SOME TOXICOLOGICAL STUDIES ON HOUSE CRICKETS

IN RELATION TO THEIR CIRCADIAN RHYTHMS

By

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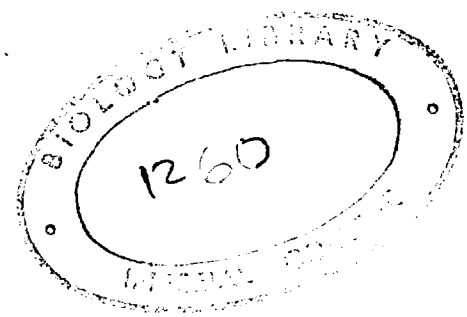

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To My Mother in

Affection and Gratitude

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ABSTRACT

The locomotor activity of the house cricket Acheta domesticus (L.) was studied in a LD 12:12 photoperiod and the persistence of its activity pattern was investigated under constant dark conditions and when the light-dark cycle was reversed.

Bioassay experiments demonstrated the presence of susceptibility rhythms to malathion, phorate and diazanon. Treatments were carried out at different times of day in relation to different levels of the insect locomotor activity.

The phenomenon of the insect's susceptibility rhythm to these compounds was studied from two aspects, one was concerned with the entry and the fate of toxicant after application on the insect, and the other dealt with the behaviour of some biochemical systems of the insect and their response to the toxication processes. Evaporation of topically applied C^{14} - phorate from different surfaces, including insect cuticle in relation to the insects locomotor activity rhythm, was also studied.

The penetration of C^{14} - phorate at different intervals after both light and dark treatments was determined and the production of the non-toxic materials of C^{14} - phorate in the insects and their frass was measured. A column chromatography technique was employed for a qualitative estimate of the parent compound and its toxic oxidative metabolites at different intervals after both light and dark treatments and a qualitative study of this fraction was also conducted, using thin layer chromatography and autoradiography.

Biochemical investigations concerned cholinesterase and carbo-

xylesterase, the enzymes most affected by the action of organophosphorus compounds. Experiments were conducted to map out daily fluctuations in their activity comparable to the insect's locomotor activity. The in-vitro inhibitory effect of phorate on cholinesterase was compared with that of phoratoxon sulfone and the in-vivo inhibitory effect of topically applied phorate on these enzymes was also determined in relation to the insects locomotor activity rhythm.

INTRODUCTION

One of the most characteristic aspects of living organisms is the dynamic nature of their environment and this is often reflected in the cyclical variations in their physiological processes. Just before the birth of Aristotle, the first written account appeared of what today is called a biological rhythm, an amateur naturalist had observed that certain plants (legumes) stand with their leaves folded to the sides of their stems at night and raise them to the sun in the morning. Day after day, throughout their entire lives, this pattern was repeated. It was only about thirty years ago that a real interest in biological rhythms began, and in this interval rhythmic physiological processes in numerous organisms have been described, almost all of these rhythms were found to persist in constant conditions (i.e., when light and temperature cycles were excluded) in the laboratory. Such a self-sustained rhythm, which in the absence of any external periodic time cues, produces a sufficient number of undamped oscillations, is called a free running or spontaneous rhythm.

So ubiquitous is the distribution of persistant rhythmic processes throughout the living kingdom, that rhythms should probably be considered a fundamental characteristic of life and should be added with such others as metabolism, growth, etc., to the elementary textbook definition of life.

In the natural habitat, organismic rhythms are strictly 24 hours in length; they are entrained to this frequency by the daily light-dark cycles generated by the rotation of earth on its axis. When plants or animals are placed in constant conditions, the rhythm persists, but the period usually becomes slightly longer or shorter than 24 hours. The term circadian (circa, about; diem, day) i.e., about a day in length was introduced by Halberg (1959) to describe free running oscillations of this period.

The search for the mechanism controlling the circadian rhythm in insects has progressed along two major directions. One suggested by Harker (1954) is that a secretion, carried by the blood or other tissues is involved in the production of the circadian rhythm activity in the cockroach. Harker (1954) also suggested that the sub-oesophageal ganglion is the centre for the control of the activity rhythm, through a hormone produced by its neurosecretory cells. No convincing confirmation has been published since this theory was postulated more than twenty years ago, despite the attempts of Robert (1966) and Brady (1967). Furthermore, it is now known that the lateral neurosecretory cells can be removed from the sub-oesophageal ganglion of an intact cockroach without stopping its locomotory activity (Brady, 1967). However, the lack of confirmation of Harker's results does not prove that the ganglion cannot act as a clock. This is particularly true since it is not known where these neurosecretory cells release their secretion. It is thus possible that later investigators have failed because they had disrupted this release mechanism in some manner which Harker managed to avoid.

The other main direction claims that the primary circadian pacemaker asserts its effect electrically through normal neural pathways in the ventral nerve cord. The work of Nishitsutsujiuwo and Pittendrigh (1968) has confirmed this claim and suggests the optic lobes of the brain, are the parts controlling the time keeping process of the cockroach. The recent results of Sokolove (1975) confirmed that in the cockroach the brain is the part controlling the time-keeping mechanism. He also suggests that the cell bodies and not the neuropile lobes of the brain are the crucial elements of the clock controlling the cockroach.

Light is a factor of special importance in circadian rhythm studies, since it has a potential role in the entrainment of rhythmicity (Hastings,

1964). Light never effects the circadian clock directly, but through complicated pathways. In vertebrates it affects the clock through light reception in the eye. However, even in vertebrates an extraretenial reception of light by the brain, or parts of the brain, has quite often been observed to be effective in synchronizing the circadian clock (Menaker, 1968; Adler, 1969).

Locomotor activity has often been employed as an indicator for the measurement of an insect circadian rhythm, mainly because of the simplicity of the methods used for detecting and measuring it. The assumption behind this is that the locomotor activity rhythm is a genuine reflection of the insect internal clock. The circadian locomotor rhythm of the house cricket has been studied by, Lutz (1932), Nowosielski and Patton (1963) and Cymborowski (1969). They showed that house crickets maintain a distinct rhythm, which persists in light/dark (LD) cycles as well as constant conditions and because of this distinct rhythm house crickets were chosen for the present study. In addition their moderate size would facilitate physiological and toxicological investigations.

Circadian susceptibility to Insecticides

Halberg (1962) suggested, that the whole organism is a circadian system in which each of a number of variables was observed to oscillate with a period of about 24 hours. Since circadian patterns are known at all levels in an organism's functions, it is not inconsistent to expect the response of a rhythmic organism, when challenged to differ as a function of its circadian phase. In one method of analysis of such systems, a fixed stimulus is administered to groups of comparable individuals at different clock hours. If the circadian system (i.e., the whole organism) is affected by the stimulus as a whole or in part, variations in response will reflect the differences in the physiological state of the animal at

the time of applying the stimulus, and the magnitude of the response will oscillate with a periodicity of about 24 hours. Thus, for example, a given dose of bacterial endotoxin, injected into mice at a specific time of the day, resulted in the death of 80 to 90 percent of the mice. However, 12 hours later or earlier a similar dose given to comparable groups of mice, may only result in the death of a small percentage or may be compatible with survival of the entire group (Halberg, 1960). Additional observations on rodents support a circadian susceptibility rhythm to an overdose of ethanol (Haus and Halberg, 1959) or drugs like librium (Matre and Halberg, 1961), similar studies on man also confirm circadian susceptibility rhythms in cutaneous sensitivity to allergens (Reinberg, et al. 1964).

The first report confirming insects circadian susceptibility to insecticides was published by Beck (1963) who found that Blatella was rhythmic in its susceptibility to dimetilan and potassium cyanide. Since 1963 other reports of insecticide susceptibility rhythms have appeared, e.g., Cole and Adkisson (1964, 1965) described the circadian susceptibility rhythm of the boll weevil, Anthonomus grandis to parathion. Fisher (1967), reported the spider mite Tetranychus uriticae of showing a circadian rhythm of susceptibility to dicofol. Fernandez and Randolph (1966), reported that in a photoperiod of LD 15:9 houseflies were less susceptible to DDT, dieldrin and endrin than in other photoperiods. But in other experiments a greater susceptibility to malathion was found when the flies were kept in LD 10:14 than in LD 14:10 (Frudden and Wellso, 1968) and although further experiments were carried out, these authors failed to detect a diel rhythm in susceptibility, whereas it does exist for other organophosphorus insecticides (Reinhardt, 1971). In general the phase relationships between the circadian locomotor rhythms of insects and their rhythms of insecticide susceptibility show no obvious physiological connections.

Both houseflies and cockroaches, for example were found to be maximally suceptible to pyrithren in the afternoon (Sullivan et al., 1970), whereas houseflies are behaviourally diurnal (Parker, 1962) and cockroaches nocturnal (Roberts, 1960). Likewise, the beetle Tenebrio molitor, was more susceptible to parathion around dawn and dusk, whereas its maximum locomotor activity extended throughout much of the night (Fondacro and Butz, 1970). A similar dawn and dusk patterns of peak susceptibility in male pink bollworm moths to azinphosmethyl was found by Ware and McComb (1970), and thus in both these insects the significance of the phase relationship with the behavioural rhythm is not obvious, except that peak susceptibility roughly coincided with the onset and offset of locomotor activity (Leppla and Spangler, 1971).

Evaporation, Penetration and Metabolism of Insecticides in Relation to Insects Circadian Activity

Despite many investigations the mechanisms whereby insecticides penetrate insects and arrive at the sites of action, have not been completely resolved. Initially the evaporation rate of a topically applied insecticide is of considerable importance to the rate of penetration. Matsumura (1963) found that rapid evaporation of the acetone solvent shortly after the application of malathion to houseflies, resulted in a decrease in the volume of the initial drop, and hence changed the permeability constant of penetration. Forgash et al. (1962) considered the reduced penetration of insecticides to be a factor of great importance to insect resistance to insecticides because there is more opportunity for loss of the toxicant from the integument by evaporation. Olson and O'Brien (1963) studied the evaporation of phosphoric acid, dimethoate, dieldrin and paraoxon from the pronotum of the American cockroach at intervals up to 150 minutes after application, but found that evaporation of these solutes was negligible

during the observation periods. Perry et al. (1964) exposing adult houseflies to residual deposits of aldrin, found that 40 to 50% of the applied dose evaporated after 24 hours. Szeicz et al. (1973) studied the rate of evaporation of different insecticides from glass rods and found that endrin and malathion were quite volatile. In 12 hours about 30% endrin and 18% malathion had been lost and in 24 hours nearly 50% of endrin and 30% of malathion had disappeared, while less than 10% of the applied dose of DDT and carbaryl were lost after 24 hours. Devonshire and Needham (1974) found that about 44% of a topically applied dose of dimethoate on aphids evaporated after 24 hours, while 25% of an equal dose of parathion evaporated after the same period.

Penetration has been always considered to be a significant factor in the susceptibility of insects to insecticides. Krueger et al. (1960) found that diazanon penetrated the integument of a resistant strain of houseflies slower than it penetrated a normal strain. Forgash et al. (1962) demonstrated that the slow penetration of diazanon into resistant houseflies was partly responsible for the level of resistance. El Bashir (1967) showed that houseflies' resistance to DDT were linked with delayed penetration of the topically applied compound. Szeicz et al. (1973) studied the rate of penetration of DDT, Carbaryl, endrin and malathion into the cuticle and the internal tissues of resistant and susceptible larvae of Heliothis virescens, and found that all the insecticides penetrated much more rapidly in the susceptible strains. Gerolt (1974), studying the mechanism of resistance to dichlorovos, suggested that this mechanism was based on reduced penetration. Apperson and Georghiou (1975) investigating the mechanisms of resistance to organophosphorus insecticides in Culex tarsalis showed consistent differences between resistant and susceptible strains in the rates of absorption of parathion at each exposure period.

There is no published work about the evaporation or penetration of topically applied insecticides in the context of insects circadian rhythms. Nevielle (1963), published the first studies about insects circadian rhythm and daily growth layers laid down in the endocuticle during early adult life of the desert locust. During the night chitin crystallites are deposited in the endocuticle in organized lamellae, while during the day the same amount of chitin is deposited, but in a non-lamellar form. Examined in section under crossed polaroids, the lamellate layers were strongly birefringent and the daily growth layers thereby are detectable as pairs of alternating light and dark bands. The same rhythm in the milkweed bug shows the same properties (Dingle, et al. 1969).

Despite this study no attempt to relate insecticide penetration and insect locomotor activity rhythm has ever been reported, though some of the available data is of relevance to the subject. Lewis (1962), reported that a thin film of oil applied on the body of blowflies, spread quicker when applied on active insects cuticle than when it was applied on dead insects. He has also shown (unpublished data) in a study of the penetration rate of topically applied insecticides on active and inactive blowflies, that penetration is faster in the active insects. Matsumura (1963) reached similar conclusion when he found that living cockroaches take up more malathion than the dead insects, and also demonstrated that starved insects have a higher rate of penetration, a phenomenon he attributed to the relatively higher level of activity of the starved insects. Contact of solutions and suspensions with the cuticular surface is an important factor in penetration, and is largely dependant on the nature of the surface the solution is applied to and the contact angle it forms with that solution (Nobell-Nesbill, 1970). The later criterion could be of some importance to the present study, since the variable locomotor activity of the insects

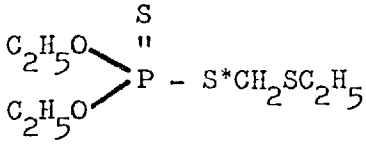
could result in different contact angles between the applied dose and the body, leading to a variable rate of penetration.

Obviously, the cuticle plays a vital role in determining the penetration of insecticides into insects. The relative importance of these routes is uncertain, but recent evidence suggesting that some insecticides move via the integument of the tracheal system (Gerolt, 1970, 1975) emphasizes the possible importance of an insect's activity in the process of insecticide penetration. The fluctuating level of insects' locomotor activity, that cause different magnitudes of elastic movement of the flexible cuticle, might also affect evaporation, the migration, and the spread of the topically applied dose to its site of action.

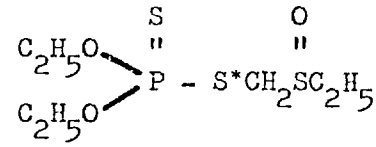
The metabolism of phorate has been thoroughly investigated in plants (Metcalf et al., 1957; Bowman and Cassida, 1957, 1958; Bowman et al., 1969) and in soils (Menzer et al., 1970; Lichtenstein et al., 1973; Schulz et al., 1973), metabolism of phorate has been studied in insects only by Bowman and Cassida (1958), Menn and Hoskins (1962). Le Patourel and Wright (1974) have studied the metabolism of phorate in the free living nematodes Panagrellus redivivus.

Five toxic metabolites are formed by the oxidation of the sulphide in the side chain to the sulphoxide and sulphone; and by the formation of a P = O amalogue, phototoxon, from the P = S group in the parent compound. The chemical structure of phorate and its toxic metabolites are as follows:

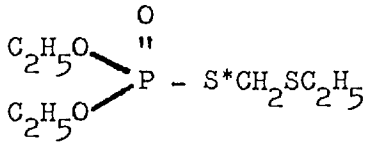
I Phorate (P=S,S)



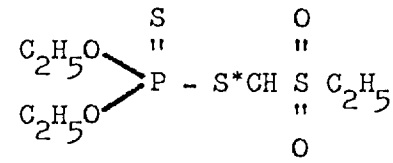
Phorate sulphoxide (P=S,SO)



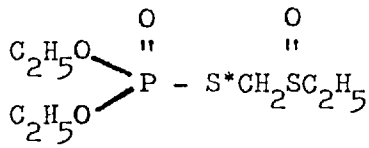
IV Phoratoxon (P=O,S)



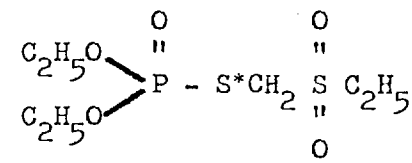
III Phorate sulphone ($P=S,SO_2$)



V Phoratoxon sulphoxide(P=O,SO)



VI Phoratoxon sulphone ($P=O,SO_2$)

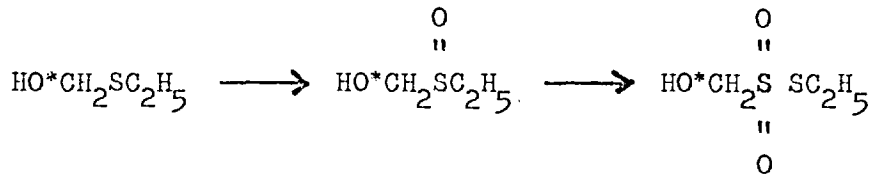


(The asterisk indicates the labelled atom in the insecticide used in this study).

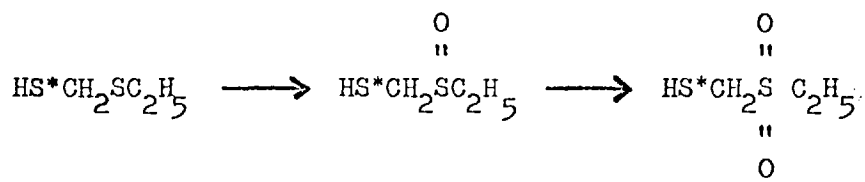
The data obtained from Bowman and Cassida (1958), and Menn and Hoskins (1962) on the metabolism of phorate in armyworms, the German cockroach, large milkweed bug and the bug Rhodnius prolixus indicated various rates of phorate metabolism, and they showed that phorate sulfone and phorate sulfoxide are the major toxic metabolites found in these insects. They also showed that phoratoxon is a very unstable compound. The toxicity of phorate and its oxidative metabolites have also been studied by these authors who found that phorate and its oxygen analogue were the most toxic compounds when topically applied to houseflies. Bowman et al. (1969) found that phorate was the most toxic compound when topically applied to 7 day old European corn borer, while its sulfoxide and sulphone were next in toxicity. On the other hand the anticholinestrase activity of phorate and its different metabolites has shown that phorate has the least inhibitory effect, while phoratoxon sulfone

is the most potent anticholinestrase metabolite (Bowman and Cassida, 1957; Menn and Hoskins, 1962; Metcalf et al., 1957; Archur et al., 1963).

Phorate is concurrently metabolised to non-toxic compounds through hydrolysis. The hydrolytic products detected by radioactive analysis will depend on the position of the labelled atom in relation to the point of hydrolysis. Phorate is predominantly hydrolysed at the thiolo sulphur and thus the hydrolytic products resulting from methylene labelled phorate will probably be represented by the sulphide-containing side chain of the parent compound with possible oxidation of the sulphide to sulphoxide and sulphone with a hydroxide or sulphydrill group at the point of cleavage as follows:



or;



This scheme is largely conjectural as very little is known about the products of hydrolysis. Some hydrolytic cleavage may also occur at the ethoxy group though this is a less common phenomenon and the non-toxic products would be expected to be water soluble also.

With the oscillating nature of different physiological functions of the insect, a study of the metabolism of the insecticide in relation to the insect rhythmicity was undertaken in an attempt to understand the interaction between the toxicant and both the activating and degrading metabolic systems. The only study dealing with metabolism

of insecticides in relation to insect circadian rhythm was conducted by Bull and Lindquist (1965). The metabolism of di-syston in fifth instar bollworms from unentrained and light synchronized populations, was compared. Results demonstrated that certain biochemical functions associated with the metabolism of organophosphorus insecticides in bollworms can be synchronized by rearing a population under a definite daily light-dark regimen. In unentrained larvae, these processes appeared random with no tendency to cyclical behaviour.

Circadian Fluctuations of Esterases and their Inhibition in Relation to the Insects Circadian Activity

The potency of the organophosphorus insecticides to inhibit mammalian cholinesterase has been known for some time but their inhibitory properties against insects cholinesterase and carboxylesterase were reported relatively recently (Chadwick and Hill, 1947; Metcalf and March, 1949). There is general agreement that organophosphorus insecticides poisoning blocks cholinesterases activity, leading to acetycholine accumulation in synapses, followed by exitation of the nervous system and finally failure of neural transmission (Smallman, 1956; Colhoun, 1959; Metcalf, 1959; Metcalf and March, 1969; Winteringham and Lewis, 1959). Lord and Potter (1950, 1951) first suggested that esterases other than cholinesterase may be involved in the organophosphorus insecticides poisoning. Their suggestions were confirmed by the work of Van Asperen and Oppenoorth (1959), Bigly and Plapp (1960).

Because the terminology of earlier workers is variable, it is appropriate here to clarify some aspects relating to the nomenclature of these enzymes. Following Pearse (1972), this study deals with esterases with a common link, their substrates are esters of carboxylic acids. These enzymes are cholinesterase and carboxylesterase.

According to O'Brien (1960) the name cholinesterase is used to describe the true or acetylcholinesterase and the pseudocholinesterase. To understand the terminology used in several papers, the following terms are synonymous (Pearse, 1972).

A - esterase = aromatic esterase = arylesterase = organophosphate resistant esterase.

B - esterase = ali-esterase = carboxylesterase = organophosphate sensitive esterase.

C - esterase = acetylesterase = sylphydrill inhibitor - resistant esterase.

Finally, according to the classification produced by the International Commission on Enzymes in 1961, the following scheme relates this classification with the previous consideration

3.	Hydrolases	
3.1.	Acting on ester bond	
3.1.1.	Carboxylic ester hydrolases	
3.1.1.1.	Carboxylic ester hydrolase = Carboxylesterase = ali-esterase	Non-specific esterases
3.1.1.2.	Aryl ester hydrolase = arylesterase	
3.1.1.6.	Acetyl ester acetyl-hydrolase = acetylesterase	
3.1.1.7.	Acetylcholine acetyl-hydrolase = acetylcholinesterase	Cholinesterases
3.1.1.8.	Acetylcholine acyl-hydrolase = Cholinesterase	

In the present experiments the following terminology will be used

ChE (acetylcholinesterase and pseudocholinesterase) and CarE (carboxylesterase)

Carboxylesterase have been always linked with resistance mechanisms to organophosphorus insecticides (Sudderuddin and Tan, 1973). Gilmour (1965) suggested that they bind toxic phosphate esters to their active sites and hydrolyse them. On the other hand Plapp and Bigley (1961) showed that carboxylesterase may be involved in the toxication processes by acting as storage sites for organophosphates and then releasing them to exert their toxic effects on ChE. Perry (1964) suggested the involvement of CarE in the detoxication processes because destruction of blood cells [non-specific esterases show unusual activity in blood of some insects (Patton, 1961)] to sub-critical levels increases the susceptibility of some insects to parathion. Esterases have been located in the cuticular pore canals of several insects, indicating a link in wax synthesis and esterases distribution (Locke, 1961).

