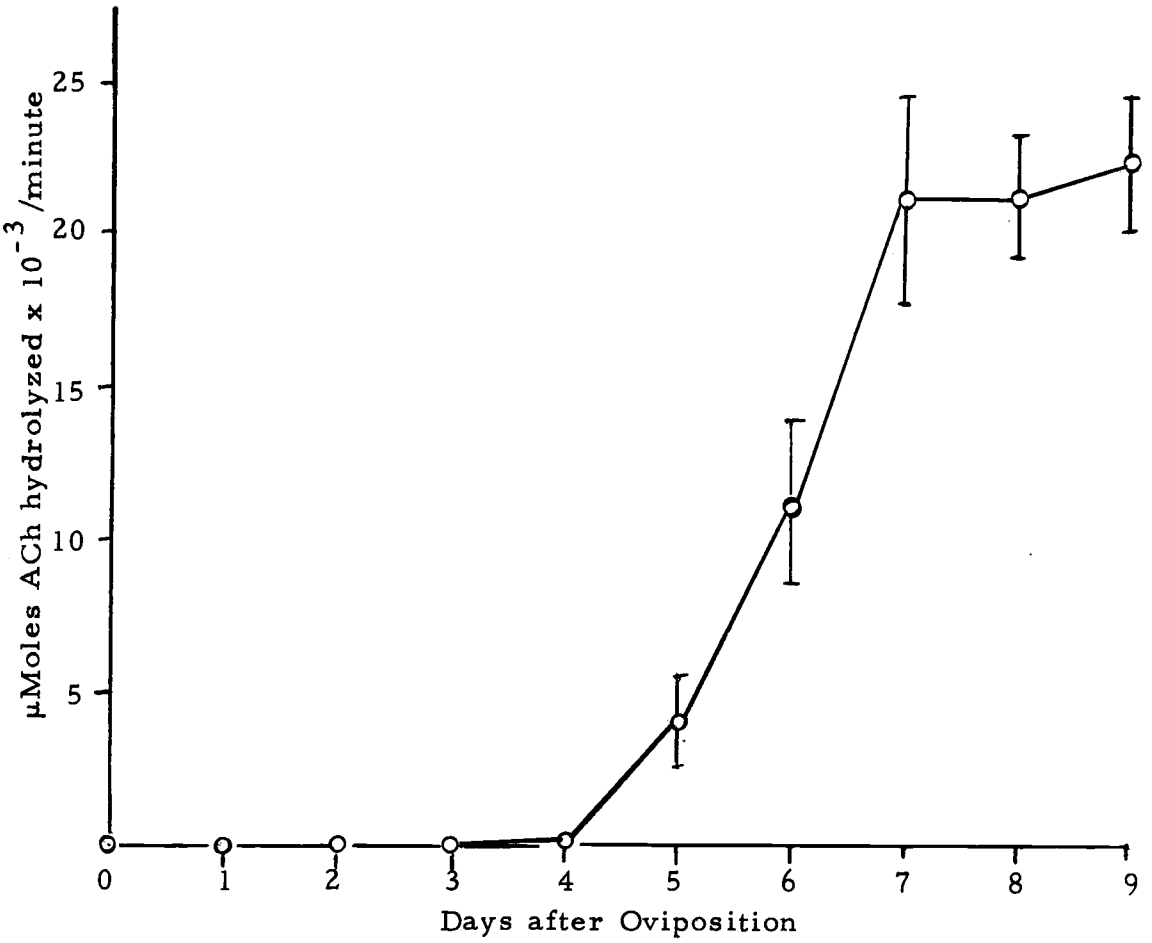


Figure 13. Development of cholinesterase in cricket eggs (radioisotopic method).

Note: vertical lines indicate 95% confidence limit.

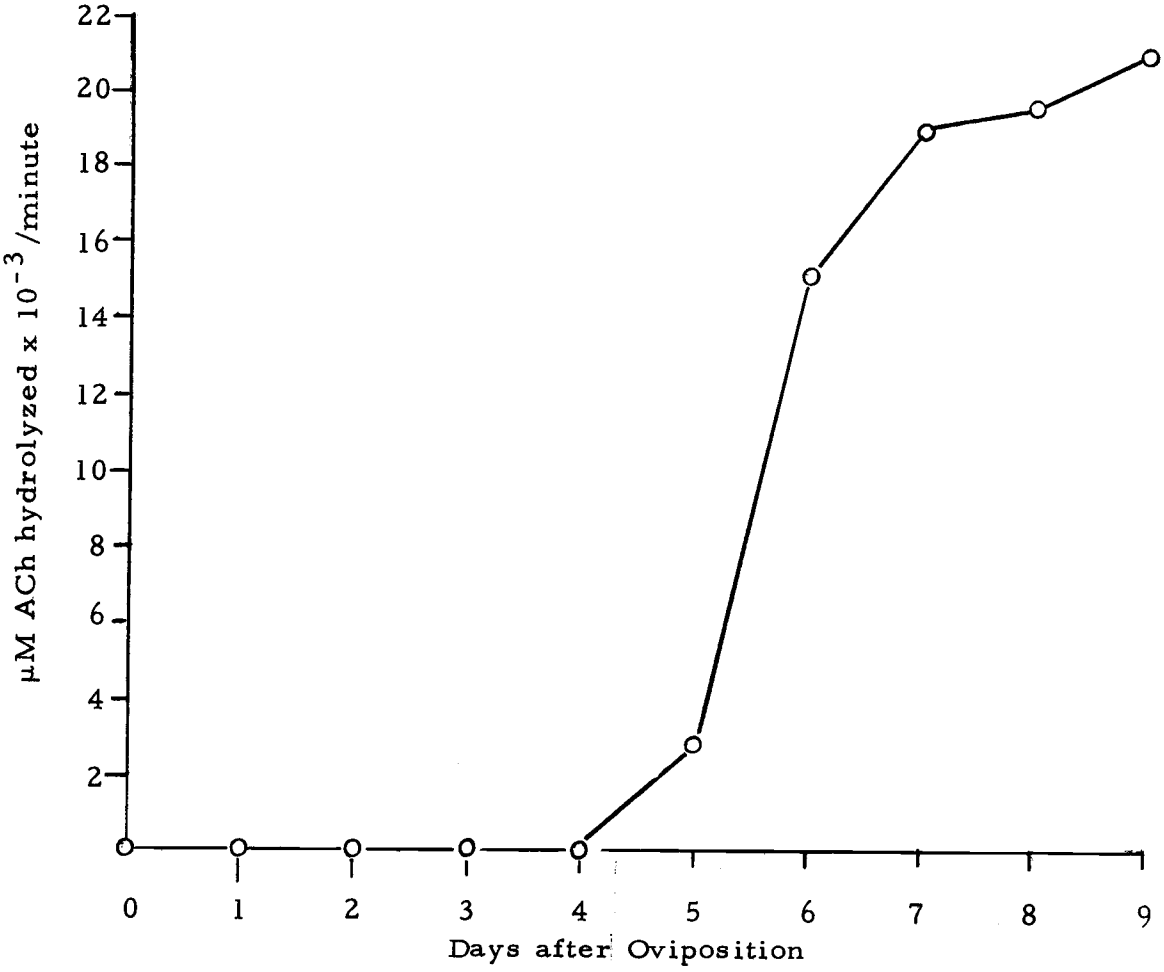


Figure 14. Development of cholinesterase in cricket eggs, based on titrametric method (substrate acetylcholine).

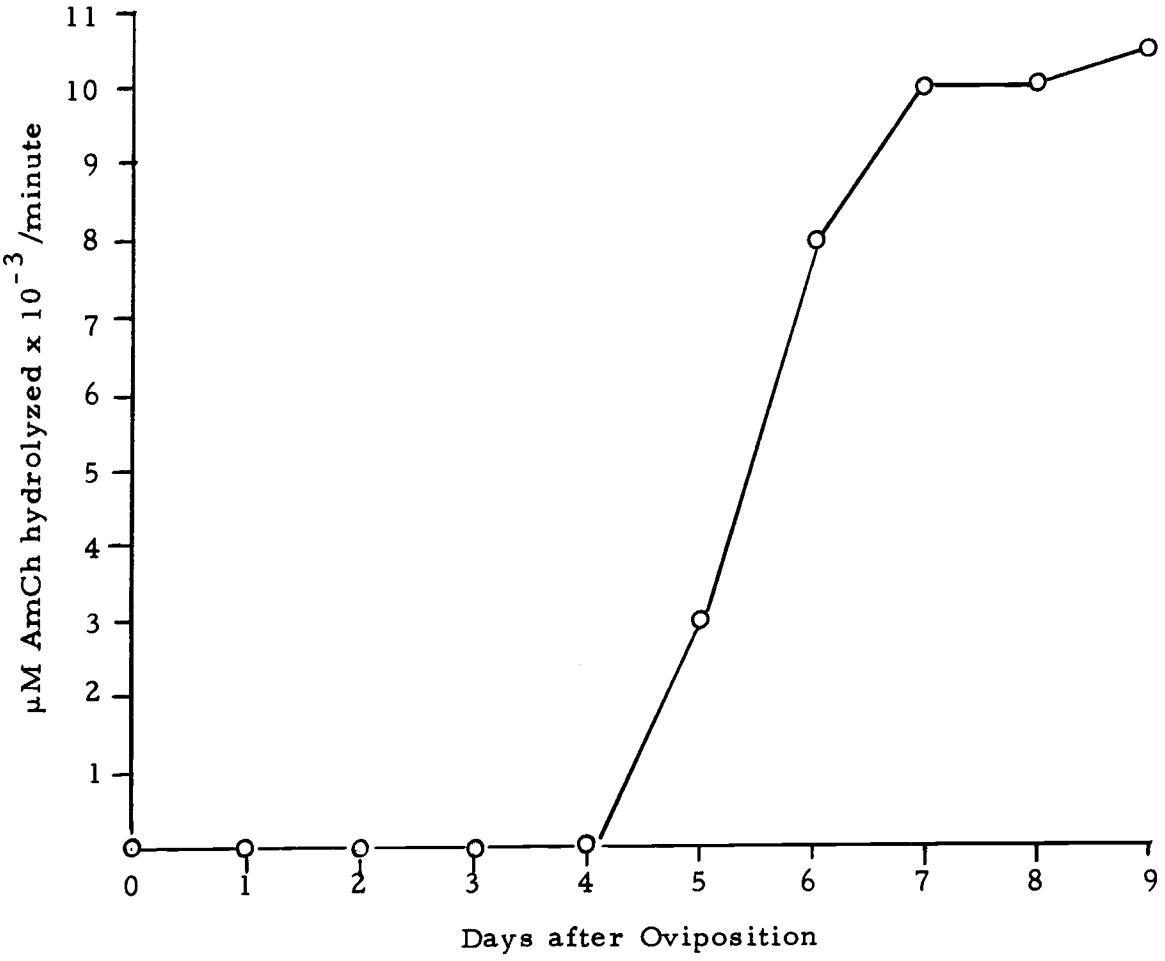


Figure 15. Development of cholinesterase in cricket eggs, based on titrametric method (substrate acetyl-methyl-choline).

proved that the radioisotopic method was much more sensitive than was the titrametric method. The former established the presence of AChE on the fourth day, while with the latter, presence of AChE was not indicated until day 5.

Degree of Development of Nervous System when AChE Appears

The first appearance of ChE in insect embryos and the relation to neurogenesis has been studied in six insects. In all of the species, the ChE appeared during or just after blastokinesis, when the nerve cord was formed into ganglia and the commissures and connectives were visible (Salkeld, 1964). In A. domesticus embryos, AChE first appeared during blastokinesis and increased until hatching.

