TOXICITY OF THE SYSTEMIC INSECTICIDE ^{14}C-PHORATE AND ITS METABOLITES TO *APHIS FABAE* SCOP. (HOMOPTERA:APHIDIDAE)

by

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September, 1978

ABSTRACT

The toxicity of ^{14}C-labelled phorate to *Aphis fabae* Scop. was investigated both in the bean plant, *Vicia faba* L., and in an artificial diet, using radiotracer techniques.

The toxic effect of ^{14}C-phorate to aphids and the accumulation of oxidised and hydrolysed metabolites in infested leaflets, aphids and their honeydew were studied in relation to the site of application of the insecticide to the bean plant. The distribution and accumulation of these metabolites in the leaves were qualitatively assessed by autoradiography and quantitatively assayed by liquid scintillation counting.

The feeding rate of the aphids on bean plants based on daily honeydew production was compared with that on a completely defined artificial diet.

Phorate metabolism in artificial diet was followed over a period of time by extraction and thin-layer chromatography. The significance of the formation of phorate metabolites in diet and in plants is discussed in relation to the relative potencies of phorate and some of its oxidised metabolites.

The relative potencies were assessed by bioassay in which phorate or one of its metabolites was incorporated in the diet. Aphid mortality was also correlated with diet uptake. The uptake of phorate treated diet, to which ^{32}P-phosphate was added, was determined by the accumulation of ^{32}P-label in the aphids and their honeydew. In the metabolite treated diets higher doses were necessary and liquid uptake was assessed using the ^{14}C-label present in the insecticide.

The results are discussed in relation to our understanding of the uptake of insecticides by sap-sucking insects, with a view to improving pest control techniques with systemic insecticides.

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1. INTRODUCTION

Systemic organophosphorus insecticides are especially useful in selectively controlling plant-sucking insect pests, and much work has been devoted to the feeding behaviour of these insects as well as their control with these compounds. The behaviour, metabolism and mode of action of these insecticides have been the focus of toxicological studies in recent years. Comprehensive reviews on this subject have been written by O'Brien (1960) and Matsumura (1975). Much attention has also been given to the translocation of systemic insecticides within plants and their relative toxicities to various insects with the aid of radiotracer, bioassay and cholinesterase inhibition techniques. Nevertheless, there is little published information on the toxicity of these insecticides in plants in relation to insecticide uptake by sap-feeding insects. Quantitative measurements of the uptake of systemic insecticides and their metabolites by such insects are of considerable importance in developing strategies for their control. Attempts have been made by Parry and Ford (1967, 1969, 1971) and Halimie and Ford (1972) to measure the liquid imbibition by *Myzus persicae* when phosphamidon was incorporated into the food. They related liquid uptake to the intraspecific differences exhibited by the aphids in their susceptibility to this insecticide, and demonstrated the depression in the level of feeding caused by phosphamidon.

In the following sections short reviews are presented in three main parts: a discussion on systemic insecticides in general, on phorate, the insecticide used in this study, with particular emphasis on its metabolism, and finally, on aphid feeding, with special reference to feeding on artificial diet.

1.1 Systemic insecticides

Systemic insecticides are a group of chemicals that are absorbed into plants and translocated primarily in the xylem in quantities biologically active against insects (Bennett, 1949, 1957). On the basis of the concept of Münch (1930) (cited by Crisp, 1972), systemic insecticides can be subdivided according to their path of translocation. A symplastic compound has been defined by Crisp (1972) as one that can pass through the plasmalemma into the living continuum and move along with the assimilate stream in the phloem tissue to other parts of the plant. An apoplastic insecticide, on the other hand, is one that is transported exclusively by the transpiration stream in the xylem or apoplast, which constitutes the non-living cell-wall continuum, and accumulates in the regions of water loss such as leaf margins.

The effectiveness of systemic insecticides is governed to some degree by the ability of the plant to absorb them in suitable amounts at the application site and translocate them to the infested part of the plant where effectiveness may be desired in specific tissues, for example, the phloem, for the control of phloem-feeding insects like aphids. Polarity is an important factor in determining the absorption and translocation characteristics of systemic insecticides. Whereas penetration and absorption of systemic insecticides into leaves, fruits and stem are influenced by the lipophilic properties of these compounds, effective translocation in the xylem depends upon their hydrophilic nature (Mitchell *et al.*, 1960). The extent and direction of translocation within the plants are therefore critical in determining insecticidal effectiveness in relation to the method of application and the pests to be controlled. The development of effective symplastic insecticides had met with little success. In the light of these facts, Crisp (1972), who

found all organophosphorus insecticides to be apoplastic, investigated the translocation characteristics of endogenous substrates within the symplast. He hypothesised that a weak acid or -COOH was a necessary functional group for effective uptake and phloem mobility of potential systemic insecticides.

It has been established that transfer of apoplastic molecules into phloem tissue does not occur to any significant degree, although there is evidence of a limited amount of downward movement of some xylem-mobile insecticides following foliar application. According to Finlayson and MacCarthy (1965), transport of systemics in general was upward with restricted downward movement. A similar conclusion was arrived at by de Pietri-Tonelli (1965) who investigated the translocation of dimethoate in plants. This insecticide moved mainly in the transpiration stream, but some downward translocation through the phloem also occurred at a slower rate. He attributed this downward movement to radial transfer from the xylem. Work on phorate transport in broad bean plants (Galley and Foerster, 1973; Foerster and Galley, 1976) has demonstrated the presence of toxic compounds in aphids placed on the lower leaves following foliar application of ^{14}C-phorate to more distal foliage, again suggesting some downward movement of this apoplastic systemic chemical.

1.2 Phorate

1.2.1. Systemic properties

The insecticide studied in this work, phorate (Thimet$^{(R)}$, or O, O, diethyl S-ethylthiomethyl phosphorodithioate), is an organophosphorus apoplastic systemic insecticide. It has been widely used for treatment of crops like cotton, alfalfa (lucerne), broad and field beans, sugar beets, flax and other agricultural crops to control a variety of pests such as

aphids, mites, thrips, whiteflies and caterpillars.

According to Mitchell *et al.* (1960) three controlling properties govern the effectiveness of a systemic insecticide. Firstly, it must be able to penetrate into the plant; secondly, it must be sufficiently water-soluble to enable it to move with the transpiration stream; and thirdly, the compound or its metabolites must be stable enough in the plant environment to exert its residual effect. These requirements are influenced by the insecticide's volatility, solubility, susceptibility to oxidation and suceptibility to hydrolysis.

Penetration and translocation are determined by the lipophlic-hydrophilic balance of the insecticide. Phorate is slightly soluble in water (17 ppm at 20°C) (Lord and Burt, 1964), but is completely miscible with most organic solvents. The partition coefficient for phorate and its oxidised metabolites in an equivolume chloroform-water system is 1.00 for phorate and all the metabolites except for phoratoxon sulfone which has a value of 0.99 (Bowman and Casida, 1957). In an equivolume hexane-water system the partition coefficient ranges from 1.00 for phorate to 0.01 for phoratoxon sulfone (Bowman *et al.*, 1969).

Lipophilic insecticides have been reported to be absorbed by the lipoid substances in the leaf cuticle (Bennett and Thomas, 1954). Likewise phorate, being lipophilic, can readily be absorbed by the cuticle and penetrate through the leaves. The lipophilic nature of phorate also facilitates its absorption by the lipoprotein layers of the roots, where it is oxidised rapidly to its sulfoxide and more slowly to its sulfone (Metcalf, 1967). The sulfoxide is easily translocated in the xylem in view of its hydrophilic property (Bowman *et al.*, 1969), compared to the lipophilic phorate and its sulfone. The oxygen analogue, phoratoxon, and

its sulfoxide and sulfone are more rapidly hydrolysed in plants than the parent compound and its derivatives (Bowman and Casida, 1958) and therefore quickly lose their insecticidal value.

Absorption of phorate by plant tissues is also influenced by its volatility. Burt *et al.* (1965) have demonstrated that sufficient could be absorbed from the vapour phase by the roots of plants to render the leaves toxic to aphids feeding on them.

The stability of a chemical in the plant environment depends on its susceptibility to metabolism, which is discussed below.

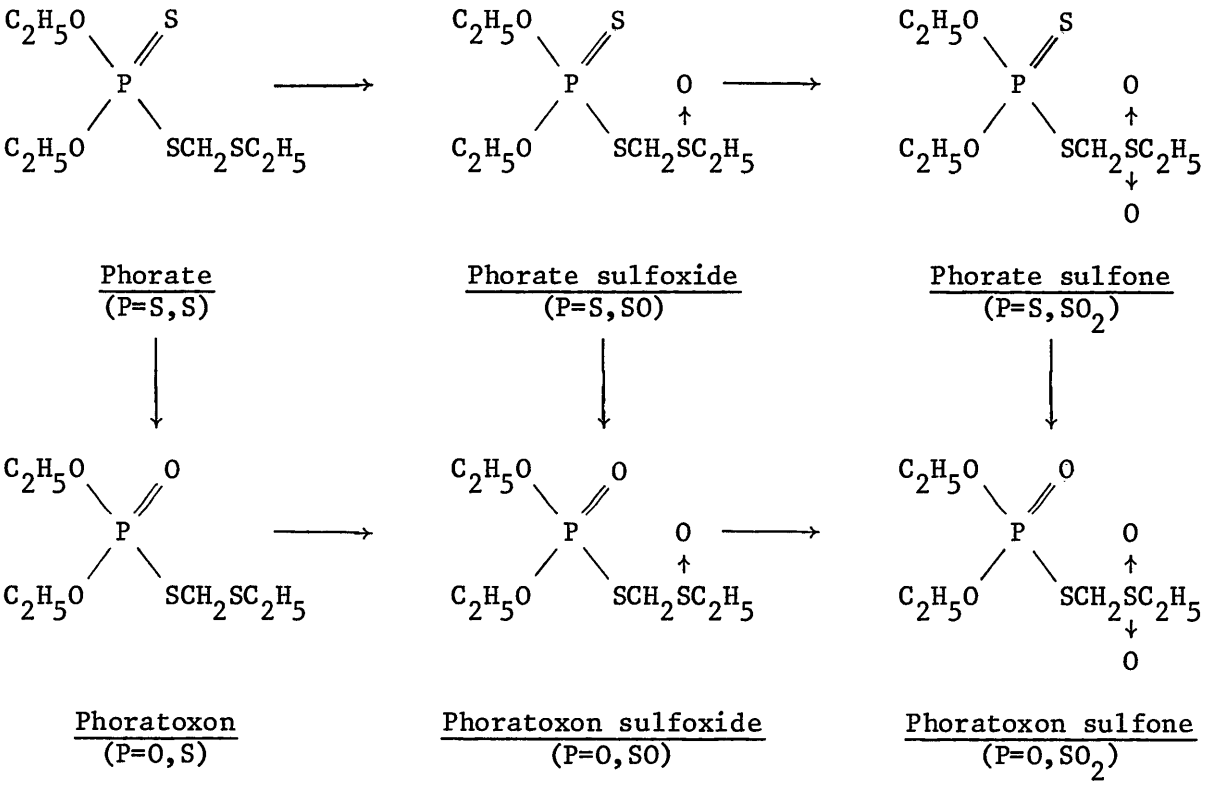
1.2.2. Metabolism

Phorate belongs to a group of chemicals that also include demeton and disulfoton. These are characterized by the presence of short alkyl chains, one of which contains a thioether sulphur that is oxidised to the corresponding sulfoxide and sulfone. The discovery of such oxidative changes in demeton isomers was made by Metcalf *et al.* (1954, 1955) and Fukuto *et al.* (1954, 1955). Phorate and other structurally related compounds are susceptible to oxidative desulphuration, thioether oxidation and hydrolysis.

1.2.2.1. Oxidation

Phorate is oxidatively desulphurated at the thiono sulphur atom so that P = S is oxidised to P = O to form the oxygen analogue, phoratoxon, which is metabolised to the sulfoxide and sulfone. Both phorate and phoratoxon can undergo thioether oxidation to give their corresponding sulfoxide and sulfone (Bowman and Casida, 1957; Metcalf *et al.*, 1957). The oxidative metabolic pathway of phorate has been established in plants, insects and mammals (Metcalf *et al.*, 1957; Bowman and Casida, 1957, 1958),

and the five toxic metabolites thus formed are shown as follows:



The oxidative metabolism of phorate may result in the formation of very potent anticholinesterase agents. For many organophosphates, oxidative desulphuration is absolutely necessary for anticholinesterase potency (Heath, 1961; O'Brien, 1967). Phoratoxon and its sulfoxide and sulfone are more effective inhibitors of acetylcholinesterase than the phorate derivatives, phoratoxon sulfone being the most powerful (Bowman and Casida, 1957; Metcalf *et al.*, 1957; Archer *et al.*, 1963). These anticholinesterase agents are also more water-soluble (Bowman, 1973; le Patourel and Wright, 1976).

1.2.2.2. Hydrolysis

Like all organophosphorus triesters, phorate and its oxidised metabolites are susceptible to hydrolysis. The rates of hydrolysis of these compounds are of toxicological significance because such reactions

lead to their detoxication. The hydrolysis rate is dependent upon the conditions of reaction, such as pH, temperature and the presence of certain catalysts like free metal ions (Eto, 1974; Fest and Schmidt, 1973; Heath, 1961).

It has been mentioned that the oxidised products are generally more toxic to acetylcholinesterase but are more readily hydrolysed, thus rapidly losing their toxic effect. In order to be effective, they must be continually formed from the less oxidised compounds to maintain low concentrations at the site of enzyme action.

Metabolism of phorate has been extensively investigated in various crops (Metcalf *et al.*, 1957; Bowman and Casida, 1957, 1958; Menzer and Ditman, 1968; Bowman *et al.*, 1969), and in soil (Menzer *et al.*, 1970; Lichtenstein *et al.*, 1973, 1974; Schulz *et al.*, 1973). However, relatively little work has been done on phorate metabolism in the pests themselves. It has only been studied in insects by Bowman and Casida (1958) and Menn and Hoskins (1962), and in nematodes by le Patourel and Wright (1974, 1976). The rates of phorate metabolism in these various systems differ, but the sulfoxide and sulfone of phorate appear to be the major oxidised metabolites found.

1.3 Aphid feeding

1.3.1. Ingestion of sap

It is well established that aphids transmit stylet-borne and persistent viruses by their probing behaviour (Leach, 1940; Esau *et al.*, 1961). In general aphids feed predominantly in the phloem, although other feeding sites have also been reported (Auclair, 1963; Lowe, 1967; Pollard, 1973). Sap intake has been extensively studied using various methods.

Kennedy and Mittler (1953) devised a cut-stylet technique which was used by Mittler (1953, 1957, 1958a, 1958b) in a series of studies on the feeding and nutrition of *Tuberolachnus salignus*. Other techniques for estimating the rate of sap ingestion include measurements of the rate of honeydew excretion alone (Auclair, 1958; Mittler, 1958a, 1958c) or combined with nutritional balance (Mittler, 1958b; Auclair, 1959; Auclair and Maltais, 1961), and the use of radiotracers (Watson and Nixon, 1953; Day and Irzykiewicz, 1953; Banks and Nixon, 1959).

It has been suggested that aphids are able to control their rate of sap uptake and excretion (Banks and Nixon, 1958, 1959; Auclair and Cartier, 1960), and these rates are not dependent solely on the turgor pressure within plants (Mittler, 1957).

1.3.2. Honeydew excretion

Honeydew excretion has been used as a crude measure of rate of sap uptake, as mentioned above. Several techniques have been employed to collect honeydew and measure excretion rates. Circular pieces of filter paper placed on revolving turn-tables for honeydew collection have been used by many workers, for example, Smith (1937), Broadbent (1951), Auclair (1958, 1959), Mittler (1958c), Mittler and Sylvester (1961). Other devices used have included long filter paper strips pulled under the feeding aphids at a constant speed (Banks and MacAulay, 1965); mineral oil (Mittler and Sylvester, 1961; Banks and MacAulay, 1964); and waxed card (Auclair, 1958, 1959; Auclair and Maltais, 1961).

The rates of honeydew excretion are affected by environmental factors such as:

1) temperature (Smith, 1937; Mittler, 1958c);

2) wind (Broadbent, 1951; Mittler, 1958c);

3) light intensity (Maxwell and Painter, 1959); and

4) diurnal changes in the total soluble carbohydrates in the food supply (Cull and van Emden, 1977).

Cessation of honeydew excretion has been reported in post-reproductive adults of *Aphis fabae* (Banks and MacAulay, 1964). Varying periods of non-excretion during ecdysis have also been shown to occur in *Myzus persicae* (Broadbent, 1951), *Acyrthosiphon pisum* and *T. salignus* (Mittler, 1958c; Auclair, 1959).

Honeydew excretion is also of toxicological interest, for it is likely to be a mechanism for the removal of toxic compounds (Eastop and Banks, 1970; Galley and Foerster, 1973; Devonshire and Needham, 1974).

1.3.3. Artificial feeding of aphids

The artificial feeding of aphids has been attempted by Hamilton (1930, 1935) and several other workers, for example, Pletsch (1937), Roach (1937), Lindemann (1948), Day and Irzykiewicz (1953), Mittler (1954), van Hoof (1958), Lampel (1958), Rochow (1960), and Marek (1961). These authors have used various gels or liquids, sometimes under pressure and contained within various types of membranes through which the aphids probed and fed. Unfortunately in these experiments few survived for more than about a week.

In 1962, Mittler and Dadd reported the first limited success in the artificial feeding and rearing of *Myzus persicae* on a chemically defined diet, presented to the aphids on a stretched Parafilm 'M' membrane. This diet was composed of 18% sucrose, a mixture of vitamins, cholesterol,

mineral salts and amino acids and amides.

Since then considerable progress has been made in terms of improving the diet composition and of techniques in making up sterile sachets. A method of making up diet sachets with Parafilm 'M' was developed by Mittler and Dadd (1963a) and subsequently improved to reduce chemical and microbial contamination (Mittler and Dadd, 1964a). Storage of the diet at -20°C was shown to reduce loss of vitamins especially ascorbic acid (Dadd *et al.*, 1967).

Sucrose was found to be essential for the aphids' survival but amino acids were essential for larval growth (Dadd and Mittler, 1965). Survival and larviposition were greatest on a complex diet (Mittler and Dadd, 1963b) and assimilation was found to occur only on such a diet and not on sucrose alone (Mittler and Dadd, 1965a). Sucrose has been found to be a phagostimulant (Mittler and Dadd, 1963a; Parry and Ford, 1967; Srivasta and Auclair, 1971b). The optimal sucrose concentrations in synthetic diets ranged from 10-20% for *M. persicae* (Mittler and Dadd, 1962, 1965a; Dadd and Mittler, 1965; Mittler, 1967a, 1967b); 10-35% for *A. pisum* (Auclair and Cartier, 1963; Auclair, 1965; Srivasta and Auclair, 1971a, 1971b); 10-15% for *A. fabae* (Dadd and Krieger, 1967; van Emden, 1967).

The search for a complete diet culminated in the development of a totally synthetic diet with the inclusion of trace minerals (Dadd and Mittler, 1966). Although this diet was qualitatively complete the aphids reared on this diet were smaller than those reared on plants. The above authors thought that nutrient imbalance was the reason for this difference in size.

1.3.4. Evaluation of feeding on artificial diet

The uptake of diet has been evaluated in several ways, for example, by differential weighing of sachets or by the increase in biomass comprising the aphids, their honeydew, water loss, production of young and exuviae during ecdysis.

The rates of feeding and excretion have been estimated using dyes such as neutral red (Mittler and Dadd, 1964b; Parry and Ford, 1967, 1969), or radioisotopes such as ^{32}P-phosphate (Parry and Ford, 1967, 1969, 1971; Danneel, 1969a, 1969b).