Although ChE inhibition is considered the main biochemical lesions the mode of action of organophosphates (O'Brien, 1967), the role of this group of enzymes in insect resistance is still uncertain, for example, Voss and Matsmura (1964) in a study on the mechanisms of malathion and parathion resistance in the two-spotted spider mite Tetranychus uritacae found that there is no detectable qualitative or quantitative differences in the properties of ChE of the resistant and susceptible strains. On the other hand Smissaert (1964) experimenting with the same animal indicated that ChE activity of its resistant strain was three times higher than that of the susceptible strain.

The activity of the inhibited esterases could be restored by reaction with water. This reaction is known as spontaneous reactivation (Reiner, 1971). Rapid recovery of inhibited ChE was reported in the American cockroach (Chamberlin and Hoskins, 1957; O'Brien, 1956) and in houseflies (Smallman and Fisher, 1958; Mengle and Cassida, 1958; Mengle and O'Brien, 1960). However, (Stegwee, 1960; O'Brien, 1961; Plapp and Bigley, 1961) suggested that only slight recovery occurred *in vivo*. More recently the work of Brady and Sternburg (1966, 1967) showed slow *in vivo* recovery of ChE in the houseflies.

Despite the extensive studies on ChE recovery, few studies have concentrated on the recovery of CarE (Plapp and Bigley, 1961; Bigley, 1966; Ahmed, 1970; Sudderuddin, 1973). These studies have shown that CarE recovery to be much slower than that of ChE.

Studies on the effect of insect rhythmicity on esterases activity was conducted by Bull and Lingquist (1968) in extracts of bollweevils. Their results indicated that the ChE activity of insects kept in LD 14:10 was about 25% higher during the light phase (when the insects were active) than during the night, while in weevils reared in constant light had a virtually constant enzyme activity across the day. Venkatachari and Dass (1968) observed a similar relationship between the locomotor activity of the scorpion and the ChE activity in its nervous system. Cymborowoski *et al*. (1970) showed that house crickets maintained under alternating light and dark underwent cyclic changes of their ChE activity, unlike insects maintained under constant light conditions who had a stable level of ChE activity throughout the day. Wan and Hooper (1967) and Hooper and Wan (1969) showed that aliesterase activity in the German cockroach and in the houseflies is cyclical, they speculated a relationship between the cyclical activity

of the corpora allata and the cyclical activity of ali-esterase.

There are no previous reports on the relationship between the inhibitory effect of organophosphates and the rhythmic activity of esterases, however this aspect was investigated in the course of the present work. Finally, a full and clear explanation of the circadian rhythm of insects susceptibility to insecticides may provide an explanation of the erratic results obtained in the bioassay of insecticides and may help to enhance the efficiency of some treatments by identifying the time of the day when insects are most susceptible. In some cases the latter factor could be of a significant consideration in drawing up schemes for insect control.

Although a number of workers had demonstrated the existence of a circadian susceptibility in some insects to different insecticides, none of these experiments have revealed much of the biochemical and toxicological bases underlying this susceptibility rhythm and there is still meagre information on this phenomenon.

The present study was carried out to confirm the existence of the circadian susceptibility of house crickets to some organophosphorus insecticides, and if possible to investigate its cause.

MATERIALS AND METHODS

1. The Insects

The house cricket Acheta domesticus (L.) was the experimental animal used in this study. A culture was started from a mixture of adults and nymphs obtained from Cambridge University. The insects were kept at 29 ± 2 C° and a relative humidity of 65 ± 5%. Since light has a vital role in entraining the rhythm, a photoperiod of 12 hr light from 0600 hr to 1800 hr GMT was provided. The illumination was provided by a fluorescent tube (80 W.).

The insects were reared in cages constructed of perspex (30 x 30 x 30 cm) with one side having a large opening covered with muslin and the muslin was lined with "velcro" strips so that flaps could be easily removed and stuck on again. Each cage was furnished with a 3 cm. layer of sterilised sand and pieces of honeycomb in which the insects could hide. Water was provided from a wet piece of cotton wool placed in deep plastic petri dishes.

Different kinds of diet were tried, the most satisfactory was the one formulated by mixing rolled oats 47.5% wt., dried skimmed milk 47.5% wt. and dried brewer's yeast 5% wt.

Eggs were laid in wet sand contained in deep dishes, the dishes were collected every 3 to 5 days and incubated at 32 C° for 14 days. When the eggs hatched the nymphs were transferred to cages where food and wet sand was provided. Groups of hatchlings were reared together so that batches of adults of known age could be used in the experiment.

2. Behaviour Studies

Insects taken at random were kept individually in delicately

balanced boxes (actographs) made of transparent polystyrene and connected with a recording apparatus (Fig. 1). The movement of the insect from one end of the actograph to the other caused the box to rock which in turn triggered the recording apparatus giving a mark on a chart recorder. Recording charts were collected at the end of each run and the frequency of the locomotor activity was measured by counting the number of marks given by the recording pen and relating it to the time of the day and the light condition, so that the pattern of the daily activity could be ascertained. Actographs with mercury dip-switches were used initially but were replaced by photo-cell switches to avoid the vapour hazard from open mercury cups.

Precautions were taken throughout to prevent the insects moving as a result of factors other than their circadian activity rhythm and water was provided by placing a piece of wet cotton wool in a small vial attached in the centre of the actograph. Another piece of light tube big enough to accommodate the insect was attached in the actograph as a shelter.

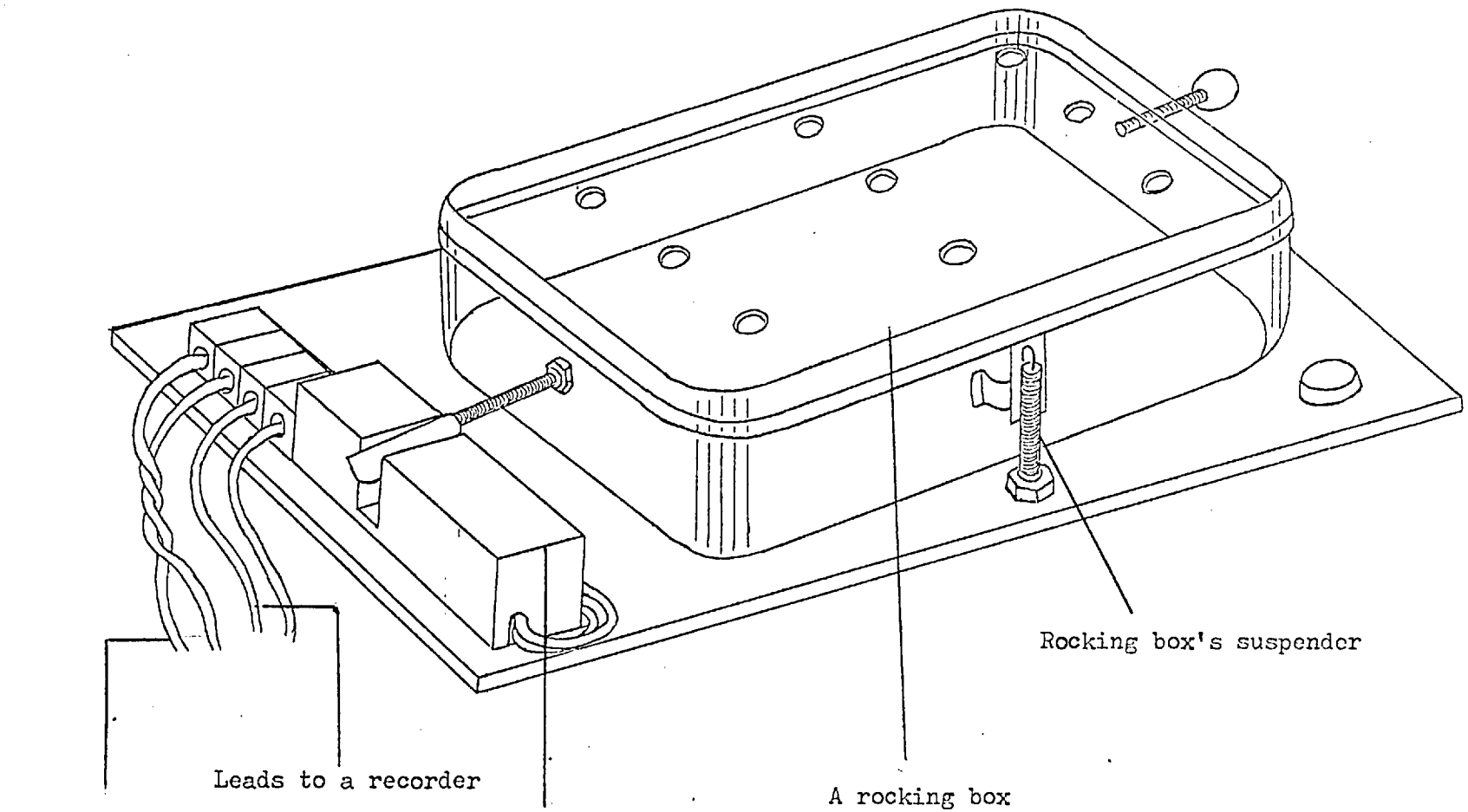
3. Bioassay Studies

3.1. The Insects

Adult insects cultured in LD 12:12 were dosed when between 5 to 14 days old. Equal numbers of males and females were treated in each assay, since some pilot experiments showed that the females were more tolerant than the males. Both sexes were used since there was insufficient of any one sex for bioassay experiments. Between 20 - 40 insects were used for each of the four or five concentration treatments. The insects were anaesthetised to facilitate handling. A mixture of 60% carbon dioxide and 40% nitrogen was used as recommended by Edwards

Fig. 1.

The photo-cell switched actograph



and Patton (1965) after Ralph (1959) had shown that 100% carbon dioxide may affect the insects' rhythmicity.

3.2. Insecticides

The insecticides used were malathion 99.0% (analytical standard sample), diazanon 99.0% (analytical standard sample) and phorate. The phorate was extracted from 10% granular formulation and was purified by column chromatography (Bowman et al., 1969). The purity of the extracted phorate was checked by a thin layer chromatography (Blinn, 1963) and the phosphorus concentration was determined by the method of Chen et al. (1956).

The insecticides were dissolved in acetone-risella oil (95-5 V/V) because preliminary experiments had shown that the risella oil enhanced the insecticides' efficiency and produced more consistent results.

3.3. Dosing Procedure

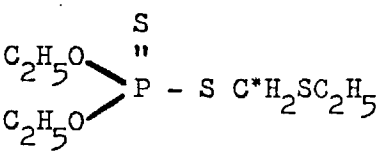
An Arnold electric microapplicator was used to deliver from an Agla syringe, 1 µl of solution to the metathoracic sternum of each insect. The insects were treated in the laboratory at room temperature but returned to the controlled environment room soon after dosing. Treated and controlled insects were kept in groups of five in plastic dishes after pilot experiments had shown that more than this number per dish induced cannabalism thus resulting in non-insecticidal mortality. When insects were dosed at night, they were collected and handled with the aid of the illumination of a red, darkroom bulb.

Insects were treated at particular times of the day coinciding with different magnitudes of locomotor activity.

Mortality data were recorded 24 hours after dosing and subjected to probit analysis (Finney, 1971) using the University of London CDC 6600 computer. The program was developed by Dr. G. Murdie.

4. Radiotracer Technique

4.1. The Insecticide



(The asterisk indicates the labelled atom).

The insecticide used C^{14} - phorate, was labelled in the methylene group, and had a specific activity of 46 µci/mg. To purify the compound, the stock solution was concentrated to 1.5 ml and eluted with 100 ml of benzene through a column 8 mm internal diameter, packed with 6 g of silicic acid (Silic AR CC7, Mallinckrodt) (Bowman et al., 1969). All the phorate was collected in the first 50 ml of the effluent. The purity of the compound was checked by thin layer chromatography (T.L.C.) using palladium chloride as a visualising agent (Blinn, 1963), by comparing the labelled compound against standards of unlabelled phorate, its sulphoxide and sulphone. The purified compound was made up to 10 ml in acetone and its specific activity was determined by spectrophotometry (Chen et al., 1956) and radio-assay in a liquid scintillation counter.

4.2. Scintillation Media

4.2.a. Solvent extracts:- C^{14} - compounds in solvent solutions were evaluated by adding samples to either butyl PBD at 8g/l. of toluene or to p-terphenyl at 6g/l. of toluene.

4.2.b. Aqueous extracts:- A slightly modified formulation of Bray's scintillant solution (Bray, 1960) was used for the water soluble fraction of the extracted insecticide. Naphthaline (60g), PPO, 2.5 diphenyloxazole (4g) and POPOP, 1.4 - Di (2 - (5-phenyloxazolyl) - benzene (0.2g) were dissolved in 100 ml of Analar grade methanol and made, up to one litre with Analar grade dioxon.

4.3. Isotope Counting Procedure

The radioactivity in each sample was measured in a Beckmann LS 250 liquid scintillation spectrometer. The activity in each sample was counted for 100 minutes or until the 2σ counting error was $\pm$ 1% whichever occurred first. Counting efficiences were assessed by the external standard method, and the results corrected for the background and efficiency.

5. Evaporation Studies

5.1. Evaporation from Glass Surfaces

Doses of 1 µl of C^{14} - phorate dissolved in acetone (Analar grade) were applied on round glass coverslips (13 mm in diameter). The application technique was the same as that described in the bioassay studies. Treated coverslips were left at room temperature for the time interval required and at the end of which they were placed in scintillation vials containing 10 ml of the scintillant solution. The radioactivity remaining on the coverslips was assessed and the rate of evaporation was calculated. Each time interval was replicated three times and the means were plotted with respect to time.

A similar set of treatments in which doses of C^{14} - phorate were dissolved in (95 - 5 V/V) mixture of acetone/risella oil, was conducted to examine the effect of risella oil on the evaporation of the carrier

solution.

5.2. Evaporation From Insect Wings

Fore-wings of house crickets were used immediately after they were severed from the insects. A dose of C^{14} - phorate in 1 µl (95 - 5 V/V) acetone/risella oil was applied on each wing. Groups of 3 treated wings were put into a small, filter paper based petri dish and kept in a fume cupboard at room temperature. At the end of each time interval the wings were washed to recover the radioactivity remaining on the surface (external fraction), then homogenized and extracted to recover the amount of radioactivity which had migrated to the inner layers (internal fraction).

Washing procedure

Each wing was rinsed with 0.5 ml of methanol, followed by another rinse with 0.5 ml of warm toluene. The warm toluene rinse was used to recover any radioactivity still present on the surface or in the superficial lipids of the epicuticle. Each rinse was collected in a separate vial and counted.

Extraction procedure

Washed wings were homogenized thoroughly with 5 ml (1-1 V/V) acetone—water in a Potter-Elvehjem hand homogenizer with a teflon plunger. The homogenate was transferred to a separating funnel, washed and separated with three 5 ml fractions of chloroform. During each chloroform wash the funnel was shaken vigorously, after which it was left until the two phases separated. Then the chloroform fraction was collected in a round-bottomed flask. Appropriate phases were combined and evaporated using a rotary evaporator. They were then transferred to scintillation vials with the appropriate scintillant

solutions and the radioactivity was counted. Knowing the initial activity in the applied dose and the residual radioactivity on and in the wings, the rate of evaporation was calculated and expressed in relation to time.

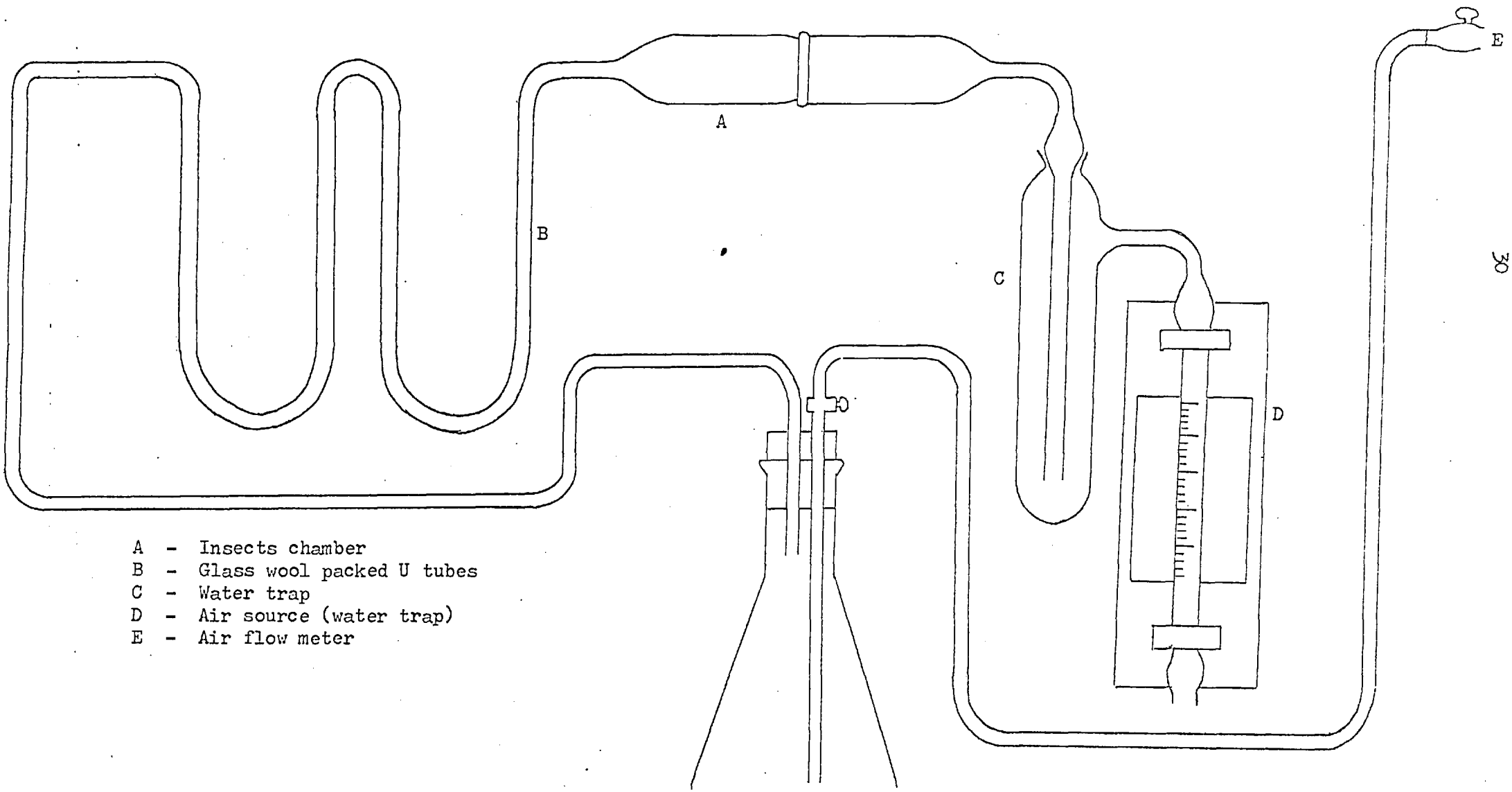
5.3. Evaporation from Silica Gel

A thin layer chromatography plate coated with 0.5 mm layer of silica gel was divided into several squares of equal size. Doses of C^{14} - phorate in 1 µl acetone-risella oil (95 - 5 V/V) were applied on each square and the plate was left at room temperature. At different time intervals the silica gel in each square was scraped off the plate and added to a vial with 10 ml of scintillant solution and the radioactivity was counted. Each time interval was repeated three times.

5.4. Evaporation from Topically Treated House Crickets in Relation to their Locomotor Activity Rhythm

The Vapour Collector:- This system was intended to capture insecticide evaporating from topically treated insects. It consisted (Fig. 2) of a horizontal glass tube in which the treated insects were kept (insects chamber). Air entered this chamber through a flowmeter and then passed through two U' - tubes packed with glass wool moistened with Risella oil. A filter pump was used to draw air through the system and the flow rate was maintained at about 100 ml/min. Earlier experiments with cold traps at -80°C showed that lower temperatures were not significantly more effective for collecting phorate evaporating from glass slides. The system was kept in a closed cupboard at room temperature. At the end of each run the insects chamber was thoroughly washed through with the scintillant

Fig. 2. The Vapour Collector



A - Insects chamber
B - Glass wool packed U tubes
C - Water trap
D - Air source (water trap)
E - Air flow meter

solution and the washings were collected in scintillation vials. The glass wool tubes were also washed through with scintillant and again the washings were collected in vials. The radioactivity in the different vials was counted and the rate of loss of the insecticide was calculated. The efficiency of the system was measured by adding a known amount of C^{14} - phorate to a glass slide which was kept in the insects chamber for specific time intervals. The slide was then removed and washed with scintillant to recover the activity still remaining on it. The radioactivity captured by the trap was also counted and then by combining the radioactivity recovered from the slide with that found in the trap and relating these to the radioactivity initially applied, the efficiency of the system was determined.

6. Phorate Penetration and Metabolism in Relation to The House Crickets Circadian Rhythm

Adults between 5-14 days old were used in this experiment. Insects were immobilized by chilling them for 2-3 minutes to facilitate handling them before a dose of C^{14} - phorate in 1 µl acetone-risella oil (95-5 V/V) was applied to each insect on the metathoracic sternum. Treated individuals were placed in the insect chamber of the evaporation trap. At the end of each pre-determined time interval, the insects were washed to recover any radioactivity remaining on the cuticle (external fraction), then homogenized and extracted to recover the radioactivity which had penetrated into or through the cuticle (internal fraction).

6.1. Washing Technique

Each insect was rinsed with 2 ml of methanol followed by a rinse of 2 ml of warm toluene. The toluene wash was used to recover any radioactivity associated with the lipid layer of the epicuticle.

Insects were held by the tibia of the hind leg in a pair of forceps, while each rinse was run over the insect from a Pasteur pipette. Each rinse was collected in a separate scintillation vial.

6.2. Internal Fraction

A number of solvent systems were tested for extracting phorate and its metabolites from the insects. When a mixture of acetone and water (1-1 V/V) was used, a stable emulsion was formed which prevented adequate separation of the organic and the aqueous phases. The chloroform-water system has been widely used with different modifications in the extraction methods for phorate and some other similar organophosphorus compounds (Metcalf et al., 1957; Metcalf et al., 1964; Coleby et al., 1972). Some modificationsof this system were tried, and the highest recovery was obtained when a mixture of chloroform and water (2/1 V/V) was used. Insects were homogenized in a mortar with 10 ml of the chloroform-water mixture (2/1 V/V) and the homogenate was transferred to a conical flask, shaken for 20 minutes and then centrifuged. The separated aqueous and organic solvent layers were withdrawn and the tissue residue extracted twice more. Each solvent layer was partitioned in a separatory funnel with the opposite solvent and the appropriate phases were combined. The solvent extract was evaporated to near dryness using a rotary film evaporator under reduced pressure (300 mm Hg.) at 45-50 C^{o}. The aqueous fraction was evaporated to 10 ml., and an aliquot of 2 ml was transferred to a scintillation vial with 10 ml of scintillation solution. Fecal pellets collected at different time intervals were similarly extracted.