In Drosophila melanogaster Meigen, AChE was found in infertile eggs. This finding seems anomalous with the rest of the data and may represent primer amount of AChE or may indicate that AChE performs a different function in early ontogeny (Smallman and Mansingh, 1969). AChE was not found in infertile eggs in this study.

Figures 16-19 illustrate the morphological development of the nervous system at the time of first appearance of the AChE. The brain and ventral nervous system were well developed. The ganglia were each divided into an interior fibrous core surrounded by a cellular cortex (Figure 16).

The supraoesophageal ganglion was subdivided into three lobes--

Figure 16. Brain and thoracic ganglia of four day old cricket embryos.

CC - Cellular cortex

FC - Fibrous core

T - Thoracic ganglia

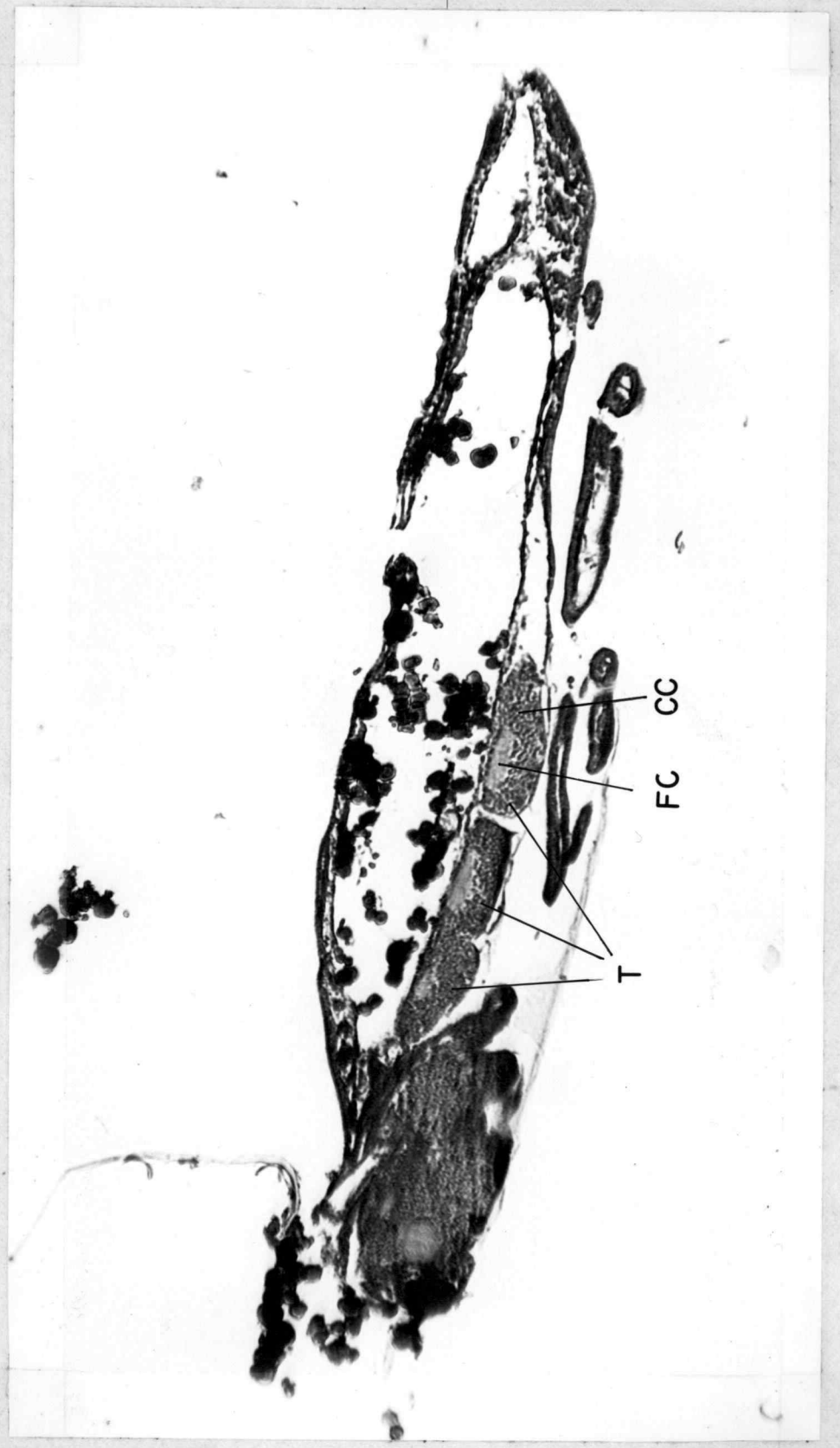

CC
FC
T

Figure 17. Circumoesophageal connectives of four day old cricket embryo.

CrC - Circumoesophageal Connectives

SG - Supraoesophageal Ganglion

SbG - Suboesophageal Ganglion

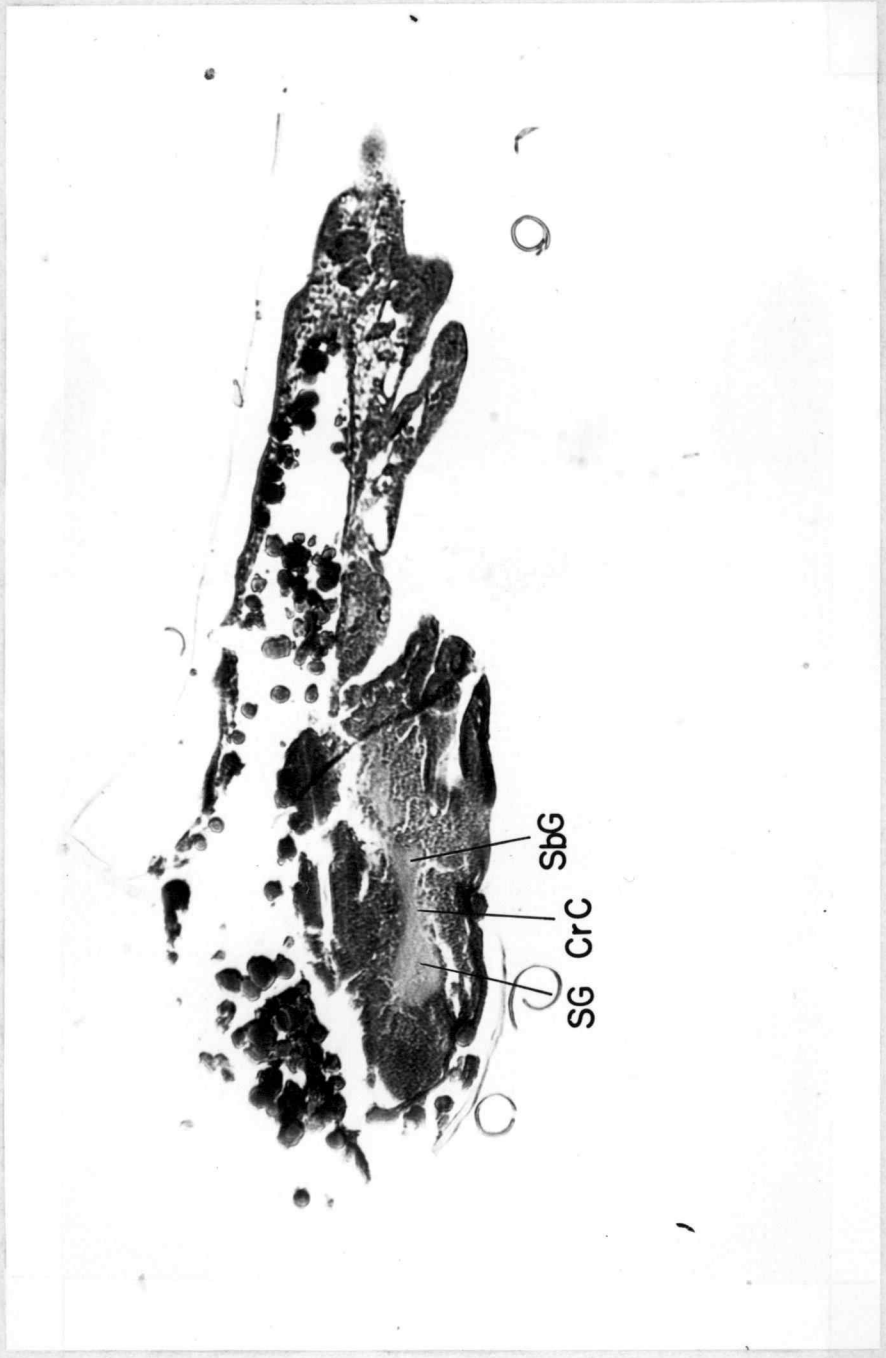

SbG
CrC
SG

Figure 18. Frontal ganglion of four day old cricket embryo.