Very little work has been carried out with insecticidal diets but attempts have been made by a few authors to assess the toxicity of some systemic compounds in artificial diets to aphids (Mittler and Pennell, 1964; Parry and Ford, 1967, 1969; Javadi, 1971; Patsakos, 1972).

It has been established that aphids feed predominantly in the phloem, but when a systemic insecticide is applied to the plant, the concentrations of the chemical in this tissue are not known since these are mainly confined to the apoplast. The total insecticide residues in the leaves in relation to that taken up by the aphids may vary enormously (Galley and Foerster, 1976b). It was hoped that an investigation of the insecticide concentrations to which the aphids are susceptible would throw some light on the order of magnitude of the doses needed to control these insects and on the relative importance of the major toxic metabolites in their food.

2. MATERIALS AND METHODS

2.1 Plants

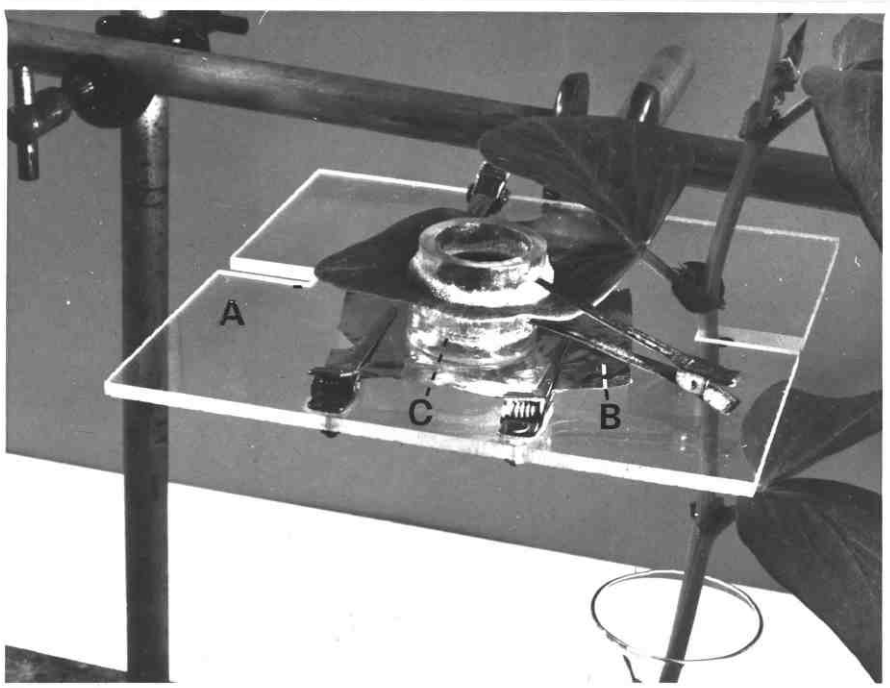
Broad bean plants, *Vicia faba* L. cv. Sutton's Aquadulce, grown in sand in a glasshouse were utilized in experiments when the pair of leaflets at the second node had fully expanded. The plants were gently loosened from the sand and the roots were carefully washed under running water to remove the sand and the testa. The removal of the testa greatly reduced microbial growth in the nutrient solution. The washed plants were then placed in specimen tubes (3.6 x 10 cm) containing Hewitt's (1966) nutrient solution with Fe-EDTA omitted. The stems of the plants were supported on perspex platforms with recesses for the stems, as shown in Fig. 2.1.

2.2 Insects

The black bean aphid, *Aphis fabae* Scop., (Homoptera: Aphididae) was used in this work. They were reared by a mass culturing method developed to provide a continuous supply of adult alatae (see Kennedy and Booth, 1950, 1964; Kennedy and Ludlow, 1974). The culture was maintained at a temperature of 15.5^{o}C and a humidity of 70-80% with a light regime of 16 hours photophase and 8 hours dark.

2.3 Aphid caging technique

Adult alates were confined to the lower surface of bean leaves by means of perspex cages, 2.5 cm in diameter, with detachable muslin caps. In experiments involving radioassay, the cages were lined with filter paper to collect the honeydew excreted (Galley, 1974). In experiments where the dry weight of the honeydew was assessed and radioisotope was not used, the muslin caps were removed and pieces of aluminium foil (ca. 5 x 4 cm), cleaned and weighed on a Mettler H20 balance, were placed beneath the cages for honeydew collection. The foils bearing the honeydew



A : Perspex platform supporting stem of bean plant

B : Aluminium foil

C : Aphid cage

Fig. 2.1. Honeydew collection on aluminium foil from caged aphids feeding on broad bean leaf.

were then dried over phosphorus pentoxide in a desiccator under vacuum for a week.

The leaves bearing the caged aphid colonies were held in position by metal clips fixed on the perspex platforms supporting the stems of the bean plants.

The experiments were carried out in a constant temperature room at a temperature of 20 ± 2°C and uncontrolled humidity. The 16 hr light and 8 hr dark cycle was maintained by 8 fluorescent tubes (1.5 m long) mounted 50 cm above the bench.

2.4 Establishment of aphid colonies on bean plants

10 adult alate virginoparae were taken from the stock culture and caged on the under surface of one of the second pair of leaflets (see aphid caging technique). They were allowed to feed and reproduce for 2 days after which the adults were removed with a pair of forceps, leaving a colony of nymphs all destined to develop into apterous virginoparae (Banks and MacAulay, 1964). All the results in this study were based on this form of *A. fabae*. These nymphs were allowed to feed and develop for a further 3 days. All treatments were carried out at this time, 6 days after the establishment of the adult alates on the leaves.

2.5 Artificial diet

An artificial diet was made up to a formula which has been described by Dadd and Krieger (1967). The water used for the diet was purified by oxidising deionised distilled water with potassium permanganate solution and redistilling it over charcoal. The components of the diet were separately weighed and dissolved in the pure water in the order amino acids : sugar : salts : vitamins to form the complete diet (van Emden,

1972). The pH was adjusted to 7.0 with KOH solution. The diet was kept in small vials in the deep freeze (-20°C), so that only small amounts needed to be thawed when required; any unused diet left in the vials was discarded.

Diet sachets were made up by enclosing a droplet of diet between two pieces of Parafilm "M" laboratory film (American Can Co.), a method devised by Mittler and Dadd (1963a) and modified by Fosbrooke, I.H.M. (private communication). A piece of parafilm membrane (ca. 5 x 5 cm) was stretched over a brass ring 93 cm diameter) resting on a mouth-operated 'vacuum' line, as shown in Fig. 2.2. About 0.5 ml of sterile diet was placed on the membrane. A tinned copper wire (ca. 90 μm) was positioned beside the droplet of diet across the brass ring, and a well was formed in the membrane by operating the vacuum line. A second piece of stretched parafilm membrane was then placed over the diet and the vacuum was slowly relaxed to force the air out of the sachet along the copper wire.

2.6 Sterilization of artificial diet

The dispensing unit for the diet consisted of a Millipore filter (pore size 0.22 μm) fitted with a rubber seal within a filter holder (Fig. 2.4). The unit was sterilized in a Little Sister autoclave for 12 min. at 2.41×10^5 Pa pressure and 138°C before use. The diet was dispensed from a syringe through this unit and then through a disposable sterile needle attached to the filter holder. The process of making up diet sachets was carried out in a Lamina Flow Work Station sterile flow cabinet. Some workers, for example van Emden (private communication), use ultraviolet light to sterilize the parafilm membrane surface in contact with the diet. However, no difference was found, as far as diet contamination was concerned, between UV - sterilized parafilm and that which had not been

Fig. 2.2. Apparatus for making up diet sachets.

a : Curtain ring resting on support

b : Plastic tubing for creating vacuum

Fig. 2.3. Completed diet sachet with aphids feeding on diet.

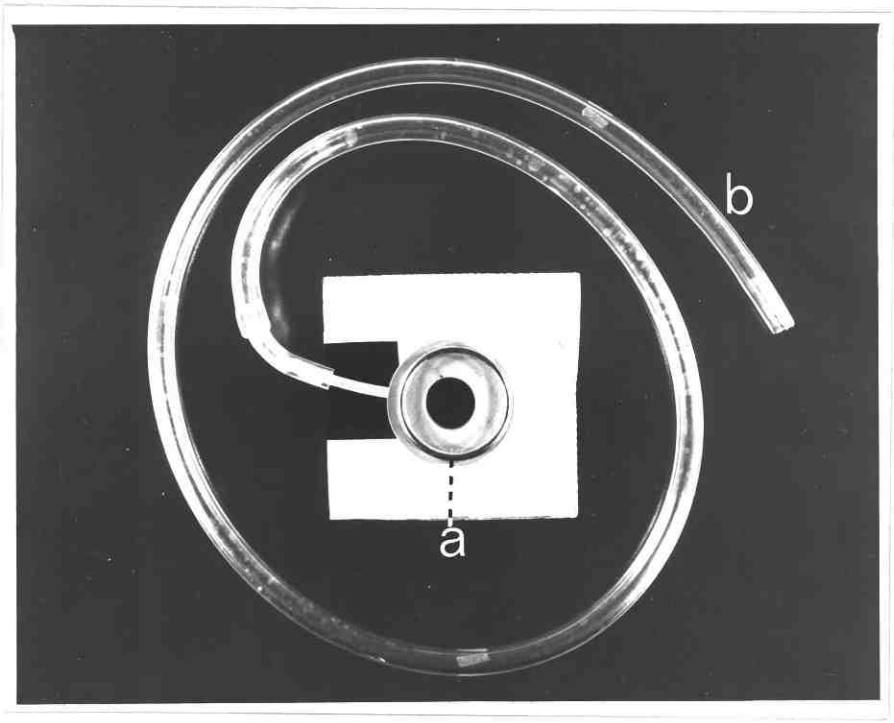
a : aphids

b : green filter

c : curtain ring supporting the sachet

d : filter paper for collecting honeydew

e : plastic petri dish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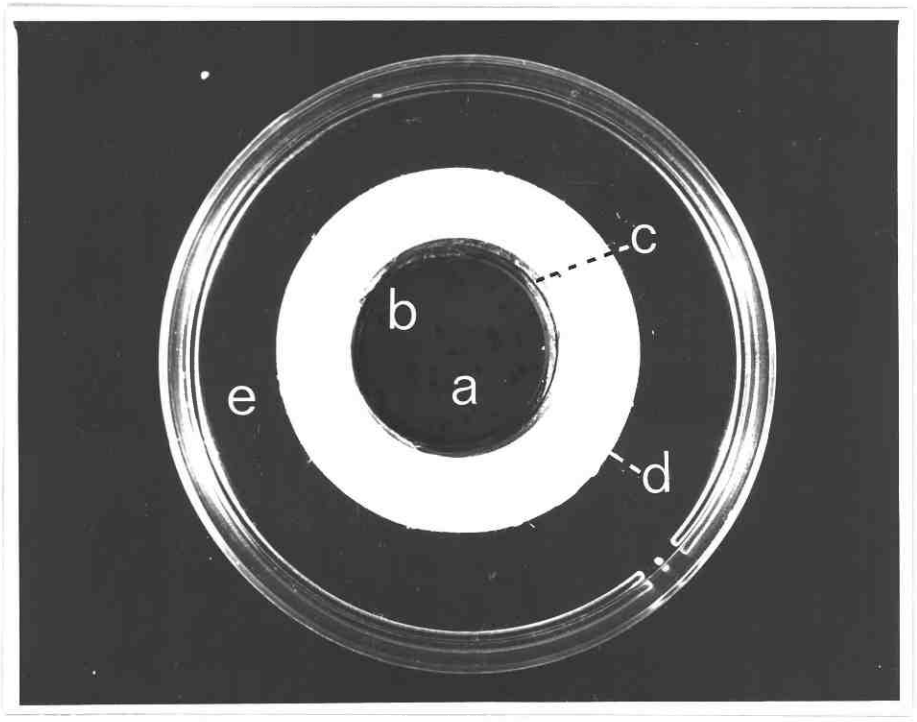
a


c
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a
e
d

Fig. 2.4. Artificial diet dispenser.

exposed to UV, provided that up to the time of use the parafilm was protected by the sterile surface of the paper with which it is interleaved in the package.

2.7 Establishment of aphid colonies on artificial diet

To facilitate changing diet sachets without undue disturbance to the feeding aphids, Frosbrooke (private communication) has devised a technique utilizing a feeding membrane separating the aphids from the sachet. This membrane was formed by stretching a piece of parafilm over a brass ring of the same size as that which supported the diet sachet. The feeding membrane was then adhered to one surface of the sachet with a film of moisture produced by blowing gently on the two surfaces.

An aphid colony was established on the feeding membrane using the procedure as that for plants. 10-12 adult virginoparae taken from the main culture were placed on the feeding membrane and allowed to feed and reproduce for 2 days. The adults were then removed and the diet sachets were renewed. With the aphid nymphs resting on the lower membrane the sachet could be detached without disturbing the colony. The nymphs fed and developed on the diet for 3 more days after which the sachets were again renewed. All treatments were added to these sachets at this stage (the 6th day) of the experiment, and for the whole run from the time the adults were first confined on the membrane a piece of green transparent filter was placed on the sachet to help the aphids settle on the diet (Fig. 2.3).

Honeydew was collected either on filter paper which was subsequently extracted for radioassay, or on weighed aluminium foil when the dry weight was assessed, as in experiments using bean plants.

2.8 Application of ^{14}C-phorate to artificial diet

A known quantity, usually 10 µl, of ^{14}C-phorate solution in acetone was applied as a droplet from a Drummond Microcap disposable pipette to the parafilm membrane stretched over a brass ring. When almost all the insecticide solution had evaporated, 0.5 ml of diet was placed on the membrane and the sachet was made up as previously described.

2.9 Bioassay

2.9.1. Technique

The appropriate concentrations of phorate or its oxidised metabolites (details are given in individual experiments) were prepared by diluting the stock solutions with acetone. The insecticide solutions were applied in 10 µl Drummond microcaps to the part of the bean plant to be treated, or to artificial diet as already described. The aphids were allowed to feed on the toxic plants or diet for 48 hours after which the number of dead aphids on each plant or diet sachet was recorded.

2.9.2. Determination of death in *Aphis fabae*

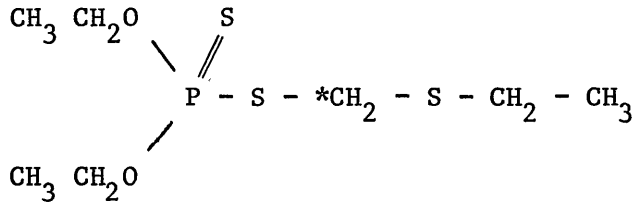
Dead and moribund aphids were both considered dead in all experiments. Aphids which have been dead for some time have a shrunken appearance. Mechanical stimulation often causes the apparently dead aphids to move normally (an observation made also by Parry (1966) in *Myzus persicae*), and the moribund ones to move only their legs in an abortive attempt to walk.

2.9.3. Bioassay analysis

The data obtained from toxicity tests were analysed by the CDC 6600 computer at the University of London Computer Centre, through the computer terminal at Silwood Park, using a program for probit analysis developed by

the Statistical Research Service, Canada Department of Agriculture.

2.10 Insecticide



Phorate ("Thimet")

O, O, diethyl S-(ethylthiomethyl) phosphorodithioate

* position of ^{14}C label

Methylene-labelled ^{14}C-phorate (above) was obtained from American Cynamid Co. and had a specific activity of 42.5 μCi/mg. It was purified before use by the column chromatographic method described by le Patourel and Wright (1974). The stock solution of phorate in benzene (2 ml) was eluted with 40 ml of benzene through a column (30 x 1.2 cm) packed with 6 g of silicic acid (Mallinckrodt Silic AR$^{(R)}$ CC-7). The eluate was collected in a 100 ml round-bottomed flask and evaporated to about 0.5 ml in a rotary film evaporator. The concentrated phorate was then made up to 10 ml in a volumetric flask with acetone and its purity determined by TLC. This solution was kept at 0^{o}C and acted as a stock from which subsequent dilution and treatments were dispensed. The chemical and radioactive concentrations were determined by Galley and Foerster (1976a) using colorimetric analysis (Chen *et al.*, 1956; Getz, 1964) and radioassay in a Beckman LS 250 liquid scintillation counter, from which the specific activity was calculated.

2.11 Thin-layer chromatography (TLC)

Thin-layer chromatographic plates used in most of this work were prepared by coating glass plates (20 x 20 cm) with a slurry of Silica gel

G (25 g) in distilled water (50 ml) using a TLC spreader adjusted to 0.25 mm layer thickness. The plates were pre-dried by blowing a current of hot air from a hair dryer for 10 min. and then heated to 110°C in an oven for 30 min. to complete the drying (Stahl, 1969). In the later stages of this study TLC aluminium sheets (20 x 20 cm) pre-coated with Silica gel 60, 0.2 mm thick (Merck) were used.

Radioactive samples were applied as spots on the plates together with aliquots of a solution of non-radioactive phorate and its oxidised metabolites to facilitate their detection. The plates were then developed in a chromatographic tank saturated for one hour with solvent by means of filter paper lining (Stahl, 1969). The solvent system used was benzene: methanol (95:5 v/v) (Grant *et al.*, 1969). The developed plates were sprayed with palladium chloride in hydrochloric acid (0.5% w/v) to locate phorate and its metabolites (Blinn, 1963).

Quantitative analysis of each metabolite was carried out by scraping the silica gel covered by each coloured spot off the TLC plate and transferring the gel into a counting vial containing 10 ml of scintillant solution. The radiocarbon was then assayed by liquid scintillation counting.

2.12 Preparation of toxic ^{14}C-phorate metabolites by bromine water oxidation

To about 2 ml of ^{14}C-phorate solution in benzene contained in a test-tube, bromine water was added dropwise with shaking, until the brown colour of bromine water, which had initially disappeared, returned and persisted. Nitrogen was bubbled into the solution for 5 min. to remove excess bromine. The solution was then transferred to a separating funnel and extracted with three 10 ml portions of hexane. The hexane layer was

collected over anhydrous sodium sulphate and concentrated to a small volume on the rotary film evaporator and the proportions of the metabolites were assayed by TLC.

The individual metabolites were recovered by column chromatography, using 6 g silicic acid, topped with 2 cm of anhydrous sodium sulphate. After addition of 2 ml benzene to the concentrated metabolite mixture, the latter was applied to the column which was then successively eluted with benzene (50 ml), 5% acetone in benzene (50 ml), 10% acetone in benzene (50 ml) and cetone (50 ml). The eluates were evaporated to small volumes and made up to required volumes in volumetric flasks with acetone. The metabolites in each fraction were identified by TLC and their radioactivity assayed by liquid scintillation counting. This column completely separated phorate (fraction I), phorate sulfone (fraction II) and phoratoxon sulfoxide (fraction IV). Fraction III contained a mixture of phoratoxon, phorate sulfoxide and phoratoxon sulfone. These three metabolites were further separated by TLC and then individually scraped off the TLC plate. They were each extracted with chloroform and concentrated by evaporation.

This method of preparing oxidised phorate metabolites gave 12.5% phorate sulfone, 6.6% phorate sulfoxide and 77.4% unoxidised phorate, with 1% or less of the other metabolites.

2.13 ^{14}C-phorate extraction procedures

2.13.1. Leaves

The method described by Menzer and Ditman (1968) and modified by Galley and Foerster (1976a) was employed in extracting ^{14}C-phorate residues from bean leaves. The solvents used were chloroform and acetone: water (1:1 v/v). 1.5 g of Charcoal ("Norit GSX") per leaflet was adequate

for decolorising the extracts. The chloroform fraction was dried over 2-3 g anhydrous sodium sulphate. 1 ml of Risella 17 oil was added and the chloroform was evaporated on a rotary film evaporator at 55°C under reduced pressure. The water-soluble extract was similarly evaporated at a higher temperature of 65°C.