6.3. Separation and Identification of Phorate and its Oxidative Metabolites

6.3.a. Column chromatography:- This method was used to separate phorate and its oxidative metabolites. The organic extract was evaporated to near dryness and the residues quantitatively transferred in 2 ml of benzene to the top of a chromatography column (20 x 1.2 cm) containing 6 g of silicic acid (Silic AR CC7, Mallinskrodt). Phorate and its oxidative metabolites were eluted with 40 ml aliquotes of benzene and acetone respectively (Le Patourel and Wright, 1974). Each effluent was collected in a 50 ml round-bottomed flask and concentrated to near dryness. The concentrated residues were quantitatively washed into counting vials with two 5 ml aliquotes of scintillant solution.

6.3.b. Thin layer chromatography:- Thin layer chromatography was employed to give a more comprehensive and qualitative idea of the full spectrum of phorate and its oxidative metabolites. Insects were homogenized as described before, but the organic fraction was concentrated, then transferred to a 10 ml centrifuge tubes so that it could be further concentrated to about 0.3 ml under a nitrogen stream. Aliquotes of known volumes were spotted on a glass chromatography plate (20 cm x 20 cm) coated with silica gel type G, 0.5 mm thick and previously activated at 100 C^{o} for two hours. Each spot was fortified with 20 ug of a mixture of unlabelled phorate and its oxidized metabolites, to facilitate the detection of the spots. The plates were developed in a benzene-methanol, (92-8 V/V) solvent system, for a distance of 15 cm from the point of application. The spots were visualized by spraying with 0.5% solution of palladium chloride (Blinn, 1963). The silica gel in the area of each spot was carefully scraped from the plate and transferred to a counting vial and 10 ml of

scintillant added for radioassay. Each spot was replicated three times and each replicate consisted of one male and one female insect.

7. Autoradiography

This method was used to confirm the location of different metabolites of phorate on the TLC plates. Aliquotes of the solvent soluble fractions were spotted and developed as described previously. They were then exposed and kept in contact with Kodak X-ray film at room temperature for nearly four months before developing in phen-X^{R} (Ilford Limited) and fixed in Amfix (Ilford Limited).

8. Enzymes Assays and Inhibition Studies

Heads of 5-14 day old insects were used throughout as a source of the insect enzymes. The insect head was removed weighed and homogenized with 2 ml of 0.1 M phosphate buffer (PH 8.0) in a Potter-Elvehjem hand homogenizer. The homogenizer was partially immersed in crushed ice.

For the measurement of cholinestrase and carboxylestrase inhibition, a dose of 1 µl of phorate (1.2 µg) was topically applied to each insect. At each predetermined time interval after application the insects were washed thoroughly with distilled water to remove any insecticide residues still remaining on the body and the heads, homogenized with the addition of acetylcholine chloride (0.12 M) to protect ChE and CarE from any further inhibition. This procedure was adopted in the inhibition studies since Van Asperen (1958, 1960) has shown that false high estimates of inhibition may result from *in vitro* inhibition during homogenization of the tissue. The homogenate was centrifuged for 2 minutes and the supernatant decanted for subsequent analysis.

Solutions and Reagents

Buffer phosphate 0.1 M PH 8.0

De-ionized water

Acetylthiocholine Iodide (0.2 M), prepared in phosphate buffer (0.1 M) PH 7.0, was used as substrate of ChE.

Thiophenyl acetate (0.15 M) was prepared in methanol as a substrate of non-specific estrases.

Acetycholine chloride (0.012 M) was prepared in phosphate buffer (0.1 M) PH 8.0.

Di-thiobis nitro benzoic acid (DTNB) (0.01 M). 39.6 mg were disolved in 10 ml phosphate buffer (0.1 M) PH 7.0 and 15 mg of sodium bicarbonate was added.

To determine the activity of ChE and CarE the method described by Ellman et al (1961) was employed. The reaction was carried out in a spectophotometer cell and the final concentration of the reaction mixture was as follows:- 3 ml of phosphate buffer (0.1 M) PH 8.0, 0.1 ml of dithiobisnitrobenzoic acid (0.01 M), 0.1 ml of head homogenate. The ChE activity was measured by adding 0.05 ml of acetylthiocholine iodide (0.2 M) while for CarE 0.03 ml of thiophenyl acetate (0.15 M) was added. The optical density of the yellow 5-thio-2-nitrobenzoic acid produced by reduction of the reagent by either thiocholine or thiophenyl was measured at 412 mu in a spectrophotomter.

EXPERIMENTS AND RESULTS

1. Behavioural Studies

These experiments were designed to establish the pattern of the insect's locomotor activity rhythm throughout the course of the day. Adult males 2-3 weeks old were used throughout, thus avoiding any complications in activity that the reproductive cycle of female insects might induce. All experiments were carried out in the constant temperature room. Care was taken not to disturb the insects during the course of the experiment to minimize any non-rhythmic interference of any kind. The insects were kept in the actographs for 1-2 days before the recording process was started, and their activity was recorded for 4-6 successive days. The experiments were carried out several times, using different insects each time in order to investigate the rhythmic nature of as many insects as possible. Experiments were conducted frequently enough through the course of study to make sure that the successive generations were maintaining an adequate rhythmic condition throughout.

The Results

1.1. Insects locomotor activity in light-dark cycle

Data accumulated from chart records are represented in Fig. 3. These results showed that the house crickets performed the majority of their locomotor activity in the dark phase. The highest level of activity was recorded 2-3 hours after the onset of darkness, and the activity persisted throughout most of the dark phase, declining slightly until it ends an hour or two before dawn. During the light phase the activity was generally low and sporadic. This pattern of activity was maintained throughout the experiments. Results showed that an average of 90% of the tested insects were rhythmic. Although a few insects died before 4-5 days, there was no marked increase in the

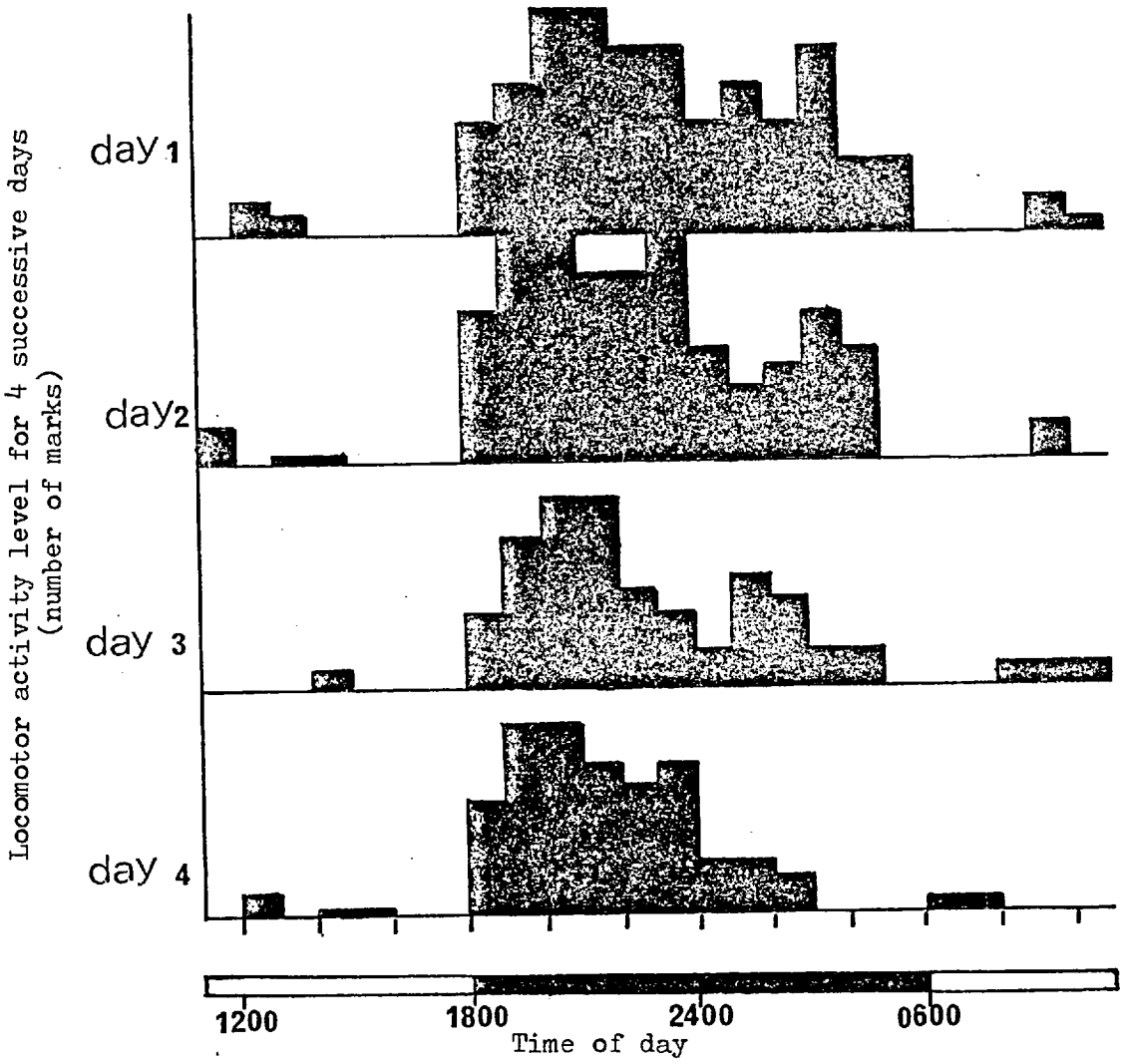


Fig. 3

Locomotor activity pattern

in L.D. cycle

activity level prior to their death as has been reported with cockroach Gunn (1940). Those that died were not included in the histograms shown in Fig. 3.

1.2. Insect locomotor activity in constant conditions

A number of insects were taken at random and placed in the actographs. The actographs were kept inside a wooden cabinet lined with black fabric and tightly sealed to prevent any light reaching the insects. Insect locomotor activity is represented in Fig. 4. The results showed that the pattern of activity that the insects had shown in the light-dark cycle persisted but that when free running under the constant dark conditions the activity peaks occurred slightly later each day.

1.3. Insect locomotor activity in inversed light cycle

A group of insects was taken at random from the stock culture and was kept for two weeks under inversed light cycle. Their locomotor activity was then recorded and data from the charts is represented in Fig. 5. These results showed that the insects responded to the reversed light system and re-entrained their activity rhythm to the new photoperiod.

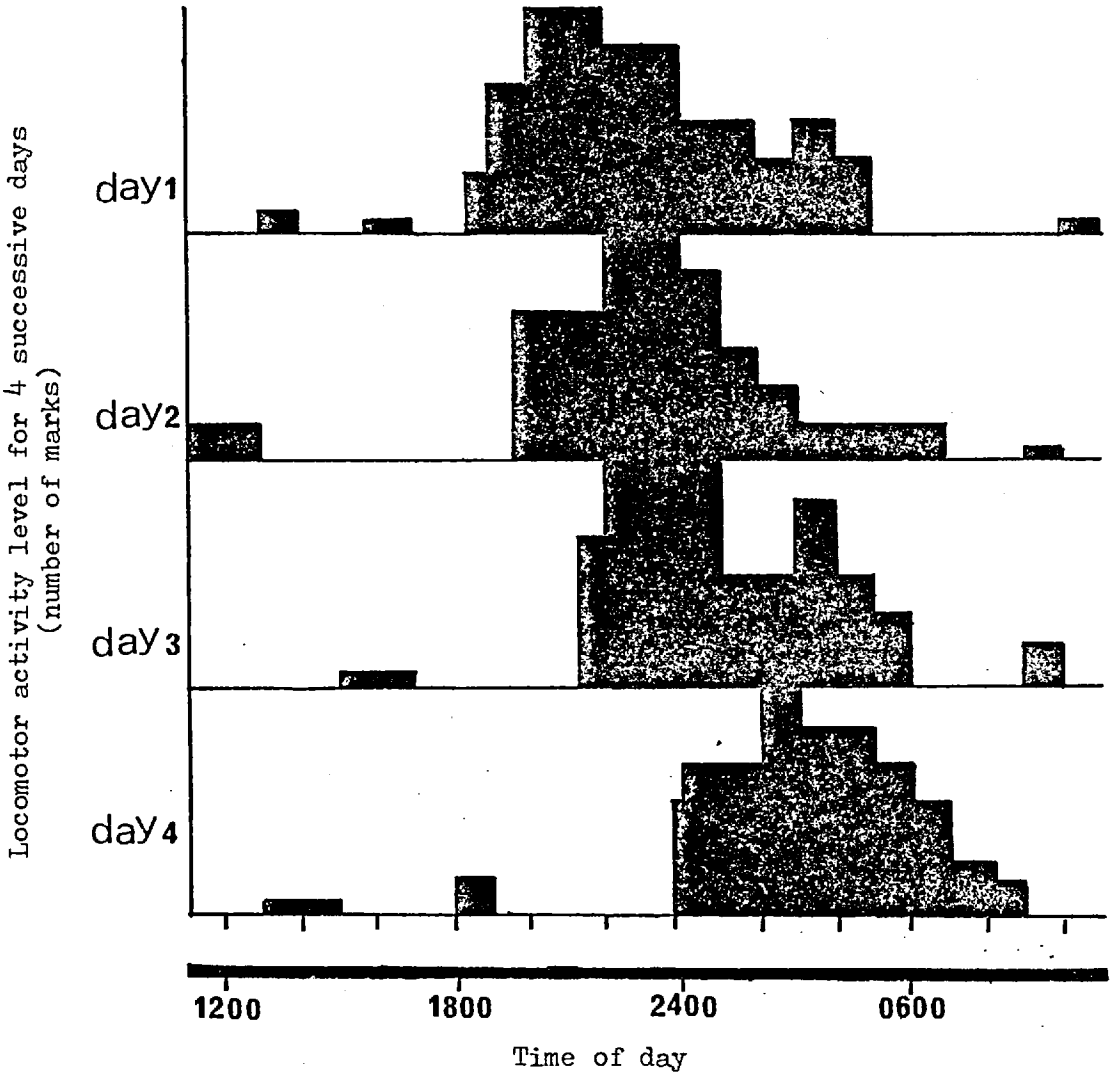


Fig. 4

Locomotor activity pattern in constant darkness

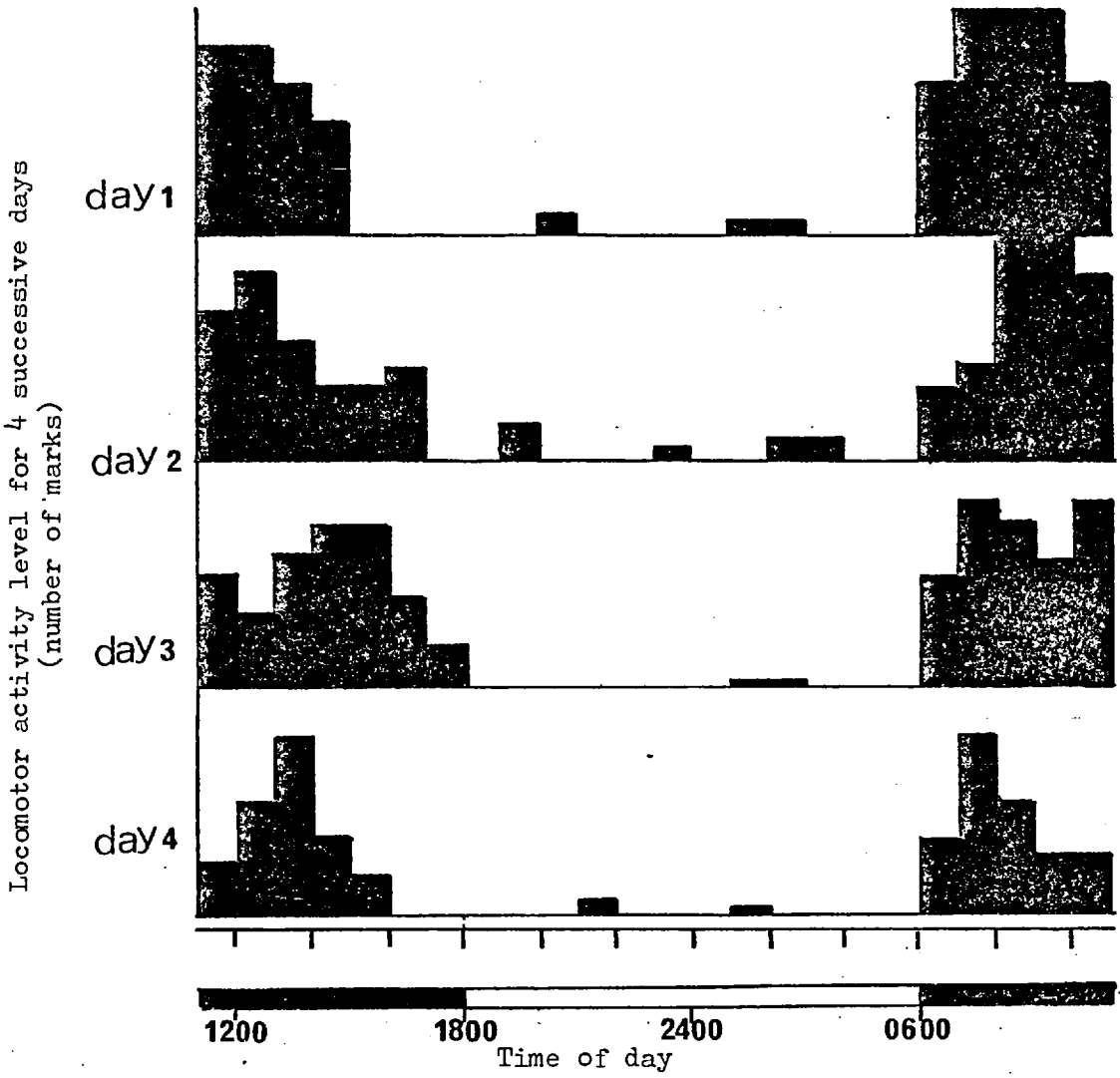


Fig. 5

Locomotor activity pattern in a reversed

LD cycle

2. Bioassay Studies

The Results

2.1. Circadian susceptibility to malathion

The LD_{50} values related to the different times of application are shown in Fig. 6 and demonstrated that the susceptibility of the house crickets varied over the course of the day. The insects were least susceptible at 1600 hr. (i.e., two hours before the onset of darkness), a period which coincided with an inactive phase of insects' locomotor activity and most susceptible when treated two hours after darkness (i.e., at 2000 hr.) a time corresponding with the highest level of locomotor activity. The next treatment at midnight showed the insects to be more tolerant, but still a little more susceptible than when treated during the light period.

2.2. Circadian susceptibility to phorate

The LD_{50} values plotted with respect to the time of application are shown in Fig. 7. The susceptibility pattern obtained is similar to that found for malathion, insects were least susceptible at midday and most susceptible at 2000 hr., with gradual return during the night and the next morning to the least susceptible condition at 1200 hr. The insects were more tolerant at each of the three times they were tested during the day than after the two night treatments, and the pattern of susceptibility correlated well with the locomotor activity rhythm.

2.3. Circadian susceptibility to diazanon

The relationship between the times of treatments and the corresponding LD_{50} values is portrayed in Fig. 7 and showed that the insects were most tolerant at midday, this level of susceptibility was fairly well maintained through the light phase. Unlike malathion and phorate the crickets were equally most susceptible when treated just after dawn and just after dusk.