F - Frontal Ganglion

SG - Supraoesophageal Ganglion

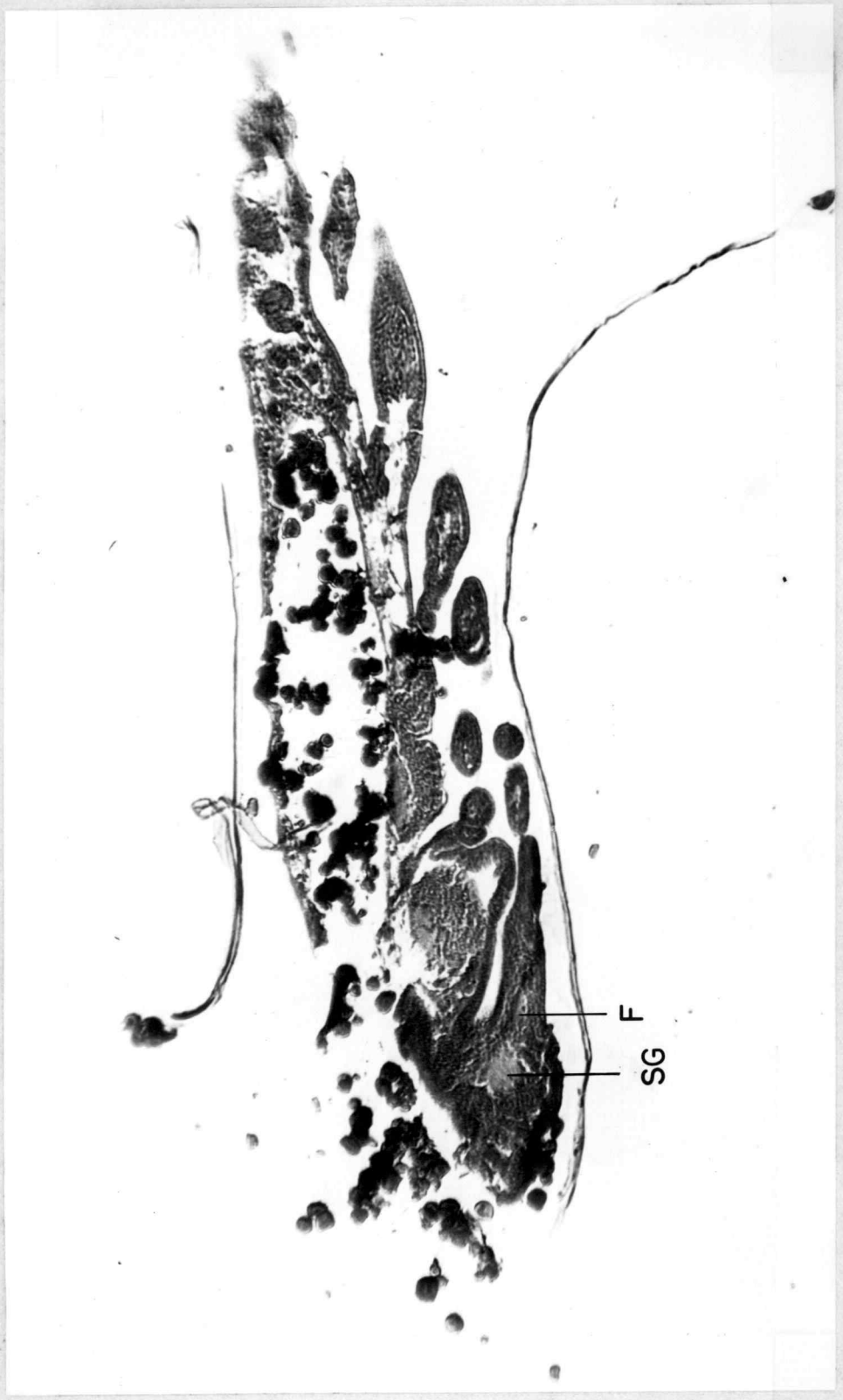
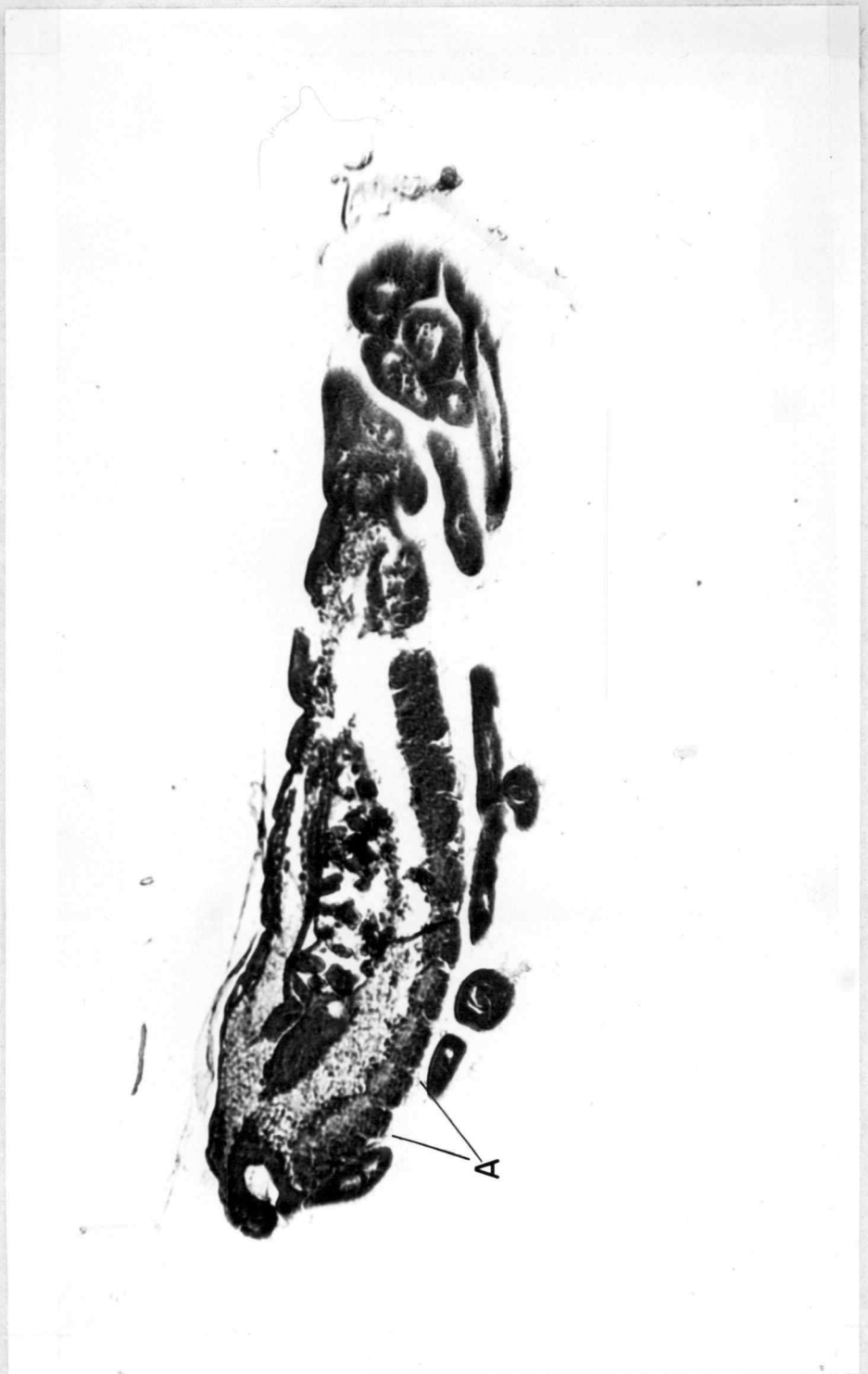

F
SG

Figure 19. Abdominal ganglia of four day old cricket embryos.

A - Abdominal ganglia


A

proto-, deuto-, and tritocerebrum. The optic lobes, attached to the protocerebrum, were present and well developed, although the eyes were not pigmented at this time. The circumoesophageal connectives were also developed (Figure 17). The suboesophageal ganglion had a trilobed fibrous cortex, indicating a fusion of the nerve ganglia of three segments. In the head region, the frontal ganglion was present, the only part of the stomatogastric system observable at this time (Figure 18).

There were three distinct thoracic ganglia, connected by fibers running between the fibrous cores of adjacent ganglia. The cellular cortex surrounded the fibrous core in the connectives also (Figure 16).

There were ten abdominal ganglia, although there was a tendency toward fusion of the last three. The ganglia each consisted of a fibrous core and cellular cortex (Figure 19).

Effect of Pesticides on Cholinesterase Activity

Ruelene

Figures 20-25 represent the effect of the three concentrations of Ruelene on the development of AChE activity.

When the pesticide was applied at day 3 (Figure 20), before the appearance of the enzyme, the enzyme activity was depressed on day 4, but rose rapidly to above normal levels on day 5. It remained elevated significantly above the mean throughout the remainder of

Figure 20. Effect of Ruelene on the AChE activity level of developing cricket eggs, when applied three days after oviposition.

Figure 21. Effect of Ruelene on the AChE activity level of developing cricket eggs, when applied four days after oviposition.

- o 0.5 μg Ruelene
- Δ 0.8 μg Ruelene
- □ 1.0 μg Ruelene
- * Significantly different from the mean at the 95% confidence level.

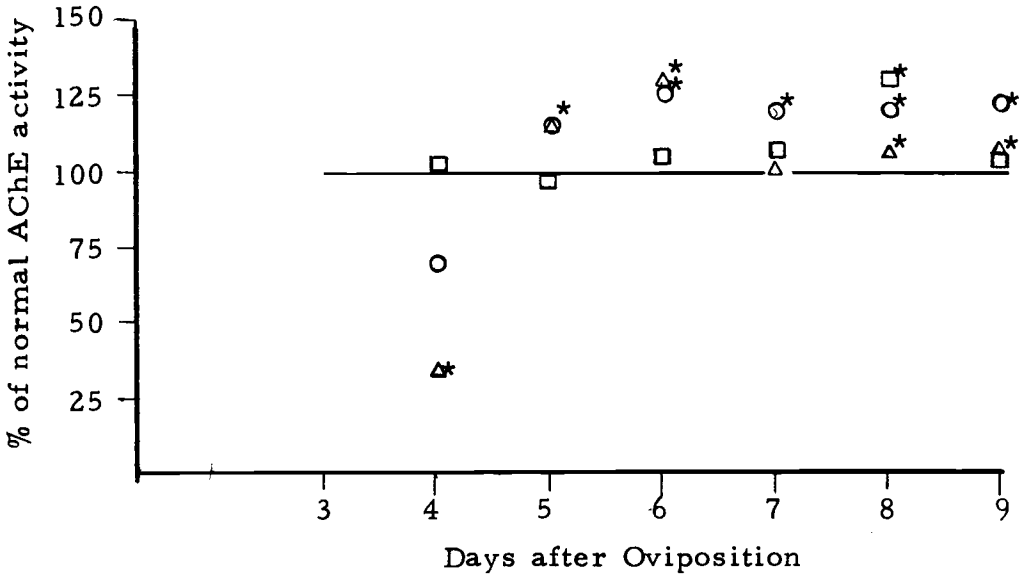


Figure 20.

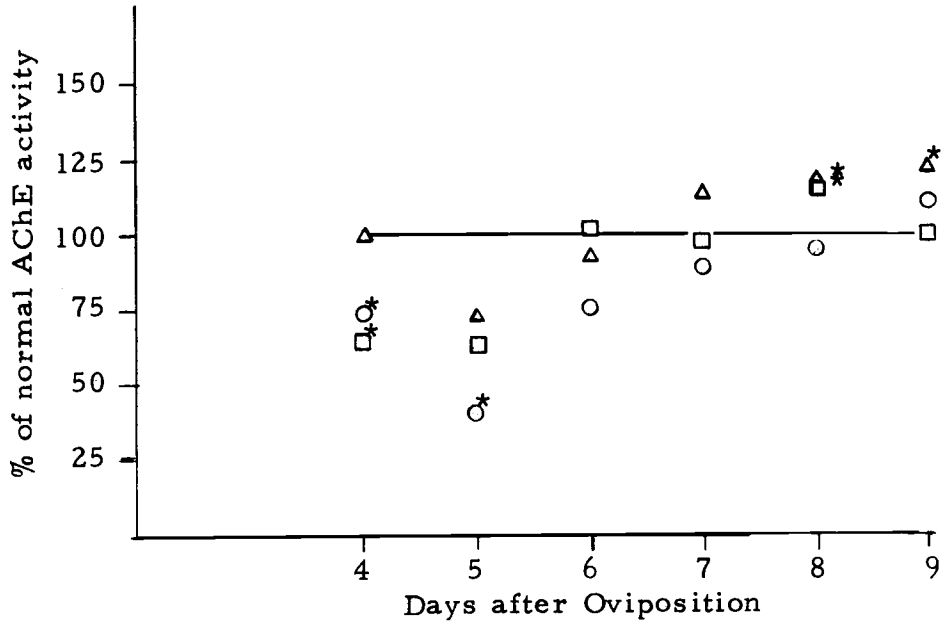


Figure 21.

Figure 22. Effect of Ruelene on the AChE activity level of developing cricket eggs, when applied five days after oviposition.

Figure 23. Effect of Ruelene on the AChE activity level of developing cricket eggs, when applied six days after oviposition.

Figure 24. Effect of Ruelene on the AChE activity level of developing cricket eggs, when applied seven days after oviposition.

Figure 25. Effect of Ruelene on the AChE activity level of developing cricket eggs, when applied eight days after oviposition.

- ○ 0.5 μg Ruelene
- △ 0.8 μg Ruelene
- □ 1.0 μg Ruelene
- * Significantly different from the mean at the 95% confidence level.

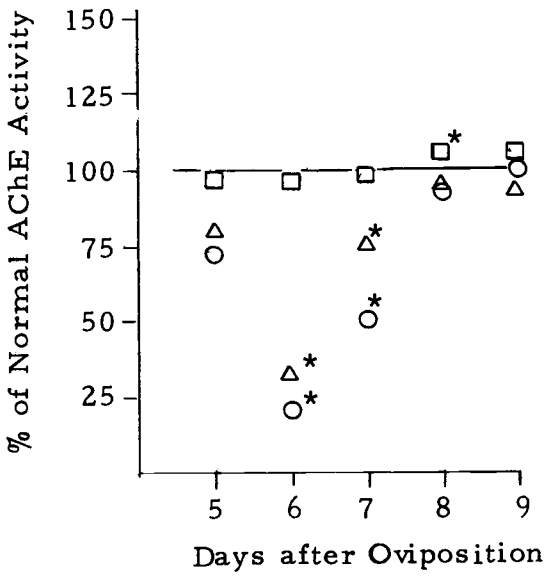


Figure 22.

