

SOME BIOLOGICAL AND BIOCHEMICAL FACTORS
AFFECTING THE TOXICITY OF CERTAIN SYSTEMIC INSECTICIDES
TO TWO APHID SPECIES

by

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ABSTRACT

The relative toxicities of some systemic organophosphorus insecticides to Acyrtosiphon pisum and Aphis fabae were studied in laboratory tests together with some of the factors affecting their toxic action.

The two species were fed on Vicia faba and the weights of the developing aphids and the rates of larviposition and honeydew production were examined.

In comparative feeding tests on ^{32}P -labelled plants the division of the ingested material between the adults, larvae and honeydew was followed.

The feeding behaviour of the two species on different artificial liquids and under different experimental conditions was examined. The acceptability of the diets in each species and the interference of dimethoate with artificial feeding were also measured radiometrically.

The significance of the feeding behaviour of each species on natural or artificial diet is discussed in relation to the observed contact or systemic toxicities.

Some of the enzymes affected during insecticidal poisoning were examined in relation to the insecticidal action and the tolerance of the aphids.

The aphid esterases were resolved electrophoretically and the cholinesterase and carboxylesterase activities were estimated with titrimetric and colorimetric techniques. Qualitative and quantitative differences in the esterases of the two species and their significance in poisoning the insect are discussed.

The interactions between the insecticides and the enzymes in the living insect were examined and the inhibitory action of the insecticides on the esterases was compared with the mortality caused. The inhibitory action of organophosphates with respect to time was also examined in the broken cell.

On the basis of the data collected, the possible physiological roles of the factors studied in the poisoning of the two aphids are discussed.

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GENERAL INTRODUCTION

Control of aphids.

Aphids cause damage as pests either directly as a result of heavy infestations of wingless individuals on various crops or with the winged forms as virus vectors. Apteræ are relatively unimportant in transmitting viruses due to their relative immobility. Thus, the problem of controlling aphids is a dual one, of preventing the wingless forms from multiplying to plant damaging numbers and of preventing the winged forms moving from virus affected to healthy plants.

Most aphid-transmitted viruses are of the "non-persistent" type. The "persistent" virus transmitted by aphids up to 1969 was found to be 38 (Gibbs, 1969). Most of the work and a considerable amount of research on aphid control has been done on preventing the spread of persistent viruses. Despite different methods used so far to prevent the spread of viruses by aphids, insecticides remain the basic control measure, though they are not without shortcomings. Contact insecticides are not especially effective in protecting plants against infection as new foliage, produced between applications is not adequately covered with insecticide and the development of systemic insecticides was hailed with as much enthusiasm as the introduction of DDT had been (Ripper et al, 1949). But as aphids must feed on sprayed plants before they can pick up the insecticide, it is not surprising that systemic insecticides have been no better than contact ones in preventing either the introduction of virus into crops or the spread of non persistent virus in crops. The need, therefore, is for potent insecticides that paralyse or kill aphids almost immediately. For maximum effective control the epidemiology of the virus and the biology of the aphids need detailed study. In this context Watson

and Plumb (1972) have reviewed the behaviour of aphids in their relationship to virus transmission.

The role of the plant in systemic insecticidal action.

The efficiency of a systemic insecticide is dependent on the active participation of the plant in three main processes; the absorption, translocation and detoxification of the chemical (Bennett, 1957).

Differences in some of the conditions in which plants are grown in the field interfere with these processes and change the potency and availability of an applied chemical. These differences are possibly responsible to some extent for the variable results obtained with systemic insecticides which are reported in the literature.

The plant can also interfere indirectly and the role of nutrition in the determination of the biochemical activities and potentialities of living organisms is rarely given enough emphasis (Gordon, 1961). There are few examples of the influence of nutrition of aphids on their tolerance to insecticides. Acyrtosiphon pisum (Harris) reared on Vicia faba L. are about 1.5 times more tolerant to rotenone than aphids reared on Tribolium pratense (Potter and Gillham, 1957) Myzus persicae (Sultz) reared on turnip (Brassica campestris) or lettuce (Lactuca sativa) are three times more tolerant to nicotine than aphids reared on nasturtium (Tropaeolum sp.) (Richardson and Casanges, 1942). All these effects are minor and difficult to interpret. Even if it could be shown that a diet that causes higher tolerance also causes larger body weight or higher fat content, one could never be certain that the increased body weight or fat is the direct cause. Some other unknown factor may be responsible. Gordon (1961) suggested that nutritional effects on insecticide tolerance can be more striking if the insecticide is present at sublethal levels in the insect body during the feeding period. Field studies often show a significant increase in the resistance of insect populations with seasonal changes (Andres et al, 1955; Kerr and Olney,

1959) and some of this increase may be attributed to the changing nutritive value during growth and maturation of the crop plants and also to adaptive changes in the insects growing in the presence of sublethal levels of insecticides.

Studies on the uptake and fate of plant material, may considerably improve our understanding of the aphid-plant relationship. The use of radioactive compounds incorporated into the plant tissues, has made it possible to study the movements of minute quantities of plant sap. Studies on the feeding response of aphids are important and this has been facilitated with the introduction of artificial feeding methods through membranes.

Selectivity of insecticides.

The difference in response between any two species is a measure of the selectivity of the insecticidal action in relation to those species. The ideal insecticide would be highly toxic to the insect pest, but quite innocuous to other forms of life with which it came into contact (Winteringham, 1969). An example of selectivity is to be found in the three systemic organophosphorus (O-P) compounds studied by Elliott (1970).

Menazon did not kill two species of anthocorids at any dosage; dimethoate and phorate killed the predators only when these insects were confined on the treated leaf-surface and not on the opposite surface. All three insecticides, especially menazon, were selective, since any dosage killed more aphids (A. pisum) than it did predators.

Among the factors that influence the physiological selectivity of organophosphorus insecticides are penetration of the chemical into the insect's body, sorption and distribution of the absorbed poison and especially metabolism. The study of O-P-susceptible and -resistant

strains of different species offer unique advantages in investigations into the separate or joint action of these factors in connection with the different sensitivities of the species or strains to the tested chemicals.

Slow penetration may be expected to increase the effect of metabolism and excretion of poison by allowing more time for loss of poison, and thereby increasing the dose needed on the outside of the insect (Lord, 1967). Differences between the LD_{50} s by topical application and by injection, is an indication of the effective barrier between the external surface and the site of action. There is considerable variation in the injected-contact toxicity ratio from one species to another and from one anticholinesterase to another (Lovell, 1963). Forgash et al (1962) Farnham et al (1965), Gwiazda and Lord (1967) and Lord (1967), demonstrated that diazinon penetrates more slowly into O-P-resistant housefly strains than into susceptible strains, indicating that slower penetration may be important although it does not account for the whole of the resistance. Sawicki and Lord (1970) have shown that the delay in knockdown of resistant M. domestica compared with susceptible flies by diazinon, parathion and chlorthionethyl was accompanied by an increase in the amounts of insecticides that could be recovered from the surface of the insects 4 hrs. after treatment, suggesting that delayed penetration was responsible. The amount of poison present on the surface diminished fastest immediately after treatment and slowed down with increasing time. Both the strain of insect and dose applied affected this rate of loss (Sawicki and Lord, 1970). Lord (1967) has found that the proportion of a chemical that penetrates the cuticle is larger for the smaller doses. Different methods of application may also affect the rate of penetration. There is also an example in Aedes aegypti (L) larvae where induced malathion tolerance was found to be due to decreased absorption (Matsumura and Brown, 1961a). Although the effect of delayed penetration on the entry of insecticides into insects

is relatively small and is of little importance in insects which lack the metabolic mechanisms for breaking down insecticides, it can have a more significant effect in insects that have detoxifying mechanisms (Sawicki and Lord, 1970).

Little is known of how the insecticide makes its way from the surface of the cuticle to the n. system, whether directly through the cuticle and haemolymph, or by some indirect route such as the tracheal system. In M. domestica (Gwiazda and Lord, 1967) and in Periplaneta americana (Burt and Lord, 1968) has been found that once diazoxon has penetrated the cuticle it appears to spread rapidly throughout the bodies of insects. The route by which the insecticide approaches the nervous system and the concentrations that occur there have been studied by Burt (1970). This author found that the haemolymph plays a major role in transport of insecticides, although other tissues are also involved. Burt and Lord (1968) also found that haemolymph can carry relatively large quantities of moderately water soluble insecticides such as diazinon and relatively small amounts are sorbed on other insect tissues. They also found that a rather insoluble insecticide such as pyrethrin I, is sorbed much more strongly on the tissues which take up a large fraction of a topically applied dose. The partition ratios, tissue to buffer solution are 3.7:1 for diazoxon and 30,000:1 for pyrethrin I. The close correspondence between dose and concentration in the haemolymph is further evidence for rapid equilibration of the poison throughout the insect. The concentration in the haemolymph determined chemically 1-2 hrs after treatment with an LD_{90} dose are remarkably similar to those producing conduction-block in ganglia in vitro in the same time, an indication that diazoxon in the central nervous system is in approximate equilibrium with that in the haemolymph (Burt and Lord, 1968).

Other routes of entry exist and Gerolt (1969) has shown that insecticides are able to reach the nervous system in another way than via the haemolymph. By using a range of insecticides (but mainly dieldrin), he found evidence that after application to the cuticle, insecticides pass over or laterally through the cuticle to the spiracles and then via the tracheae and tracheoles to the nervous system and other tissues. Burt (1970) does not exclude the possibility that some insecticide passes from the outer surface of the cuticle into the haemolymph via the tracheae which is a more likely route for less water-soluble insecticides.

Gwiazda and Lord (1967) and Lord (1967) have shown that diazinon is sorbed at places other than the site of action in susceptible and resistant strains of houseflies. This non specific sorption, of the same degree in the two strains, would be at least partly reversible and concentration-dependent, and diminishes the amount of the chemical available for poisoning by some 5 to 40 times (Gwiazda and Lord, 1967). One of the toxicological significances of this sorption, according to Lord (1967) is that in this way the insecticide is protected from being converted to less toxic substances, though Weiden (1968) points out that lipophilic compounds which are more likely to be taken up by microsomes are thus more susceptible to microsomal oxidation. Sorption may also be important in the penetration of insecticides through tissues or membranes because the properties of rapid and complete reversible sorption might be expected to facilitate movement of materials in contrast to lack of "solubility" which would present a barrier.

There are claims that resistance can be conferred by storage of the insecticide in the fat reserves of the insect (Hoskins and Gordon, 1956) or excretion of unaltered insecticide (Perry and Buckner, 1959).

However, Gordon (1961) states that it has not always been shown that storage and excretion are not normal processes in the susceptible strains or that equal doses of a non-toxic analogue of the insecticide are more efficiently stored or excreted by the resistant strain than by the susceptible strain.

The mechanism of the preadaptation for resistance in most cases is, according to Brown (1964) mainly due to detoxification of the insecticide. Preadaptations involving reduced absorption through the cuticle and alimentary canal are of relatively minor importance. There is now abundant evidence that differences in enzymic metabolism are one of the most important factors in selectivity (O'Brien, 1967) and the most studied group of insecticides in this context is the organophosphates (reviewed by Hope and Ordish, 1961; O'Brien, 1961b; Metcalf, 1964).

Enzymic metabolism of the absorbed insecticide may involve an increase (intoxication) or a decrease (detoxication) in the toxicity of the molecule. Enzymic metabolism of the insecticide usually involves detoxication (Winteringham, 1969). The relationship between the rate of detoxication and the rate of intoxication, determines the toxic effect to the insects (Yang, 1971). Resistance to O-P insecticides in insects is caused by higher rates of degradation and correspondingly higher levels of hydrolytic enzymes in the O-P-resistant strains than in susceptible strains (O'Brien, 1966; Brown, 1968). It was found that degradation of diazoxon (Gwiazda and Lord, 1967) and paraoxon (Oppenoorth and Asperen, 1961; Nolan, 1970) was higher in resistant strains than in susceptible strains of M. domestica. When larvae of malathion-susceptible or-resistant strains of Culex tarsalis Coquillett are exposed to equal concentrations of malathion (1 ppm), the resistant strain has only about 1/3 as much malaoxon as the normal strain after 30 minutes (Matsumura and Brown, 1961b).

This difference is partly caused by its greater ability to detoxify malaoxon, for when larvae are exposed to malaoxon, this toxicant is eliminated by the resistant but not the susceptible strain, and homogenates of resistant larvae destroy malaoxon almost five times as fast as those of susceptible larvae. It is widely recognised that resistance ratios which may be large with topical applications may be very small with injected applications, though this does not necessarily mean that the strains differ in the rapidity with which a chemical may penetrate. Thus a 2-fold difference in metabolic degradation could have a disproportionately large protective effect if, in both strains, the topical doses penetrated at identical rates, sufficient to overwhelm the degrading system of the susceptible strain (Nolan, 1970).

The role of esterases in insecticidal action.

Because of the implication of esterases in the poisoning of insects by insecticides, studies on the insecticidal action of O-P compounds have raised considerable interest in insect esterases, both those poisoned by the toxin and those involved in detoxication.

The cholinergic system comprises three components: the synaptic transmitter, acetylcholine (ACh); the acetylcholine hydrolysing enzyme, acetylcholinesterase (AChE); and the acetylcholine synthesizing enzyme, choline acetyltransferase or choline acetylase (ChA) (Smallman and Mansingh, 1969). Despite early uncertainty the evidence for the presence of the system in insects is now unequivocal and its function in insects has been reviewed by Smallman (1958), O'Brien (1960), Chadwick (1963), Colhoun (1963), Treherne (1966). In insects as in other animals, the cholinergic system is associated primarily with nervous tissue, though there is strong evidence that this system is not the only neural transmitter in insects. Evidence from toxicological studies suggest also that the muscular movements are controlled by the

same system (Mehrotra and Smallman, 1957). Various experiments have been done to attempt to explain the action of O-P insecticides on different species and the stages of development with differences in the levels of cholinergic components. The comparative value of the available data however is restricted due to the differences in precision of the variety of methods were used by various experimenters with different species.

It is generally thought that cholinesterase inhibition is the primary cause of poisoning by organophosphates (Spencer and O'Brien, 1957; Smallman and Fisher, 1958; Smith and Salkeld, 1965; Metcalf and March, 1969). However considerable doubt exists as to whether the O-P insecticides exert their insecticidal action by the inhibition of ChE activity in the nervous system, and many workers have stressed the possibility that inhibition of other esterases might also be involved, even if they are not necessarily the cause of death (Lord and Potter, 1951, 1954a; Hopf, 1952, 1954; Potter et al, 1957; Asperen, 1958b, 1960a; Brown, 1961; Bigley, 1966).

The most studied groups of esterases involved in the insecticidal action of O-P compounds are the cholinesterases and carboxylesterases. Cholinesterases (ChEs) are of two types, Acetylcholinesterase (AChE) and cholinesterase (ChE). They were classified by the International Commission on Enzymes in 1961 under the systematic names Acetylcholine acetylhydrolase (3.1.1.7) and Acetylcholine acylhydrolase (3.1.1.8) respectively. AChE is the predominant acetylcholine-decomposing enzyme of nervous tissue and the erythrocytes of most species, whereas ChE predominates in the blood plasma of most species. Each enzyme was found to hydrolyse both choline and non-choline esters, but the rate of hydrolysis of each ester by each of the two enzymes differs. They can also hydrolyse aromatic as well

as aliphatic esters, often quite rapidly. The enzyme from houseflies, although regarded as an AChE, is intermediate in properties.

Carboxylesterases (Car-Es), formerly known as "aliesterases" or "B-esterases" were classified under the system^{atic} name carboxylic ester hydrolase (3.1.1.1). It is probable that a number of Car-Es exist. The enzyme attacks both aliphatic and aromatic neutral esters, so that the old name "aliesterase" was incorrect (Dixon and Webb, 1964). The term carboxylesterases (Car-Es) is used throughout this work for the carboxylic ester hydrolase.

To understand fully the action of an insecticide it is necessary to know the lethal mechanism, the critical site of action, how the poison reaches this site, and any protective mechanism the insect may possess (Burt and Lord, 1968). Strong arguments have been put forward that the toxic action of insecticidal O-P compounds is due to the inhibition of ChEs within the n. system of insects and thus disorganization of synaptic conduction, but this has not been proved (Metcalf, 1955; Burt and Lord, 1968).

In resistant and susceptible strains of houseflies it was found that after external application of poison the extent of ChE inhibition was related in both strains to the toxic symptoms (Lord et al, 1963). A strain of the mosquito C. tarsalis specifically resistant to malathion and malaoxon alone (Darrow and Plapp, 1960) was characterized by a 2- to 3-fold increase in ChE activity (Matsumura and Brown, 1961b). Using a number of p-nitrophenyl phosphates and a phosphorothioate Dauterman and O'Brien (1964), studied the correlation of the sensitivity of Apis mellifera to these poisons in vivo and their ChE inhibition in vitro. There was a high correlation between in vitro inhibition and in vivo toxicity. Asperen (1958a) and Plapp and Bigley (1961) reported that Car-E inhibition was higher in poisoned houseflies. But this was not coincident with symptoms of poisoning as was the case with

ChEs. Furthermore, maximum Car-E inhibition preceded symptoms and reactivation continued as poisoning advanced to point of death. O'Brien (1961a) showed that inhibition values for Car-Es were higher than those for ChEs in houseflies treated with parathion and 3 other phosphorothionates. However, because of the coincidence of symptoms with inhibition of ChEs, it was concluded that death was due to ChE inhibition. Lord et al (1963) have found that houseflies are killed by doses that inhibit only about 1/3 of the total activity of the thoracic ganglion. Further, although "superficial" and "peripheral" ChEs are almost completely inhibited, activity in the neuropile is little affected. The vital function of ChE is normally considered to be at the synapses, which are in the neuropile. If this is the only vital function for ChEs then the thoracic ganglion cannot be a critical site of toxic action, or inhibition of ChEs is not the primary cause of death. This does not rule out the possibility that inhibition of ChEs in other parts of the n. system may be critical. According to Burt et al (1966) and Booth and Metcalf (1970), localized sites of ChE inhibition in houseflies may be responsible for the action of the toxicants used by these authors (organophosphates and carbamates), rather than a generalized inhibition. The peripheral areas of the thoracic ganglion of the central nervous system appear to be one of these sites. The thoracic ganglion, controls segmental reflex activity and it is not surprising to find the locomotory apparatus nonfunctional when peripheral inhibition of ChE occurs in the ganglion (Pringle, 1940; Roeder, 1953; Molloy, 1961). The exact function of this non synaptic peripheral enzyme as it relates to death of the insect is not known. Experiments with houseflies, with and without addition of eserine showed that 60% of ChE activity and 20% of Car-E activity to α -naphthyl acetate is contributed by the heads (Asperen, 1962).

Lord and Potter (1950, 1951) were the first to suggest that esterases other than ChEs may be important in O-P-poisoning. According to Hopf (1958) there is at least one other distinct enzyme, a phenylesterase, present in locust ganglia. The inhibition of this enzyme seems to be more correlated to toxic symptoms than that of ChEs. However, he accepts that in the absence of information on its natural substrate or on the occurrence of other enzymes it is not possible to speculate on its role in the mode of action of phosphoric insecticides. In vivo and in vitro experiments conducted by Matsu-mura and Brown (1961b), proved the Car-E inhibitor EPN (Ethyl 4-nitrophenyl phenylphosphonothionate) to be a strong synergist to malathion for the resistant strain, whereas the phosphatase inhibitor NaF showed only a weak synergistic effect. They, therefore, concluded that it is mainly the Car-E and not phosphatase activity that is the deciding factor in malathion metabolism. On the other hand, selective inhibition of Car-Es in houseflies by DEF carried out by Bigley (1966) did not result in death of treated susceptible or resistant flies. Although Car-Es apparently play a nonvital role in housefly general metabolism (Bigley, 1966), its role in degrading toxic O-P compounds is highly important, especially when resistance is present. After DEF pretreatment there was a greater accumulation of paraoxon in both strains.

Several reports have shown that AChE of susceptible and resistant strains of houseflies do not differ (Asperen and Oppenoorth, 1959; Bigley and Plapp, 1960; Forgash et al, 1962) but Mengle and Casida (1960) suggested that resistant flies may contain a factor which protects the enzyme. Asperen (1960b) has pointed out that after poisoning houseflies with various organophosphates, there was a very high percentage of Car-E inhibition, in comparison with a low percentage of ChE inhibition at the time of prostration. This could mean that inhibition

of Car-Es is more important than ChE inhibition in causing the death of the fly. On the other hand Stegwee (1960) showed that considerable Car-E inhibition in houseflies treated with tri-o-totyl phosphat (cited as Tri-o-cresylphosphate, TOCP), which is a selective inhibitor not affecting ChEs, did not cause death. When those flies were treated 24 hrs later with tetraethyl pyrophosphate (TEPP), typical symptoms of O-P-poisoning developed.

Asperen and Oppenoorth (1959) found that in 5 parathion-resistant and 2 malathion-resistant strains of houseflies the activity of Car-Es, measured by the hydrolysis of esters such as methyl butyrate, was only about 20% of the Car-E activity of normal strains. Further study (Asperen and Oppenoorth, 1960) showed that resistant flies contain a modified Car-E which has the capacity of degrading the oxygen analog^{ue} to which resistance has developed. Two parathion-resistant strains could break down paraoxon and diazoxon more rapidly than the normal strains. These authors (Oppenoorth and Asperen, 1960) concluded that in the O-P-resistant strains the Car-E has been converted into a breakdown enzyme which thus functions as a phosphatase with very high affinity for its substrates paraoxon and diazoxon and it seems difficult to correlate the low Car-E activity with resistance in houseflies. The main cause of O-P-resistance in houseflies is therefore degradation of the toxic oxygen analogues of the poisons by some altered Car-E, although other mechanisms are present in some strains (Oppenoorth and Asperen, 1961). This is corroborated by the strong synergistic action of organophosphates that block these breakdown enzymes. At this time it appeared that carboxylesterase-like degrading enzymes were able to prevent the toxicant from reaching a fatal concentration at the site of action whereas most of the total elimination was brought about by other mechanisms present in both susceptible and resistant strains

(Oppenoorth and Asperen, 1961).

Since then, differences in the Car-E activity between susceptible and resistant strains of different species have been found, though the ChE activities were similar. Lower Car-E activity was found in parathion- and malathion-resistant strains of houseflies (Bigley, 1966) and also in malathion-resistant blowfly Chrysomya pytoria (Wild) (Substrate α -naphthyl acetate; Ozaki, 1969). On the other hand, Matsumura and Brown (1961b) have ascribed malathion resistance in the larvae of the mosquito C. tarsalis to a higher Car-E activity. Strains of Drosophila melanogaster Meigen showing resistance to parathion and to other insecticides, proved to have 7-8 times more Car-Es than normal strains (Ogita, 1961). Resistance to several organophosphates in the hemipterans, the green rice leaf hopper Nephotettix cincticeps Uhler (Ozaki, 1969) and the small brown leaf-hopper Lao-delphax striatellus Fallen (Kojima et al, 1963; Ozaki, 1969) is linked with high Car-E activity (substrate β -naphthyl acetate). Similarly, resistance to O-P compounds in M. persicae is linked with high Car-E activity (Needham and Sawicki, 1971; Sudderuddin, 1972) and the loss in resistance in an unselected strain of this species was reflected by a corresponding loss of Car-E activity (Needham and Sawicki, 1971).

Combined effect of factors affecting toxicity

All results indicate that several factors are involved in the development of resistance. However, the effects seem to be so complex that no attempt has been made to develop a theoretical treatment of these results (Sawicki and Lord, 1970). Knowledge of how a substance affects a sensitive mechanism is not enough to predict its biological activity (Lewis and Lord, 1969). Thus, O-P insecticides kill by inhibiting ChEs, but anti-ChE activity is not a reliable guide to toxicity, because toxicity is much affected by other phenomena.

By applying ^3H -dimethoate to 5 insect species it was found that in the highly sensitive housefly penetration was particularly rapid and in the initial 30 min. there was a 5-fold increase in production of the oxygen analog^{ue} and the ChEs were 100 times more sensitive (Uchida et al, 1965). However, variations in these factors could not explain all the variations in toxicity to the other 4 species. According to these authors, additional factors will have to be studied to account fully for the remarkable species specificity of dimethoate. Lord et al (1963) found that to produce equal symptoms and ChE inhibition in resistant and susceptible strains of houseflies it required up to 40 times as much diazoxon for the resistant as for the susceptible strain when the poison was applied externally. This difference between the two strains may mean that the ChEs of the resistant flies is less susceptible to inhibition or that a smaller proportion of the externally applied poison reaches the site of action, perhaps because of differences in permeability or detoxication, or a combination of both (Lord et al, 1963). Initial studies of parathion-resistant (Oppenoorth, 1958) and malathion-resistant (March, 1959) strains of houseflies showed that they contained no more ChEs than the normal and that their ChEs were of normal susceptibility to inhibition by O-P compounds in vitro. The resistance was still present if the O-P compound was injected and was therefore not due to differences in absorption. The parathion-resistant strains accumulated less para-oxon in the body, not because it was produced more slowly from parathion but because it was degraded more rapidly than in the normal strains (Oppenoorth, 1958). Tissues of malathion-resistant flies also detoxified mala-oxon twice as fast as susceptible flies (March, 1959). However, in a strain of diazinon-resistant flies, which absorbed less diazinon and contained less diazoxon than normal, the difference in degradation rates seemed insufficient to account for the resistance (Krueger et al, 1960).

Factors that influence susceptibility by modifying the effect of metabolism of poisons are not only differences in the distribution of the poison, but also differences in the distribution and sensitivity of detoxication mechanisms (Gwiazda and Lord, 1967). Little is known about specificity at the site of action within the Insecta as a whole, because the effect of insecticides has been examined electrophysiologically on very few species of insects (Burt, 1970). Nearly all the work has been done on one species of cockroach, and on locusts and houseflies. Insecticides that interfere with synaptic conduction (e. g. O-P compounds) are likely to show some specificity at the site of action, but with all insecticides physiological specificity is mostly associated with such factors as penetration and metabolism (Burt, 1970). Smissaert (1964) found higher enzyme activity in homogenates of a susceptible strain of Tetranychus urticae than in resistant strains. Moreover, he found that the ChEs of the ^{susceptible} λ -strain were more sensitive than those of the ^{resistant} λ -strain to inhibition by diazoxon or paraoxon. He concluded that the lower ChE activity of the ^{resistant} λ -strain was an incidental effect but the lower sensitivity to inhibition by the O-P ^{compounds} λ was a factor in the lower in vivo toxicity. Quantitative tests conducted by Gwiazda and Lord (1967) indicated that the mechanisms for decomposing diazinon are located differently in the two strains of houseflies. The susceptible strain seems to have a detoxication mechanism both internally and in the integument, whereas the resistant strain has possibly one only in the integument. Resistance or tolerance to O-P compounds due to AChE insensitivity was reported by Schuntner and Roulston (1968) in blowflies and by Zahavi (1970) in Ceratitis capitata Wied. from a locality where organophosphates had been used extensively. AChE from resistant Medfly heads was found to be 20% less sensitive to phosphamidon than in normal strains.

Insects showing generalized tolerance to insecticides, are unlikely to have exceptionally efficient detoxication mechanisms for all

organic toxins. It seems probable that several adaptations exist for these insects to withstand toxic compounds. For example, Gordon (1961) suggests that the larval forms of many insects with less well organized nervous systems than the adults are more insecticide-tolerant. Gordon continues that the larvae must endure variable and severe biochemical stress, presumably arising in their more sessile existence and with a less vulnerable nervous system and more active defense mechanisms than the adults they possess this "endurance vigor" which is of value in survival. Adult forms of some insect species would be able also to endure a deprivation of food and water for long periods, that would kill other insect species and thus avoid taking in any toxic material present. In Aphid species this is attributable to the sap-feeding mechanisms, by which they avoid intake of many deleterious plant biochemicals or added toxic materials.

New approaches to insect control.

The need for new insecticides is made apparent by the increasing number of resistant strains which are arising in the field. In the area of aphid control the need is for rapid poisoning to prevent the spread of aphid-born viruses and selective action to interfere as little as possible with predator and parasite populations. These pressures have recently led to new approaches to insect control being attempted.

O'Brien (1970) has reported work describing the prospect of using the acetylcholine receptor as a new target for insect control. The best known target so far for poisoning is acetylcholinesterase, the enzyme which destroys acetylcholine and in so doing prepares the neuron for a new excitation. But there is a completely different macromolecule called the receptor, which is of even more central importance to cholinergic neurons. This is the protein with which acetylcholine normally combines in order to produce its excitatory

effect. This acetylcholine receptor has never been isolated. Its properties are only recognized by its action in the intact cell. In vertebrates very many agents are known which act upon the receptor, like curare, succinylcholine and atropine, and it is assumed that nicotine acts by combining with the receptor in insects. O'Brien describes the first study of the properties of the acetylcholine receptor at the broken cell level in Torpedo electroplax, housefly head and rat brain. A thorough study of this receptor, he believes, may make it possible to design new toxicants operating on the receptor. It is quite likely that, just as in the case of acetylcholinesterase, there will be important differences between the receptors of insects and mammals and that these differences might be a basis for new selective toxicants.

Maddrell and Casida have reported that paralytic doses of insecticides representing each of the major types, applied to the fifth instar nymphs of Rhodnius prolixus Stål, initiate massive secretion by their Malpighian tubules. As a result there is in each case a very large increase in the volume of rectal fluid and a corresponding reduction in the haemolymph volume. The mode of action appears to involve the liberation of a diuretic factor, probably the diuretic hormone from the central nervous system into the haemolymph at the paralytic stage of poisoning. The disturbances in water distribution and cation balance in the haemolymph possibly contribute to the eventual death of treated insects (Casida and Maddrell, 1971; Maddrell and Casida, 1971). These authors suggest that these findings on the excretory physiology of insects are of potential importance in the use and design of insecticidal chemicals. Because of the rapid loss of a large volume of haemolymph not only is the ionic balance likely to change drastically, but the insecticide concentration in the haemolymph may increase as a result of the

fluid loss and so enhance its disruptive action on the nervous system. These changes can be initiated by chemicals that paralyse the insect for only brief periods and they suggest that possibly insecticidal chemicals that are non persistent in the environment could be used not as acutely lethal paralytic agents but as initiators of disadvantageous excretion (Maddrell and Casida, 1971; Maddrell, 1972).

INSECT MATERIAL AND INSECTICIDES

Insect Material. Laboratory strains of the species Acyrtosiphon pisum (Harris) and Aphis fabae Scop. (Homoptera:Aphididae) were used as the experimental insects in this study. The aphids were reared on pot-grown broad bean plants, Vicia faba (L.), a dwarf variety "The Sutton" was used. Bean seeds were germinated in wet vermiculite or sand in a glasshouse and when 5 to 10 cm high were transferred to pots also containing vermiculite with 2 to 3 plants per pot. The aphid species were kept separately and to prevent mixing of the two species and different age groups each pot was covered with a cellulose acetate cylinder, 11 cm diam. x 15 cm high, topped with a terylene voile. The pots were kept in plastic trays, 55 x 28 x 7 cm deep, 8 pots per tray on a vermiculite bed, through which water was supplied to the plants.

The aphid cultures were maintained in an air conditioned room at $20 \pm 1^{\circ}\text{C}$. The relative humidity was not controlled, being usually between 40 and 50%. Light was provided 16 hrs a day by four 85W daylight fluorescent tube lights 1.20 m long and 15 cm apart, arranged 35 cm above the plants.

Insecticides. The following analytical grade organophosphorus systemic insecticides were used:

- a) Dimethoate (dimethyl S-(N-methylcarbamoylmethyl) phosphorothiolothionate), 99.5% active ingredient.
- b) Menazon (S-(4,6-diamino-1,3,5-triazin-2-ylmethyl) dimethyl phosphorothiolothionate).
- c) Phorate (diethyl S-(ethylthiomethyl) phosphorothiolothioate), 98.9% a.i.
- d) Phoratoxon (diethyl S-(ethylthiomethyl) phosphorothiolate).

2% w/v stock solutions were made up, dimethoate in acetone and the rest chemicals in 2-ethoxy^ethanol. Dilutions of the above stock

solutions with acetone or deionized distilled water in the case of dimethoate and 2-ethoxyethanol in the case of the other chemicals were prepared as necessary. The analytical material of the above compounds and the solutions prepared, were stored at -15°C to minimize breakdown. In all these experiments the water used was first distilled and then deionized.

PART ONE

BIOLOGICAL ASPECTS

SECTION A. GROWTH AND REPRODUCTION OF THE TWO SPECIES

Introduction

Different aphid species settle on the undersurface of V. faba leaves in characteristic patterns. Some species, like A. fabae, Megoura viciae Buckt., prefer to settle mainly on the main veins of leaves, others on peripheral parts of the leaf lamina (Myzus spp) or on intermediate areas [A. pisum, Aulacortum solani (Kltb.)] (Lowe, 1965, 1967a). This author also demonstrated a close correlation between the observations of settling and probing for many aphid species. A. fabae feed in the phloem of the larger veins whereas other species, for example A. pisum, do not settle preferentially on big veins, but feed from alternative food sources in the bean leaf, the smaller vein or mesophyll (Lowe, 1967a). It has been suggested therefore by Lowe (1967b) that a gradient of properties exists from the leaf base to the lamina margin, which exercises this behavioural pattern on the species. The differential settling and feeding of aphids appears to influence their growth. A. fabae and A. solani grew larger when caged on the peripheral areas of mature bean leaves than when caged on basal areas of the leaves (Lowe, 1967b). The performance of systemic insecticides in controlling aphids may well be affected also by the preferred food source of the insects.

In the present work observations on the growth of the two species were carried out after they were left to settle freely on their preferred sites of V. faba leaves. Differences in their rates of growth may indicate quantitative or nutritional differences in the plant material taken up by the aphids. Insecticidal material distributed in the plant will also be taken up in different proportions.

Larvae born during any period of adult life are associated with

nutrients ingested earlier. Banks and Macaulay (1965) found that 70% of the total nitrogen ingested by the adults of A. fabae appeared in the larvae. It has been found also in these studies (see page 56) that a significant quantity of radioactive phosphorus taken up by the adults of the two species was transferred to the larvae. Transfer of nutrient materials to unborn larvae occurs readily and the ability to transfer compounds less useful nutritionally may be important in toxicological studies of aphids, since in this way the larvae constitute a route by which absorbed insecticides or their metabolites, may be removed from the adult as has been already established in Glossina pallidipes Aust. (Irving, 1968). The capacity to eliminate compounds by this larval route will be in direct relationship to the number of the larvae produced. In the present work the rate of larval production in the two species was measured during the major part of their reproductive life.

Closely associated with food intake by aphids is the honeydew they excrete. Auclair (1963) has said that a quantitative determination of honeydew excretion "... constitutes a most valuable tool in aphid physiology. It may be used in studies of the rates of aphid feeding, food assimilation and growth, in nutritional experiments involving food balance and nitrogen economy, in assessing the degree of resistance of host plant, and it may serve as a crude measure of the general metabolic activity of the insect...". The rate of excretion depends not only on the aphid species but also on the stage of the insect, and the species and variety of the host plant (Auclair, 1958, 1959; Auclair and Maltais, 1961; Banks and Macaulay, 1965). Various environmental factors also influence the growth of the plant and the periodicity with which aphids excrete honeydew droplets (Auclair, 1958; Mittler, 1958b). Honeydew production is an indication

of aphids feeding, however it can follow different forms in different species. Mittler (1958a) observed that Tuberolachnus salignus (Gmelin) continues to excrete large quantities of honeydew during post-reproductive life, but it is remarkable that A. fabae does not do so, although it continues to feed (Banks and Macaulay, 1965).

Through the honeydew the aphids excrete a great part of the plant sap taken up. Auclair (1959) has found that A. pisum adults fed on the susceptible variety "Perfection" of Pisum sativum, ingested 10.65 mg per individual in 71 hrs. Of this quantity, the body weight was increased by 1.47 mg to a final weight of 2.57 mg, 1.60 mg was lost by evaporation, 0.05 mg in 2 exuviae and as much as 7.53 mg per individual as honeydew. The same species when reared on resistant and susceptible varieties of P. sativum excreted as honeydew 47% and 58% respectively of the total ingested (Auclair and Maltais, 1961).

In toxicological studies of aphids the study of honeydew excretion is one of great importance not only because it gives a rough picture of the feeding rate of the insects and therefore the rate of ingestion of a toxic compound in the plant sap, but also because through the honeydew the insect has a mechanism of elimination of an absorbed chemical and its metabolites. Quantitative and qualitative measurements of honeydew production have been carried out so far on several aphid species by many workers. The rate of honeydew excretion has been estimated by most authors as a product of the number of droplets and the mean volume per droplet (as for example by Auclair, 1958, 1959). The collection and exact measurement of honeydew volumes is necessary in order for it to be used in toxicological studies. In the present work, the honeydew excreted by the two species was collected in its original form. Also, a simple method was used to measure the specific gravity of the honeydew.

Materials and Methods

Rate of growth. A number of adults of each species were put separately per species on plants. 24 hrs. later all the adults were removed and the larvae produced during this period were weighed using a VDF torsion balance 'United' type 2.5, with a sensitivity of 5 μ g-division. The weighings were performed every 2 days and the aphids were transferred to new plants every 4 days. The insects were allowed to settle on their preferred sites. Larvae of both species were weighed initially in groups of 5 confined within a small gelatine capsule of known weight. From the 4th day onwards the aphids were weighed individually. Weighings were continued up to the 18th day and covered all the pre-reproductive life and about 10 days of the reproductive period of the aphids.

Rate of larval production. Early adults of the two species were caged individually on the undersurface of leaves under small rubber rings of 1 cm internal diameter covered with a terylene net. Each ring was held with a spring clip onto the plant. Two pots, each with 3 plants, were used with 5 cages of each species to each pot. The larvae produced by each adult were counted daily and removed from the cages.

Collection and measurement of honeydew. In order to collect honeydew, it is necessary to restrict the insects to the undersurface of a leaf. A number of ways of preventing them from leaving a test leaf were tried. Firstly, a string ring supported on a cellulose acetate base which surrounded the insects and restricted them within a certain area of the leaf was tried. The ring was kept in position by means of a spring clip (Fig. 1.1a) Secondly, a foam plastic ring around the petiole of a single leaf that was inclined slightly upwards was often sufficient to prevent aphids migrating from the leaf. (Fig. 1.1b).

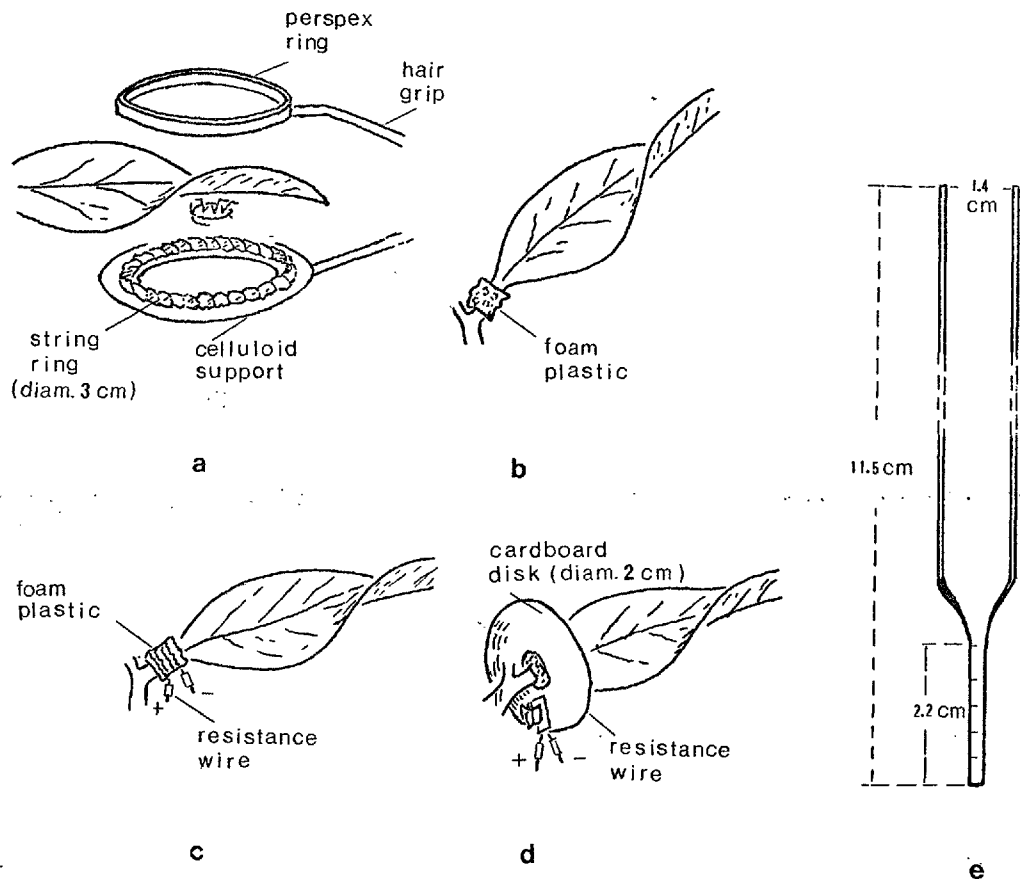


Fig. 1.1. a-d. Methods used to restrict aphids on the leaves for honeydew collection; e. Hopkins vaccine centrifuge tube for the separation of honeydew from the oil.

The third method consisted of a thermal barrier produced by a short length of resistance wire surrounding a single leaf petiole and carrying an electric current. A foam plastic ring (Fig. 1.1c) or a cardboard disc (Fig. 1.1d) were used to prevent heat damage to the plant. The resistance wire was glued on the periphery of the cardboard disc, which had a radial slit allowing it to be placed around the petiole from which it was further isolated by a thin foam plastic ring. A small clip joined the two ends of the slit and prevented the barrier being dislodged. The string ring was not effective in restricting the insects to one leaf and the resistance wire, isolated from the leaf petiole simply by a foam plastic ring produced leaf decay at the temperatures necessary to prevent aphids migrating to other parts of the plant. This was especially so with A. fabae. More satisfactory was the use of a simple foam plastic barrier around the petiole of an upwardly directed leaf, or the thermal barrier around a cardboard disc. Aphids occasionally fell, when they approached the hot wire and neither of these methods was entirely satisfactory in restricting the movements of the aphids. Thus, frequent counts of the number of adults on the undersurface of the leaves were necessary in order to assess the number of adults present and the duration of their feeding. Occasionally new adults were added during an experiment when the original number was reduced by migration or falling. These new adults were considered to be feeding and were counted only after they had settled. The sum of the mathematical products of the number of adults and the duration of feeding in each experiment, gives the total number of aphids-hours for honeydew production.

After the insects had settled on the lower surface of a leaf about 10 ml of Risella 17 oil, specific gravity 0.7, was placed in a disposable polystyrene petri-dish 9 cm diameter beneath them.

The degree of 'sticking', of honeydew droplets on the disposable petri-dish was lower than on a glass surface. The oil surface was positioned 4-5 cm below the aphids so that the droplets had a small distance to fall through which evaporation could occur. A little Sudan IV dye was dissolved in the oil to make the honeydew droplets easier to see as clear beads. To ensure insignificant loss of water from the droplets to the oil, the oil was first shaken with and left standing over a 10% sucrose solution. Any droplets were removed by centrifugation before use. (Honeyborne^{C.H.B.} and Gibson^{R.W.}, 1967, personal communication).

At the end of the collection period excess oil was removed from the petri-dish with a pasteur-pipette. The small remaining volume of the oil with the honeydew droplets were transferred to a Hopkins vaccine centrifuge tube which had a capillary base about 2 mm diameter and a capacity of about 0.05 ml (Fig. 1.1e). The droplets of honeydew were spun down from the oil into the capillary by using a fly-out centrifuge head run at about 1,500 g for 10 min. The honeydew collected in the base of the capillary and was measured using a travelling microscope and vernier scale. The volume of honeydew was estimated from a calibration curve produced beforehand by measuring the lengths and weights of small volumes of distilled water added to the tube and centrifuged in a similar manner. The weights of water were converted to μl and plotted against their corresponding lengths. The density of water was taken as 0.99823 g/ml at 20°C, the temperature of the calibration (International Critical Tables). The corrected volume of honeydew was divided by the total aphids-hours to give the mean volume produced per aphid per hour.