2.13.2. Aphids

The chloroform - acetone - water system described for leaf extraction was adopted for extracting ^{14}C-phorate from aphid colonies consisting of 100-150 individuals. The amount of charcoal used was reduced to 0.2-0.5 g per colony.

2.13.3. Honeydew

Foerster's (1974) procedure for honeydew extraction was tried. The filter paper bearing the excreted honeydew was found to tear into shreds within the separating funnel on shaking, and tended to block the tap of the funnel. A modification of this method was employed which consisted of shaking the filter paper with 20 ml of chloroform: water (1:1 v/v) in a 100 ml round-bottomed flask for 5-10 minutes, and then filtering the contents of the flask under reduced pressure into a separating funnel. The flask and the filter paper were washed with 5 ml of chloroform and then 5 ml of water and the washings were added to the separating funnel. The two phases were separated. The chloroform layer was collected over anhydrous sodium sulphate. The aqueous layer was re-extracted with another 15 ml of chloroform. The chloroform and the aqueous fractions were separately concentrated to small volumes under reduced pressure in a rotary film evaporator. Recovery of radio-label by this method was over 99%.

2.13.4. Diet

200 µl aliquots of the artificial diet containing ^{14}C-phorate were withdrawn from the sachet and thoroughly shaken with 20 ml of chloroform: water (1:1 v/v). The two phases were then separated and the aqueous phase was extracted with two further 10 ml portions of chloroform. The solvent-soluble fraction, dried over anhydrous sodium sulphate, and the water-soluble fraction were separately concentrated as described.

2.13.5. Parafilm membrane

The diet was completely withdrawn from the sachet with a micro-capillary tube leaving behind the two layers of parafilm membrane. The surfaces of the membrane which had been in contact with the diet were rinsed with 1 ml of acetone: water (1:1 v/v). The membrane was then shaken in a 50 ml round-bottomed flask with 5 ml acetone and 5 ml chloroform for 15 minutes. The contents were filtered under reduced pressure into a 100 ml separating funnel. The flask was washed with 5 ml of chloroform followed by 15 ml of water, and the washings were added to the separating funnel. The two phases were shaken and allowed to separate and the aqueous phase was extracted twice more with 15 ml portions of chloroform used in washing the flask and the parafilm membrane residue. The solvent-soluble and the aqueous phases were concentrated as previously described.

All concentrated extracts were transferred to counting vials with washings totalling 10 ml of the appropriate scintillant solution.

2.14 ^{32}P extraction procedures

2.14.1. Aphids

Each colony of aphids were homogenised with 20 ml of distilled water

and filtered through a buchner funnel into a 50 ml round-bottomed flask. The extract was evaporated to near dryness on the rotary film evaporator and transferred to a scintillation vial. The flask was washed with 10 ml of Bray's scintillant solution which was then added to the vial.

2.14.2. Honeydew

Filter paper containing honeydew was shaken with 5 ml of distilled water in a conical flask for 10 min. The extract was filtered through a buchner funnel and the flask and filter paper were washed down with three 5 ml aliquots of water. The filtrate was similarly evaporated and transferred with 10 ml of scintillant to the counting vial.

2.15 Radioassay techniques

2.15.1. Radioassay by Geiger-Müller counter

In certain experiments where the absolute quantities of radiolabel was not essential, e.g. where only the proportion of radioactivity in various metabolites on TLC plates was required, the activity was assessed with a thin end-window G-M tube, Mullard MX 168, connected to a Panax scaler (102 ST). Samples were placed about 0.5 cm from the tube window (2.0 cm diameter), and were counted for 30 min. The results were corrected for background and counting efficiency which was approximately 4% for ^{14}C.

2.15.2. Liquid scintillation assessment of ^{14}C- and ^{32}P-labelled compounds

Radioactivity present in leaves, aphids, honeydew and artificial diet was measured in scintillant solutions using a Beckman LS 250 liquid scintillation counter. The following scintillant solutions were used:

(1) 10 ml of 2 - (4' - t - Butyl phenyl) - 5 - (4" - biphenyl) - 1, 3, 4 - Oxadiazole (Butyl-PBD) in toluene (4 g/l) (Turner, 1971). This scintillant was used for solvent extracts.

(2) 10 ml of modified Bray's scintillant solution (Bray, 1960) consisting of naphthalene (60 g), 2, 5 - Diphenyl oxazole (PPO) (4.0 g), 1, 4 - Di [2 - (5 - phenyloxazolyl)] - benzene (POPOP) (0.2 g), and methanol (100 ml) made up to 1 litre with dioxan. This cocktail was used for aqueous extracts.

The activity in samples was assessed in glass scintillation vials having a low ^{40}Potassium content. Each sample was counted either for 100 min. or until a 2σ error of $\pm$ 1.0% of the observed radioactivity in the sample was reached, whichever occurred earlier. The results were corrected for background and counting efficiency. Counting efficiencies were determined by the external standard channels ratio method. A ^{14}C-hexadecane internal standard was used for highly quenched samples.

2.15.3. Autoradiography

Leaf samples for autoradiography were severed from the plants and were immediately arranged on a polythene sheet. To facilitate handling a cardboard backing was used and the leaves were held in place with self-adhesive tape. In a dark room using a Kodak safe light with a 6B filter, the samples were placed on Kodirex X-ray film in a film holder (16.2 x 14.2 cm) which was then wrapped in a black polythene bag and stored at -20^{o}C under lead bricks to maximise contact between samples and film. After a suitable period of exposure (usually several weeks), the image was developed in Kodak D-19 developer for 5-8 min. The developer residue was removed with water and the film was fixed in a solution of Amfix for twice the length of time taken for the film to clear. The film was washed under running water for half an hour and allowed to dry.

3. EXPERIMENTS AND RESULTS

3.1 Estimation of ^{14}C-phorate metabolites in bean leaves, aphids and honeydew

In most of the experiments in this work, phorate treatment lasted 48 hours. In bean plants, phorate was allowed to translocate for 48 hours during which the aphids fed on the plant sap through the phloem and excreted honeydew containing various toxic as well as non-toxic metabolites of phorate. To investigate the toxic metabolic products formed by the aphids in relation to those oxidised by the plants, 5 μg of ^{14}C-phorate (>97% purity) was administered to the upper surface of the infested leaflet on the second node. Honeydew collection was then initiated. After 48 hours the treated leaflets were removed from the plants and together with the aphids and filter paper bearing honeydew were stored at $-20^{o}C$ until analysed. 5 replicates were carried out for the bean leaf analysis, and one colony selected at random consisting of about 50 aphids was analysed.

The leaves, aphids and honeydew were extracted and subsequently assayed by TLC. The difference in the proportion of oxidised phorate metabolites present in the bean leaves, the aphids and their honeydew after 48 hours is noteworthy (Fig. 3.1). In bean leaves, phorate sulfoxide was the major oxidised product formed, comprising more than 60% of the total metabolites. Phorate sulfone and phoratoxon sulfoxide, the two other metabolites present in an appreciable amount in that order, were only 28% and 20% respectively of phorate sulfoxide. The other minor metabolites were found in minute quantities, and only 4% of the original phorate remained in this state at the end of 48 hours.

In contrast to the leaflets, the toxic label found in the aphids was

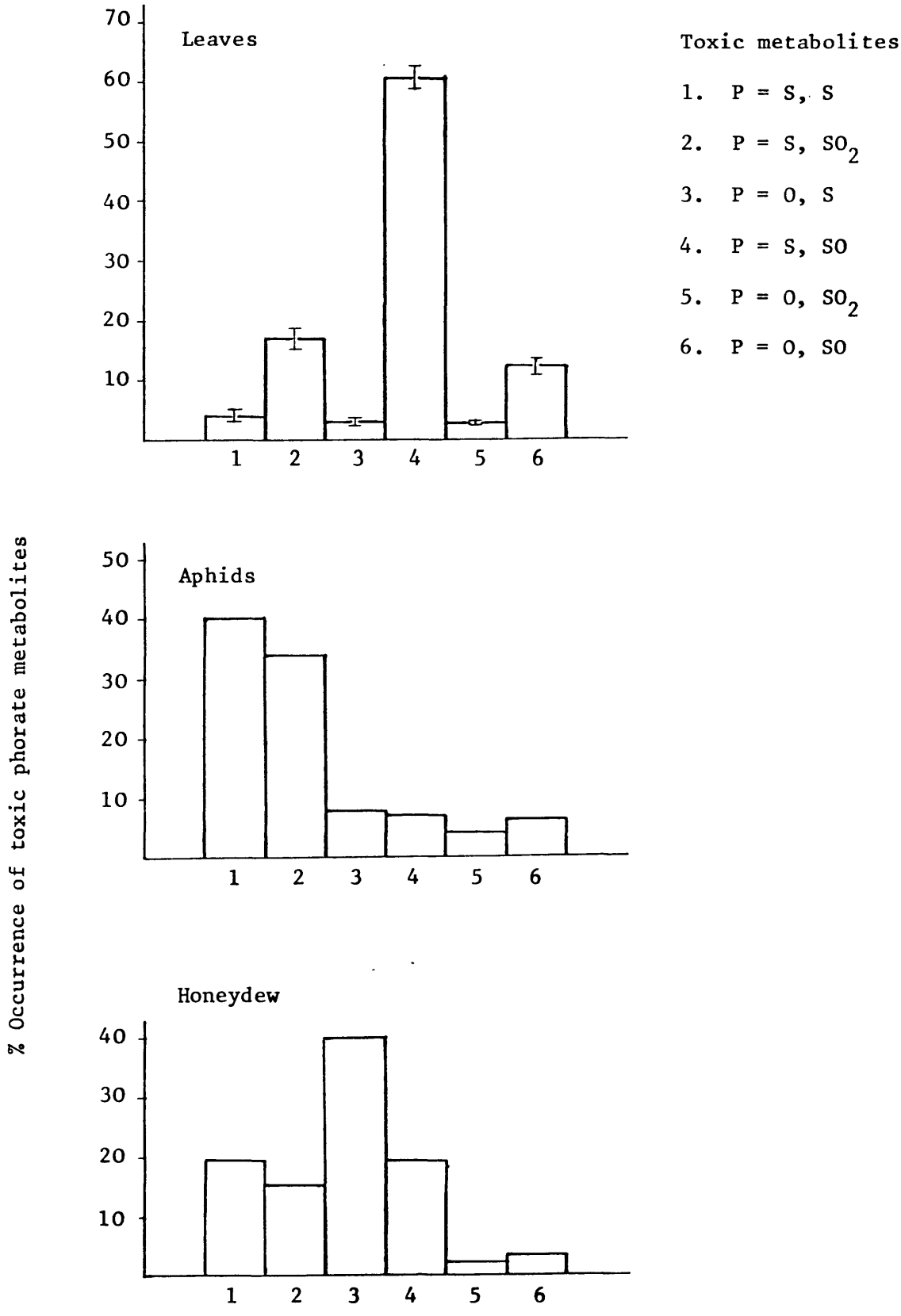


Fig. 3.1. Toxic ^{14}C-labelled metabolites found in bean leaves, aphids and honeydew 48 hours after foliar application of ^{14}C-phorate. (Vertical bars represent the standard errors of individual means)

mainly in the form of unchanged phorate and phorate sulfone. In the honeydew, phoratoxon was the major product, with lesser amounts of unoxidised phorate, phorate sulfoxide and phorate sulfone in that order.

A subsidiary experiment was carried out to estimate the ^{14}C-phorate metabolites in bean leaves 18 hours after foliar treatment, using the same procedures as the 48 hour assay. The following toxic metabolites were recovered from the leaves: 64% phorate sulfoxide, 12% each of phorate sulfone and unoxidised phorate, with small amounts of phoratoxon and its sulfoxide and sulfone (Fig. 3.2).

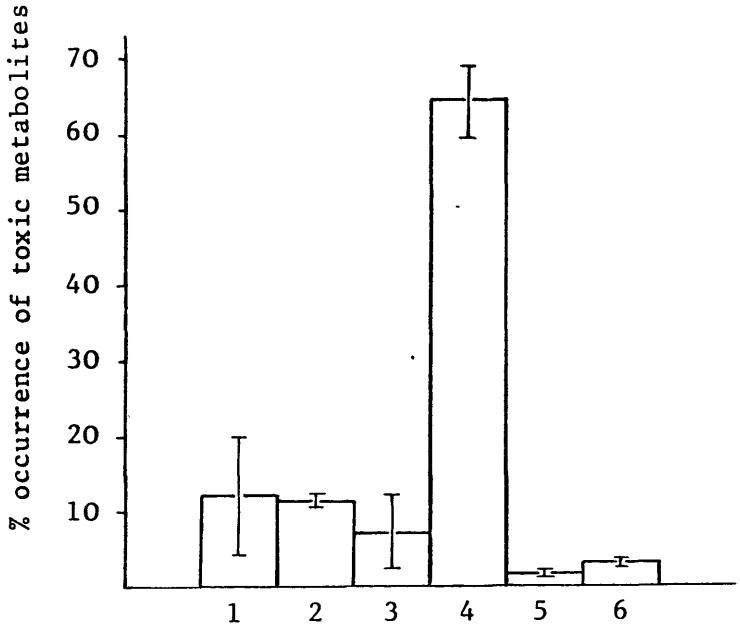


Fig. 3.2. Toxic ^{14}C-labelled metabolites found in bean leaves 18 hours after foliar application of ^{14}C-phorate, with standard errors of individual means.
(Compounds 1-6 as in Fig. 3.1)

3.2 Effect of foliar application of phorate on its toxicity to *A. fabae*

Preliminary experiments showed that very high concentrations of phorate needed to be applied to the tip of the leaflet before any aphid mortality could be observed. In this experiment non-radioactive phorate was used because large quantities were required.

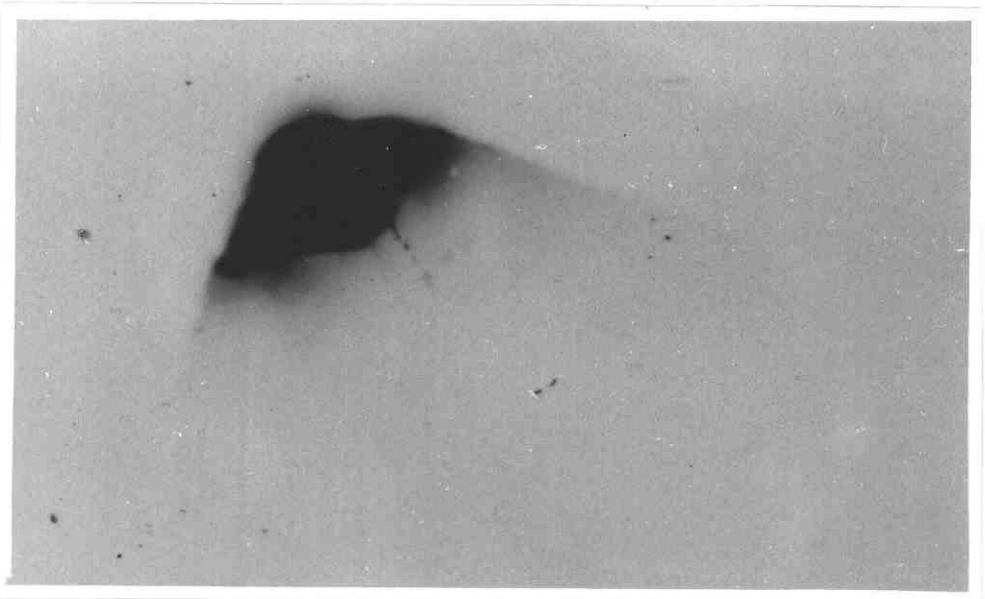
The following amounts of phorate in acetone solution were applied at the tip of the second leaflet on which the aphids had been born: 20, 40, 80, 200, 400, 500, 1000 µg. Each dose was replicated three times.

After 48 hours the mortality levels were recorded, the data being shown in Table 3.1. It was not possible to establish a value for LD_{50} in this experiment as the results indicate a tremendous amount of variation in mortality between replicates. Moreover, an increase in the amount of phorate applied to the leaflet did not cause a corresponding increase in aphid mortality.

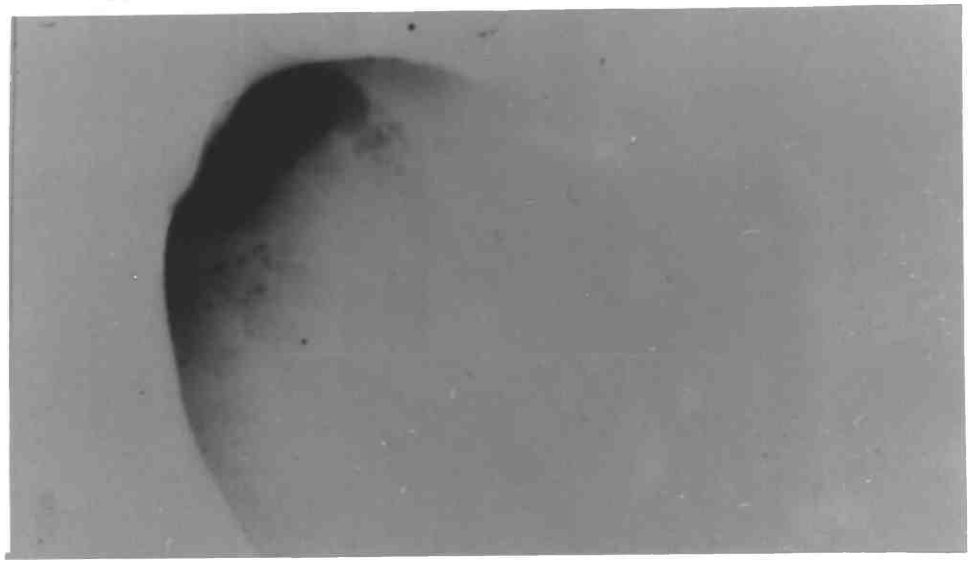
The autoradiographs of an uninfested leaflet and an infested one 48 hours after application of 5 µg ^{14}C-phorate to the leaf apex show little difference in ^{14}C-label distribution between the two (Fig. 3.3). Most of the radiocarbon remained at the leaf tip, and none was found in the adjacent leaflet.

Table 3.1. Aphid mortality at various phorate concentrations in leaf tips.

µg phorate/leaflet	% mortality (mean ± s.e.)
Control	0
20	8.5 ± 2.9
40	6.6 ± 3.1
80	5.6 ± 0.5
200	66.5 ± 16.8
400	9.6 ± 3.6
500	20.4 ± 14.3
1000	69.3 ± 30.7



A. Uninfested leaflet



B. Infested leaflet

Fig. 3.3. Accumulation of ^{14}C label in uninfested and infested leaflets 48 hours after ^{14}C-phorate treatment to leaf apex.

3.3 Aphid mortality and phorate residues in bean leaves, aphids and honeydew in relation to the site of phorate application

An attempt was made to compare the mortality levels induced by phorate application to various sites on the bean plant.

^{14}C-phorate was applied to the following regions of the bean plants: 5 µg at the base of the leaflet, 10 µg at the tip of the leaflet, and 20 µg to the roots via the nutrient. The aphid colonies were established on the second leaflet as before. After 48 hours of feeding and honeydew collection, the aphid mortality on each plant was noted as shown in Table 3.2. The aphids, the filter paper bearing honeydew and the leaflets on which the aphids had been feeding were stored at -20°C until they were extracted for radiocarbon assay. Where the phorate was applied to the foliage, the insecticide remaining on the leaf surface was washed with 10 ml of Butyl-PBD into a counting vial. The leaf tissue was then divided into 2 parts, the area where phorate had been applied and the remaining portion of the lamina. These pieces were weighed before freezing and extracted separately. The data are given in Tables 3.3a, b and 3.4a, b.

When phorate was applied on the leaf base the mortality observed ranged from 4% to 90% (Table 3.2). Extremely low mortality levels were recorded for aphid colonies where the insecticide was applied to the leaf tip or to the roots via the nutrient solution, even though twice the amount was used in the former and three times in the latter compared to the leaf base.