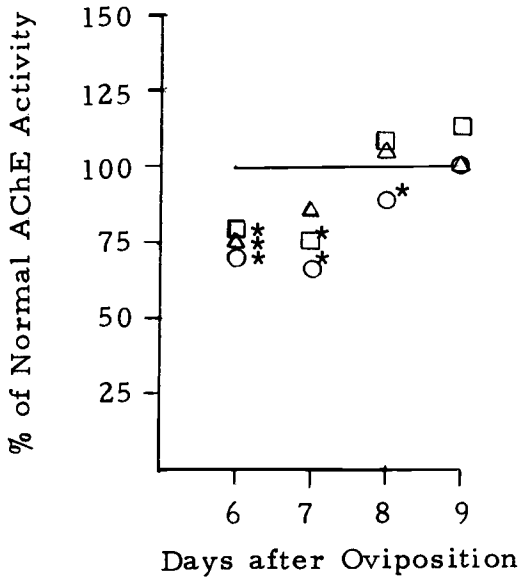


Figure 23.

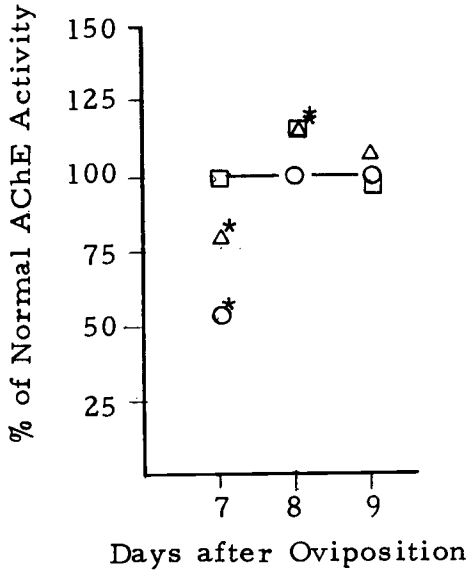


Figure 24.

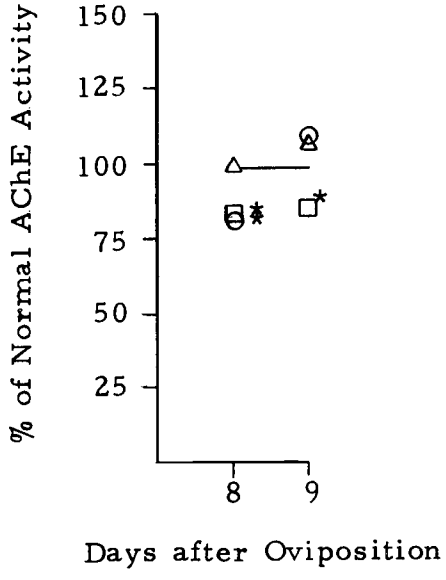


Figure 25.

embryogenesis. First instar nymphs also had an AChE activity level higher than did undosed controls.

When the pesticide was applied at day 4 (Figure 21), there was an immediate depression of AChE activity with the two higher dosages. By the next day, the AChE activity level was depressed at least 25% in all cases. After day 5, the AChE activity began to increase. The AChE activity passed significantly above the mean at day 8, but returned to normal, in all except the intermediate dosage, after hatching.

When the pesticide was applied at day 5 (Figure 22), the AChE activity level was sharply depressed by the next day, but returned to normal within three days. When the pesticide was applied six days after oviposition (Figure 23), AChE activity depression was almost immediate and lasted two or three days. When the pesticide was applied at day 7 (Figure 24), in two cases the depression was immediate, but lasted only one day. This also occurred when the eggs were dosed at day 8 (Figure 25) but, in this case, the nymphs resulting from the eggs dosed with the highest concentration of pesticide had a significantly lowered AChE activity.

Tiguvon

Figures 26-31 illustrate the effect of different concentrations of Tiguvon on the development of AChE.

Figure 26. Effect of Tiguvon on the AChE activity level of developing cricket eggs, when applied three days after oviposition.

Figure 27. Effect of Tiguvon on the AChE activity level of developing cricket eggs, when applied four days after oviposition.

- o 0.1 μg Tiguvon
- Δ 0.2 μg Tiguvon
- □ 0.3 μg Tiguvon
- * Significantly different from the mean at the 95% confidence level.

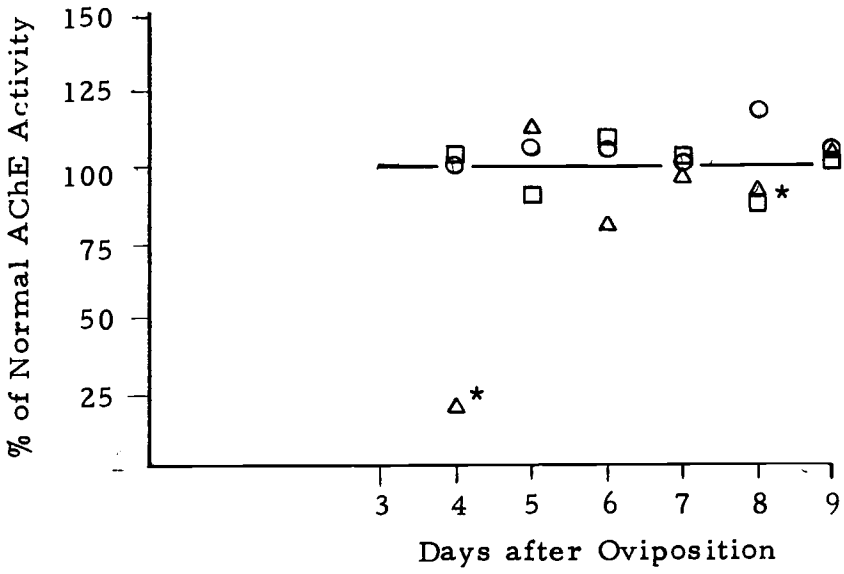


Figure 26.

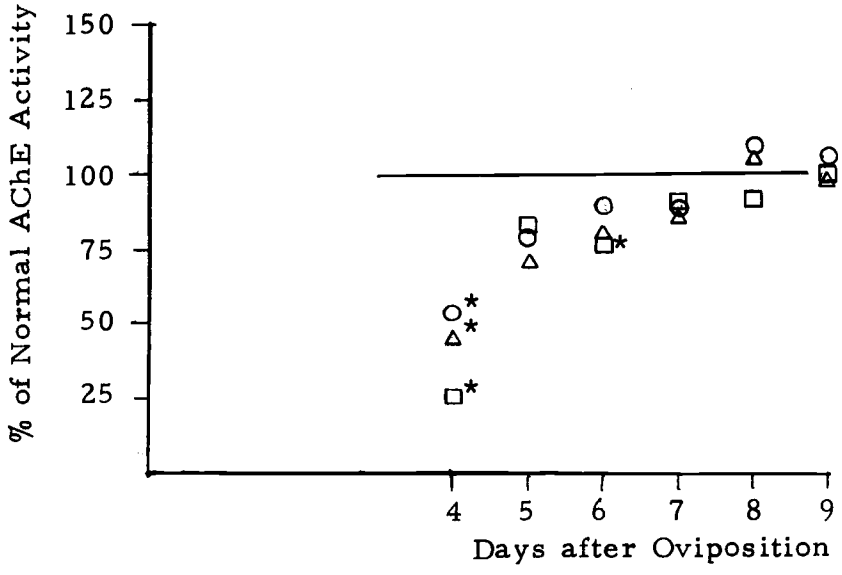


Figure 27.

Figure 28. Effect of Tiguvon on the AChE activity level of developing cricket eggs, when applied five days after oviposition.

Figure 29. Effect of Tiguvon on the AChE activity level of developing cricket eggs, when applied six days after oviposition.

Figure 30. Effect of Tiguvon on the AChE activity level of developing cricket eggs, when applied seven days after oviposition.

Figure 31. Effect of Tiguvon on the AChE activity level of developing cricket eggs, when applied eight days after oviposition.

- ○ 0.1 μg Tiguvon
- △ 0.2 μg Tiguvon
- □ 0.3 μg Tiguvon
- * Significantly different from the mean at the 95% confidence level.

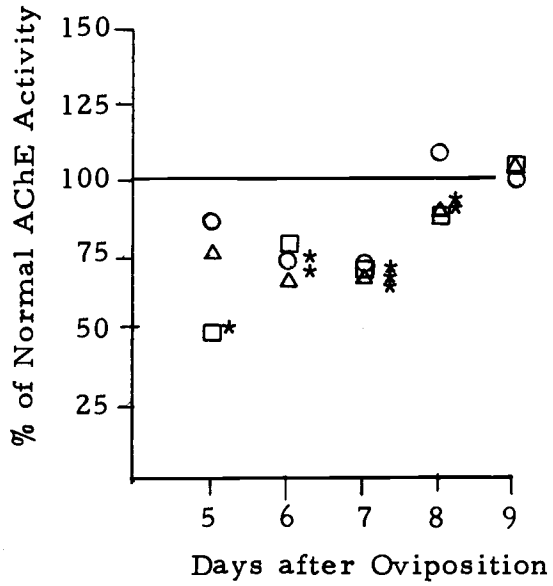


Figure 28.

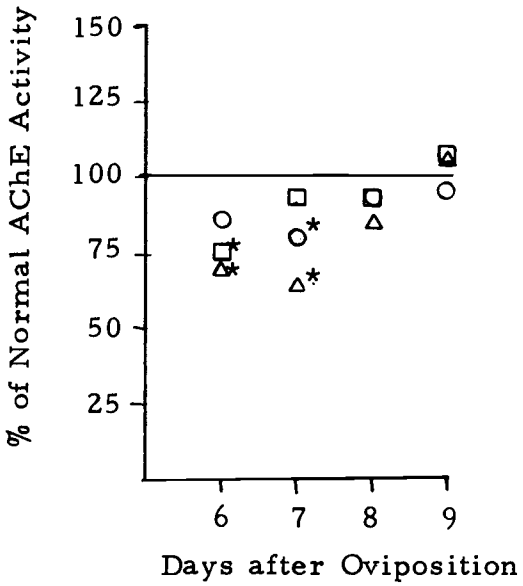


Figure 29.

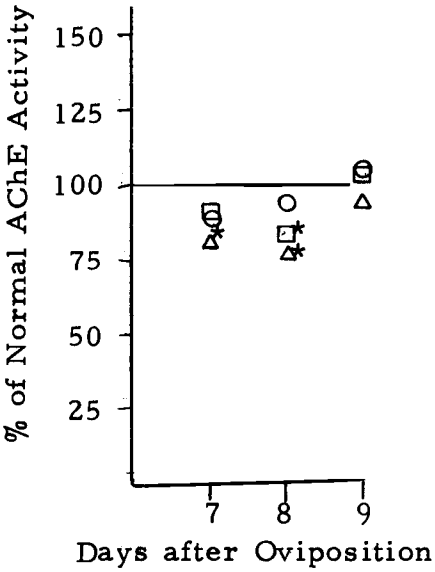


Figure 30.

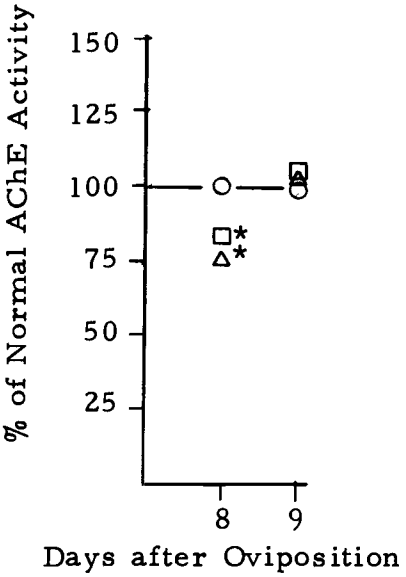


Figure 31.