Results

1. Rate of growth of the two species. The mean weights of the aphids and standard errors are presented in the Fig. 1.2. It shows that

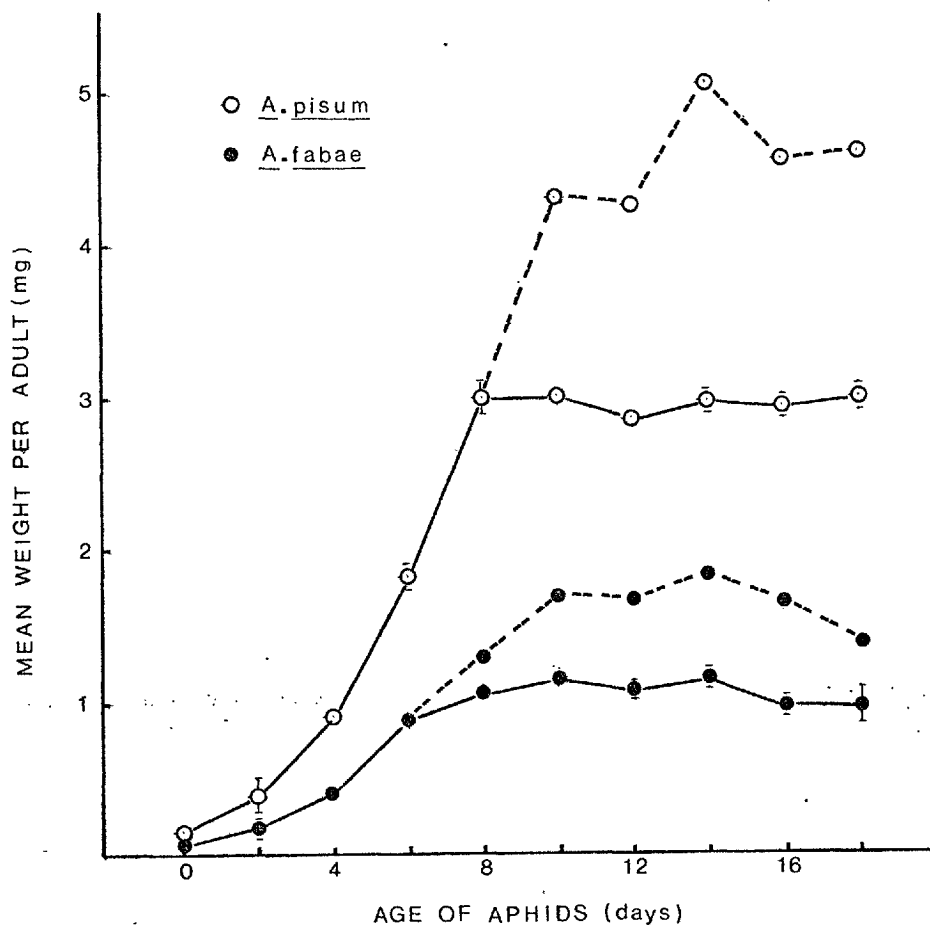


Fig. 1. 2. Rate of growth of the two species (see Appendix 1)

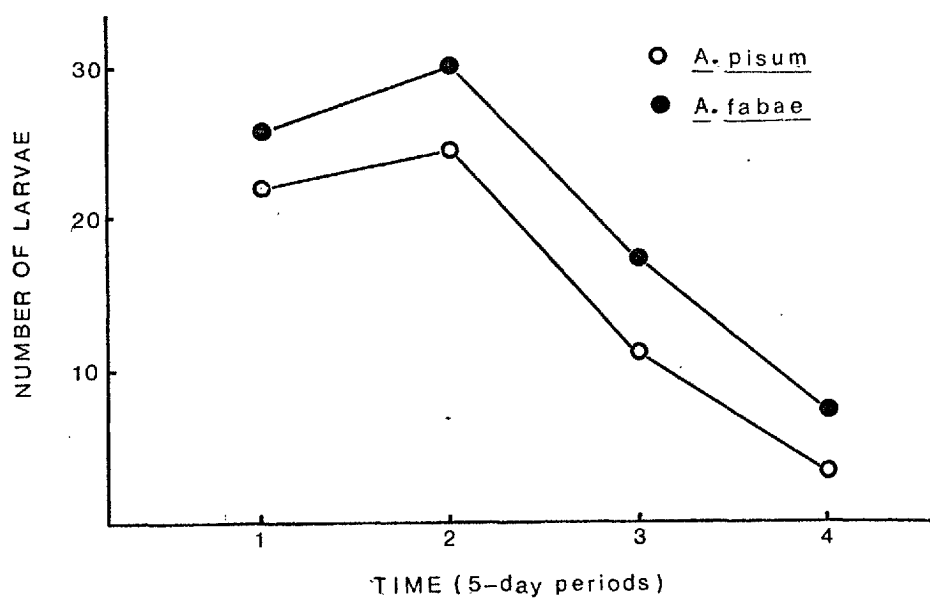


Fig. 1. 3. Larviposition per adult in five-day periods (see Appendix 2)

there was a rapid increase in the aphids weight up to the 8th day in both species. Though the weight of A. pisum increases more rapidly than A. fabae, the species are more similar when the data is expressed as an increase relative to the weight of the 1st instar nymphs. Larviposition started on the 7th day in A. fabae and 8th day in A. pisum. During the reproductive period of the adults no further increase of the body weight in A. pisum occurred, while in A. fabae there was a slight decrease. However if the weight of the larvae produced is added to the mean weight of the adults during the reproductive period we have a further increase up to the 14th day in both species.

2. Rate of produced larvae. The Fig. 1.3 shows that the number of larvae born to both species followed parallel patterns. The maximum larviposition occurred between the 6th and 10th day in both species. The number produced per adult was higher in the case of A. pisum than in A. fabae. This difference was kept stable throughout the whole period of the 20 days larviposition. Within this period the total number of larvae produced was in A. pisum 81.3 per adult and in A. fabae 61.5 per adult.

3. Produced honeydew.

Volume of honeydew. The data in Table 1.1 shows that the volume of honeydew produced by A. pisum adults was nearly 6 times greater than by A. fabae adults.

Table 1.1. Volume of honeydew produced by the adults of the two species (see Appendix 3).

Case	<u>A. pisum</u>			<u>A. fabae</u>	
	Exp. 1	Exp. 2	Exp. 3	Exp. 1	Exp. 3
Total volume (μl)	29.82	22.88	22.36	1.48	2.99
Aphids-hours	635.50	486.50	515.90	165.50	467.40
Honeydew (μl)/aphid/hr.	0.0469	0.0470	0.0433	0.00892	0.00640
Mean volume (μl/aphid/hr.)	0.046 ± 0.0012			0.0077 ± 0.0013	

Specific gravity of honeydew. To estimate the specific gravity of the honeydew, a capillary of 5 μl was weighed when filled with distilled water and again filled with honeydew. At the prevailing temperature of 21°C the density of water is 0.99802 g/ml (International Critical Tables). Thus, the volume of the capillary was $5 \times 0.99802 = 4.99$ μl. The weight of A. pisum honeydew in the same capillary was 5.2 mg and of A. fabae 5.4 mg. The specific gravity, therefore, of the honeydew produced by the two species was in A. pisum $5.2/4.99 = 1.04$ and in A. fabae $5.4/4.99 = 1.08$.

Discussion

An important factor in relative toxicological studies, is the quantity of plant material absorbed and therefore the quantity of insecticide ingested with the plant sap. In order to use the relative increase of body weight as a criterion of uptake it must be known how much of the total quantity taken up has been used to increase the body weight in each species. From the figures given previously for A. pisum (Auclair, 1959, page³²/above) the body weight increases by 1 mg for

every 7.2 mg taken up. This ratio alters during the insect's development. The adult aphid continues to feed but does not increase in weight. There is however a continued increase in biomass which can be derived from the weight of larvae produced, as the dotted lines of Fig. 1.2 show when the weight of the larvae produced was added to that of the adults. This increase finally stops when the reproductive period is over, though in A. fabae, continued feeding without the production of honeydew would be expected to cause a continued gain in weight in post-reproductive life (Banks and Macaulay, 1965).

Absorption of plant material and conversion to aphid tissue either in the adult or developing embryos indicates also that systemic insecticide absorbed with the plant food could similarly be available to exert a toxic action.

Over 5 days intervals of their reproductive period A. pisum adults produced 3.8 to 6.3 larvae per adult more than A. fabae. In the 20 days of larviposition A. pisum produced about 20 larvae per adult more than A. fabae, though in these experiments production by A. fabae was lower than that found by Banks and Macaulay (1965) on other varieties of broad beans. Thus, A. pisum adults have the potential to excrete a greater quantity of the assimilated material and with it any toxic compound that may be present, through the greater number of larvae they produce.

Unwanted and possibly toxic material can also be excreted through the honeydew. Estimation of honeydew production by counting the number of droplets and mean droplet volume has the disadvantage that there is often a wide range of droplet sizes produced. Auclair (1958) quoted a three-fold range in honeydew droplet volume for A. pisum reared on P. sativum. With the technique used here it was possible to collect and measure the volume of honeydew produced by the two species directly. Unsuccessful attempts to prevent adults moving to

other parts of the plant were overcome by the frequent counting of adults on the undersurface of the leaves.

The volume of honeydew excreted by the adults of A. pisum was 0.046 μ l per aphid per hr. and represents about 2.3% of the body weight. The volume excreted by A. fabae, 0.0077 μ l/aphid/hr. represents about 0.8% of the body weight. Thus, A. pisum adults produced more honeydew than A. fabae and even though more than twice the size of A. fabae, took about 40 hours to produce their own weight of honeydew, while A. fabae took as long as 127 hrs. to produce their own weight in honeydew. The results for honeydew production by A. pisum agree with those found by Shaefer (1938), where this species excreted 3.14% of the body weight/aphid/hr. and by Auclair (1959), where the same species was feeding on P. sativum; however in earlier experiments Auclair (1958) found slightly higher feeding rates with considerable variability of honeydew production. Aphids of A. fabae species reared on two varieties of V. faba during development from larva to adult (mean duration 7.5 days) excreted 2.83 μ l and 3.60 μ l or 0.0168 μ l and 0.0216 μ l/aphid/hr (Banks and Macaulay, 1965). They produced therefore 1.7% and 2.2% of the adult-body weight per hour in the two varieties, two to three times the rate of excretion found in these experiments. Other species were found to excrete higher quantities of honeydew. T. salignus (Mittler, 1958b) and M. persicae (Day and Irzykiewicz, 1953) excreted as much as 33% of their own weight per aphid per hour.

The specific gravity of honeydew was found to be nearly the same in the two species, 1.04 in A. pisum and 1.08 in A. fabae. Auclair (1958) found specific gravity of A. pisum honeydew reared on P. sativum to be about 1.3 and Srivastava and Auclair, in unpublished results referred to by Auclair (1963), about 1.03. These are comparable to the specific

gravity measured in these experiments, though the figure of 1.3 quoted by Auclair seems rather high.

Auclair (1963) has concluded that when aphids are reared on the same host-plant and under the same environmental conditions, differences in honeydew production suggest different aphid feeding and metabolic processes. Greater honeydew production must be correlated with increased feeding rate, thus A. pisum which will take in relatively more toxic material has the potential to excrete greater quantities also. However, a factor which considerably complicates the toxicological significance of the feeding behaviour is the well established interference of some systemic insecticides present in the feeding medium in changing the feeding response of the aphids. Studies on this aspect with M. persicae have been carried out by Mittler and Pennell (1964), Parry and Ford (1967, 1969, 1971) and Javadi (1971) using the artificial feeding technique. An attempt was made also in the present study to examine the inhibitory action of O-P compounds to A. pisum and A. fabae and is described below in Section C.

SECTION B. UPTAKE AND EXCRETION STUDIES OF A. PISUM
AND A. FABAE FEEDING ON ^{32}P LABELLED
LEAF-TISSUE.

Introduction

O'Brien and Wolfe (1963) refer to non specific labelling of food-stuffs as a widely used technique to study feeding as a social function, as in the transfer of food within insect colonies, or on an individual basis in the study of food or water intake and transfer of plant sap containing virus from plant to plant. There has long been an interest in following the feeding behaviour of phytophagous insects particularly disease vectors such as aphids.

The feeding and excretion behaviour of several aphid species has been studied by allowing the insects to feed on leaf tissues made radioactive, usually with ^{32}P -phosphate, and then relating the radioactivity of the aphids, honeydew and larvae to that of the leaves. The results obtained are mostly of value for comparative purposes only, since the concentration of the radioactive materials in the actual plant fluid ingested was unknown (Watson and Nixon, 1953; Day and Irzykiewicz, 1954; Banks and Nixon, 1959). It has been suggested that the rate of excretion by the aphids is equal to the rate of ingestion (Banks and Nixon, 1959). A great deal of work has therefore been done in the study of the excretion through the honeydew and it has been found that different factors influence its production.

At one time turgor pressure was thought to determine the rate of feeding in the plant. But using radioisotope techniques A. fabae and Aphis sambuci L. were found to produce twice as much honeydew when attended by ants (Herzig, 1937; Banks and Nixon, 1958). This enhanced honeydew production was the result of increased intake of radioactive sap from the plants. It follows therefore, as Herzig (1937) supposed, that attendance by ants directly stimulates both

the excretion and feeding rates of the aphids. At these higher feeding rates the number of larvae produced by A. fabae and A. sambuci was doubled or trebled (Herzig, 1937). However no increase in larviposition was observed by Banks (1958) and Banks and Nixon (1958) when A. fabae feeding on bean plants was stimulated similarly. These authors have also demonstrated that the reproductive rate is significantly affected by the age of the plant tissue on which the aphids feed and accepted that the reproductive rate of the aphid is affected more by the quality than by the quantity of the nutrients which the aphid receives.

It has been found that much of the ^{32}P ingested by the adults appears in the young larvae when they were born (Watson and Nixon, 1953; Banks and Nixon, 1958, 1959). In radioautographs made from serial sections of aphids fed for 24 hrs. or more on ^{32}P labelled leaves, the dense blackening in the region of the developing embryos, indicated that much of the phosphorus taken up by the parents is deposited in this tissue (Watson and Nixon, 1953). Since adults lose radioactivity when they give birth to young, some workers have preferred to study uptake and excretion in nymph (Banks and Nixon, 1958, 1959).

Most of the studies on uptake and excretion rates by aphids that have been carried out so far were designed to investigate the performance of aphids as virus vectors and their relationship to susceptible and resistant varieties of host plant. It is perhaps surprising that there are few reports of work on the uptake and excretion behaviour of aphids on plants directed towards an understanding of systemic insecticide performance.

The present work investigates some of the relationships between the susceptibility of the two aphid species to systemic insecticides and their feeding behaviour on plant tissues. The ability

of the two species to take up radioactive sap was estimated by referring the radioactivity of insects directly to that of the labelled leaf-tissues. The relative rates of assimilation and excretion of ^{32}P by the two species was also examined by allowing the adults of the two species to feed alternately on the same radioactive areas. Finally, the rate of ingestion of radioactive material by the two species was followed in time and the fate of the isotope after the insects had been removed from the labelled plants was followed. The toxicological significance of the results are discussed. Since the aphids were confined to a certain leaf-area, little choice was given them to select their preferred sites on the undersurface of the leaf (van Emden, 1967) or to feed from places with large nutritional differences (Lowe, 1967a).

Materials and Methods

Radioactive compound. A standard ^{32}P -sodium ortho-phosphate solution was used (PBS 1, Radiochemical Centre, Amersham).

Application. Plant tissue was labelled in two different ways. When aphids were to be caged on whole leaves the isotope was injected into the petiole in portions of 5 μl up to a total volume of 10 to 20 μl per petiole with activities ranging from 3.7 to 7.1 μCi . These multiple applications of the isotope solution were spread over a 2 hours period in order to facilitate absorption by the plants. Aphids were also fed on leaf discs and in these experiments 1.7 to 3.6 μCi of isotope was applied in 5 μl to the wicks supplying the discs with water. Further details of the leaf disc technique are given below (page 92).

Feeding experiments. Groups of 5 to 10 adults of each species were caged separately on the labelled plant areas 24 hrs. after application of the radioactive phosphate. The aphids were caged on the under-surface of the treated leaves of the plant in perspex cages 2.5 cm in diameter, with a muslin top (Fig. 1.4a). In the leaf disc experiment

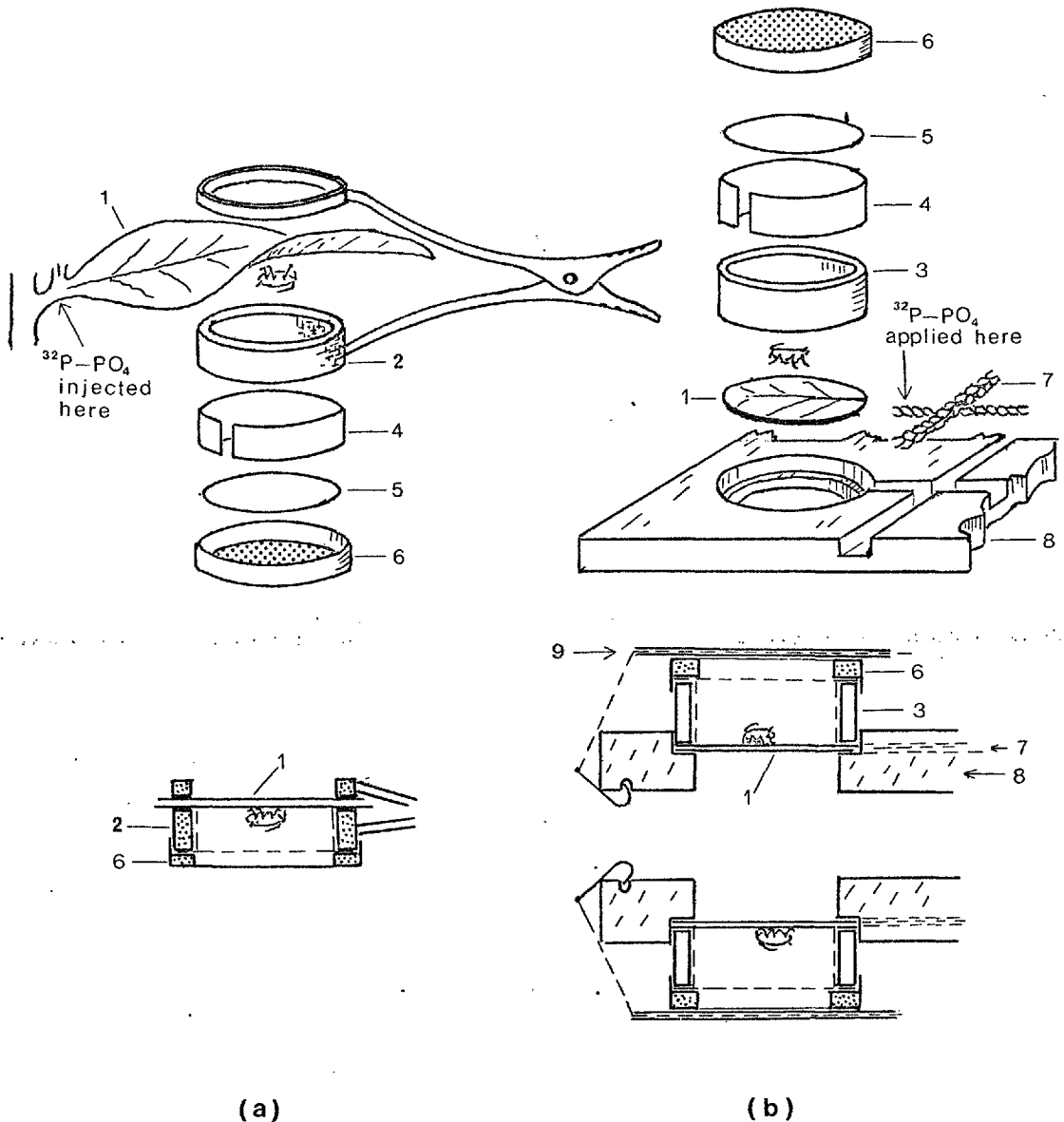


Fig. 1. 4. Exploded and cross-sectional diagrams of the aphid cages (a) on intact leaf and (b) on leaf-disc of broad bean plant, after application of ^{32}P -compound.

1. leaf, leaf-disc; 2. "perspex" cage attached to "crocodile" hair grip; 3. stainless steel cage; 4. strip and 5. disc of filter paper treated with bromocresolgreen; 6. celluloid retaining cap; 7. cotton wick for water supply to the leaf disc; 8. "perspex" tray; 9. rubber band through glass tube to keep the cages in position. (after D.J. Galley, personal communication, 1969).

stainless steel rings were used topped with a muslin cover and held in position with a rubber band. The perspex trays carrying the discs were kept inverted (Fig. 1.4b).

Honeydew produced by the captive aphids was collected on filter paper lining the inverted cages. To locate the spots of honeydew, the filter paper was previously dipped in a 2% bromocresolgreen ethanolic solution to which a few crystals of citric acid were added. The yellow colour of the dry bromocresolgreen-treated paper changes to blue in the places where the honeydew droplets are present.

The adults were confined to the labelled leaf-surfaces for up to 24 hrs. To minimize the errors caused by uneven distribution of label in different leaves of the plant, the species were alternated at the end of each 24 hrs. feeding period. That is, leaf areas on which A. pisum were caged initially were subsequently used for A. fabae and vice versa.

In experiments where the rate of loss of ^{32}P from labelled aphids was examined, 10 adults of each species were caged together on 4 contaminated leaf discs and after 4, 8, 12 and 24 hrs, 2 adults of each species were collected from each of the 4 labelled discs and caged separately, by species, on clean leaf-discs.

Counting techniques. Samples of adults, larvae and honeydew droplets were collected every 24 hrs. for counting. In cases where the experiment lasted more than 24 hrs. the counting of all the samples was carried out at the end of the experiment. Adults and larvae were killed by freezing immediately after collection. Adults and larvae were counted intact, without squashing, since ^{32}P is a strong β -emitter and easily penetrated the small barrier of the aphid tissue. The increase in efficiency gained by homogenizing the aphids was barely worthwhile. Where ^{32}P was injected into the petiole of the leaves 2.5 cm diameter discs were cut at the end of the experiment

from the places where the insects had been caged. These leaf-disc samples were cut in small pieces and homogenized in a glass, hand operated homogenizer in 2 ml of phosphate buffer solution 0.1 M, pH 7. After decanting, 0.2 ml portions of the homogenate were counted with a scintillation counter in 5 ml scintillation fluid (N.E. 240). The counts of the aphid samples collected from these treated leaves were then expressed in mg of plant material utilized and excreted by the insects.

Two counting procedures were used:

1. Liquid scintillation counting. A PANAX Equipment Ltd. scintillation head and associated PX equipment giving efficiency for ^{32}P of 88.3% was used. Dry samples were counted in 2 ml N.E. 213 (Nuclear Enterprise, Edinburgh) scintillation fluid. Aqueous homogenates were counted in 5 ml of N.E. 240.
2. Geiger-Müller counting. A thin end window G-M tube, Mullard MX 168, connected to an Isotope Development Ltd. 1700 Scaler was used. A range of voltages from 250 to 800 volts were tested and operating plateau was found to be 400 to 550 volts. The samples were counted at 400 V in the centre of a planchet at a distance 14 mm from the end window. At this distance and voltage the efficiency of the counter for the ^{32}P was 6.5%.

Counts were corrected for background and decay and were expressed in nCi per mg of plant material or per adult.

Honeydew Samples. The honeydew produced by radiolabelled insects was collected on bromocresolgreen-treated paper and the honeydew spots cut out, as described above, and counted in the centre of an aluminium planchet.

To investigate the extent, if any, to which filter paper prevented the ^{32}P β emission reaching the Geiger-Müller detector, the following tests were carried out:

(a) The same sample of labelled honeydew was counted after each of three different arrangements in the centre of a planchet. The counts obtained, 15906 ± 282 , 15764 ± 281 and 15589 ± 279 c.p.m., show that the reproducibility is good. This indicates that whatever the arrangement of the filter paper pieces, similar counts are obtained provided that the same counting conditions are maintained.

(b) The above sample was entirely covered with one, two or three large pieces of filter paper and the counts obtained were 15582 ± 279 , 15470 ± 278 and 15110 ± 275 c.p.m. respectively. These counts compared with 15589 ± 279 c.p.m. of the uncovered sample represent a fall in the counting efficiency of only 0.3% and show that the high energy β emission from ^{32}P can easily penetrate the sheets of filter paper covering the sample and provided the pile of filter paper pieces is not too deep, no serious loss in efficiency will occur.

This method of counting labelled honeydew was a time consuming procedure. A quicker counting technique was tested which avoided cutting out the individual honeydew spots. 10 μl of a dilute solution of ^{32}P -sodium phosphate containing 25 nCi were put on each of five filter paper discs 2.5 cm in diameter. When dry, they were counted with a Geiger-Müller counter to check their activity and 4 of them, after they were cut into small pieces, put separately in 2 ml of the following solutions:

Solution	(1)	50% aqueous acetone
"	(2)	50% " ethanol
"	(3)	0.03 M $\text{NaHPO}_4 \cdot 12\text{H}_2\text{O}$
"	(4)	1 M HCL

After 1 hour all the papers were taken from their solutions and immersed in 2 ml of fresh solvent. This procedure was repeated again after 1 hour. 0.2 ml aliquots of each extract were dried on a planchet and counted under a Geiger-Müller counter. All radioactivity

was found to have been extracted within the 1st hour by all the solutions tested. The results are given in the table 2.1.

Table 1.2. ^{32}P -compound extracted from filter paper, within the first hour.

Solution	counts per minute		
	In the measured sample (.2 mL)	In the whole sample	Counting efficiency
Control	-	1131.0	11.6%
sol. (1)	129.9	1299.0	13.3%
" (2)	116.1	1161.0	11.9%
" (3)	114.9	1149.0	11.8%
" (4)	134.4	1344.0	13.7%

As can be seen from this table, similar amounts were extracted in all the cases and compared with the unextracted sample the counting efficiency was slightly but not significantly increased. Accordingly this simple technique can be useful in routine experiments where a great number of labelled honeydew samples on filter paper have to be counted. There is a disadvantage with this elution technique, in that although it functioned satisfactorily for labelled phosphate, the extent to which the isotope in a leaf and in aphid honeydew is combined with larger less mobile molecules was unknown. The indications in preliminary experiments on the extraction of honeydew labelled with ^{32}P were that a small proportion was not leached from the filter paper. Thus, in order to keep counting conditions similar to earlier experiments and to obviate the risk of losing a proportion of the isotope, the labelled honeydew samples were counted, as has already been described, by cutting out the spots and positioning them in the centre of an aluminium planchet.

Results

1. Quantity of plant sap taken up and excreted by the adults during 24 hrs. feeding. The results of the experiments in which aphids were fed on leaves labelled by petiole injection are shown in Fig. 1.5. It can be seen that there was an uneven distribution of ^{32}P between the two leaves. The activity present in the leaves (a) and (b) was 1.2 and 7.0 nCi per mg of plant material respectively.

If it is assumed that the distribution of ^{32}P was uniform, A. pisum adults removed about 8 times their body weight of plant sap or 15.8 mg per adult of which 7.2 mg were retained in the adult and 8.6 mg excreted in the honeydew during the 24 hrs. of feeding. The quantity of plant sap taken up by A. fabae in the same period, 1.1 mg per adult, was nearly equal to the body weight. Half of this was found in the adult and half excreted through the honeydew.

Since last instar nymphs and early adults were used, no larviposition occurred during the experiment.

2. Relative quantity of ^{32}P taken up and excreted by the adults during 24 hrs. feeding on labelled plant tissue. The radioactive compound in these experiments was applied both by injection 7.1 μCi into the petiole of two leaves and in the water supply to leaf-discs at the rate of 3.6 μCi per disc. Adults were caged by species for a 24 hrs. period. In the whole leaf experiments one leaf was used per species and in the disc experiments three leaf-discs per species were used. Fresh insects of each species were caged on the other leaf or leaf-discs during the second 24 hrs. of the feeding period. Thus, both species were allowed to feed in turn at each feeding site. After 48 hrs. the adults, larvae and honeydew were counted separately under a Geiger-Müller counter. The results are shown in the Table 1.3 and are expressed graphically in Fig. 1.6.

Table 1.3. Removed ^{32}P from labelled leaf-tissues per adult and distribution to adults, larvae and honeydew during 24 hr. feeding (see Appendices 5 and 6).

Way of treatment with ^{32}P	Species	Total quantity removed (nCi)	Distribution %		
			Adult	Larvae	Honeydew
Injection	<u>A. pisum</u>	32.9	57.3	6.7	36.0
	<u>A. fabae</u>	19.2	47.8	21.8	30.4
Wick application	<u>A. pisum</u>	59.4	43.7	7.1	49.1
	<u>A. fabae</u>	49.6	39.8	13.8	46.4

Judging from the quantities removed from each feeding site by both species the levels of activity found in the two leaves in the injection experiment and in the 6 leaf-discs in the wick application experiment varied considerably.

The graph shows that:

1. The total quantity of ^{32}P taken up by the adults of A. fabae was less than that taken up by the adults of A. pisum in both applications. However A. fabae excreted a greater proportion of the activity removed from the plant, in the honeydew and larvae than A. pisum.
2. Both species excrete labelled material in their honeydew. 36.0 to 49.1% of the quantity taken up by A. pisum adults appeared in the honeydew and 30.4 to 46.4% appeared in A. fabae honeydew. In contrast, the quantity of ^{32}P excreted in the larvae laid during the feeding period was very low. In A. pisum, only 6.7 to 7.1% of the total consumed and in A. fabae 13.8 to 21.8% appeared in the larvae. Even so it is perhaps surprising that even these quantities of the isotope have found their way into the larvae in so short a time.

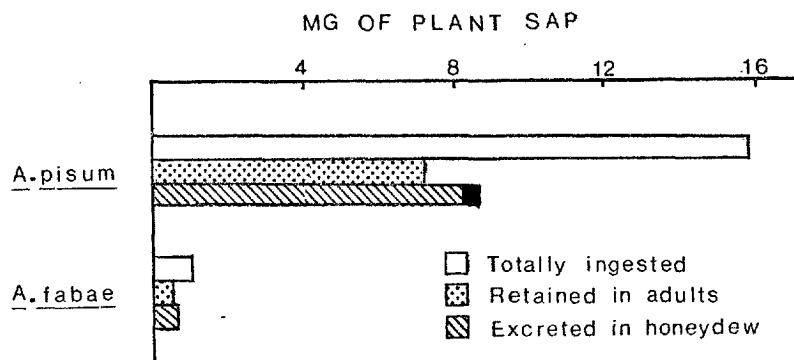


Fig. 1.5. Distribution of ingested plant material after 24 hrs. feeding, per adult (see Appendix 4).

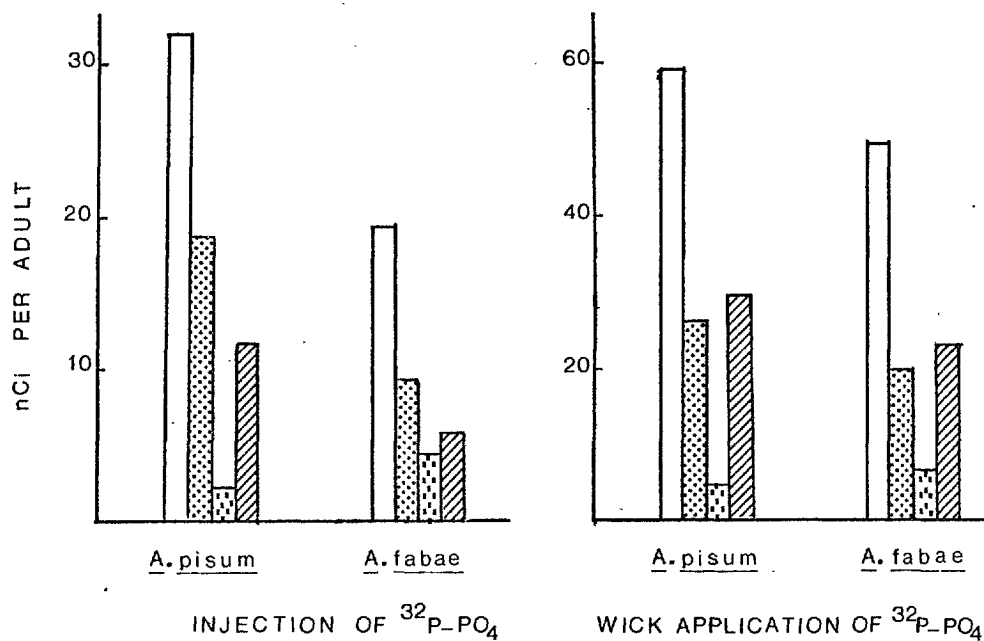


Fig. 1.6. Total quantity of ^{32}P removed from plant tissues during 24 hrs. feeding and its distribution in adults, larvae and honeydew (see Appendices 5 and 6).

totally ingested ^{32}P
 retained in adults
 ^{32}P passed to larvae
 excreted in honeydew

3. Uptake of ^{32}P with time and distribution in the larvae and honeydew. ^{32}P -phosphate was applied to the wicks at the rate of 1.7 μCi per leaf-disc. Adults were caged on the treated discs to feed for 4, 8, 12 and 24 hrs. After these periods the adults were transferred and caged separately by species on untreated leaf-discs. 24 hrs. later samples of adults and of the larvae and honeydew produced were collected and were counted with the Geiger-Müller counter. The untreated leaf discs were also checked for possible gross contamination arising from the aphids feeding. The results are shown in the Table 1.4 and the Fig 1.7.

Table 1.4. Distribution of ^{32}P 24 hours after feeding on labelled discs for different times. During the 24 hrs. the aphids fed on unlabelled discs (see Appendix 7).

Species	Time(hrs) spent on labelled leaf-discs	Total Quantity removed (nCi/adult)	Distribution %			
			Adults	Larvae	Honeydew	Leaf discs
<u>A. pisum</u>	4	3.9	66.7	5.1	25.6	<2.6
	8	8.0	65.0	5.0	28.7	<1.2
	12	10.8	71.3	12.0	15.7	<0.9
	24	19.0	73.1	14.7	11.6	<0.5
<u>A. fabae</u>	4	3.5	68.6	11.4	14.3	5.7
	8	2.5	52.0	16.0	24.0	8.0
	12	8.8	52.3	21.6	26.1	0.0
	24	13.9	66.9	23.0	7.9	<2.1

As was expected, the radioactivity found in the adults of the two species increased with the time of their feeding on the labelled plant tissues and, as with the previous experiments, the overall quantities of ^{32}P absorbed by A. pisum were greater than that absorbed by A. fabae at each time period.

A high portion of the quantity of ^{32}P absorbed was still in the

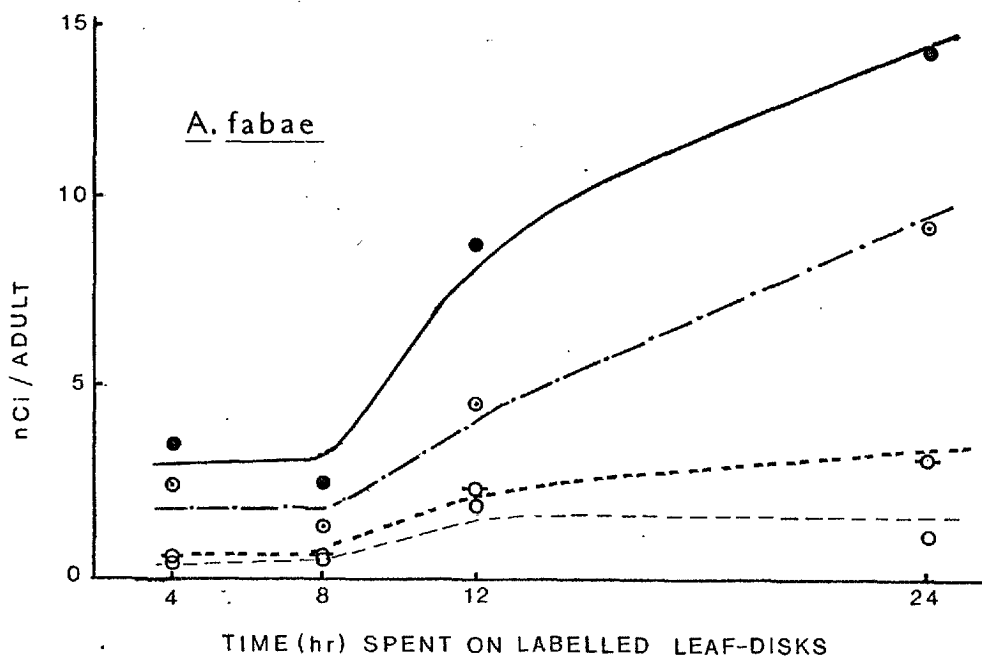
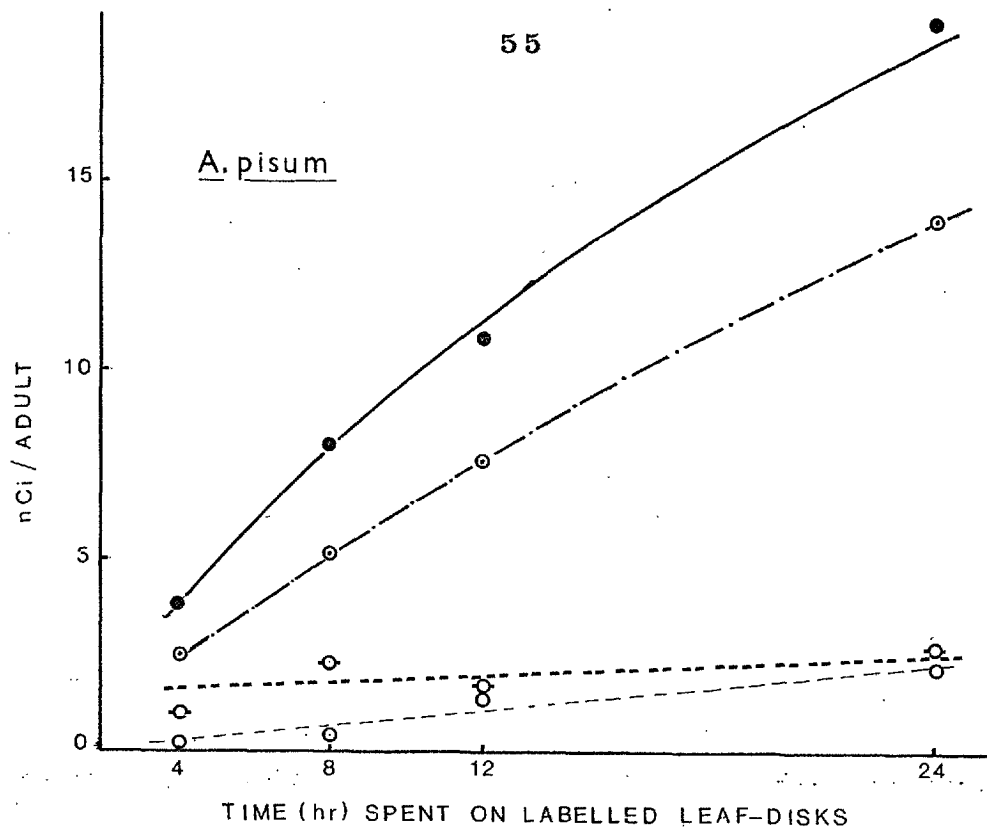


Fig. 1. 7. Distribution of ^{32}P 24 hours after feeding on labelled discs for different times. During the 24 hrs. the aphids fed on unlabelled discs (see Appendix 7)

●—● ^{32}P totally taken up ○—○ ^{32}P passed to larvae
 ○—○ retained in adults ○—○ excreted in honeydew

adult's body at the end of the 24 hrs. period during which the insects were feeding on untreated leaf discs. This portion ranged from 65 to 73% in A. pisum and 52 to 68.6% in A. fabae.

The loss of the radiolabel from the adults during their 24 hrs. feeding on clean leaf-discs was almost entirely through the honeydew and larvae in both species. Insignificant traces were injected by the labelled adults into the plant tissue. More ^{32}P was excreted in the honeydew in both species fed for up to 12 hrs. on labelled plants, but after feeding for 24 hrs., loss through the larvae predominated, especially in A. fabae. The loss of ^{32}P in the larvae increased with the time spent feeding on the labelled discs. Again the portion of the ^{32}P loss in the larvae was higher in A. fabae than in A. pisum, 5.1 to 14.7% in A. pisum and 11.4 to 23.0% in A. fabae of the total consumed. Although the amount of radiolabel was higher per larva of A. pisum than of A. fabae (0.43 and 0.24 nCi/larva respectively), A. fabae produced more larvae than A. pisum adults in 24 hrs.

Discussion

The calculations were made on the assumption that ^{32}P is uniformly distributed in the tissues of each leaf or leaf-disc and that it is equally available to the aphids at all times. This is not necessarily true but the aphids feed at similar sites in or close to the phloem. Thus, the disparity between the two species' diets will probably be small and as these experiments were conducted to investigate relative differences in uptake rather than to evaluate an absolute feeding rate, any minor variations must be accepted.

Differences in uptake between individuals have been found even when they were fed on the same radioactive surfaces and for the same time (Watson and Nixon, 1953) and these authors used groups of 8 to 10 aphids for each experiment. Similarly in these experiments individual feeding rates were not considered but only mean rates of groups

of 8 to 40 adults.

The levels of radioactivity were not the same in all the leaves or leaf-discs of each experiment. A large difference appeared between the leaves when the labelled phosphate was injected into the petiole and there was often very much more radioactivity in one leaf than in the other. This almost certainly arises from the position of the label in the petiole after injection. The vascular tissue adjacent to the label will remove greater quantities than those at a greater distance. The leaf to which these vascular bundles run will then become labelled to a greater extent. In the case of systemic application of the radioactive compound through the wicks, only slight differences between the discs were observed. Because of these differences the relative rates of ingestion and excretion of radioactive compound by the two species were estimated after allowing the adults of each species to feed on all the treated plant tissues.

The quantities of plant material ingested were estimated from the radioactivity found in the adults and honeydew samples related to the radioactivity levels in the leaves where the adults of each species were confined. The radioactivity in the adults and honeydew of A. pisum was higher than in A. fabae, indicating that the former species removed more plant sap than the latter. If it is assumed that ^{32}P distribution is uniform, then the mean quantity of plant sap absorbed by the adults of A. pisum was 0.66 mg per aphid per hour, corresponding to about 33% of the body weight, while that absorbed by A. fabae was 0.045 mg/aphid/hr, which is equal to about 4.5% of the body weight. It must be mentioned here that A. fabae adults were fed on tissue with a ^{32}P concentration 5.8 times higher than that in the leaf on which A. pisum were fed and it is not known if this high concentration of the radioisotope had any influence on the feeding response of this species. Any radiation effect is extremely unlikely however, as the dose rate from the small

activities of isotope used were far below the levels at which radiation syndromes begin to appear in insects. Both species excreted nearly half of the ingested material through their honeydew. No loss of the radioactive compound occurred in larvae, since only early adults were used. Where a few last-instar nymphs were included a diminution of feeding activity will have occurred at the moult but no activity was lost with the excuviae, an observation also recorded by Auclair (1959). Banks and Nixon (1959) found that the maximum rate of feeding for A. fabae reared in an open air insectary without controlled environmental conditions was 0.2 mg sap/aphid/hr. and Watson and Nixon (1953) found the adults of M. persicae took 0.042 mg sap/aphid/hr. which is about equal to 10% of the body weight of this species.

In experiments of relative uptake and excretion of ^{32}P by the two species, it was found that the adults of A. fabae ingested 16.5 to 41.5% less radioisotope than ingested by A. pisum. Of this quantity A. fabae adults excrete more than A. pisum and retain in their body 7.3 to 9.7% less than the A. pisum adults. Data on rates of sap intake for several aphid species are summarized by Auclair (1963). These data show that differences of considerable magnitude (up to almost 100 times) occur between aphids, differences which are also reflected in the rates of excretion.

Both species lost most of the radioactivity with their honeydew. A. fabae adults excreted more radioisotope in their larvae than A. pisum. Though the larvae of A. fabae contained less label, possibly a reflection on the lower rate of absorption by their mothers, the higher reproductive rate, up to three times than of A. pisum, over the period of the experiment, allowed a greater proportion of isotope to be excreted in the larvae.

It is possible that part of the ^{32}P in the young larvae was taken directly from the labelled leaf surfaces on which they were born.

However, it appears that ^{32}P was taken up mostly from their mothers body than from the labelled plant tissues. Thus, where larvae of A. pisum were laid on labelled leaves or leaf-discs they contained 6.7 to 7.1% of the totally ingested ^{32}P by the adults, and 13.8 to 21.8% was found in larvae of A. fabae. Where larvae of the two species were laid by labelled adults on unlabelled leaf-discs, they contained in A. pisum 14.7% and in A. fabae 23.0% of the total ^{32}P ingested by the adults. The above data strongly indicate that the greater proportion of the isotope found in the larvae was taken, before birth, from the mother and that insignificant feeding occurred over the period of the experiment.