Following leaf base treatment 0.2 ± 0.03% of the initial phorate applied remained on the leaf surface as toxic metabolites, whereas leaf tip treatment resulted in 8 ± 1.2% appearing as surface toxic metabolites (Table 3.3a).

Table 3.2. Mortality levels of aphids in relation to the site of application of phorate.

Treatment	% mortality
5 µg to leaf base	38.8
	3.7
	89.5
10 µg to leaf tip	2.1
	5.2
	1.0
20 µg to roots	2.2
	2.6
	0.0

Table 3.3a. Total toxic metabolites found in host leaflets, aphids, honeydew and on leaf surfaces 48 hours after ^{14}C-phorate treatment.

Treatment	Mean ± s.e. (pmol phorate equivalent/leaflet or 100 aphids)			
	In leaflet		In aphids	In honeydew
	Surface	Lamina		
5 µg (19 x 10^3 pmol) to leaf base	42.3 ± 5.2	881 ± 56.9	6.6 ± 4.6	25.7 ± 14.9
10 µg (38 x 10^3 pmol) to leaf tip	311.5 ± 47.5	4780 ± 1992	29.6 ± 8.4	8.9 ± 2.1
20 µg (77 x 10^3 pmol) to roots	-	206.5 ± 125	3.4 ± 1.4	11.7 ± 6.5

Table 3.3b. Concentration of toxic metabolites in the two areas of the leaf lamina.

Treatment	Mean ± s.e. (pmol phorate equivalent/g leaf tissue	
	Lamina (a)	Lamina (b)
5 µg (19 x 10^3 pmol) to leaf base	465 ± 97.5	2358 ± 339
10 µg (38 x 10^3 pmol) to leaf tip	359900 ± 206900	157 ± 59.2

(a) site of ^{14}C-phorate application.

(b) remaining portion of the lamina.

Table 3.4a. Total non-toxic metabolites found in host leaflets, aphids and honeydew 48 hours after ^{14}C-phorate treatment.

Treatment	Mean ± s.e. (pmol phorate equivalent/leaflet or 100 aphids		
	In leaflet	In aphids	In honeydew
5 µg (19 x 10^3 pmol) to leaf base	220 ± 28.3	9.0 ± 0.3	43.1 ± 21.2
10 µg (38 x 10^3 pmol) to leaf tip	141 ± 52.3	18.4 ± 1.7	312.2 ± 72.9
20 µg (77 x 10^3 pmol) to roots	76.7 ± 41.3	8.8 ± 1.1	160.7 ± 83.4

Table 3.4b. Concentration of non-toxic metabolites in the two areas of the leaf lamina.

Treatment	Mean ± s.e. (pmol phorate equivalent/g leaf tissue)	
	Lamina (a)	Lamina (b)
5 µg (19 x 10^3 pmol) to leaf base	433 ± 76.6	485 ± 19.3
10 µg (38 x 10^3 pmol) to leaf tip	7960 ± 4370	75.1 ± 33.1

(a) and (b) as in Table 3.3b.

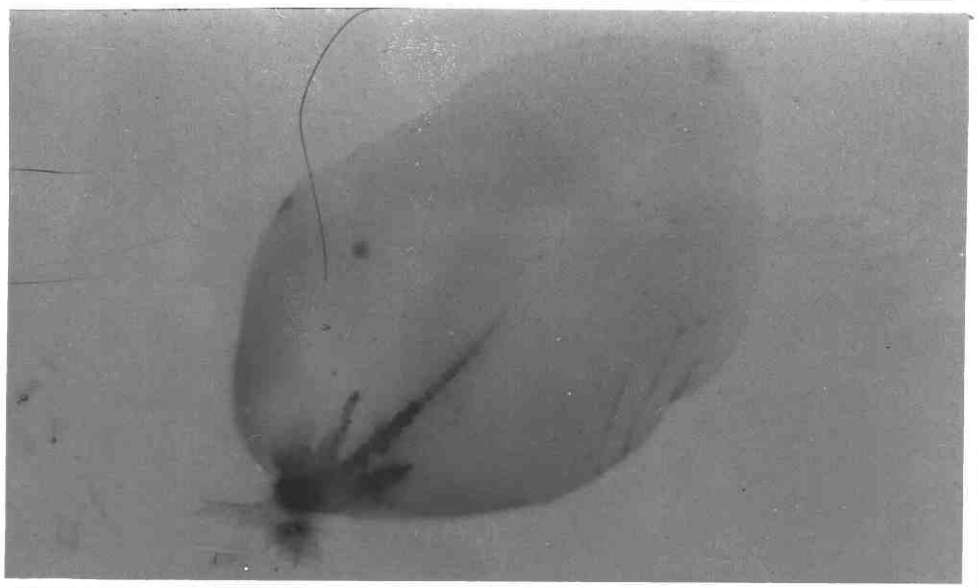
More toxic residues were found in host leaflets receiving phorate from the tips than in those treated at the leaf base. In the former the major proportion of toxic metabolites accumulated at the site of application, while in the latter most of the metabolites were translocated to other parts of the leaflet (Table 3.3b). This clearly shows that movement of phorate metabolites occurs more readily from the base to the tip of the leaflet (acropetal) than in the reverse direction (basipetal). Root treatment led to relatively small quantities of both toxic and non-toxic metabolites in the leaflets. In these experiments, leaflets undoubtedly contained more toxic than non-toxic residues regardless of the site of application.

The data obtained from aphid and honeydew extraction showed that the radiolabel found in the honeydew excreted by aphids feeding on tip-treated leaflets was mainly in the non-toxic form, although it was the toxic residues that predominated in the leaflets. Aphids feeding on leaflets that received insecticide from the leaf base contained smaller quantities of toxic and non-toxic label in their bodies as well as their honeydew. As with tip-treated leaflets, root treatment led to greater accumulation of non-toxic than toxic metabolites in the honeydew.

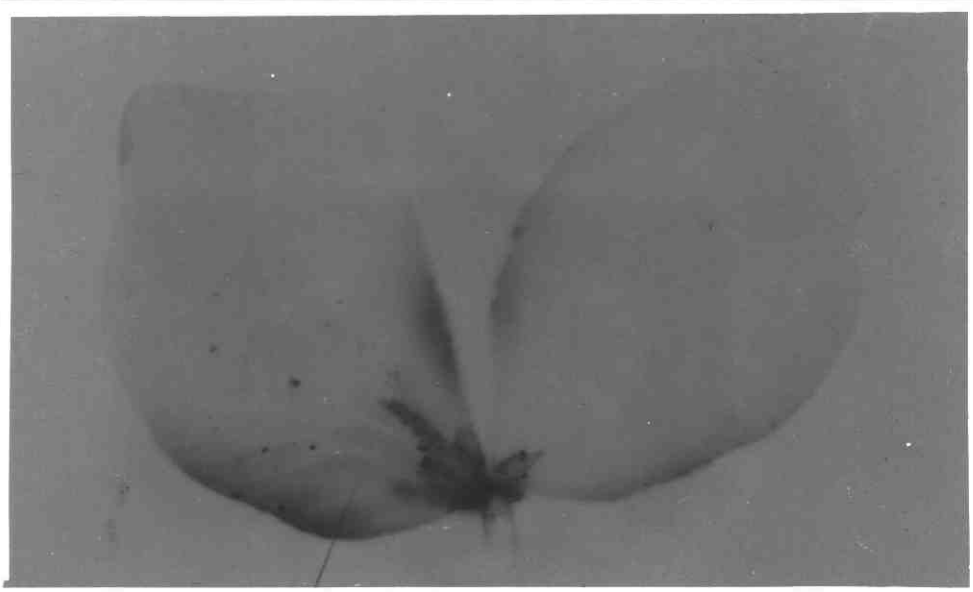
The total radioactivity recovered from the infested leaflets, aphids and honeydew after phorate treatment to the foliage represented only a small proportion of the initial phorate applied (ca. 6% after leaf base treatment, ca. 15% after leaf tip treatment).

^{14}C-phorate was applied to the roots of one plant, the base of an uninfested second leaflet of another, and the base of an infested second leaflet of a third plant. The pairs of second leaflets in all 3 plants were autoradiographed after 48 hours and are presented in Figs. 3.4 and

3.5. In the root treatment, the radiolabel in the leaflets had accumulated more around the leaf margin. Following leaf-base treatment, most of the radiocarbon moved in an acropetal direction and very little appeared in the adjacent leaflet. No visible difference in the ^{14}C distribution between the uninfested and the infested leaflet can be discerned, indicating that the aphids did not cause an accumulation of radiocarbon at their feeding site. These autoradiographs of ^{14}C-phorate treatment to the various sites on the bean plant (Figs. 3.3 - 3.5) confirm the translocation characteristics of this apoplastic compound.



A. Uninfested



B. Infested

Fig. 3.4. Accumulation of ^{14}C label in uninfested and infested leaflets 48 hours after ^{14}C-phorate treatment to leaf base.

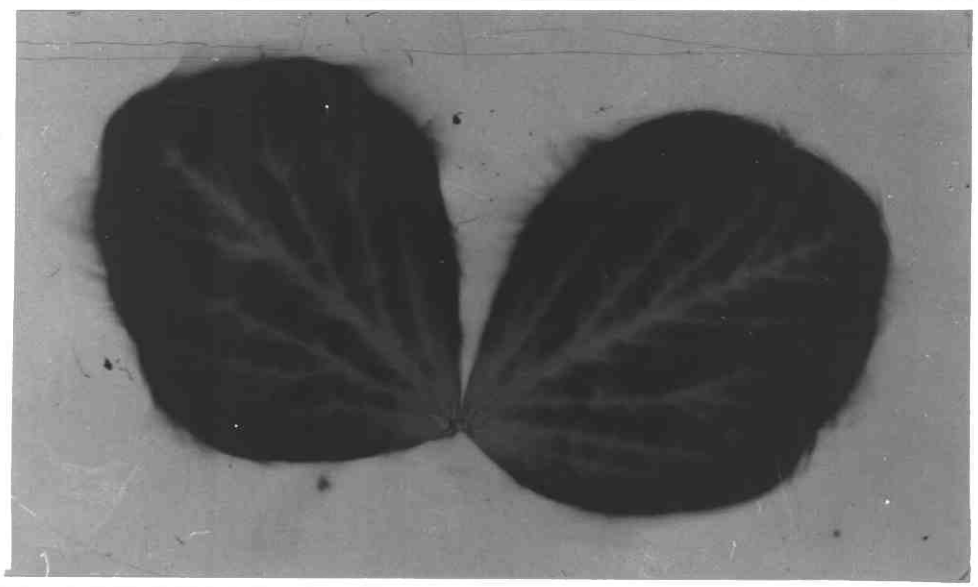


Fig. 3.5. Accumulation of ^{14}C label in second pair of leaflets 48 hours after ^{14}C-phorate treatment to roots.

3.4 Measurement of feeding rates of aphids on bean plants and on artificial diet

3.4.1. Feeding rate

Aphid colonies were born on one of the pair of leaflets on the second node of 5 plants. When the adults were discarded on the third day, honeydew collection was begun. The foils were renewed daily over a period of 5 days. The dry weight of the honeydew was recorded, and the feeding rate was expressed in terms of μg honeydew/aphid/day over a period of 6 days.

The results are shown in Fig. 3.6. The dry weight of the honeydew produced each day by an individual aphid represents the total solid matter excreted by the aphid per day. A linear relationship exists between the feeding rate and the age of the aphids within the experimental period ($P<0.001$), with a slope of 13.12 ± 1.19.

3.4.2. Feeding rate on diet

Colonies of aphids were established on 6 diet sachets. When the adults were removed on the third day honeydew collection was initiated. The foils were renewed daily for the following 4 days and the dry weight of the honeydew excreted was recorded as for plants.

It is evident from Fig. 3.7 that the feeding rate in terms of honeydew production bears a linear relationship to the age of the aphids within the experimental period ($P<0.001$), with a slope of 12.17 ± 1.04.

3.4.3. Comparison of feeding rate on plants with that on diet

Statistical analysis (see Table 3.5) shows that there is no significant difference ($P>0.9$) between the slope in Fig. 3.6 and that in Fig. 3.7. Hence there is no significant difference ($P>0.9$) between the bean plants and the artificial diet as far as the feeding rate of aphids is

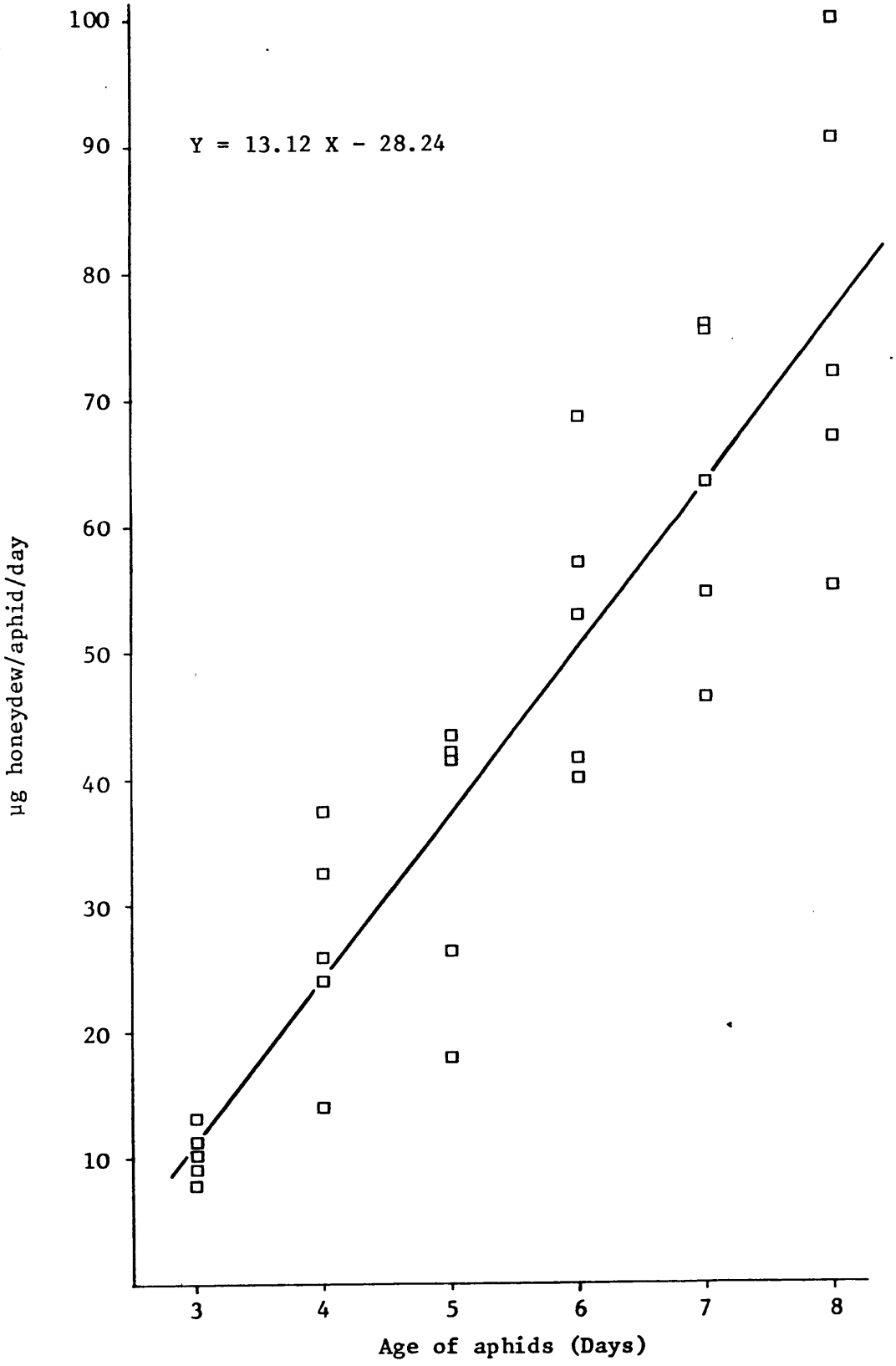


Fig. 3.6. Feeding rate of aphids on bean plants.

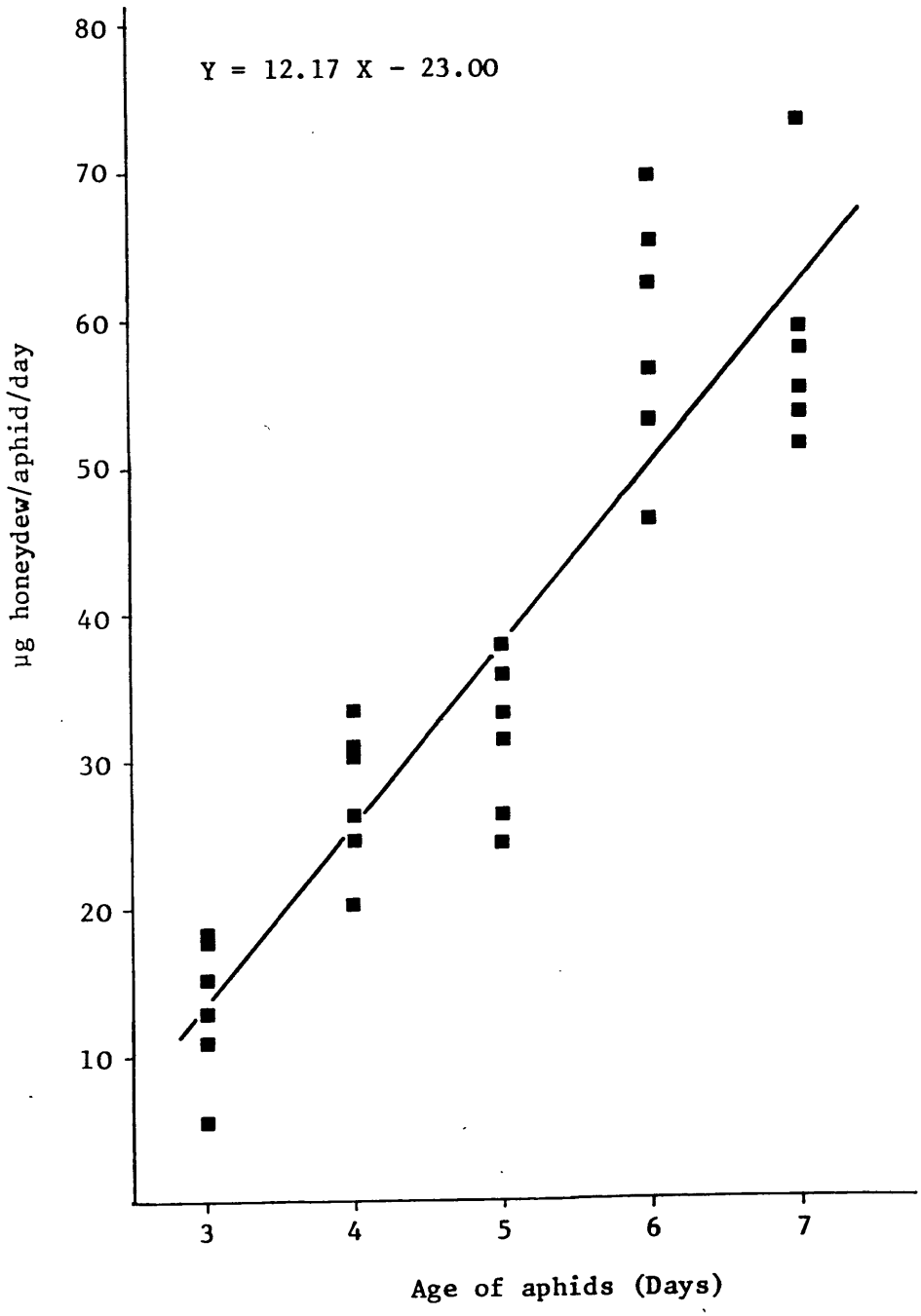


Fig. 3.7. Feeding rate of aphids on artificial diet.

concerned.