When the eggs were dosed before the appearance of AChE (Figure 26), the AChE activity level was affected the following day at only one dosage level. The level returned to normal by day 5 and remained within normal limits until the day before hatching, when it was depressed significantly in the two higher concentrations and became significantly above normal in those eggs dosed with the lowest concentration. In those nymphs which hatched successfully, there was no significant difference in the AChE activity level from the control.

When the eggs were dosed on the fourth day after oviposition, there was an immediate reduction of AChE activity with all of the dosages (Figure 27). However, the AChE activity returned to normal limits by day 5 and remained within normal limits through hatching.

When Tiguvon was applied five days after oviposition, there was an immediate reduction with any dose, however, the decrease was significant only for the highest pesticide concentration (Figure 28). The AChE activity level remained significantly below normal until day 8. The nymphs which hatched successfully had AChE activity levels within the normal range.

When Tiguvon was applied at day 6 or day 7 (Figure 29, 30), the AChE activity was depressed immediately and remained depressed at least at some dosage levels for one more day, then returned to normal levels.

Tiguvon had very little effect on immediate AChE activity when

applied at day 8 except at 0.2 μg (Figure 31).

The AChE activity levels for nymphs that came from eggs which were dosed with Tiguvon were based on data from surviving nymphs. Samples of eggs which had failed to hatch were also analyzed for AChE activity. It was found that the nymphs which hatched had an average AChE activity of 22.8×10^{-3} μM substrate hydrolyzed/mg cricket and those which did not hatch had an average AChE activity of 15.35×10^{-3}. This difference was significant at the 99.9% level. Therefore, it can be said that those embryos which failed to hatch had a distinctly lower cholinesterase activity level than those which did hatch.

There has been a controversy as to whether or not there is a correlation between mortality and AChE activity depression in insects poisoned with anticholinesterase agents (Smith and Goulding, 1968, 1970; Morallo and Sherman, 1967). The research covered by this thesis indicated that in the case of cricket eggs, those eggs that did not hatch had a lower AChE activity level than those eggs that did hatch.

Attempts were made to correlate the mortality caused by Tiguvon with the AChE activity level of the late stage embryos. This was difficult, because although mortality is significantly greater than normal in some cases, mortality is rarely greater than 20% (Table 3). Fifteen eggs were analyzed together, and 20% mortality would mean approximately three eggs out of the 15 would fail to hatch due to low

cholinesterase activity. Low AChE activity in only 1/5 of the eggs in a sample may not make enough difference in total AChE activity to indicate a significant activity decrease. However, when the percent mortality was great (e. g., with eggs treated with 0. 2 μg on day 8, or with 0. 3 μg on day 7 or day 8), the AChE activity was depressed significantly below normal (Figure 30, 31) the day before hatching. However, there were other times when the AChE activity was depressed significantly below normal on day 8, but increased to within normal bounds by the time of hatching. With the limited mortality induced by Tiguvon, it was not possible to predict the resultant nymphal mortality on the basis of the AChE activity in the late embryonic stages.

Previous data in which anticholinesterase agents have been tested on insect eggs have indicated that in eggs which do not hatch, ChE activity is 0% in the house fly (Mehrotra and Smallman, 1957), 15% of normal in the peach tree borer (Smith and Wagenknecht, 1956) and 18% of normal in the cabbage butterfly (David, 1959). Cricket embryos from eggs which did not hatch had AChE activity levels depressed only 30%, that is 70% of normal. This comparatively low figure might be explained by the fact that the amount of pesticide used in the above mentioned papers was more than enough to cause 100% insect mortality, whereas in this research, mortality was considerably less than 100%.

Sevin

Figure 32 illustrates the effect of Sevin applied three days after oviposition on AChE activity. It was significantly depressed at days 4 and 5 and in one case at day 6, returned to normal, then was significantly above normal after eclosion.

When the embryos were dosed four days after oviposition (Figure 33), the AChE activity was depressed immediately by the two higher concentrations. All levels were depressed on day 5, but returned to normal after that time. With the two higher dosages, the AChE activity level rose to a level significantly above the mean, but this high level was not maintained in the first instar nymph.

Dosages of varying concentrations applied at five, six, or seven days (Figures 34, 35, 36) after oviposition showed little difference in their effect on AChE activity levels. The AChE activity was significantly depressed immediately but gradually recovered to approximately normal levels by the time of hatching. In only one case (Figure 34) did the AChE activity levels of dosed eggs rise significantly above normal, however this phenomenon was not maintained in the nymphs.

When the embryos were dosed with Sevin one day before hatching (Figure 37), the AChE activity level was depressed immediately, but again had returned to normal at the time of hatching.

Figure 32. Effect of Sevin on the AChE activity level of developing cricket eggs, when applied three days after oviposition.

Figure 33. Effect of Sevin on the AChE activity level of developing cricket eggs, when applied four days after oviposition.

- o 0.6 μg Sevin
- Δ 0.8 μg Sevin
- □ 1.0 μg Sevin
- * Significantly different from the mean at the 95% confidence level.

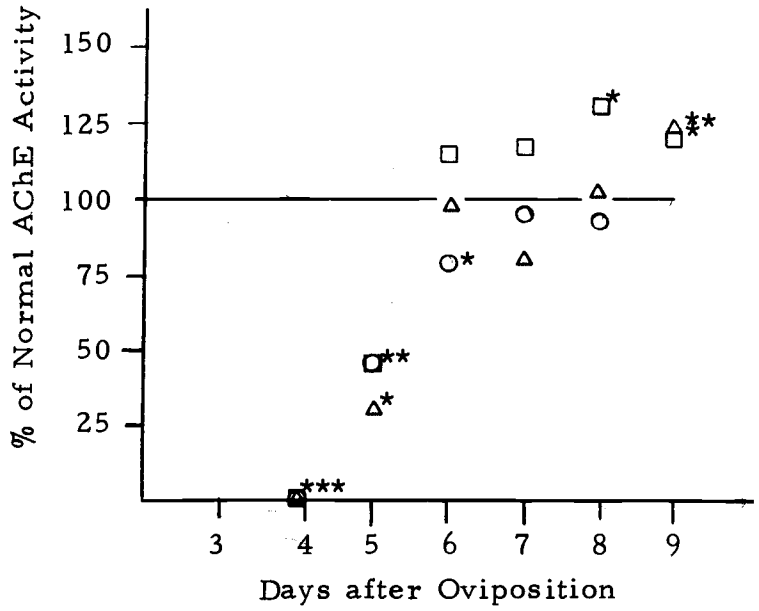


Figure 32.

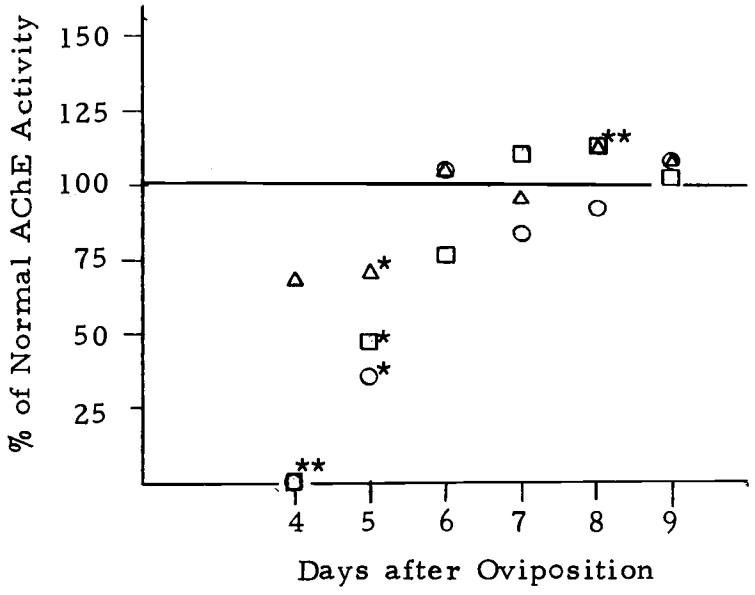


Figure 33.

Figure 34. Effect of Sevin on the AChE activity level of developing cricket eggs, when applied five days after oviposition.

Figure 35. Effect of Sevin on the AChE activity level of developing cricket eggs, when applied six days after oviposition.

Figure 36. Effect of Sevin on the AChE activity level of developing cricket eggs, when applied seven days after oviposition.

Figure 37. Effect of Sevin on the AChE activity level of developing cricket eggs, when applied eight days after oviposition.

- ○ 0.6 μg Sevin
- △ 0.8 μg Sevin
- □ 1.0 μg Sevin
- * Significantly different from the mean at the 95% confidence level.

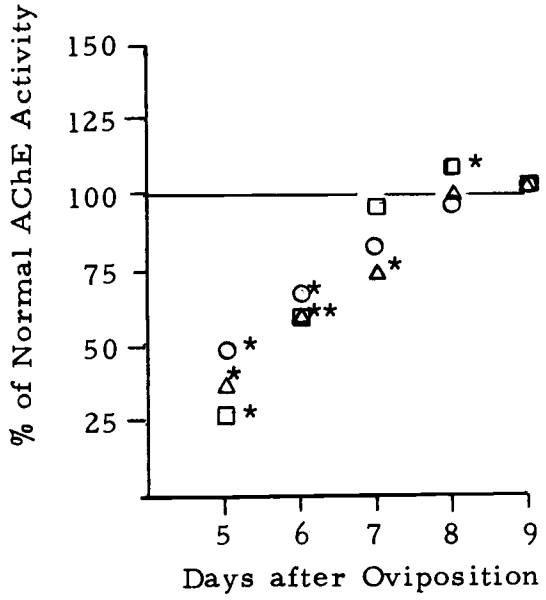


Figure 34.

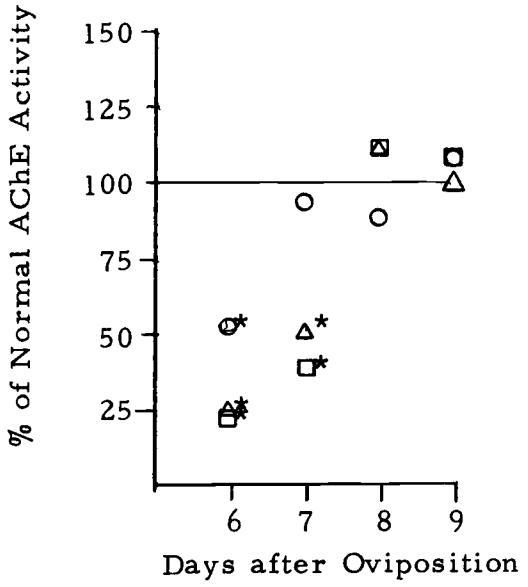


Figure 35.

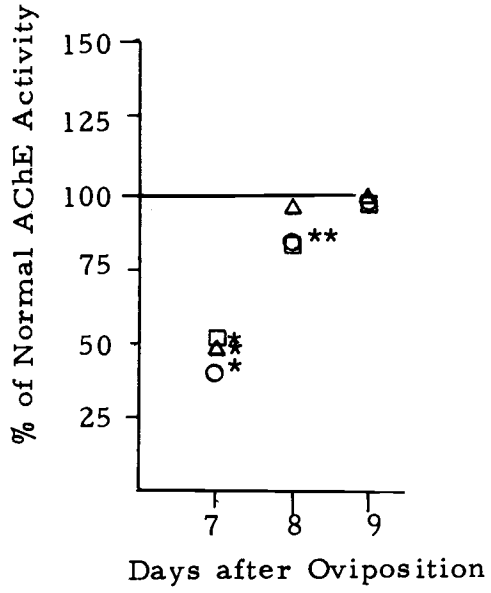


Figure 36.