Fig. 6

Circadian susceptibility of the house crickets to malathion

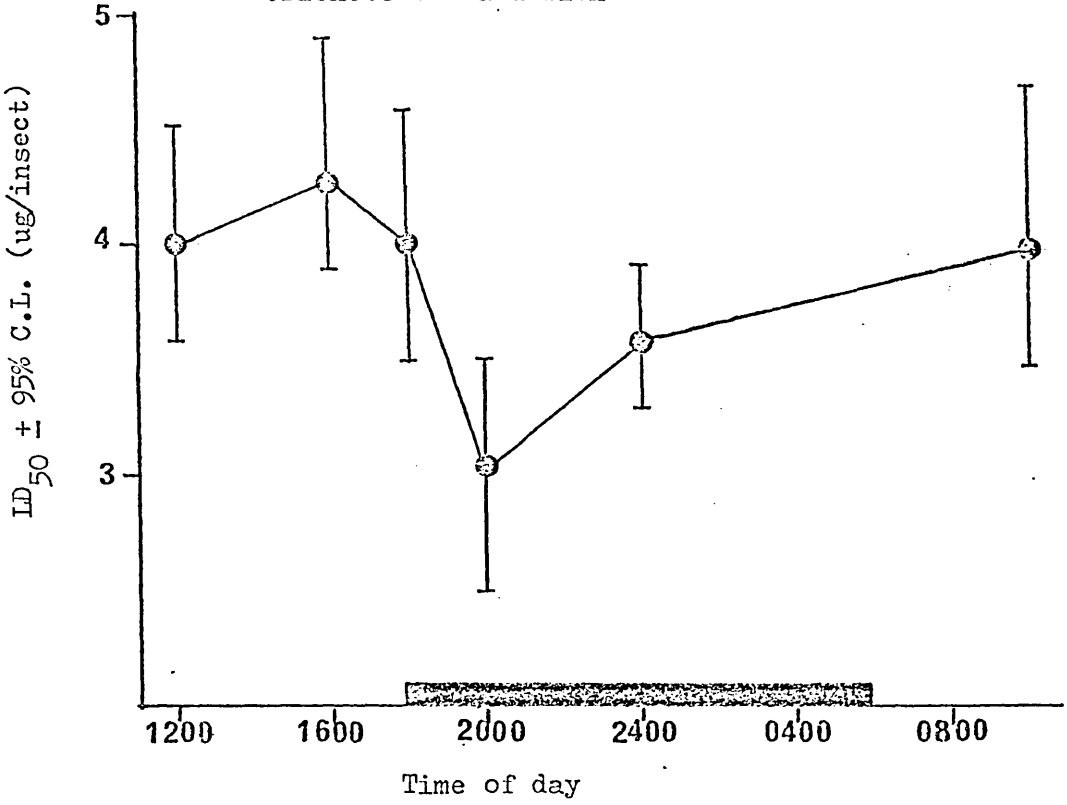
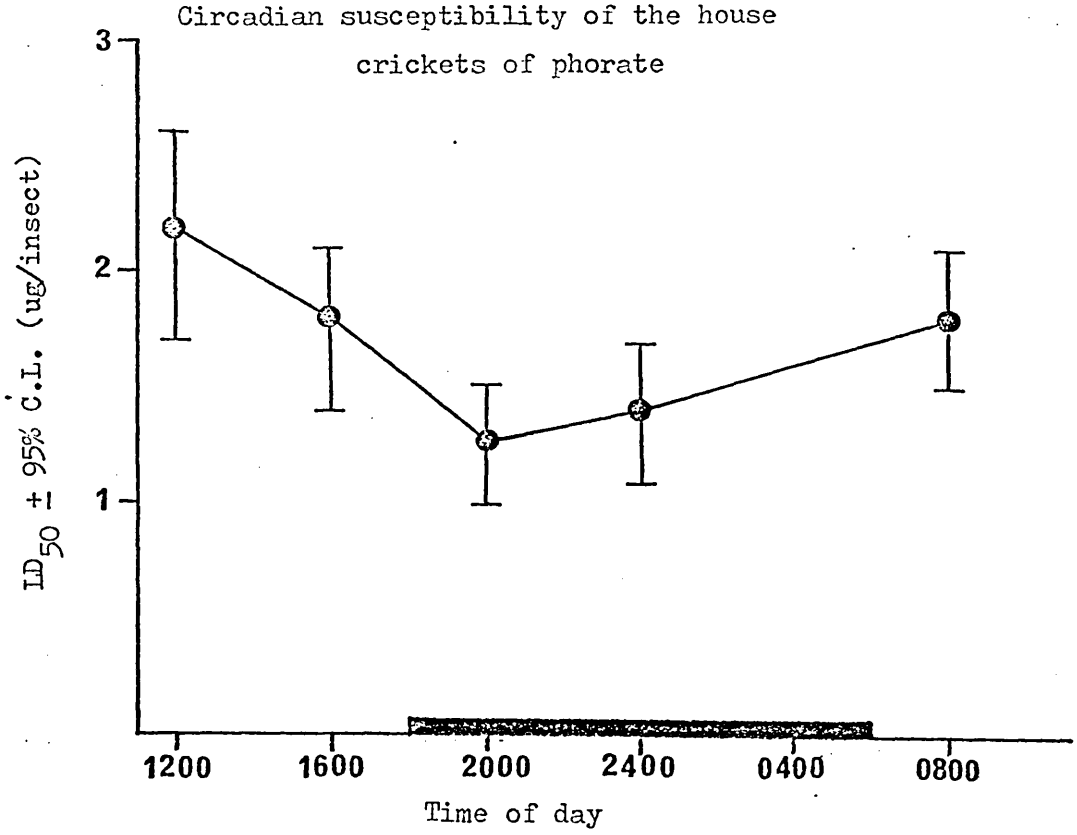


Fig. 7

Circadian susceptibility of the house crickets of phorate



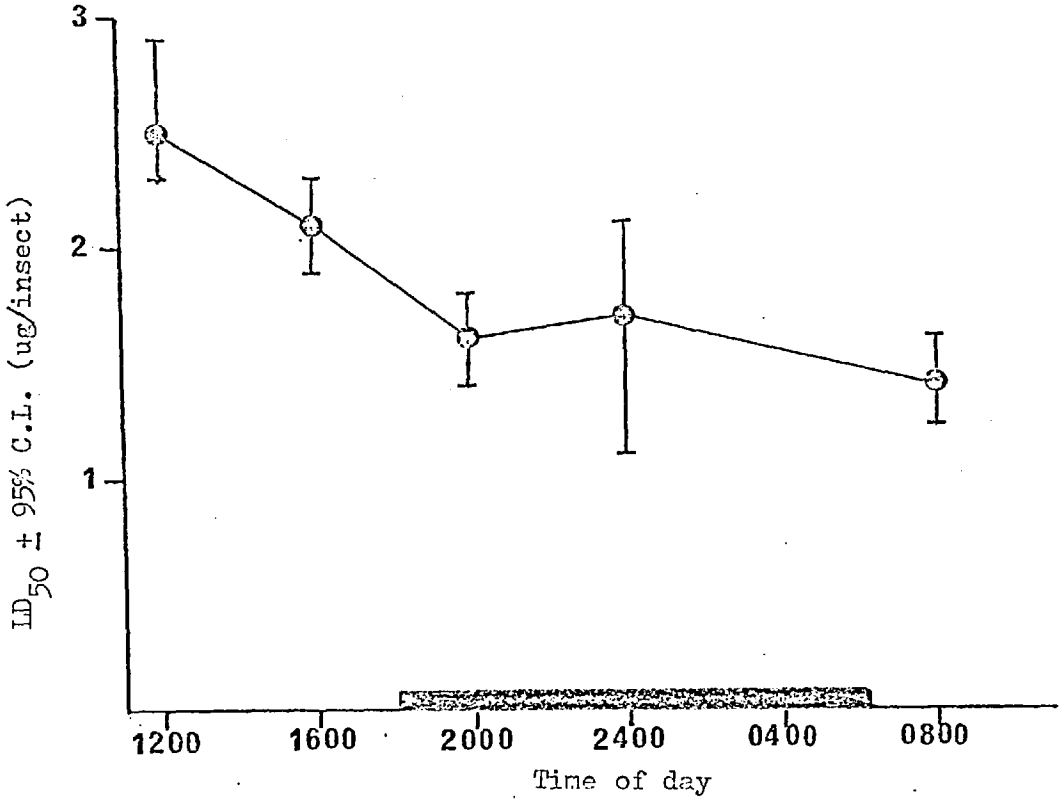


Fig. 8

Circadian susceptibility of the house crickets to Diazanon

3. Evaporation Studies

C^{14} - Phorate Evaporation

An investigation was carried out to study and measure the rate at which topically applied phorate evaporates from different surfaces. These surfaces tested were glass, fresh insect's wings and silica gel. C^{14} - phorate - evaporation was also studied from topically treated house crickets in relation to their locomotor activity rhythm, the rate of evaporation was measured during both the active and inactive phase. Four insects (2 males and 2 females) were topically treated at the beginning of each phase and the volatile compounds from treated insects were trapped, measured and a relationship between different time intervals and rate of evaporation was obtained for each of the two phases of locomotor activity.

The Results

3.1. Evaporation from glass surface

Fig. 9 shows the evaporation rate when phorate was applied in 100% acetone, while Fig. 10 shows evaporation of the same compound when applied in an acetone-risella oil (95-5 V/V) mixture. The figures showed the substantial effect of the oil in reducing the evaporation rate of topically applied phorate, for example 30 minutes after application only 47% of the dose applied in 100% acetone remained, while after the same interval 87% of the dose applied in (95-5) acetone-risella oil was recovered, after 60 minutes 3% were left on the glass surface and 85% respectively.

3.2. Evaporation from fresh wings

Fig. 10 also shows the evaporation of C^{14} - phorate from fresh wings after application in acetone-risella oil mixture. The results showed that the rate of evaporation from fresh wings was higher than

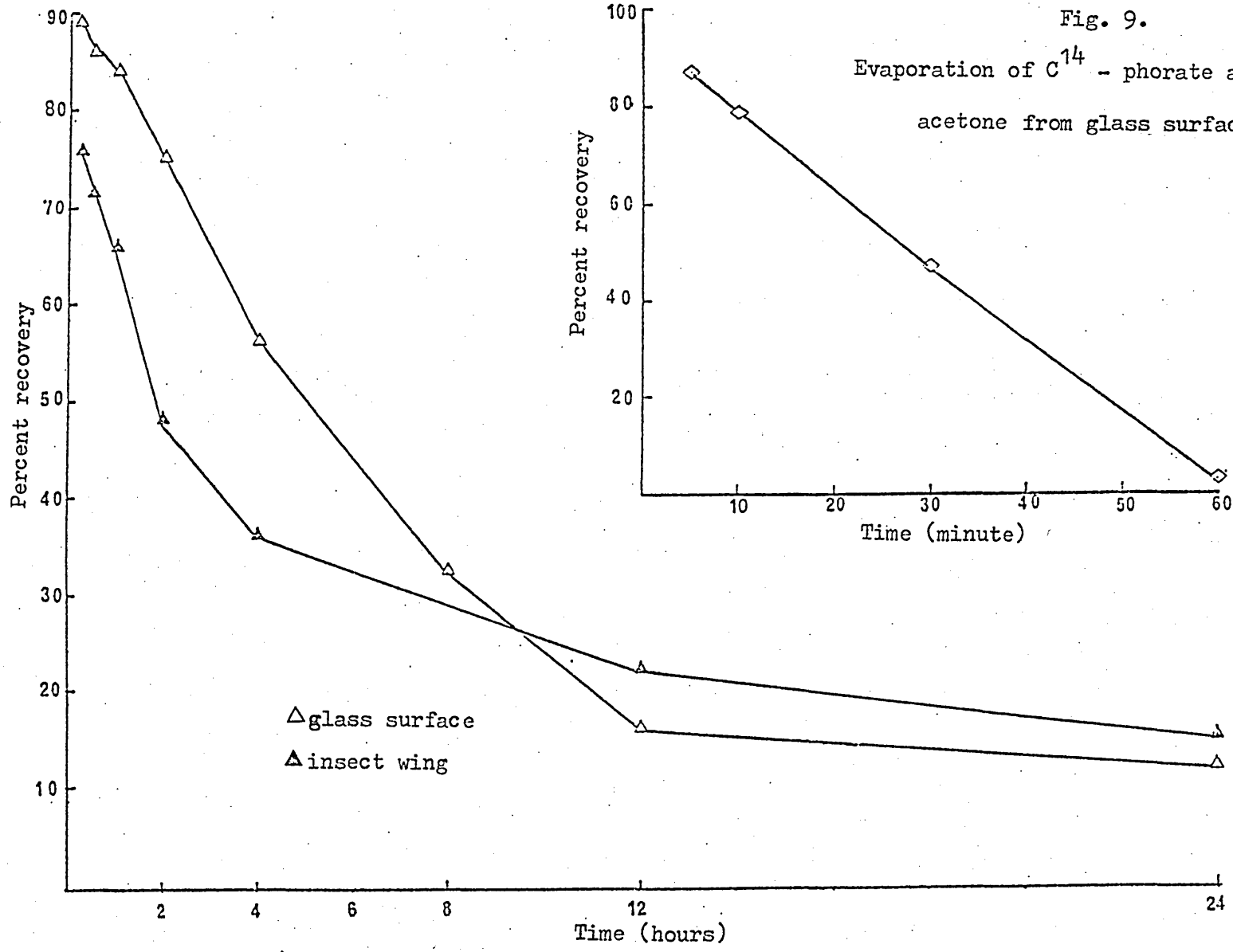


Fig. 9.

Evaporation of C^{14} - phorate applied in acetone from glass surface

Fig. 10. Evaporation of C^{14} - phorate in acetone-risella oil from glass surfaces and insect wings.

from glass slides for the first 12 hours, after which the rate of evaporation was reversed and reduced.

3.3. Evaporation from silica gel

There was no detectable loss of C^{14} - phorate from plates coated with 0.5 ml layer of silica gel. Thus it seems that the absorptive qualities of the silica gel enabled it to retain the applied dose of phorate without any significant evaporation taking place.

3.4. Evaporation from house crickets in relation to their circadian rhythm of locomotor activity

Fig. 11 shows the evaporation rate of C^{14} - phorate in acetone-risella oil (95-5 V/V) during the active and inactive phases of the house crickets locomotor activity rhythm. The result showed that at no time significant differences in the rate of evaporation were detected. The results indicated that the locomotor activity rhythm had no significant effect on the evaporation of topically applied phorate. Results also showed that up to 10% of the applied dose was lost by evaporation 3 hours after the dose was applied, and this loss increased gradually with time until after 24 hours about 18% of the applied dose had evaporated.

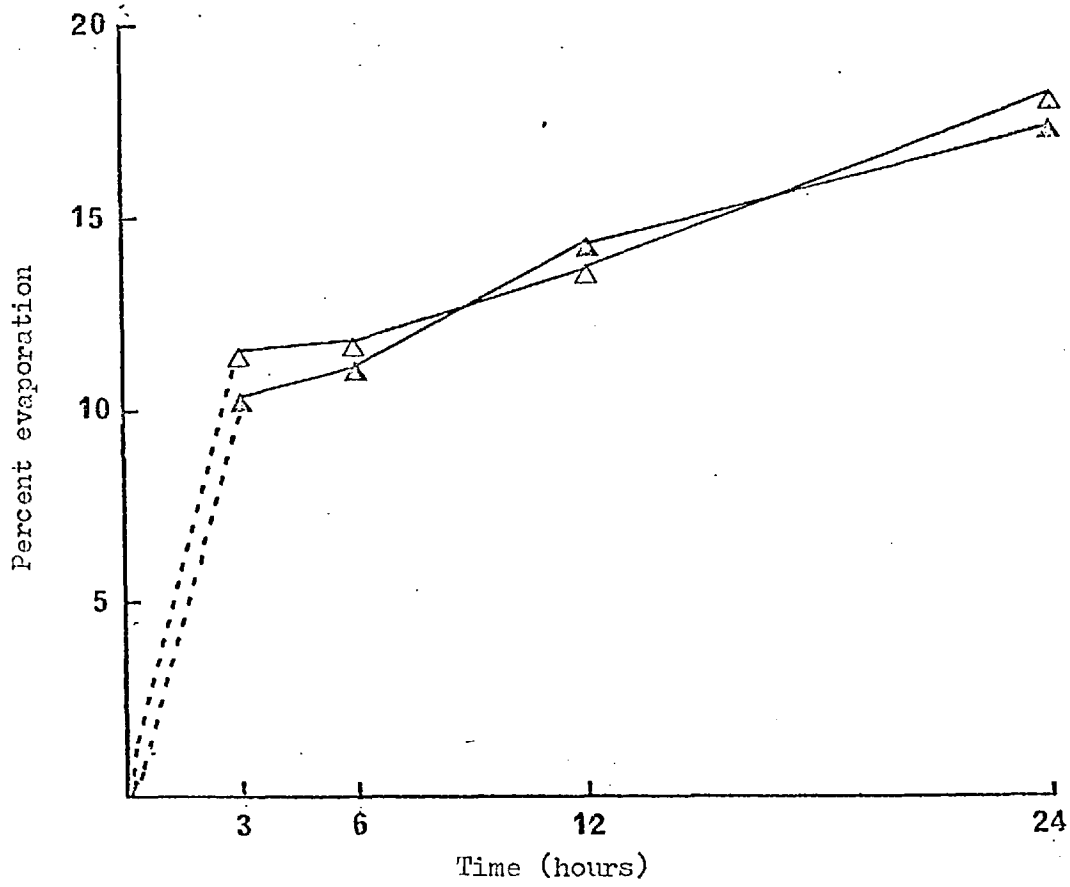


Fig. 11

Evaporation of topically applied C^{14} phorate in relation to the insect locomotor activity

▲ Active insects

△ Inactive insects

4. Phorate Penetration and Metabolism in Relation to House Crickets' Circadian Rhythm

This experiment was conducted to ascertain the relationship between the house cricket's locomotor activity rhythm and the rate of penetration and metabolism of topically applied phorate (0.5 µg/insect). Penetration and metabolism studies were carried out during both the active and inactive phases of the insect's locomotor activity rhythm. Insects were treated topically at 12 noon, the time at which the insects exhibit their lowest level of locomotor activity and susceptibility to insecticides, and again at 6 p.m. the beginning of the insect's highest level of locomotor activity and susceptibility to the toxicants. The rate of penetration and metabolism was measured at 0, 3, 6, 12 and 24 hour intervals after application in each of the activity phases. Each time interval was replicated four times, each replicate consisted of 2 insects (a male and a female).

The Results

4.1. Penetration

Fig. 12 and 13 show the amounts of radioactivity remaining on the insect cuticle in both the dark (active) and light (inactive) phases at the different times after application. The rate of penetration was significantly higher at the 6 hours interval in the active phase ($P > 0.02$), indicating a higher rate of penetration in the early interval of the dark phase, when the insects were most active.

Results also showed that the amounts of radioactivity recovered by the warm toluene wash were always higher in all dark intervals except the 6 hours one.