The radioactivity found in the adults of both species increased with the time spent on labelled leaf-discs. With A. fabae this increase started only after 8 hrs. feeding. Watson and Nixon (1953) also found differences in time, in the activity of adult M. persicae fed on radioactive turnip and sugar-beet plants. These authors found the mean rate of uptake to be 10 μg of plant sap per hour in the first hour of feeding, 40 $\mu\text{g/hr.}$ between one and four hours feeding and 17 $\mu\text{g/hr.}$ between 6 and 24 hrs. This decrease with the longer feeding times is attributed, according to the above authors, to loss of ^{32}P in nymphs born during the feeding period. Auclair (1963) in his review on aphid feeding, states that it took from 2 to 3 hours for the majority of aphids (82 to 97%) to become radioactive, presuming that it took them this time to reach the phloem.

After removing the aphids from the labelled leaf-discs the ^{32}P level in their bodies declined slowly. Even when labelling occurred for only 4 hours considerable proportions of the ^{32}P taken up was still found in the adult body 24 hrs. later. The proportions in each species were similar, A. pisum retaining 65.0 to 73.1% and A. fabae 52.0 to 68.6% of the ingested quantity.

During the 24 hrs. following the uptake of ^{32}P , the distribution of the label between the larvae and honeydew varies slightly between the species. In A. pisum the levels of ^{32}P in the honeydew declined slightly with increased time spent feeding on the labelled plant, more being passed out in the larvae. In A. fabae the loss via the larvae was also increased from 11.4 to 23.0% of the total absorbed with increasing time spent feeding on the labelled plant. The increasing loss via the larvae in A. pisum was mainly due to the increased number of larvae produced by this species, whereas in A. fabae it was the result of increasing quantities of ^{32}P per larva. The pattern of gradual excretion of ^{32}P by the two species via the honeydew and larvae is to be expected with an element so active biologically as phosphorus, which takes part in many reactions especially those relating to energy transfer processes. These processes are in a state of dynamic balance, in which individual ions are continually gained and lost. Labelled material added to the system will slowly be depleted as it is replaced by fresh unlabelled material. One possible reason for the rapid and extensive uptake of ^{32}P by the embryos is that here in the developing tissues phosphorus forms an essential part of the DNA molecule. Auclair (1958) found that apterous adults of A. pisum fed on P. sativum excreted 1.21 μl of honeydew per aphid per hour, while apterous reproducing adults of the same species excreted 0.81 μl /aphid/hr. In the case of the reproducing aphids a part of the material which would otherwise have been excreted was apparently absorbed by the larvae.

The relative fecundity of the two species found in these experiments did not agree with that found in the comparative larviposition experiments as has been referred above (p. 38), where during a productive period of 20 days the number of larvae born to A. pisum adults was greater than to A. fabae ones. The presence of the added quantities of phosphate in the adults body could be a reason for the

above difference. A more likely explanation however lies in the short duration of the radiotracer experiments and that some of the adults could have been at different stages of their larviposition period.

Relative retention of the label by the adults and its distribution in developing larvae fall rather beyond the scope of this present study. More detailed experiments could show if rates of incorporation of ^{32}P into the larvae varied with stage of development and also to what extent incorporated label was lost by the larvae in the honeydew when they commenced feeding.

A small portion of the ^{32}P absorbed by the adults was re-injected into clean leaf-discs, 0.5 to 2.6% in A. pisum and 0 to 8.0% in A. fabae. However part of this contamination of the clean leaf discs could have been caused by honeydew which failed to fall onto the filter paper cage lining, or occurred during aphid handling.

When a toxicant is distributed in the plant it is unlikely that the rates of uptake by the two species will be exactly similar to those obtained in this study with ^{32}P . However these results do provide information on the initial ability of the two species to ingest plant sap and of its subsequent distribution. The presence of a toxicant is known to alter the feeding rates in M. persicae (Parry and Ford, 1967, 1969, 1971; Javadi, 1971). This effect found also to occur in A. pisum and A. fabae, and is referred to later in this work, where insecticides were offered to the aphids in artificial diets. The same effect occurs when poisons are incorporated in plant tissue (Halimie and Ford, 1972).

It is not known whether an organophosphorus insecticide in the aphid is distributed similarly to ^{32}P -phosphate. However these results do indicate the relative ease with which a phosphorus compound can be distributed to the honeydew and larvae.

SECTION C. UPTAKE STUDIES ON ARTIFICIAL DIET.

Introduction

Artificial Feeding.

The main disadvantage in assessing aphid uptake from radio-labelled plants is the uncertainty of the concentration of the label at the feeding sites. Successful artificial feeding overcomes this problem and also provides the means for numerous investigations on the influence of nutritional and other factors on the physiology of aphids.

The artificial feeding of aphids has progressed considerably since the first attempt by Hamilton (1935) to rear M. persicae artificially for short periods through the epidermis of cabbage leaves instead of the animal membranes used previously. Mittler and Dadd in 1962 reported what appears to be the first limited but significant success in the artificial feeding of an aphid (M. persicae) on a chemically defined diet. The authors used a membrane of stretched Parafilm "M" retaining an aqueous diet of 18% sucrose and small amounts of vitamins, cholesterol, mineral salts and aminoacids and amides. The rearing of a limited number of aphid species on artificial solutions has been attempted by many investigators with varying degrees of success. However the maintenance of permanent cultures of M. persicae and A. fabae on chemically defined diets now seems possible (Dadd and Mittler, 1966, Dadd and Krieger, 1967).

Sterile preparations of chemically defined diets have been achieved by filtration, and membranes sterilized by U.V. irradiation or ethanol washing (Auclair and Cartier, 1963; Mittler and Dadd, 1964a; Auclair, 1965, 1967a).

Many of the special feeding requirements of the aphids have been studied, especially the roles played by carbohydrates, amino-

acids, vitamins, salts, trace metals, lipids and cholesterol, in survival, growth, reproduction and behavioural responses (Mittler and Dadd, 1962, 1965; Auclair and Cartier, 1963; Auclair, 1965; Mittler, 1967a, 1967b, 1970; Danneel, 1969b; Srivastava, 1971). Of the carbohydrates studied, sucrose seems to be an essential nutrient as well as a phagostimulant (Srivastava, 1971a). Little sucrose was excreted in the honeydew of aphids fed on diets containing this sugar, thus aphids are able to efficiently absorb and metabolise sucrose (Dadd and Mittler, 1965a, 1965b). Also in diets where sucrose was replaced in part or totally by any one of the following sugars; cellobiose, fructose, galactose, glucose, lactose, melezitose, raffinose, ribose, sorbose or trehalose, the survival Aphis gossypii Glover adults was drastically reduced (Auclair, 1967b).

The sucrose requirement varies in the different species, whether they are reared for several generations on more complex diets, or for several days on a simple sucrose solution. Optimum sucrose concentrations in complex diets were found to be between 10-20% for M. persicae (Mittler and Dadd, 1962, 1965; Mittler, 1967a, 1967b), 10-35% for A. pisum (Auclair and Cartier, 1963; Auclair, 1965; Srivastava, 1971, 1971a), 10-15% for A. fabae (Dadd and Krieger, 1967; Emden, 1967), and 20-30% for A. gossypii (Auclair, 1967a, 1967b). In plain sucrose diets the optimum concentration for M. persicae was still in the 10 to 20% range (Mittler and Dadd, 1962, 1965; Mittler and Pannell, 1964; Parry and Ford, 1967, 1969), whereas with A. fabae stronger sucrose solutions up to 18% were better (Danneel, 1969b).

Different factors producing optimum conditions for maximum food uptake by aphids have also been studied.

The pH of the diet has been found to influence aphid growth, reproduction and survival, as well as the selection of diets by

aphids (Auclair, 1965, 1967a; Cartier, 1968). A. pisum, A. gossypii and Macrosiphum euphorbiae (Thomas) performed better on slightly alkaline diets (pH 7.3 to 7.6) and A. pisum does not accept diets with pHs less than 7 or more than 8. A. gossypii appears to tolerate a wider range of dietary pH than A. pisum, and will grow and reproduce fairly well on diets of pH ranging from 6.5 to 8.0.

It appears that on chemically defined diets the quantity and quality of light may influence the settling and feeding responses of aphids and in turn the rates of growth and reproduction. Intense white illumination of 600 foot candles deters feeding (Auclair, 1967b). A. pisum and A. gossypii generally grew, survived and reproduced better on diets receiving 90 to 330 ft-c. than on those receiving 405 to 900 ft-c. (Cartier, 1965). Very weak illumination or darkness have little effect on feeding. A. gossypii preferentially settled on and colonized diets receiving non or only a weak light of 5 to 50 ft-c. (Auclair, 1967a, 1967b). A. pisum, A. gossypii and M. euphorbiae preferred diets lit with orange or yellow light than diets lit with white, red or blue or in darkness (Cartier, 1965, 1966; Auclair, 1967b; Srivastava, 1971). The wavelengths 610 to 570 nm, the red to yellow portion of the visible spectrum, apparently exerted a definite attraction, whereas the blue to violet end, 485 to 420 nm had a repellent effect. The intermediate green area of the spectral range had little influence on the feeding behaviour of aphids. Slight variations from these preferences may occur among the three aphid species or between biotypes within one species.

The relative humidity in which the cages with artificial diet were kept is another factor which may influence the feeding behaviour of aphids. The available data refer to a few aphid species fed artificially in quite different relative humidity conditions. A. pisum has been fed on full diets in "high" (possibly about 100%) r.h. (Auclair and Cartier

1963; Auclair, 1965) and in about 50% (Srivastava, 1971). A. gossypii was fed also in a r.h. about 50% (Auclair, 1967a), while A. fabae in only 17 to 32% (Danneel, 1969a). M. persicae, mainly fed on a simple sucrose solution (Javadi, 1971), was kept at about 100%, 50% or in "very low" relative humidity. No conclusive evidence of any effect of relative humidity on feeding behaviour can be drawn from the literature.

It has been postulated that the turgor pressure within plant cells contributes significantly to the normal rate of sap intake by aphids, since it was shown by Kennedy and Mittler (1953) that when the stylets of feeding willow aphids, T. salignus, were severed, sap exuded from the stylet stumps left in the plant. That aphids however can imbibe liquids that are not under pressure has been amply demonstrated during the past few years from studies on artificial feeding (Auclair, 1969). However van Emden (1967) and Wearing (1968) reported slight increases in the longevity of adults of A. fabae, Brevicoryne brassicae(L) and M. persicae, when they were fed on sucrose solutions at pressures of about 2 atm. And van Emden (1967) suggested that the lack of pressure could limit the uptake by aphids even on nutritionally adequate diets and might explain why some nutrients have to be incorporated into these diets at unexpectedly high levels to sustain aphid growth. It is perhaps worth mentioning here that in the present work during the process of sealing of the small quantities of sucrose solutions between two sheets of parafilm membrane, a substantial pressure was applied on the diet which permitted the diet to exude freely when tiny holes were made in the membrane even 24 hrs. later. No doubt, a pressure on the diet existed in artificial feeding experiments carried out by other workers and was applied inevitably during the process of sealing the sachets and fixing them on the feeding cages. This is an area in which further study could clarify any beneficial influence that pressure may exert on feeding.

Evaluation of Feeding tests.

The total quantity of any food taken up by aphids, whether plant sap or artificial diet, is represented by a gain in weight, an amount of honeydew excreted, evaporation of water, production of young larvae in the case of reproductive adults and excuviae during ecdysis in the case of nymphs. All these fractions must be measured quantitatively in order to obtain true values of the amount of food taken in.

Different methods have been used so far to estimate the feeding rate and excretion of aphids fed on artificial diets. Dyes or radio-tracers have been incorporated into diets as indicators of feeding. For example, neutral red dye (0.1%) has been used and after feeding each aphid subsequently examined under a binocular microscope. Each was allocated a colour grade of 0, $\frac{1}{2}$, 1, 2 or 3 and colour scores and percentage inhibition values were calculated (Mittler, 1964b; Parry and Ford, 1967, 1969). The dye used however, was insufficient to show small differences in feeding, also it had an adverse effect on liquid uptake by M. persicae (Mittler and Dadd, 1963b; Parry and Ford, 1967, 1969).

Radioactive isotopes have also been used as an accurate method to measure the quantity of artificial diet taken up. Hamilton (1935) worked with polonium in an attempt to show that M. persicae was capable of transmitting virus particles from plant to plant. In more recent studies (Parry and Ford, 1967, 1969, 1971; Danneel, 1969a, 1969b; Javadi, 1971) compounds containing a radioisotope of phosphorus were used as indicators. Known amounts of ^{32}P -phosphate were incorporated into artificial diets and after different feeding times samples of adults, honeydew and larvae were counted to find the quantities taken in and excreted and thus the corresponding volume of the artificial diet consumed. The use of radiotracers is of great

value, when quantitative estimations on the movement of the diet is attempted within short periods of time.

Since honeydew production is correlated with the feeding rate of aphids, the number of droplets (frequency) or the volume (rate of excretion) of the honeydew produced by a known number of aphids in a certain period of time may be used to measure, though not very accurately the rate of feeding. In some cases, samples of honeydew have been analysed to determine the constituents remaining, which from a knowledge of the levels present in the diet has provided information on the utilization of different compounds incorporated in the diet (Maltais, 1957; Auclair and Cartier, 1963; Auclair, 1967a).

When an artificial feeding experiment has lasted several days, periodical weighing of adults and/or progeny, individually or in groups has been carried out to estimate the rate of feeding by differences in weight (Auclair, 1967a, 1967b; Srivastava, 1971).

In long term experiments or in experiments with insecticidal diets, the mortality or longevity of adults was assessed (Mittler and Pannell, 1964; Parry and Ford, 1969; Srivastava 1971; Javadi, 1971).

A rather indirect method was the weighing of sachets with artificial diet immediately before and after a known number of aphids had fed on them for a known period of time. Differences in weight gave the amount of diet ingested (Auclair, 1965; Mittler, 1970; Srivastava, 1971a).

Effect of insecticides on the feeding behaviour of aphids.

Treatment of plants with insecticides can change the plant-insect relationship by the interaction of a number of different factors such as various sublethal effects, change in food acceptability and changes in the physical properties of the treated area. Similarly the behaviour of different insect species to the plant changes. In the case of aphids a number of characteristic examples have been reported. A repellent action of BHC to M. persicae has been observed which could affect

virus spread by this species (Chang, 1949). An increase in the incidence of potato virus Y in potato fields sprayed with insecticide has been reported by Chapman (1951), McEwen (1953) and Roland (1953), and was presumed to be due to both increased wandering and frequency of probing by aphids. The introduction of systemic insecticides has in many cases failed to prevent the spread of certain viruses which may even increase after spraying with schradan and with the contract insecticide parathion (Münster and Murbach, 1952).

Very little is known about the effects of systemic insecticides on aphid feeding behaviour and a great deal of clarification is necessary. Many of the variables difficult to quantify in plants can be standardised to a large extent with the artificial feeding technique. A variety of studies have been carried out on the effects of different non nutrient additives present in artificial diets. For example, 0.1% neutral red in the diet has been found to influence the feeding behaviour of M. persicae with varying degrees of repellence or toxicity to different strains of this species (Mittler and Dadd, 1963a, 1963b; Parry and Ford, 1967, 1969). In the presence^{ce} of phosphamidon, the number of salivary plugs produced on membranes is significantly higher than that for diets without insecticide, showing that more probing has been carried out by the aphids on those membranes containing toxic diet (Mittler and Pannell, 1964; Parry and Ford, 1967, 1969, 1971). Thus, artificial feeding techniques make it possible to study the effect on aphids of toxic material ingested in known form and quantity.

Although there are many reports of work on the nutrition of aphids, particularly of the two species M. persicae and A. pisum using artificial feeding techniques, there are only a few using these techniques to study the influence of systemic insecticides on feeding behaviour. The investigations reported so far refer to M. persicae and the influence of phosphamidon or dimethoate on the feeding behaviour of this species

on artificial diets (Mittler and Pannell, 1964; Parry and Ford, 1967, 1969, 1971; Javadi 1971).

In the present work simple artificial diets of different sucrose solutions were tested on both species. Comparisons of these diets were also carried out with a complex diet. Different experimental conditions such as lighting and relative humidity, which can affect the feeding behaviour of aphids on a sucrose solution were studied.

Toxicological aspects of the effects of dimethoate and phorate incorporated in the diet of the two aphid species were also investigated.

In these studies the longevity of the insects was considered and uptake of sucrose solution with or without dimethoate was assessed radiometrically.

Materials and Methods

The Artificial membrane. Parafilm "M" membrane was used first by Mittler and Dadd (1962) for the artificial feeding of aphids and has since been adopted by many other workers. A commercially available parafilm membrane "M" in sheets made by "Marathon" American Can Co. (Menasha, Wisconsin), consisting of an homogeneous blend of rubber and wax, was also used in this work. Small squares, 5 x 5 cm, of this membrane were stretched by hand in two directions at right angles to each other, but not up to their breaking point. In this stage, the membrane was stable for long periods and not permeable to water. In experiments where the influence of stretched and unstretched membranes was examined, unstretched ones were also used. The membrane, because of its structure, does not allow the feeding liquid to come out on withdrawal of the stylets by the aphids and it is self-sealing on to perspex or stainless steel feeding cages. It appears to be a fair substitute for the plant epidermis and there are no indications

so far of any interference with the feeding response of the aphids.

Feeding Solutions. The objective of this work was to find a simple but adequate artificial diet. A series of sucrose solutions were tested, diluted in boiled deionized distilled water. A complex diet, as described by Dadd and Mittler (1966), was also tested. These authors used this diet to rear aphid of M. persicae for 30 successive generations. The same diet was also used by Dadd and Krieger (1967) for the rearing of A. fabae for 25 generations. It consisted of 20 aminoacids, 15% sucrose, vitamins, mineral salts and the whole diet was adjusted to pH7. A 15% W/V sucrose solution was finally chosen as a feeding liquid for both species. This solution was kept in the deep freeze and was renewed every 10 days. A volume of 0.2 ml was employed per feeding sachet.

Feeding cages. Stainless steel or perspex rings 2.5 cm diam. x 1 cm long were used as feeding cages. A stretched sheet of parafilm membrane was attached to the ring by pressing gently with the fingers and 0.2 ml of the diet delivered from a disposable syringe in the centre. The drop was covered by a second membrane and a mouth operated suction device used to lower the level of the first membrane and drop, thus avoiding leakage of the liquid while the second membrane was fixed in place. In early experiments a wire thread was placed by the droplet to allow the egress of air from the space between the two films, while the covering membrane was fixed over the drop (Fosbrooke,^{I.H.M} 1971, personal communication). The use of the wire thread was omitted in later experiments, since the air could be removed easily by keeping the upper sheet in a horizontal position and gradually pressing onto the drop of diet while reducing the pressure below, followed by gentle release of the reduced pressure to exclude all the air between the two sheets whereupon the upper film was fixed by pressing the edges of the parafilm on the walls of the ring (Fig. 1.8).

Insect material. Apterous adult virginoparae of the same age were used in these experiments. No starvation or anaesthetization of the adults preceded their caging under the feeding sachets and depending on the objective of each experiment, the adults of each species were caged separately, or with both species in the same feeding cage. 2 to 6 replicates and 5 to 15 adults per replicate (cage) were used in the different experiments.

Insecticides. A series of concentrations of the systemic insecticides dimethoate and phorate were prepared usually immediately before they were offered to the aphids. Stock solutions of 0.02% of dimethoate or phorate in deionized distilled water or 2-ethoxyethanol respectively were used. Small volumes of these solutions were added from a Hamilton microsyringe to 2 ml 15% w/v sucrose diet to make in a series of concentrations up to 16 ppm with dimethoate and up to 8 ppm with phorate. Additional quantities of solvent (distilled water or 2-ethoxyethanol) were added to each insecticide dilution and to the control, so that the amount of solvent present was the same in each treatment.

Radioactive solutions. For an accurate estimation of the amount of diet taken up, a ^{32}P -sodium phosphate solution (1 mCi/ml, with 3 mg PO_4 /ml) supplied by the Radiochemical Centre, Amersham, U.K. (PBS 1), was incorporated in the liquid fed to the aphids. 1 or 5 μl of the radioactive compound was added by a Drummond Microcap capillary (Shandon Co.) to the 0.2 ml drop of the poisoned or insecticide-free sucrose solutions, before the upper sheet of the artificial membrane was fixed in place.

Experimental conditions. All these experiments were carried out in the same conditions in which the aphid cultures were kept except where the influences of lighting and humidity were being studied.

Results

1. Comparison of different artificial diets.

Sucrose solutions up to 40% w/v and the complex diet were offered to the adults of the two species. Ten adults were caged separately by species in two replicates for each diet. The acceptability of the different solutions by the two species was studied in terms of longevity of the adults. The living adults were counted daily for 5 days. As the Fig. 1.9 shows, of all the liquids offered those least acceptable to the aphids were deionized distilled water and 5% sucrose. In these tests the complex diet was no better than the sucrose 10-40% solutions. The longevity of A. pisum adults was higher in 10% and 20% sucrose and for A. fabae adults 10% and 40% sucrose, while this species also accepted sufficient of the 20% sucrose concentration. Adults of A. pisum failed to survive for more than 2 days on the high sucrose concentration of 40%. With the exception of A. fabae feeding on distilled water all the solutions tested succeeded in keeping a high number of both species of adults alive for 24 hrs. Observations indicated that all the liquids tested were more acceptable to A. fabae than to A. pisum.

When 10, 15 and 20% sucrose solutions were offered to the adults in preliminary experiments both species survived better on the 15% solution. In a comparison of the acceptability of a 15% sucrose and the complex diet, the adults of both species survived longer on the sucrose solution (Fig. 1.10).

From the series of experiments with 15% sucrose solution in which ^{32}P -phosphate was incorporated, it may be seen from Fig. 1.11 that the quantity of the diet taken up differed in the two species. A. fabae took more than A. pisum. Already within the 1st 12 hours of feeding the ingested quantity of the diet in the former species

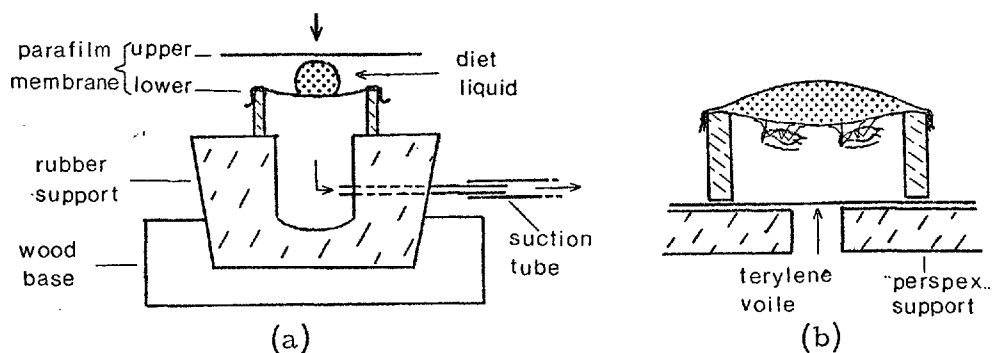


Fig.1.8 Cross sectional diagrams showing (a) the preparation of a feeding cage and (b) a cage during feeding.

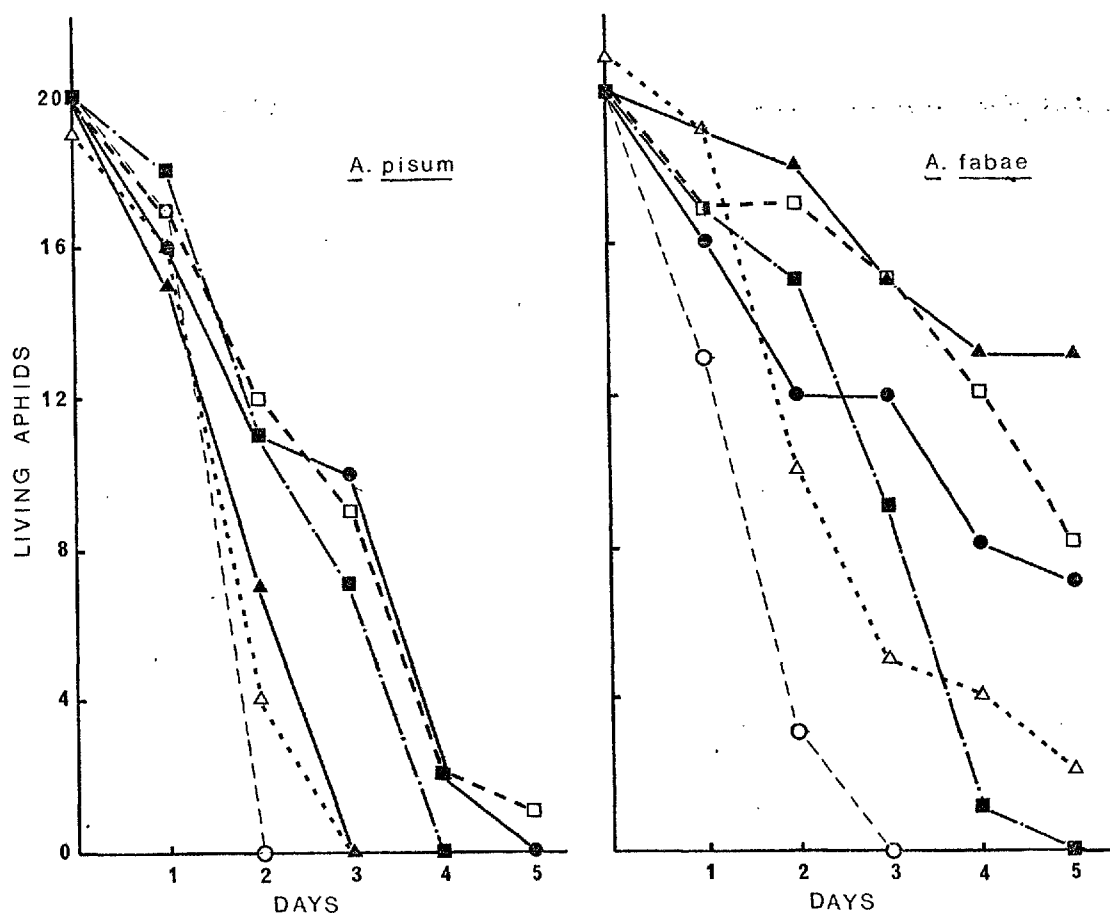


Fig.1.9 Longevity of adults of the two species fed with different artificial diets, through parafilm membrane (see Appendix 8).

○-○ distilled water
 △-△ 5% sucrose soln.
 □-□ 10% sucrose soln.

●-● 20% sucrose soln.
 ▲-▲ 40% sucrose soln.
 ■-■ complex diet.

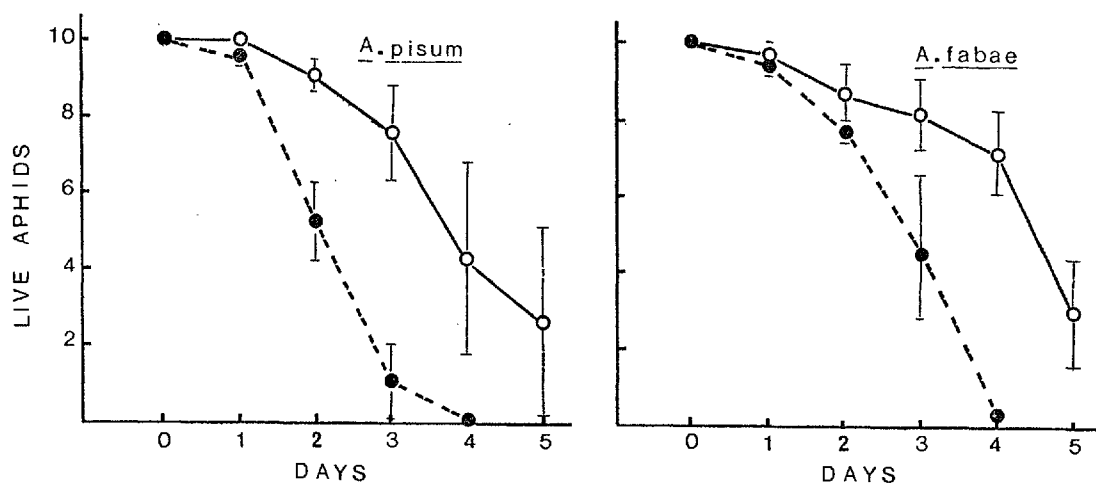


Fig. 1.10. Longevity of adults of the two species fed on 15% sucrose solution or with the complex diet through parafilm membrane (see Appendix 9)

○—○ 15% sucrose sol.

●---● complex diet

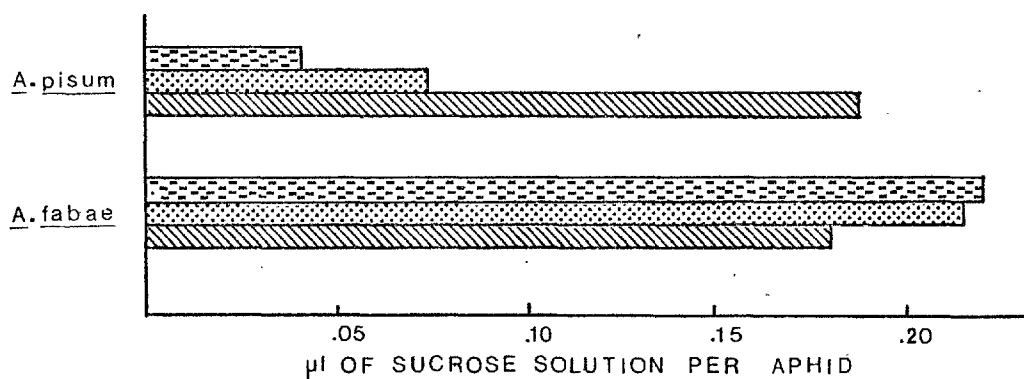


Fig. 1.11. Quantity of 15% sucrose solution taken up at different times by the adults of the two species feeding through parafilm membrane (see Appendix 13)

▨ 12 hr.

▤ 18 hr.

▩ 24 hr. feeding.

was 0.220 μ l per adult and this quantity remained at the same level when adults were checked after 18 and 24 hrs feeding. A. pisum took very little, 0.04 μ l per adult, in the first 12 hours, which increased only to 0.074 μ l/adult in 18 hrs and after 24 hrs feeding the ingested quantity of diet per adult was 0.187 μ l.

The time during which sucrose solutions have been kept in the sachets before being offered to the adults also seems to be important for A. pisum. The quantity of sucrose solution taken up was measured radiometrically and it was found that when the diet was fresh and after it was kept for 24 hrs in the sachets, the ingested quantity per adult in 24 hours was respectively:

in A. pisum 0.420 μ l and 0.040 μ l (mean of 10 and 20 adults)

in A. fabae 0.216 μ l and 0.154 μ l (mean of 20 and 22 adults)

2. Some factors affecting the feeding response of the two species.

The thickness of the parafilm membrane, relative humidity and lighting conditions were studied with respect to their influence on the feeding behaviour of the two species. Throughout these experiments 15% sucrose solution was used and the standard test conditions as described above were established. The acceptability of the diet was estimated in terms of longevity of the adults.

Thickness of the feeding membrane. The diet was offered to the adults through unstretched and stretched parafilm membranes. As the Fig. 1.12 shows, no difference in the feeding response of the two species was observed.

Relative humidity. Three r.h. conditions were established:

One about 100%, one about 0% and an intermediate value between 40 to 50% which was the uncontrolled r.h. of the insect-rearing room. The dry test was carried out with the feeding cages and the adults in a desiccator with silica gel and the humid tests in a wet desiccator. The insects were removed from the extreme r. humidities for only

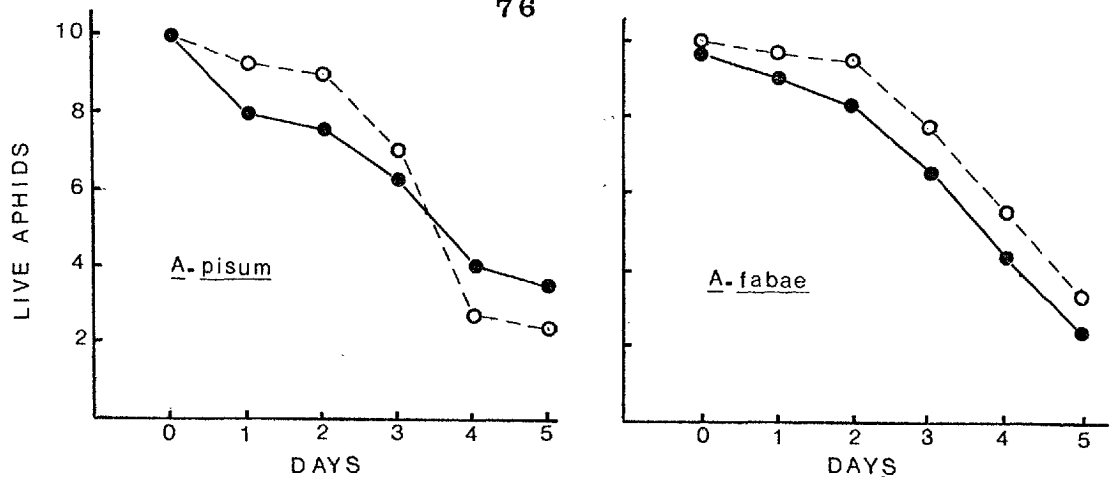


Fig. 1.12. Longevity of adults, fed on 15% sucrose solution through stretched or unstretched membrane (see Appendix 10).

○ stretched, ● unstretched membrane.

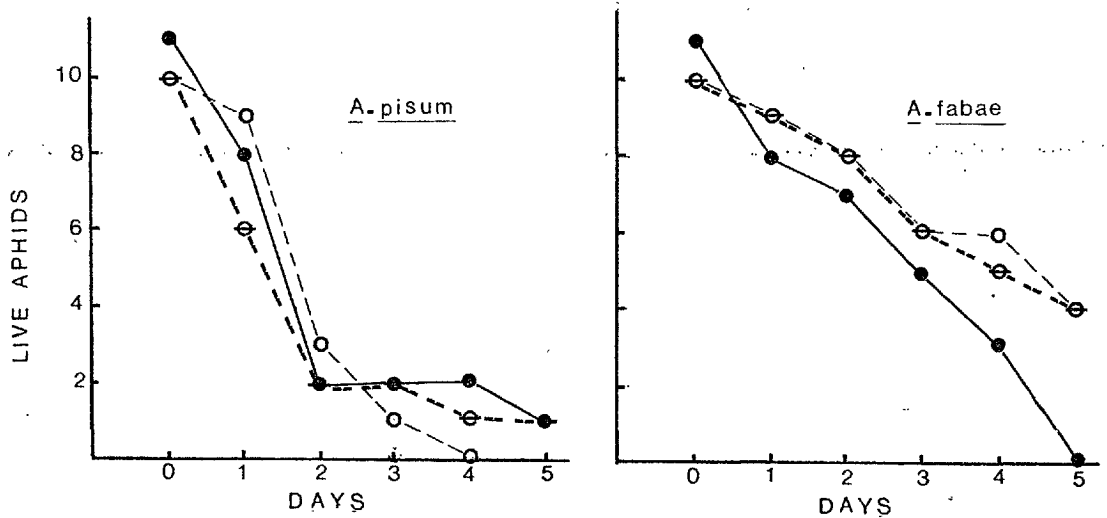


Fig. 1.13. Longevity of adults, fed on 15% sucrose solution in three different relative humidities (see Appendix 11)

○ 0%, ◐ 40-50%, ● 100% relative humidity.

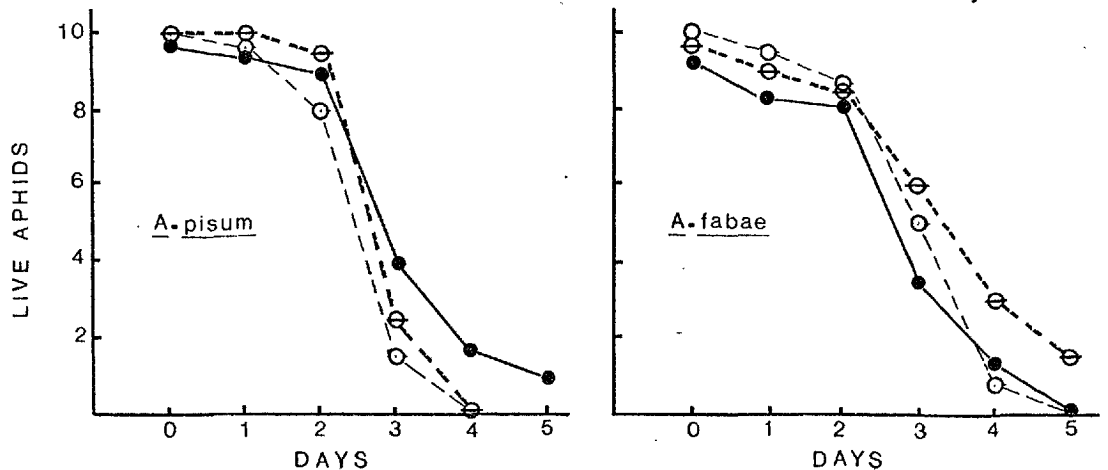


Fig. 1.14. Longevity of adults fed artificially on 15% sucrose solution under three different lighting conditions (see Appendix 12)

○ full artificial light, ◐ dimmed light, ● darkness.

a few minutes every day, to count the living insects in each cage. From the Fig. 1.13 can be seen that the feeding behaviour of A. pisum was similar under all three tested conditions, while there was rather better acceptance of the diet by A. fabae at 0% and 40 to 50% relative humidity.

Lighting conditions. The sachets with the feeding liquid were lit for 16 hrs a day with white illumination about 125 ft-c, by a dimmed light, or kept continuously in darkness. The Fig. 1.14 shows that there was no difference in the acceptability of the feeding solution by the two species under these lighting conditions.

3. The effect of dimethoate on the feeding behaviour.

a. Adults of both species were caged on a range of dimethoate solutions in a 15% sucrose diet. Each sachet also contained 1 μCi of ^{32}P -phosphate. One batch of aphids were fed for 24 hrs. immediately after the preparation of the sachets, after which fresh insects were caged for a further 24 hrs. After each 24 hrs feeding period the living adults were counted and the quantity of diet they had taken up was calculated.

Table 1.5. Quantities of sucrose solution, taken up per adult in 24 hrs, containing different concentrations of dimethoate (radiometric measurement in live adults).

Species	Dimetho- ate concentr. (ppm)	Freshly prepared diet		Diet 24 hrs. old	
		No. of adults	µl/adult	No. of adults	µl/adult
<u>A. pisum</u>	0	10	0.420	20	0.040
	1.0	8	0.102	20	0.028
	2.0	10	0.085	19	0.024
	4.0	9	0.040	18	0.014
	8.0	-	-	20	0.008
<u>A. fabae</u>	0	20	0.216	22	0.154
	0.5	17	0.142	17	0.128
	1.0	17	0.116	10	0.114
	2.0	6	0.112	12	0.102
	4.0	5	0.070	5	0.098
	8.0	2	0.064	-	-

From the Table 1.5 it can be seen that the adults of both species avoided poisoned diets even at the lowest concentrations used where the mortality was also low. There was a proportional decrease of the quantity of diet taken up with increasing concentrations of the chemical. When adults of A. pisum were given poisoned or non-poisoned diets which had been made up 24 hrs. previously, the rate of feeding was much lower than when the diet was freshly prepared in the sachet. The rate of feeding of A. fabae adults was nearly the same on fresh or old diets. It seems therefore that A. pisum were sensitive to some change that occurred in the diet once it was prepared and they did not feed readily whether it was contaminated with dimethoate or not.

b. The lowest concentrations of dimethoate which gave about 100% kill in 24 hrs, 16 ppm in A. pisum and 8 ppm in A. fabae, were incorporated into the sucrose solution. 1 to 2.6 μCi of ^{32}P -phosphate was added per sachet and the adults of the two species were allowed to feed for 12, 18 and 24 hours. Sachets without insecticide were also set up as controls. Living and dead adults were counted and from their radioactivity the μl of artificial diet ingested per adult were calculated.

From the Fig. 1.15 it can be seen that the taken up diet in the untreated controls was more in the case of A. fabae adults than in A. pisum as already has been described. When poisoned diet was offered the quantity taken up by A. pisum within the 1st 12 hrs. was the same as in the non poisoned diet (0.041 μl /adult), whereas A. fabae adults preferred not to accept this diet (0.220 μl /adult and 0.062 μl /adult in untreated and poisoned diets respectively). When the feeding time increased to 18 hrs. both species accepted less of the poisoned diet than the untreated one. When the quantity of the poisoned diet taken up exceeded a certain threshold, mortality was produced. This threshold quantity was nearly the same for each species, whether it was taken up during 18 or 24 hrs. feeding (0.066 to 0.070 μl /adult in A. pisum and 0.076 to 0.091 μl /adult in A. fabae).

4. Toxicity of poisoned sucrose solutions.

Dimethoate and phorate were incorporated separately into sucrose solutions and bioassays were carried out on them. Dimethoate concentrations ranged from 0.5 to 30.0 ppm and phorate from 0.5 to 8.0 ppm (see Appendices 14 & 15). With dimethoate it was found that nearly 100% mortality was achieved in 24 hrs. at a concentration of 16 ppm in A. pisum and 8 ppm in A. fabae adults. Phorate was more

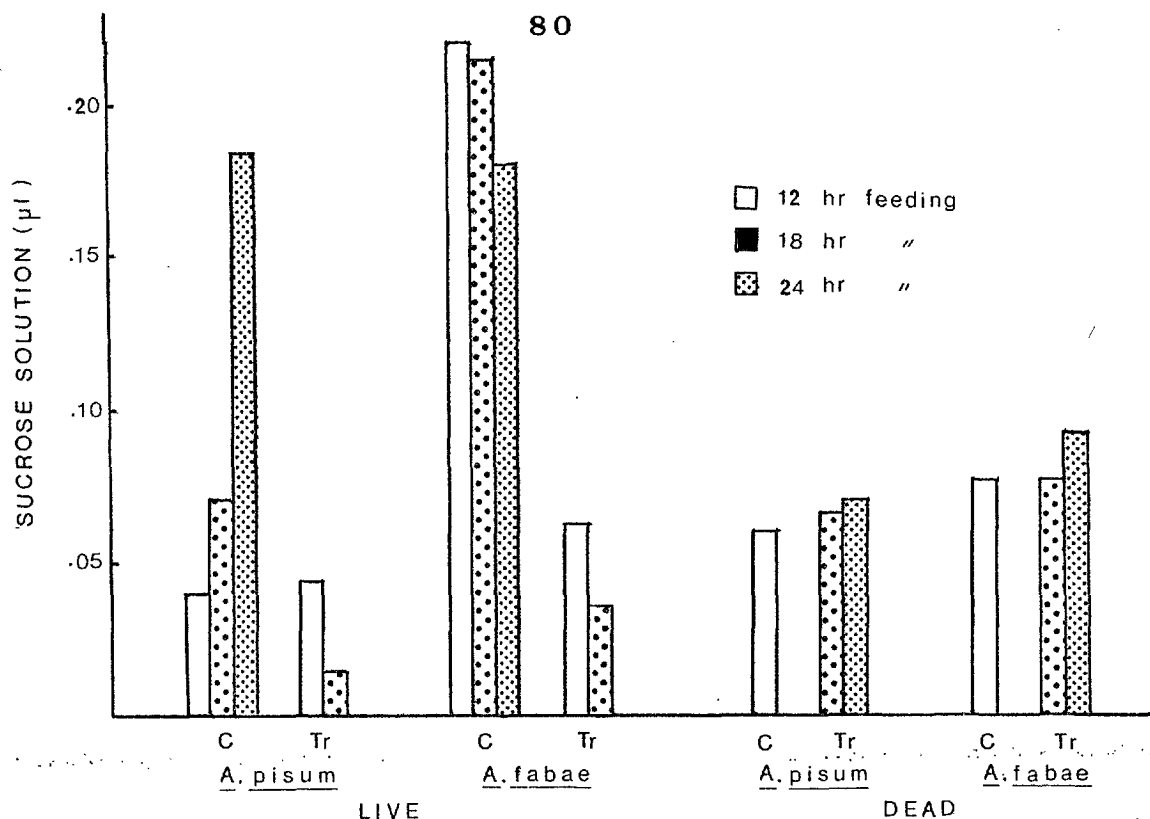


Fig.1.15. Quantity of 15% sucrose solution found per adult after different times of feeding on unpoisoned and dimethoate-poisoned diet (see Appendix 13)

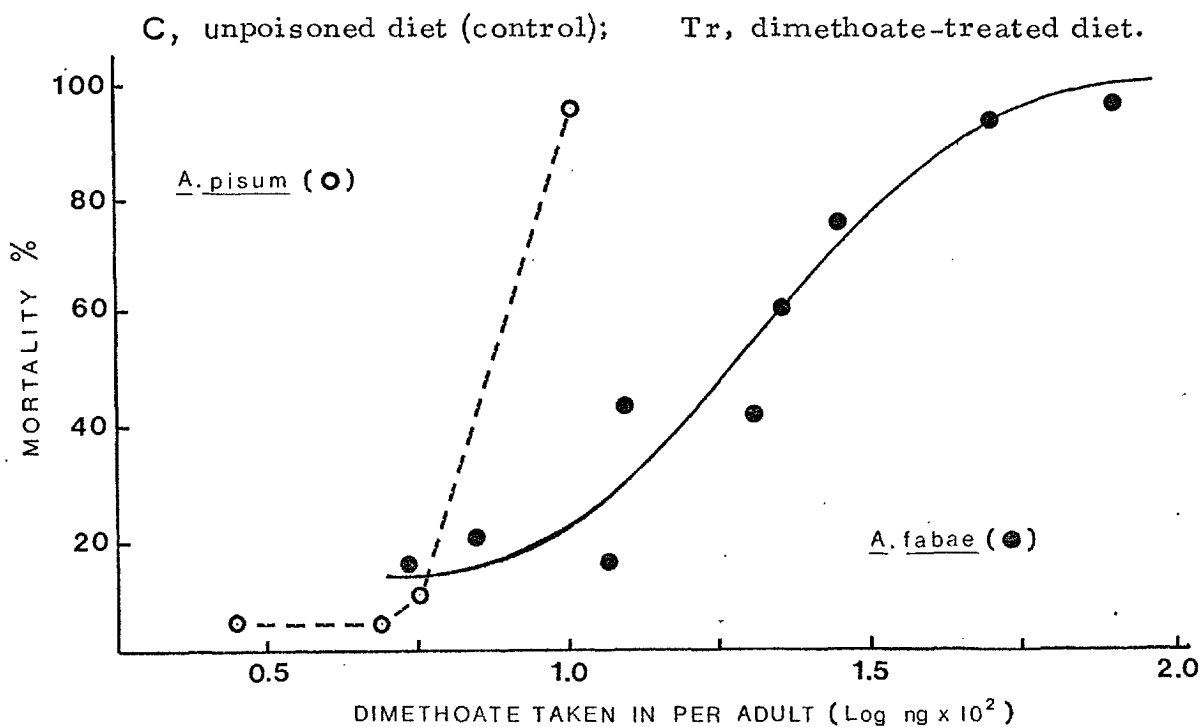


Fig.1.16. Correlation between mortality and the quantity of dimethoate taken in with 15% sucrose solution (see Appendix 16)

toxic and the lowest doses causing 100% mortality were 8 ppm for A. pisum and 4 ppm for A. fabae. Since the presence of dimethoate in the diet strongly inhibited uptake, there was little correlation between the observed mortalities and the concentrations of the chemical in the sucrose solution (Appendix 14). In experiments where the sachets also contained ^{32}P , the adults were collected and counted with a Geiger-Müller counter at the end of each 24 hr. bioassay. From the amount of radioactivity found the amount of dimethoate taken up per adult was calculated. As the Fig. 1.16 shows, there is positive correlation between the quantity of dimethoate imbibed and the observed mortalities especially with A. fabae. In the above experiments with radiotracers, the live adults were also counted at the end of each bioassay. Differences in uptake were found in the living insects since none had reached the mortality threshold and uptake was more closely correlated with concentration.