Table 3.5. Comparison of aphid feeding rates on bean plants and on diet

	Slope	Standard error	
Plants	13.12	± 1.19	(N = 60)
Diet	12.17	± 1.04	

No significant difference was found (P>0.9, t test) between the feeding rate on bean plants and that on artificial diet.

However, as the feeding rate was calculated on the basis of the dry weight of honeydew, the quantity of liquid excreted was not taken into consideration. Furthermore, the aphids fed on artificial diet appeared to be smaller than those fed on bean plants.

3.5 Effect of acetone on aphids' feeding rate

The aim of this experiment was to investigate the feasibility of using acetone as a solvent for phorate to be added to the artificial diet. Acetone was incorporated into the diet in two different ways as follows:

I. 10 µl acetone were added directly to 0.5 ml of diet in the sachet.

II. 10 µl acetone were spread out on the parafilm membrane stretched over the brass ring, and allowed to evaporate completely before 0.5 ml of diet were added.

These sachets together with those containing only the diet were fed to the aphids 5 days after the establishment of the colonies on the complex diet. Honeydew was collected and the dry weight recorded after 48 hours. The mortalities and feeding rates of these treatments were compared with those of the control.

The incorporation of acetone into the diet did not appear to increase aphid mortality (Table 3.6).

Table 3.6. Effect of acetone on aphid mortality

Treatment	% mortality (Mean ± s.e.)	
	24 hr.	48 hr.
I. Acetone (not evaporated)		
Control	2.1 ± 1.1	4.1 ± 1.3
Treated	1.2 ± 0.6	1.9 ± 0.6
II. Acetone (evaporated)		
Control	1.7 ± 1.5	2.7 ± 2.1
Treated	1.9 ± 0.5	3.1 ± 0.7

When the feeding rate was compared (Table 3.7) the addition of acetone directly into the diet resulted in a significant drop in feeding rate ($0.001 < P < 0.01$). However, when the acetone was evaporated from the parafilm membrane before adding the diet, no significant difference ($P>0.2$) was found between this and the control with respect to the feeding rate. Henceforth, when insecticide was incorporated into the diet the acetone in which the insecticide was dissolved was evaporated on the parafilm membrane before the diet was added.

Table 3.7. Aphid feeding rate with respect to the method of incorporation of acetone into artificial diet.

a. Effect of applying 10 µl acetone directly to 0.5 ml diet.

Replicates	µg honeydew/aphid/48 hr.	
	Control	Treated
1	107.2	89.3
2	105.9	88.4
3	156.3	89.2
4	131.8	83.9
5	134.8	87.2
Mean	127.2	87.6
± s.e.	9.4	1.0

The difference between the control and the treated is statistically reliable ($0.001 < P < 0.01$, t test).

b. Effect of evaporating 10 µl acetone on parafilm membrane before adding 0.5 ml diet.

Replicates	µg honeydew/aphid/48 hr.	
	Control	Treated
1	48.5	53.2
2	54.3	60.0
3	63.0	57.0
4	56.1	46.7
5	54.4	51.9
6	69.9	47.7
Mean	57.7	52.8
± s.e.	3.1	2.1

No significant difference ($P>0.2$, t test) was found between the control and the treated.

3.6 Recovery of toxic and non-toxic ^{14}C-labelled metabolites from ^{14}C-phorate treated diet sachets

3.6.1. Recovery from diet

A series of ^{14}C-phorate concentrations ranging from 3.85×10^2 - 1.30×10^4 nmol l^{-1} was added to diet sachets and kept at 20^oC for 48 hours. 200 µl aliquots of diet from each sachet were partitioned between chloroform and water. Each fraction was then analysed for radiocarbon by liquid scintillation counting. The results are expressed in Table 3.8. It can be seen here that only 1% toxic and 0.5% non-toxic label was recovered from the diet.

3.6.2. Absorption of ^{14}C-labelled metabolites by parafilm membrane

1 µg ^{14}C-phorate was added to 0.5 ml artificial diet in a sachet. After 2 hours 200 µl of the diet were withdrawn and extracted with chloroform and water as already described. The ^{14}C label in the toxic and non-toxic fractionswas assayed in the liquid scintillation counter. The ^{14}C content in the parafilm membrane was similarly assayed after removal of the diet from the sachet. This procedure was repeated at 4, 10 and 48 hours after ^{14}C-phorate incorporation into the diet, with 2 replicates at each time interval.

The per cent original ^{14}C-phorate recovered from the diet and the membrane as toxic and non-toxic compounds is shown in Table 3.9. A large proportion, about 75%, of the initial phorate was found to be absorbed by the parafilm membrane in toxic form, and this was consistent over 48 hours. The non-toxic metabolites bound to the membrane were present in negligible amounts (0.1%). The level of toxic label in the diet decreased rapidly from 1.8% at 2 hours to almost half the quantity after 10 hours and then remained constant thereafter.

Table 3.8. Recovery of toxic and non-toxic ^{14}C-labelled phorate metabolites from diet 48 hours after ^{14}C-phorate application.

Initial phorate concentration (nmol l^{-1})	Toxic metabolites		Non-toxic metabolites	
	nmol l^{-1} phorate equivalent	% initial phorate	nmol l^{-1} phorate equivalent	% initial phorate
3.85×10^2	4.0	1.0	1.1	0.3
1.00×10^3	13.5	1.3	5.3	0.5
2.00×10^3	25.4	1.3	6.4	0.3
4.31×10^3	39.3	0.9	26.4	0.6
4.85×10^3	37.2	0.8	25.4	0.5
5.54×10^3	46.8	0.8	31.4	0.6
9.54×10^3	80.2	0.8	57.7	0.6
9.90×10^3	95.5	1.0	58.5	0.6
1.19×10^4	97.4	0.8	49.3	0.4
1.30×10^4	113.4	0.9	71.6	0.6
Mean		0.96		0.50
± s.e.		0.06		0.04

Table 3.9. Proportions of toxic and non-toxic ^{14}C-label found in diet and parafilm membrane at various time intervals after phorate treatment.

Time (Hr)	Per cent original phorate			
	Diet		Membrane	
	Toxic	Non-toxic	Toxic	Non-toxic
2	1.8	0.4	75.0	0.1
4	1.5	0.4	76.7	-
10	1.0	0.5	75.1	0.1
48	1.1	0.5	74.9	0.1

3.6.3. Partitioning of ^{14}C-labelled hydrolysis products in an equivolume chloroform-water system

As the ^{14}C-phorate used in this work was methylene-labelled, the following experiments were carried out to estimate the proportion, if any, of ^{14}C-labelled hydrolysis products that partitioned into the chloroform fraction. This was investigated both in diet and in an aqueous medium.

3.6.3.1. Diet

1 µg of ^{14}C-phorate was added to 0.5 ml diet in the usual manner, and the sachet was kept at 20°C for 48 hours. Two 200 µl aliquots of the diet were then withdrawn from the sachet and partitioned between chloroform and water. The ^{14}C content in the aqueous extracts was assessed in Bray's scintillant solution. The radioactivity in the concentrated chloroform extract from one aliquot was assayed in scintillant while the

other was analysed by TLC, followed by liquid scintillation counting. The results are presented in Table 3.10. The amount of radiocarbon that remained at the origin on the TLC plate was assumed to represent the ^{14}C-labelled hydrolysed metabolites of phorate that had partitioned into the chloroform phase. Most of the radioactivity was detected around the coloured spots on the TLC plate. The silica gel between the spots was also counted and very low radioactivity was found associated with it. This precluded the possibility that any chloroform-soluble hydrolysis products might have migrated up the TLC plate with the solvent system used to develop the plate.

These results in Table 3.10 show that only 0.9% of the total radioactivity recovered from the TLC plate was found at the origin. This amounted to less than 2% of the total water-soluble ^{14}C-label extracted from the diet. A comparison of the total radioactivity recovered from the TLC plate with that of the chloroform extract analysed in the liquid scintillation counter indicates that about 14% of radiocarbon had been lost in the TLC technique.

3.6.3.2. Aqueous medium

2.5 µg of ^{14}C-phorate in 40 µl acetone solution were added to 1 ml of water and the pH was adjusted to 12. The solution was thoroughly shaken and left at room temperature for 2 days to allow hydrolysis to take place. The solution was then adjusted to pH 7.0 and extracted with chloroform and water. The chloroform fraction was concentrated and analysed by TLC followed by estimation of ^{14}C-label in the liquid scintillation counter. The aqueous extract was counted in 10 ml of Bray's scintillant solution.

Table 3.10. Recovery of radioactivity from diet.

Metabolites	Dpm	% ^{14}C-label recovered from TLC plate
Radioactivity at origin	4.4	0.9
P = O, SO	98.5	21.2
P = O, SO_2	21.2	4.6
P = S, SO	74.5	16.1
P = O, S	44.8	9.7
P = S, SO_2	65.7	14.2
P = S, S	154.6	33.3
Total activity on plate	463.7	
Chloroform-soluble fraction	540	
Water-soluble fraction	254	

The results are tabulated (Table 3.11) to show the proportion of hydrolysis products which had partitioned into the chloroform layer.

Table 3.11. Recovery of radioactivity from hydrolysed metabolite mixture.

	Dpm	% radioactivity recovered from TLC plate
Radioactivity at origin	637	2.9
Radioactivity associated with toxic metabolites	22299	97.1
Aqueous fraction	95900	-

About 3% of the radiocarbon remained at the origin, and this represented

only 0.7% of the total water-soluble label recovered from the reaction mixture.

The results of these two experiments have shown that the major portion (> 99%) of ^{14}C-labelled hydrolysis products of methylene-labelled ^{14}C-phorate partitioned into the aqueous phase in an equivolume chloroform-water system.

3.7 Phorate metabolism in artificial diet

^{14}C-phorate was added to artificial diet at a concentration of approximately 11 µmol l^{-1} in a glass vial, and the system was maintained at 20 ± 2°C. At time intervals of 0, 1, 2, 6, 9, 24, 30, 48, 72 and 96 hours, 20 µl aliquots of diet were taken and partitioned between chloroform and water. After concentration the ^{14}C-labelled components of the toxic chloroform-soluble fraction were separated by TLC and quantitatively analysed in liquid scintillant. The ^{14}C content of the non-toxic water-soluble fraction was assessed in 10 ml of Bray's scintillant solution. No analysis of its components was carried out.

The amounts of phoratoxon as well as its sulfoxide and sulfone were very small mostly less than 5%; these are shown in Appendix 5.

The breakdown of phorate and the formation of phorate sulfoxide and phorate sulfone in artificial diet are shown as functions of time in Fig. 3.8. Hydrolysis of phorate is presented in Fig. 3.9. The results are expressed as percentages of the total radioactivity recovered from the diet samples at various time intervals on the assumption that no loss of insecticide occurred through evaporation, absorption on the glass surface of the vial or during the extraction procedure. It must be mentioned, at this juncture, that such loss of radiocarbon did occur and will be discussed in detail.

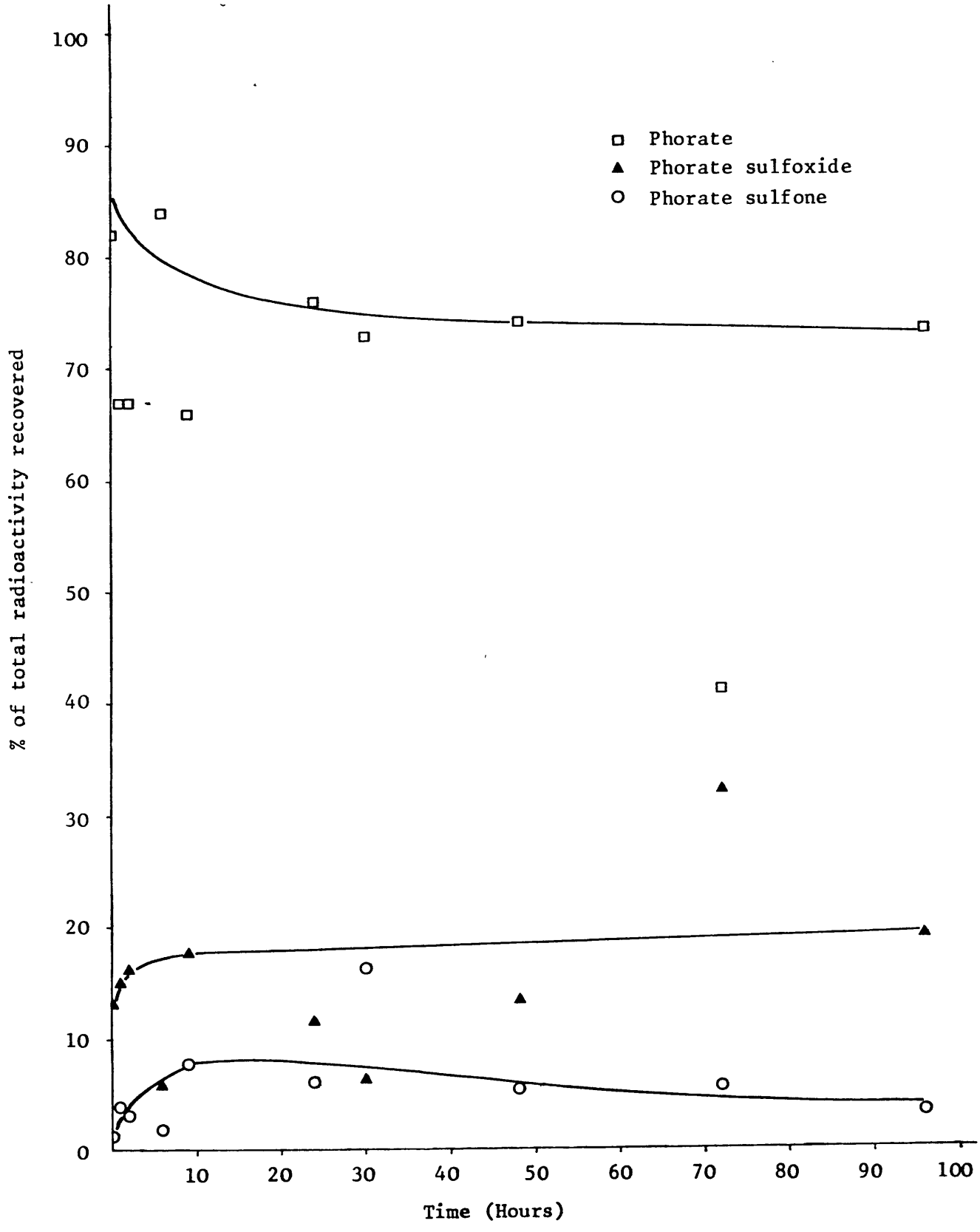


Fig. 3.8. Metabolism of phorate and its sulfoxide and sulfone in artificial diet.

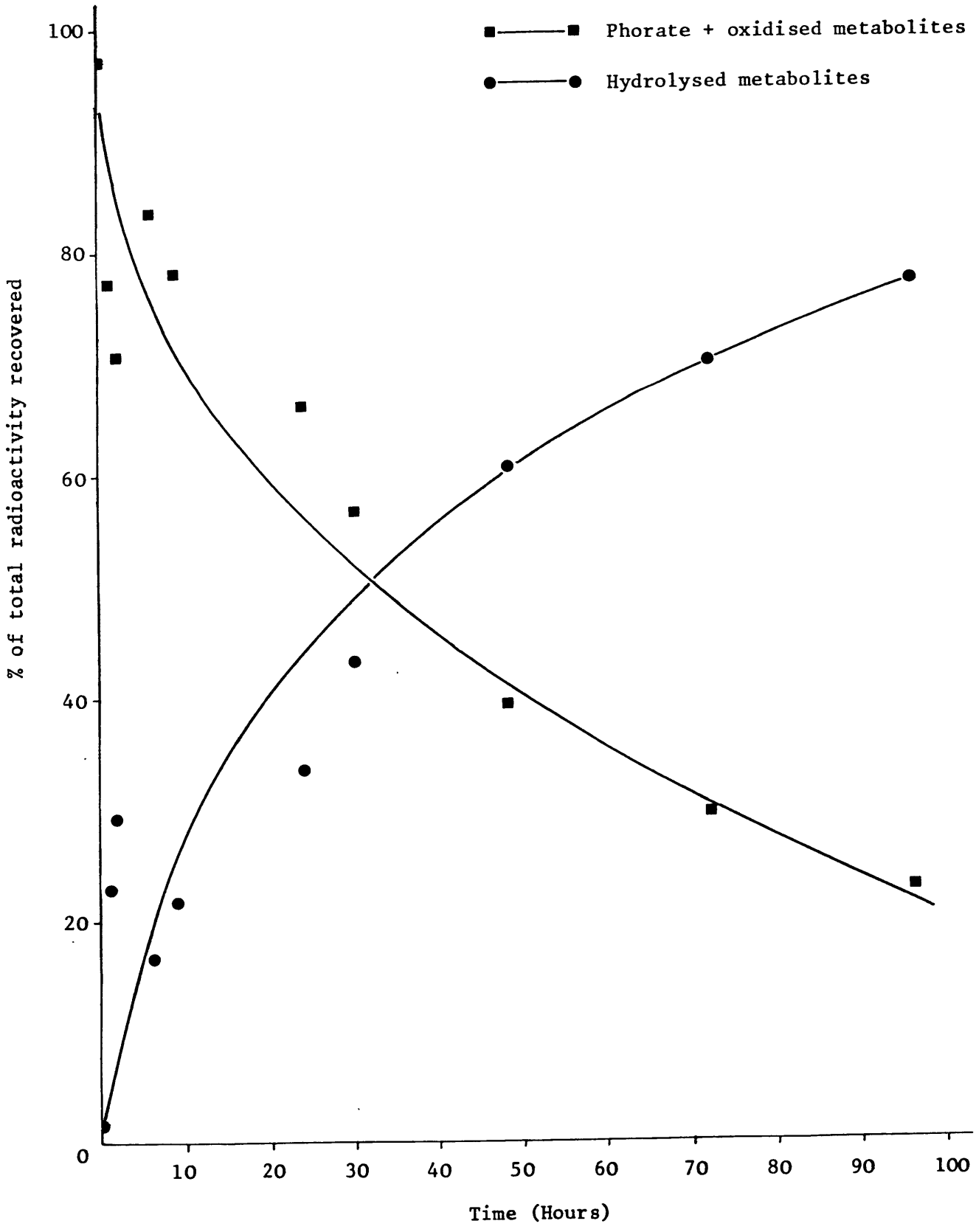


Fig. 3.9. Oxidation and hydrolysis of phorate in artificial diet.

3.8 Relative toxicity of phorate and some of its metabolites to *A. fabae*

In all toxicity tests involving dose and response, various concentrations of the metabolites were incorporated into the diet. The aphids were then fed on the toxic diet. Mortality was recorded after 48 hours. Each treatment was replicated at least three times. The dose unit used was the concentration of the toxic radiocarbon extracted from the diet at the end of the 48 hour period.

3.8.1. Phorate

Using the data obtained from the computed probit analysis, the probit kill (having been adjusted by the computer for control mortality employing Abbott's formula, 1925) was plotted against the log concentration of the toxic residues in the diet. The best fit regression line was then drawn (Fig. 3.10). The LC_{50} value was 19 nmol l^{-1}, and the LC_{95} was 38.4 nmol l^{-1}.