Fig. 12

Penetration of C^{14} phorate applied to inactive crickets

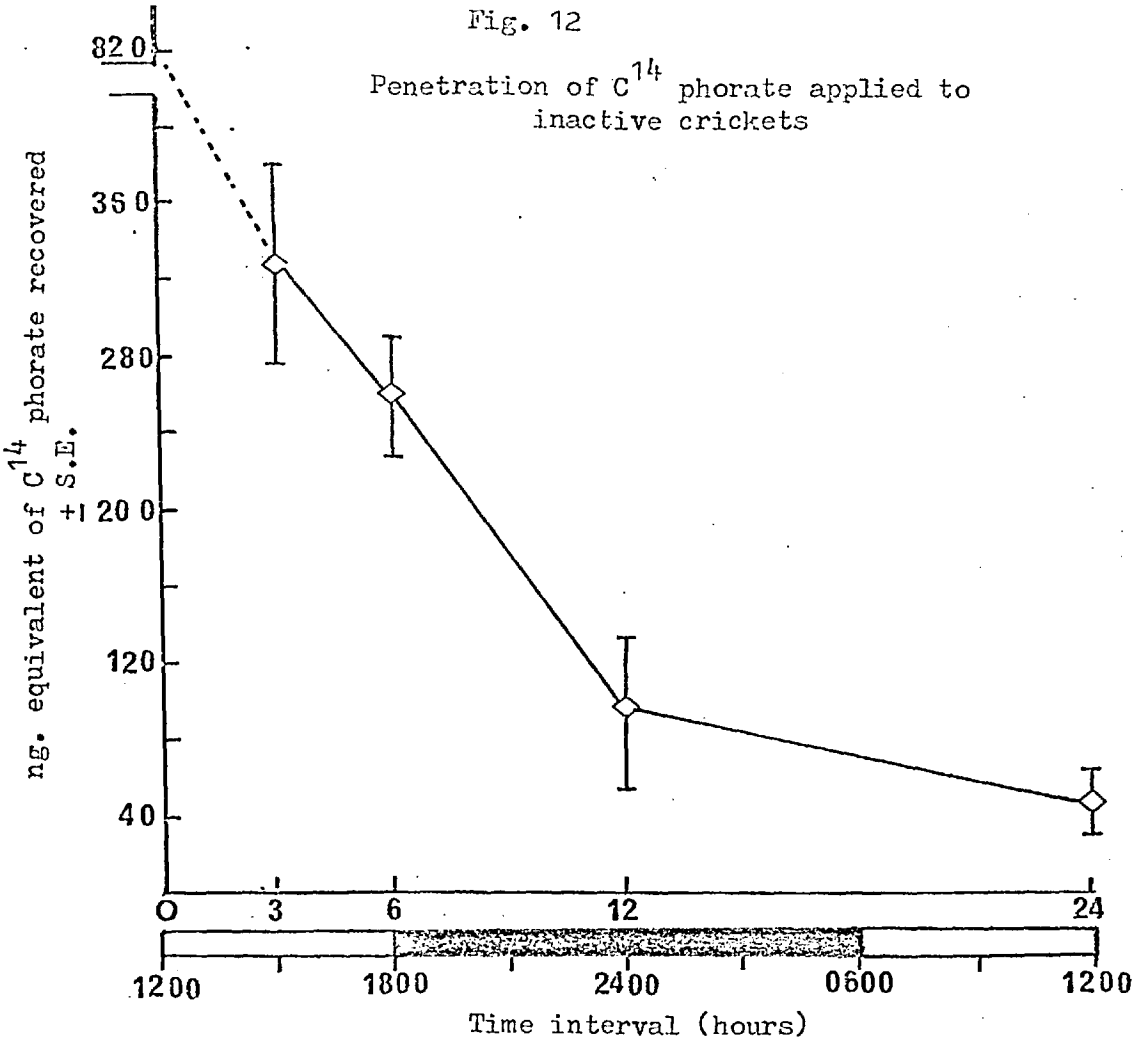


Fig. 13

Penetration of C^{14} phorate applied to active crickets

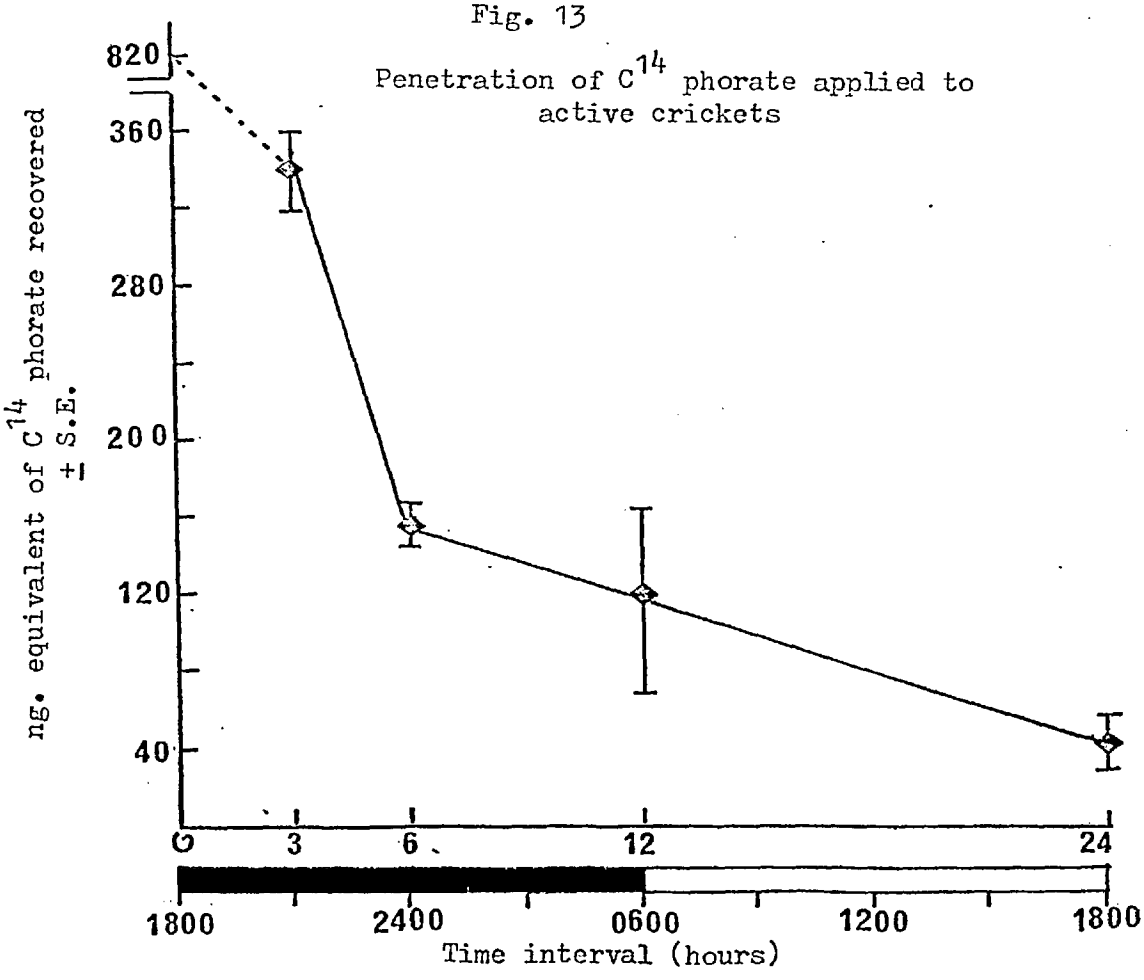
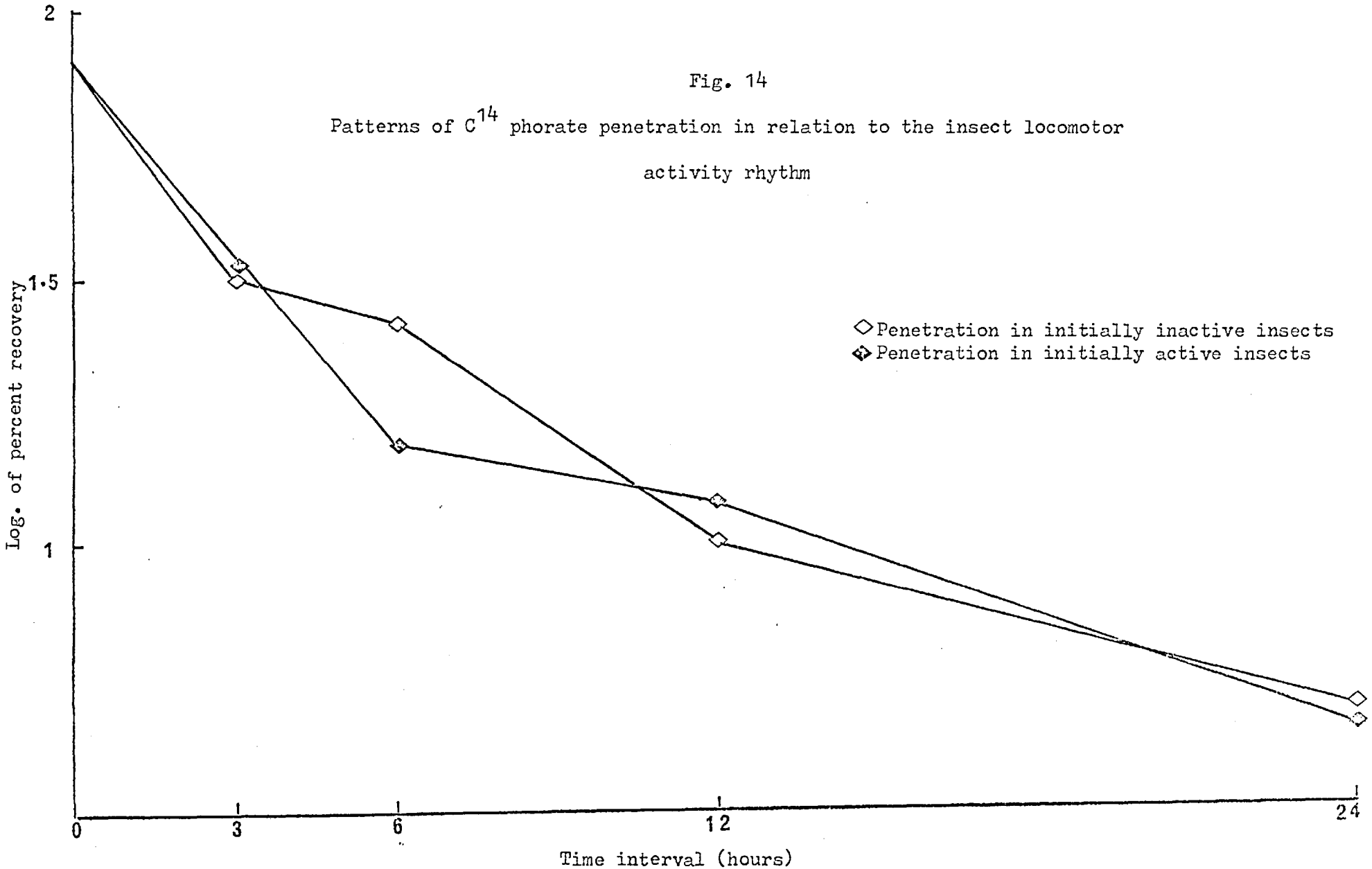


Fig. 14

Patterns of C^{14} phorate penetration in relation to the insect locomotor activity rhythm



4.2. Hydrolytic metabolites

The concentrations of the hydrolytic metabolites recovered at different time intervals in both the internal and frass extracts are shown in Fig. 15 and 16. Internal concentrations recovered following the light and dark treatments showed no significant differences up to 12 hours after the insecticide was administered, while the amount recovered at the 24 hours interval was significantly higher in the dark treatment, but this result was not of considerable importance because by that time the major part of the non-toxic metabolites was already excreted. The later observation explains the sharp decline in the amounts of the internal fractions 12 hours after application in both the light and dark treatments, which coincided with the increase in the non-toxic contents of the frass extracts. The results showed that the non-toxic metabolites in the frass extracts were higher in the light treatment at all intervals, for example at the 6 hours interval the amount recovered at the light (21.5% of the applied dose) was twice as much as the amount recovered in the equivalent interval of the dark treatment (10.4% of the applied dose).

A full statistical analysis of the frass extracts was not feasible, mainly because insects production of frass was erratic, more over, it was not possible to distinguish between the frass of individual treated insects. Despite the considerable scatter of data, results showed an evident trend which suggested that the accumulation of the non-toxic metabolites was higher during the insect's inactive phase.

4.3. Solvent-Soluble (Toxic) metabolites

The amounts of the solvent soluble metabolites recovered after the light (Fig. 17) and dark (Fig. 18) treatments were not significantly different. In both treatments the highest amounts of the toxic material

Fig. 15

Non-toxic products of C^{14} metabolism in initially inactive crickets

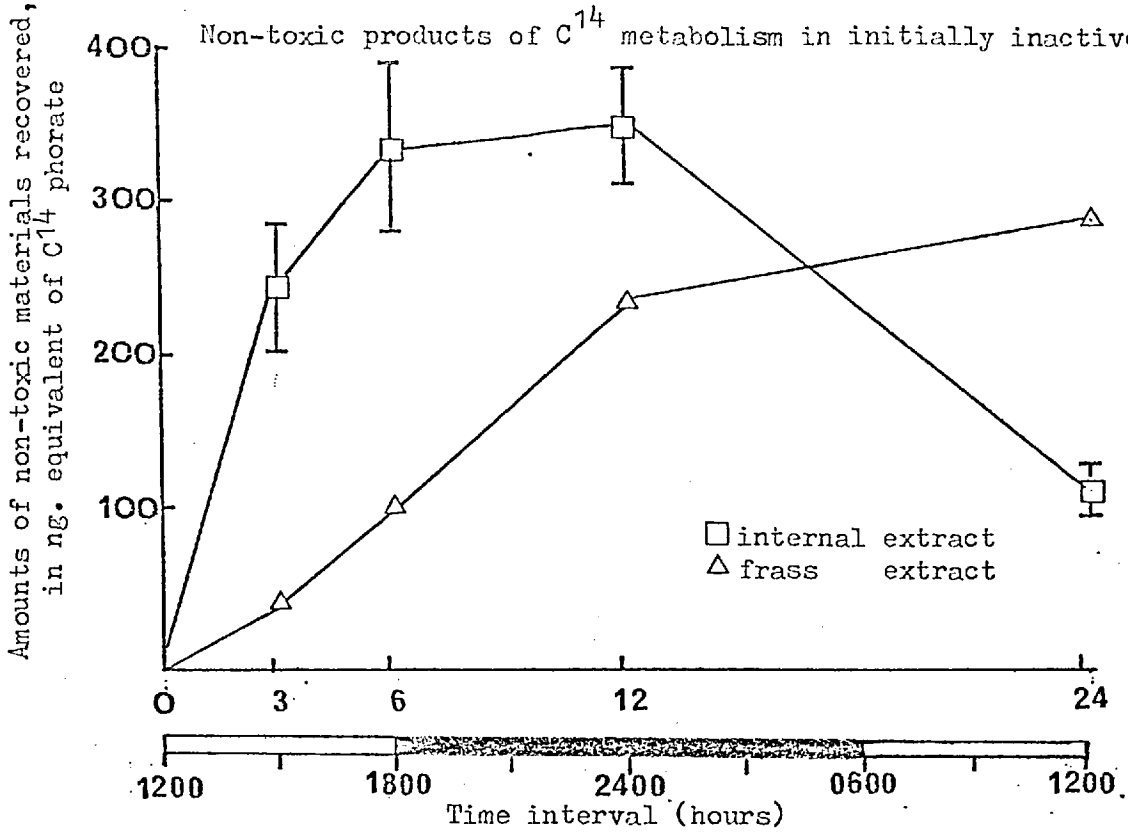
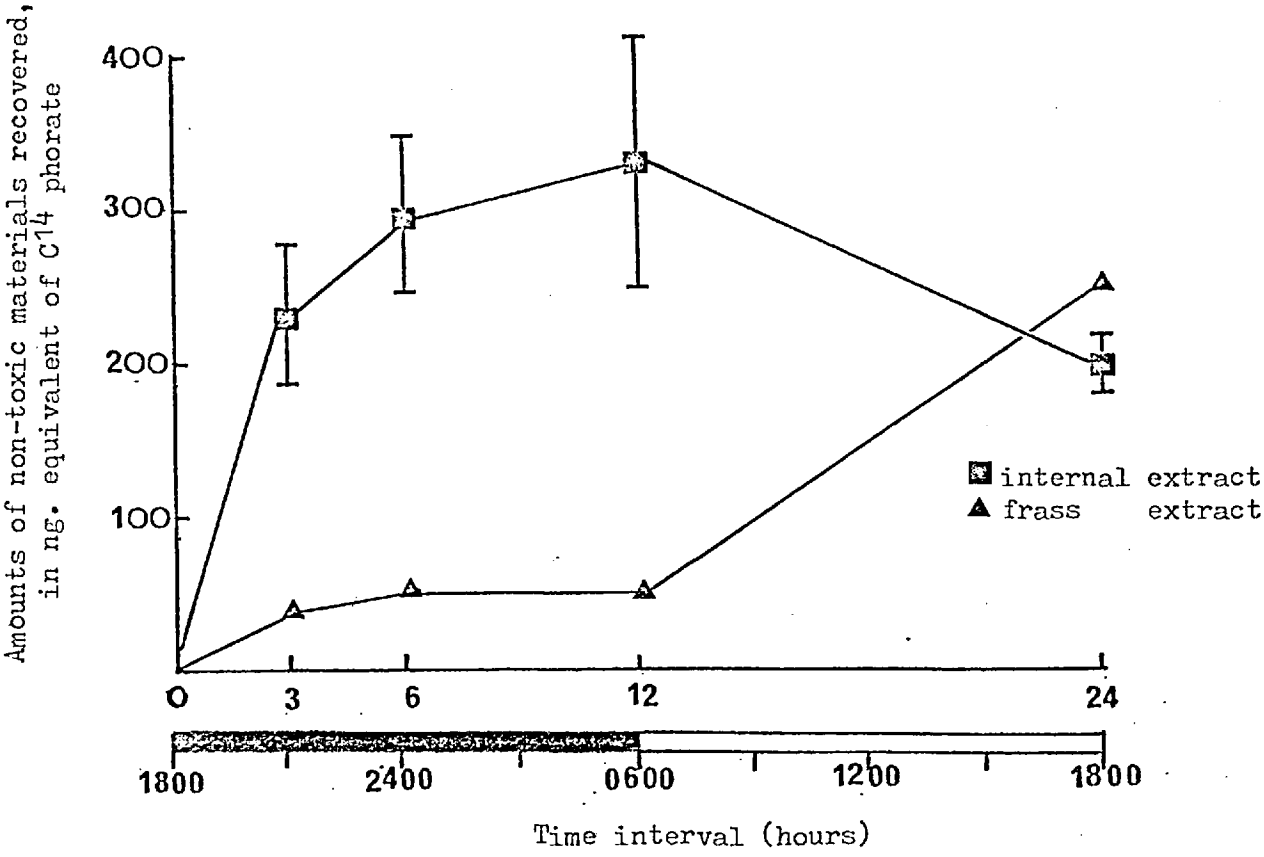


Fig. 16

Non-toxic products of C^{14} metabolism in initially active crickets



in the internal fractions were 24.8% and 18.4% of the applied dose, recovered 3 hours after application in the dark and light treatments respectively.

Phorate degradation was relatively slow during the first 12 hours after application, while it was much faster in the following 12 hours.

Insignificant amounts of the toxic materials were excreted in the frass, the highest quantity recovered was 0.03% of the applied dose, 24 hours after application.

When the internal fraction was analysed, using column chromatography results showed that phorate was the major component of this fraction at all time intervals in both treatments. Phorate concentration ranged from 14.3% to 1.6% of the applied dose during the course of the light treatment and from 20% to 2.6% during the dark treatment.

Fig. 19 and 20 show the amounts of phorate and its oxidative metabolites recovered at different intervals of the light and dark treatments respectively. Statistical analysis showed no significant difference in the degradation of phorate or the build-up of its oxidative metabolites between the two treatments, however, phorate degradation in the first 6 hours of the dark phase was more rapid than that of the light treatment and the oxidative products reached their highest level 3 hours after application in dark treatment, while in the light treatment the highest amount was recovered 6 hours after application.

Fig. 17

Toxic fraction recovered from insect homogenates following the application of C^{14} phorate to inactive crickets

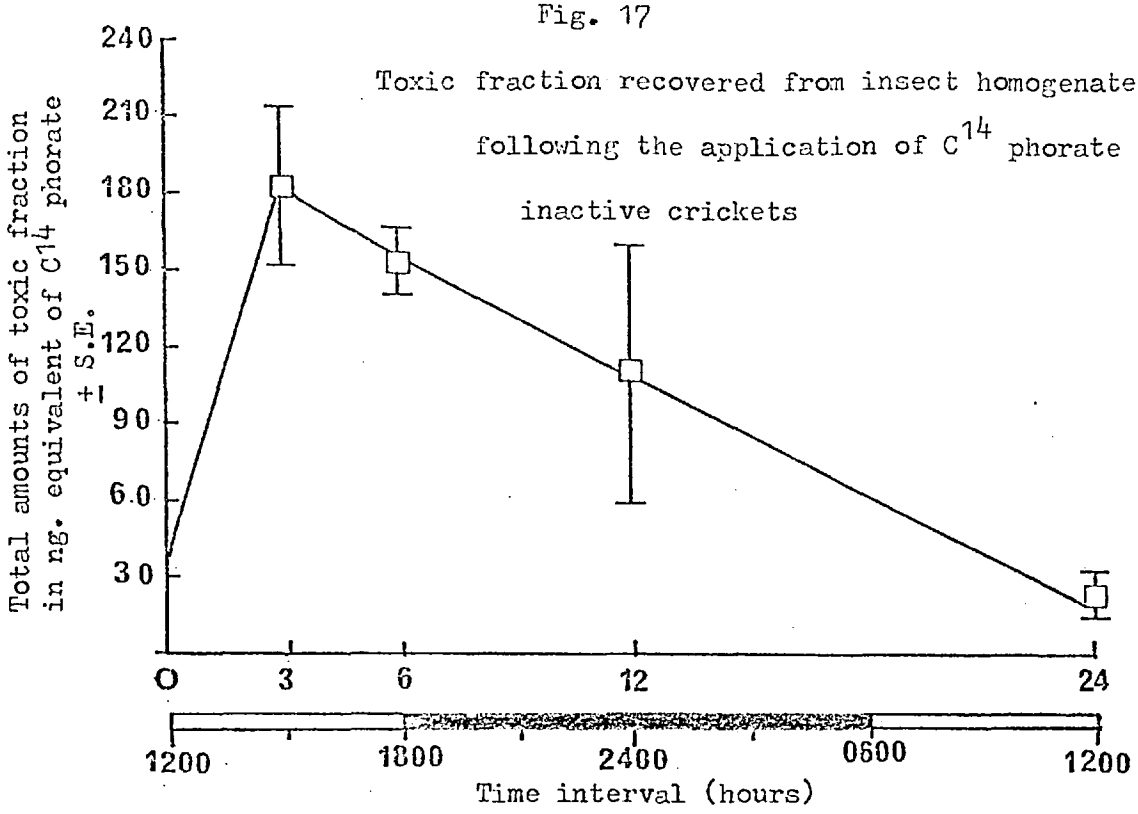


Fig. 18

Toxic fraction recovered from insect homogenates following the application of C^{14} phorate to active crickets