Discussion

The 15% w/v sucrose solution used for the artificial feeding of both species in these experiments was also found to be the optimum for feeding M. persicae (Parry and Ford, 1967). It is surprising that this solution was also superior to the more complex diet tested in these experiments. However the complete diet had been stored at -15°C before use and it is possible that during storage some change occurred which could be detected and deterred the aphids from feeding. (Dadd et al., 1967)

A complex diet is not necessary for short periods either with or without insecticides and a simple sucrose solution may indeed be preferable as has been pointed out by Danneel (1969a). In toxicity studies, the use of a simple diet as a medium for the insecticide ensures a minimum of interference with the properties of the poison. Some

systemic insecticides are sparingly soluble in water; for example a solubility in water of 15 ppm at 15°C and 19 ppm at 26°C has been quoted for phorate (Lord and Burt, 1964), and according to data referred to by Martin (1971), the solubility of the same chemical at room temperature is 50 ppm. Any increase in other solutes in the diet will lower the insecticide solubility still further. The solubility of other systemic insecticides is, however, higher. For menazon a solubility of 240 ppm at 20 to 25°C is referred, and dimethoate is as much as 2.5% soluble in water at 21°C (Martin, 1971).

An important aspect of any diet for short term feeding experiments is that it should be taken readily by the insects and as the 15% sucrose solution proved satisfactory in this respect, it was used as the vehicle in the toxicity and feeding studies. Even so the acceptability by the two species of this solution followed quite different patterns. Uptake of labelled diet by A. pisum adults increased with time. Of the total quantity taken up in 24 hrs, only 22% was imbibed in the first 12 hrs. and at 18 hrs. only 40% had been taken. This increased uptake after some time, suggests that a threshold reaction involving a "hunger stimulus" was necessary before the aphids began taking large quantities of the diet (Parry and Ford, 1969). This feeding reaction did not occur in A. fabae and nearly the same quantity of diet was taken up, whether the feeding time was 12, 18 or 24 hrs. The readiness with which the aphids took diet also appeared to be a function of the diets age. A. pisum was especially sensitive in this respect and there were strong indications that this aphid fed less readily on the artificial diets than A. fabae thus supporting the findings of Auclair (1965) who estimated that the feeding and excretion rates of A. pisum on artificial diets were usually much lower than those recorded on plants.

The different humidity and lighting conditions tested did not materially affect the feeding behaviour of the two species in these experiments and a relative humidity 40 to 50% and artificial light for 16 hrs. with 8 hrs. darkness were maintained throughout subsequent tests. The artificial light used here was far below the levels which have been found to reduce feeding in some aphids. The thickness of parafilm membrane did not exert any influence on the feeding response of the two species. Auclair (1965) also was unable to demonstrate any significant effect of membrane thickness. Stretched membranes were used in these experiments because they adhered to the cages better when the sachets were made up.

Different concentrations of dimethoate markedly inhibited feeding in the two species. As the concentration of insecticide in the diet increased, the amount of liquid taken up decreased. This inhibiting action of dimethoate was very strong even in the lowest concentration tested which produced the minimum mortality. The decrease in amounts of liquid taken up was not proportional to the increasing concentration of the chemical. Inhibiting action to M. persicae has been found also in diets containing phosphamidon (Parry and Ford, 1967, 1969, 1971) and dimethoate (Javadi, 1971) though Mittler and Pannell (1964) question this influence of phosphamidon on this species.

One problem that arose in this work was the considerable differences in feeding behaviour exhibited by the insects. Though considerable care was taken to ensure that aphids of similar ages not near the end of their life span were used and experimental conditions reproduced as carefully as possible, fluctuations in feeding rates were observed. These differences were more frequent in A. pisum and may be due to different factors, one such factor being

the possible deterioration with time of the feeding solutions in the sachets.

The inhibition of feeding by dimethoate for periods shorter than 24 hrs, was different in the two species. In the first 12 hrs. a considerable reduction of the quantity of diet taken up by A. fabae adults occurred. Thus, this species imbibed only 28% of the quantity it had taken up in the same time from non poisoned diet and 16.2% of the corresponding unpoisoned diet after 18 hrs. feeding. A. pisum adults had taken in nearly the same quantity from the poisoned diet as in the control after 12 hrs, but after 18 hrs they had imbibed 19% of the control. The increased inhibition of feeding in the two species after 18 hrs is possibly the result of some sublethal action arising from the intake of small quantities of dimethoate and leading to a reduction in the feeding rate in addition to any deterrent effect the insecticide has already imposed. In the first 12 hrs the adults of both species avoided accepting poisoned diet at the lethal level. But as the time of feeding was increased, the influence of the "hunger stimulus" caused them to take in more diet and sufficient dimethoate to cause mortality after 18 and 24 hrs feeding. The striking feature in these experiments was the similar quantities of diet found in the dead insects irrespective of the duration of feeding. This suggests that the insects have at this stage taken in lethal doses of poison which were for A. pisum 1.06 to 1.12 ng/adult and for A. fabae 0.61 to 0.73 ng/adult.

In toxicity tests with the insecticides dimethoate and phorate by the artificial feeding method no correlation was found between the insecticide concentrations and mortality. This was especially so with dimethoate. The two factors influencing the result are probably the variability of the two species in feeding through parafilm membranes and the strong inhibition of the feeding response by insecticide which

increases, but not at a proportional rate with the concentration of the chemical in the diet. Parry and Ford (1967) also point out the meaninglessness of any direct comparison of their LC_{50} values for phosphamidon to three strains of M. persicae, owing to the differences in uptake that occurred. To establish an actual dosage-mortality relationship by this method it is necessary to obtain the amounts of insecticide ingested by the aphids (Mittler and Pannell 1964). These amounts were estimated when a ^{32}P -compound was added into the sucrose solutions with dimethoate and so give a more meaningful dose-response relationship (Fig. 1.16). A point which needs further study is whether there is any influence on mortality of the volume of diet from which the dimethoate is absorbed. Aphids are capable of considerable throughput especially when attended by ants (Herzig, 1937; Banks and Nixon, 1958). The modifications to their gut which enable this to occur may well affect the degree of absorption that occurs at different feeding rates and insecticide concentrations.

PART TWO

RELATIVE TOXICITY STUDIES

Introduction

The performance or activity of an insecticide depends upon the interaction between the chemical and the organism. The result of this interaction may be affected by many factors, including the effect of different test methods.

There are many properties an insecticide must possess for it to be effective when applied topically. The waxy layer of an insect's epicuticle is the first barrier to entry, and in order that an insecticide should penetrate it, it must be to some extent lipophilic. In a second phase the insecticide has to penetrate the more aqueous regions of the exo- and endo-cuticle, for this, an oil-water partition coefficient favouring more polar compounds is desirable (Olson and O'Brien, 1963; Hadaway and Barlow, 1966). Treatments which tend to destroy or disrupt the epicuticular layer accelerate penetration and increase the emphasis on this second phase. The third stage of penetration involves transport to the site of action, which for the organophosphorus insecticides is mainly the central nervous system. It seems that the haemolymph has a major role in transport of insecticides to the nervous system (Burt, 1970). However other routes of entry have been postulated in which the insecticide, soluble in the epicuticular wax moves by diffusion to the spiracles and then via the rest of the respiratory system, into the nervous system (Gerolt, 1969).

During penetration different factors interfere which depend on the chemical and the treated insect. Sorption of the insecticide at sites in the insect's body other than the site of action is likely to result in increased amount of the poison necessary to kill an insect (Lord, 1967).

Metabolism of the chemical in the insect's body differentiates the toxicity of the chemicals to different species (Matsumura and Hogendijk, 1964; Nakatsugawa et al., 1969). Sanderson and Edson (1964) after experiments with locusts poisoned with labelled dimethoate conclude that variations in the rates of loss of active dimethoate metabolites probably account for much of the selective toxicity to these insects. Yang (1971) has emphasized that the relationship between the rate of detoxication and the rate of intoxication of diazinon, determines the toxic effect to susceptible and resistant strains of house flies. Enzymic detoxication of the toxicant prevents it reaching a fatal concentration at the site of action (Oppenoorth and van Asperen, 1961). The two factors most frequently postulated to account for variabilities in the susceptibility of insects to insecticides are differences in penetration and metabolism (Lord et al., 1963; Farnham et al., 1965; Uchida, et al. 1965; Gwiazda and Lord, 1967; Lewis and Lord, 1969; Nolan and O'Brien, 1970; Zahavi, 1970).

It is widely recognized that resistance ratios which may be large with topical application may be very small with injected applications (Nolan and O'Brien 1970). However, when two phosphorothioate insecticides (Ciba : C-9491 and C-8874) were applied topically or by injection to two insect species [Boettcherisca peregrina (Robineau-Desvoidy) and Chrysomya megacephala (F.)], the toxicities of the two compounds to the adults were similar; it appears therefore, that the cuticle of both species is not an effective barrier in preventing the penetration of these compounds (Chuo and Sherman, 1971). Similar results were obtained by Metcalf and March (1949) with parathion and paraoxon. Both were readily absorbed by the cuticle of P. americana, resulting in almost identical LD₅₀ values after topical application or injection.

Differential responses of the same species to different chemicals has been reported. For instance, malaoxon was 30 times more potent to Medfly AChE than phosphamidon (Zahavi, 1970). Selection and breeding from resistant individuals when malathion was applied to larvae of two strains of A. aegypti resulted in a 5-fold increase of tolerance in 5 generations; this tolerance was associated entirely with a decrease in absorption of malathion by the larvae. Selection with parathion however applied to 3 strains of this species, resulted in a 2-to 3-fold increase in tolerance with no decrease in absorption (Matsumura and Brown, 1963).

When several factors may be involved (activation, degradation, penetration), interactions make it very difficult to establish the role of any one factor. Sometimes it is possible to eliminate some of these factors, for example Nolan and O'Brien (1970) in working on the ratio of toxicities for susceptible and resistant strains of houseflies used paraoxon, which requires no activation and injected it to avoid differences in penetration.

With bioassays it is feasible to measure quite accurately the potency of an insecticide and to a certain extent study some of the factors involved. Bioassays have been criticised for variability, though credited with greater sensitivity and specific response to the actual toxicants than many chemical methods. However, careful rearing technique, standardization of insect colonies and planned test design have reduced much of the unreliability of the technique. At the same time, modern physico-chemical analytical techniques, such as gas liquid chromatography, have enormously increased the sensitivity and precision of insecticidal assay by other means. The subject of pesticide assay has been reviewed by many authors, for example, Sun (1957); Nagasawa (1959); Needham (1960); Hoskins and

Craig (1962); Busvine (1971).

The technique of topical application in biological assay has become quite popular. Reasonable precision can be achieved with very simple apparatus and there are certain advantages in applying a known dose to individual insects. Volumes much less than 1 μ l can be applied, if desired. Harrison (1961) describes a simple glass capillary applicator which, calibrated radiometrically with an 18% coefficient of error, can apply volumes down to a minimum of 0.00005 μ l. In choosing the solvent for topical application, factors to be considered apart from the solubility of the insecticide, are the toxicity to the insect, effects on insecticide potency and general convenience for the application (Busvine, 1971).

When systemic insecticide is offered to aphid species through their food, whole plants, a single leaf or leaf-discs can be used (David and Cardiner, 1951, 1953a, 1953b, 1955; Matteson et al., 1963; Burt et al., 1965; Abdellatif et al., 1967; Galley, 1968). In this case the insecticide is absorbed from the aphid gut after varying degrees of alteration in the plant tissues.

In recent studies of the influence of the chemicals on the feeding response and mortality of aphids, bioassays with aphids have been carried out with the systemic insecticide, phosphamidon or dimethoate, incorporated in artificial diets (Mittler and Pannell, 1964; Parry and Ford, 1967, 1969, 1971; Javadi 1971).

The effect of body weight, age, starvation and post-treatment

temperature on the toxicity of organophosphates has been examined in bioassays with aphids (Potter and Gillham, 1957; Murdie, 1965; Ratanawaraha, 1968)

The object of the present work was to determine the relative susceptibility of laboratory strains of A. pisum and A. fabae to phorate dimethoate and menazon, when these compounds were applied topically or systemically. Different methods were used to apply the chemicals systemically through the leaf-tissue. The poisoning produced by the three chemicals after the aphids had fed on treated leaf tissue is demonstrated. Observed differences in the toxicity of each chemical to the same species are discussed.

Materials and Methods

Insecticides. For topical or systemic application acetone solutions of dimethoate and 2-ethoxyethanol solutions of phorate and menazon were used. Immediately before they were applied, solutions of the three chemicals were prepared by dilution from stock solutions stored at -15°C . In the topical applications 0.08 to 5.5ng of the chemicals were applied per adult in 6 doses per bioassay with usually 3 to 4 replicates per dose. In systemic application, depending on the chemical and the treatment method, the doses applied ranged from 0.02 to 500 μg of the chemical per leaf-disc, in 5 to 6 doses per bioassay with 3 to 4 replicates per dose.

Insects. Adult apterous virginoparae of the species A. pisum and A. fabae were used. In each bioassay the adults of each species were

of the same age and size. To facilitate handling, the aphids were anaesthetized with carbon dioxide and in each bioassay 5 to 16 adults of both species were caged on the same leaf-discs.

The adults were kept for 24 hrs on inverted leaf-discs 2.5 cm in diameter on perspex trays as described by Galley (1968), and were caged under perspex or stainless steel rings topped with thin muslin. Early experiments had soon shown that these aphids especially A. fabae could climb from the cages when a polytetrafluoroethylene (PTFE) dispersion (Fluon II, I.C.I.) was used to paint the inside of the rings to prevent the insects escape (Muir, 1958; Needham and Dunning, 1966; Galley, 1968).

Topical application. Solutions of the insecticides were applied to the insects with glass microcapillaries constructed in a way similar to that described by Hewlett and Lloyd (1960). Glass tubing of internal diameter about one-half the external one was drawn out after heating. The long pieces of microcapillary formed were cut into short lengths between 1.2 and 1.5 cm long. These short capillaries were inserted into a rubber plug at the end of a glass holder, 0.5 cm diam. x 8.0 cm long, with the aid of a hypodermic needle (Fig 2.1a).

Each microcapillary was calibrated before use, as the volumes delivered cannot always be estimated from their length and internal diameter since the bore may not be uniform. Calibration was carried out colorimetrically using a nearly saturated solution of the dye Sudan Black "B" in acetone. 0.5g of the dye were added to 50 ml acetone and 24 hrs later undissolved dye was removed by centrifuging at about 2,500 g for 30 minutes. 45 ml of this solution was made up to 50 ml with more solvent. This solution did not change its optical density for at least 1 year when stored in darkness at -15°C . Two series of dilutions in acetone were made from this original stock

solution and the optical densities measured in a Beckman DB spectrophotometer at a wavelength of 670 nm (see Appendix 17). These values were plotted against the corresponding concentration to provide a calibration curve for the dye. The volumes of microcapillaries were estimated by repeated deliveries of the stock dye solution to 2 ml of acetone in 3 replicates, until sufficient color was developed to be accurately measured in the spectrophotometer. The mean optical density for the replicates of each capillary were referred to the calibration curve to find the corresponding concentration and from this and the number of deliveries, the volume of each microcapillary was calculated.

Microcapillaries of 0.05 and 0.06 μ l capacity were chosen to apply the insecticide solutions to the insects. These capillaries were filled by dipping into the insecticide solution, care being taken not to dip more deeply than necessary, thus preventing the retention of excess liquid on the external surface. The liquid was expelled from the capillary by pressure, applied by mouth, through a rubber tube fitted onto the other end of the glass holder. Pressure was maintained until after the tip of the capillary had been removed from the insect's body.

Systemic application. A number of different assay techniques were used to assess the relative systemic toxicity of the chemicals to aphids on leaf-discs. In preliminary experiments dimethoate solutions were applied to the upper surface of the leaf-discs with a 5 μ l pipette ("Drummond Microcap", Shandon Co.). After treatment the leaf discs were positioned upside down on the perspex tray and adult aphids caged on the abaxial surface of the leaf-disc, now uppermost. In alternative applications 5 μ l aliquots of solutions of the chemicals were applied to the cotton wicks close to the leaf-discs, after positioning

on the perspex tray, or 1 μ l of the chemical solution was injected into the main vein of the leaf-disc (Fitton, 1972) with a microsyringe, before the discs were set in position. Another technique to maintaining leaf-disc turgidity was also developed. From pieces of blotting paper 3.5 cm square, circles of 2.5 cm diameter were removed with a sharp cork borer. The squares were then placed in rows on a 20 x 20 cm glass plate and held in position with small strips of masking tape. The blotting papers were connected by cotton wicks to a water reservoir which maintained the turgidity of the leaf discs which were placed upside down in the circular space within each blotting paper. The insecticide was applied in 5 μ l of solvent to the blotting paper in the space between the end of the cotton wick and the edge of the leaf disc (see Fig 2.1 b). The insects were caged on the leaf-discs with stainless steel rings having muslin tops. The cages were held in position by rubber bands passing through a glass tube over the cages.

In this series of experiments the aphids were introduced to the leaf-discs of V. faba 8 to 10 hours after the application of the insecticides. It is probable that reasonable distribution of the insecticides in the discs had occurred in this time. Labelled phorate was well distributed within 12 hrs in brussels sprout leaves (Galley, 1968).

Evaluation of relative toxicity. Aphid response to the applied insecticide was recorded after 24 hrs. Only aphids capable of normal walking were counted as alive; moribund aphids and those capable of unco-ordinated movement were counted as dead.

The data from each bioassay were analysed by the London University CDC 6600 Computer, using a program for probit analysis developed by the Canadian Department of Agriculture.

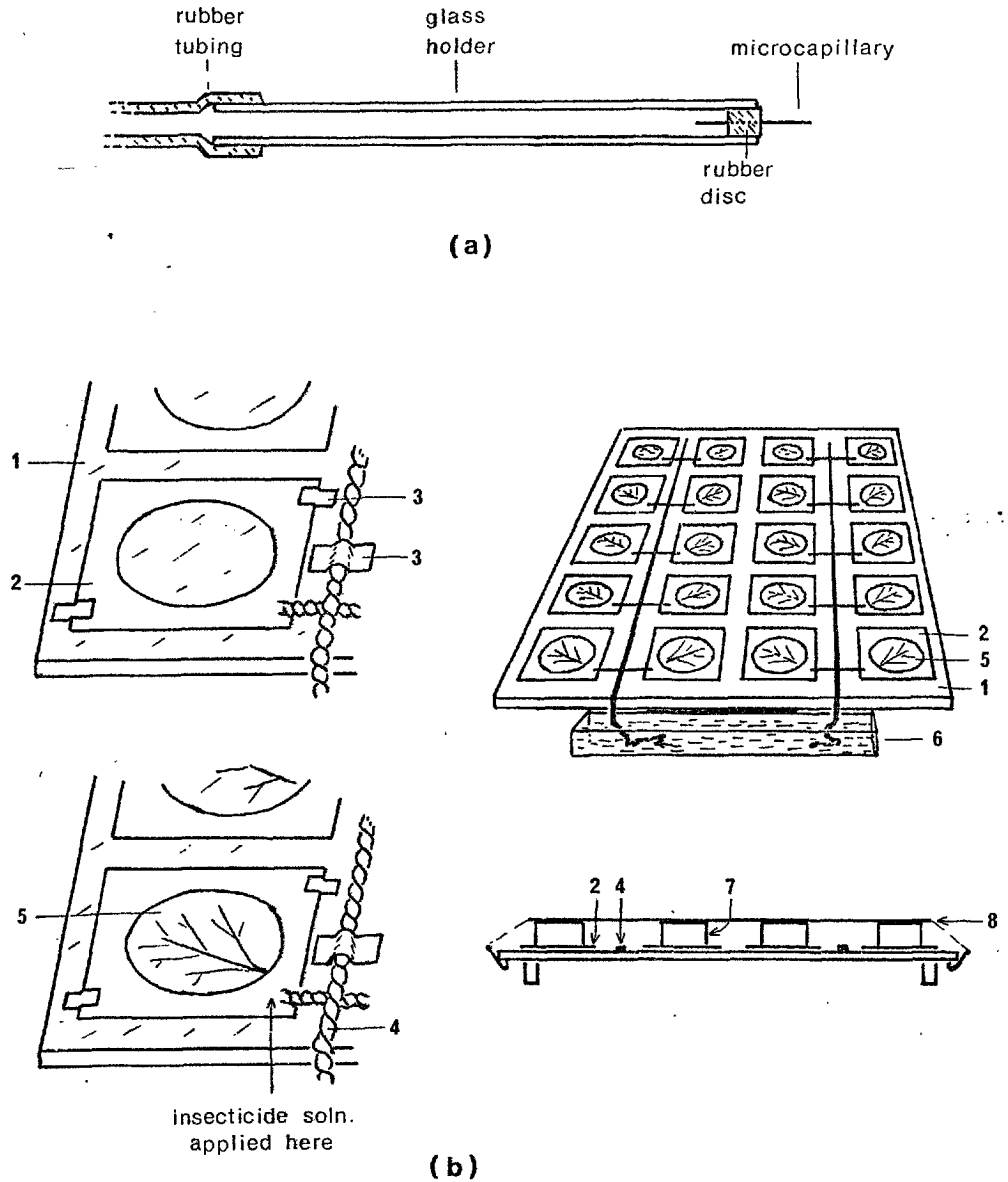


Fig. 2.1. (a) Microcapillary used for the topical application
 (b) Diagram of the technique for systemic application with blotting paper.

1. glass plate; 2. blotting paper; 3. masking tape;
4. cotton wick for water supply; 5. leaf-disc;
6. water reservoir; 7. stainless steel retaining cage ; 8. rubber band to keep the cages in position.

Results

Tests for parallelism were carried out in similar bioassays and from these groups the toxicities for the chemicals and methods of application evaluated for each species. A chi-square test was computed for heterogeneity. Since the regression coefficient (b) from the joint analysis was significant, estimates of effective dose for a 50% response level and the corresponding confidence limits (95% level) were computed. For all the dosage levels tested, probit mortalities, after correcting for control deaths (Abbott, 1925), were plotted against the log dose. The "best fit" regression lines were drawn using the data supplied by the computer.

1. Contact toxicity. The LD₅₀ values of the topical applications are given in Table 2.1 and the probit lines for each chemical are illustrated in the Figs 2.2, 2.3 and 2.4. These results show that dimethoate and menazon as contact poisons were about 4 times more toxic to A. pisum than to A. fabae, whereas phorate was less toxic to A. pisum than to A. fabae adults.

Table 2.1. Medium lethal doses of the three chemicals tested by topical application to the two species (ng/adult) (see Appendix 18)

INSECTICIDE	<u>A. pisum</u>	<u>A. fabae</u>	RELATIONSHIP.
Dimethoate	0.266	1.090	1 : 4.1
Phorate	0.972	0.431	1 : 0.44
Menazon	0.384	1.634	1 : 4.2

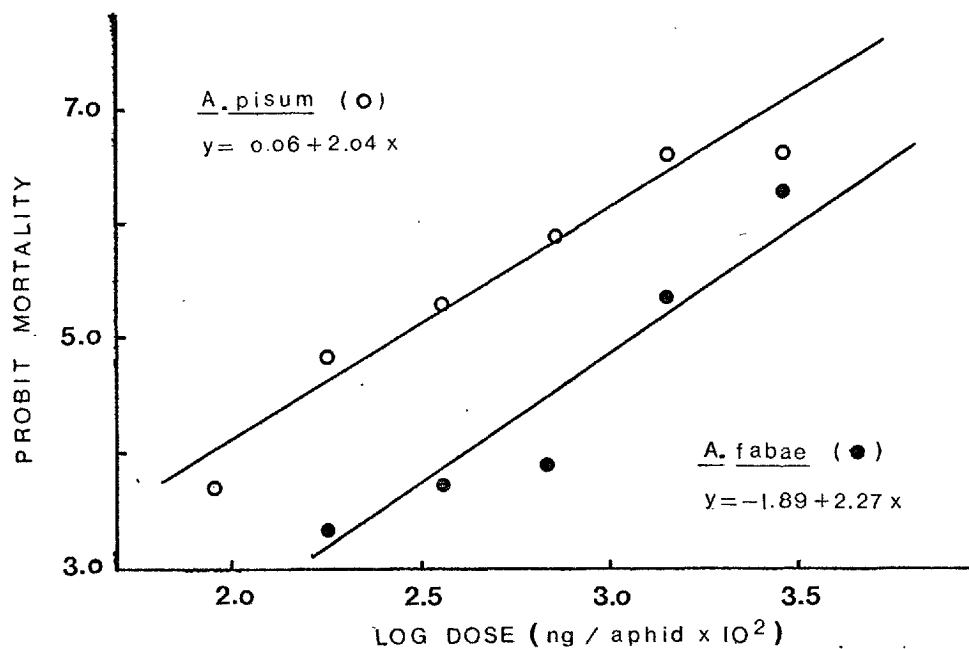


Fig. 2.2. Dose/response regression for the two species to the contact action of dimethoate (see Appendix 18)

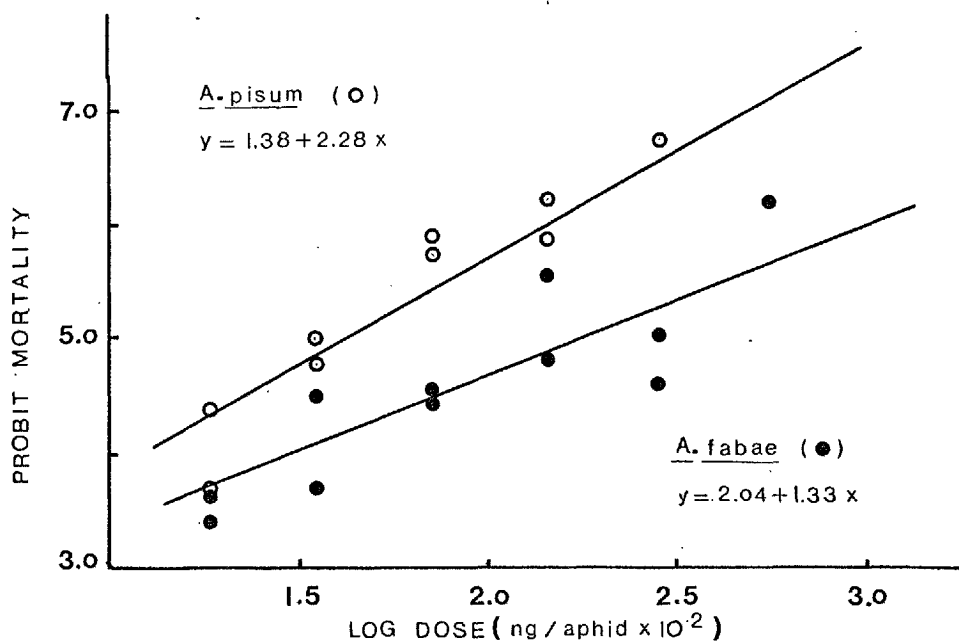


Fig. 2.3. Dose/response regression for the two species to the contact action of menazon (see Appendix 18).

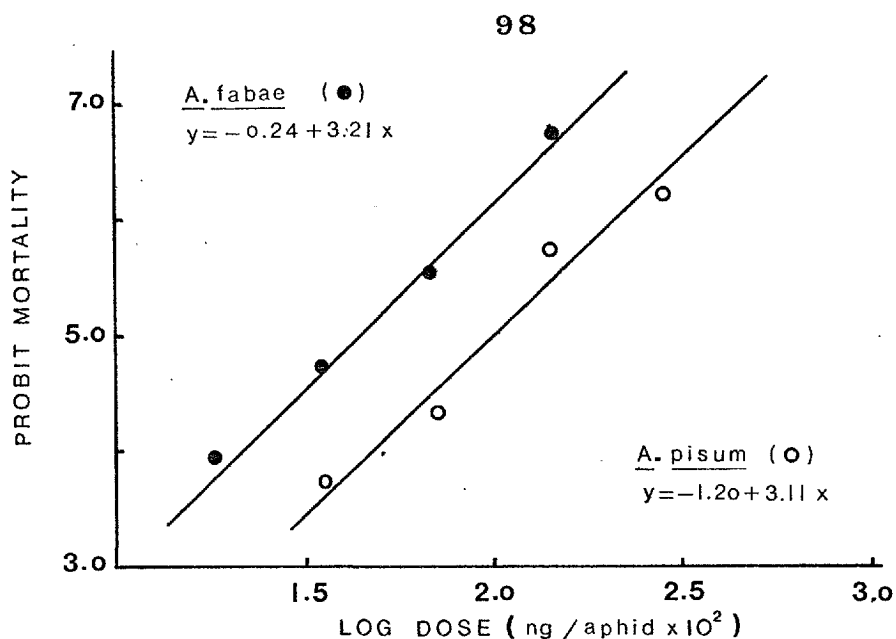


Fig. 2.4. Dose/response regression for the two species to the contact action of phorate (see Appendix 18).

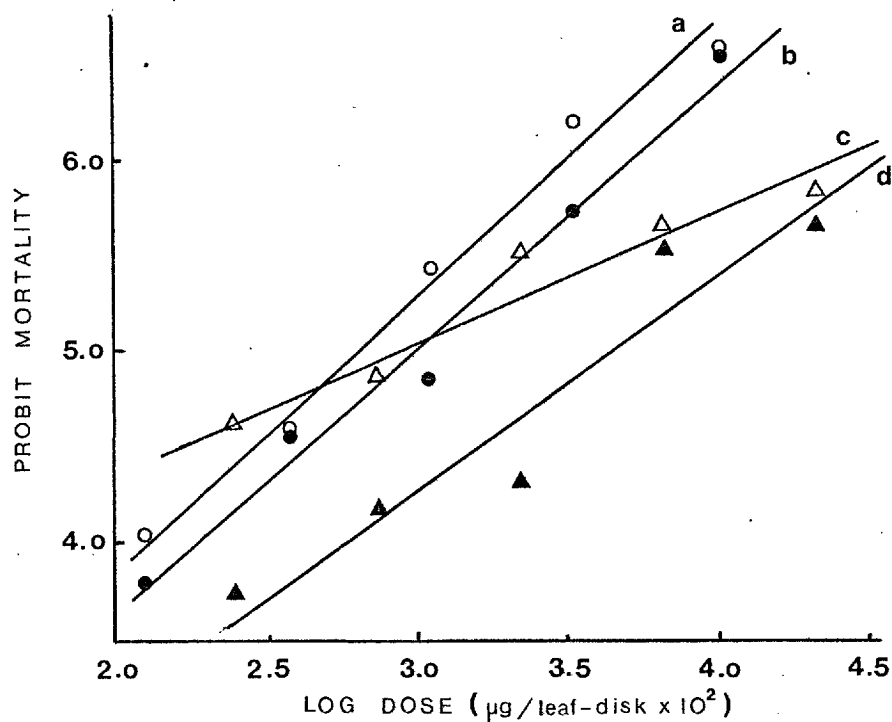


Fig. 2.5. Dose/response regression for A. pisum and A. fabae to the systemic action of dimethoate (see Appendix 19).

Wick application	a. <u>A. pisum</u> (○) $y = 0.92 + 1.46x$	b. <u>A. fabae</u> (●) $y = 0.79 + 1.41x$
Leaf surface appl.	c. <u>A. pisum</u> (△) $y = 2.99 + 0.69x$	d. <u>A. fabae</u> (▲) $y = 0.88 + 1.13x$

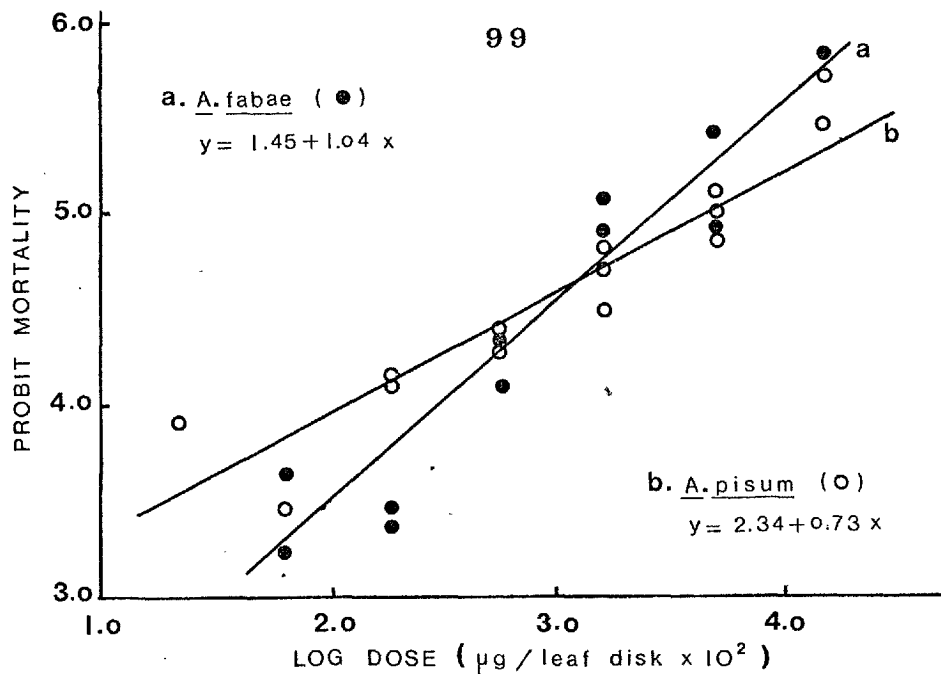


Fig.2.6. Dose/response regression for the two species to the systemic action of menazon (see Appendix 19).

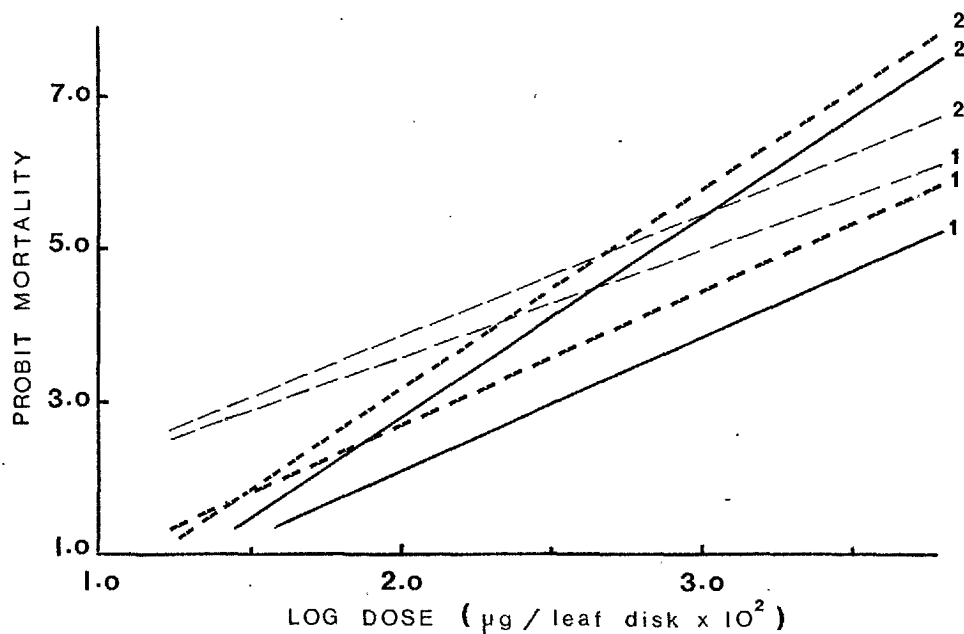


Fig2.7. Dose/response regression for A. pisum and A. fabae to the systemic action of phorate (see Appendix 19)

		<u>A. pisum</u> (1)	<u>A. fabae</u> (2)
wick application	(——)	$y = -9.30 + 4.22x$	$y = -2.38 + 2.62x$
injection	(---)	$y = -0.88 + 1.79x$	$y = -2.18 + 2.67x$
blotting paper appl.	(----	$y = 0.79 + 1.41x$	$y = 0.65 + 1.61x$

2. Systemic toxicity. Dimethoate was 5 to 6 times more toxic to A. pisum than to A. fabae, when it was applied on the upper surface of the leaf-discs. The observed difference in susceptibility of the two species was very small when this chemical was applied through the wicks. In A. pisum the LD₅₀ dose of dimethoate was nearly the same in both methods of systemic application, whereas quite different doses were applied on the wicks and on the leaf-surface to achieve 50% mortality in A. fabae, 0.973 and 4.306 µg/disc respectively (Table 2.2 and Fig 2.5).

Table 2.2 Medium lethal dose of the chemicals tested by different systemic application methods (µg/disc) (see Appendix 19)

Insecticide	Way of application	<u>A. pisum</u>	<u>A. fabae</u>	Relation-ship
Dimethoate	Wick appl.	0.613	0.973	1 : 1.6
	Leaf-surface app.	0.793	4.306	1 : 5.4
Phorate	Wick appl.	2.598	0.651	1 : 0.25
	Injection	1.962	0.492	1 : 0.25
	Blotting paper appl.	0.952	0.502	1 : 0.52
Menazon	Blotting paper appl.	4.110	2.548	1 : 0.62

With menazon and phorate the toxicities caused were nearly the same in the two species when each of these chemicals was applied on the blotting paper (see Table 2.2 and Figs 2.6 and 2.7). When phorate was applied on the wicks or injected in the leaf-discs, the relative toxicities were reversed when compared to dimethoate. A. fabae was nearly four times more susceptible to phorate than A. pisum in the LD₅₀ level (Fig. 2.7). The quantity of phorate required to achieve a 50% mortality in 24 hrs. in A. fabae was similar, 0.492 to 0.651 µg/disc, when the chemical was applied in three different ways. In A. pisum the LD₅₀ of this chemical were 0.95 µg/disc in the blotting

paper application, 1.96 $\mu\text{g}/\text{disc}$ by injection and 2.60 $\mu\text{g}/\text{disc}$ when applied through the wicks.

Discussion

Considerable difficulty was experienced during many of the bioassays with inexplicable mortalities among the insects. One possible reason was contamination of the perspex trays from previous bioassays especially as many insecticides are soluble to differing degrees in some plastic materials. This contamination was extremely variable and even though stringent precautions were taken, it was never completely eliminated. It was because of these difficulties that the assay technique in which blotting paper was used to keep the leaf-discs turgid, was developed. In this technique only the stainless steel cages and glass plate were used repeatedly and these could be more thoroughly cleaned than perspex. The blotting paper method was used only as a replacement for the wick application and to keep insects after their topical treatment with insecticides (see page 142). The technique could also be used to maintain injected leaf-discs, but would be unsuitable for surface treatments as the moisture in contact with the glass enables potentially systemic deposits to penetrate the leaf tissue abnormally rapidly (Galley, 1968).

The quantities of the three insecticides required to achieve a 50% mortality after their topical application was different in each species. This difference arises in the interaction of the many different factors involved in the toxic action of the compounds. These factors being the ability of each chemical to penetrate the aphids' cuticle, the ability to reach the site of action (Winteringham, 1969) the rates of activation and degradation and the differential ability of the compound

and its toxic metabolites to inhibit cholinesterases. Which of these factors is predominant at each stage in the process of intoxication cannot be assessed on these results alone, though it is evident that considerable differences exist between the species, since dimethoate and menazon were 4 times more toxic to A. pisum than to A. fabae whereas phorate was more toxic to A. fabae.

In the bioassays where the compounds act systemically, uptake of the chemical is oral, thus the species selectivity of each toxicant is likely to be influenced also by other factors which are related more to the manner of application, the particular feeding site and the rates of uptake and absorption by each species. An additional factor which influences the toxicity of the chemical is the extent to which activation or degradation occurs in the leaf tissue before it is removed by the insects.

The relative toxicity of the insecticides to the two species after systemic application was different in the different methods of application. A. pisum was 5 to 6 times more susceptible than A. fabae to dimethoate when this chemical was applied on the upper leaf surface but slightly more susceptible when the application was to the wicks. The toxic levels of each chemical applied by different routes to each species are not absolutely comparable, owing to possible changes occurring mainly in the biological material used (insect, plants). However it is evident from the data obtained that the variability of the LD₅₀ values of dimethoate in A. pisum was greater in leaf-surface application than in wick application and in A. fabae this chemical was much less toxic when applied on the leaf-surface. Elliott(1970) has found that phorate, dimethoate and menazon killed over 50% of A. pisum on bean leaves at $1.6 \mu\text{g}/\text{cm}^2$ whether the aphids were on the treated or untreated surfaces. However, the interference of the leaf-epidermis

reduces the entry of the chemical into the leaf-tissue, as has been demonstrated by Galley (1968). This author working with the species B. brassicae and M. persicae has found that insecticides applied to the wick act much faster. After 48 hrs. the LD₅₀ of dimethoate applied to the leaf-surface was found to be thirty times greater than when applied to the wick.

The relative toxicity of phorate and menazon was the same in the two species when these chemicals were applied on the blotting paper, but A. pisum adults proved 4 times more susceptible than A. fabae to phorate when it was applied on the wicks or injected in the leaf-disc. The quantity of phorate necessary to produce a 50% mortality in the two species was different in the three different systemic application methods. With A. pisum the LD₅₀s were in the ratio 1 : 2.1 : 2.7 after application on the blotting paper, injection and on the wicks respectively; but in A. fabae the LD₅₀ values, in the ratio 1 : 0.98 : 1.3, were achieved with more or less the same quantity of phorate in the three ways of systemic application. This difference between the two species may be a reflection of the different feeding behaviour. Soon after wick application or injection of phorate, it is likely that higher concentrations of the chemical appear first in the largest veins before spreading out into the parenchyma. Thus, A. fabae which prefers to feed on the largest veins of the leaf is feeding in areas where the concentration of insecticide is higher than in the secondary veins and parenchyma, the areas preferred by A. pisum. When the chemical is applied on the blotting paper it may be transferred into the leaf-tissue through the edges of the disc and be more uniformly distributed between the larger veins and the small veins and parenchyma, so a smaller dose will produce a 50% mortality to A. pisum and have a similar effect on A. fabae.