3.8.2. Phorate sulfone

As with phorate, probit kill (after correction for control mortality using Abbott's formula, 1925) was plotted against the log dose of phorate sulfone. The best fit regression line calculated by the computer was then drawn. The probit regression lines for phorate and phorate sulfone appear in Fig. 3.10. The LC_{50} value for phorate sulfone was 2.4×10^3 nmol l^{-1} and the LC_{95} was 11×10^3 nmol l^{-1}. A test for parallelism between the two lines carried out by the computer showed that the data contradicted the hypothesis of parallelism. Thus their relative potency could not be compared. However, for practical purposes there is a considerable separation between the lines with phorate being the more toxic. A comparison made of the potencies using the values of LC_{50} and LC_{95} as criteria show that at the LC_{50} level, phorate was 127 times as toxic as

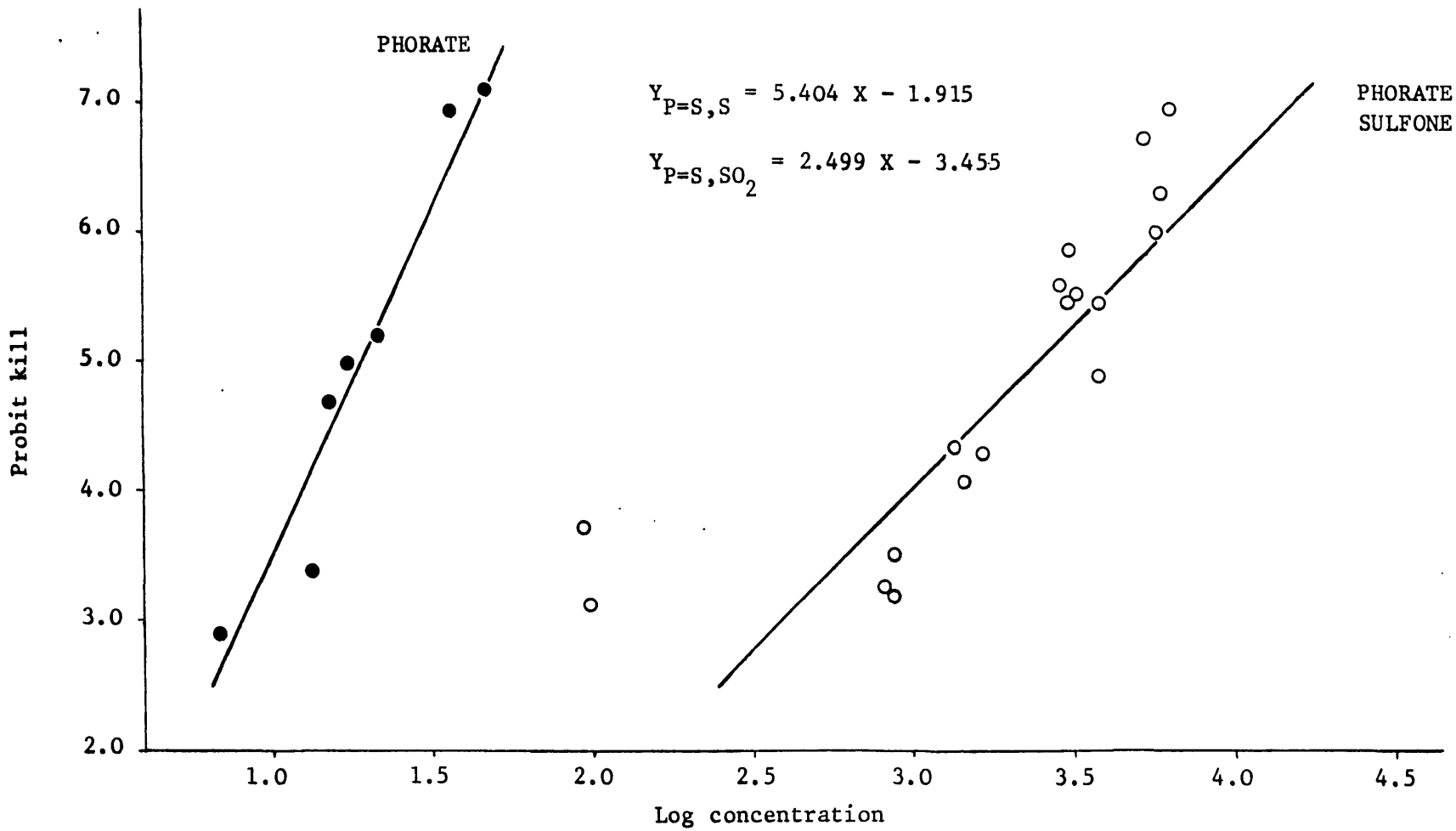


Fig. 3.10. Dose/response regressions for *A. fabae* treated with phorate and phorate sulfone.

(Concentration is expressed in nmol l^{-1} phorate equivalents)

phorate sulfone, and at the LC_{95} level phorate was even more effective by a factor of 287.

3.8.3. Phorate sulfoxide and phoratoxon sulfoxide

Although phorate was quickly oxidised to its sulfoxide in bean leaves (Figs. 3.1 and 3.2) and in artificial diet (Fig. 3.8), this compound could not be prepared in sufficient quantity to carry out a bioassay with a range of doses. Phoratoxon sulfoxide was also obtained in small quantity using the bromine water oxidation method. Therefore, only two concentrations each of the above metabolites were used in this toxicity test.

The results are given in Table 3.12. No mortality was found at the two concentrations of phorate sulfoxide used in this experiment, even though the higher of the two doses was more than six times the LC_{50} of phorate. Extremely low mortality levels were recorded for phoratoxon sulfoxide the concentrations of which were 117 and 650 times respectively greater than the LC_{50} of phorate.

At the higher of the two doses, the aphids were seen to be wandering within the confine of the brass ring cages, suggesting that the diet in these sachets was less acceptable to them.

The order of toxicity therefore appeared as follows: phorate > phorate sulfone > phorate sulfoxide > phoratoxon sulfoxide.

The remaining oxidised metabolites of phorate, viz. phoratoxon and its sulfone, were not studied. These compounds were present in such minute quantities in the metabolite mixture that enough of the chemicals for the bioassay experiments could not be prepared by the methods employed in this work.

Table 3.12. Effect of phorate sulfoxide and phoratoxon sulfoxide on aphid mortality.

Phorate metabolite	Initial metabolite concentration in diet (nmol l^{-1} phorate equivalent)	Mean ± s.e.	
		Toxic fraction in diet (nmol l^{-1} phorate equivalent)	% mortality
Control	0	-	0.9 ± 0.5
Phorate sulfoxide	41	22.0 ± 4.6	0.4 ± 0.4
	205	118 ± 18	0.6 ± 0.4
Phoratoxon sulfoxide	3870	2220 ± 97	1.8 ± 0.8
	19400	12300 ± 409	1.3 ± 0.05

3.8.4. Relationship between the initial insecticide concentration and the toxic metabolite concentration after 48 hours

Both phorate and phorate sulfone exhibit a linear relationship between the initial concentration applied to the diet and the final concentration of toxic residues extracted from the diet at the end of a 48 hour experimental period ($P < 0.001$ in both cases) (Figs. 3.11 and 3.12). However, much more toxic residues were recovered from diet containing phorate sulfone (83.4 ± 2.3%) than from that containing the parent compound, phorate (1.1 ± 0.1%) (see Appendices 8 and 9).

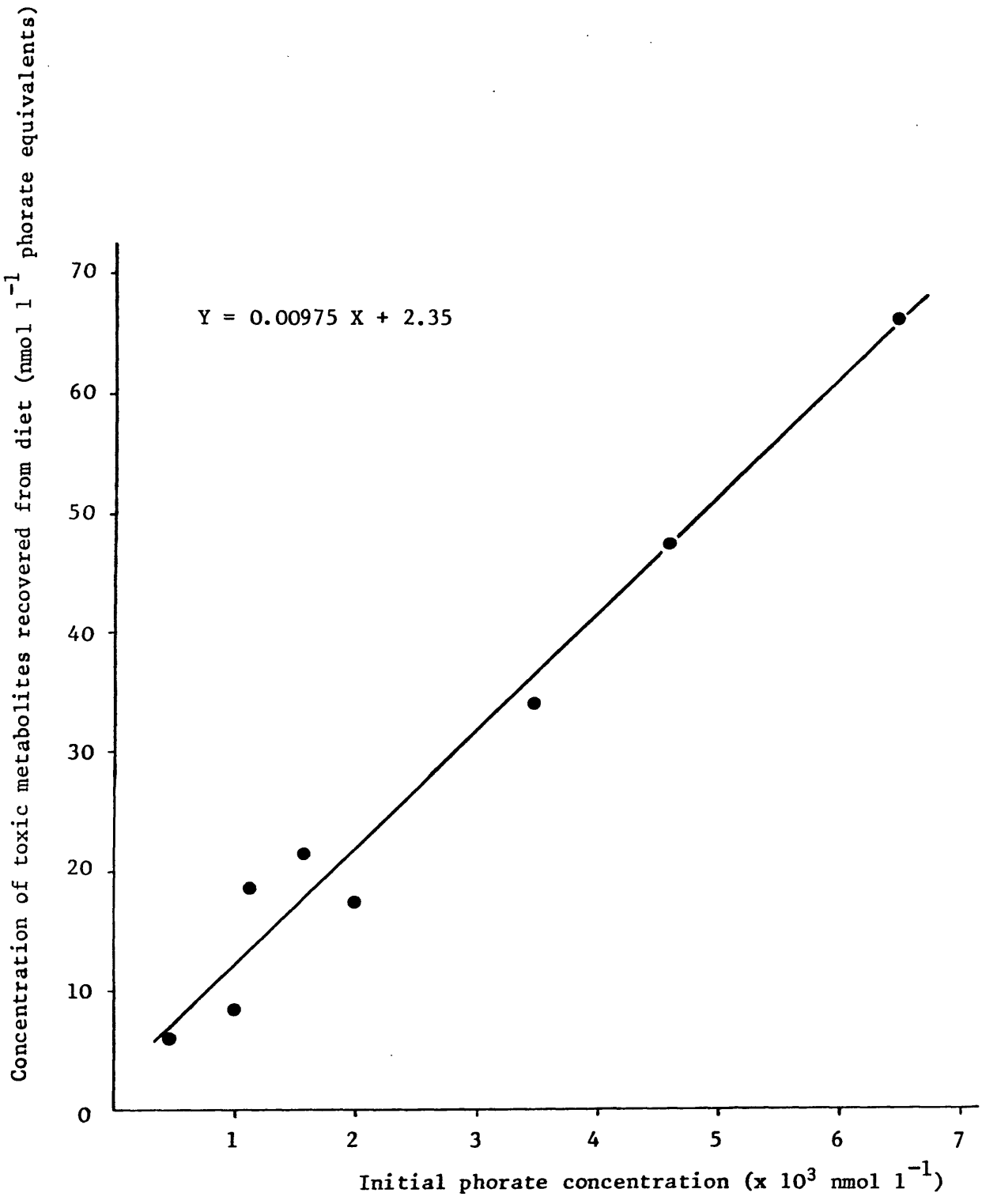


Fig. 3.11. The relationship between initial phorate concentration and concentration of toxic metabolites recovered from diet after 48 hours.

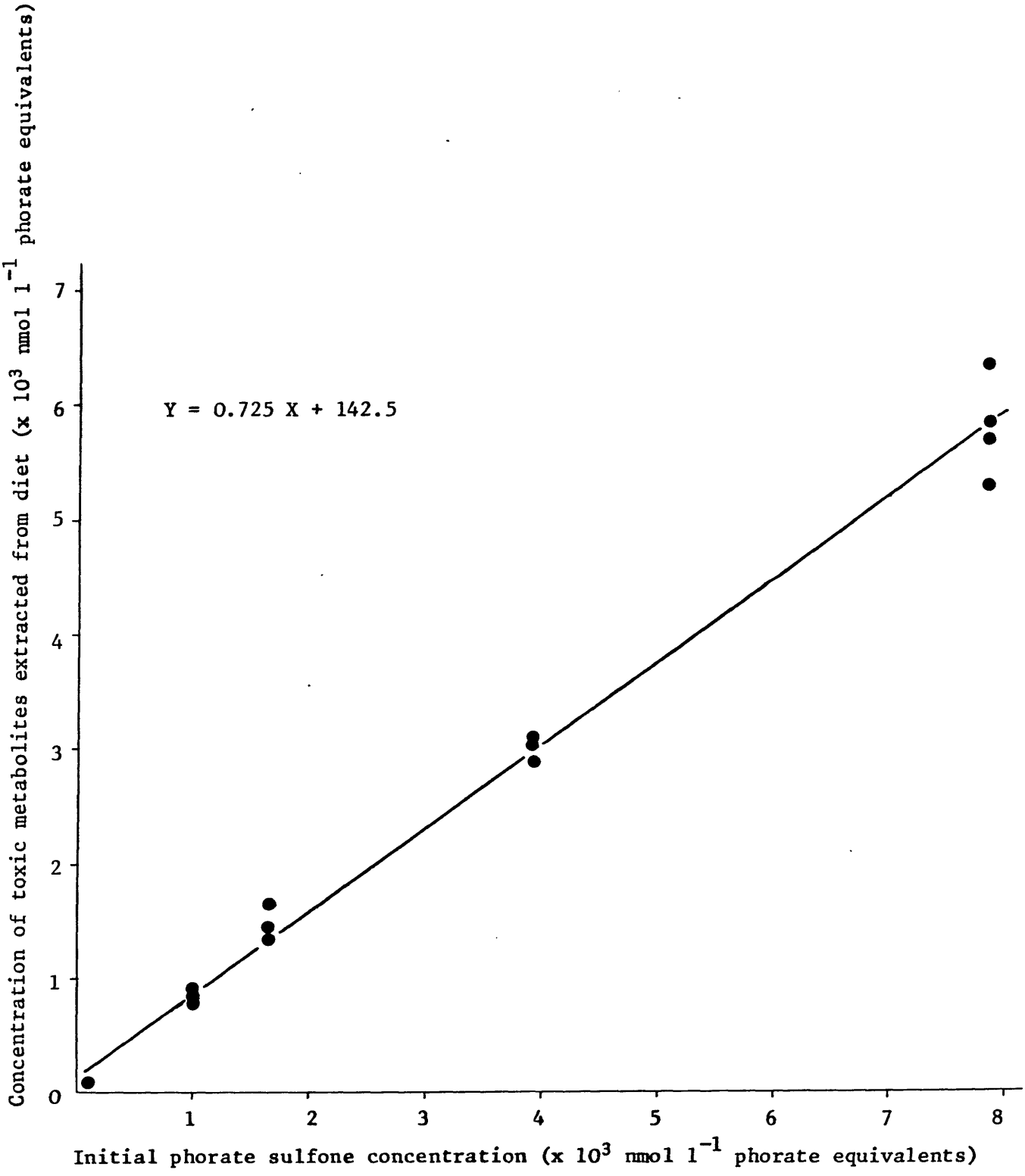


Fig. 3.12. The relationship between initial phorate sulfone concentration and concentration of toxic metabolites recovered from diet after 48 hours.

3.9 Effect of the incorporation of phorate and its metabolites into the diet on liquid uptake by aphids

The following experiments were carried out to evaluate the diet uptake by the aphids at various concentrations of phorate and its metabolites.

3.9.1. Phorate, using ^{32}P-phosphate as a feeding indicator

The radiocarbon extracted from the aphids and their honeydew following uptake of diet containing ^{14}C-phorate was present in such low amounts that it was impossible to assay the diet uptake with accuracy. A second tracer, ^{32}P-phosphorus in the form of Sodium ^{32}P-phosphate, was incorporated into the diet at an activity of 91980 dpm/ml diet to measure the volume of liquid imbibed by the aphids at various concentrations of ^{14}C-phorate. After 48 hours the aphids and their honeydew were extracted and the radioactivity assessed. Having corrected the ^{32}P activity for decay, the volume of diet imbibed by the aphids was calculated for each insecticide concentration. 3 replicates were carried out wherever possible.

Fig. 3.13 indicates a tendency for a reduction of feeding with increasing phorate concentrations. At sublethal concentrations there was only a slight but definite drop in diet uptake. At low toxic levels the graph shows a sharp drop in feeding, showing that the aphids were able to detect the presence of the poison. Then the liquid uptake became constant between LC_{15} and LC_{50}. Fig. 3.13 also shows a drastic reduction in diet uptake between LC_{50} and LC_{95} levels, beyond which the graph levelled off again, suggesting that this was due to the poisoning and subsequent death of the aphids.

3.9.2. Phorate sulfone

As higher doses of ^{14}C-phorate sulfone were used in this experiment,

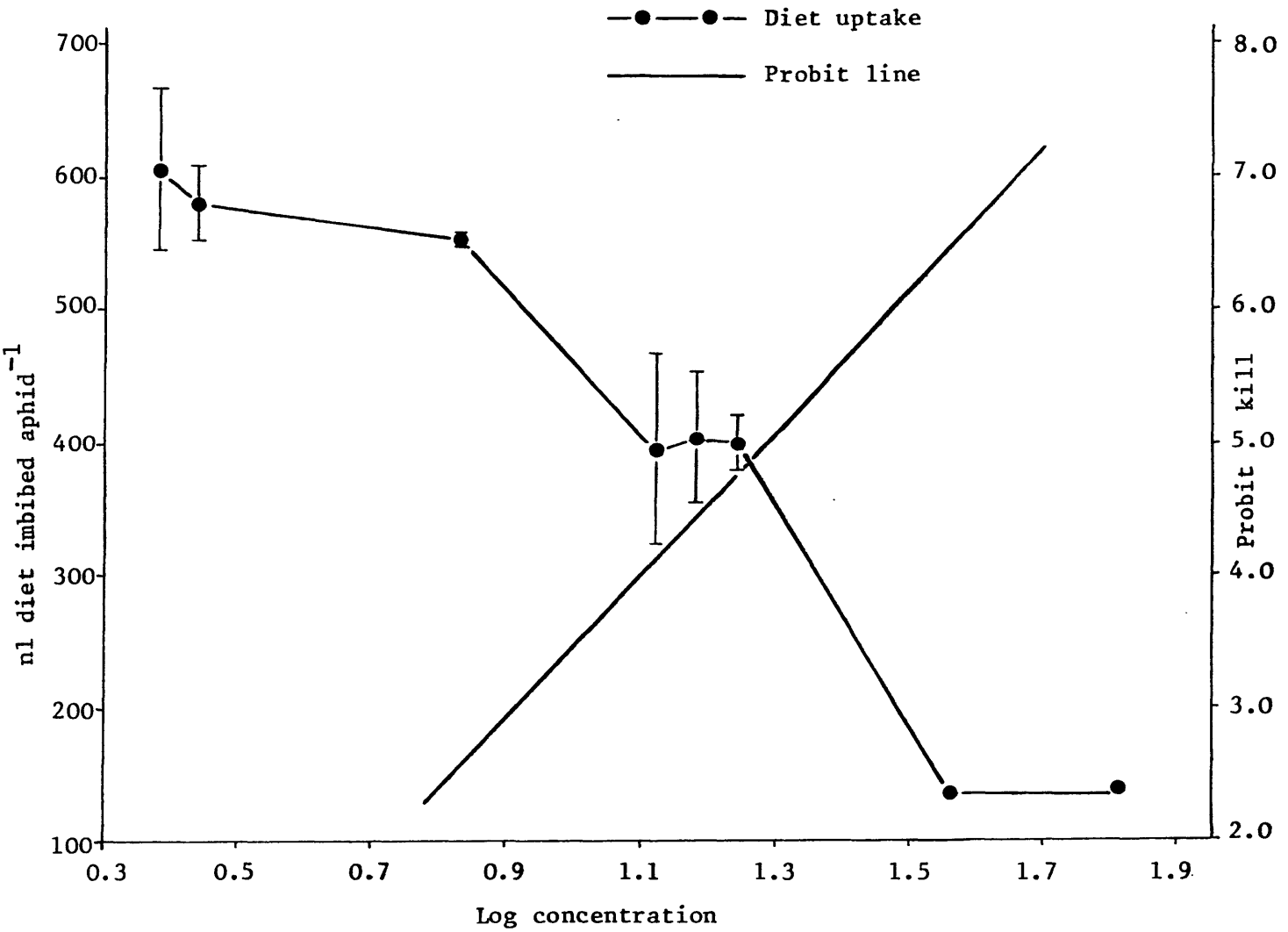


Fig. 3.13. Effect of phorate concentration on diet uptake in relation to aphid mortality.
(Concentration is expressed as mmol l^{-1} phorate equivalents; vertical bars represent standard errors of individual means; probit line is the same as that in Fig. 3.10).

the ^{14}C content in the aphids and honeydew was detectable. The volume of diet ingested by the aphids was determined by the total toxic and non-toxic radiolabel imbibed by the aphids over 48 hours, using the concentration of total toxic and non-toxic metabolites extracted from the diet in the calculation.