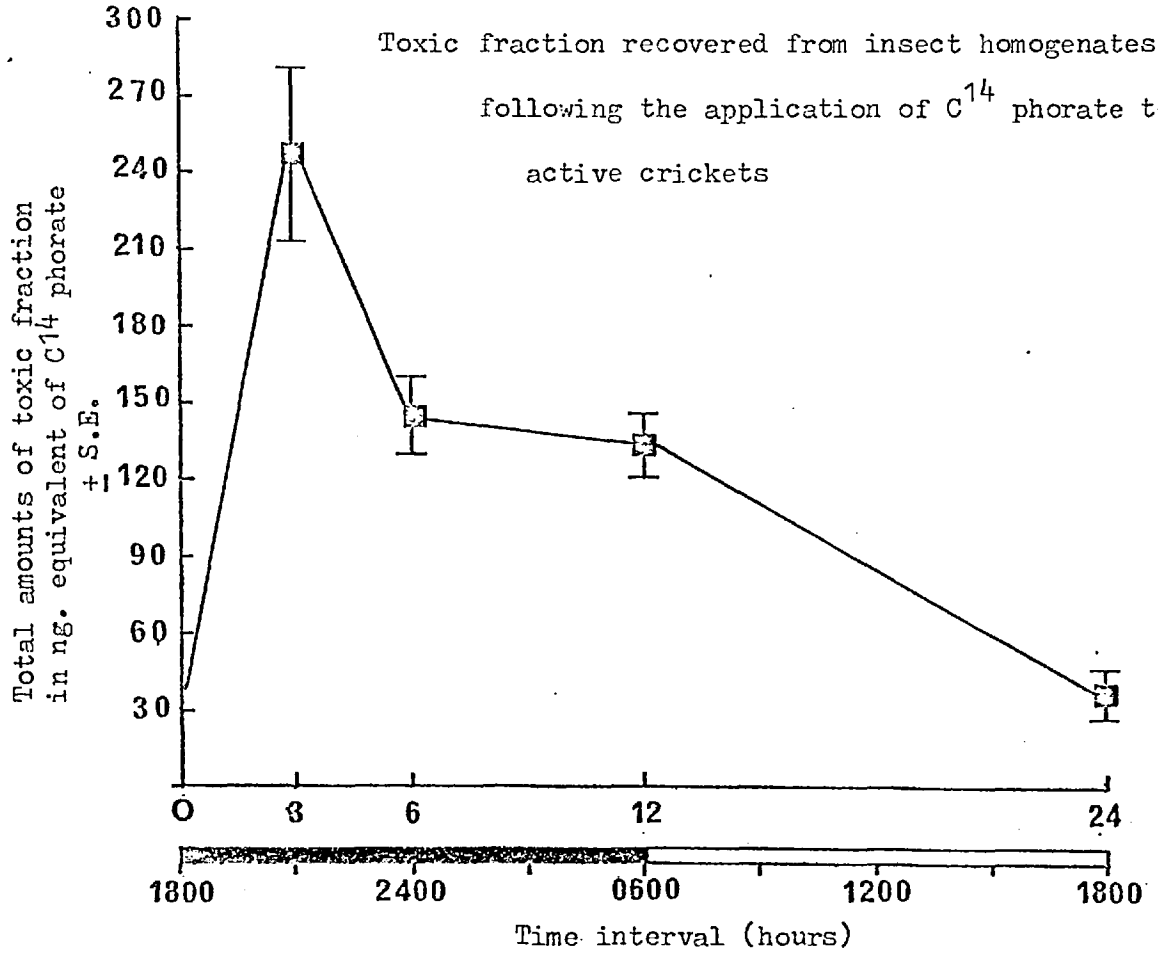


Fig. 19

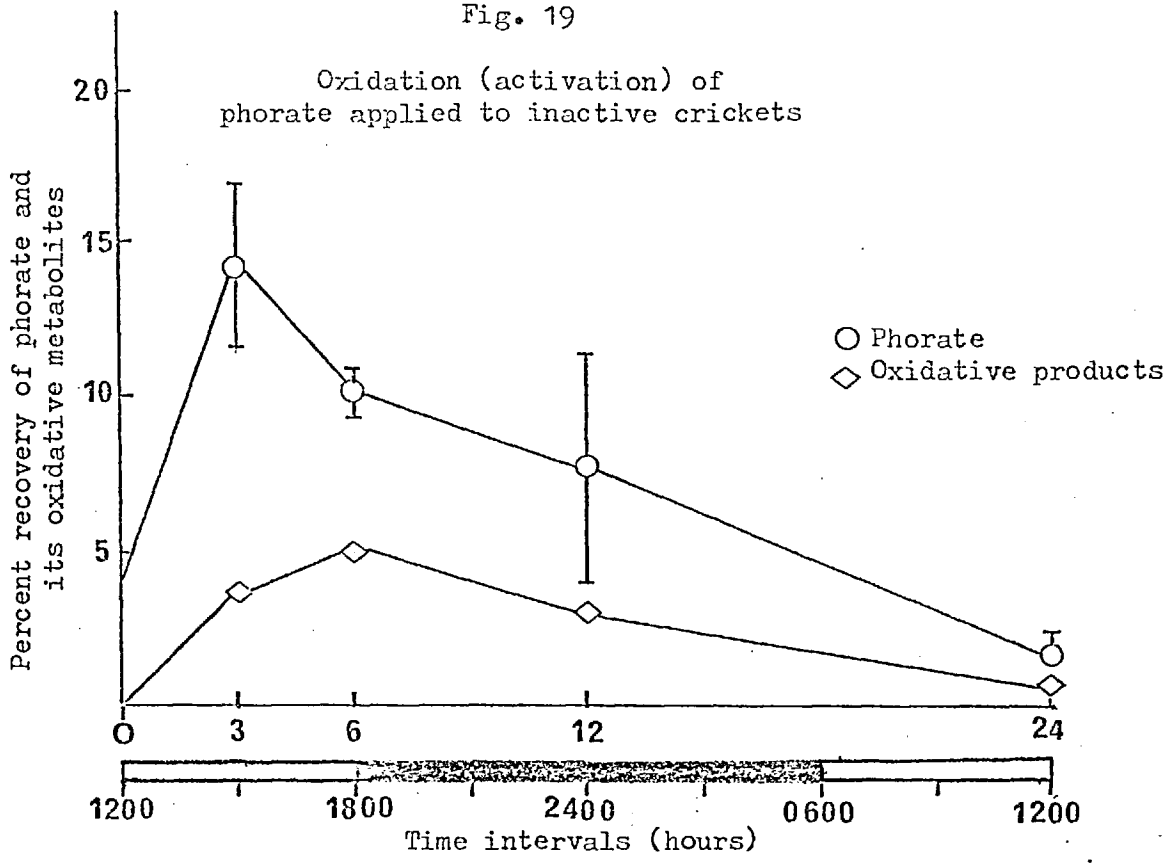
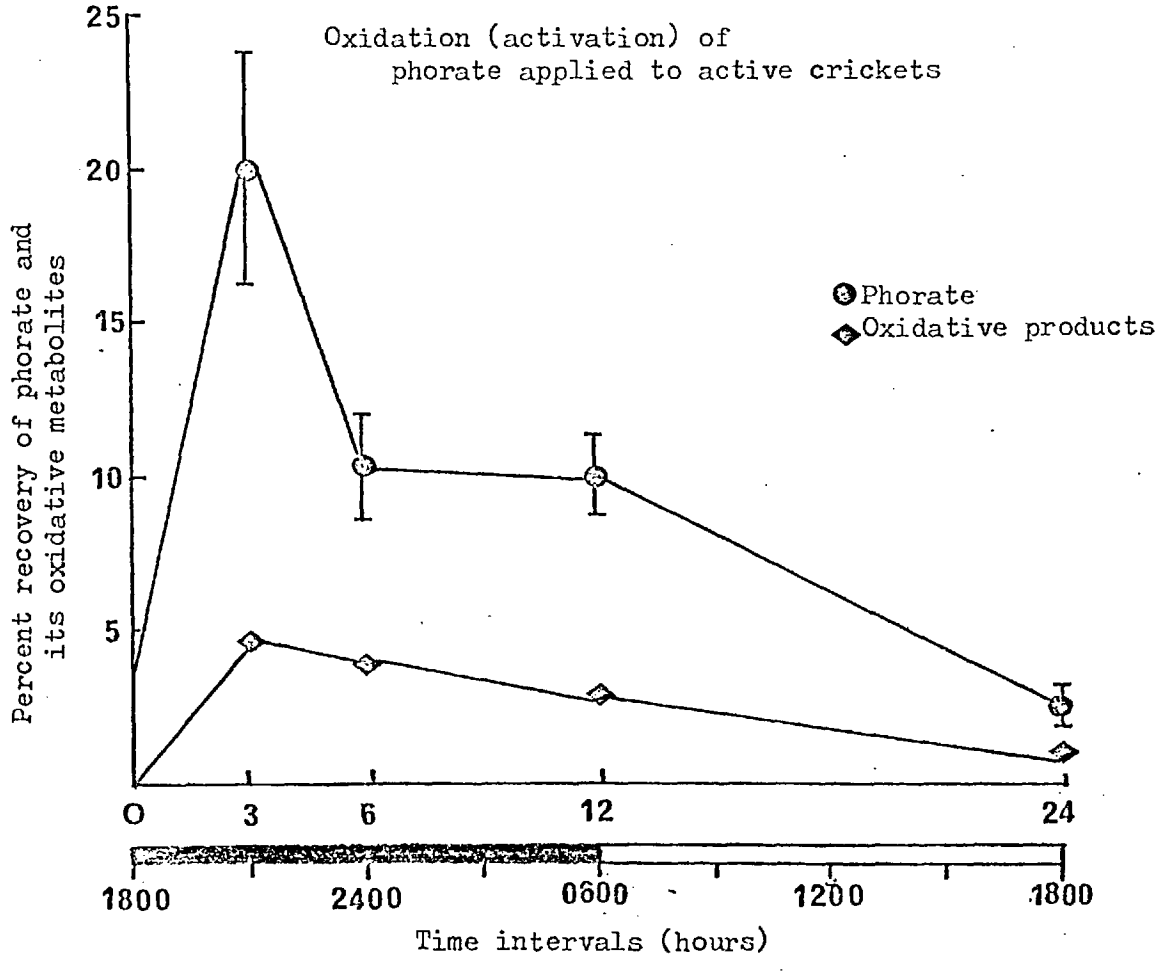

Oxidation (activation) of
phorate applied to inactive crickets
Percent recovery of phorate and its oxidative metabolites
20
15
10
5
Phorate
Oxidative products
0
3
6
12
24
1200
1800
2400
0600
1200
Time intervals (hours)

Fig. 20


Oxidation (activation) of
phorate applied to active crickets
Percent recovery of phorate and its oxidative metabolites
25
20
15
10
5
Phorate
Oxidative products
0
3
6
12
24
1800
2400
0600
1200
1800
Time intervals (hours)

5. Thin Layer Chromatography Studies on the Fate of C^{14} - Phorate in the House Crickets in Relation to their Circadian Rhythm

Table 1 shows a more detailed analysis of the oxidative metabolites of topically applied C^{14} - Phorate. Phorate and its five metabolites were extracted and then analysed by TLC. The location of the different metabolites was confirmed by autoradiography Fig. 21. The results showed that phorate sulfoxide was the major product at all time intervals of the two treatments followed by phoratoxon sulfoxide at the 3 hours intervals of both treatments, while phoratoxon sulfone was the second major metabolite recovered at both 12 hours intervals. Traces of the oxygen analogue (Phoratoxon) were detected after the 3 hours dark interval and at 6 hours interval of both the dark and light treatments (1.2% and 0.5% of the applied dose respectively). Phoratoxon was not detected 12 hours after either treatment.

The above results agreed with those of Menn and Hoskins (1962) who studied the metabolism of phorate in the German cockroach, Bull et al. (1963) who studied the metabolism of dimethoate in Boll weevil and Bull (1965) investigating the metabolism of di-syston (a compound similar to phorate) in Boll worm. These authors found the thiolo-phosphate analogue to be a highly unstable compound, it did not accumulate in high concentrations possibly because of its extensive degradation or conversion to other products. In common with the results obtained by the above authors with phorate, dimethoate and di-syston, this study has also shown that phorate sulfoxide is the most stable of the active intermediate compounds formed in vivo.

The overall concentration of the oxidative metabolites was higher in the dark treatment, and this also applied to the parent compound. This suggested that phorate underwent a more rapid oxidation process in the dark phase while the detoxication process was operating at a lower intensity.

Table 1.

Percent of phorate and its oxidative metabolites

as a proportion of the applied dose

Time after application	P = S,SS	P = S,SO	P = S,SO_2	P = O,S	P = O,SO	P = O,SO_2
3 L (1200-1500 h)	23.4	14.6	1.5	-	3.5	2.4
3 D (1800-2100 h)	25.7	8.8	2.58	0.8	3.5	2
6 L (1200-1800 h)	8.4	3.4	0.5	0.5	1.9	1.7
6 D (1800-2400 h)	9.6	4.5	2.2	1.2	2.1	2.5
12 L (1200-2400 h)	2.7	3.2	1.22	-	1.1	1.35
12 D (1800-0600 h)	7.2	13.7	2.3	-	3.44	3.72

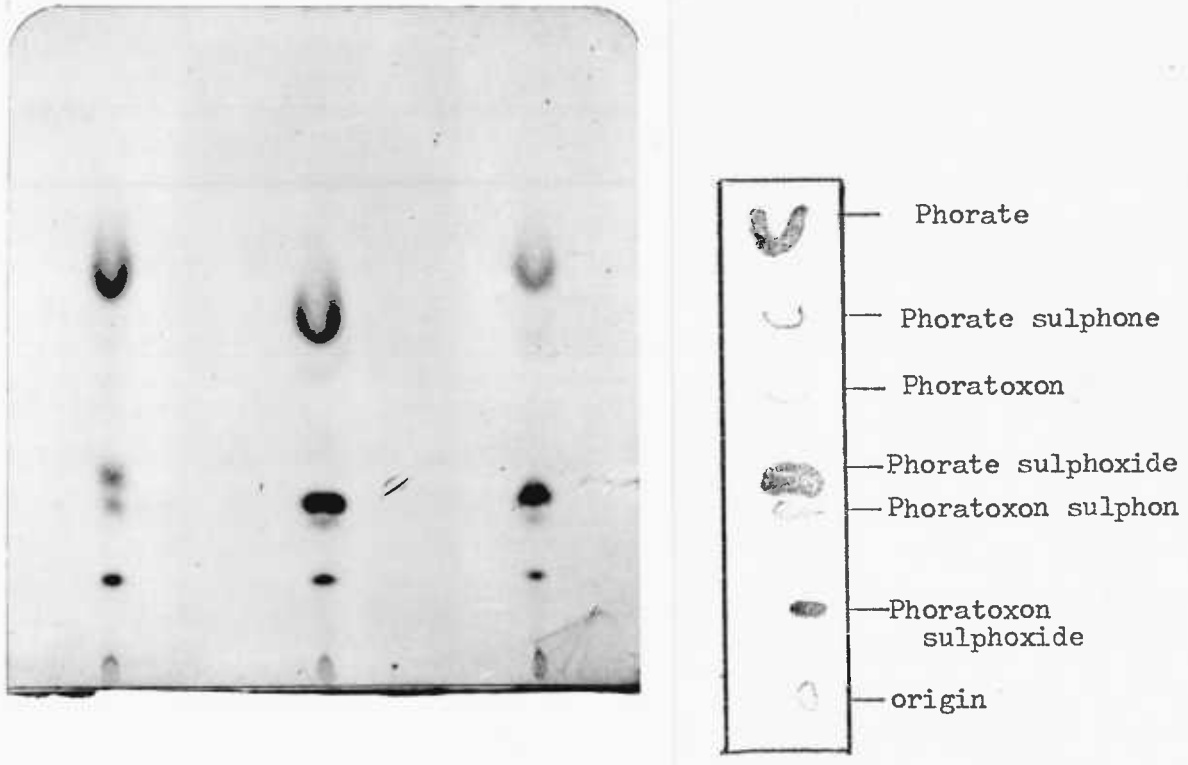


Fig. 21

Autoradiograph of TLC plate showing the separation of the toxic metabolites of phorate extract from insects

6. Biochemical Studies

One experiment was carried out to detect the presence of daily fluctuations in the activity levels of the cholinesterase and carboxylesterases of the house cricket's head since the head was considered the richest source of these enzymes (O'Brien, 1967). At different time intervals a head was removed, weighed and its content of ChE and CarE was measured. Each time interval was replicated 10 to 12 times with equal numbers of male and female heads. In another experiment designed to study the inhibitory effect of phorate on ChE and CarE in relation to the insect's circadian rhythm, amounts of inhibited ChE and CarE were determined after the insects had been treated topically with a sub-lethal dose of phorate (1.2 µg). Phorate was applied twice to coincide with the crest and trough of the insect's susceptibility rhythm and the amounts inhibited were measured 3, 6 and 12 hours after each application. In each of the inhibition measurements the ChE and CarE activity of the treated insects were compared with that of control insects of the same age, sex, maintained under identical conditions from the same batch as the treated insects. A series of pilot experiments were conducted in order to establish a satisfactory procedure for the enzymes assay and to ascertain its optimum conditions.

The Results

6.1. Substrate concentration

The influence of substrate concentration on the activity of ChE and CarE was studied. To a standard volume of the homogenate (100 µl) a series of substrate concentrations was added and the rate of hydrolysis for each concentration was measured. Results portrayed in Table 2 shows that the ChE activity increased with increasing the concentration

Table 2.

The influence of substrate concentration on the activity of ChE and CarE

Substrate	Concentration (M)	Rate, in moles of substrate hydrolysed per minute per gram tissue
Acetylthiocholine Iodide	0.1×10^{-6}	1.9×10^{-6}
	0.5×10^{-6}	2.5×10^{-6}
	1.0×10^{-6}	2.8×10^{-6}
	2.0×10^{-6}	2.4×10^{-6}
	4.0×10^{-6}	1.9×10^{-6}
Thiophenyl acetate	2.0×10^{-7}	8.5×10^{-6}
	4.0×10^{-7}	10.0×10^{-6}
	6.0×10^{-7}	10.68×10^{-6}
	8.0×10^{-7}	10.7×10^{-6}

Table 3.

Phoratoxon sulfone in-vitro inhibitory effect on ChE of the house crickets

Time/minute	ChE activity*	% inhibition
0	2.09×10^{-6}	0
10	1.49×10^{-6}	28.7
20	0.996×10^{-6}	52.3
30	0.697×10^{-6}	66.6
60	0.498×10^{-6}	76.1

* ChE activity is expressed as the rate in moles of substrate hydrolysed per minute per gram tissue.

of acetylcholine iodide up to ($1.0X\ 10^{-6}M$) a concentration which attained the maximum velocity of the reaction. Any further increase in the substrate concentration caused a decline in the ChE activity. It was also shown that increasing the concentration of thiophenyl acetate caused a proportional increase in the CarE activity up to a concentration of ($6.0X\ 10^{-7}M$). Any further increase in the substrate had little further effect on the CarE activity.

6.2. Substrate specificity

This experiment was carried out to ensure that ChE was not acting on thiophenyl acetate and thus would not be included when the non-specific esterases are assayed. The homogenate of one head was divided into two equal parts and the non-specific esterases activity of one part was measured. To the other part eserine ($10^{-6}M$) was added to inhibit the ChE (Booth and An-Horng Lee, 1971), then the non-specific esterases activity were measured. The results showed that the two parts exhibited the same level of activity, indicating that ChE was not hydrolysing the thiophenyl acetate.

6.3. Aryl esterases "A type"

This experiment was conducted to examine the existence of type A esterases, a group which hydrolyses organophosphorus compounds but are not inhibited by them and to distinguish them from type B esterases. Because the substrate used is hydrolysed by the two groups, it was impossible to estimate solely the B type. Thus the homogenate of one head was divided into two equal parts and the non-specific esterases of one part were measured. The other part was incubated with paraoxon ($10^{-3}M$) for 30 minutes to inhibit the B type esterases, thus ensuring that only the A type was measured. The results showed that paraoxon had inhibited 99% of the non-specific esterases activity. This result

indicated that the A type esterases do not exist in the house crickets head homogenate, or exist in an insignificant amount, while the main bulk of the non-specific esterases is made up of B type esterases.

6.4. The efficacy of the protective technique and the in-vitro inhibitory effect of phorate and phoratoxon sulfone

This experiment was conducted to examine the effect of the protective technique suggested by Van Asperen (1958, 1960). In this study, results were also obtained on the in-vitro inhibitory effect of phorate and phoratoxon sulfone on ChE. The homogenate of several heads was divided into 1 ml aliquots. To three of these, a dose of phorate (10 µl of 0.12% in acetone) was added and to the fourth aliquot 10 µl of acetone alone was added. Tubes containing the mixtures of the homogenate and phorate were incubated at 20°C for 10, 30 and 60 minutes. At the end of each time interval the ChE activity was measured and compared with the blank tube. The results showed that no inhibition had taken place at any of these time intervals. The experiment was repeated with a higher dose of phorate (100 µl of 0.12% in acetone) and the mixture incubated for 2 hours, the results showed an inhibition percentage of 55%. The above experiment was repeated using phoratoxon sulfone (20 µl of 5.5 µg/100 ml of acetone) and the activity of ChE was measured at the end of each time interval and compared with the activity of a mixture of 1 ml homogenate and 20 ul of acetone. Table 3 shows the ChE activity at different time intervals and the inhibition percentages, indicating that phorate had a very weak in-vitro inhibitory effect on ChE in comparison to phoratoxon sulfone, results which agree with the findings of many workers (Bowman and Cassida, 1958; Menn and Hoskins, 1962; Archur et al., 1963). The effect of the protective technique was examined by homogenizing the heads in the

presence of acetylcholine chloride (the protective substrate). Phoratoxon sulfone was added to an aliquot of this homogenate and the ChE and CarE activity levels estimated after 45 minutes. The results showed that the activity of both enzymes was the same as the controls, thus indicating that this procedure provided a 100% protection to both ChE and CarE.

6.5. Circadian changes of ChE activity in the head of house crickets

Results concerning the daily changes in ChE activity in the head of house crickets cultured in LD 12-12 are shown in Fig. 22. The activity of ChE changed significantly in the course of 24 hours, with a maximum value at 12 noon, while the lowest level of activity was found 4 hours after the onset of darkness (2200 hrs). There was a 2 fold difference between the highest and lowest levels of activity.

6.6. Circadian changes of CarE activity in the head of house crickets

Fig. 23 shows the extent of the daily fluctuations of the activity level of CarE in the house cricket's head. With two peaks of activity occurring at 12 noon and 12 midnight. The onset of darkness marked a gradual fall of the activity to the lowest recorded level, 4 hours after the onset of the dark period. The difference between the highest and lowest level of CarE activity was also about 2 fold. Results also showed that the CarE activity was about 4 times higher than that of ChE.

6.7. Phorate in-vivo inhibitory effect of ChE and CarE of the house cricket head in relation to the insects circadian rhythm

Results showing the inhibition of ChE and CarE in the house cricket's head are represented in Table 4 . The results showed no significant differences between the dark and light treatments in the

Fig. 22

Daily fluctuations in cholinesterase activity in the house cricket's head

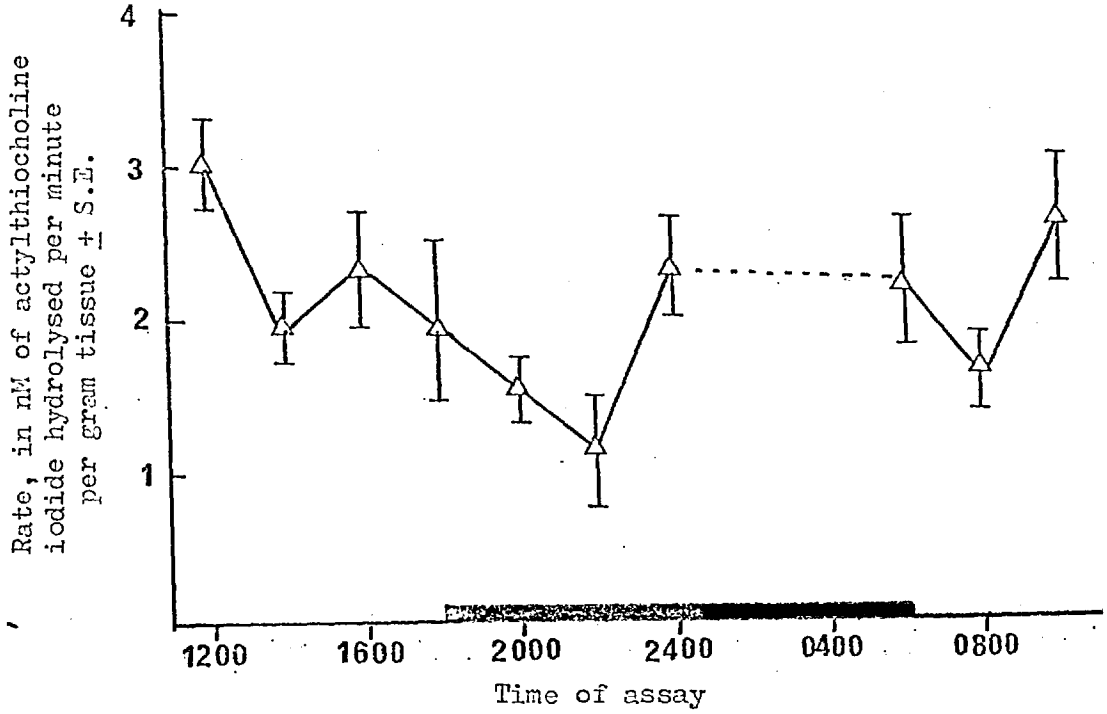
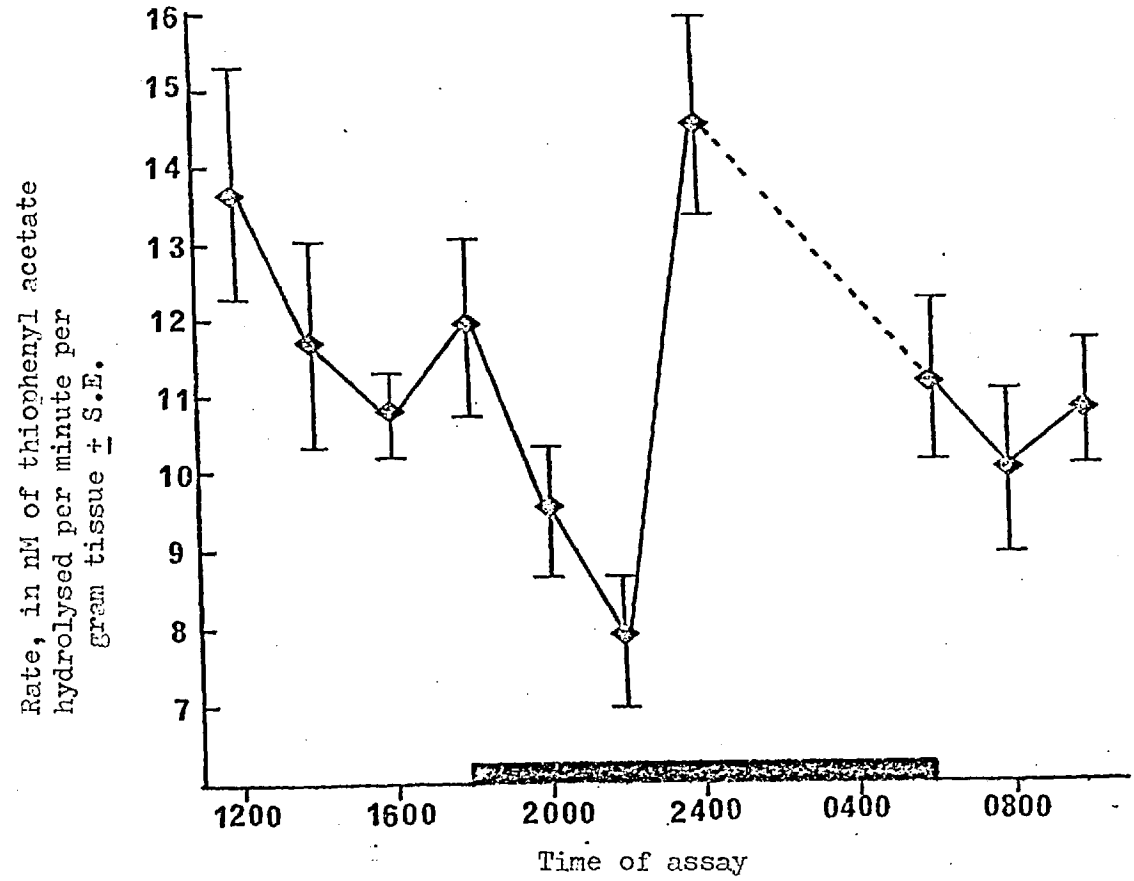


Fig. 23

Daily fluctuations in carboxylestrase activity in the house cricket's head



concentrations of ChE inhibited 3 and 6 hours after application of phorate. However, because the control levels were different at these times, inhibition percentages varied noticeably in the two treatments. The amount of ChE inhibited was doubled 6 hours after application in the two treatments, but inspite of this increase, the percentage of ChE inhibited in the dark treatment was comparatively small owing to the high level of ChE activity normally present at this time. The amounts of CarE inhibited showed no significant difference between the two treatments 3 hours after application, nor were they different when expressed as a proportion of the control level. However, the concentration inhibited 6 hours after application was significantly higher in the dark treatment ($P > 0.01$) and this inhibition continued consistently with the time, indicating a progressive nature of the inhibitory effect of phorate and its toxic metabolites on CarE.