PART THREE

BIOCHEMICAL ASPECTS

SECTION A. STUDY OF THE ESTERASES OF THE TWO SPECIES

A.1. ELECTROPHORETIC SEPARATION OF CHOLINESTERASES AND CARBOXYLESTERASES ON POLYACRYLAMIDE GEL.

Introduction

Although electrophoretic methods of protein separation lack the precise localization ability of cytochemical methods, they are capable of isolating tissue components for further biochemical analysis.

The use of the electrophoretic techniques to study insect esterases was limited until a good starch gel supporting medium was developed by Smithies (1955). Although agar and starch gels have yielded good resolution of insect esterases, subsequent kinetic studies on the separated components have been prevented by the low carrying capacity of these gels and the difficulties encountered in recovering the enzymes. A new synthetic medium, acrylamide, was introduced as a supporting medium for zone electrophoresis by Raymond and Weintraube (1959) and subsequently developed by Raymond in collaboration with others (Raymond and Nacamuchi, 1962; Raymond, 1964; Raymond and Jordan, 1966). Polyacrylamide gels were found far superior to agar and starch gels because of the better resolution and definition that is often achieved. They are easy to handle, being less fragile even in concentrations as low as 3% and they provide a rather more uniform average pore size at a given concentration. At a concentration of 7% for example, they have an effective pore size of about 50 Å (Ornstein, 1964). The pore size is inversely proportional to the square root of the gel concentration (Anonymous, 1967) and can be "tailored" to the dimensions of the molecules to be separated, by varying the gel concentrations. In contrast to both starch and agar gel, polyacrylamide gel is relatively inert

chemically, is non-ionic and does not react with colorimetric reagents. Ornstein (1964) and Davies (1964) (after Williams and Riesfield, 1964) further extended the use of this gel by developing disc electrophoresis techniques.

Markert (1959), Augustinsson (1961), Echobichon and Israel (1967) and Venkov et al. (1967), working on the electrophoretic separations of various esterases of vertebrates, laid the foundation of the characterization of these enzymes through the use of specific substrates and inhibitors. In studies of insect esterases, the enzymes have also been classified according to their reactions with certain carbamates or organophosphorus inhibitors (Mounter and Whittaker, 1953; Salkeld, 1965; Eldefrawi, 1970). Cholinesterases (ChEs) are inhibited by both carbamates and O-P compounds, carboxylesterases (Car-Es) by O-P compounds but not by carbamates and aromatic esterases by neither.

Enzyme electrophoresis patterns are remarkably species specific and could possibly be considered as an extension of the species specificity of protein electrophoresis patterns in the haemolymph of a number of insects and spiders described by many workers (Whittaken and West, 1962; Hubby and Throckmorton, 1965; Salkeld, 1969; Trebatoski and Haynes, 1969; Couch, 1971), although Whittaken and West (1962) concluded that there is no such thing as a "type" pattern for an individual species, except under the experimental conditions usually common to the work of a single investigator. Gel composition and experimental procedure are factors that influence not only the rate, but also the direction of esterase migration; for example, using the same insect species (housefly), substrate and pH, three studies have yielded vastly different patterns: Mengel et al. (1963) on starch gel, in a potential gradient of 5.5 V/cm for 4 hrs found all esterases to act as cations, Velthuis and van Asperen (1963) on agar gel at 30 V/cm for 12 mins reported approximately equal proportions to act as cations and anions, whereas Collins and Forgash (1968) on acrylamide gel at

12 V/cm for 3.5 hrs have found all but one to act as anions.

Electrophoresis of insect esterases on polyacrylamide gel has been relatively less widely used. Sims (1965) used it to study esterases of 2 species of Drosophila. Price and Bosman (1966) employed the medium to investigate non-esteratic proteins of the blowfly, Calliphora erythrocephala. Arurkar and Knowles (1967, 1968) conducted a more extensive research on esterases using polyacrylamide gel when they studied the enzymes in homogenates of 7 insect species. Ahmad (1968) and Collins and Forgash (1968) used polyacrylamide gel and demonstrated carboxylesterases in susceptible and resistant strains of houseflies.

Studies on aphid esterase zymograms appears to be very limited. Sudderuddin (1970, 1972) has worked on aphid zymograms in the vetch aphid M. viciae and on susceptible and resistant strains of the green peach aphid M. persicae. There are no reports of electrophoretic studies of A. pisum and A. fabae esterases. The present work is therefore an attempt to study the pattern of the esterases, especially those involved in organophosphorus poisoning of these two species and to contribute to the very limited work which has been done so far on the esterases of aphids.

Materials and Methods

Electrophoretic apparatus. A disc electrophoresis apparatus was used (Davies, 1964) as modified by Sudderuddin (1971), holding eight rectangular, perspex running "tubes", 0.6 x 0.7 x 7.8 cm long, instead of the cylindrical ones (0.5 cm bore x 7.5 cm long) normally supplied. Two buffer tanks, were connected with the vertical running columns. The one above was made the cathode and the one below the anode.

Buffer solutions. The following buffer solutions were used:

(a) Tris - EDTA - Borate buffer, recommended by Raymond (1964) was made up with analytical grade materials to the following formula: 5.5 g trishydroxymethyl amino methane, 2.5 g boric acid, 1.0 g ethylene diamine tetracetic acid (di-sodium salt), made up with deionized distilled water to a volume of 1 litre. This solution had a pH of 8.5 ± 0.05 .

(b) Phosphate buffer, prepared in a way similar to that suggested by Gomori (1955), was made up of 2.678g of di-potassium hydrogen orthophosphate and 1.31g of potassium dihydrogen phosphate each diluted in 250 ml deionized distilled water to give 0.1 M solutions. 61.5 ml of the former and 38.5 ml of the latter solution were mixed to make 100 ml of 0.1 M phosphate buffer at pH 7.

Preparation of gels. Since a uniform gel of 5% w/v failed to separate the esterases, a variable system was employed consisting of an initial portion of 4% and the main portion of 8% w/v of polyacrylamide. The gels were prepared as described by Price (1968). 0.8g or 1.6 g "Cyanogum 41" (BDH) were dissolved in 20 ml of Tris-EDTA-Borate buffer to give the 4% and 8% gel concentrations. Two catalysts, 90 mg of ammonium persulphate and 0.1 ml of 2-dimethyl aminoethyl cyanide were carefully placed separately on the bottom of a 200 ml beaker. The buffer containing the Cyanogum was then added while rotating the beaker in order to rapidly mix all the contents. With the bases of the running tubes sealed with plasticine the 8% gel mixture was run in from a pipette to occupy the first 5.6 cm of the tube. A few drops of distilled water were added and drawn off with filter paper just before the 4% gel was added. This procedure ensured a flat meniscus and a smooth interface between the gels. Enough of the 4% gel was added to occupy 1.2 cm of the tube above the main gel. A length of about 1 cm was left to receive the sample. A small volume

of distilled water was dropped onto the surface of the second gel and was again removed before the sample was applied. In all these procedures care was taken to avoid the entrapment of any air bubbles. The gels were allowed to polymerize for 2 hrs, before the plasticine plugs were removed. The unit was then ready to receive the sample for subsequent electrophoresis.

Insect homogenates. 230 mg of the adults of each species were homogenized in 3 ml of ice-cold deionized distilled water in a hand operated glass homogenizer. Homogenates were centrifuged at 17,000 g for 30 min at 8°C. About 0.2 ml of the supernatant was put on the top of each running tube and a small quantity of Tris-EDTA-Borate buffer was also added. Folded filter papers (3 x 0.7 cm) were used in the sample space to prevent the sample from flowing back into the reservoir.

Conditions of electrophoresis. The apparatus was moved into a cold room (8°C) and the appropriate amount of Tris-EDTA-Borate buffer was poured in the lower and upper buffer reservoirs, about 210 ml and 170 ml respectively. With the reservoirs connected to a Shandon Vokam constant voltage/current DC power supply, the electrophoretic separation of the esterases was performed under constant current conditions at 5 mA per column. During a run the potential rose from 110 to 200 volts. It took 1¼ hrs. for the electrophoresis to be completed, after which the gels were removed from the tubes by a rectangular perspex plunger, which closely fitted the tubes. The gels slide out easily with gentle pressure on the plunger at the top end.

Esterase detection and identification. The detection of esterases was based on enzymatic hydrolysis of α -naphthyl acetate to α -naphthol, which was coupled to a diazodye to form a highly insoluble pigment, α -naphthol diazoate.

The substrate solution was prepared at a concentration of 1.3 x

10^{-2} M by diluting 250 mg of α -naphthyl acetate in 100 ml of a mixture of 25 to 30 ml acetone and 75 to 70 ml deionized distilled water. To prepare the staining reagent 200 mg of Fast Blue RR salt (Michrome No. 608, E. Gurr Ltd.) were dissolved in a mixture of 50 ml phosphate buffer (0.1 M at pH 7) and 50 ml of deionized distilled water.

After electrophoresis the gels were put in a glass staining dish and the sides sprayed with the substrate solution. The gels were then left at room temperature in the dark for 20 minutes. After this incubation period the gels were washed with 3 changes of dist. water to remove the substrate. Sufficient stain reagent was poured into the dish to cover the gels and the dishes placed in an incubating bath at 37°C in dim light. The reaction starts immediately and therefore after the first 5 min the gels must be checked for the degree of coloration in the bands. After about 20 min. the reaction was stopped by washing the gels in many changes of dist. water and clearing in a 7 to 10% acetic acid. They were then stored in a 7% acetic acid solution. The positions on the gels where active esterase enzymes occur, appear as dark bands easily distinguishable against a rather light background of the rest of the gel.

As a standard practice in each electrophoresis, samples of one species were used at a time, and of the eight gels obtained after electrophoresis, 4 controls were used to detect all the esterase bands, 2 were treated with eserine which inhibits cholinesterases and 2 treated with dichlorvos which inhibits cholinesterases and carboxylesterases.

a. Treatment with eserine (physostigmine) sulphate. Cholinesterases are inhibited at 10^{-4} M eserine but carboxylesterases are not affected at this concentration. The gel strips after electrophoresis were covered with a solution of 10^{-4} M eserine sulphate and left for 20 min at

20°C. The gels were then given a quick rinse and stained for esterases as described above. Bands which appear on the controls but not in the eserine treatments are cholinesterases.

b. Treatment with dichlorvos (2,2-dichlorovinyl dimethyl phosphate) Organophosphates inhibit cholinesterases and carboxylesterases at dilute concentrations. This selective inhibition has been used so far to distinguish these two groups of enzymes from other esterases like arylesterases (formerly known as A-esterases) and acetyleresterases (formerly known as C-esterases) which are not affected by O-P compounds. 10^{-4} M dichlorvos was used for the treatment of gels after electrophoresis in the same way as the eserine treatment. All esterase bands appear except the cholinesterases and carboxylesterases.

Mobility measurements. Since the front was easily detected during and after the electrophoresis no marker dye was added with each sample before a run. The distance travelled by the central point of each band was divided by the distance travelled by the front gave an Rf value which is an indication of the average mobility of the individual esterases.

Results

A total of 5 esterases in A. pisum and 6 in A. fabae, all hydrolysing α -naphthyl acetate were obtained in the variable 4% and 8% polyacrylamide gel. In each species one of the bands was inhibited by eserine and was thus categorized as cholinesterase and a further band inhibited by dichlorvos was a carboxylesterase (Fig.3.1) The mobility of both ChEs and carboxylesterases in A. fabae, was higher than in A. pisum. The average mobility values for ChE and

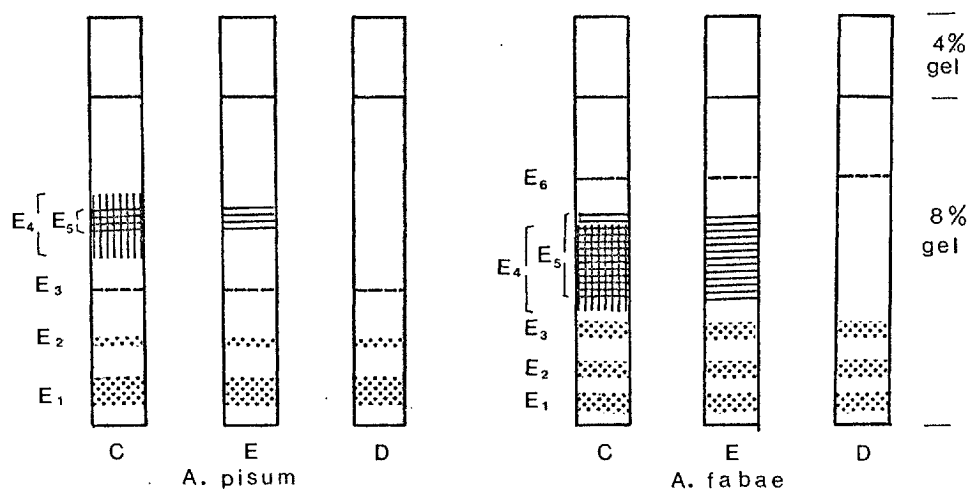


Fig. 3.1. Diagrams of esterase patterns in the adults of *A. pisum* and *A. fabae* obtained electrophoretically in a variable polyacrylamide gel.

C, untreated gels (controls); E, gels treated with eserine sulphate (ChEs inhibited); D, gels treated with dichlorvos (ChEs and Car-Es inhibited).

Table 3.1. Average mobilities of esterase bands of the two species, separated in a variable, polyacrylamide gel system (4% and 8%)

Esterase Bands	<i>A. pisum</i>				<i>A. fabae</i>	
	Distance (mm) from the origin		Average mobility		Distance (mm) from the origin	
	Minimum	Maximum			Minimum	Maximum
E ₆	-	-	-	0.39	25.0	26.0
E ₅	31.5	34.0	0.52	0.62	40.0	42.0
E ₄	33.0	35.0	0.53	0.66	43.0	44.0
E ₃	44.0	44.0	0.69	0.77	50.0	52.0
E ₂	52.0	54.0	0.83	0.89	57.0	60.0
E ₁	59.0	62.0	0.95	0.96	62.0	65.0
Front	63.0	64.0	1.00	1.00	65.0	67.0

carboxylesterase bands were in A. fabae 0.66 and 0.62 whereas in A. pisum were 0.53 and 0.52 respectively (Table 3.1.)

Discussion

When polyacrylamide gel at a uniform concentration of 5% was used, a single band was formed after electrophoresis of homogenates of the two species. By using a variable gel concentration, the gradation of gel pores provided a sharper resolution of the esterase bands. Thus, 5 and 6 bands were separated in A. pisum and A. fabae respectively, staining as dark brown areas of varying intensities.

Of the esterases which hydrolysed α -naphthyl acetate and moved to the anode under the experimental conditions used, one was identified as cholinesterase and one as carboxylesterase in each species. The bands due to the activity of these esterases in both species partly overlapped. The mobility of nearly all the esterases revealed in A. fabae homogenates was higher than in A. pisum.

The possibility that some esterases moved to the cathode was not ruled out. However previous tests in aphid esterases in the same conditions used here showed that there were none that behaved as cations (Sudderuddin^{K.I.}, personal communication).

It has been shown, that enzymes other than ChEs and carboxylesterases can also split α -naphthyl acetate. These enzymes may be arylesterases (3.1.1.2) (Menzel et al., 1963), acid (3.1.3.2) or alkaline (3.1.3.1) phosphatases (Cook and Forgash, 1965). According to the classification of Oosterbaan and Jansz (1965), the dichlorvos-insensitive enzymes must be labelled as C-type esterases (or arylesterases) which neither hydrolyse nor are inhibited by O-P compounds.

Accordingly the bands E_1 , E_2 and E_3 in A. pisum and E_1 , E_2 , E_3 and E_6 in A. fabae are most possibly predominantly esterases of this group.

Apart from differences in mobility other qualitative differences in the cholinesterases and carboxylesterases can be seen in the zymograms obtained from the two species. The band due to cholinesterase activity was much more strongly stained in the case of A. pisum than in A. fabae. In the latter species however this esterase occupied a larger area. In contrast, carboxylesterase activity in A. fabae appears to be higher since the corresponding band is larger and more darkly stained than in A. pisum. It is not known if the ChE and carboxylesterase activities revealed in these experiments are due to a single form of each esterase or not. Work on housefly brain ChE by Eldefrawi (1970) has proved the polymorphism of this group of esterases.

Esterase patterns of two other aphid species, studied also with the use of polyacrylamide gel by Sudderuddin (1970, 1972), have shown that of the esteratic zones from M. viciae, one was typified as a ChE and two, one predominantly and one partly, as carboxylesterases. In organophosphorus-susceptible and -resistant strains of M. persicae he also found that of a total of 6 esteratic bands of each strain one was due to ChEs, two to carboxylesterases and one exhibiting both carboxylesterase and arylesterase action. The remaining two esteratic zones were formed because of arylesterase and possibly some cholinesterase activity. Comparing the esterase profiles in all four "susceptible" aphids it is evident that considerable variability exists in the different genera. Ahmad (1968) and Collins and Forgash (1968) in studying the carboxylesterases of susceptible and resistant strains of houseflies concluded that esterase patterns varied a lot between and within strains and "no clear-cut consistent differences were observed between the

two strains". However the last part of this statement contradicts the conclusions of other workers and is referred to above (page 106)

A.2. QUANTITATIVE MEASUREMENTS OF ESTERASE ACTIVITIES

A.2.1. TITRATION OF CHOLINESTERASES AND CARBOXYLESTERASES.

Introduction

The titrimetric method for measurement of esterases, relies on the fact that when an enzyme acts upon an ester a carboxylic acid is produced which is directly proportional quantitatively to the amount of the ester (substrate) hydrolysed and can thus, under suitable experimental conditions, give an indication of the activity of the esterase. This activity can be expressed as microlitres of alkali needed to neutralize the acid produced or as μM of substrate split by the homogenate in a given period. For substrates like acetylcholine and ethyl butyrate, this has the following relationship (Ahmad 1968) : $100 \mu\text{l}$ of $0.05 \text{ M NaOH} \equiv 5 \mu\text{M}$ of substrate hydrolysed.

In the present work the esterase activity was assayed following the titrimetric method described by Glick (1937) and Alles and Hawes (1940) and adopted by Shatoury (1963), Ahmad (1968) and Sudderuddin (1972).

Materials and Methods

Insect homogenates. 145 mg of adult aphids of each species were homogenized with 3 ml deionized distilled water, as described in the previous experiments. Vacuum assisted filtration through a muslin cloth followed and the volume of the homogenate was made up to 4.5 ml

Assay of esterase activity. Acetylcholine chloride was used as the substrate for the ChEs and ethyl butyrate for carboxylesterases. In both cases two electrolytes, MgCl_2 and NaCl , were used as activators of the enzymes (Shatoury, 1963). Reaction vessels of 15 ml capacity were used and the total initial working volume per vessel was 12 ml made up as follows:

Phosphate buffer (0.1M)	1.2 ml ,	final conc. 10^{-2}M
MgCl_2 (0.1M)	1.2 ml ,	" 10^{-2}M
NaCl (1.0M)	2.4 ml ,	" $2.0 \times 10^{-2}\text{M}$
Acetylcholine chloride (0.15M)	1.2 ml ,	" $1.5 \times 10^{-2}\text{M}$
or Ethyl butyrate (0.3 M)	1.2 ml ,	" $3.0 \times 10^{-2}\text{M}$
De-ionised, dist. water	4.0 ml ,	
Insect homogenate	1.0 ml ,	supernatant from 322 mg of insects

The homogenate was always added last and after mixing was incubated initially for 5 minutes at 37°C in a water bath and then adjusted to pH 7 by adding 0.05 M NaOH . It was then incubated for 30 minutes under continuous shaking at the same temperature and the released acid titrated with 0.05 M NaOH solution, until pH 7 was reached. The amount of alkali utilized per ml of homogenate was recorded as an index of esterase hydrolysis. Two replicates of each estimation were carried out.

Results

The results are presented in Tables 3.2 and 3.3. It is evident that the amount of alkali needed to titrate the free acid derived from the hydrolysis of acetylcholine chloride by the action of ChEs was higher in A. pisum than in the A. fabae homogenates (relationship 1:0.57),

whereas the activity of the carboxylesterases was nearly the same in both species (relationship 1:1.04). The activity of the carboxylesterases in splitting ethyl butyrate was much higher in comparison to the ChEs activity in both species, covering 95.5% of the combined ChE and Car-E activity in A. pisum and 97.5% in A. fabae.

Table 3.2 Cholinesterases, hydrolysing acetylcholinechloride, and carboxylesterases, hydrolysing ethyl butyrate, in homogenates of the two species.

Substrate	μl of 0.05M NaOH						Relation- ship
	<u>A. pisum</u>			<u>A. fabae</u>			
	I	II	mean	I	II	mean	
Acetylcholine chloride	30	40	35	20	20	20	1:0.57
Ethyl butyrate	830	670	750	820	740	780	1:1.04
Relationship	1:21.4			1:39.0			

Table 3.3 Percentage of esterase activities in the two species.

Esterases	<u>A. pisum</u>	<u>A. fabae</u>
Cholinesterases	4.5	2.5
Carboxylesterases	95.5	97.5

A.2.2. COLORIMETRIC MEASUREMENT OF ESTERASE ACTIVITY

Introduction

The introduction of colorimetry to the study of esterases marks an important advance in the refinement of enzyme classification, which up till then had relied on titrimetric or gasometric methods. Simplicity and sensitivity are the main advantages of colorimetric techniques. The substrates used are either directly chromogenic nitrophenol esters, or the colour is developed in a second step by azo coupling to phenolic and naphtholic substrates.

In 1949 the hydroxamic acid technique was published by Hestrin. This method is based on the discovery that hydroxylamine at an alkali pH in water rapidly converts acetylcholine stoichiometrically to hydroxamic acid throughout a wide range of ester concentrations according to the reaction:

$$\text{RCOOR}' + \text{H}_2\text{NOH} \rightarrow \text{RCONHOH} + \text{R}'\text{OH}$$

(acetylcholine) (hydroxylamine) (hydroxamic acid)

The hydroxamic acid produced is estimated colorimetrically. Although the action of ChEs and Car-Es on specific substrates such as acetylcholine chloride and ethyl butyrate can be studied by this direct method, it is on the other hand less sensitive when hydrolysis rates are low (Gomori, 1953).

In 1953, Gomori published a sensitive method for the quantitative assay of esterases in mammalian material, based on the colorimetric estimation of naphthol that is formed from the hydrolysis of naphthylesters. Asperen (1962) using the same technique with slight modifications studied the hydrolysis of naphthyl esters by housefly esterases and the interaction of these enzymes with some inhibitors. To study the esterase activity of S- and R-strains of M. persicae Needham and Sawicki (1971) and Sudderruddin (1972) also used this

technique. In this study this colorimetric method was used for quantitative determinations of esterase activities in A. pisum and A. fabae

The chief factors which determine the initial velocity of a particular reaction are enzyme and substrate concentration, pH, temperature and the presence of activators or inhibitors (Dixon and Webb, 1964). To find the optimum conditions for assessing the enzyme activities in the aphids used in this study, tests were carried out in which the enzyme activities were measured when temperature, enzyme and substrate concentration were varied. The effect on inhibition by eserine sulphate acting at different concentrations and for varying times was also measured.

Materials and Methods

Enzyme preparations. The insect homogenates were prepared in deionized distilled water and for the preliminary experiments on the effect of homogenate concentration, were diluted to give concentrations ranging from 0.125 to 4.0 mg of aphid fresh weight per ml. In subsequent experiments dilutions of 2 mg A. pisum or 1 mg A. fabae per ml were used. These quantities represent approximately the body-weights of the adults.

Substrate solution. A series of α -naphthyl acetate concentrations were tested to measure the hydrolytic activity of the esterases. As a usual practice this substrate was used at the concentration of 2×10^{-4} M. β -naphthyl acetate was also tested at the concentration 2×10^{-4} M. The above naphthyl esters were diluted in 1 ml acetone and the volume was made up to 100 ml with 0.04 M phosphate buffer at pH 7. The substrate solution was always freshly prepared before each assay.

Inhibitor. Eserine sulphate was used to inhibit the cholinesterase fraction of the total esterase activity. Different concentrations of this inhibitor in phosphate buffer and different preincubation times of the enzymes with the inhibitor were tested. Following these preliminary tests this inhibitor was used at 10^{-6} M and was allowed to react with the enzymes for 5 min. before the substrate was added.

Experimental conditions. The quantities of the above constituents which were added per test-tube were as follows:

	<u>Blank</u>	<u>Control</u>	<u>Inhibitor</u>
Deionized distilled water	1 ml	-	-
Insect homogenate	-	1 ml	1 ml
Phosphate buffer	1 ml	1 ml	-
Eserine solution	-	-	1 ml
Substrate solution	<u>4 ml</u>	<u>4 ml</u>	<u>4 ml</u>
Total	6 ml	6 ml	6 ml

Deionized distilled water was added to the blank to replace the insect homogenate. Phosphate buffer was added to the blank and control tubes instead of the inhibitor solution, as preliminary experiments showed that the blank with or without eserine solution gave the same optical density. The incubation was carried out in a water bath at 37°C under continuous shaking. This temperature was found to be within the optimum range for the hydrolytic activity of the esterases of the two aphids on α - and β -naphthyl acetate. The incubation of the mixtures was carried out for 30 minutes.

Staining solution. At the end of the incubation time the reaction was stopped by the addition of diazoblue-sodium lauryl sulphate solution (DBLS). This solution consisted of 2 parts of 1% diazoblue B solution (tetrazotized di-o anisidine, E. Gurr, London, Michrome No. 250) and

5 parts of a 5% solution of Na-lauryl sulphate (pure, BDH). The mixture was always freshly prepared and kept in the dark.

α -naphthol gives an intense red colour with the DBLS solution which quickly changes to a fairly stable blue one, whereas β -naphthol gives a stable red colour. A "Unicam" SP1300, series 2 colorimeter was used to measure the optical densities after the DBLS solution was added. A spectrum red filter, transmission range from 620 nm upwards (Ilford No. 608) was used for α -naphthol and a spectrum bright yellow filter having a transmission range 545 to 685 nm (Ilford No. 626) for β -naphthol was used.

Estimation of the esterase activity. The cholinesterase activity was estimated by subtraction of the activity found in the samples treated with eserine from the overall esterase activity measured in the controls. Since only comparative measurements are necessary, the readings of optical density derived from the α - or β -naphthol produced, are taken as a measure of the reaction (Dixon and Webb, 1964). However the observed optical densities can be transformed to μ g of naphthol produced. A calibration curve for α -naphthol was worked out plotted from the optical densities of a series of α -naphthol dilutions in 6 ml of 1% acetone and 0.04M phosphate buffer at pH 7 to which 1 ml of DBLS solution was added. The line obtained proved to be linear (Appendix 20). In these esterase assay experiments there were two to three replicates for each estimation.

Results

1. Effect of homogenate concentration. Homogenates containing 0.125, 0.25, 0.5, 1.0, 2.0 and 4.0 mg of insect per ml were used. The results are given in Fig.3.2. The increase in enzyme concentration leads to an increase in the activity of carboxylesterases in both species. In A. pisum the ChE activity was increased up to 2.0 mg of insect material per ml, while in A. fabae the activity of these esterases was low and nearly the same at all the enzyme concentrations tested.

2. Effect of incubation temperature. The ChE and Car-E activities of both species towards 2×10^{-4} M α - or β -naphthyl acetate were studied at 22, 27, 32, 37 and 42°C incubation temperatures. The hydrolytic activity of these two groups of enzymes in both species on the two substrates tested was in general increased up to temperatures of 32 or 37°C. The ChE activity on α -naphthyl acetate in both species was higher at 32 to 37°C, and at 42°C was significantly reduced. The Car-E activity, especially in A. pisum increased steadily with increasing temperature (Fig.3.3). The ChEs, especially of A. pisum, failed to hydrolyse β -naphthyl acetate at all the temperatures tested. In contrast the Car-E activity of both species on this substrate was higher than with α -naphthyl acetate. The optimum temperature for Car-E activity with β -naphthyl acetate layed also between 32 and 37°C (Fig.3.4). Accordingly, α -naphthyl acetate was used as the substrate in subsequent experiments and the incubation temperature maintained at 37°C.

3. Effect of α -naphthyl acetate concentrations on the esterase activity of the two species. The rate of hydrolysis of α -naphthyl

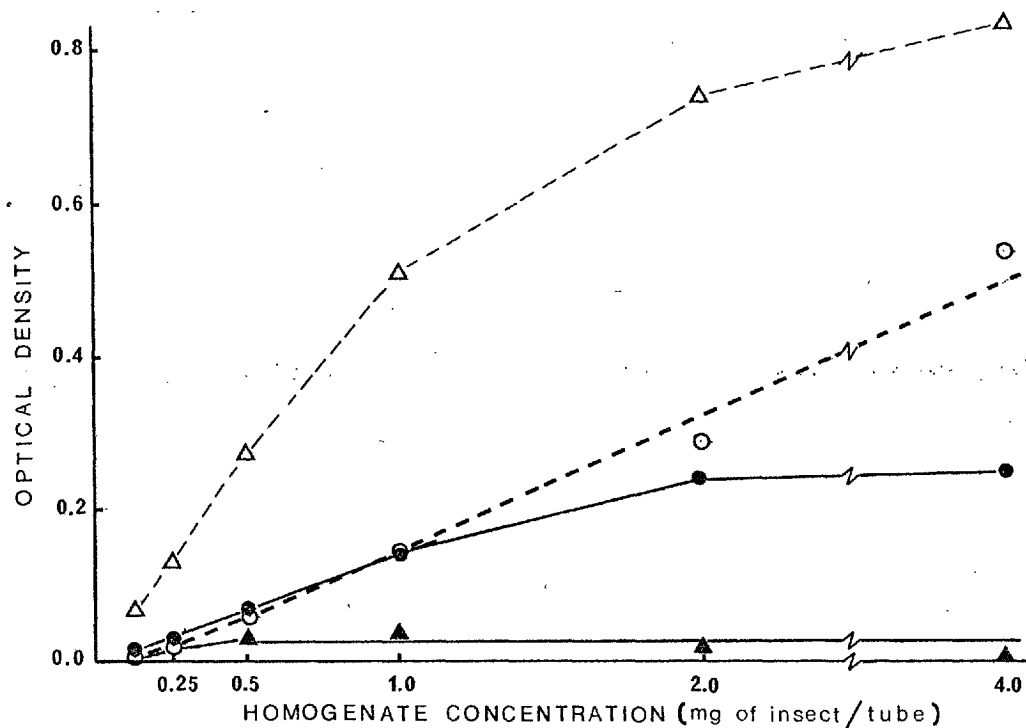


Fig. 3. 2. The influence of homogenate concentration on the esterase activity in A. pisum and A. fabae to α -naphthyl acetate $2 \times 10^{-4}M$ (see Appendix 21)

	<u>A. pisum</u>	<u>A. fabae</u>
ChE activity	●—●	▲—▲
Car-E "	○---○	△---△

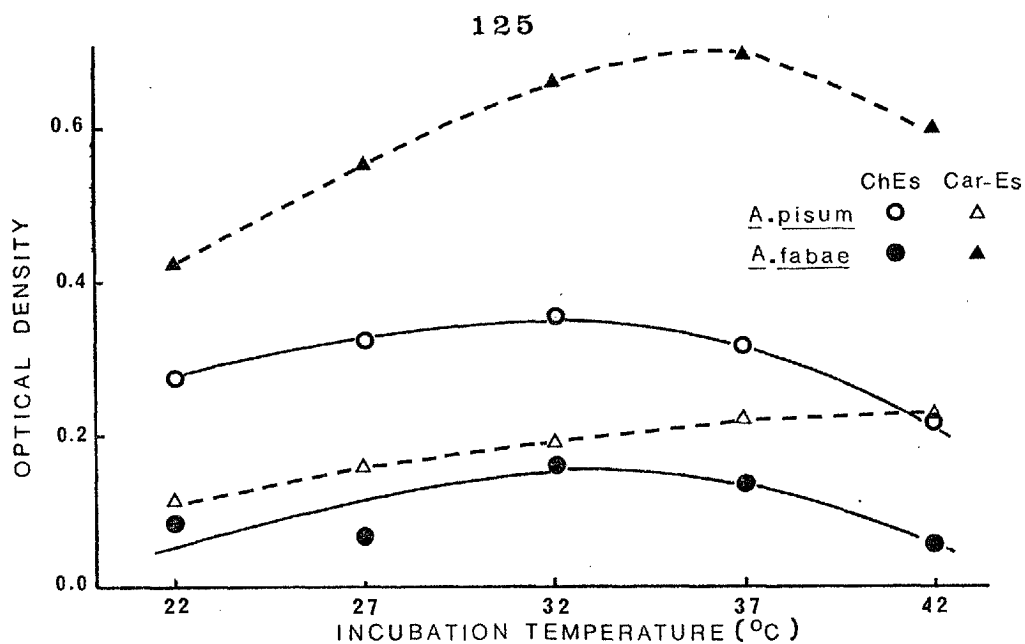


Fig. 3.3. Effect of incubation temperature on esterase activities in A. pisum and A. fabae to α -naphthyl acetate $2 \times 10^{-4}M$ (see Appendix 22)

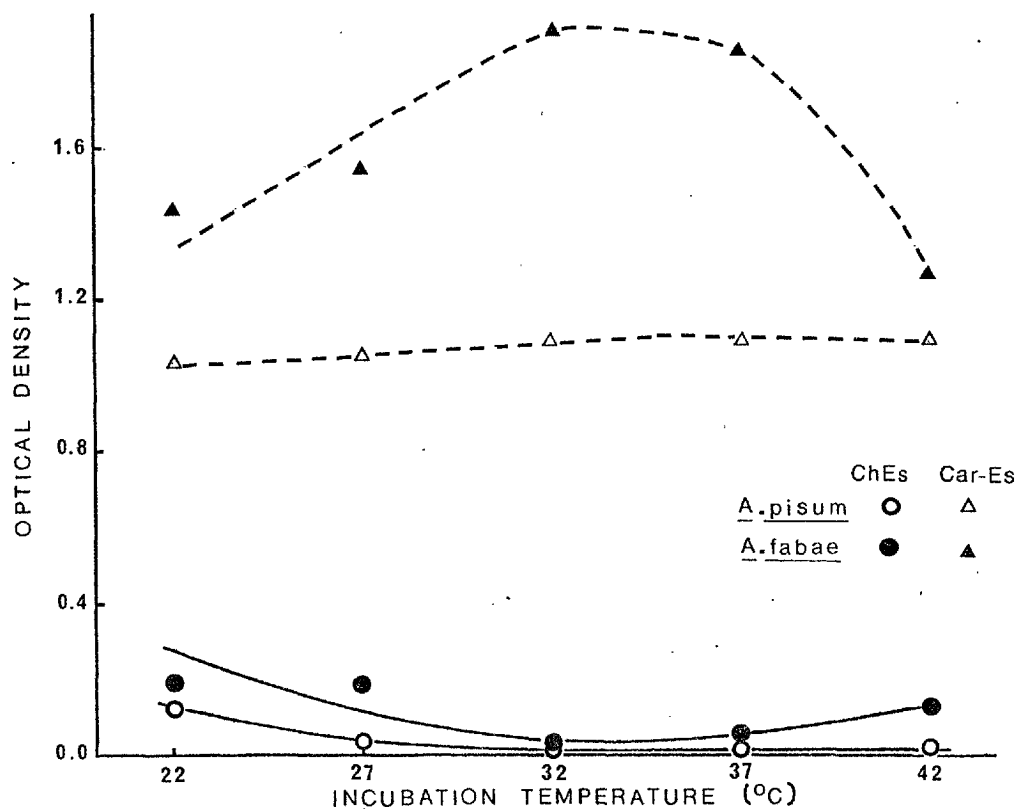


Fig. 3.4. Effect of incubation temperature on esterase activities in A. pisum and A. fabae to β -naphthyl acetate $2 \times 10^{-4}M$ (see Appendix 22).

acetate by the ChEs and Car-Es was determined by incubating homogenates of the two species with a series of substrate concentrations, 0.5, 1.0, 2.0, 4.0, 8.0 and 16.0×10^{-4} M. The data obtained and the respective substrate concentrations were transformed to their reciprocals and plotted in a Lineweaver-Burk plot as described by Dixon and Webb (1964). From these graphs Michaelis-Menten constant (K_m) for α -naphthyl acetate were obtained for each esterase type. The reciprocal O.D. values where $1/S = 0$ correspond to the maximum velocity of the reaction in each case. These values and the slope, K_m/V , of each line were obtained.

According to the results the ChE and Car-E activities of the two species towards α -naphthyl acetate increased as the substrate concentrations increased, approaching the asymptote with substrate concentrations above than 4.0×10^{-4} M (Fig. 3.5.) The rates of hydrolysis of α -naphthyl acetate by the ChEs of the two species were very different and were higher in the case of A. fabae, whereas the hydrolysis by the Car-Es of the two species took place at the same rate (Fig. 3.6.). The K_m value for the above substrate for the ChEs of A. pisum was 0.30×10^{-4} M and of A. fabae 0.83×10^{-4} M whereas for the Car-Es of both species K_m was 0.48×10^{-4} M.

4. Effect of eserine sulphate on the inhibition of esterase activity.

Two aspects of the inhibitory action of eserine sulphate were studied. In one series of tests the influence of different concentrations of this inhibitor on the esterases was examined. Concentrations ranging from 10^{-9} to 10^{-5} M eserine sulphate were used with a 5 minute preincubation time. In the other series the importance of the preincubation time of the homogenate/inhibitor mixture was studied. A single concentration 10^{-4} M of eserine was allowed to react with homogenates of the two species for different times up to 80 minutes

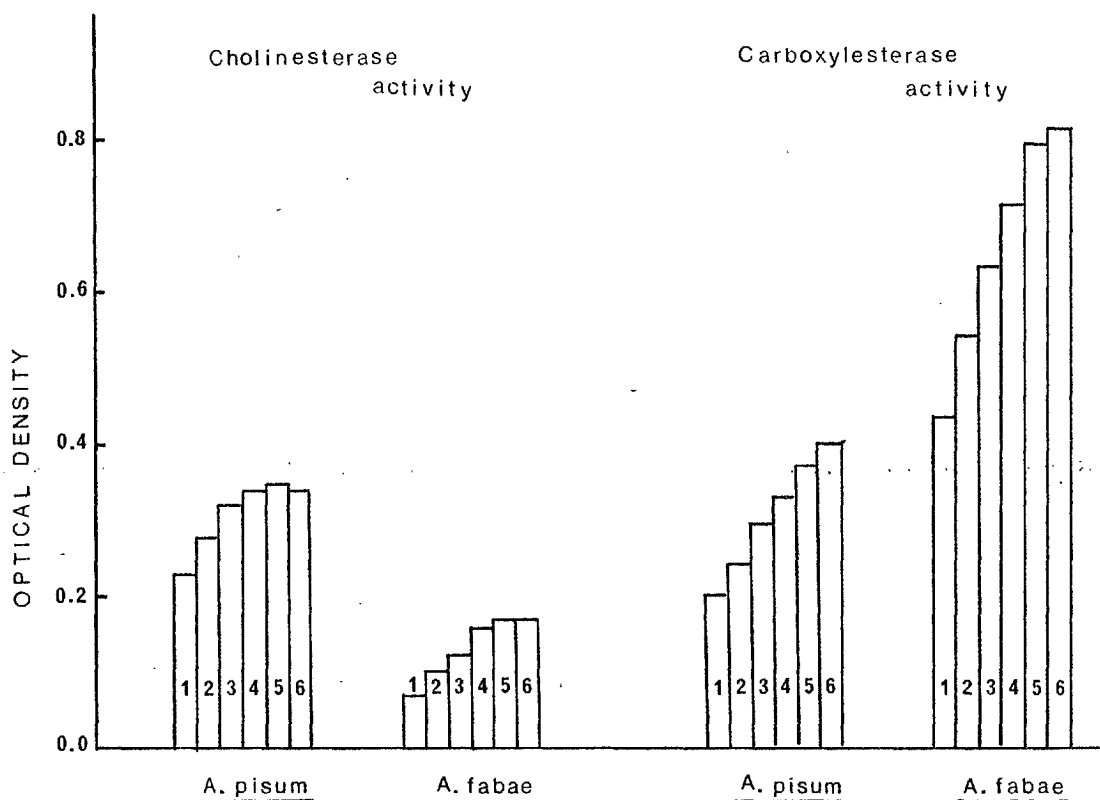


Fig. 3.5. The influence of substrate concentration on the esterase activities in A. pisum and A. fabae (see Appendix 23)

α -naphthyl acetate concentrations	1:	$0.5 \times 10^{-4} \text{M}$
	2:	$1.0 \times 10^{-4} \text{M}$
	3:	$2.0 \times 10^{-4} \text{M}$
	4:	$4.0 \times 10^{-4} \text{M}$
	5:	$8.0 \times 10^{-4} \text{M}$
	6:	$16.0 \times 10^{-4} \text{M}$

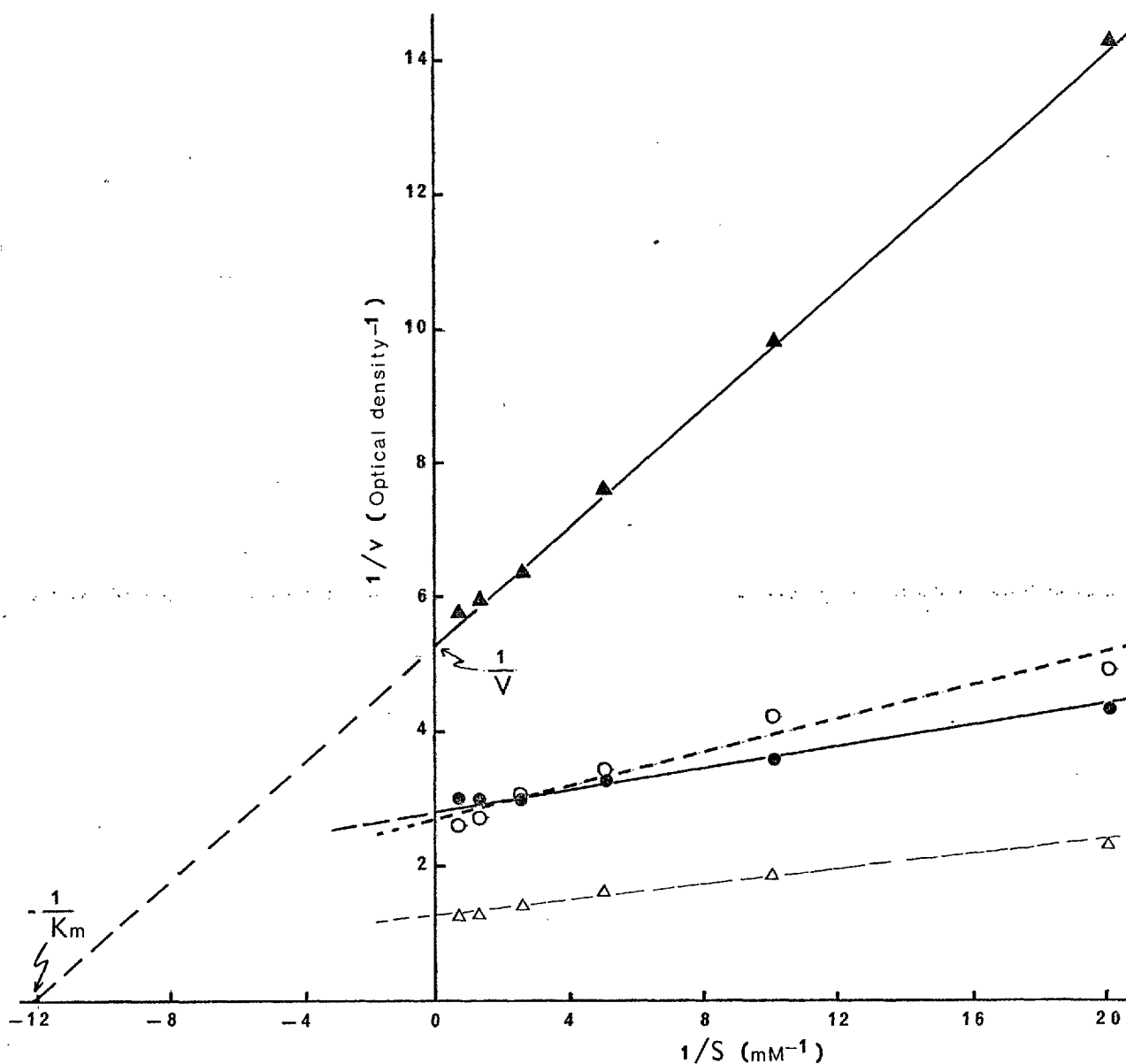


Fig. 3. 6. Double reciprocal plot, $1/v$ vs. $1/S$, for the reactions catalysed by the esterases of A. pisum and A. fabae (substrate α -naphthyl acetate; incubation at 37°C for 30 mins; see Appendix 23)

		K_m	V	Slope (K_m/V)
ChEs:	<u>A. pisum</u> (●—●)	$0.30 \times 10^{-4} \text{ M}$	0.35	0.084
	<u>A. fabae</u> (▲—▲)	$0.83 \times 10^{-4} \text{ M}$	0.18	0.440
Car-Es	<u>A. pisum</u> (○-○)	$0.48 \times 10^{-4} \text{ M}$	0.37	0.130
	<u>A. fabae</u> (△-△)	$0.48 \times 10^{-4} \text{ M}$	0.77	0.062

at room temperature, before the substrate was added. Incubation to assess esterase activity was carried out for 30 min. at 37°C.