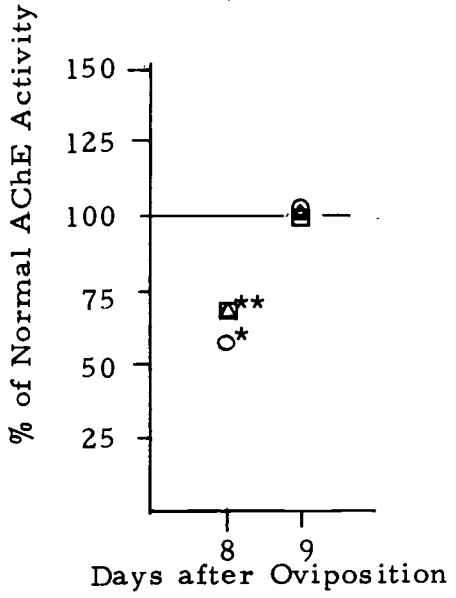


Figure 37.

Baygon

Figures 38-43 illustrate the effect of Baygon on AChE activity levels of the cricket embryos.

When dosed three days after oviposition (Figure 38), the lowest concentration seemed to have little effect on AChE activity. The two higher dosages caused a depression of AChE at day 4, or caused a delay in its formation. The AChE remained depressed through day 5. However, by day 8, AChE activity had risen significantly above normal, and in one case the high level was maintained in the nymphs.

When eggs were dosed four days after oviposition (Figure 39), AChE activity depression occurred with all three dosages although the depression was not as great as the one brought about when dosage was a day earlier. The AChE level remained depressed from one to three days depending on the dosage rate, but rose to normal by day 8.

The pattern of AChE activity depression brought about by dosages of Baygon administered five, six, seven, or eight days after oviposition (Figures 38-41) was essentially the same as that of Sevin at comparable times.

Comparison of the Effects of the Four Pesticides

In vivo. The AChE activity was depressed within one day after application of the pesticide, indicating that at least part of the action of

Figure 38. Effect of Baygon on the AChE activity level of developing cricket eggs, when applied three days after oviposition.

Figure 39. Effect of Baygon on the AChE activity level of developing cricket eggs, when applied four days after oviposition.

- o 0.6 μg Baygon
- Δ 0.8 μg Baygon
- □ 1.0 μg Baygon
- * Significantly different from the mean at the 95% confidence level.

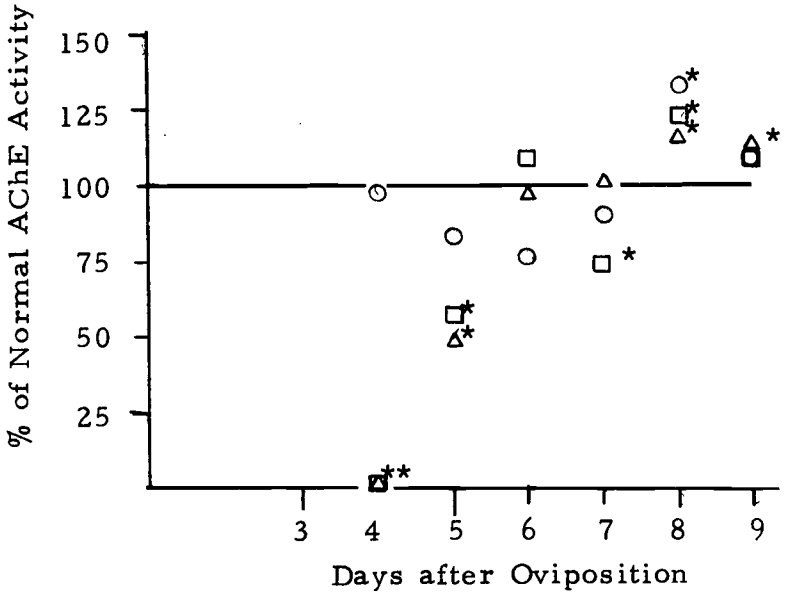


Figure 38.

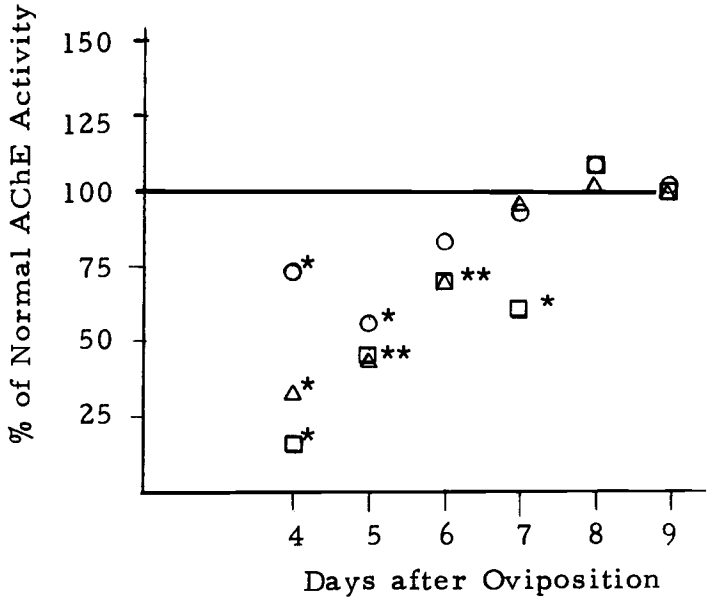


Figure 39.

Figure 40. Effect of Baygon on the AChE activity level of developing cricket eggs, when applied five days after oviposition.

Figure 41. Effect of Baygon on the AChE activity level of developing cricket eggs, when applied six days after oviposition.

Figure 42. Effect of Baygon on the AChE activity level of developing cricket eggs, when applied seven days after oviposition.

Figure 43. Effect of Baygon on the AChE activity level of developing cricket eggs, when applied eight days after oviposition.

- o 0.6 μg Baygon
- Δ 0.8 μg Baygon
- □ 1.0 μg Baygon
- * Significantly different from the mean at the 95% confidence level.

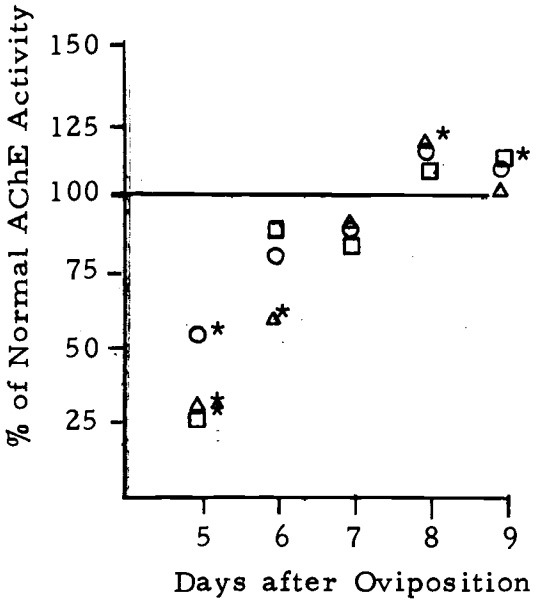


Figure 40.

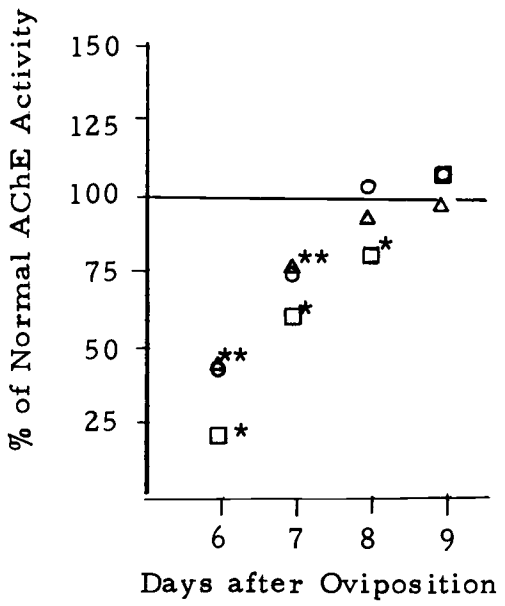


Figure 41.

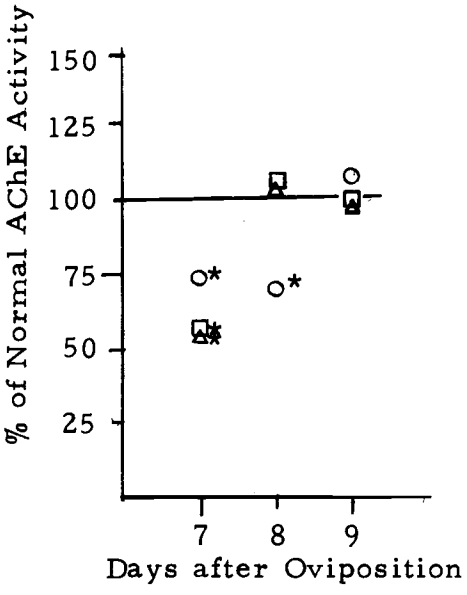


Figure 42.

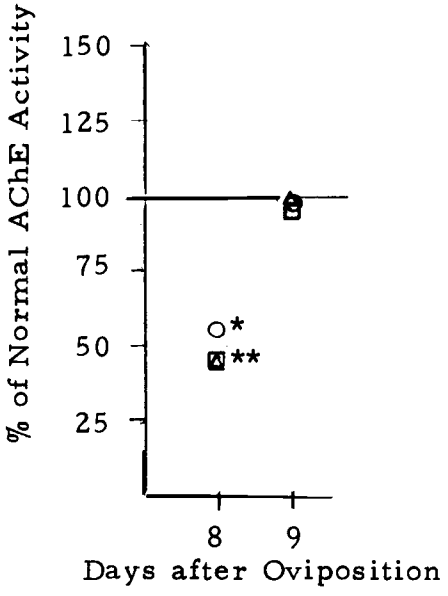


Figure 43.

the pesticide was immediate. With eggs dosed at day 3 or day 4, the two carbamates depressed and held the AChE activity level significantly below normal for a longer period of time than did the organophosphates. However, when dosage occurred later in embryonic life, the difference in longevity of action largely disappeared.

In almost all cases, the AChE activity was depressed for only a few days, and gradually returned to normal levels before the eggs hatched. In these cases, no mortality occurred. But in cases where high mortality did occur, the AChE activity was depressed significantly below normal on the day prior to hatching. Upon analysis, the AChE activity levels of all cricket eggs failing to hatch was significantly lower than the AChE activity of those that did hatch.

It was found that when either Ruelene (0.5 or 0.8 μg/egg) or Sevin (0.6, 0.8, or 1.0 μg/egg) was applied to eggs three days after oviposition, the AChE activity levels of the emerging nymphs was significantly above the levels of the undosed controls.

In vitro. Inhibitor constants (K_i) were determined for all four of the pesticides with pure acetylcholinesterase as the enzyme source (Figure 44) and cricket homogenate as the enzyme source (Figure 45). K_i is the reciprocal of the affinity of the enzyme for inhibitor (Dixon and Webb, 1959).

With pure enzyme, Tiguvon had the lowest K_i value (0.3), therefore the enzyme had the highest affinity for this inhibitor. This

Figure 44. Graphical determination of dissociation constants of the pure AChE-pesticide complexes (K_i).