In the ChE experiments a reduction in the amounts of ChE inhibited occurred after 12 hours in both treatments. This reduction which was substantially higher in the light treatment may be interpreted as a recovery of the enzyme, a phenomenon which has been reported by many workers (Brady and Sternburg, 1966; Ahmed, 1970; Zettler and Brady, 1970; Reiner, 1971).

Table 4

Phorate in vivo inhibitory effect of ChE and CarE

in the house cricket's head in relation to the insects

locomotor activity rhythm

Time of Treatment	Time of Assay	Amount of ChE in control insect ± S.E.	Amount of ChE inhibited ± S.E.	Percent of Inhibition	Amount of CarE in control insects ± S.E.	Amount of CarE inhibited ± S.E.	Percent Inhibition
1200 (12 noon)	1500 (3 p.m.)	3.2 ± 0.25	0.812 ± 0.21	25.3	10.9 ± 1.4	3.8 ± 0.7	35
1800 (6 p.m.)	2100 (9 p.m.)	2.5 ± 0.32	0.83 ± 0.20	33	8.3 ± 0.96	3.2 ± 0.6	38.5
1200	1800	3.4 ± 0.37	1.6 ± 0.27	47	9.9 ± 1.2	5.1 ± 0.5	51.5
1800	2400	4.4 ± 0.49	1.7 ± 0.3	38	13.4 ± 1.3	7.9 ± 0.87	58.5
1200	2400	3.2 ± 0.23	0.84 ± 0.27	25.2	11.1 ± 0.87	5.4 ± 0.62	48.2
1800	0600	3.06 ± 0.24	1.3 ± 0.18	42	13.2 ± 1.1	8.0 ± 0.69	60.0

Amounts of ChE and CarE are expressed as nM substrate hydrolysed/1 minute/1 g. tissue

DISCUSSION

Locomotor Activity Studies

The present series of locomotor activity experiments confirmed the results of Lutz (1932), Nowosielski and Patton (1963) and Cymborowski (1969), that the house cricket maintains a rhythm of locomotor activity which persisted under constant conditions and can be reversed by reversing the light/dark cycle. Although these authors showed that some of the insects with which they experimented, had an afternoon peak of activity in addition to the post-dusk one, the insects used in the present work showed only one distinctive post-dusk peak of activity.

The term circadian rhythm implies that endogenous processes when entrained, continue to oscillate (free running) with a periodicity of approximately 24 hours, after the removal of external cues, in what is commonly called constant conditions (Kalmus, 1966). In the present work, the experiments carried out under constant darkness, demonstrated the circadian nature of the house crickets activity rhythm. Furthermore, one of the important features demonstrated in that experiment was the daily drift in the activity peaks. This particular feature has been suggested as the strongest piece of evidence that timing is endogenous and a result of a response to an underlying physiological clock (Brady, 1974). The locomotor activity rhythm obtained in the inversed light-dark cycle, demonstrated the vital importance of this cycle in the entrainment of the circadian rhythm. The insects' ability to shift their activity in phase with the prevailing regime, is one of the features favouring the interpretation that the circadian rhythm is an endogenous rather than an exogenous phenomenon (Bunning, 1967).

Bioassay Studies

Bioassay results illustrate the existance of a circadian rhythm of susceptibility of house crickets to malathion, diazanon and phorate, which agree with many studies on other insect species, demonstrating a circadian changes in susceptibility to various organophorphates (Beck, 1963; Cole and Adkisson, 1964, 1965; Frudden and Willso, 1968; Ware and McComb, 1970).

Although crickets have not previously been shown to have a circadian susceptibility rhythm to insecticides, Nowosielski et al. (1964) demonstrated the presence of a circadian rhythm of sensitivity to narcotics (e.g. ether, chloroform and carbon tetrachloride), and they also found that the crickets were most sensitive during the first half of the night, coinciding with the locomotor activity peak, a pattern similar to the one found in the present study, in spite of the difference in the mode of action of narcotics and organophosphorus insecticides.

The results suggest that the susceptibility rhythm to malathion and phorate was regulated, or at least in synchrony, with the photoperiod under which the insects are maintained, but the diazanon results differed from the pattern malathion and phorate have shown, with two peaks of susceptibility, one soon after the onset of darkness, when the insects were most active, and another in the early hours of the light phase, when the insects were least active. These results indicated that the susceptibility rhythm was not always in synchrony with the locomotor activity rhythm, a result which agreed with the conclusion of Brady (1974), that there is no obvious physiological connection between the circadian behavioural rhythm of insects and their susceptibility rhythm to insecticides. The diazanon results have weakened the assumption that the locomotor activity is the sole

factor behind the insects circadian susceptibility to insecticides and indicated that biochemical, physiological and behavioural interactions are more likely to be the cause of that phenomenon. The insecticides used, malathion, phorate and diazanon are known to undergo different metabolic processes in the tissues of the treated insects. One of these reactions is the conversion by the catalytic effects of some oxidative enzymes to metabolites more toxic than the parent compounds. The products of the conversion mechanisms as well as the residues of the parent compounds (malathion, phorate and diazanon) are thought to cause death mainly by their inhibitory effect on the acetylcholinesterase system (O'Brien, 1967). Since two enzyme systems are involved in this process, namely the system converting the parent compound to its metabolites and the acetylcholinesterase inhibition system; it may be hypothesized that qualitative and/or quantitative rhythmic fluctuations of these enzymes or their products could lead to the results observed in these experiments.

Other factors based on the behavioural activity of the insects may influence the insects' susceptibility to these compounds. These factors include, the volatility of the applied dose of the insecticide and the speed at which it penetrates to the site of action, each of which may be influenced by the insects' locomotor activity.

Evaporation Studies

Evaporation rate from fresh wings was higher than that from glass surface within the first 12 hours after treatment. Such a trend may arise as a result of the numerous invaginations and sclerotizations on the wing surface which tend to increase the surface area of the wing and therefore of the applied insecticide, thus aiding the evaporation rate. Harris and Lichtenstein (1961) showed that the evaporation of some insecticides is proportional to the moisture content of the treated surface, in the present study, since fresh wings are more humid than glass coverslips at least for the first few hours after removing them, such differences in the rate of evaporation might be expected. After 12 hours the rates of evaporation, though declining, were reversed, in that, loss occurred at a faster rate from the glass surfaces than from the wings. This reversal is probably the result of the progressive penetration of the applied dose to inner layers of the cuticle, a process which decreased the amount of free insecticide left on the wing surface and consequently decreased the rate of evaporation. Moreover, wings as biological surfaces, with their lipophilic nature and affinity for absorption of insecticides (Galley, 1967) are more likely to retain an applied dose of insecticide longer than a glass surface, a property which may delay subsequent evaporation irrespective of the amounts evaporating in the early hours of the experiment.

The results also showed that the insects locomotor activity did not significantly affect the rate of volatile loss of topically applied insecticide. Nevertheless, the results showed that up to 18% of the applied dose had evaporated 24 hours after application, these results agree with the findings of Devonshire and Needham (1974) who demonstrated that large amounts of parathion and dimethoate were lost from the surface of topically treated aphids, their findings emphasized that evaporation from the cuticle can greatly influence results when insecticides are applied topically.

Penetration and Metabolism Studies

The present work showed that penetration of topically applied insecticides was more rapid when the insects were active. Thus, the fast uptake of phorate during the first 6 hours in the dark treatment coincided with a period of a high level of the insects' locomotor activity and the later slower penetration (i.e. from 6-12 hours after application) took place when the locomotor activity level was declining. Penetration during the light phase was rather slow in the first 6 hours after application, a period which was also characterized by a low level and sporadic locomotor activity. The following 6 hours showed a noticeably higher rate of penetration, coinciding with a substantial increase in the locomotor activity.

When the logarithm of the percentage of the amounts of phorate remaining on the insects were plotted against time (Fig. 14) was used to compare the pattern of penetration as recommended by (O'Brien, 1967); penetration during the dark phase showed a biphasic pattern, while penetration during the light phase was more linear. Because of the scatter of values in these experiments, this must be considered more a trend than a significant effect and a more detailed study of this aspect is necessary before a definite conclusion is reached.

In other reports different types of penetration have been shown to occur with one compound in one organism, though in some experiments the differences reported were due to the techniques employed (O'Brien, 1967). However, this cannot be the explanation of differences reported in the present work since the same technique was used for the light and dark treatments. Several suggestions have been put forward to interpret differences between linear and non-linear types of penetration. Matsumura (1963) explained non-linear penetration of malathion into the

American cockroach by the occurrence of binding to a component of the cuticle, so that immediately after application, a portion of malathion could not be washed off. However, this possibility does not occur for the present study because the washing technique used for the light and dark treatments was the same. Buerger and O'Brien (1965) suggested that biphasic penetration occurs when the applied compound is rapidly metabolized, so that one is actually examining penetration of more than one compound. O'lson and O'Brien (1963) studying the penetration of dimethoate (a close relation of phorate) in the cuticle of the American cockroach, found that no conversion occurred on the surface of the cuticle. On the other hand Ahmed and Gardener (1968) reported that malathion was activated to malaoxon (a more potent anticholinesterase compound) by the locust body wall. In the present study, the possibility of some fluctuations in phorate metabolism in the house cricket cuticle cannot be totally eliminated, moreover, some activation processes may occur in the absence of any biological catalyst, for example some oxidation of the parent compound (Phorate) may occur in the presence of atmospheric oxygen (Brown, 1975). However, this is an aspect of penetration which requires further investigations.

The results have shown more radioactivity recovered by the warm toluene wash after 3,12 and 24 hours in the dark treatment than that at the same intervals in the light treatment. Since the toluene wash was intended to recover any radioactivity contained in the epicuticular wax layers of the cuticle, this result would suggest a wider and deeper spread of the dose applied in the dark phase. The later results are consistent with the trend the present work has shown, relating the penetration of topically applied insecticides to the insects locomotor activity.

Although there are no reports relating the penetration of insecticides to the insect's activity rhythm, the present results can be reconciled with the results of Lewis (1962) who demonstrated that the extent of the movement of a thin film of oil over Phormia terraenovae cuticle was greater in active insects and he suggested that this was brought about by the small elastic deformations of the cuticle surface. In a study of the penetration of topically applied compounds, he (Lewis, unpublished data) has also reported a faster rate of penetration into the active insects. Gerolt (1969, 1970) after a series of experiments concluded that an organic insecticide spreads laterally within the integument probably primarily in the endocuticle and reaches the site of action via the integument of the tracheal system. Although this is probably not the only route of entry, the results of the present work agreed with this general principle since different levels of the insects locomotor activity affected the rate of penetration of topically applied phorate.

The results showing the concentrations of phorate and its toxic metabolites recovered at the light and dark treatments (Fig. 19 and 20) suggest a faster rate of phorate activation in the early hours of the dark phase, a period which coincided with a high level of insects' locomotor activity and susceptibility to the toxicant, with the highest concentration of the oxidative metabolites recovered 3 hours after application. Phorate activation following the light treatment was slower and the highest concentration of the oxidative products was recovered 6 hours after application. The results obtained with thin layer chromatography agreed with this trend. Leesch and Fukoto (1972) found that the amount of P = O esters recovered at any time after treatment was the result of the difference in formation and degradation

of these esters. In the present study (Table 1) greater amounts of the P = O esters were found throughout the dark treatment, indicating that the conversion of P = S to P = O esters took place more rapidly in the dark phase or that hydrolysis of these compounds was slower. Phoratoxon was detected 3 hours after application in the dark treatment, while no traces of it were detected in the equivalent interval after the light treatment and the proportion of phoratoxon detected 6 hours after application in the dark treatment was more than twice the amount recovered 6 hours after application in the light period. Since phoratoxon is known to be an unstable compound (Menn and Hoskins, 1962), its presence in greater amounts in extracts of insects treated in the dark phase may suggest that the house cricket metabolism of phorate differed, depending on the time that the phorate was administered.

Wilkinson (1973), suggested that the anticholinesterase activity of the organophosphorus insecticide is normally associated with a high degree of electrophilicity at the phosphorus atom, this property was enhanced by the presence of the electron drawing group. Menn and Hoskins (1962) showed that the toxicity of phorate and its oxidative metabolites is influenced by the polarity of these compounds, similar results were obtained by Bowman et al. (1969). In view of the present work, the presence of the oxidative metabolites in higher concentration in the dark treatment extracts was consistent with the high susceptibility the house crickets showed to phorate during the dark phase. These results agreed with the findings of Menn and Hoskins (1962) who, after a study of phorate metabolism in three insect species, suggested that insect mortality was dependent on the in vivo activation of phorate to its oxidative metabolites. Brady and Arthur (1963) and Ushida et al. (1965) studied the toxicity and metabolism of dimethoate in different

insects and suggested that the higher sensitivity houseflies showed to dimethoate was based on a higher rate of P = O production recovered from their extracts in comparison to other insects.

Sun (1968) suggested that after application of an insecticide to insects penetration, detoxication and activation of the insecticide proceeded simultaneously, but at different rates. The relationship of these rates to one another determines the apparent toxicity of a substance. The results presented here (Table 1) showed that in addition to the higher concentrations of oxidative metabolites recovered in the dark treatment, the concentrations of the parent compound were also higher in this treatment, suggesting that the hydrolytic processes proceeded at a slower rate in the dark treatment and this would also be a factor which could have influenced the higher rate of activation of the parent compound. Although the concentrations of the internal extracts of the hydrolytic metabolites did not show any significant difference in favour of this interpretation, the hydrolytic contents of the frass extracts showed a substantial difference between the light and dark treatments, suggesting a higher rate of hydrolysis and excretion of the toxic compound during the light treatment. Moreover, the presence of a high concentration of the parent compound during the dark intervals may have affected the metabolic processes and hindered the detoxication mechanisms, resulting in the higher level of susceptibility the crickets showed in the dark phase. In a comparable study of the metabolism of diazanon in houseflies Yang et al. (1971) found that the housefly microsomes hydrolysed diazoxon (the oxygen analogue of diazanon) quite readily when it was supplied alone, but when the parent compound (diazanon) was also present, it competed for or blocked the detoxication mechanism

and prevented diazanon metabolism. Different hydrolysis routes of dimethoate have been found to occur in the liver of three mammalian species depending on the concentration of this compound. In the rat more degradation took place at the C-N bond than at the S-C bond at a high concentration of dimethoate (10^{-3}M), but the reverse occurred at the low concentration (10^{-6}M) (Ushida et al., 1964). However, in these experiments the concentration range was much greater than in the present work.

The significance of penetration as a parameter reflecting the intensity of the interaction between the insect and the topically applied insecticide is still uncertain. This uncertainty is based on the fact that the rate of disappearance of applied insecticide from the surface is no evidence of the rate of penetration into the body and may measure only the penetration into the integument. However, it seems logical that the rate of surface disappearance may be proportional to penetration into the insect body.

In the present work, penetration appeared to be higher in the first 6 hours in insects treated in the dark phase, a period which was also characterized by a higher rate of activation (P = O production). One way of interpreting this correlation is to consider that the higher rate of activation occurred as a result of the higher amount of toxicant penetrating into the insect. Conversely, the higher rate of penetration may be caused by the faster activation processes during the dark phase, converting the compound to its different metabolites, with more of the parent compound diffusing through the cuticle in order to restore the dynamic equilibrium of the toxicant. The later hypothesis agrees with the results of Ahmed and Gardener (1970) who found that rapid penetration was accompanied by a higher rate of malathion

conversion to malaoxon. Elliot et al. (1970) suggested that detoxication is the rate determining factor of pyrethrins penetration into the mustard beetle, on the other hand Sawiki and Lord (1970) showed that detoxication mechanisms did not measurably affect the penetration of diazanon into houseflies.

Farnham et al. (1965) emphasized the importance of slow penetration as a factor of substantial importance in the insecticides' effect on insects, but Ushida et al. (1965) and Lord (1968) suggested that a cuticle of low permeability would only be an advantage to an insect which possessed a mechanism for disposing of the toxicant, because the disposal process would tend to be more effective at a slower rate of penetration. The present results have suggested that a correlation between the insects' locomotor activity and the magnitude of the metabolic reactions which the compound undergoes in the treated insect is the penetration determining factor and thus might be expected to govern the ultimate effect of the toxicant on the insect. These results are in harmony with Lewis (1965), who suggested that the rate of absorption of a contact insecticide is basically a function of the cuticle permeability and of the concentration gradient across the cuticle.

Although the metabolism studies have indicated some measureable differences in the toxic and non-toxic products between light and dark treatments, a full interpretation of these results in relation to the insect's circadian rhythm was not achieved, mainly because of the noticeable shortage of literature on insecticide metabolism in the context of insect's circadian rhythms, coupled with the complexity of the metabolic processes involving different suggested pathways, each of which is catalysed by different groups of enzymes. Recent studies have shown the presence of highly specific enzymes capable of combating

the toxic effect of different insecticides. Matsumura and Hogendijk (1964), demonstrated the presence of thionases (i.e. enzymes which hydrolyse thiono-esters) in the houseflies, Bull (1965) suggested the presence of an enzyme system hydrolysing di-syston sulfone; Krueger (1975), extracted an enzyme system responsible for the oxidation of the alkylsulphur in the phorate molecule to phorate sulfoxide. Although no experiments relating to these specific enzymes and insect rhythms have been reported, studies on the oxidative activities of liver mitochondria in rats (Glick and Cohen, 1964) and on transamidinase (an enzyme involved in energy metabolism in mouse) (Van Pilsum and Halberg, 1964), have shown that these enzymes undergo daily changes in their activity comparable to the hosts circadian locomotor rhythms. Microsomal multi-function oxidases play a major part in the metabolism of insecticides (O'Brien, 1967; Eto, 1974), recent investigations have shown that the activity of these enzymes is changeable, depending on various physiological factors such as age and sex (Al-Aziz, et al. 1969), Benke and Wilkinson (1971) also showed that in crickets the activity of these enzymes changed with age, a change corresponding to different levels of susceptibility which the insects showed to carbaryl. Beck (1968) proposed a scheme for the physiological basis of circadian rhythms, he suggested that physiological processes such as susceptibility, resistance and locomotor activity may be governed by enzymatic systems. In turn, biological rhythms, physiological or behavioural may be associated with specific groups or systems of enzyme-dependent metabolic reactions. Thus in the present work it might be hypothesized that rhythmic fluctuations in these enzyme systems is one of the factors affecting the outcome of the toxicant metabolism resulting in the quantitative and qualitative differences detected between light and dark treatments.

Biochemical Studies

The results of the investigation into the daily fluctuations in ChE and CarE levels of activity showed that there was a substantial depression in their activities following the onset of the dark phase, coinciding with the period of greatest locomotor activity and a high level of susceptibility to the toxicants. On the other hand these enzymes attained their highest level of activity at 2400 hrs and again at 1200 hrs despite the magnitude of locomotor activity and the susceptibility of the insects at these two periods. Thus a complete correlation of this fluctuating level of esterases activity to the susceptibility of the insects was not deduced.

Gamouri (1965) pointed out the role of CarE in binding the toxic phosphate esters to their sites and carrying out their hydrolysis, other workers have shown that resistance to organophosphorus compounds was linked with high levels of CarE activity (Krueger and O'Brien et al., 1959; O'Brien, 1967; Sudderuddin, 1973). It can be seen from the results showing CarE inhibition (Table 4) that the amounts inhibited 6 and 12 hours after the application of phorate were significantly higher in the dark treatment, although control insects had similar levels of activity at the light and dark treatments of these intervals. These results suggested that the intensity of the CarE inhibition was governed by the rate of the insecticide activation rather than the activity level of the enzymes involved, results comparable to the findings of Krueger et al. (1960). The inhibited concentrations of ChE did not show any significant difference at the 3 and 6 hours intervals of the light and dark treatments, but the percentages of inhibition varied as a result of the fluctuation in ChE daily level and the highest level of inhibition was 47%, recorded in insects surviving 6 hours after the

application of phorate in the light phase. Results showed that in dead insects the ChE inhibition rate amounted to 98% of the total activity, the later results agreed with the findings of An-Horng Lee et al. (1973) who found that when house crickets were knocked down, only traces of ChE could be detected. However, the relationship between the intensity of ChE inhibition and different stages of crickets poisoning was not examined in this study since only alive insects were investigated. However, Brady and Strenburg (1966) showed that various levels of ChE inhibition followed sub-lethal dosages of different organophosphorus compounds, indicating no standard levels of ChE inhibition results in poisoning symptoms.

The insects surviving the 12 hours intervals in the light treatment showed a substantial recovery of inhibited ChE in comparison to those treated in the dark treatment in the dark phase, meanwhile CarE showed no signs of recovery after the same interval in the two treatments. Recovery has been attributed to synthesis of new ChE and/or reactivation of inhibited ChE (Brady and Strenburg, 1967; Ahmed, 1970) and although the synthesis of ChE has not been studied in relation to insects' circadian rhythms, studies have been conducted relating insects' rhythmicity with the synthesis of other relevant compounds. Cymborowski (1970) showed that the synthesis of protein and the accumulation of neurosecretion in the house crickets occur in a cyclic manner comparable to their locomotor activity, Kono (1975) suggested the presence of secretory cycles through the fifth instar of Pieris larvae entrained by short and long days, his suggestion was based on daily changes in the ultrastructure of the neurosecretory cells.