Table 3.13 shows that a nine-fold rise in the insecticide concentration resulted in a seven-fold decrease in diet uptake, although there was little difference in the mortality levels at these two concentrations. This suggests that the diet became less suitable and so the aphids fed less. When the dose was further increased by a factor of 3.5 there was a marked increase in mortality but the diet uptake remained the same. When this dose was then doubled, the diet uptake was halved.

Table 3.13. Diet uptake by aphids following ^{14}C-phorate sulfone incorporation into the diet.

Metabolite concentration in diet after 48 hours (nmol l^{-1} phorate equivalent)	Total ^{14}C ingested per aphid (nmol phorate equivalent)	nl diet $aphid^{-1}$	% mortality
9.9 x 10	2.8×10^{-4}	2.8×10^{-6}	4.4
8.8×10^{2}	3.3×10^{-4}	3.8×10^{-7}	4.9
3.1×10^{3}	1.2×10^{-3}	3.9×10^{-7}	67.5
6.0×10^{3}	1.2×10^{-3}	2.0×10^{-7}	92.1

3.9.3. Phorate sulfoxide and phoratoxon sulfoxide

Diet uptake by the aphids in this experiment was assessed in a similar manner to that in 3.9.2, using the radiocarbon in ^{14}C-phorate

sulfoxide and ^{14}C-phoratoxon sulfoxide as a feeding indicator.

It is clear from Table 3.14 that a five-fold increase in phorate sulfoxide concentration led to an approximately five-fold reduction in diet imbibition by the aphids. In the case of phoratoxon sulfoxide increasing the dose by five times corresponded to only about a three-fold drop in liquid uptake. In spite of the low mortality observed at these doses, there was a significant decrease in the volume of diet taken up by the aphids, once again giving evidence of the lowered acceptability of the diet rendered by the presence of toxic metabolites.

Table 3.14. Diet uptake by aphids following addition of phorate sulfoxide and phoratoxon sulfoxide to the diet.

Phorate metabolite	Metabolite concentration in diet (nmol l^{-1} phorate equivalent)	Total ^{14}C ingested per aphid (nmol phorate equivalent)	nl diet $aphid^{-1}$	% * mortality
Phorate sulfoxide	22	3.1×10^{-4}	1.4×10^{-5}	0.4
	118	3.3×10^{-4}	2.8×10^{-6}	0.6
Phoratoxon sulfoxide	2.2×10^{3}	1.6×10^{-3}	7.2×10^{-7}	1.8
	1.2×10^{4}	2.8×10^{-3}	2.3×10^{-7}	1.3

* Control mortality = 0.9%

4. DISCUSSION

4.1 Phorate treatment of bean plants and its toxicity to *A. fabae*

The toxic effect of ^{14}C-phorate on *A. fabae* and the accumulation of radiocarbon in bean leaves, aphids and honeydew were investigated. This insecticide is metabolised at a rapid rate to form oxidised and hydrolysed products. The toxic effect is therefore exerted by a mixture of phorate and its toxic oxidised metabolites rather than by the parent compound alone. The metabolism of phorate in broad bean plants following uptake by roots has been studied previously (Foerster, 1974; le Patourel, unpublished results). The latter worker has demonstrated the ability of the bean plants to actively convert phorate to its sulfoxide and to traces of other oxidised metabolites. The activating mechanism is probably present in the microsomal fraction of the plant homogenate.

For the purpose of this present study, in which all phorate treatments were analysed after 48 hours, the proportions of various toxic metabolites 48 hours after foliar application of ^{14}C-phorate were estimated in the treated leaves, aphids and honeydew. A considerable proportion of phorate was converted in the leaves to its sulfoxide (61%) and, to a lesser extent, its sulfone (17%) (Fig. 3.1). A comparison of these results with those of the 18 hour analysis of bean leaves (Fig. 3.2) shows little change in the proportions of these two metabolites between 18 and 48 hours after treatment.

The proportions of toxic metabolites in the aphids and their honeydew did not follow the same pattern as those in the leaves (Fig. 3.1). However, too much reliance should not be placed on the data, since very small quantities of the toxic residues were found (see Appendix 1). There were mainly unoxidised phorate (40%) and its sulfone (34%) in the aphids,

but in the honeydew 40% of the toxic fraction was phoratoxon, with equal amounts of phorate and its sulfoxide (ca. 20% of each) and 15% of phorate sulfone. The reasons for this difference are obscure but several possible factors may be involved. These levels of radioactivity were too low to be detected with accuracy by the Geiger-Müller counter. The soft beta emission from ^{14}Carbon is absorbed significantly in even the thinnest mica windows (Thornburn, 1972). Thus such low-energy beta particles especially when in small quantities are difficult to detect using this device. The limitations of the TLC technique must also be mentioned. The toxic fraction extracted from the aphids and honeydew could not be completely separated into discrete spots, for the organic matter in the extracts often interfered with the separation, each component acting as an individual solvent or absorbent for the metabolites. Hence the detection of a high percentage of phoratoxon could be an artifact of the work.

Nevertheless, it is possible that the rate of oxidation in the aphids may have been more rapid than in the plants, thus leading to greater proportions of phorate sulfone and the oxygen analogue, phoratoxon, in the honeydew at the time of extraction. There is evidence to show that oxidative desulphuration takes place in the fatbody of insects (Fest and Schmidt, 1973), and that such activation is catalysed by activating enzymes present in the fatbody microsomes of certain insects (Nakatsugama and Dahm, 1962, 1965). On the other hand, there could have been an apparent, faster loss of toxic label within the aphids via hydrolysis, since toxic material that was hydrolysed was not replaced in the insects from the parent compound as in the leaves, but only by that arriving in the aphids' food. These aphids fed in the phloem, where the concentrations of the individual metabolites were not known. The proportions of these

metabolites in the phloem could be different from those in a gross extract of the whole leaf, and may have been reflected in the aphids and their honeydew.

These hypotheses could not be proved and therefore the real reasons remain doubtful. The answer to this question lies in the development of techniques to analyse the phloem sap for the concentrations of the individual metabolites available to the aphids, and to accurately measure the small quantities of radiolabelled metabolites in the aphids and honeydew. More replicates and larger sample sizes could increase the degree of accuracy in the assay.

Having established that oxidised metabolites were found in bean leaves, aphids and honeydew in different proportions 48 hours after foliar application of phorate, the toxicity of this insecticide (or more likely the combined toxicity of a mixture of phorate and its metabolites) to *A. fabae* following foliar treatment was now investigated.

Phorate has been shown to move in the transpiration stream and accumulate in the leaf margins (Galley and Foerster, 1976a), but a small amount of leakage of toxic material from xylem to phloem may occur (Crisp, 1972). The low mortality levels observed following treatment of the leaf apex with high concentrations of phorate was probably due to such leakage.

The quantitative analysis by autoradiography indicates that most of the radioactivity has remained near the site of application, and two conclusions may be drawn from this evidence. Firstly, very little transfer of toxic radiocarbon from xylem to phloem occurred because mortality levels were low; also, though less significant, the aphids caused little or no accumulation of label at their feeding sites. Secondly, most of

the radiocarbon that transferred to the phloem probably did so in non-toxic form, which was then exported from the leaflet or taken up by the aphids (Galley and Foerster, 1973, 1976a). This label was unlikely to have appeared in an autoradiograph, because, in these situations, the concentration of ^{14}C at any particular time would be so low.

When toxicity was concerned, there was no correlation between dose and response, and much variation existed between replicates. This variability might be expected to some extent, since each plant constituted an independent system differing slightly from the others in the rate of evaporation, absorption, translocation and metabolism of phorate. Hence the amount of insecticide translocated and available to the aphids varied from plant to plant. Such variability after foliar application has been shown to be greater than after root absorption (Bennett, 1957).

A comparison was then made between foliar and root treatments with phorate in terms of aphid mortality and distribution of phorate residues in leaves, aphids and honeydew. In foliar application, leaf base treatment and leaf tip treatment were also compared.

The translocation pattern seen from autoradiography and radioassay data following ^{14}C-phorate treatment to various sites on the bean plants confirmed the apoplastic characteristics of this compound. Phorate and its metabolites were more readily translocated following root uptake than after foliar applications. Mitchell *et al.* (1960) reached similar conclusions about systemic insecticides. There was also a greater tendency for phorate to translocate from the proximal to the distal part of the leaflet than in the opposite direction, a trend also found to occur with dimethoate in bean leaves and lemon leaves (de Pietri-Tonelli, 1965).

Amounts of toxic and non-toxic label in the aphids and their honeydew differed between the colonies on tip-treated and those on base-treated leaflets. Colonies on tip-treated leaflets excreted much more non-toxic than toxic label in their honeydew than did those on base-treated leaflets (Tables 3.3a and 3.4a). Phorate is translocated mainly in the transpiration stream towards the leaf margins, and in the base-treated leaflets leakage into the phloem may have occurred along this route, especially near the aphids' feeding site. This relatively rapidly translocation of phorate to the aphid colonies caused a greater proportion of the toxic metabolites to be imbibed by the aphids. In the tip-treated leaflets relatively little of the toxic compared to the non-toxic label was ingested by the insects, because leakage of label into the phloem was confined to the leaf tip, since most of the radiocarbon remained around the site of application. The extent of phloem transport of toxic material therefore would be small compared to leaf-base treatment. This distribution pattern is a feature in agreement with the translocation characteristics of the two types of compounds, namely toxic and non-toxic metabolites.

The higher mortalities in the base treatment again indicates that more toxic material was available to these insects. In these colonies the small quantities of ^{14}C label, especially the non-toxic form, accumulated in the aphids and honeydew were probably due to the cessation of feeding when death ensued.

4.2 Loss of ^{14}C label following foliar application of ^{14}C-phorate

Following ^{14}C-phorate treatment to the leaf base, about 6% of the applied dose was recovered as total toxic and non-toxic metabolites from the infested leaflets, aphids and honeydew 48 hours later. In the leaf

tip treatment, about 15% of radiolabel was recovered from these tissues. Loss of radiocarbon can occur in various ways, such as evaporation from leaf and insect surfaces, hydrolysis followed by export out of the treated leaves, and the extraction procedures.

A considerable amount of insecticide can be lost from leaf surfaces, depending on the vapour pressure of the insecticide, the physical conditions prevailing at the time of application (Bennett, 1957), and the surface area over which the insecticide is spread. As phorate is volatile to a certain extent (vapour pressure 0.11 Pa at 20°C) (Martin and Worthing, 1977) a significant amount would evaporate into the atmosphere after application. Air movement and relative humidity were not controlled in these experiments. When leaf surfaces on which phorate had been applied were washed with scintillant solution and their radioactivity assayed, more residues were found on the leaf surfaces that received the insecticide at the apex than those that received it at the base. This difference was attributable probably to the different areas over which the phorate solutions were administered. The acetone solutions applied to the leaf base were found to spread over a larger area than those applied to the leaf tip. These differences may be directly responsible for the differing degrees of evaporation of the insecticide from the leaf surfaces.

Evaporation of insecticides has been shown to occur also from the body surfaces of insects (Ahmed Aly, 1976), although in the experiments reported here loss of ^{14}C label from the insects was more likely to have occurred through evaporation from the honeydew and possibly through respiration in the form of $^{14}CO_2$. However no experiments were carried out to verify this.

Another route for the loss of ^{14}C label could have been the export

of predominantly hydrolysed products from the treated leaves to other parts of the plants (Foerster and Galley, 1976). The amounts thus exported must be small compared to amounts in the treated leaves, for this export can only occur when some phorate metabolites from the xylem enter the phloem possibly by radial transfer (de Pietri-Tonelli, 1965).

A small proportion of radioactivity, usually less than 10%, may be lost also in the extraction procedures (Foerster, 1974), and about 5% is usually adsorbed on glass surfaces, depending on the nature of the glass material (G.N.J. le Patourel, private communication). Preliminary experiments had shown that when a sample with high radioactivity was evaporated using the rotary film evaporator, some carry-over of ^{14}C label into the next sample occurred, showing that some radioactivity could be lost when samples were evaporated.

Thus, leaf surfaces are the principal site of loss of ^{14}C label. Leaf surfaces are known to be the site of evaporation of water in plants, and the evaporation of some insecticides are proportional to the moisture content of the treated surface (Harris and Lichtenstein, 1961).

4.3 Comparison of feeding rate of aphids on bean plants with that on artificial diet

The use of bean plants to study the toxicity of phorate to aphids posed two major problems. One was the marked variation in the experimental results discussed previously; the other was that the actual quantities of the insecticide available to the aphids were not known, so that it was impossible to calculate doses accurately, and as such LD_{50} values are not totally reliable. These difficulties were overcome in this work by the use of a completely defined artificial diet which had supported 30 generations of *Myzus persicae* and 25 generations of *Aphis*

fabae (Dadd and Krieger, 1967). As aphids born on the diet settled more readily than those transferred from bean plants, the former were utilized as experimental material.

A knowledge of the performance of the aphids fed on artificial diet is essential before results derived from such techniques with diet could be related to those with plants. If the composition of the synthetic diet closely resembles that of the natural food, aphids would feed better, and the susceptibility of diet-feeding aphids to systemic insecticides would then be comparable with that of plant sap-feeding ones. One way of assessing aphid performance is to measure the feeding rate in terms of the dry weight of honeydew produced per aphid per day over a number of days. When a comparison was made between the feeding rate of diet-fed aphids and that of the plant-fed ones, no significant difference was found between the two within the experimental period. However, the aphids fed on diet were smaller in size than those fed on bean plants, showing that the latter must have taken up a larger quantity of solid matter and used up more of the food ingested for growth. Dadd and Mittler (1966) attributed the smaller size to nutrient imbalance. Mittler (1970) found that *Myzus persicae* feeding on mustard or radish seedlings ingested almost twice as much plant sap as those feeding on synthetic diet, and they also grew to twice the size on the plants. Llewellyn and Leckstein (1978) compared the energy budgets and growth efficiency of *Aphis fabae* reared on synthetic diets with aphids reared on broad beans, and they reported that the former utilized only 20% of the energy content of their food for growth, while the latter had a growth efficiency of 50%. Another possible reason for the difference in size could be due to the longer duration of each instar reared on artificial diet than those reared on broad bean (Tsitsipis and Mittler, 1976). One other possible

factor may be the difference in the acceptability of the diet to different strains of a species of aphid owing to different nutritional requirements, and on this basis Griffiths *et al.* (1975) developed a diet with improved acceptability to 3 strains of *M. persicae*.

An interesting point was noted in aphids feeding on artificial diet in that there was a slight reduction in honeydew production on the days when the diet sachets were changed, indicating that excretion was interrupted during diet renewal, which must therefore have caused more than a very short interruption in their feeding.

Obviously further work could have been done towards the development of a perfect diet, but this was not the concern of this study. The present diet on which the aphids survived adequately was considered satisfactory for these toxicological experiments.

4.4 Phorate metabolism in artificial diet.

Prior to the investigation of phorate metabolism in artificial diet, it was important to determine the amount of radioactivity that could be recovered from the diet after application of ^{14}C-phorate.

Acetone was found to be a suitable solvent for phorate application provided it was evaporated from the parafilm membrane before adding the diet.

It was intriguing to find that when samples of diet containing ^{14}C-phorate enclosed within parafilm sachets were extracted after 48 hours and analysed for radioactivity the total toxic plus non-toxic label recovered amounted to only 1.5% of the initial phorate applied to the diet (Table 3.8). This led to the suspicion that the major portion of the original lipophilic phorate molecules had been absorbed by the parafilm membrane.

When the membrane was extracted for radioactivity a large quantity of radiocarbon was found associated with it. This membrane-bound ^{14}C label consisted mainly of toxic label partitioning into the chloroform phase, and comprised approximately 75% of the initial phorate. This proportion was consistent over a period of 48 hours, as shown in Table 3.9.

Taking into consideration the total radiocarbon recovered from both diet and membrane, there was still about 20% of the initial phorate that was unaccounted for. It is very likely that most of this 20% was lost through evaporation when the diet sachets were made up. When phorate was applied to the diet using the method mentioned above, a considerable amount of this volatile insecticide could have evaporated into the atmosphere. Although Risella-17 oil has been found to reduce such loss, this oil could not be used in the diet because it was found to dissolve the parafilm membrane even when present in very small quantities. A small proportion of the insecticide may also have evaporated from the completed sachets, but this probably was negligible in comparison with losses by other routes. In addition, some 10% or less as in other extractions is usually lost during this procedure.

The contact toxicity to aphids of the toxic compounds in the parafilm membrane was not assessed. However, it can be assumed that the toxic effect, if any, would be minimal because the aphids, except for their stylets, were not in direct contact with the toxic membrane since they were separated from the sachets by the feeding membrane. The possibility of death of aphids owing to the fumigant or contact action of insecticides penetrating the parafilm feeding membrane has been ruled out by the experiments conducted by Mittler and Pennell (1964), who demonstrated a high survival of aphids which were prevented from feeding on sachets

containing high insecticidal concentrations.

At this juncture, the relationship between the initial concentrations of phorate and phorate sulfone and the final concentrations of toxic metabolites recovered from the diets containing the two respective compounds was investigated 48 hours after insecticide treatment of the diets. Linear regression analyses by computer showed a linear relationship between the initial concentrations and the final toxic metabolite concentrations for both phorate and its sulfone (Figs. 3.11 and 3.12). On comparison of these two figures it can be seen that appreciably more of the toxic residues was recovered from diet containing phorate sulfone (ca. 83%) than from that containing phorate (ca. 1%) after 48 hours. This disparity must be attributable to the different partition coefficients of these toxic components between the membrane and the diet. Phorate is known to be more lipophilic than its sulfone, for in an equivolume hexane-water system, for example, the partition coefficient for phorate is 1.00 while that of its sulfone is 0.98 (Bowman *et al.*, 1969). However, the partition coefficients of these two compounds between the membrane components and water may be very different.

Owing to the difficulty in preparing large amounts of phorate metabolites, and the limited time available for this work, the absorption of phorate sulfone and other toxic metabolites by parafilm membrane was not investigated. Further work on this needs to be carried out in order to achieve a fuller understanding of the relative toxicity of phorate and its metabolites to aphids.

The hydrolysis products formed from phorate metabolism were not analysed for their individual components in this work. It should be borne in mind, however, that the position of the radiolabel in the insecticide

molecule determines which of the hydrolysis products will be detected and into which fraction the radiolabelled compounds will partition when extracted with chloroform and water. The phorate used in this study was methylene-labelled. Therefore the determination of the partitioning properties of the hydrolysed radiocarbon was fundamental to the study of phorate metabolism. An attempt was made to quantify the radioactivity associated with the hydrolysis products that might have partitioned into the chloroform fraction. The chloroform fractions extracted from both diet and an aqueous solution of phorate hydrolysate were analysed by TLC. The amount of radioactivity at the origin together with that not associated with the toxic radiocarbon on the TLC plate was found to be less than 1% in both diet and the aqueous medium. Although this does not prove conclusively that only less than 1% of the hydrolysis products formed from methylene-labelled phorate partitioned into chloroform, it goes some way to showing that the major proportion of the hydrolysis products partitioned into the aqueous phase. This means that the radioactivity associated with the chloroform fraction can be assumed to consist almost completely of toxic label, and that associated with the aqueous phase is present almost completely as non-toxic label.