From Fig. 3.7. it is evident that partial inhibition of the esterase occurred in both species at concentrations above 10^{-8} M eserine. The maximum inhibition of activity was achieved at a concentration 10^{-6} M in A. pisum whereas in A. fabae the inhibition continues to increase from 11.4% inhibition at 10^{-6} M eserine to 20.9% at 10^{-5} M. The time of preincubation with 10^{-6} M eserine solution did not appear to have any effect on the esterase activity of A. fabae homogenates, as can be seen from Fig. 3.8. Thus, the hydrolytic activity of these homogenates on α -naphthyl acetate was decreased by the same small proportion, less than 10%, after preincubation for different times up to 80 min. In A. pisum increased preincubation times did affect the hydrolytic activity on α -naphthyl acetate. There was a 21% decrease of the activity when no preincubation period was allowed and maximum inhibition about 46% was reached after a 20 minutes preincubation. In subsequent experiments 10^{-6} M eserine was used and the enzyme was preincubated for 5 min. prior to the reaction with the substrate solution.

5. Progress of esterase activity with time. The hydrolysing activity of ChEs and Car-Es on α -naphthyl acetate was measured with time. A number of reaction tubes were set up and the reaction was stopped after 5, 10, 15, 20, 25 and 30 min. of incubation by the addition of 1 ml per tube of DBLS solution. The optical densities obtained were transformed to μ g of α -naphthol produced, following the calibration curve of Appendix 20. The results are demonstrated in the Fig. 3.9. The cholinesterase activity in A. pisum adults was higher than in A. fabae. Thus after 30 minutes incubation the ChE activities in A. pisum and A. fabae were in the ratio 1:0.17 per adult or 1:0.34

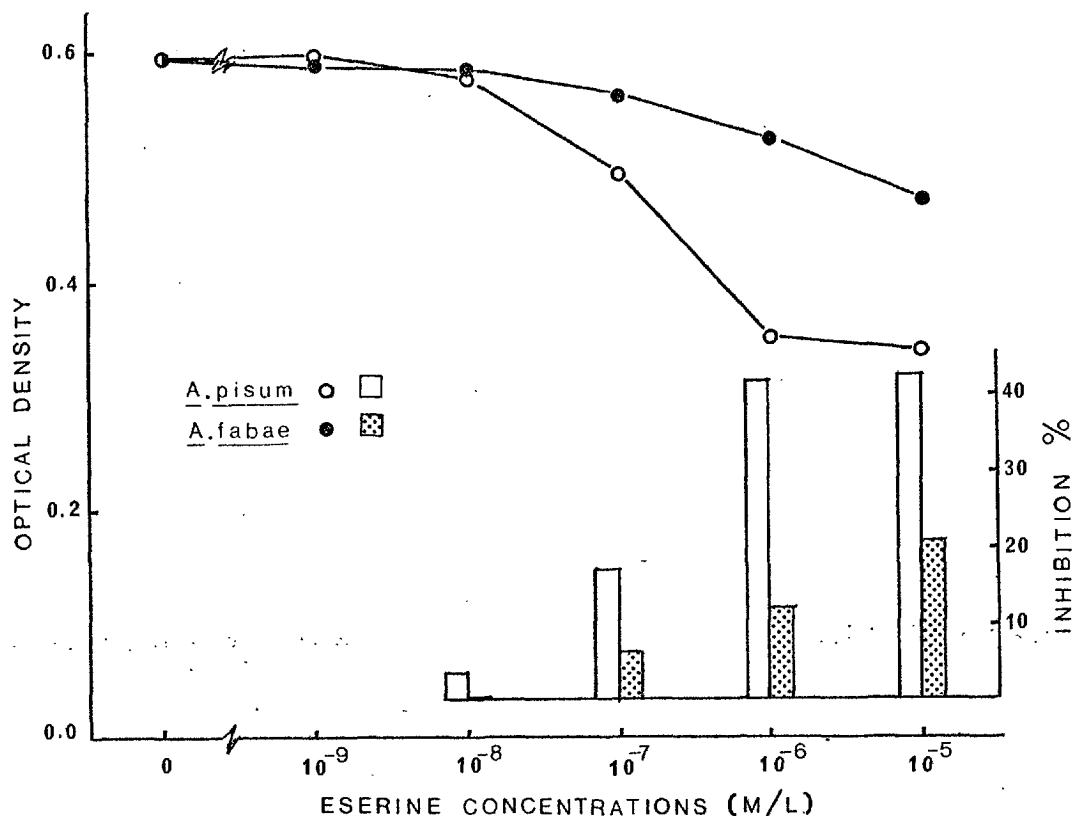


Fig. 3.7. Esterase activity and degree of inhibition after treatment of homogenates of the two species with different concentrations of eserine sulphate (substrate, α -naphthyl acetate 2×10^{-4} M; incubation at 37°C for 30 min; see Appendix 24).

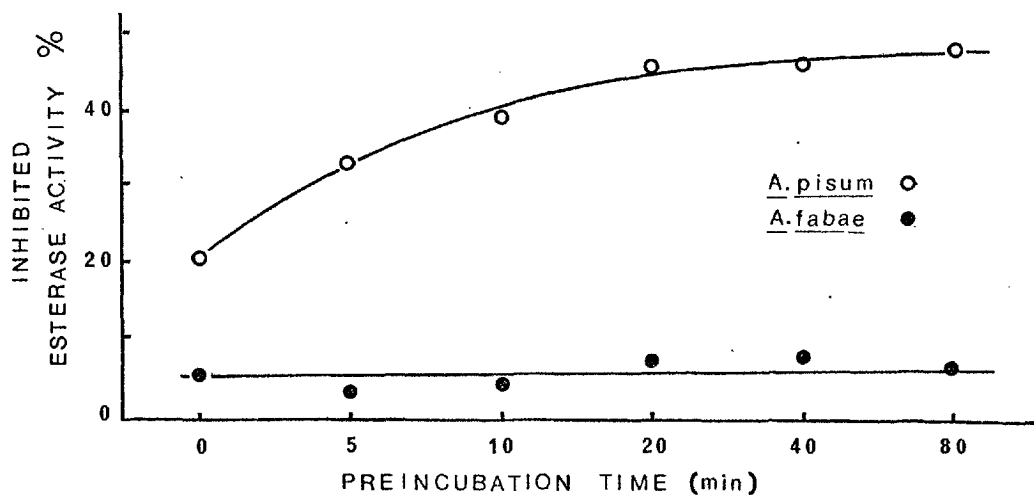


Fig. 3.8. Inhibition of esterase activity by 10^{-6} M eserine at different preincubation times (substrate, α -naphthyl acetate 2×10^{-4} M; see Appendix 25)

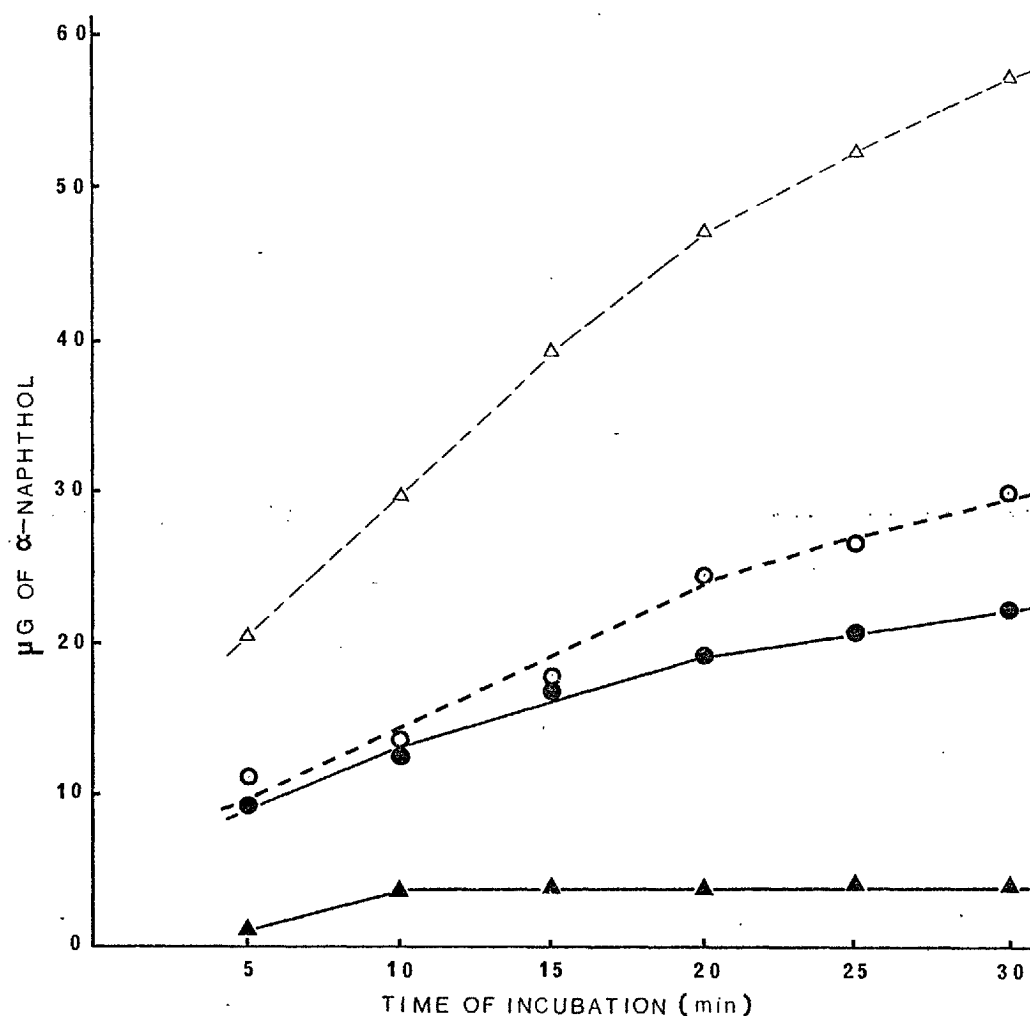


Fig. 3.9. Esterase activities of *A. pisum* and *A. fabae* for different incubation times at 37°C towards α-naphthyl acetate (conc. 2×10^{-4} M; see Appendix 26).

	<u><i>A. pisum</i></u>	<u><i>A. fabae</i></u>
ChE activity	●—●	▲—▲
Car-E "	○---○	△---△

per mg of adult. The activity of these esterases (ChEs) in A. pisum was increased 6.8 to 19.1% of the observed activity after 30 min. incubation, for every 5-minute increase of incubation time. In A. fabae the low ChE activity was stable, after a small increase in the first 10 minutes.

The level of carboxylesterase activity was the reverse in the two species and higher in A. fabae than in A. pisum. After 30 min. this activity in the two species was in the ratio A. pisum : A. fabae = 1:1.91 per adult and 1:3.82 per mg of adult. The Car-E activity increased with time in both species. For every 5-minute increase of the reaction time the activity was increased from 7.1 to 22.5% in A. pisum and from 8.4 to 16.7% in A. fabae.

Discussion

In both methods used for quantitative measurement of the esterase activity, the cholinesterases in A. fabae was found to represent only a small fraction of the combined ChE and carboxylesterase activity, 2.5% with the titrimetric procedure and 6.1 to 17.3% in the colorimetric method. The activity of these esterases with the two methods of estimation also proved to be lower in A. fabae than in A. pisum (relationship 1:0.55 by titration and 1:0.13 to 1:0.31 by colorimetry). In contrast in A. pisum the esterase activity was found to be quite different with the two methods. Thus, the ChEs represented only 4.5% by titration and as much as 42.5 to 59.1% of the combined ChE and Car-E activity in the colorimetric method (see Table 3.4.)

Table 3.4. Relative cholinesterase (ChE) and carboxylesterase (Car-E) activities within the same species and between the two species after incubation of the same weight of insect material per assay, for 30 min. at 37°C.

Method of Assay	Esterase activities %				Relationship	
	<u>A. pisum</u>		<u>A. fabae</u>		<u>A. pisum</u> : <u>A. fabae</u>	
	ChE	Car-E	ChE	Car-E	ChE	Car-E
Titration	4.5	95.5	2.5	97.5	1:0.55	1:1.02
Colorimetry	42.5	57.5	6.1	93.9	1:0.14	1:1.63
"	50.0	50.0	6.4	93.6	1:0.13	1:1.87
"	59.1	40.9	15.9	84.1	1:0.27	1:2.05
"	55.8	44.2	17.3	82.7	1:0.31	1:1.86

The very high Car-E activity found with the titrimetric method shows possibly that these esterases hydrolyse the specific substrate ethyl butyrate more efficiently.

In both methods the incubation was carried out at the same temperature and for the same time. The initial pH was also the same in both techniques. However different substrates were used in the two methods and as Dixon and Webb (1964) state, crude preparations of enzymes from different sources show different specific patterns and it is not clear whether this is to be interpreted as due to variants of the enzymes themselves, or to mixtures of different enzymes in various proportions. It is possible therefore, that in the homogenates of the two species there were different affinities of each type of esterase to the specific substrates used in the titrimetric method.

In the colorimetric method the activity corresponding to ChEs was the one which could be inhibited with eserine. The two types of esterases therefore, both acting on α -naphthyl acetate were distinguished after treatment of the homogenate with 10^{-6} M eserine. The higher ChE activity measured with the colorimetric method in both species, especially in A. pisum, is due possibly to an extended inhibitory action of eserine to other esterases and not only ChEs. It is unlikely however, that any inhibitory action of eserine to esterases other than ChEs could be found to occur at the concentration of the inhibitor used here. This inhibitor has been used extensively by many workers for the selective inhibition of ChE activity and in addition the most clear-cut and distinctive feature between ChEs and Car-Es is the markedly different sensitivity of these esterases to eserine and similar alkaloids (Mendel and Rudney, 1943; Gomori, 1953), to which ChEs are about a thousand times more sensitive than Car-Es. Also Easson and Stedman (1937)

and Richter and Croft (1942) in their conclusions on the effect of eserine sulphate on esterases hydrolysing α -naphthyl acetate, noted that Car-Es were very little influenced even by 10^{-3} M eserine, whereas according to van Asperen (1959) concentrations of eserine sulphate up to 10^{-5} do not affect Car-E activity. In the present work it was found also that eserine sulphate added to A. pisum homogenate at a concentration 10^{-5} M failed to increase the proportion of inhibited esterases above that found at 10^{-6} M. This last point will be discussed again later. However Sudderuddin (1972) found that 10^{-7} M eserine sulphate more or less completely blocks ChE activity of susceptible and resistant strains of M. persicae and concludes that higher concentrations of this inhibitor must also inhibit carboxylesterases.

In connection with these it is perhaps worth noting here that the rough quantitative picture of the two groups of esterases obtained in the electrophoresis zymograms of the two species was in closer agreement with the results of the colorimetric method, where A. pisum was found to have a much higher ChE activity and lower Car-E activity than A. fabae.

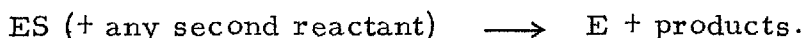
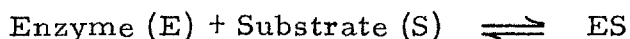
In studying the hydrolytic activity of increasing concentrations of esterases on α -naphthyl acetate it was found that the Car-Es of both aphids showed substantially increased activity while of the ChEs, only those of A. pisum increased in activity with increasing concentrations and even then only slightly. For any enzyme there is an optimum concentration at which maximum activity occurs on a certain substrate under a given temperature and other experimental conditions. It seems that the optimum concentrations of ChEs for the hydrolysis of α -naphthyl acetate is not the same in the two aphid species.

The optimum temperature in enzyme reactions is determined by the balance between the effect of temperature on the rate of the enzyme reaction and its effect on the stability of the enzyme. The rate of Car-E activity of the two species on α -naphthyl acetate was increased with the increase of temperature, whereas the activity of these esterases on β -naphthyl acetate was quite different in the two species. In A. pisum Car-E activity remained at the same level at all the tested temperatures, but in A. fabae it was reduced in high temperature (42°C). The ChE activity of both species to α -naphthyl acetate was reduced at temperatures higher than 32° to 37°C , whereas the hydrolytic activity of these esterases towards β -naphthyl acetate remained low in both species and nearly at the same level at all the tested temperatures.

Differences in the Car-E activity in organophosphorus-susceptible and -resistant strains of houseflies to different substrates were found also by van Asperen (1962). Thus, the activities to α -naphthyl acetate were much higher for O-P-susceptible than for O-P-resistant flies (relationship 1:7), but no such difference was found if β -naphthyl acetate or indophenyl acetate were used as substrates. Also in O-P-susceptible and -resistant strains of M. persicae differences in Car-E activity existed only when α -naphthyl acetate was used as substrate. There was no appreciable difference in the Car-E activity of the two strains towards β -naphthyl acetate (Sudderuddin 1972). Because the esterase activity revealed is dependant upon the substrate involved, Augustinsson (1968) expressed the view that all work on esterases must involve at least two substrates.

Substrate concentration is one of the most important factors which determine the velocity of enzyme reactions. The theory developed by Michaelis and Menten in 1913, and referred to by

Dixon and Webb (1964), adopts the suggestion that the enzyme first forms a complex with its substrate and this subsequently breaks down giving the free enzyme and the products of the reaction:



Although the level of ChEs activity in A. fabae was found to be lower than in A. pisum, the rate of hydrolysis of α -naphthyl acetate by the former is much higher, the corresponding K_m values were $0.83 \times 10^{-4} \text{ M}$ and $0.30 \times 10^{-4} \text{ M}$ respectively. This is a strong indication that in addition to the quantitative differences already found, qualitative differences also exist between the ChEs of the two species. Thus, much lower concentrations of A. fabae ChEs hydrolysed increasing quantities of the substrate α -naphthyl acetate more rapidly than higher concentrations of A. pisum cholinesterases. In contrast, the differences in the carboxylesterases of both species, which have the same K_m values ($0.48 \times 10^{-4} \text{ M}$) were only quantitative.

Studies of the effect of the carbamate inhibitor eserine sulphate on the esteratic activity of the two species, give further indications of qualitative differences existing between the ChEs of these aphids. Thus, while the inhibitory action of this compound on A. pisum esterases was complete at 10^{-6} M eserine, in the case of A. fabae esterase inhibition was still increasing with concentration at 10^{-5} M eserine. If we assume that ChEs are mostly inhibited by eserine up to concentrations of the order of 10^{-5} M , then the ChEs of A. fabae are more insensitive to the inhibitor than the ChEs of A. pisum and higher concentration of the inhibitor (10^{-5} M) can only improve the inhibitory action. Furthermore, when preincubation time of homogenates with the inhibitor was increased from 0 to 80 min, no increased inhibition was observed in A. fabae, while in A. pisum esterases were more liable to be inhibited with increased preincubation time.

SECTION B. INTERACTION BETWEEN INSECT MATERIAL AND
ORGANOPHOSPHORUS INSECTICIDES

Introduction

The whole aspect of the toxic action of many insecticides has been thrown open to discussion recently by the work of O'Brien (1970), Casida and Maddrell (1971), Maddrell and Casida (1971). However the toxic effect of organophosphorus compounds to both vertebrates and invertebrates generally is considered to be due to their ability to inhibit cholinesterases with subsequent disruption of nervous activity caused by the accumulation of acetylcholine at the nerve endings. The role of carboxylesterases also seems to be very important in the intoxication mechanism and differences of this type of esterase were demonstrated in susceptible and resistant strains of many insect species.

In order to demonstrate the enzyme inhibition aspects of the toxic effect of a compound it is necessary to show that the compound has a strong inhibitory action on the enzyme in vitro and that this enzyme in the living insect is actually inhibited at the time that symptoms of intoxication appear (Asperen, 1958a).

If an O-P compound is added to an enzyme homogenate it will compete with ranging degrees of success for the esterases present. Consequently the inhibition which any particular esterase undergoes will not only depend on the characteristics of its own reaction with the O-P compound, but also on the presence of other esterases and phosphatases in the homogenates. In crude homogenates of O-P - susceptible and -resistant houseflies, several components reacting

with O-P compounds have been found: A ChE, splitting acetylcholine, a Car-E, splitting methyl and phenyl butyrate, a phosphatase, splitting diazoxon and paraoxon, and other compounds most probably proteins (Asperen and Oppenoorth, 1960).

Esterases react with O-P compounds in a mole to mole reaction, resulting in both inactivation of the enzymes and elimination of the O-P compounds. Although this reaction is complex, it may be depicted simply as occurring in two steps: 1) phosphorylation of the enzyme and 2) hydrolysis of the phosphorylated enzyme. In many cases the latter reaction is extremely slow and the enzyme is very slowly regenerated, thus the O-P compounds are considered as inhibitors (Dixon and Webb, 1964).

In in vitro reactions an additional substance besides the enzyme and substrate is required in many cases in order that the reaction may proceed. Such "cofactors" are found unchanged at the end of the reaction and may therefore be regarded as an essential part of the catalytic mechanism. The majority of enzyme cofactors may be broadly divided into two classes: a) specific coenzymes, organic rather complicated molecules such as Nicotinamide adenine dinucleotide (NAD), Nicotinamide adenine dinucleotide phosphate (NADP), which act by undergoing reduction and oxidation, etc. and b) activators, frequently of a very simple nature, e.g. inorganic ions, which in various ways bring the enzyme itself into a catalytically active state (Dixon and Webb, 1964). A mixture of cofactors was used in the study of oxydative metabolism of malathion by Matsumura and Brown (1961b), and reduced NADP and/or reduced glutathione (GSH) were used by Yang (1971) as cofactors in the in vitro metabolism of diazinon and diazoxon in O-P-susceptible and -resistant houseflies. When diazoxon was assayed enzymically by Gwiazda and Lord (1967) in susceptible

and resistant strains of houseflies, no cofactors were used.

O-P esters have been shown to be inhibitors of ChEs and also a number of other esterases, lipases, and proteolytic enzymes (O'Brien, 1960, 1961a, 1961b). Most of the strong selective organophosphorus insecticides are phosphorothionates (i. e. containing P=S group) and are relatively poor inhibitors of cholinesterases. However, in vivo they undergo enzymatic oxidation to the more potent anticholinesterases phosphate (P=O) analog^{ues}, in several tissues of insects. The presence of an O-P compound in the insect tissue can therefore result in both the inhibition of insect esterases and the enzymatic degradation of the compound. The unchanged compound or the esterase activity in the system can express the rate of the reaction.

In many insect species this interaction has been studied after application of O-P compounds in vivo and in vitro. For example, the insecticidal activity of 2 halogenated dialky phosphorothioates applied topically and by injection to B. pelegrina and C. megacephala were related to inhibition of fly-head ChEs in vivo and in vitro. (Chuo and Sherman, 1971). A direct correlation was found between insecticidal toxicity and head ChE inhibition in vitro but not in vivo. In all cases, maximum inhibition was somewhat higher in dead flies, than in surviving flies. When susceptible strains of D. melanogaster were poisoned with the O-P insecticides parathion and mintacol, their ChE activity declined very quickly; In resistant strains on the other hand, the activity of ChEs decreased only slowly. If however, ChE preparations from these different strains were incubated with the insecticides, no differences in inhibition rates were found between susceptible and resistant strains (Rusmuson and Holmstedt, 1958)

Although oxidation of O-P insecticides to the more potent anti-cholinesterase oxygen analogues usually is expected when the insecti-

cide is applied to the living insect, studies in different species have shown that the insecticidal toxicity is not always correlated to the reduction of the ChE activity in vivo. This possibly indicates different rates of oxidation between the species and differences in enzyme molecules which determine differential affinity for O-P compounds. In insect homogenates the tissue structure is destroyed and possibly when organophosphorus insecticides are applied to an in vitro homogenate oxidation in the mitochondria may not occur. It is quite likely therefore that the oxygen analogues of the O-P compounds cannot be formed in vitro to inhibit the esterase activity. However in some cases when O-P compounds were applied in homogenates they did produce reduction of esterases activity. This possibly indicates the variation of oxidative mechanism between the species.

In this study, the inhibition of esterase activity by O-P insecticides was examined after they were applied to living insects. The inhibitory action in vivo was related to the mortality produced by these compounds. Solutions of insecticides were also added to homogenates of the two species and the resulting inhibition of the ChE and Car-E activity was studied with time.

B.1. IN VIVO EFFECT OF ORGANOPHOSPHORUS INSECTICIDE
INHIBITORS ON CHOLINESTERASES AND CARBOXYLESTERASES.

Materials and Methods

Insecticidal treatment. Apterous adults of the two species were used in these experiments. Dimethoate in acetone and phorate or menazon in 2-ethoxyethanol were applied in volumes of 0.05 μ l/aphid or 5 μ l/disc. The insects to which insecticide had been applied topically were caged on leaf-discs kept moist with blotting paper (see page 94). Where the chemicals were applied systemically, the aphids were put on the discs 8 hrs. after the application of the insecticides to the blotting paper.

The doses of dimethoate applied topically were 0.375 ng/adult A. pisum and 1.50 ng/adult A. fabae. Samples of live and dead treated and untreated insects were collected 24 hrs. after application for esterase assay. Adults of A. pisum were treated also with 0.19 ng dimethoate per adult and samples of alive insects were taken immediately after application and then after 2, 4, 6, 18 and 24 hrs.

Phorate was applied to the two species both topically in doses ranging from 0.16 to 5.12 ng/insect and systemically ranging from 0.02 to 4.86 μ g per leaf disc. Samples of live and dead insects were collected 24 hrs. after their exposure to the chemical.

Menazon solutions were applied systemically to both species in treatments ranging from 0.06 to 1.62 μ g/disc. The LD₅₀ dose, 4.86 μ g/disc in A. pisum and 1.62 μ g/disc in A. fabae, was also applied. Samples of treated and untreated live and dead insects of both species were collected 24 hrs. after their exposure to insecticide.

Esterase assay. The insect samples were homogenized in a hand operated glass homogenizer in sufficient ice-cold deionized distilled water, to give 2 mg of A. pisum or 1 mg of A. fabae per ml of homogenate. Measurement of the esterase activity was performed by the colorimetric method as described in page 120, in which 2×10^{-4} M α -naphthyl acetate was used as substrate, 10^{-6} M eserine sulphate as inhibitor and the mixtures incubated at 37°C for 30 minutes.

Results

From preliminary experiments it was found that no significant reduction of the ChE and Car-E activity occurred in dead untreated insects of both species within 24 hrs. It is safe, therefore, to consider the reduction of the activity found in samples of dead insects after treatment with insecticide as being caused by these chemicals. This is further supported by the higher esterase activity found in untreated dead insects compared to dead treated ones. The results obtained are given in Figs. 3.10, 3.11, 3.12, 3.13, 3.14 and 3.15.

1. Treatment with dimethoate. The topically applied doses of dimethoate caused 48% mortality in A. pisum and 75% in A. fabae. The esterase activities were reduced almost equally in samples of live and dead insects 24 hrs. after their treatment in comparison to untreated ones. The reduction in activity of both esterases was greater in A. pisum than in A. fabae (Fig. 3.10). At different time intervals, after topical application of a sublethal dose of the above chemical to A. pisum a reduction only of the ChE activity was observed in the living insects. The Car-E activity remained the same in treated and untreated insects for the whole 24 hr. period (Fig. 3.11.).

2. Treatment with phorate. The ChE and Car-E activities of treated insects of both species, still living 24 hrs. after their exposure to a series of doses, were similar to the control insects, whether the compound was applied topically or systemically. However, reduction in activities of these enzymes occurred in samples of dead insects and the reduction was in general related to the dose applied (Figs. 3.12 & 3.13). Thus, a rapid decrease of ChEs was observed in samples of A. pisum which died of higher concentrations of phorate, whichever way the chemical was applied. In A. fabae, the ChE activity, already very low, was not so rapidly reduced at the higher phorate doses. In dead insects of this species however, the Car-E activity was reduced quickly and proportionally to the increase in dose.

3. Treatment with menazon. The cholinesterase activity in live treated adults of both species 24 hrs. after systemic application was slightly reduced, as the applied doses of menazon were increased (Fig. 3.14). When the LD₅₀ dose was applied systemically to both species, no difference in the ChE and Car-E activities of A. pisum was observed in live or dead insects between treated and untreated adults. In A. fabae, the ChE activity was reduced considerably (Fig. 3.15).

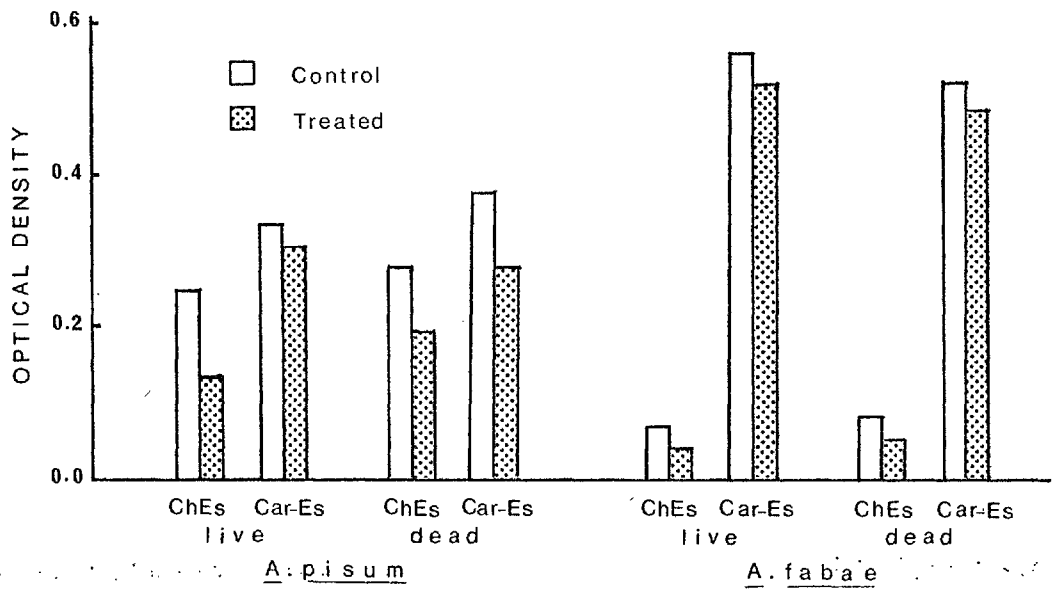


Fig. 3.10. Esterase activities in live and dead aphids 24 hours after topical application with dimethoate (see Appendix 27)

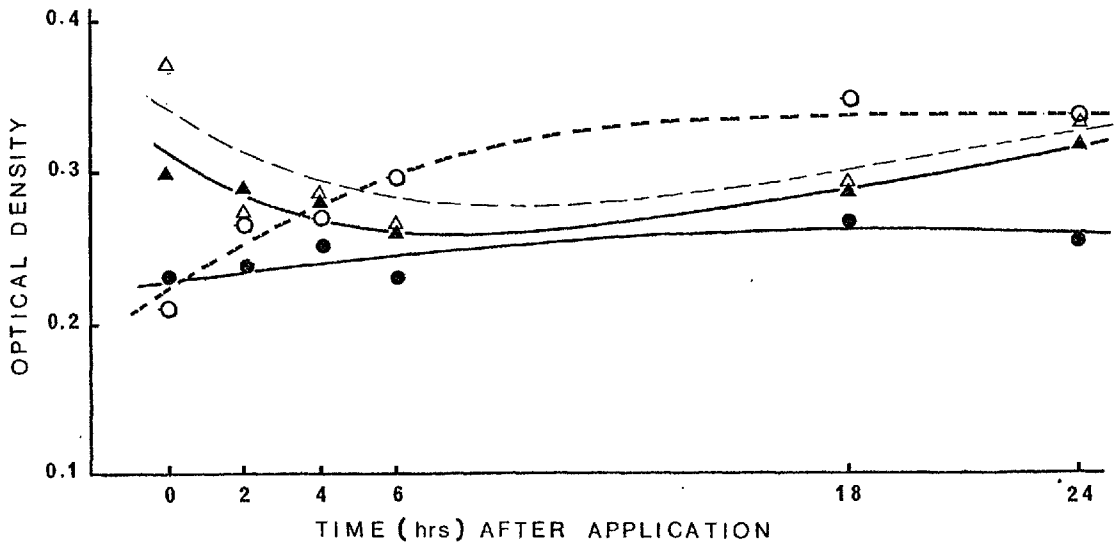


Fig. 3.11. Esterase activities in adults of *A. pisum* still living, at different time intervals after topical application with dimethoate (see Appendix 28)

	Control	Treated
ChE activity	○ — ○	● — ●
Car-E "	△ — △	▲ — ▲

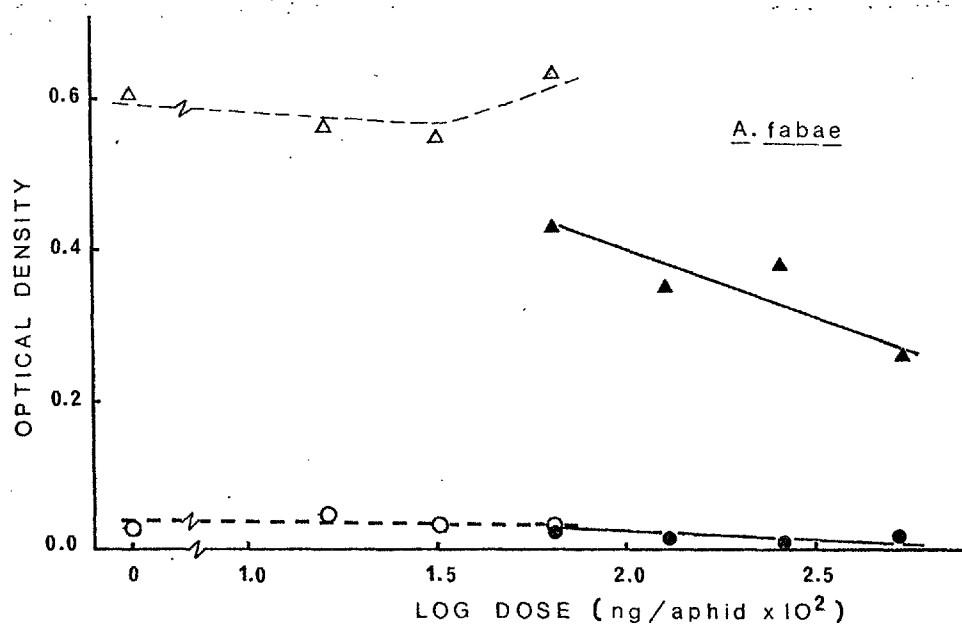
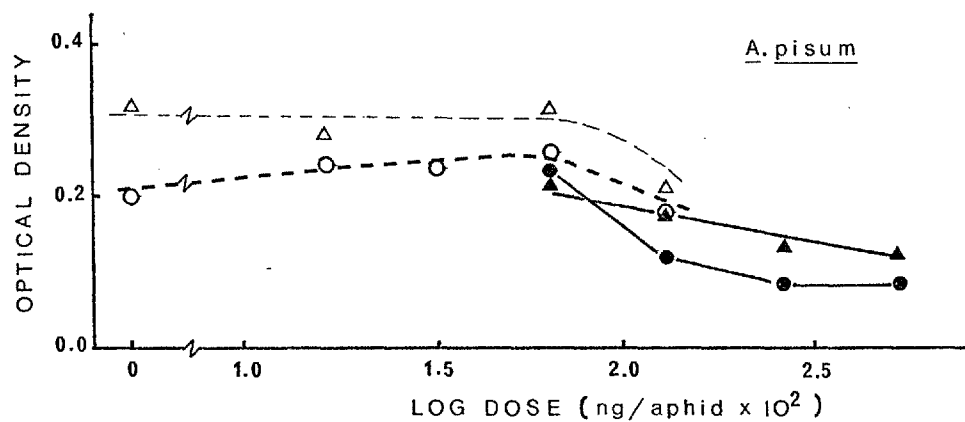


Fig. 3.12. Esterase activities in live and dead aphids 24 hours after topical application with phorate (see Appendix 29)

	<u>Live</u>	<u>Dead aphids</u>
ChE activity	○-○	●-●
Car-E "	△-△	▲-▲

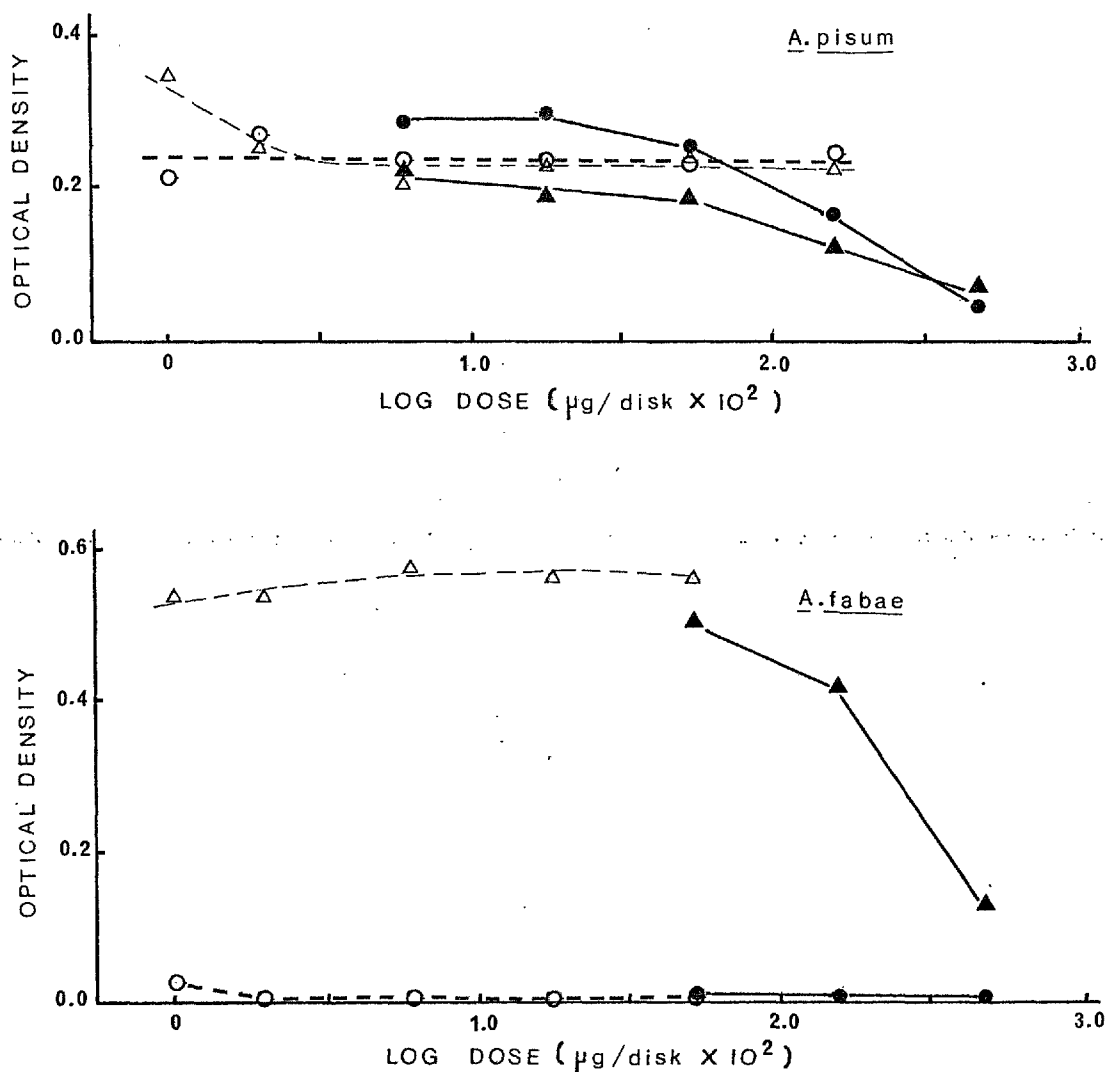
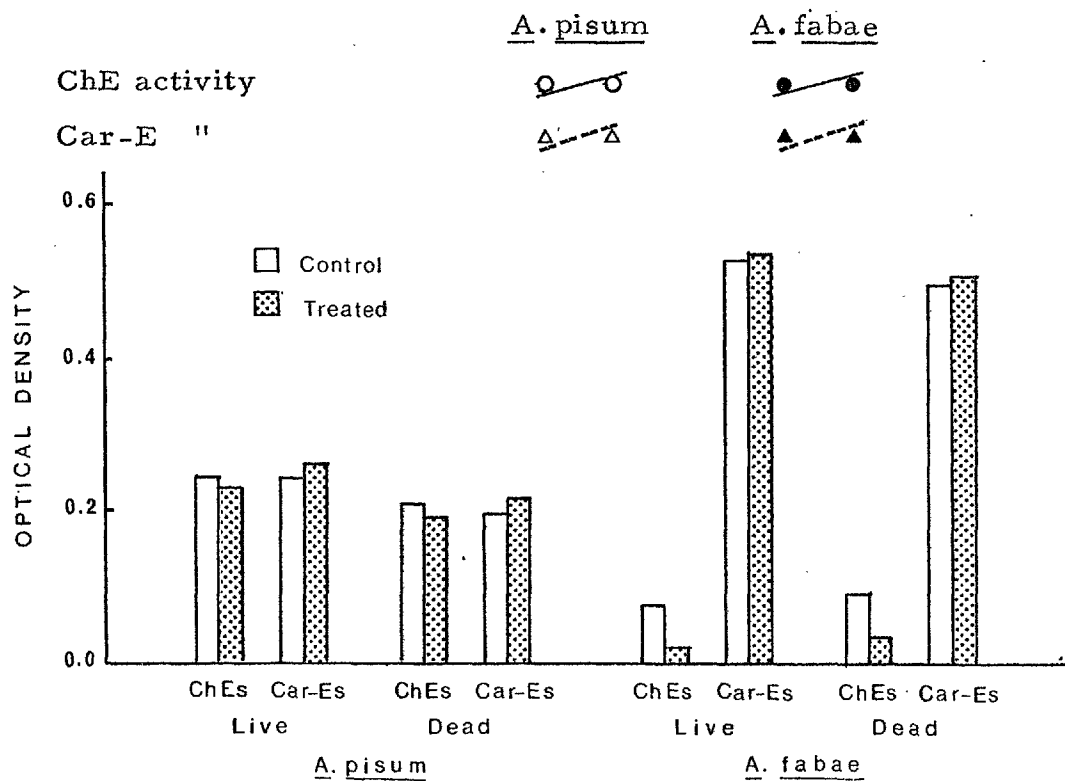
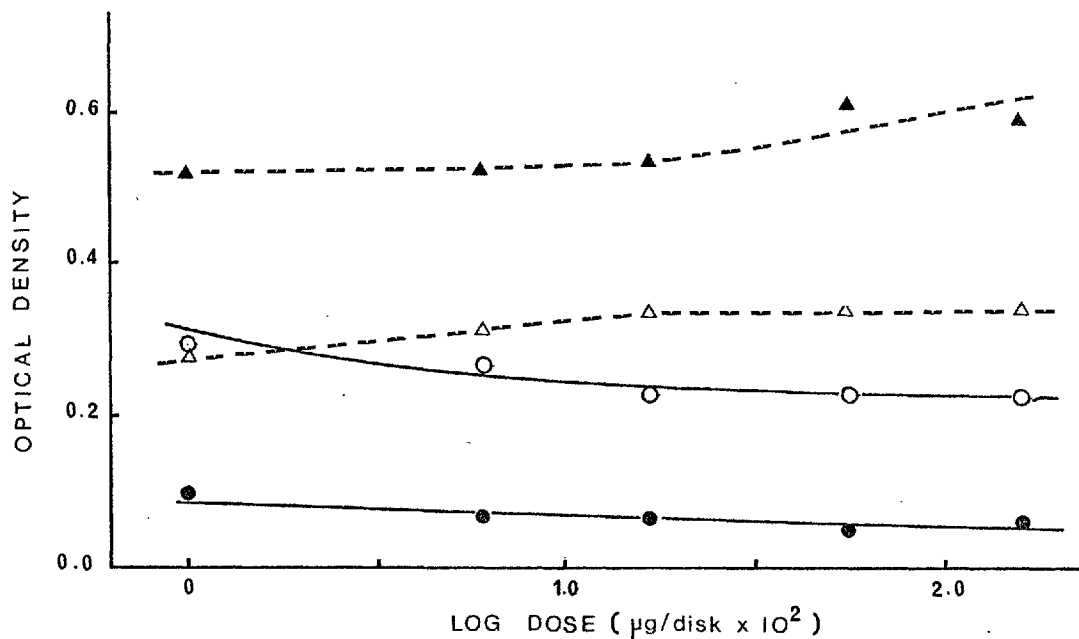


Fig. 3.13. Esterase activities in live and dead aphids, 24 hrs. after exposure to phorate applied systemically (see Appendix 30)

	Live	Dead aphids
ChE activity	○-○	●-●
Car-E "	△-△	▲-▲



B. 2. EFFECT OF ORGANOPHOSPHORUS INSECTICIDE INHIBITORS
ON CHOLINESTERASES AND CARBOXYLESTERASES OF THE
TWO SPECIES IN VITRO.