The present work did not involve any investigation concerning the synthesis of ChE, however, it is possible that this synthesis in the

house crickets proceeds in a rhythmic pattern and thus differences in the rate of recovery of inhibited ChE found in the present study could be interpreted accordingly. Further work along these lines would be a valuable extention in this study.

Finally, in view of the results obtained in this work, it would seem that the phenomenon of the insect's susceptibility rhythm to organophosphorus compounds is a result of a collective effect of different factors, each of them proceeding rhythmically, governed by the physiological state of the insect and reflected in the locomotor activity level of the insects. Although no significant differences in the rate of penetration was detected at most of the intervals between the light and dark treatments there was an evident trend suggesting that the locomotor activity has influenced the rate of penetration of the topically applied dose. The insects treated in the light period showed a higher rate of excreting the hydrolysed toxicant while the insecticide activation proceeded at a higher rate in those treated in the dark phase, resulting in greater accumulation of the oxidative metabolites which could have led to the higher inhibition level of CarE noticed 6 and 12 hours after the application of phorate in the dark phase. ChE inhibition was not related to the insects circadian rhythm, nevertheless, its recovery was substantially higher in insects treated in the light phase and that might have contributed effectively to the fluctuating level of the insects' susceptibility to the toxicant.

SUMMARY

A culture of house crickets (Acheta domesticus L.) was kept under LD 12:12 h photoperiod. A series of locomotor activity experiments showed that the crickets maintained a locomotor activity rhythm, the insects were most active in the dark while the activity was weak and sporadic during the light phase. Confirmation was obtained that this pattern of activity persisted when the insects were maintained under constant dark conditions but that the peaks of activity drifted, being slightly delayed on each successive day. When the light-dark cycle was reversed, the insects activity rhythm was re-entrained such that their peaks of activity coincided with the dark phase of the new light régime.

Topical treatments with malathion, phorate and diazanon at different times of the day chosen in relation to the different levels of the locomotor activity showed the insects to have susceptibility rhythms to these compounds. In general the insects were least susceptible when treated around midday and most susceptible when treated early in the dark phase. However, insects treated with diazanon showed two peaks of susceptibility, one which coincided with the commencement of the dark phase and the other, soon after the onset of the light phase.

Evaporation studies showed that topically applied phorate was lost by evaporation more rapidly from a glass surface than from the insect cuticle. Although the insects' locomotor activity had no significant effect on the evaporation of topically applied phorate, it was demonstrated that there could be a considerable loss of the topically applied compound by this route and evidence was obtained of the importance of

using oil in the carrier solution in bioassay experiments to minimize this loss.

In pursuit of the aspects underlying the insects susceptibility rhythm to toxicants, the role of various factors was investigated. Studies on the penetration of the insecticide showed that this occurred more rapidly into insects treated in the dark phase six hours after application, there was also an indication suggesting that the toxicant spread more over the cuticle in most of the dark phase intervals. Metabolic studies showed that the insects treated in the light phase excreted more of the non-toxic residues from applied C^{14} - phorate. On the other hand the activation of phorate proceeded at a faster rate in insects treated in the dark and analysis of the toxic fraction by (T.L.C.) showed greater amounts of phorate and its oxidative metabolites in the extracts of these insects with phorate sulfoxide as the major metabolite recovered at all time intervals after application in both dark and light treatments. Traces of phoratoxon were recovered three hours after application in the dark phase, none was detected at the same intervals of the light phase treatments.

Enzyme assay experiments demonstrated the presence of daily fluctuations in the activities of ChE and CarE. The activity of these enzymes in the heads of the house crickets were at their lowest at 2200 h. a period characterized by a high level of locomotor activity, these results emphasized that time of day is a factor of considerable importance when enzyme assays are conducted. The in-vitro inhibitory effect of phorate on ChE was very weak in comparison with that of phoratoxon sulfone. The amounts of ChE inhibited after an application of a sub-lethal dose of phorate did not differ significantly in relation to the time of phorate application, but CarE inhibition was

significantly higher after 6 and 12 hours of application in the dark phase, suggesting a greater inhibitory effect of the toxicant on CarE when applied in the dark phase, meanwhile CarE did not show any signs of recovery during the 24 hours of the experiments. The inhibited ChE showed a rapid recovery in insects treated in the light phase, unlike those treated in the dark where recovery was only slight. The later result contributes speculation about the recovery and/or the synthesis of ChE and the possibility of a rhythmic fluctuations governing this process.

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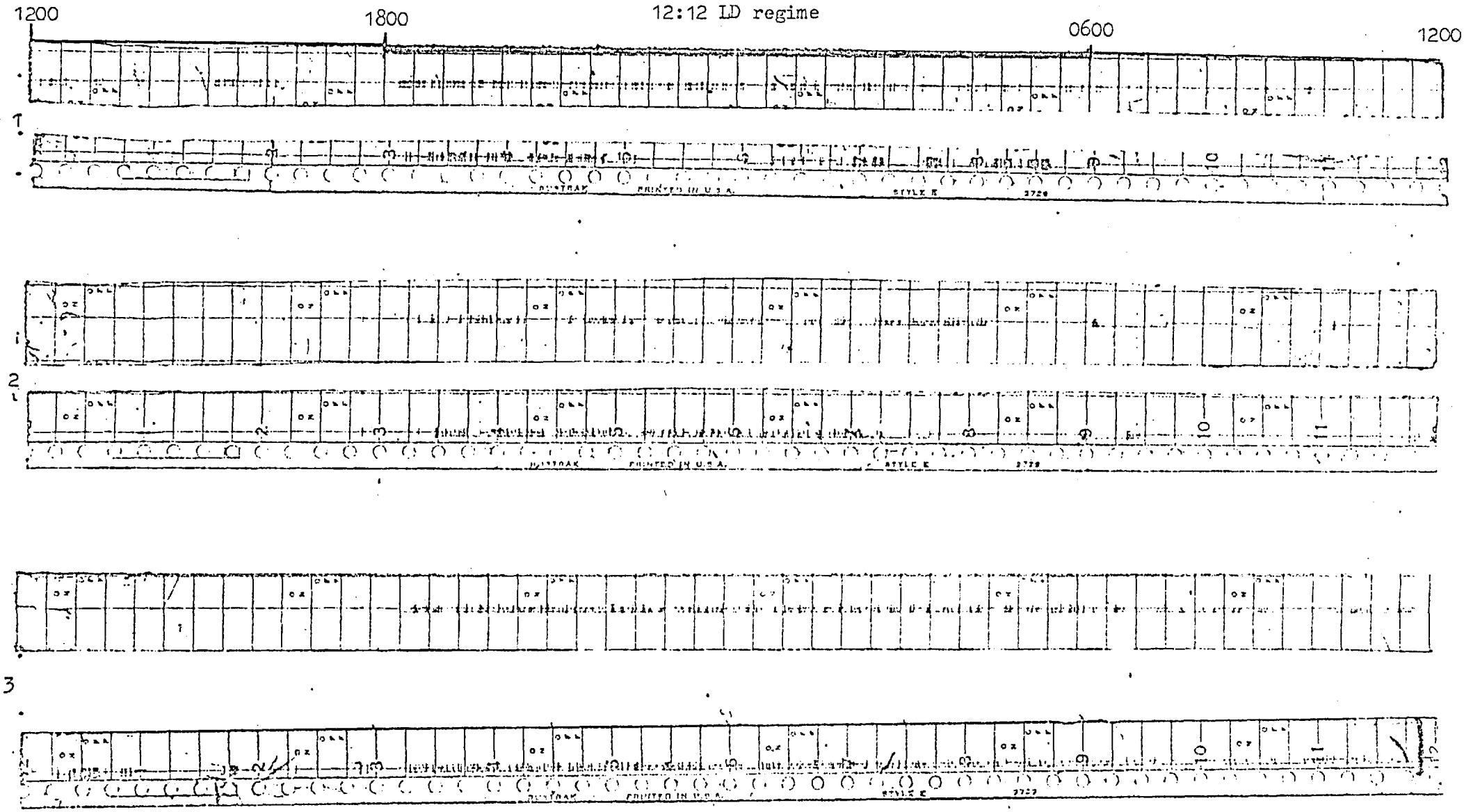
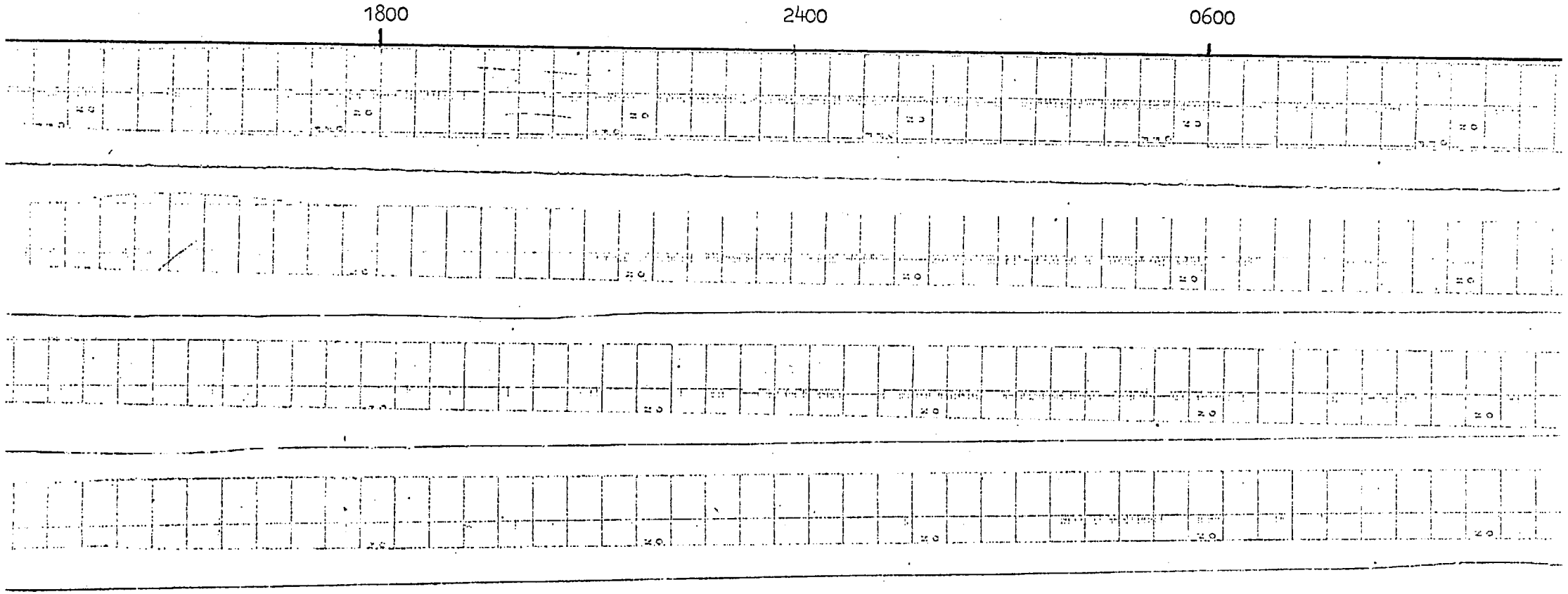
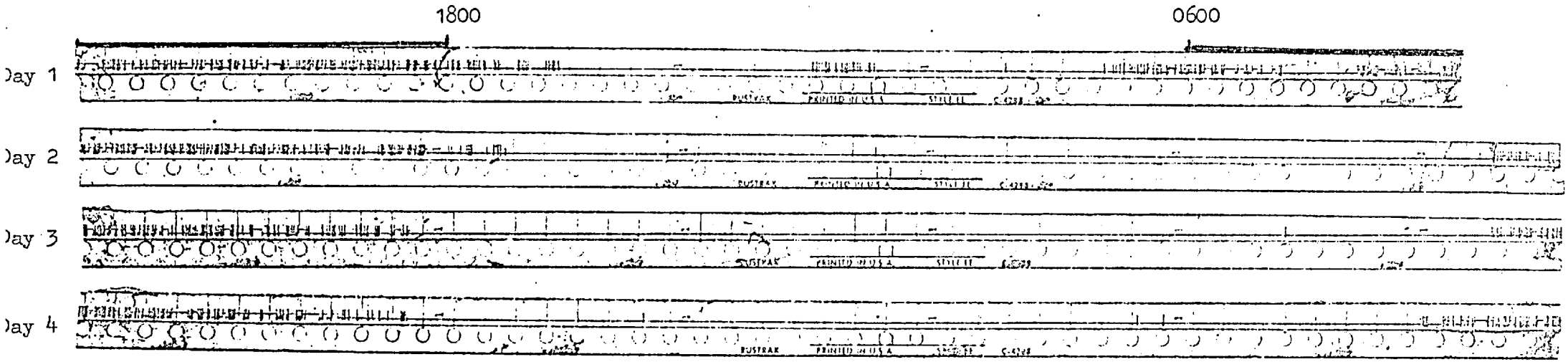
Typical recording of the locomotor activity of house crickets under 12:12 LD regime



Typical recording of the locomotor activity of a house cricket over four successive days under constant dark conditions



Typical recording of the locomotor activity of house crickets under reversed light regime



Appendix 1 Fig. 6

Susceptibility of house crickets to malathion at different times of the day

Time of Application	LD_{50} ug/insect	95% C.L. of LD_{95}		a	b ± S.E.	Heterogeneity		
		Lower	Upper			χ^2	d.f.	P
1200 h - 12 noon	4.0	3.6	4.6	7.27	5.7 ± 1.19	0.76	2	> 0.5
1600 h - 4 p.m.	4.3	3.9	4.9	7.18	6.0 ± 1.15	0.40	2	> 0.8
1800 h - 6 p.m.	4.0	3.5	4.6	6.9	4.9 ± 1.1	1.7	2	> 0.3
2000 h - 8 p.m.	3.0	2.5	3.5	7.4	4.6 ± 1.02	3.05	2	> 0.2
2400 h - 12 midnight	3.6	3.3	3.9	7.9	6.6 ± 1.15	1.2	2	> 0.5
1000 h - 10 a.m.	4.0	3.5	4.7	7.07	5.2 ± 1.3	2.82	2	> 0.2

Appendix 2 Fig. 7

Susceptibility of house crickets to phorate at different times of the day

Time of Application	LD_{50} ug/insect	95% C.L. of LD_{50}		a	b ± S.E.	Heterogeneity		
		Lower	Upper			χ^2	d.f.	P
1200	2.2	1.7	2.6	3.4	4.4 ± 1.11	0.582	2	> 0.7
1600	1.8	1.49	2.17	3.97	3.9 ± 0.73	0.599	2	> 0.7
2000	1.2	1.05	1.5	4.5	3.8 ± 0.99	2.1	2	> 0.3
2400	1.4	1.1	1.7	4.4	3.4 ± 0.68	1.53	2	> 0.3
0800	1.8	1.5	2.1	3.8	4.1 ± 0.70	2.1	2	> 0.3

Appendix 3 Fig. 8

Susceptibility of house crickets to diazanon at different times of the day

Time of Application	LD_{50} ug/insect	95% C.L. of LD_{50}		a	b ± S.E.	Heterogeneity		
		Lower	Upper			χ^2	d.f.	P
1200	2.5	2.3	2.9	7.8	4.8 ± 1.02	2.06	3	> 0.5
1600	2.1	1.9	2.3	8.4	5.1 ± 0.98	0.97	3	> 0.8
2000	1.67	1.41	1.81	8.7	4.8 ± 0.89	1.8	3	> 0.5
2400	1.76	1.1	2.1	7.5	3.3 ± 1.13	1.12	3	> 0.7
0800	1.4	1.2	1.6	8.4	4.1 ± 1.08	1	2	> 0.5

Appendix 4 Fig. 9, 10, 11

a. Evaporation of C^{14} - phorate applied in 1 ul of acetone from glass surface.

Time (min.)	Amount of radioactivity recovered in D.P.M. (mean)	% Recovery
0	747	100
5	648	87
10	588	79
30	348	47
60	21	3

b. Evaporation of C^{14} - phorate applied in 1 ul of acetone-risella oil from glass slide.

Time	Amount of radioactivity recovered in D.P.M. (mean)	% Recovery
0	1063	100
10 min.	957	90
30 min.	925	87
60 min.	909	85
120 min.	809	76
4 hr.	602	57
8 hr.	345.7	33
12 hr.	178.4	17
24 hr.	139	13

c. Evaporation of C^{14} - phorate applied in 1 ul of acetone-risella oil (95/5 V/V) from insects wings

Time	Amount of radioactivity recovered in D.P.M.	% Recovery
0	3339	100
10 min.	2542	76
30 min.	2398	72
60 min.	2240	67
120 min.	1634	49
4 hr.	1245	37
12 hr.	758.3	23
24 hr.	529	16

d. Evaporation of C^{14} - phorate applied in 1 ul of acetone-risella oil (95/5 V/V) from topically treated house crickets.

Time (hour)	Amount of radioactivity recovered in D.P.M. (mean)	
	light	dark
3	2035	1797
6	2083	2038
12	2422	2578
24	2747	2598

Appendix 5 Fig. 11 - 12

The amounts of radioactivity recovered in the surface wash

Time (hours)	Amounts of radioactivity recovered in D.P.M., mean and S.E.	
	Light	Dark
3	2487 4206 3144 2048 mean = 2971 S.E. = 469	3313 2546 3165 3418 mean = 3110 S.E. = 195
6	2519 2212 1650 3067 mean = 2362 S.E. = 295	1582 1371 1503 1220 mean = 1419 S.E. = 79
12	723 1570 364 mean = 885 S.E. = 357	482 746 1999 mean = 1075 S.E. = 467
24	770 571 248 163 mean = 438 S.E. = 141	446 399 552 248 mean = 411 S.E. = 63

Appendix 6 Fig. 12, 13

The amounts of water soluble (non-toxic fraction) recovered in the internal fraction

Time (hours)	Amounts of radioactivity recovered in D.P.M., mean and S.E.	
	Light	Dark
3	2537 3155 1759 1605 mean = 2264 S.E. = 360	3018 2420 1719 1097 mean = 2063 S.E. = 417
6	4460 3225 2032 2659 mean = 3094 S.E. = 516	3445 3441 1490 2305 mean = 2670 S.E. = 476
12	2687 2900 3153 4144 mean = 3221 S.E. = 322	4855 3442 2099 1606 mean = 2995 S.E. = 728
24	1011 1000 1214 1157 mean = 1095 S.E. = 53	1390 1746 1922 2146 mean = 1801 S.E. = 159

Appendix 7 Fig. 14, 15

The amounts of solvent soluble (total toxic fraction) recovered in the internal fraction

Time (hours)	Amounts of radioactivity recovered in D.P.M., mean and S.E.	
	Light	Dark
3	2131 2080 1357 1062 means = 1675 S.E. = 265	1633 1968 3106 2236 mean = 2235 S.E. = 315
6	1509 1665 1095 1312 means = 1395 S.E. = 123	1002 1663 1166 1457 mean = 1322 S.E. = 147
12	2303 303 588 834 means = 1007 S.E. = 445	1223 1525 937 1091 mean = 1194 S.E. = 124
24	228 153 153 355 means = 222 S.E. = 47	392 200 234 541 mean = 341 S.E. = 78

Appendix 8 Fig. 16, 17

The amounts of the parent compound (phorate) separated by column chromatography

Time (hours)	Amounts of radioactivity recovered in D.P.M., mean and S.E.	
	Light	Dark
3	1680 648 1119 1717 means = 1291 S.E. = 254	1430 1174 1914 2732 mean = 1812 S.E. = 342
6	948 1093 965 726 means = 933 S.E. = 76	666 1062 718 1330 mean = 934 S.E. = 153
12	1760 184 329 591 means = 716 S.E. = 358	845 1182 796 831 mean = 913 S.E. = 90
24	95 270 144 83 means = 148 S.E. = 42	134 467 118 246 mean = 241 S.E. = 80

Appendix 9

Percentages of different fractions recovered

calculated in relation to the applied dose

± S.E.

Time interval (hour)	Surface Wash			Homogenate fraction	
	methanol wash	Toluene wash	Total	Solvent soluble	water soluble
3 L (1200 - 1500 h)	28.6 ± 5.1	4.3 ± 0.1	33 ± 5.2	18.4 ± 2.9	25.1 ± 4.0
3 D (1800 - 2100 h)	29.3 ± 1.9	5.2 ± 0.2	34.5 ± 2.1	24.8 ± 3.5	23.0 ± 4.6
6 L (1200 - 1800 h)	21.7 ± 3.6	4.5 ± 5.4	26.2 ± 3.2	15.5 ± 1.3	34.3 ± 5.7
6 D (1800 - 2400 h)	12.0 ± 0.8	3.6 ± 0.17	15.7 ± 0.8	14.6 ± 1.6	29.6 ± 5.1
12 L (1200 - 2400 h)	8.3 ± 3.2	1.5 ± 1.0	9.8 ± 3.9	11.1 ± 4.9	35.7 ± 3.5
12 D (1800 - 0600 h)	9.1 ± 3.9	2.8 ± 1.2	11.9 ± 5.1	13.2 ± 1.3	33.2 ± 8.0
24 L (1200 - 1200 h)	3.8 ± 1.0	1.0 ± 0.28	4.8 ± 1.5	2.4 ± 0.5	12.1 ± 0.5
24 D (1800 - 1800 h)	3.2 ± 0.6	1.3 ± 0.14	4.5 ± 0.7	3.7 ± 0.8	20 ± 1.7

Appendix 9 Fig. 18 - 19

Daily fluctuations on the activity of cholinesterase and carboxylesterase in the head of the house cricket

Time of assay	rate in nM acetylthiocholine iodide hydrolysed per min. per g. tissue ± S.E.	rate in nM thiophenyl acetate hydrolysed per min. per g. tissue ± S.E.
1200	4.0 ± 0.3	13.8 ± 1.5
1400	2.94 ± 0.22	11.7 ± 1.3
1600	3.35 ± 0.35	10.7 ± 0.54
1800	2.97 ± 0.47	11.89 ± 1.1
2000	2.55 ± 0.22	9.5 ± 0.79
2200	2.1 ± 0.37	7.86 ± 0.76
2400	3.37 ± 0.32	14.6 ± 1.3
0600	3.2 ± 0.41	11.26 ± 0.95
0800	2.55 ± 0.24	10.0 ± 0.98
1000	3.6 ± 0.38	10.9 ± 0.79