Figure 42A. Ruelene
Figure 42B. Tiguvon
Figure 42C. Sevin
Figure 42D. Baygon

1/v	Inverse of velocity of reaction
I	Concentration of inhibitor
$-K_i$	Inhibitor constant
s_1	1.1 μM ACh/sample
s_2	2.2 μM ACh/sample

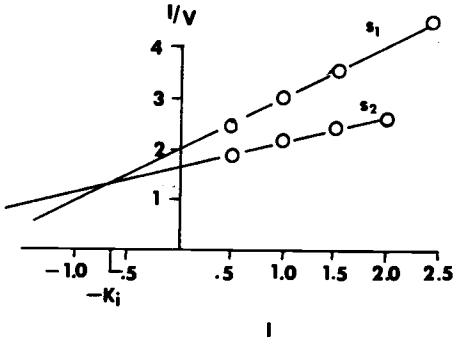


Fig 44A

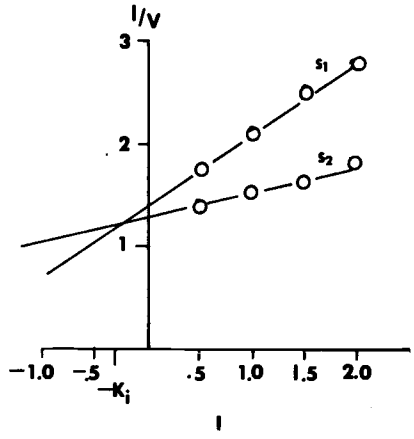


Fig 44B

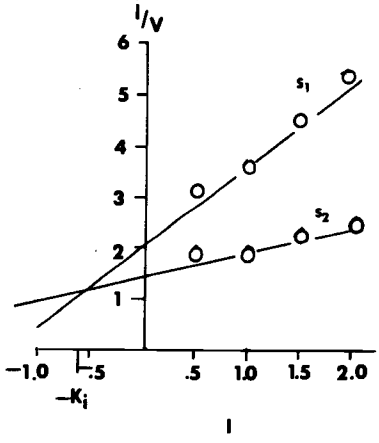


Fig 44C

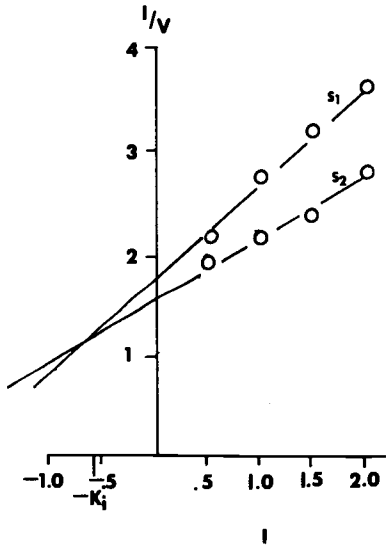


Fig 44D

Figure 45. Graphical determination of dissociation constants of cricket homogenate enzyme-pesticide complexes (K_i).

Figure A. Ruelene
Figure B. Tiguvon
Figure C. Sevin
Figure D. Baygon

1/v	Inverse of velocity of reaction
I	Concentration of inhibitor
$-K_i$	Inhibitor constant
s_1	0.55 μM ACh/sample
s_2	1.1 μM ACh/sample

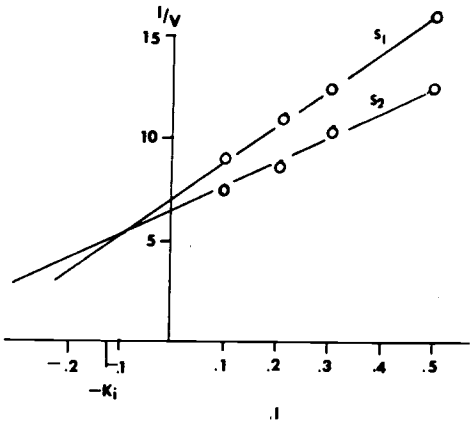


Fig 45A

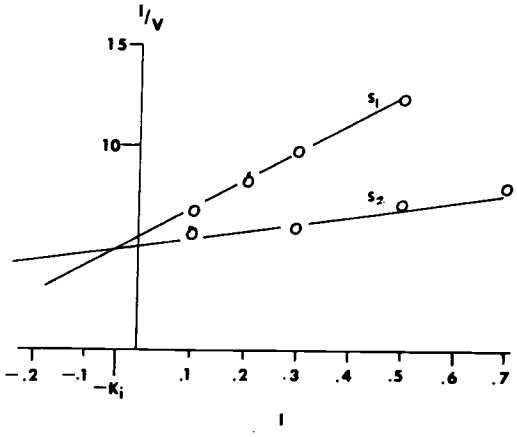


Fig 45 B

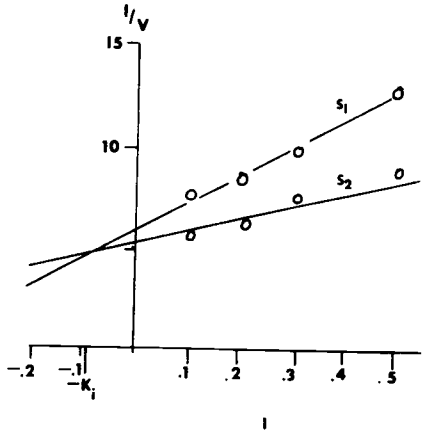


Fig 45C

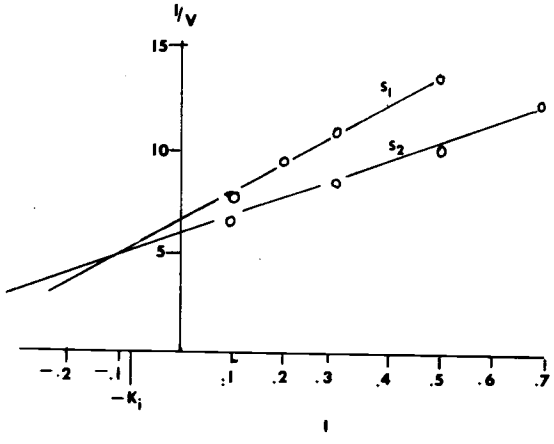


Fig 45D

agreed with mortality data since Tiguvon was the only pesticide tested causing significant mortality in the egg. Ruelene had the lowest affinity for pure enzyme of the pesticides tested (K_i = 0.68). Sevin and Baygon both had intermediate K_i values (K_i = 0.6, 0.55, respectively).

With cricket homogenate (from day 8 embryos) a similar relationship was noticed. Tiguvon had the lowest K_i value (0.04), Baygon and Sevin had an intermediate K_i value (0.08), and Ruelene had the highest K_i value (0.12).

The K_i values obtained with the pure AChE and the cricket AChE cannot be compared because of the great difference in purity and source of the two enzymes (Dixon and Webb, 1959).

In all cases, the pesticides were found to be competitive inhibitors of both types of AChE. This is evidenced by the fact that the K_i for s_1 and s_2 do not intercept on the x-axis (Dixon and Webb, 1959).

Rate of Penetration of the Pesticides at Various Ages

Embryonic Versus Nymphal Tolerance

Observations indicated that eggs were more tolerant of the four pesticides than were first instar nymphs. To substantiate this observation, different pieces of filter paper were treated with 35 μg of Sevin; 35 μg of Baygon; 35 μg of Ruelene or 3.5 μg of Tiguvon. In all

cases, cricket eggs placed on this filter paper for 24 hours did not have mortality significantly different from undosed controls (< 10%) upon hatching. When nymphs were placed on filter paper dosed with Sevin, mortality was 76%; with Baygon, 96%; with Tiguvon, 96%; with Ruelene, 2%.

Based on this test, it was concluded that first instar nymphs were much more susceptible to Tiguvon, Sevin, and Baygon than were eggs. Lack of susceptibility of nymphs to Ruelene may be partially due to low affinity of the pesticide for AChE.

Percent Penetration Through the Chorion

Tests were run to determine the role of the chorion as a physical barrier preventing the pesticides from reaching the active sites in the embryo. Data from these tests are presented in Table 6. It can be seen that from day 3 through day 7, age of the embryo had no appreciable effect on the percent penetration of either Sevin or Baygon. With the organophosphates, however, there was a much greater percent penetration at day 3 than at the later days.

With all pesticides used, penetration was lowest at day 8. The difference in the action of the two types of compounds at day 3 cannot be correlated with the physical or chemical properties of the pesticides.

Water absorption occurred very rapidly during early stage V and

Table 6. Percent penetration of pesticides through the chorion.

Days after Oviposition	Time after Dosage (min)	Ruelene	Tiguvon	Sevin	Baygon
3	0	0	0	0	0
	15	0	10	0	0.8
	30	6	10	2.0	1.6
	60	10	8	2.0	1.8
	120	11	10	3.0	2.5
4	0	0	0	0	0
	15	7.1	7.3	0	0.7
	30	4.3	5.1	1.5	0.8
	60	4.5	4.9	2.0	1.5
	120	4.3	5.2	3.0	2.5
5	0	0	0	0	0
	15	0	0	0	0
	30	8.0	8.4	1.5	0
	60	5.0	5.0	1.6	1.0
	120	5.0	5.0	2.4	2.5
6	0	0	0	0	0
	15	0	0	0	0
	30	3.8	5.1	1.3	3.5
	60	4.0	4.9	3.0	3.0
	120	3.9	4.7	2.4	2.7
7	0	0	0	0	0
	15	0	0	0	0
	30	3.0	5.3	2.5	3.4
	60	3.5	5.3	2.5	2.3
	120	2.5	5.0	3.1	3.0
8	0	0	0	0	0
	15	0	0	0	0
	30	3.5	3.5	1.4	1.3
	60	2.0	4.5	1.6	1.8
	120	1.5	3.0	1.9	1.6

any liquid placed on the chorion prior to that time would be absorbed (McFarlane, Ghouri and Kennard, 1959; McFarlane and Kennard, 1960). Embryos dosed at day 3 were usually in late stages of water absorption or water absorption had already stopped. Slight differences in times of application could and did result in great differences in rates of penetration.

After Stage V, the permeability of the chorion fluctuated slightly, and seemed to be lowest at stage X (McFarlane and Kennard, 1960). This was corroborated by the present work in that the lowest percent pesticide penetration was found at day 8 (Stage X).

Comparison of Pesticide Penetration to Maximum AChE Depression

A comparison was made between the amount of pesticide which penetrated through the chorion of the egg and the resultant maximum depression of AChE activity in the cricket embryos. Results are summarized in Table 7.

With each pesticide, there was a relationship between the amount of pesticide penetration and the AChE activity depression. With Baygon, small changes in concentration seem to make quite a large change in maximum AChE depression.

The relationship between pesticide and percent AChE activity varied for each pesticide. This can be explained by the different affinities of each pesticide for cricket AChE.

Table 7. Amount of chorion penetration by pesticides compared with resultant maximum AChE activity depression.

Day after Oviposition	Ruelene		Tiguvon		Sevin		Baygon	
	Pesticide (μg)	Max. ChE depression	Pesticide (μg)	Max. ChE depression	Pesticide (μg)	Max. ChE depression	Pesticide (μg)	Max. ChE depression
4	.035	65%	.021	40%	.030	65%	.025	75%
5	.04	75%	.024	50%	.024	65%	.025	65%
6	.02	30%	.015	35%	.024	60%	.035	75%
7	.015	50%	.015	25%	.031	60%	.03	60%
8	.015	25%	.009	20%	.019	40%	.018	50%

Comparison of Percent Penetration to Mortality

Tiguvon had the highest rate of penetration of any of the pesticides on any day (Table 6). This fact, in conjunction with the higher enzyme affinity of Tiguvon, would account for the higher mortality rate due to Tiguvon.

Mortality was highest (greater than 20%) when Tiguvon was applied three, seven, or eight days after oviposition (Table 3). Percent penetration was highest (10%) at day 3 (Table 6). The comparatively short time for recovery before hatching was probably one of the factors influencing mortality at the later stages (day 7 or 8).

Effect of Embryonic Pretreatment on Subsequent Tolerance of Nymphs to Anticholinesterases

Eggs which had been dosed five days after oviposition were allowed to hatch. The nymphs were fed chicken mash containing 10 ppm methyl parathion. Table 8 contains data on the resulting mortality. At least four replicates were run for each test. The data were anlyzed using the paired difference test (Mendenhall, 1967).