Materials and Methods

Insect homogenates. Adults of each species were homogenized in ice-cold 0.05 M Tris (hydroxymethyl) aminomethane (Tris) buffer, pH 7 (Gwiazda and Lord, 1967), in a glass homogenizer. The buffer solution was prepared in a way similar to that described by Gomori (1955) as follows:

(a) 24.2 g of Tris (hydroxymethyl) aminomethane in 1000 ml (0.2 M)

(b) 0.1 M HCl

6.25 ml of the solution (a) + 11.5 ml of (b) made up to 200 ml gave a 0.05 M buffer solution of pH 7. Deionized distilled water was used throughout. Homogenized samples were centrifuged at 30,000 g for 30 min at 0°C. The supernatant was decanted and made up with cold buffer to give 110 mg of insect per ml of extract. Each homogenate was divided into two parts of 2.25 ml each, one was treated with insecticide solution, the other was used as a control.

Insecticidal treatment. 0.02% solutions of dimethoate in deionized distilled water and menazon, phorate or phoratoxon in 2-ethoxyethanol were used. 1.25 ml of each of the above solutions were made up to 5 ml with deionized distilled water and of these solutions 0.25 ml containing 12.5 µg of insecticide were added to each 2.25 ml sample of insect homogenate. Each treated sample contained therefore 5 µg insecticide per ml and the relationship of insecticide to insect material was 50 ppm (Gwiazda and Lord, 1967). In the control samples 0.25 ml distilled water or distilled water + 2-ethoxyethanol, prepared in the

same way, were added to make a total volume of 2.5 ml per case. Treated and untreated samples were kept in a water bath at 27°C for a total period of 5 days.

Sampling. Immediately after the preparation of the above mixtures and then every 24 hrs. samples of 0.2 ml from the A. pisum and 0.1 ml from the A. fabae incubations were taken for esterase assay. These aliquots were made up to 5 ml and 10 ml respectively, with de++dist. water, and thus contained 4 mg of A. pisum or 1 mg of A. fabae per ml and the enzyme activities were estimated as before.

Results

In untreated homogenates (controls), the cholinesterase activity of A. pisum was reduced steadily during the 5 day period. In A. fabae the activity of these esterases stayed at nearly the same level throughout. A rapid decrease of the carboxylesterase activity occurred within the first 1 or 2 days in A. pisum following by a slow decline over the rest of the period. In A. fabae samples a steady decrease of the Car-E activity occurred (Figs. 3.16, 3.17, 3.18 & 3.19).

In insecticide-treated homogenates the esterase activities found are compared to those in the corresponding controls.

1. Treatment with dimethoate or phorate. The decrease of ChE and Car-E activities in both species treated with these chemicals was very similar to the controls, throughout the 5 day period (Figs. 3.16 & 3.17).
2. Treatment with menazon. A strong reduction of the cholinesterases appeared in the homogenates of A. pisum within the first day, and after 48 hrs. the activity of these esterases was reduced to zero. In contrast, no reduction of the ChEs in A. fabae or of the Car-Es in either

species was observed (Fig. 3.18).

3. Treatment with phoratoxon. In A. pisum there was a rapid reduction of the cholinesterase activity immediately after the addition of the chemical to the homogenates, detectable within 15 to 20 min, and within the 1st day all ChE activity had disappeared. In A. fabae where the ChE activity is normally low, the reduction was not so marked but the activity had also disappeared after 1 day.

The carboxylesterases activity was also highly reduced in both species. In A. pisum most of the inhibition occurred immediately after the addition of the chemical, whereas in A. fabae the reduction of Car-Es occurred within the 1st day. The activities of Car-Es in both species remained at the depressed level for the rest of the 5 days period (Fig. 3.19).

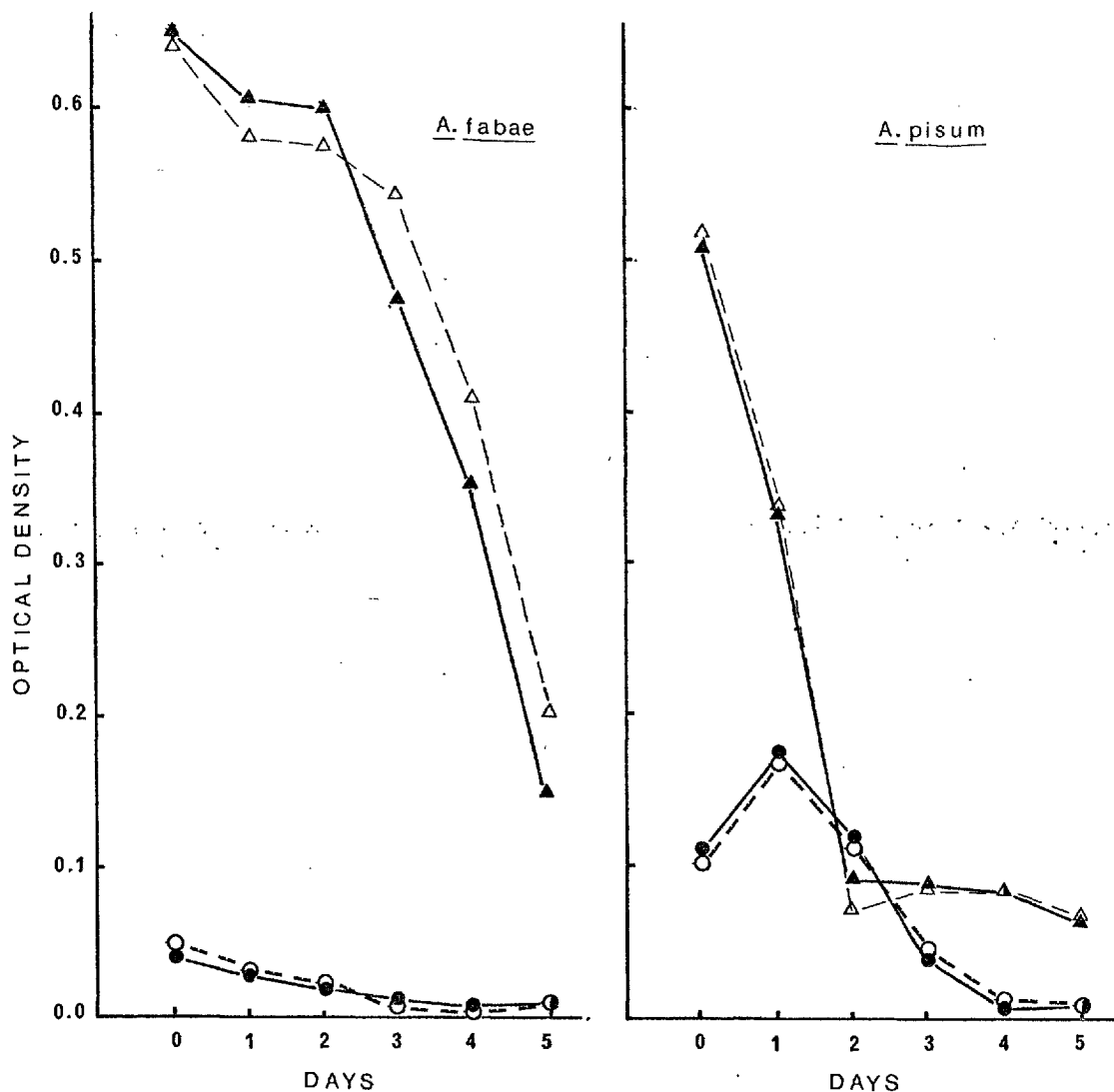


Fig. 3.16. Esterase activities at different times, in homogenates of the two species untreated or treated with dimethoate solution (final conc. of dimethoate, 5 ppm; substrate, α -naphthyl acetate 2×10^{-4} M; see Appendix 33)

	<u>Control</u>	<u>Treated</u>
ChE activity	○- -○	●- -●
Car-E "	△- -△	▲- -▲

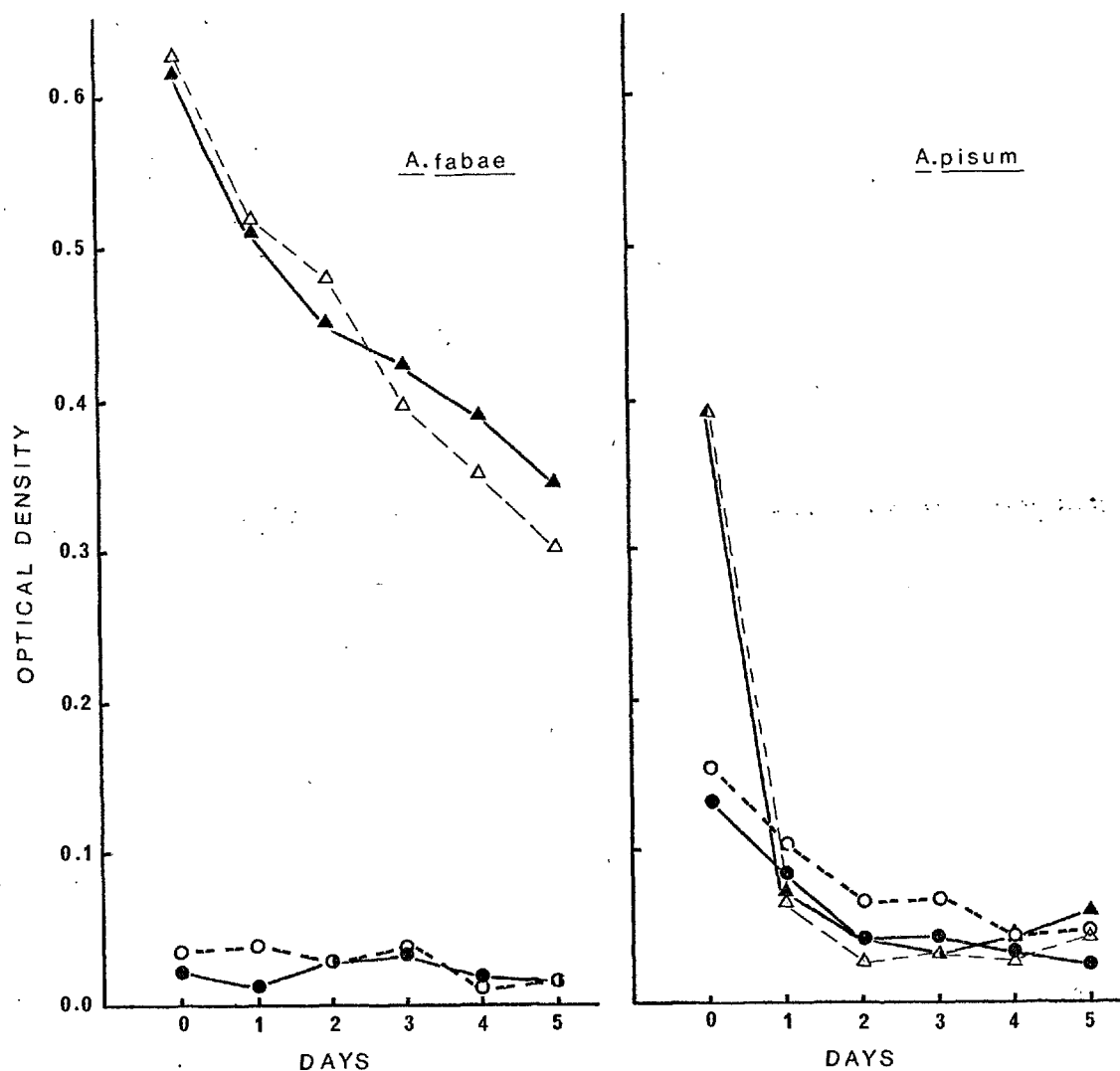


Fig. 3.17. Esterase activities at different times, in homogenates of the two species untreated or treated with phorate solution (Final conc. of phorate, 5 ppm; substrate, α -naphthyl acetate 2×10^{-4} M; see Appendix 33)

	Control	Treated
ChE activity	○—○	●—●
Car-E "	△—△	▲—▲

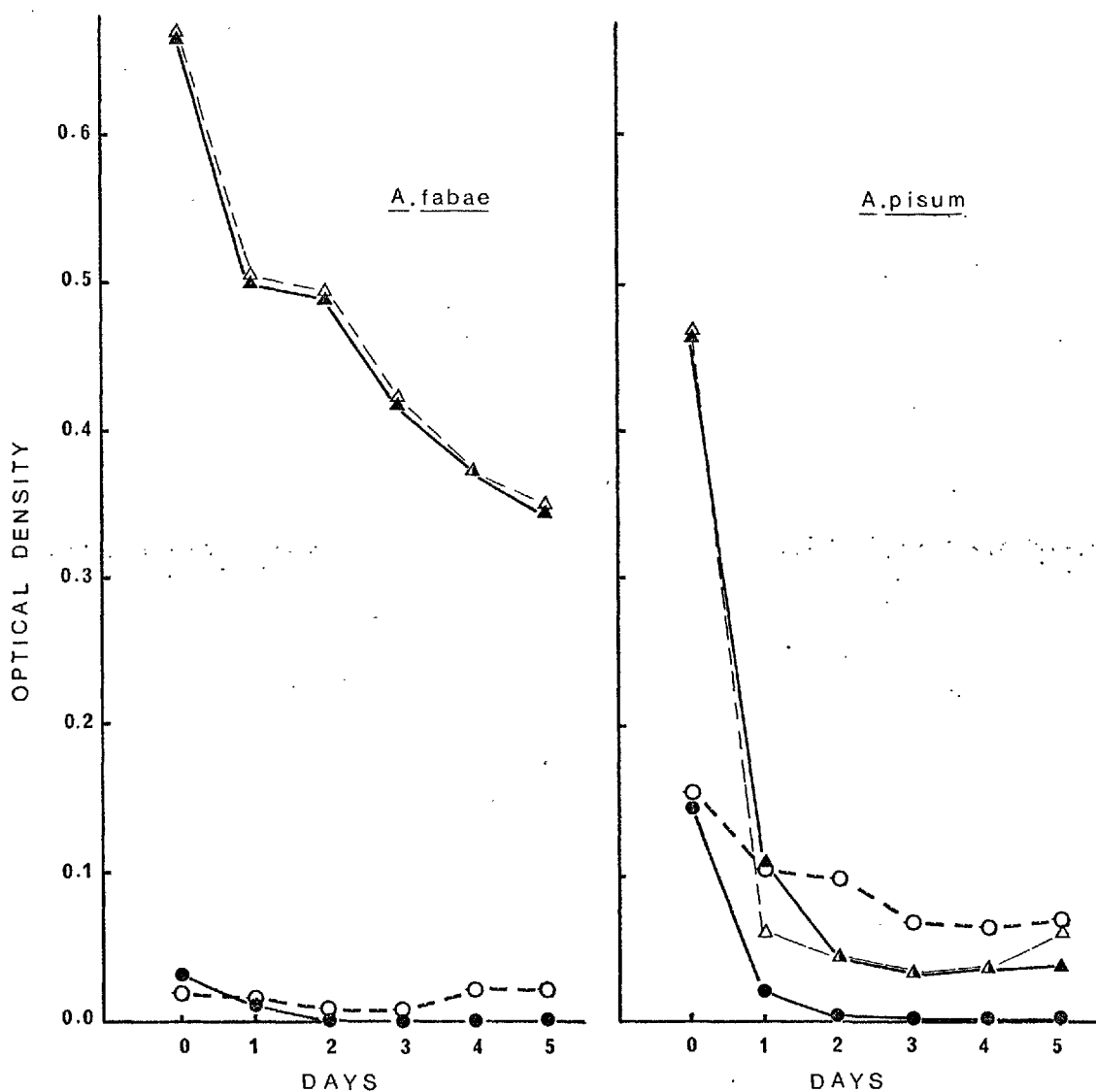


Fig.3.18. Esterase activities at different times, in homogenates of the two species untreated or treated with menazon solution (Final conc. of menazon, 5ppm; substrate, α -naphthyl acetate 2×10^{-4} M; see Appendix 33)

	Control	Treated
ChE activity	○—○	●—●
Car-E "	△—△	▲—▲

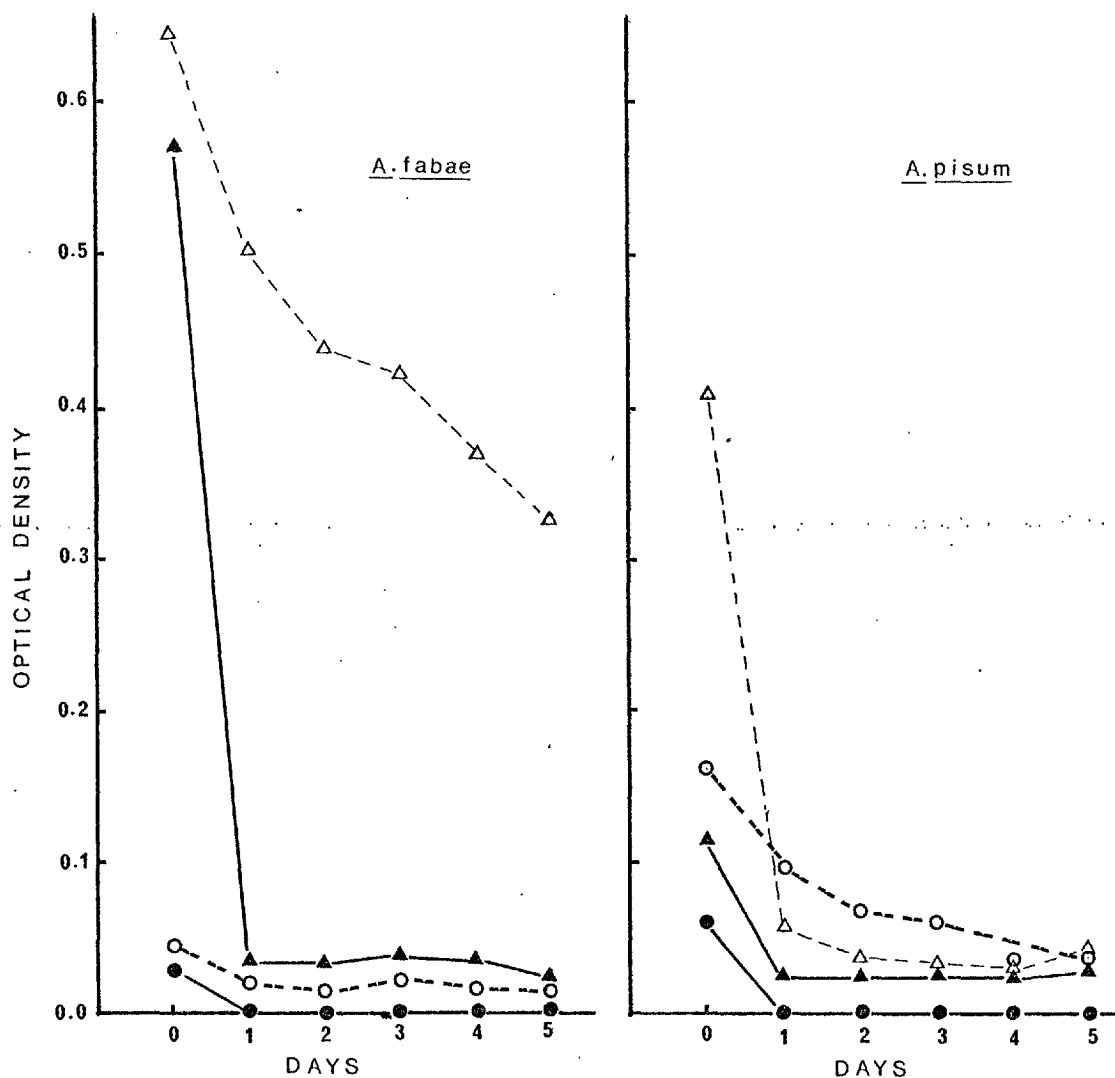


Fig. 3.19. Esterase activities at different times, in homogenates of the two species untreated or treated with phoratoxon solution (Final conc. of phoratoxon, 5 ppm; substrate, α -naphthyl acetate 2×10^{-4} M; see Appendix 33) .

	Control	Treated
ChE activity	○—○	●—●
Car-E "	△—△	▲—▲

Discussion

Dimethoate solutions applied topically to the adults caused inhibition of a significant part of the ChE activity which was of similar extent in both species, 33% and 31% in live and dead insects of A. pisum and 40% in live or dead A. fabae when compared to the controls. However the mortality caused cannot be related to any measurable difference in the activity of this group of esterases within the treated insects since the inhibition of ChEs was about the same in both dead and live insects. Also the reduction of the ChE activity, up to 24.5% in A. pisum by the same insecticide in vivo, was not correlated with any mortality. The Car-Es of both species were much less sensitive to dimethoate. In A. fabae these esterases in samples of live or dead insects were 7% inhibited and also do not seem to be related to the mortality produced. There are strong indications however, that in the case of A. pisum the inhibition of these esterases may be closely correlated with mortality, since in samples of dead insects the Car-E activity was reduced to as much as 26%, whereas there was only an 8% reduction in samples of living insects.

The reduction of ChE activity with menazon did not appear to contribute to the observed mortality, since the inhibition of these esterases was nearly of the same level in live and dead samples of treated insects. It is worth noting that the ChEs of A. fabae were far more sensitive to this chemical than the ChEs of A. pisum. The activity of these esterases in the former species was reduced by 74% and 67%, in living and dead insects, while in the latter species only 5% and 8% reduction respectively was found.

Both topical and systemic applications of phorate caused mortality that could be related to the reduction of ChEs and Car-Es. The degree of inhibition of the two groups of esterases in samples of dead insects was increased as the doses of the chemical to which the insects were exposed were increased. In samples of live insects the esterase activity in general was not reduced, and over the dose range over which they survived remained at the same level as in the control. This could indicate that until the inhibition of these enzymes reaches a fatal level, the insect is able to reverse the enzyme inhibition. This recovery action may possibly be different at different concentrations of the chemical.

O'Brien (1956) has shown that cockroaches poisoned by malathion show little in vivo reduction of ChEs and similar results have been obtained by Asperen (1958a) when 0,0-dimethyl-0-2,2-dichlorovinyl phosphate was applied on house flies. Many workers using different methods for the estimation of the ChE activity in house flies, have also found that O-P compounds acting in vivo do not completely inhibit ChEs. Furthermore no correlation has been found between insecticidal toxicity and enzyme inhibition in vivo (Lord and Potter, 1954 b ; Asperen, 1958b; O'Brien, 1960; Chuo and Sherman, 1971).

In the in vitro over extended time intervals experiments, the esterases in untreated homogenates of A. pisum appeared more unstable than those of A. fabae. This was especially so, with the carboxylesterases.

Treatment of homogenates of the two species with dimethoate or phorate did not reduce the esterase activities compared with the controls, while treatment with menazon also had little effect when compared with the controls, except to reduce considerably the ChE activity in A. pisum.

When homogenates were treated with phoratoxon, the oxygen analogue of phorate, the reduction in the esterase activities, especially

the ChEs rapidly followed and within the first 15 to 20 min. the ChE activity in A. pisum was reduced by 62% and in A. fabae by 33% when compared to the controls. The carboxylesterase activity in A. pisum was reduced also immediately after application with phoratoxon by 71%, while in A. fabae a strong reduction (93%) appeared within the first day.

The high degree of esterases inhibition by oxygen analogues of O-P compounds has been confirmed by Matsumura and Brown (1961b). They found that the ChEs of malathion-susceptible and -resistant strains of Culex tarsalis larvae, were 2,000 times as sensitive to malaoxon as to malathion both in vivo and in vitro. Oppenoorth and Asperen (1961) proposed that the mechanisms for elimination of O-P compounds which are present both in susceptible and resistant strains of houseflies are not active in homogenates. Therefore, there is little chance that the activities of ChEs or Car-Es will be reduced in vitro. It has been concluded that highly toxic O-P insecticides were not always the most effective in vitro ChE inhibitors (Fukuto, 1957; Lovell, 1963; Dauterman and O'Brien, 1964; Bigley, 1966; Morallo and Sherman, 1967). Any one O-P compound on the other hand exhibits a considerable range of potencies and will inhibit enzymes from many species to different extents. Chuo and Sherman (1971), as mentioned above, did find a correlation between toxicity and in vitro head ChE inhibition with two phosphorothioates applied to B. peregrina and C. megacephala, and in A. melifera and M. domestica a similar correlation has also been found (Metcalf and March 1950)

GENERAL DISCUSSION AND CONCLUSIONS.

The outcome of the application of an insecticide to an insect is dependent on those many physiological processes that are at all related to the movement of the compound to its site of action or to the detoxication or elimination of the poison from the system. The rate of these processes in each species whether metabolic (uptake, excretion) or reproductive, could be important in explaining the susceptibility of the insects. The comparative rate of plant sap uptake in A. pisum and A. fabae indicates their initial ability to feed on broad bean plants. This feeding ability can also be related to the rate that the imbibed food is converted to aphid tissue in the adult or to the production of larvae and a picture of the intensity of these physiological processes is given by the honeydew production. The feeding rate will greatly influence the mortality which is directly dependent on the quantity of any toxic compound taken in with the plant sap. On the other hand the potential of each species to excrete part of the ingested material in the larvae or with the honeydew provides a route by which the aphid may excrete the assimilated toxicant or its metabolic products.

The weights of the growing instars of A. pisum and A. fabae increased at the same relative rate, but since the weight per individual is higher in A. pisum than in A. fabae, the quantity of plant material absorbed per aphid was higher in the former than in the latter species. The weight of developing aphids increased upto the time that larviposition started. There was however a continued increase in biomass which can be derived from the weight of larvae produced. In comparative tests with ^{32}P it was found that A. fabae ingested 16.5 to 41.5% of the amounts of ^{32}P ingested by A. pisum. Also with the use of ^{32}P -phosphate it was found that in 24 hrs. feeding, A. pisum absorbed plant

material equivalent to 33% of their body weight whereas in A. fabae only 4.5% of their body weight per hour was absorbed. The quantity of the plant material taken in by A. pisum was therefore many times that taken in by A. fabae and does not correspond to the difference of the body weights of the two species. The radioactivity found in the adults of both species increased with the time spent on labelled leaf-discs. A continuous absorption therefore of plant material and conversion to aphid tissue either in the adult or in the developing embryos occurred.

The two species excreted different quantities of honeydew and produced, per adult, different numbers of larvae. A part of the ingested material therefore, passed to the larvae and the quantity depends on the number of larvae produced. During a reproductive period of 20 days more larvae were produced by A. pisum than by A. fabae. However larviposition seems to be related to the stage of the adults and occasionally in feeding experiments lasting 1 or 2 days a higher number of larvae were produced by A. fabae. It has been said (Banks and Macaulay, 1965) that larvae use the materials previously ingested by their mothers. Here it was found also that when the time of feeding of the aphids on ^{32}P -labelled plants was increased, the quantity of ^{32}P found was higher not only in the adults body but also in the larvae. This increase of ^{32}P in the larvae of A. pisum was due to the increased number of larvae produced, whereas in A. fabae there was an increased quantity of the label per larva. In contrast the quantity of ^{32}P that was excreted with the honeydew did not increase with the time of labelling; instead the majority of this was found to be transferred to the larvae. The possible importance of the larvae produced by poisoned insects as routes for elimination was shown by Irving (1968) where reproductive females of the fly G. pallidipes withstood higher quantities of chlorinated hydrocarbons than virgin

females and this ability increased with the number of in utero larvae.

A. pisum produced more honeydew than A. fabae. Of the plant sap taken up a quantity corresponding to 15% of the body weight was excreted with the honeydew in A. pisum, but in A. fabae only an equivalent of 2.2% of the body weight was excreted. A significant part of the material ingested is excreted with the honeydew and in comparative tests, after 24 hrs. feeding of the two species on ^{32}P labelled leaf-tissue, it was found that most of the radioactivity taken from the plant was lost with the honeydew.

Insecticides may not and almost certainly do not behave in the same way as orthophosphate either in the plant or in the insect. Further investigation is required in this field for which radioisotope labelled material would be required. With autoradiographic techniques it should be possible to follow the passage of an insecticide into the insect and to determine the extent to which the larvae provide a sink for sublethal doses and the extent to which the aphid is able to excrete insecticide in the honeydew.

The greater absorption of the food material in A. pisum than in A. fabae possibly contributes to the higher susceptibility of A. pisum towards some of the tested insecticides. However these results show that the feeding activity in each species was partly dependent on the food offered to them and on other factors, as for example the presence of other compounds in the diet. When artificial diet was offered to the two species through parafilm membranes the relative feeding rates were reversed and A. pisum accepted the sucrose solution less readily than A. fabae did and they were also more easily influenced by a stale diet, possibly responding to some deleterious change in the components of the diet. This response of A. pisum is supported by Auclair (1965) who found that the feeding and excretion rates of

this species on artificial diets were usually much lower than those he recorded on plants.

The presence of dimethoate in the diet resulted in a considerable reduction of feeding activity in both species. In A. pisum the feeding inhibition appeared to be mainly a result of some sublethal poisoning by this chemical, whereas in A. fabae it was caused predominantly by a repellent action of dimethoate. The repellent action of dimethoate to A. fabae possibly occurs also when the chemical is incorporated in the plant tissue and the reduction of feeding could be partly responsible for the higher tolerance shown by this species towards dimethoate.

The inhibitory action of dimethoate in the two species although higher at higher concentrations of the chemical was not proportional to the concentration. Thus, in bioassays with the artificial feeding technique it was not possible to correlate the mortality caused to the concentrations of dimethoate. With ^{32}P label incorporated into the diet, the exact amount of insecticide imbibed from different concentrations was estimated. The mortality caused can be then compared to the actual quantity of dimethoate ingested, and the greater sensitivity of A. pisum to dimethoate established here (Fig. 1.16) is in agreement with the relative toxicity of this chemical towards the two species found in bioassays with plants. The mortality caused by phorate incorporated in the artificial diet was in both species proportional to the concentration of the chemical. This indicates that either no inhibitory action was exercised by this chemical or this action if it occurred, was similar in all the tested concentrations.

The initial ability of the aphids to feed from a certain medium can change with the mere presence of a chemical in it. This change was established in the present work with the technique of artificial feeding and was more apparent in A. pisum. The same inhibitory

effect has been shown also to occur in the case of M. persicae fed artificially on phosphamidon-poisoned (Parry and Ford, 1967, 1969) or dimethoate-poisoned diet (Javadi 1971). Halimie and Ford (1972) have described the inhibitory action of phosphamidon incorporated in radish plants, on the feeding of two strains of M. persicae. This action was related to the concentration of insecticide applied to the plant in both strains but not at the same level in the two strains. The reduction of feeding could be caused according to these authors by sublethal toxic effects or alternatively the insecticide could be repellent. The latter effect might even be progressively reduced if increasing hunger subsequently counteracted the repellency.

The toxicological results obtained in this study could be related not only to feeding differences. Lowe (1969) has reported that there is no simple relationship between the feeding behaviour of A. pisum and A. fabae fed on V. faba and their susceptibility to systemic insecticides vamidothion and schradan. Some properties of the applied insecticides may also be responsible for the poisoning of the aphids. The physical and chemical properties of the insecticides used are important factors not only because they determine the entry of the poison into the plant tissue or insect body and the movement in these media, but also because they govern the changes the original form of each insecticide may undergo. The toxicity after topical application was partly related to the rate of cuticular penetration, though movement to the site of action is also a limiting step as is the rate of oxidation, to the more toxic phosphate and the stability of this analog^{ue} once formed. When the systemically applied compound is lipophilic it may not disperse so readily through the leaf discs and thus aphids could avoid it by feeding at sites less contaminated by insecticide. Little is known however of the microdistribution and movement of pesticides in plants.

Many thio and dithio phosphate compounds are thought to exert their toxic action after oxidation to phosphates. Certainly the oxygen analog^{ues} are more potent inhibitors of esteratic enzymes than the parent thio-phosphates. This clearly was shown in the in vitro studies of the esterase inhibition by insecticides in this work. Phorate did not show any inhibitory action on the esterases when it was incubated over a period of 5 days with homogenates of the two species. However its oxygen analog^{ue} phoratoxon reacted rapidly with the cholinesterases and carboxylesterases of the two species and entirely eliminated an activity in these esterases within the first day. With phoratoxon, oxidation of the sidechain sulphur also confers a greater toxicity to the molecule. If oxidation in the plant occurred at a different rate than in the insect then systemic application might be expected to give results differing qualitatively and quantitatively from topical applications.

When phorate was applied topically to Blatella germanica L. O. fasciatus and Rhodnius bugs, analyses showed the presence of phorate sulphoxide and/or sulfone; but there was no evidence for the "oxon" pathway in these insects (Menn, 1962). According to many investigations referred to by Menzie (1969) the total anti-ChE activity of the highly lipophilic phorate was increased for several days, after it was applied to plants and there was an increase in the amount of material that is more polar in its solubility properties.

The highly water soluble dimethoate, although possessing negligible lipophilic properties, was rapidly absorbed when applied to plants. According to many works referred to by Menzie (1969), this chemical decomposed both on the surface and within the plant by hydrolysis and oxydation. On the plant surface dimethoate underwent non-enzymatic oxidation to the oxygen analog^{ue} and hydrolysis to water soluble derivatives. Within the plant, all derivatives were also found. The

desmethyl analog^{ue} was predominant inside the plant, whereas on the surface the oxycarboxy compound predominated. When bollworms, Heliothis zea, were treated by injection with labelled dimethoate, the highly toxic oxygen analog^{ue} was found in highest concentration (Sanderson and Edson, 1964). A series of derivatives and among them the oxygen analog^{ue} were also found after application of dimethoate to Prodenia litura F. (Zayed et al, 1968), P. americana, B. germanica and M. domestica (Morikawa and Saito, 1966).

The ease with which an insecticide can enter the plant or insect body and the nature of the derived compounds after its decomposition is of vital importance in the interaction between these compounds and the insect enzymes which are involved in the organophosphorus poisoning.

Sublethal differences may be expected also in investigation on the progress of intoxication with menazon, to which aphids are much more susceptible. Here again oxidation is important in the poisoning process. Whether oxidation occurs more readily in aphids than in other insects or whether the plant is more important is open to conjecture.

The toxicological results depend however not only on the anti-ChE properties of the toxic compound but also on the quantity and quality of the esterases the insect possesses. In the study of the esterases of A. pisum and A. fabae quantitative differences were established between the two species. In A. pisum the activities of ChEs and Car-Es remained nearly in the same level with only a slightly higher Car-E activity. The variability of the ChE activity found in this species with titration and colorimetry was due possibly to some specific nature of these esterases in A. pisum determining

a differential reaction towards the different substrates used. In A. fabae the Car-E activity predominated, whereas the ChE activity 6.1 to 17.3% of the combined esterase activities of this species, was only a fraction of the ChE activity measured in A. pisum.

The esterases of the two species did not differ only quantitatively. Differences in quality were also established. In the electrophoretic separation although in the two species one band was identified as ChE and one as Car-E, the area occupied by each type of esterases and the colour density were different. Also, the mobility of both esterases through the polyacrylamide gel was greater in A. fabae than in A. pisum. The ChE activity in A. fabae although much lower than in A. pisum, hydrolysed α -naphthyl acetate more rapidly however, than the ChEs of the latter species. The difference in the Car-E activities between the two species seems to be only quantitative, as far as their reaction with α -naphthyl acetate is concerned. The overall esterase activities in homogenates of the two species responded differently in the presence of the ChE inhibitor eserine sulphate. The esterases of A. fabae proved to be more insensitive to eserine than those of A. pisum.

The above mentioned quantitative and qualitative differences in the two types of esterases between the two species cannot however predetermine the toxic effect of a particular compound. The toxic effect seems to be a complex phenomenon in which the affinity of the compound and its derivatives to the esterases, and the importance of the elimination of a particular esterase in a particular site of the insect body, are both factors of some importance. These statements are borne out by the various degrees of inhibition and activity of the esterases and the corresponding mortalities from the three insecticides used in these experiments.

When dimethoate was given systemically to the two species by wick application it was nearly equally toxic to the two species. The high solubility of this compound in water and the possible absence of any strong lipophilic property has possibly some connection with the different contact toxicity of dimethoate to the two species and the different systemic toxicity, when the chemical was applied on the upper surface of the leaf-discs. Dimethoate was 4 times less toxic to A. fabae when it was applied dorsally, probably because of differences in penetrating the cuticles of the two species. The plant epidermis could also constitute a serious barrier to the entry of this chemical into the mesophyll and only a small fraction of the applied quantity may have entered the plant. However the higher rate of feeding by A. pisum on plants resulted in greater quantities of poisoned plant sap being ingested by this species than by A. fabae and a higher mortality in A. pisum in the 24 hrs. exposure period. The similar inhibition of ChEs of the two species by dimethoate in vivo does not exclude however the existence of some difference in susceptibility of the two aphids towards this chemical. Possibly differences in ChE sensitivity to dimethoate exist in localized vital sites of the insect bodies. However the lack of correlation between ChE inhibition and mortality makes this possibility rather remote. There was a difference in the reaction of dimethoate with the Car-Es in the two species in vivo. In A. pisum these esterases were reduced by 26% and the reduction was correlated to the observed mortality, thus it is possible that these esterases are also responsible for the higher susceptibility of this species to dimethoate. The inhibitory action of dimethoate which has been demonstrated with the artificial feeding technique to be higher in A. pisum than in A. fabae may occur also when the chemical is incorporated into the plant tissue, as has been established in the case of M. persicae with phosphamidon

(Halimie and Ford, 1972). If this is so, then this inhibitory action constitutes one factor which protects the insect from taking large quantities of poisoned plant sap as its greater feeding ability declines.

The stronger lipophilic properties of phorate possibly aided a more rapid penetration of the cuticle of the two species by this chemical leading to a simiral contact toxicity to both species. The LD₅₀ values of this chemical were similar for the same body-weight in the two species. The systemic toxicity of the compound to the two species seems to be influenced by their differing feeding behaviour. Phorate, as a chemical with very limited water solubility is less readily moved from one part of the plant tissue to neighboring areas, unless transformed to the toxic but more polar compound, phorate sulphoxide. When applied on the wicks or injected into the main vein of the leaf-disc it probably occurred in higher concentrations in the large and small veins. A. fabae feeding mainly at these sites had the opportunity to imbibe plant sap with higher concentrations of poison. A. pisum on the other hand fed preferentially on small veins and the mesophyll thus avoiding high concentrations of this compound and as a result the mortality produced was 4 times lower than that produced in A. fabae. When phorate was applied on the blotting paper it was possibly distributed in a different pattern and the median lethal dose of the chemical was proportional to the body weight of the aphids. It was shown that both ChEs and Car-Es in the two species were sensitive to phorate and they were strongly inhibited in vivo after topical or systemic application of the chemical. Furthermore, the mortality caused was correlated to the in vivo inhibition of these esterases. The lack of in vitro inhibitory action of phorate towards the two types of esterases in the two aphids, indicates the importance of the structural form of the living insect.

The observed in vivo inhibition of esterases occurred possibly only after phorate was first oxidized to the stronger anti-ChE phorate sulphoxide, which did not occur once the structure of the living insect had been destroyed by maceration.

Menazon, intermediate in lipophilic properties showed differences in contact toxicity. It was, like dimethoate, as a contact poison, less toxic to A. fabae than to A. pisum. This possibly occurred because of an inherent difficulty in penetrating the cuticle of A. fabae. Although it was shown that the ChEs in A. fabae were more sensitive to menazon, the ChE inhibition in both species in vivo was not correlated to the mortality. In contrast, in vitro this chemical was able to inhibit the ChE activity only in A. pisum. However menazon is a specific aphicide being many times less toxic to other insects and until the nature of this specificity has been clarified one cannot justifiably make general comparisons with other compounds.

It was not known to which extent the topically or systemically applied insecticides had metabolized in the V. fabae tissue or within the insects to other compounds with lower or higher anti-esterase properties. This has been shown to occur in some cases by many workers as has been mentioned above. However the extent to which this occurred in these experiments is not known and the investigation of this aspect, beyond the scope of this study, would be an attractive topic for further research.

SUMMARY

PART ONE

Studies on the growth and reproductive ability of A. pisum and A. fabae were carried out.

The weigh of developing aphids in both species increased up to the time the larviposition commenced. There was however a continuing increase in biomass, because of the produced larvae. During a reproductive period of 20 days adults of A. pisum produced more larvae than those of A. fabae. The difference was 3.8 to 6.3 larvae in each 5-day period. The honeydew produced by the two species, indicative of the feeding rate, was collected and measured in its original form. It was found that A. pisum produced 0.046 μl /aphid/hr whereas A. fabae produced 0.0077 μl /aphid/hr. The specific gravity of the honeydew measured with a simple technique was found to be 1.04 in A. pisum and 1.08 in A. fabae.

The feeding behaviour of the two species was studied in broad bean plants and on artificial diet. These studies were facilitated with the use of ^{32}P -phosphate incorporated into the plant tissue and in the artificial diet.

The studies with plants showed that the average feeding rate over a 24 hrs feeding period was 0.66 mg of plant sap per A. pisum adult per hr and 0.045 mg per A. fabae adult per hr. In both species nearly half of the absorbed ^{32}P was found in the honeydew produced during the 24 hrs of feeding.

In comparative tests it was found that A. fabae ingested 16.5 to 41.5% less ^{32}P than A. pisum and retained in their body 7.3 to 9.7% less than A. pisum. Though most of the radioactivity was lost in the honey-

dew in both species more ^{32}P was found in the larvae of A. fabae than in the larvae of A. pisum. This difference was a result of a 3-fold number of ^{Larvae} produced by A. fabae during the short period of these experiments, as compared to A. pisum.

When adults of the two species were fed for a series of different times up to 24 hrs on ^{32}P -labelled leaf-tissue it was found that in both species the ^{32}P taken in increased with the time of feeding. The distribution of the ^{32}P absorbed during the differential labelling was examined in the two species 24 hrs after the labelling, in the adults and the larvae and honeydew. The ^{32}P level in the adults declined slowly and after 24 hrs 65.0 to 73.1% of the total absorbed initially by A. pisum and 52.0 to 68.6% in A. fabae was still present in the adult bodies. In both species the loss via the honeydew decreased slightly as the labelling period was increased. In contrast the loss of ^{32}P in the larvae produced increased with the labelling time.

A series of sucrose solutions and a complex diet were tested for artificially feeding the two species through parafilm membrane. For a few days maintenance of the adults a 15% sucrose solution was proved more satisfactory and in addition this simple diet ensured a minimum of interference with the properties of the poisons administered in the diet.

A. pisum adults accepted the 15% sucrose solution less readily than A. fabae and a hunger stimulus was necessary before the aphids began taking large quantities. The same species was also especially sensitive to diets which were not freshly prepared.

Dimethoate incorporated in the feeding solution markedly inhibited feeding in both species. The inhibition was higher with higher concentrations of dimethoate, though it was not proportional to the concentrations of this chemical. No evidence was obtained that phorate

inhibited the artificial feeding. The inhibitory action caused by dimethoate was examined at different times and was found in A. fabae to be due possibly to a repellent action whereas in A. pisum it appeared to be due more to some sublethal effect with alternatively a repellent effect. Mortality was produced in both species when under the influence of a hunger stimulus they took in more poisoned diet thus absorbing a lethal quantity. At this stage in the dead insects of each species similar quantities of poisoned diet were found, irrespective of the duration of feeding. The dimethoate in these quantities was between 1.06 and 1.12 ng per aphid in A. pisum and 0.61 to 0.73 ng per aphid in A. fabae. Because of the repellent and sublethal effect of dimethoate there was no correlation between the observed mortality and the concentrations of the chemical. A dose-mortality correlation could only be established when the actual amounts of the insecticide ingested were obtained.

PART TWO

The relative contact and systemic toxicities of the insecticides dimethoate, menazon and phorate to the two species were examined.

After topical application to the dorsum dimethoate and menazon were 4 times more toxic to A. pisum than to A. fabae, whereas phorate was twice as toxic to A. fabae than to A. pisum.

Different methods were used for the systemic application of the insecticidal solutions. They were applied on the upper surface of leaf-discs, on the wicks, or on the wet blotting paper maintaining leaf disc turgidity or they were injected into the main vein of the leaf discs. The mortalities produced by each chemical were usually different for each method of systemic application. Dimethoate was 5 to 6 times more toxic to A. pisum than to A. fabae when it was applied to the leaf-

surface, but nearly of the same toxicity to the two species when it was given through the wicks. Each one of the chemicals phorate and menazon exerted almost the same toxicity to the two species, when they were applied on the blotting paper. However phorate in the wick application or by disc injection was nearly 4 times more toxic to A. fabae than to A. pisum.

PART THREE

Zymograms of the two species were obtained by electrophoretic separation of aphid homogenates on polyacrylamide gel of variable concentration. In each species one of the bands was identified as cholinesterase and one as carboxylesterase. The remaining bands, 3 in A. pisum and 4 in A. fabae, were possibly predominantly arylesterases. The mobility of ChE and Car-E bands was found to be higher in A. fabae than in A. pisum.

The esterase activities were measured with a titrimetric and a colorimetric method. With the two methods the ChE activity in A. fabae represented a small fraction of the combined esterase activity, 2.5% by titrimetry and 6.1 to 17.3% by colorimetry, and was much lower than the ChE activity in A. pisum. In A. pisum the ChE activity was found to be different with the two methods. With the titrimetric method the ChE activity was found to represent only 4.5% of the combined esterase activities, whereas with colorimetric method it was 42.5 to 59.1%. This difference was probably due to the different substrates used with the two methods and their correspondingly different affinities for the enzymes. Most of the esterase activity in A. fabae was due to Car-Es, while in A. pisum with the colorimetric technique the Car-E activity was found to be similar to the ChEs.

Different experimental parameters that could affect the esterase

activities were studied using the colorimetric technique. Differing homogenate and substrate concentrations, incubation time and temperature and the influence of the ChE inhibitor eserine sulphate were studied. From the study of these variables it was established that apart from the quantitative differences between the two types of esterases in the two species, qualitative differences also existed. Thus, much lower concentrations of ChEs in A. fabae hydrolysed α -naphthyl acetate more rapidly than the much higher concentrations of these esterases in A. pisum. In contrast, the differences in Car-Es between the two species were only quantitative. The ChEs in A. fabae proved to be less sensitive than the ChEs in A. pisum to increasing concentrations of eserine or to increasing preincubation time with this inhibitor.

The three systemic insecticides were applied topically or systemically to A. pisum and A. fabae and the mortality produced after 24 hrs was examined in relation to the esterase activities. The esterases were assayed colorimetrically.

Dimethoate applied topically inhibited the ChE activity in the two species similarly. However the mortality caused could not be related to the ChE inhibition. The Car-Es in A. fabae were shown to be more sensitive to this chemical than the Car-Es of A. pisum and in this latter species the reduction of activity of these esterases was correlated with mortality.

The ChEs in A. fabae were far more sensitive to menazon 24 hrs after systemic application than in A. pisum. However the reduction of ChE activity in both species could not be related to mortality.

Phorate applied topically or systemically caused mortality in the two species which was related to the reduction of ChE and Car-E activities. These reductions increased as the concentrations of the

chemical and the mortality increased.

Homogenates of the two species after their treatment with these three insecticides and with phoratoxon, were kept over a period of 5 days at 27°C. The esterase activities were estimated colorimetrically at daily intervals, in comparison to untreated homogenates (controls). The esterases in the controls were in general more unstable in A. pisum than in A. fabae. Treatment with dimethoate or phorate did not reduce the esterase activities more than in the controls, though treatment with menazon caused considerable reduction of the ChE activity in A. pisum. After treatment with phoratoxon the ChE activity in A. pisum was reduced dramatically and in A. fabae very strongly within the first 15 to 20 minutes. The Car-E activity in A. pisum was also reduced strongly in the same time, whereas in A. fabae a very strong reduction of these esterases occurred within the 1st day.

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Appendix 1. (Fig.1.2)

Mean weight of aphids according to age and larvae produced per adult. (A. pisum larva = 0.142 mg, A. fabae larva = 0.052 mg)

Age of aphids (days)	Number of aphids	Mean weight (mg) $\pm$ S.E.	Mean no. of larvae	Weight of larvae (mg)	Weight of adult + larvae (mg)
<u>A. pisum</u>					
0	30	0.142 $\pm$ 0.053			
2	30	0.391 $\pm$ 0.145			
4	30	0.896 $\pm$ 0.035			
6	30	1.835 $\pm$ 0.063			
8	30	3.004 $\pm$ 0.109			
10	30	3.008 $\pm$ 0.060	9.2	1.3	4.308
12	30	2.851 $\pm$ 0.042	10.1	1.4	4.251
14	30	2.964 $\pm$ 0.069	13.9	2.1	5.064
16	30	2.941 $\pm$ 0.066	11.5	1.6	4.541
18	30	3.003 $\pm$ 0.075	11.5	1.6	4.603
<u>A. fabae</u>					
0	30	0.052 $\pm$ 0.019			
2	30	0.174 $\pm$ 0.065			
4	30	0.407 $\pm$ 0.009			
6	30	0.875 $\pm$ 0.023			
8	30	1.065 $\pm$ 0.028	4.2	0.22	1.285
10	30	1.147 $\pm$ 0.050	10.4	0.54	1.687
12	16	1.072 $\pm$ 0.052	11.4	0.59	1.662
14	13	1.147 $\pm$ 0.055	13.1	0.68	1.827
16	12	0.967 $\pm$ 0.049	13.0	0.68	1.647
18	6	0.968 $\pm$ 0.117	7.9	0.41	1.378

Appendix 2. (Fig. 1.3)

Mean number of nymphs produced per adult during the first 20 days of the reproductive period.

Larvi- position day	Mean number of nymphs		Larvi- position day	Mean number of nymphs	
	<u>A. pisum</u>	<u>A. fabae</u>		<u>A. pisum</u>	<u>A. fabae</u>
1	4.2	4.4	11	3.7	4.7
2	5.0	5.3	12	3.6	3.3
3	5.4	4.1	13	3.2	1.7
4	4.7	4.8	14	2.7	1.5
5	6.6	3.5	15	5.3	0.0
Sum	25.9	22.1	Sum	17.5	11.2
6	7.3	4.0	16	1.0	0.0
7	5.8	4.8	17	2.3	1.5
8	5.7	7.3	18	2.3	0.0
9	7.3	5.3	19	2.0	1.0
10	4.2	3.3	20	0.0	1.0
Sum	30.3	24.7	Sum	7.6	3.5

Appendix 3 (Table 1.1)

Number of aphids and time during which honeydew was produced.

<u>A. pisum</u>			<u>A. fabae</u>		
No. of aphids	Hours	Aphids - hrs.	No. of aphids	Hours	Aphids - hrs
<u>Experiment 1.</u>					
22	1.0	22.0	10	1.0	10.0
23	1.5	34.5	5	1.5	7.5
23	1.0	23.0	6	1.0	6.0
24	1.0	24.0	13	1.0	13.0
23	1.0	23.0	10	1.0	10.0
24	1.0	24.0	10	1.0	10.0
24	1.0	24.0	9	1.0	9.0
27	1.0	27.0	7	1.0	7.0
28	14.0	392.0	6	14.0	84.0
28	1.5	42.0	6	1.5	9.0
		<u>635.5</u>			<u>165.5</u>
<u>Experiment 2.</u>					
19	0.5	9.5			
21	17.0	357.0			
20	6.0	120.0			
		<u>486.5</u>			
<u>Experiment 3.</u>					
6	1.0	6.0	31	1.0	31.0
10	1.33	13.3	28	1.33	37.3
14	1.5	21.0	27	1.50	40.5
14	1.08	15.2	25	1.08	27.1
25	17.17	429.2	18	17.17	309.0
25	1.25	31.2	18	1.25	22.5
		<u>515.9</u>			<u>467.4</u>

Appendix 4. (Fig. 1.5)

Distribution of ingested plant material, after 24 hrs feeding, per adult (Activity of ^{32}P compound 15.9%; efficiency of scint. counter 88.3%; mean weight per leaf-disc of 2.5 cm diam., 160 mg).

^{32}P in the leaf disc extracts				Distribution of ^{32}P					Corresponding mg of plant per adult.
leaf	c. p. m.	nCi	nCi per mg of leaf-disc	Species	Sample	c. p. m.	nCi	nCi per adult	
a	62,280	199.8	1.2±1.12	<u>A. pisum</u>	Adults	13372.8	42.9	8.6±2.93	7.2
					Honey-dew	16141.2	51.8	10.3±3.22	8.6
					Total	29514.0	94.7	18.9±4.35	15.8
b	350,928	1125.9	7.0±2.65	<u>A. fabae</u>	Adults	4236.0	13.6	4.5±2.13	0.6
					Honey-dew	3442.8	11.0	3.7±1.92	0.5
					Total	7678.8	24.6	8.2±2.86	1.1

Appendix 5. (Table 1.3 and Fig.1.6)

Radioactivity found in the adults and their excretion products during 24 hrs feeding on ^{32}P -labelled leaves (Activity of the ^{32}P compound 61.6%; efficiency of the G-M counter 6.5%)

Species	Samples	1st day				2nd day				Mean per adult	
		No. of in-sects	c.p.m.	cpm per adult	nCi per adult	No. of in-sects	c.p.m.	cpm per adult	nCi per adult	nCi	nCi %
<u>A. pisum</u>	adults	leaf a				leaf b				leaves a+b/2	
	larvae	6	14129.4	2354.9	26.5±5.15	7	6946.8	992.4	11.2±3.34	18.8	57.3
	honeydew	26	1958.4	326.4	3.7±1.92	15	458.1	65.4	0.7±0.86	2.2	6.7
	Total		7250.4	1208.4	13.6±3.69		6308.4	901.2	10.1±3.18	11.8	36.0
<u>A. fabae</u>	adults	leaf b				leaf a				leaves b+a/2	
	larvae	6	2599.8	433.3	4.9±2.21	6	7194.0	1199.0	13.5±3.67	9.2	47.8
	honeydew	67	2074.8	345.8	3.9±1.97	40	2392.8	398.8	4.5±2.12	4.2	21.8
	Total		1168.5	194.7	2.2±1.48		5049.6	841.6	9.5±3.08	5.8	30.4
				973.8	11.0			2439.4	27.5	19.2	100.0

Appendix 6. (Table 1.3 and Fig.1.6)

Radioactivity found in the adults and their excretion products during their feeding for 24 hrs. on ^{32}P labelled leaf discs. (Activity of the ^{32}P -compound 71.2%; efficiency of G-M counter 6.5%)

Species	Samples	1st day				2nd day				Mean per adult	
		No. of in-sects	c. p. m.	cpm per adult	nCi per adult	No. of in-sects	c. p. m.	cpm per adult	nCi per adult	nCi	nCi %
<u>A. pisum</u>		leaf-discs a, b, c.				leaf-discs d, e, f.				leaf-discs a to f/2	
	adults	21	75522.6	3596.3	35.0±5.92	21	36770.1	1750.9	17.0±4.13	26.0	43.7
	larvae	72	12713.1	605.4	5.9±2.43	59	5676.0	270.3	2.6±1.62	4.2	7.1
	honeydew		68120.1	3243.8	31.6±5.62		57923.7	2758.3	26.8±5.18	29.2	49.1
	Total			7445.5	72.5			4779.5	46.4	59.4	99.9
<u>A. fabae</u>		leaf-discs d, e, f.				leaf-discs a, b, c.				leaf-discs a to f/2	
	adults	21	51063.0	2431.6	23.7±4.86	19	31073.4	1635.4	15.9±3.99	19.8	39.8
	larvae	145	16106.7	767.0	7.5±2.73	93	12165.0	640.3	6.2±2.50	6.8	13.8
	honeydew		43192.5	2056.8	20.0±4.47		51014.1	2684.9	26.1±5.11	23.0	46.4
	Total			5255.4	51.2			4960.6	48.2	49.6	100.0

Appendix 7. (Table 1.4 and Fig 1.7)

Distribution of the ^{32}P 24 hours after feeding on labelled discs for different times.
During the 24 hours the aphids fed on unlabelled discs (Activity of the ^{32}P compound 31%; efficiency of G-M counter 6.5%)

Sample	A. pisum					A. fabae				
	No. of in-sects	c. p. m	cpm per adult	nCi/adult		No. of in-sects	c. p. m.	cpm per adult	nCi/ adult	
				absolute	%				absolute	%
4 hrs feeding										
Adults	8	920.7	115.1	2.6±1.6	66.7	7	767.4	109.6	2.4±1.6	68.6
Larvae	10	76.2	9.5	0.2±0.5	5.1	31	134.7	19.2	0.4±0.6	11.4
Honeydew		364.5	45.6	1.0±1.0	25.6		157.2	22.4	0.5±0.7	14.3
Leaf-disc		25.8	3.2	0.1±0.3	2.6		54.0	7.7	0.2±0.4	5.7
Total			173.4	3.9	100.0			158.9	3.5	100.0
8 hrs feeding										
Adults	8	1850.2	231.3	5.2±2.3	65.0	8	470.4	58.8	1.3±1.1	52.0
Larvae	6	142.8	17.8	0.4±0.6	5.0	52	149.7	18.7	0.4±0.6	16.0
Honeydew		838.2	104.8	2.3±1.5	28.7		236.1	29.5	0.6±0.8	24.0
Leaf-disc		39.0	4.9	0.1±0.3	1.2		72.0	9.0	0.2±0.4	8.0
Total			358.8	8.0	99.9			116.0	2.5	100.0
12 hrs feeding										
Adults	8	2759.1	344.9	7.7±2.8	71.3	8	1634.1	204.3	4.6±2.1	52.3
Larvae	24	473.1	59.1	1.3±1.2	12.0	55	682.8	85.3	1.9±1.4	21.6
Honeydew		615.0	76.9	1.7±1.3	15.7		825.9	103.2	2.3±1.5	26.1
Leaf-disc		38.4	4.8	0.1±0.3	0.9		0	0	0	0
Total			485.7	10.8	99.9			392.8	8.8	100.0
24 hrs feeding										
Adults	8	4971.6	621.4	13.9±3.7	73.1	8	3318.9	414.9	9.3±3.0	66.9
Larvae	27	1011.0	126.4	2.8±1.7	14.7	47	1151.1	143.9	3.2±1.8	23.0
Honeydew		785.1	98.1	2.2±1.5	11.6		406.8	50.8	1.1±1.1	7.9
Leaf-disc		19.8	2.5	0.1±0.2	0.5		117.6	14.7	0.3±0.6	2.1
Total			848.4	19.0	99.9			624.3	13.9	99.9

Appendix 8. (Fig. 1.9)

Longevity of adults fed with different artificial diets through parafilm membrane.

Repli- cates	Days of feeding											
	Live <u>A. pisum</u>						Live <u>A. fabae</u>					
	0	1	2	3	4	5	0	1	2	3	4	5
<u>deionized distilled water</u>												
I	10	9	0	0	0	0	10	6	1	0	0	0
II	10	8	0	0	0	0	10	7	2	0	0	0
Total	20	17	0	0	0	0	20	13	3	0	0	0
<u>5% sucrose solution</u>												
I	9	9	3	0	0	0	12	10	5	3	3	2
II	10	7	1	0	0	0	9	9	5	2	1	0
Total	19	16	4	0	0	0	21	19	10	5	4	2
<u>10% sucrose solution</u>												
I	10	9	7	3	2	1	10	8	8	7	5	4
II	10	8	5	4	0	0	10	9	9	8	7	4
Total	20	17	12	7	2	1	20	17	17	15	12	8
<u>20% sucrose solution</u>												
I	10	9	5	4	0	0	10	7	5	5	4	4
II	10	7	6	6	2	0	10	9	7	7	4	3
Total	20	16	11	10	2	0	20	16	12	12	8	7
<u>40% sucrose solution</u>												
I	10	5	2	0	0	0	10	9	8	6	6	6
II	10	10	5	0	0	0	10	10	10	9	7	7
Total	20	15	7	0	0	0	20	19	18	15	13	13
<u>Complex diet</u>												
I	10	9	7	7	0	0	10	8	6	1	0	0
II	10	9	4	0	0	0	10	9	9	8	1	0
Total	20	18	11	7	0	0	20	17	15	9	1	0

Appendix 9 (Fig. 1.10)

Longevity of aphids fed on 15% sucrose solution or with the complex diet through parafilm membrane.

days of feeding	Live <u>A. pisum</u>					Live <u>A. fabae</u>				
	I	II	III	IV	mean	I	II	III	IV	mean
<u>15% sucrose solution</u>										
0	10	10	10	10	10.0±0	10	10	10	10	10.0±0
1	10	10	10	10	10.0±0	9	10	10	10	9.75±0.26
2	9	9	10	8	9.0±0.41	7	10	10	8	8.7±0.76
3	9	6	10	5	7.5±1.2	6	9	10	8	8.2±0.86
4	7	0	10	0	4.2±2.53	4	9	8	8	7.2±1.11
5	0	0	10	0	2.5±2.50	2	2	7	1	3.0±1.36
<u>complex diet</u>										
0	10	10	10	10	10.0±0	10	10	10	10	10.0±0
1	10	10	9	9	9.5±0.28	9	10	9	10	9.5±0.29
2	7	4	3	7	5.2±1.04	8	8	8	7	7.7±0.26
3	0	0	0	4	1.0±1.0	6	6	6	0	4.5±1.5
4	0	0	0	0	0.0	1	0	0	0	0.25±0.25
5	0	0	0	0	0.0	0	0	0	0	0.0

Appendix 10 (Fig. 1.12)

Longevity of adults, fed on 15% sucrose solution through stretched or unstretched membrane.

Feeding time (days)	Live <u>A. pisum</u>					Live <u>A. fabae</u>				
	I	II	III	IV	mean	I	II	III	IV	mean
<u>stretched membrane</u>										
0	10	10	10	10	10.0±0	10	10	10	10	10.0±0
1	9	10	9	9	9.25±0.25	10	10	9	10	9.75±0.26
2	9	9	9	9	9.0±0.0	9	10	9	10	9.5±0.28
3	6	7	9	6	7.0±0.71	8	9	8	6	7.75±0.63
4	1	0	9	1	2.75±2.10	8	7	6	1	5.5±1.55
5	0	0	9	0	2.25±2.25	5	6	2	0	3.25±1.38
<u>unstretched membrane</u>										
0	10	10	10	10	10.0±0	10	9	10	10	9.75±0.26
1	8	7	9	8	8.0±0.41	10	8	9	9	9.0±0.41
2	7	7	8	8	7.5±0.29	9	8	8	8	8.25±0.25
3	5	6	7	7	6.25±0.48	8	6	6	6	6.5±0.50
4	1	6	7	6	5.0±1.35	6	3	4	4	4.25±0.63
5	0	4	7	3	3.5±1.44	5	1	0	3	2.25±1.48

Appendix 11 (Fig. 1.13)

Longevity of adults, fed on 15% sucrose solution in three different relative humidities.

Feeding time (days)	Relative humidity					
	100%	40-50%	about 0%	100%	40-50%	about 0%
live <u>A. pisum</u>				live <u>A. fabae</u>		
0	11	10	10	11	10	10
1	8	6	9	8	9	9
2	2	2	3	7	8	8
3	2	2	1	5	6	6
4	2	1	0	3	5	6
5	1	1	0	0	4	4

Appendix 12. (Fig.1.14)

Longevity of adults fed artificially on 15% sucrose solution, under three different lighting conditions.

Feeding time (days)	Live <u>A. pisum</u>					Live <u>A. fabae</u>				
	I	II	III	IV	mean	I	II	III	IV	mean
<u>Full artificial light</u>										
0	10	10	10	10	10.0±0	10	10	10	10	10.0±0
1	10	10	10	9	9.75±0.26	10	9	10	9	9.5±0.28
2	5	10	9	8	8.0±1.08	10	8	9	8	8.75±0.48
3	0	0	6	0	1.5±1.5	4	3	8	5	5.0±1.08
4	0	0	0	0	0	0	1	2	0	0.75±0.48
5	0	0	0	0	0	0	0	0	0	0
<u>Dimmed light</u>										
0	10	10	10	10	10.0±0	10	10	9	10	9.75±0.26
1	10	10	10	10	10.0±0	9	9	8	10	9.0±0.41
2	9	9	10	10	9.5±0.28	9	8	8	9	8.5±0.29
3	5	4	0	1	2.5±1.19	9	7	6	2	6.0±1.47
4	0	0	0	0	0	6	4	2	0	3.0±1.29
5	0	0	0	0	0	4	0	2	0	1.5±0.96
<u>Darkness</u>										
0	10	10	9	10	9.75±0.26	10	8	9	10	9.25±0.46
1	9	10	9	10	9.5±0.28	10	4	9	10	8.25±1.44
2	8	10	9	9	9.0±0.41	10	4	8	10	8.0±1.41
3	4	3	7	2	4.0±1.08	6	2	2	4	3.5±0.96
4	0	0	6	1	1.75±1.44	2	1	0	2	1.25±0.48
5	0	0	4	0	1.0±1.0	0	0	0	0	0

Appendix 13. (Figs. 1.11 & 1.15)

Quantity of 15% sucrose solution found per adult after different times of feeding on unpoisoned and dimethoate-poisoned diet (16 ppm dimethoate in A. pisum, 8 ppm in A. fabae)

Time of feeding (hrs.)	<u>A. pisum</u>				<u>A. fabae</u>			
	Live		Dead		Live		Dead	
	No. of in-sects	µl per adult	No. of in-sects	µl per adult	No. of in-sects	µl per adult	No. of in-sects	µl per adult
<u>Unpoisoned diet</u>								
12	8	0.041	-	-	8	0.220	-	-
18	12	0.074	-	-	12	0.215	-	-
24	*	0.187	12	0.060	*	0.180	2	0.076
<u>Poisoned diets</u>								
12	8	0.044	-	-	8	0.062	-	-
18	9	0.014	20	0.066	8	0.035	63	0.076
24	-	-	64	0.070	-	-	94	0.091

(* means from a number of experiments)

Appendix 14

Mortality of adults, caused by dimethoate taken in through parafilm membranes from different concentrations in 15% sucrose solutions.

Dime- thoate conc. (ppm)	Experiments									
	A		B		C		D		E	
	Treated insects	Mort. %	Treated insects	Mort. %	Treated insects	Mort. %	Treated insects	Mort. %	Treated insects	Mort. %
	<u>A. pisum</u>									
0	37	14	30	23	20	20			20	0
1.0	-	-	-	-	-	-			20	5
2.0	-	-	-	-	-	-			20	5
2.5	-	-	-	-	20	85			-	-
4.0	-	-	30	10	-	-			20	10
5.0	35	37	-	-	20	100			-	-
8.0	-	-	30	37	-	-			20	0
10.0	36	53	-	-	-	-			-	-
16.0	-	-	30	53	-	-			20	10
20.0	36	64	-	-	-	-			-	-
30.0	36	64	-	-	-	-			-	-
	<u>A. fabae</u>									
0	36	19	30	7	17	12	22	9	22	0
0.5	-	-	-	-	-	-	20	20	20	15
1.0	-	-	-	-	-	-	20	15	23	43
2.0	-	-	31	71	-	-	15	60	22	41
2.5	-	-	-	-	20	40	-	-	-	-
4.0	-	-	29	93	-	-	20	75	20	95
5.0	37	95	-	-	20	70	-	-	-	-
8.0	-	-	30	93	-	-	27	93	22	100
10.0	36	100	-	-	19	95	-	-	-	-
20.0	32	94	-	-	22	100	-	-	-	-
30.0	39	95	-	-	-	-	-	-	-	-

Appendix 15.

Mortality of adults, caused by phorate taken in through parafilm membranes from different concentrations in 15% sucrose solutions.

case		Phorate concentrations (ppm)					
		0	0.5	1.0	2.0	4.0	8.0
<u>A. pisum</u>	Treated	20	20	20	20	20	20
	Mort. %	10	10	15	20	70	85
<u>A. fabae</u>	Treated	20	20	20	20	20	20
	Mort. %	10	5	10	20	90	100

Appendix 16. (Fig 1.16)

Imbibed quantities of dimethoate with 15% sucrose solutions by the adults and caused mortality %. The quantity of dimethoate into live insects was measured, after 24 hrs. feeding on poisoned diet, radiometrically (Two replicates per concentration were used).

<u>A. pisum</u>				<u>A. fabae</u>			
Dimethoate con. (ppm)	Mortality %	Ingested per adult		Dimethoate con. (ppm)	Mortality %	Ingested per adult	
		µl of diet	ng dimethoate			µl of diet	ng dimethoate
1.0	5.0	0.028	0.028	1.0	15.0	0.116	0.116
2.0	5.0	0.024	0.048	0.5	15.0	0.108	0.054
4.0	10.0	0.014	0.056	0.5	20.0	0.142	0.070
1.0	95.0	0.100	0.100	2.0	41.0	0.102	0.204
				1.0	43.5	0.124	0.124
				2.0	60.0	0.112	0.224
				4.0	75.0	0.070	0.280
				8.0	92.6	0.064	0.512
				4.0	95.0	0.198	0.792

Appendix 17.

Optical densities of different dilutions of the stock solution of Sudan Black "B" dye in acetone.

Light absorption (nm)	Concentrations % in stock solution					
	10.0	5.0	2.5	1.25	0.625	0.312
I	1.450	0.750	0.385	0.212	0.105	0.050
II	1.450	0.750	0.378	0.212	0.110	0.060
mean	1.450	0.750	0.382	0.212	0.108	0.055

Appendix 18 (Table 2.1; Figs 2.2, 2.3 & 2.4)

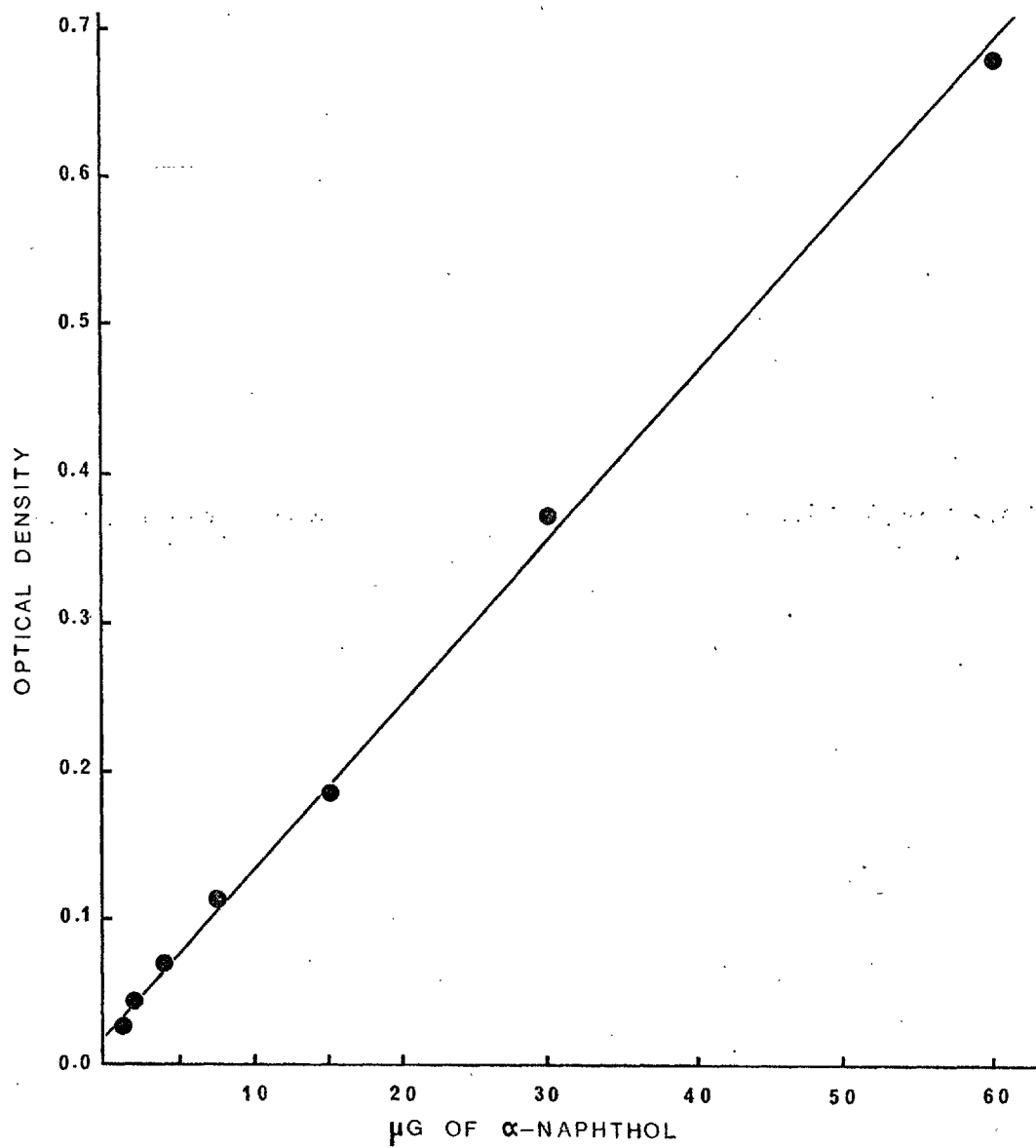
Probit analyses data for the topically applied insecticides.

Insecticides	Species	Parameters of Probit lines		Heterogeneity		LD ₅₀ ± s.e. (ng/aphid)	95% confidence limits of LD ₅₀	
		a	b ± s.e.	χ ² (d.f.)	P		Lower	Upper
Dimethoate	A. pisum	0.06	2.04 ± 0.33	0.42(1)	0.52	0.266 ± 0.069	0.186	0.361
	A. fabae	-1.89	2.27 ± 0.63	11.04(4)	0.03	1.090 ± 0.110	0.481	4.771
Phorate	A. pisum	-1.20	3.11 ± 0.53	1.26(1)	0.26	0.972 ± 0.052	0.739	1.221
	A. fabae	-0.24	3.21 ± 0.52	0.15(1)	0.70	0.431 ± 0.049	0.333	0.535
Menazon	A. pisum	1.38	2.28 ± 0.28	5.36(5)	0.38	0.384 ± 0.049	0.298	0.471
	A. fabae	2.04	1.33 ± 0.30	20.24(8)	0.01	1.634 ± 0.111	0.953	3.916

Appendix 19 (Table 2.2; Figs. 2.5, 2.6 & 2.7)

Probit analyses data for the systemically applied insecticides (* regression not significant)

Insecticides & Applications	Species	Parameters of Probit lines		Heterogeneity		LD ₅₀ ± s. e. (µg/disc)	95% confidence limits of LD ₅₀	
		a	b ± s. e.	x ² (d. f.)	P		Lower	Upper
<u>Dimethoate</u>								
Wick appl.	A. pisum	0.92	1.46 ± 0.30	0.43(3)	0.93	0.613 ± 0.121	0.310	1.030
	A. fabae	0.79	1.41 ± 0.29	0.73(1)	0.41	0.973 ± 0.121	0.504	1.676
Leaf-surface app.	A. pisum	2.99	0.69 ± 0.20	0.70(2)	0.71	0.793 ± 0.220	0.144	1.879
	A. fabae	0.88	1.13 ± 0.23	1.72(1)	0.18	4.306 ± 0.129	2.453	8.791
<u>Phorate</u>								
Wick appl.	A. pisum	-9.30	4.22 ± 0.60	6.49(4)	0.17	2.598 ± 0.033	2.236	3.036
	A. fabae	-2.38	2.62 ± 0.39	2.52(2)	0.28	0.651 ± 0.054	0.498	0.831
Injection	A. pisum	-0.88	1.79 ± 0.31	0.82(3)	0.84	1.962 ± 0.070	1.445	2.848
	A. fabae	-2.18	2.67 ± 0.43	0.46(1)	0.50	0.492 ± 0.060	0.359	0.637
Blotting paper appl.	A. pisum	0.79	1.41 ± 0.27	1.35(2)	0.52	0.952 ± 0.104	0.588	1.621
	A. fabae	0.65	1.61 ± 0.85	12.88(1)	*	0.502 ± 0.080	-	-
<u>Menazon</u>								
Blotting paper appl.	A. pisum	2.34	0.73 ± 0.09	7.82(15)	0.93	4.110 ± 0.106	2.676	7.246
	A. fabae	1.45	1.04 ± 0.14	6.53(8)	0.58	2.548 ± 0.092	1.721	4.077



Appendix 20

Standard curve for the determination of α -naphthol (Each value is a mean of two replicates).

μg of α -naphthol	0.937	1.875	3.75	7.5	15.0	30.0	60.0
optical densities	0.025	0.042	0.067	0.110	0.185	0.367	0.675

Appendix 21 (Fig. 3.2)

The influence of homogenate concentration on the esterase activity of A. pisum and A. fabae to α -naphthyl acetate 2×10^{-4} M (Optical density)

Esterases	Species	Insect material (mg/tube)					
		.125	.25	.5	1.0	2.0	4.0
Cholin- esterases	<u>A. pisum</u>	.017	.024	.064	.137	.237	.242
	<u>A. fabae</u>	.010	.020	.026	.035	.013	.005
Carboxyl- esterases	<u>A. pisum</u>	.007	.022	.060	.138	.286	.533
	<u>A. fabae</u>	.066	.120	.271	.509	.740	.835

Appendix 22 (Figs. 3.3 & 3.4)

Effect of incubation temperature on the esterase activity of the two species to α - or β -naphthyl acetate 2×10^{-4} M. (Optical density)

Esterases	Species	Incubation temperature (°C)				
		22	27	32	37	42
<u>α - naphthyl acetate</u>						
Cholinest- erases	<u>A. pisum</u>	.272	.322	.354	.319	.223
	<u>A. fabae</u>	.082	.068	.160	.132	.058
Carboxyl- esterases	<u>A. pisum</u>	.110	.154	.189	.220	.225
	<u>A. fabae</u>	.426	.552	.660	.698	.592
<u>β - naphthyl acetate</u>						
Cholinest- erases	<u>A. pisum</u>	.125	.035	.005	.005	.018
	<u>A. fabae</u>	.180	.180	.030	.046	.134
Carboxyl- esterases	<u>A. pisum</u>	1.025	1.055	1.085	1.085	1.085
	<u>A. fabae</u>	1.430	1.546	1.900	1.850	1.262

Appendix 23. (Figs. 3.5 & 3.6)

Data for the double reciprocal plot of $1/v$ vs $1/S$ for the hydrolysis of α -naphthyl acetate by cholinesterases and carboxylesterases of the two species, at 37°C .

Species	mM/L (S)	$\frac{1}{S}$	ChEs		Car-Es	
			Optical density (v)	$\frac{1}{v}$	Optical density (v)	$\frac{1}{v}$
<u>A. pisum</u>	0.05	20.00	0.228	4.39	0.202	4.95
	0.10	10.00	0.278	3.60	0.238	4.20
	0.20	5.00	0.320	3.12	0.292	3.42
	0.40	2.50	0.338	2.96	0.328	3.05
	0.80	1.25	0.345	2.90	0.368	2.72
	1.60	0.62	0.340	2.94	0.398	2.51
<u>A. fabae</u>	0.05	20.00	0.070	14.29	0.432	2.31
	0.10	10.00	0.102	9.80	0.540	1.85
	0.20	5.00	0.132	7.58	0.630	1.59
	0.40	2.50	0.158	6.33	0.712	1.40
	0.80	1.25	0.168	5.95	0.792	1.26
	1.60	0.62	0.168	5.95	0.812	1.23

Appendix 24. (Fig. 3.7)

Esterase activity (optical density) and inhibition after treatment of homogenates of the two species with different concentrations of eserine sulphate.

Eserine concentra- tions(M/ L)	<u>A. pisum</u>		<u>A. fabae</u>	
	Activity	Inhibition %	Activity	Inhibition %
0	0.595	0	0.594	0
10 ⁻⁹	0.595	0	0.590	0
10 ⁻⁸	0.578	2.8	0.590	0
10 ⁻⁷	0.493	17.1	0.560	5.7
10 ⁻⁶	0.345	42.0	0.526	11.4
10 ⁻⁵	0.339	43.0	0.470	20.9

Appendix 25. (Fig. 3.8)

Inhibition of esterase activity (optical density) by 10⁻⁶ M eserine at different preincubation times.

Preincu- bation time (mins)	<u>A. pisum</u>			<u>A. fabae</u>		
	Overall activity	Inhibited action	Inhibi- tion %	Overall activity	Inhibited action	Inhibi- tion %
0	0.517	0.108	20.9	0.607	0.037	6.1
5	0.560	0.190	33.9	0.648	0.021	3.2
10	0.561	0.223	39.7	0.650	0.029	4.5
20	0.561	0.258	46.0	0.652	0.047	7.2
40	0.600	0.278	46.3	0.690	0.055	8.0
80	0.568	0.274	48.2	0.631	0.039	6.2

Appendix 26 (Fig 3.9.)

Esterase activity for different incubation times, studied with the colorimetric method (substr. α -naphthyl acetate 2×10^{-4} M).

Incubation time (mins)	<u>A. pisum</u>			<u>A. fabae</u>		
	Optical density	μ g of α -naphthol	Acti-vity %	Optical density	μ g of α -naphthol	Acti-vity %
Cholinesterases						
5	.121	9.2	41.8	.032	1.0	27.0
10	.159	12.5	56.8	.057	3.5	94.6
15	.204	16.7	75.9	.062	3.7	100.0
20	.230	19.0	86.4	.060	3.6	97.3
25	.249	20.5	93.2	.061	3.6	97.3
30	.265	22.0	100	.062	3.7	100
Carboxylesterases						
5	.142	11.0	37.0	.247	20.4	35.8
10	.169	13.5	45.4	.351	29.7	52.1
15	.216	17.7	59.6	.457	39.2	68.8
20	.291	24.4	82.1	.545	47.0	82.4
25	.314	26.5	89.2	.604	52.2	91.6
30	.352	29.7	100	.655	57.0	100

Appendix 27 (Fig. 3.10)

Esterase activities in live and dead aphids of the two species 24 hrs. after topical application with dimethoate (Doses applied and mortalities: in A. pisum 0.37 ng/aphid, mort. 48%; in A. fabae 1.50 ng/aphid, mort. 75%)

Species	State	Replicates	Optical density					
			Combined activity		Car-Es		ChEs	
			Control	Dimethoate	Control	Dimethoate	Control	Dimethoate
<u>A. pisum</u>	Live	I	.600	.475	.330	.310		
		II	.565	.470	.335	.305		
		III	.580	.473	.330	.300		
		M	.582	.473	.332	.305	.250	.168
<u>A. pisum</u>	Dead	I	.658	.480	.379	.280		
		II	.662	.465	.381	.280		
		M	.660	.473	.380	.280	.280	.193
<u>A. fabae</u>	Live	I	.630	.560	.560	.520		
		II	.625	.560	.560	.520		
		III	.625	.560	.560	.520		
		M	.627	.560	.560	.520	.067	.040
<u>A. fabae</u>	Dead	I	.600	.525	.518	.490		
		II	.600	.540	.523	.480		
		M	.600	.533	.520	.485	.080	.048

Appendix 28. (Fig. 3.11)

Esterase activities in adults of A. pisum still living at different time intervals after topical application with dimethoate. (dose applied, 0.19 ng/aphid; each estimation is a mean of two replicates).

Time after application(hrs)	Optical density			
	ChEs		Car-Es	
	Control	Dimethoate	Control	Dimethoate
0	.210	.230	.370	.298
2	.266	.238	.274	.292
4	.271	.252	.284	.278
6	.295	.230	.265	.255
18	.347	.267	.293	.283
24	.335	.253	.330	.315

Appendix 29 (Fig. 3.12)

Mortality and esterase activities in live and dead aphids 24 hrs. after topical application with phorate.

Dose of phorate (ng/aphid)	State of aphids	<u>A. pisum</u>			<u>A. fabae</u>		
		Mortal. %	<u>Optical densities</u>		Mortal. %	<u>Optical densities</u>	
			ChEs	Car-Es		ChEs	Car-Es
0	live	0	.203	.315	0	.030	.600
0.16		6	.242	.278	14	.047	.568
0.32		10	.243	.225	38	.031	.542
0.64		25	.262	.258	71	.030	.630
1.28		77	.182	.208	-	-	-
0.64	dead	25	.234	.216	71	.025	.425
1.28		77	.121	.182	96	.012	.348
2.56		88	.087	.143	100	.006	.370
5.12		100	.085	.121	100	.012	.252

Appendix 30 (Fig. 3.13)

Mortality and esterase activities in live and dead aphids 24 hrs. after exposure to phorate, applied systemically.

Dose of phorate (µg/disc)	State of aphids	<u>A. pisum</u>			<u>A. fabae</u>		
		Mortal. %	<u>Optical densities</u>		Mortal. %	<u>Optical densities</u>	
			ChEs	Car-Es		ChEs	Car-Es
0	live	0	.218	.351	0	.030	.538
0.02		2	.272	.250	7	.000	.540
0.06		12	.237	.208	21	.003	.570
0.18		20	.239	.231	16	.000	.560
0.54		30	.230	.235	25	.010	.560
1.62		48	.245	.220	-	-	-
0.06	dead	12	.288	.232	-	-	-
0.18		20	.295	.190	-	-	-
0.54		30	.252	.183	25	.005	.500
1.62		48	.162	.120	93	.007	.416
4.86		100	.039	.069	100	.000	.133

Appendix 31 (Fig. 3.14)

Mortality and esterase activities in live adults of the two species 24 hrs. after exposure to menazon, applied systemically (Each estimation is a mean of two replicates).

Dose of menazon ($\mu\text{g}/\text{disc}$)	Log dose ($\mu\text{g} \times 10^2$)	<u>A. pisum</u>			<u>A. fabae</u>		
		Mortality %	Optical densities		Mortality %	Optical densities	
			ChEs	Car-Es		ChEs	Car-Es
0	0	0	.295	.280	0	.100	.520
0.06	0.778	4	.267	.313	3	.065	.520
0.18	1.255	7	.225	.335	4	.063	.537
0.54	1.732	25	.225	.335	26	.045	.600
1.62	2.209	43	.220	.335	46	.058	.582

Appendix 32 (Fig. 3.15)

Esterase activities in live and dead adults of the two species, 24 hrs. after exposure to menazon applied systemically (doses applied and mortalities: in A. pisum 4.86 $\mu\text{g}/\text{disc}$, mort. 51%; in A. fabae 1.62 $\mu\text{g}/\text{disc}$, mort. 53%)

Species	State	Replicates	Optical densities					
			Combined activity		Car-Es		ChEs	
			Control	Menazon	Control	Menazon	Control	Menazon
<u>A. pisum</u>	live	I	.500	.505	.245	.260		
		II	.490	.490	.250	.265		
		M	.495	.498	.248	.263	.247	.235
<u>A. pisum</u>	dead	I	.408	.415	.202	.222		
		II	.412	.410	.199	.215		
		M	.410	.413	.200	.219	.210	.194
<u>A. fabae</u>	live	I	.610	.560	.540	.540		
		II	.610	.560	.525	.540		
		M	.610	.560	.533	.540	.077	.020
<u>A. fabae</u>	dead	I	.600	.540	.502	.510		
		II	.596	.540	.502	.505		
		M.	.598	.540	.502	.508	.096	.032

Appendix 33. (Figs. 3.16, 3.17, 3.18 & 3.19)

Esterase activities at different times, in homogenates of the two species untreated (controls) or treated with insecticide solutions (Each estimation is a mean of two replicates)

Time (days)	Optical densities							
	A. pisum				A. fabae			
	ChEs		Car-Es		ChEs		Car-Es	
	Control	Treated	Control	Treated	Control	Treated	Control	Treated
<u>Treatment with dimethoate</u>								
0	.102	.113	.518	.505	.050	.040	.640	.650
1	.169	.178	.336	.332	.030	.028	.580	.605
2	.112	.117	.073	.092	.023	.015	.575	.600
3	.076	.063	.088	.091	.007	.013	.543	.470
4	.011	.007	.083	.082	.005	.010	.408	.350
5	.009	.007	.068	.065	.012	.008	.202	.151
<u>Treatment with menazon</u>								
0	.155	.145	.470	.465	.020	.035	.670	.660
1	.104	.021	.063	.108	.015	.012	.505	.500
2	.100	.004	.045	.046	.010	.003	.495	.490
3	.070	.000	.034	.036	.006	.005	.424	.418
4	.066	.000	.036	.033	.022	.000	.375	.374
5	.071	.000	.062	.040	.024	.004	.350	.345
<u>Treatment with phorate</u>								
0	.155	.133	.388	.390	.037	.023	.628	.615
1	.103	.083	.065	.075	.040	.012	.520	.513
2	.067	.041	.036	.039	.028	.030	.480	.450
3	.067	.039	.032	.034	.039	.033	.398	.422
4	.042	.030	.029	.042	.011	.018	.351	.391
5	.045	.023	.043	.059	.016	.016	.302	.345
<u>Treatment with phoratoxon</u>								
0	.164	.063	.409	.117	.045	.030	.645	.570
1	.098	.000	.059	.025	.020	.000	.503	.035
2	.069	.000	.036	.023	.015	.000	.440	.034
3	.060	.000	.034	.022	.022	.000	.423	.038
4	.037	.000	.033	.023	.016	.000	.357	.036
5	.037	.000	.044	.027	.015	.000	.326	.025