It can be seen that those nymphs pretreated with either of the organophosphorus compounds, Tiguvon or Ruelene, had a mortality significantly higher than those which had not been treated. Those nymphs pretreated with carbamates at day 5 (Sevin or Baygon) had no significant change in mortality level from the control.

Table 8. Effect of pretreatment on exposure of nymphs to anticholinesterase agents.

Embryonic dose (μg)	Day of dosage	Level of ChE in nymph before nymphal dose	% Mortality of treated nymphs	% Mortality of control nymphs	% Difference
Tig. 0.1	5	Normal	80%	50%	30% higher*
Ruel. 0.5	5	Normal	93%	50%	43% higher*
Bay. 1.00	5	Normal	59%	60%	1% lower N.S.
Sev. 1.0	5	Normal	54%	58%	4% lower N.S.
Ruel. 0.5	3	Above normal	77%	54%	23% higher*
Ruel. 0.8	3	Above normal	76%	52%	24% higher*
Sev. 0.6	3	Above normal	26%	51%	25% lower*
Sev. 0.8	3	Above normal	30%	49%	19% lower*
Sev. 1.0	3	Above normal	33%	50%	17% lower*

*Significantly different from the control at the 95% confidence level.

N.S. - not significant at the 95% confidence level.

From the studies of the effects of pesticides on AChE activity levels, it was found that when either Ruelene (0.5 or 0.8 μg/egg) or Sevin (0.6, 0.8, or 1.0 μg/egg) was applied to eggs three days after oviposition, the AChE activity levels of the emerging nymphs was significantly above the levels of the undosed controls. To determine if this increased AChE activity had any effect on the tolerance of the nymphs to a second application of an anticholinesterase, a similar test was run for nymphal mortality when the nymphs were pretreated at day 3 of embryonic development. Results are presented in Table 8.

In the case of Sevin, as the AChE activity of the nymphs was raised above normal levels, nymphal mortality was decreased below normal levels. This would indicate that the increased AChE activity brought about by sublethal doses of Sevin does cause an increased tolerance to another anticholinesterase.

With Ruelene, the results are more difficult to explain. Nymphs, pretreated as eggs with a normal nymphal AChE activity, had a mortality of 93% as compared to control mortality of 50%. However, if nymphs which were pretreated as eggs at day 3 and thus having an AChE activity level above normal levels were used in mortality studies, the nymphal mortality was only 23% above normal. Therefore, although the Ruelene has some kind of residual effect on the nymphs, the presence of an increased AChE activity has a tendency to dampen this effect.

Tests were also run to determine how long the AChE activity remained above normal after hatching. Embryos were dosed at day 3 and allowed to develop to second or third instar nymphs before they were analyzed for AChE activity. The AChE activity level of undosed second instar (five day old nymphs) crickets was 19.9×10^{-3} μM/mg and the level of undosed third instar (nine day old nymphs) crickets was 19.4×10^{-3} μM/mg. There was no significant difference between the second or third instar crickets which had been treated as day 3 embryos and untreated controls.

The effects of sublethal doses of anticholinesterases on insect embryos had not been studied previously. However, with studies of adult insects, sublethal poisoning with anticholinesterases caused an eventual return to normal levels (Mengle and Casida, 1958; Mengle and O'Brien, 1960) or almost normal levels (O'Brien, 1956, 1961; Stegwee, 1960). No reference in the literature was found of a case in which the anticholinesterase compounds caused an eventual increase of AChE activity above normal levels.

SUMMARY

Data were collected on the following aspects of cricket development and mortality: (1) the effect of two organophosphate pesticides (Ruelene and Tiguvon) and two carbamate pesticides (Sevin and Baygon) on development and hatching of the cricket embryos; (2) characterization of acetylcholine-hydrolyzing enzyme in the cricket embryos; (3) development of activity of the enzyme during embryonic development and correlation to morphological development of the nervous system; (4) the effect of four pesticides given at various days on the cholinesterase (ChE) activity of the embryos; (5) percent penetration of the pesticides through the chorion, (6) the effect of embryonic pretreatment on the ability of the nymphs to withstand a dose during nymphal life.

Embryonic development took about nine days at 35°C. When eggs were dosed 3, 4, 5, 6, 7, or 8 days after oviposition, development continued until just prior to hatching. Eggs dosed earlier than day 3 were killed immediately, but this could be caused by the lethal effects of high concentration of carrier (DMSO). Although this thesis was concerned with the effects of sublethal doses, one of the pesticides, Tiguvon, caused some mortality if applied 3, 7, or 8 days after oviposition.

Analysis by means of specific substrates indicated that the only acetylcholine-hydrolyzing enzyme in the embryo and one day old first

instar nymph was acetylcholinesterase (AChE). The enzyme first appeared at day 4 and increased to maximum embryonic levels by day 7, increasing again only slightly at the time of hatching. It was found that AChE first appeared at the time of blastokinesis. At that time, the brain and ventral nerve cord were developed, the stomatogastric system was only beginning to differentiate.

Pesticides caused an immediate decrease in the AChE activity level, without initiating mortality. AChE activity levels tended to return to normal within three days and remained normal through hatching. The two carbamate pesticides used depressed the AChE activity longer than the two organophosphates during early stages of embryonic life, but there was little difference in later stages.

When mortality greater than 20% occurred in embryos, the AChE level was always significantly below normal on day 8. However, there were other times when the AChE activity level was significantly below normal on day 8, but no significant mortality occurred, and the nymphs had normal AChE activity levels.

In vitro tests of the four pesticides with AChE indicated that Tiguvon had the highest affinity for AChE and Ruelene had the lowest affinity. Baygon and Sevin were intermediate in this respect. The pesticides were all found to be competitive inhibitors of AChE.

It was found that, with the exception of Ruelene, nymphs were much more susceptible to the pesticides than were the eggs. Tests of

penetration rates indicated that the percent penetration through the chorion was never more than 10% and usually about 3%. Tiguvon had the highest penetration rate.

Tests were run to determine if pretreatment of eggs with pesticide would change the susceptibility of nymphs to another cholinesterase inhibitor. It was found that those nymphs from eggs treated with carbamates at day 5, when fed food containing pesticide, exhibited the same mortality as nymphs from eggs that had not been pretreated. However, those nymphs from eggs which had been dosed with organophosphates at day 5 exhibited a mortality 30-40% higher than did nymphs from undosed eggs.

When either Sevin (carbamate) or Ruelene (organophosphate) was applied to three day old eggs, AChE activity was observed to be higher at hatching than in undosed controls. Nymphs from eggs pretreated with Sevin at day 3 had 20% less mortality than did nymphs from untreated eggs, when both were fed food containing pesticide. Under the same conditions, nymphs from eggs pretreated with Ruelene at day 3 had 20% greater mortality than did nymphs from untreated eggs. However, nymphs from eggs dosed at day 3 that exhibited a higher than normal AChE activity level sustained less mortality than did nymphs from eggs dosed at day 5 that exhibited normal AChE activity level, when both fed on pesticide-treated food.

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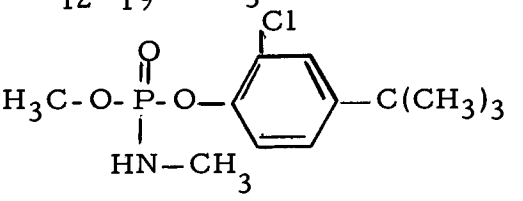
APPENDICES

APPENDIX I

Chemical formulae taken from Kenaga and Allison (1969).

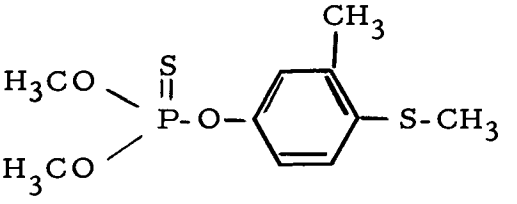
Ruelene: 4-tert-butyl-2-chlorophenyl methyl methylphosphoramidate.

$C_{12}H_{19}ClNO_3P$



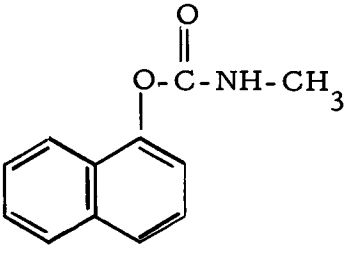
Tiguvon: 0, 0-dimethyl 0-(4-(methylthio)-m-tolyl)phosphorothioate.

$C_{10}H_{15}O_3PS_2$



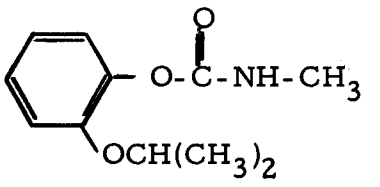
Sevin: 1-naphthyl methylcarbamate.

$C_{12}H_{11}NO_2$



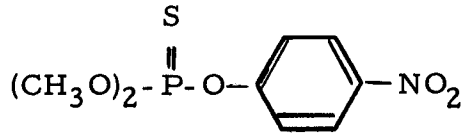
Baygon: 0-isopropoxyphenyl methylcarbamate.

$C_{11}H_{15}NO_3$



Methyl Parathion: 0, 0-dimethyl 0-p-nitrophenyl phosphorothioate.

$C_8H_{10}NO_5PS$



APPENDIX II

Reagents Used for ChE Activity Analysis

1. Phosphate Buffer - pH 7.4

   (a) Mono-sodium Phosphate - $NaH_2PO_4 \cdot H_2O$

   For a 0.2M solution, 13.9 g of $NaH_2PO_4 \cdot H_2O$ were placed in a 500 ml volumetric flask and brought to volume with glass distilled water.

   (b) Di-sodium Phosphate - $Na_2HPO_4 \cdot 12H_2O$

   For a 0.2M solution, 35.85 g of $Na_2HPO_4 \cdot 12H_2O$ were placed in a 500 ml volumetric flask and brought to volume with glass distilled water.

   (c) For one liter of a buffer solution of pH 7.4 140 ml of mono-sodium phosphate solution (a) were mixed with 360 ml of di-sodium phosphate solution (b) and 500 ml of glass distilled water.

2. Substrate

   (a) NonRadioactive Material

   A cold (carrier) substrate solution was made by placing 226.12 mg of acetylcholine bromide (Eastman Kodak Co.) in a 100 ml volumetric flask and was brought to volume with phosphate buffer, pH 7.4.

   (b) Radioactive Material

   A total of 5 ml of hot substrate was prepared which

contained 2.5 μC of activity. Using a microsyringe, 62.5 μl of acetyl-1-C^{14}-choline iodide (New England Nuclear Corp.) from a stock solution containing 40 μC/ml was placed in 0.5 ml of cold substrate. To this mixture, 4.44 ml of phosphate buffer (pH 7.4) were added.

3. Scintillation Counting Solution

The scintillation counting solution was made by placing 3.0 g of ϕ-p-terphenyl and 30 mg of POPOP [1,4-bis-2-(5-phenyloxazolyl) benzene] (Packard Instrument Co.) in a one liter volumetric flask and bringing it to volume by the addition of toluene (reagent grade).

4. Amberlite CG-120, sodium salt, 200-400 mesh (Mallinckrodt Chemical Co.), analytical-reagent grade.

An amberlite, CG-120, resin-ethanol suspension for stopping the hydrolysis reaction was made in the following steps:

1. The amberlite, CG-120, was washed by filtering 100% ethanol through 100 g of amberlite with the aid of a Buchner funnel.
2. The white film on the top of the washed amberlite, CG-120, was scraped off and discarded.
3. The washed amberlite, CG-120, was dried in a dessicator for at least 24 hours and stored therein until used.