The metabolism of phorate and its metabolites in diet was studied at various intervals over a period of 96 hours. One striking feature of phorate metabolism both in bean plants and in artificial diet is the rapidity with which the parent compound is oxidised to its sulfoxide and sulfone, although oxidation in plants is catalysed by enzymes while that in diet is non-enzymic. A knowledge of the chemical, physical and toxicological properties of phorate and its metabolites is of great significance to aphid control. The importance of phorate metabolism cannot be overemphasized. It has been known that aphids normally reach a maximum

rate of ingestion of plant sap between 12-16 hours (Banks and Nixon, 1959) or of diet after approximately 12 hours (Parry and Ford, 1969). During this time the aphids would be taking up a mixture of phorate, its sulfoxide and sulfone, three major products in bean plants (Fig. 3.1 and 3.2) and in artificial diet (Fig. 3.8) following phorate treatment.

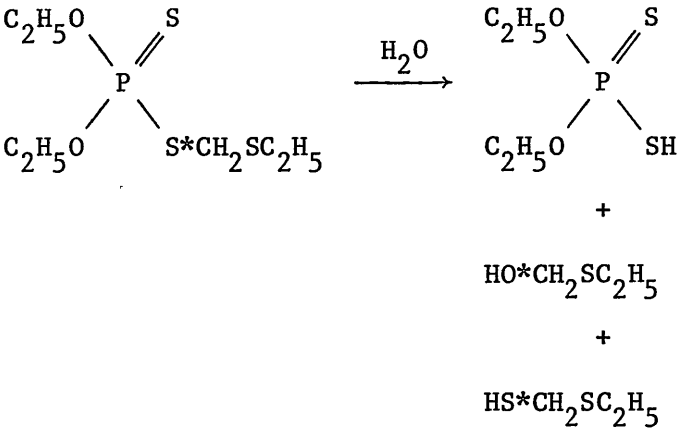
The amounts of phoratoxon and its sulfoxide and sulfone found in artificial diet were in the region of 5% or less. These values were likely to be due to experimental errors as these metabolites are less stable and more readily hydrolysed than the corresponding phorate derivatives (Faust and Gomaa, 1972), and hence were present in such low quantities that they were impossible to detect with any accuracy.

Fig. 3.8 indicates the extent of apparently instantaneous oxidation of phorate to its sulfoxide and a small amount of sulfone. Metal ion systems such as EDTA-Fe^{++} complexes (Undenfriend *et al.*, 1954; Brodie *et al.*, 1954; Ullrich and Staudinger, 1969) are known to catalyse the hydrolysis and oxidation of some organophosphorus insecticides (Knaak *et al.*, 1962). The diet used in this work contains traces of EDTA-Fe^{++} and other metal ion complexes which in the presence of oxygen may well catalyse the oxidation of the phosphorotriester, phorate.

The decline of phorate concentration and the buildup of its sulfoxide and sulfone are assumed to be first-order rate processes, and can best be treated as the three-compartment open model in biological systems described by Atkins (1969) and Notari (1975). The rate of accumulation of phorate sulfoxide is equal to the rate of formation from phorate minus the rate of dissipation via oxidation to phorate sulfone and via hydrolysis. Similarly, the rate of accumulation of phorate sulfone is equal to the rate of formation from phorate sulfoxide via oxidation minus the rate of

dissipation via hydrolysis and oxidation to phoratoxon sulfone. Assuming first-order kinetics, the rate of formation of phorate sulfoxide is proportional to the concentration of phorate. Likewise, the rate of formation of phorate sulfone is proportional to the concentration of phorate sulfoxide. These processes are diagrammatically illustrated in Fig. 4.1. The actual shapes of the curves depend on the values of the rate constants. Phorate metabolism in soil at 20°C has been studied over a period of 128 days by Abou-Donia (1976). A comparison of his data with the results obtained from this two-day diet experiment indicates that the latter represent only the portion A in the above model.

The diet provides an aqueous environment for hydrolysis of phorate and its oxidised metabolites, and these processes also follow first-order kinetics. The components of the hydrolysed products were not identified, but the following compounds were assumed to be formed from the methylene-labelled ^{14}C-phorate:



*Position of radiolabel

On the basis of the results in Tables 3.10 and 3.11, these ^{14}C-labelled hydrolysis products must have partitioned almost completely into the aqueous fraction in a chloroform-water system.

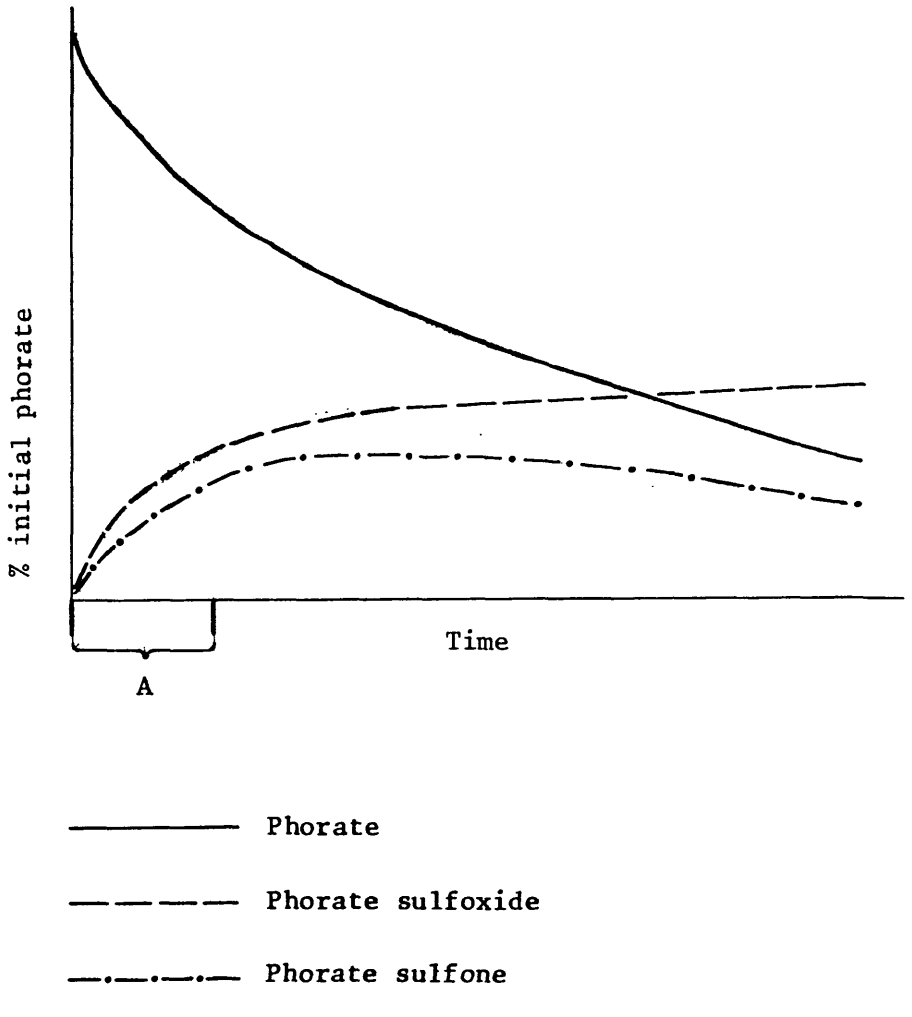


Fig. 4.1. Model for metabolism of phorate, phorate sulfoxide and phorate sulfone.

In Fig. 3.8 and 3.9 the percentages of radioactivity recovered from the diet at various time intervals are probably an underestimate of the true values. There are several reasons for this unrecoverable radioactivity. Loss of radiolabel could have occurred through evaporation of the volatile metabolites from the diet which in this particular experiment was not enclosed within sachets. Some 14% (see experiment 3.6.3.1.) could have been lost from the TLC plate through evaporation and absorption by the silica gel. A small proportion was probably lost through adsorption on the surfaces of the vial containing the diet as well as surfaces of the glassware used in the extraction and concentration procedures as discussed earlier. The limitations of the TLC technique as a source of experimental error have also been discussed. Other more accurate but time-consuming techniques could have been employed, for instance, gas-liquid chromatography and column chromatography. However, these methods were not necessary for the purpose of this experiment, which was to estimate the proportions of various metabolites formed in the diet as a function of time. From these data the proportions of the various metabolites ingested with the diet by aphids during the experimental period would be known.

4.5 Relative toxicities of phorate and some of its metabolites to *A. fabae* in relation to diet uptake by the aphids

Satisfactory bioassays were carried out with different concentrations of phorate and phorate sulfone only, because the latter was more readily prepared from phorate by the bromine water oxidation method than the other metabolites. Phorate sulfoxide, despite being present in the largest proportion in plants and artificial diet, could not be prepared in sufficient quantities, using this technique, for a proper toxicity test to be performed. Notwithstanding these limitations, a certain amount of information obtained from dose/response experiments with phorate, phorate

sulfone, phorate sulfoxide and phoratoxon sulfoxide indicated that the parent compound, phorate, was the most toxic to aphids when added to the diet. Next in toxicity was its sulfone, followed by its sulfoxide and phoratoxon sulfoxide in that order.

Probit analysis of the dose/response data for phorate and its sulfone (Fig. 3.10) showed that the two lines were not parallel. Thus, a comparison of the relative potencies using this type of analysis could not be drawn. However, such a comparison could be made of the LC_{50} and LC_{95} values derived from the individual regression lines (Bánki, 1978). These values give an idea of how effective the two compounds were at these mortality levels, and show that phorate was considerably more toxic generally than the sulfone. In the interest of crop protection and insect pest control, LC_{95} values would be of greater significance than LC_{50} values when comparing compounds whose probit lines are not parallel to each other. It is clear from Fig. 3.10 that phorate is even more effective than its sulfone at higher concentrations.

Had time permitted, a search for other more suitable methods to prepare larger quantities of ^{14}C-labelled toxic metabolites would have been undertaken to enable a range of doses to be tested. Unfortunately these radiolabelled compounds were not available commercially.

Some work on the relative toxicities of phorate and its metabolites has been done previously. For example, Bowman *et al.* (1969) has determined the relative toxicities of phorate and five of its metabolites in corn to the European corn borer, and found the order of toxicity to be phorate > phorate sulfoxide > phorate sulfone, with comparatively low effective toxicities of the three O-analogues. Using fly-brain cholinesterase inhibition to study the toxicities of the oxidised products of phorate,

Metcalf *et al.* (1957) has shown that the replacement of P = S with P = O markedly increased the anticholinesterase activity by increasing the instability of the molecule. Thus P = O, SO_2 (phoratoxon sulfone) was the most potent cholinesterase inhibitor.

The less stable oxygen analogues are more toxic as anticholinesterase agents, and may well be more toxic *in vitro*. When phorate is reacted with cholinesterase *in vitro* it has to be broken down to the oxygen analogues to exert their full effect at the site of action. *In vitro* studies of cholinesterase inhibition would therefore show phoratoxon sulfone to be the most toxic and phorate the least. When phorate is added to the diet a low concentration of phoratoxon may be maintained by the parent compound to give a continuous supply of phoratoxon sulfoxide and sulfone until the phorate is depleted. When ingested the penetration of toxic compounds to the site of enzyme action is affected very much by their partition coefficients and their susceptibility to hydrolysis. The more toxic metabolites are generally more hydrophilic and would not penetrate to the enzyme so readily. They are also more easily hydrolysed and thus may lose their insecticidal effect before reaching the target site. When oxidised metabolites are individually fed to the aphids as such, they would be rapidly hydrolysed, and their levels are not maintained by the parent compound or by less oxidised products. They would therefore appear to be less toxic *in vivo* than phorate. It would be interesting to assess the inhibition of cholinesterase activity of *A. fabae* by phorate and its metabolites. The findings of the bioassays together with inhibition studies may confirm the above.

It can be seen in Table 3.12 that when relatively high doses of phorate sulfoxide and phoratoxon sulfoxide were incorporated in the diet

no mortality or extremely low mortality levels were recorded. There were insufficient data to calculate the LC_{50} values but it is evident that these two metabolites were of low toxicity to the aphids. The wandering behaviour observed at 1.2×10^4 nmol l^{-1} phorate equivalent of phoratoxon sulfoxide after 48 hours suggested that the apparently low toxicity of this metabolite might be due to a change in the acceptability of the diet and hence the rejection of the diet by the aphids. The insects might not have ingested sufficient quantities of the diet to be poisoned. Other workers have shown that there was a decrease in diet uptake when phosphamidon was incorporated. This decrease was a consequence of a change in acceptability of the diet, and was the reason for a higher LC_{50} value for the darker strain of *Myzus persicae* when compared with the light strain, rather than a difference in the susceptibility to the insecticide between the two strains (Parry and Ford, 1967, 1969).

When phorate was added to the diet ^{32}P-phosphate was used as a feeding indicator to overcome the difficulty in measuring small amounts of ^{14}C-label in the aphids and honeydew. A general trend can be seen from Fig. 3.13 in which the aphids fed less as the insecticide concentrations were increased. At sublethal concentrations between 2.5 and 6.0 nmol l^{-1}, there was a small but definite drop in diet uptake from about 600 nl/aphid to 550 nl/aphid. This reduced uptake may in part be caused by a lowered acceptability of the diet as was shown for phosphamidon by Parry and Ford (1967, 1969).

At the lower toxicity levels up to LC_{15} the sharp decline in feeding is likely to be due to the onset of poisoning rather than the aphids' increasing 'dislike' for the diet. However, the distinction between these aspects might be resolved by choice-chamber type experiments such as those

carried out by Mittler and Dadd (1964b) and Parry (1966). The ability of the aphids to discriminate between pairs of liquids as demonstrated by these workers and the probing responses elicited by different feeding solutions behind a parafilm membrane (Mittler and Dadd, 1965b) give sufficient evidence that aphids do avoid unsuitable feeding liquids.

It may be seen in Fig. 3.13 that liquid uptake between LC_{15} and LC_{50} remained more or less constant. However, the variation between replicates was too great for the curve in this region to be defined. This pattern was found to be repeated with phorate sulfone. This general trend probably arises because the major portion of the radiophosphorus ingested by the aphids was present in the honeydew. The ^{32}P-label, and hence the volume of diet imbibed by the total number of insects in each colony, was divided by this number to give the average diet imbibed per aphid. At the lower concentrations the survival was higher and most of the aphids were feeding and producing honeydew, whereas at higher doses the dead aphids had fed and excreted some honeydew before dying. The number of live aphids responsible for the bulk of honeydew production progressively decreased with increasing doses. It follows from the above argument that the constant level of diet uptake over this range of doses is attributed to the fact that the surviving aphids at the higher doses had excreted more honeydew than those fed on less toxic diets. The poisoned aphids could have excreted possibly a larger volume of honeydew as a detoxication mechanism.

With a further increase in phorate concentration the corresponding decrease in liquid uptake must have been, to a larger extent, due to the high mortality levels. Many wandering aphids were found at these concentrations, and this behaviour, together with other signs such as

loss of body co-ordination, was evidence of poisoning. It is, however, difficult to know for certain whether all the aphids which died were poisoned or whether some of them had starved to death. There might have been a point at which hunger overcame the insects' avoidance for the toxic diet and they began to feed sufficiently to become poisoned.

When all or nearly all the aphids were dead on the most toxic diets, they must have ingested lethal quantities of the insecticide even though the volumes of liquid imbibed were small. A further increase in the doses did not have any more effect on diet uptake.

The effect of phorate sulfone concentrations on diet uptake in relation to aphid mortality is given in Table 3.13. Sublethal doses were not studied in this experiment. The volumes of diet imbibed by the aphids were calculated from the ^{14}C-label present in the aphids and their honeydew on the basis of the recoverable ^{14}C label in the diet 48 hours after treatment. These results cannot be compared in absolute terms with diet uptake influenced by phorate incorporation, because in the latter the volumes imbibed were calculated on the basis of the original ^{32}P-phosphate concentration in the diet, and it was assumed that there was no loss of radioactivity apart from decay which has been corrected for. There was a similarity in the pattern of diet uptake following addition of either phorate or its sulfone. The constant volume of diet ingested between mortality levels of 5% and 68% associated with phorate sulfone was similar to the results obtained with phorate. The same hypothesis may apply here, that the surviving insects excreted larger amounts of honeydew as part of a physiological mechanism to eliminate the toxic products. At the 92% mortality level the reduction in liquid uptake was again due to the death of the aphids.

When phorate sulfoxide was incorporated in the diet a five-fold increase in concentration gave rise to a corresponding five-fold decrease in diet uptake as shown in Table 3.14. Since there was no effective mortality here, the decline in feeding must have been attributed to the detection by the aphids of the chemical in the diet, which rendered it less acceptable. Increasing the concentration of phoratoxon sulfoxide by five times only caused a three-fold drop in uptake. The low mortality at a concentration of 1.2×10^4 nmol l^{-1} of phoratoxon sulfoxide, an amount which greatly exceeded the proportion normally present in a metabolite mixture derived from phorate, suggests that this compound has no insecticidal value. On the other hand, the wandering behaviour and the reduction in feeding again indicate an altered acceptability of the diet to the aphids, with a trend towards the aphids tolerating phoratoxon sulfoxide more than phorate sulfoxide.

This wandering behaviour as a result of a lowered acceptability of the diet may cause the aphids to probe more when feeding on the plant in an attempt to find suitable food. In so doing aphids can increase the incidence of virus transmission.

The artificial feeding experiments in this work have demonstrated the susceptibility of *A. fabae* to phorate and some of its oxidised metabolites and the effect of different concentrations of these compounds on the diet uptake by the aphids. The reduction in feeding when these toxic compounds were present in the diet indicated a lowered acceptability of the diet, and this caused the aphids to wander away from the food. These findings are of immense importance to virus transmission and aphid control. The reasons for the unsuccessful attempts to determine the dose/response relationship in bean plants are now clear. The concentrations of toxic

compounds available to the aphids feeding in the phloem were not known, and are dependent upon the site of insecticide application and the rates of absorption, translocation, oxidation and hydrolysis in the plant. Although the concentrations effective against the aphids have been evaluated in the diet, higher concentrations may be needed in plants because of the small amounts of toxic material partitioning into the phloem from the xylem. Physiological factors such as the size of the aphids may also have some influence on their susceptibility to the insecticide. The smaller aphids fed on diet may be more susceptible to phorate than the larger plant-fed ones.

The results obtained in this study conducted over a limited period of time open up several possible aspects of further research. The most pressing problem is the need for developing suitable techniques to measure the concentrations of systemic insecticides in the phloem. These concentrations can then be correlated with insecticide uptake by aphids from the phloem and the partitioning of this compound into this tissue from other parts of the plant. There is scope for further work on the effect of systemic insecticides on the feeding behaviour of aphids in relation to virus transmission. A fuller investigation into the relative potencies of phorate and its oxidised metabolites in terms of systemic toxicity and cholinesterase inhibition would be an important step in toxicological studies. Knowledge derived from the effect of metabolism on the toxicity of a compound would be useful in developing new systemic insecticides with greater potential toxicity.

SUMMARY

1. An attempt was made to assess the toxicity of phorate to *Aphis fabae* in broad bean plants in relation to the site of insecticide application. With the aid of radiotracer techniques, the accumulation of radiocarbon in the leaves, aphids and honeydew was quantitatively determined using a Geiger-Müller counter and a liquid scintillation counter.

2. No correlation was found between insecticide concentrations applied to the plants and mortality in the aphids, and an LC_{50} value could not be obtained. There was much variability between replicates. The reasons for this were thought to be due to the apoplastic characteristics of this compound which reached the aphids only via leakage into the phloem.

3. The difficulty in obtaining consistent results in the bean plant experiments was overcome by the use of an artificial diet as a tool for subsequent toxicological studies.

4. A method of making up sterile diet sachets and of changing the sachets with minimum disturbance to the aphids is described.

5. The performance of the aphids on this diet was assessed by comparing the feeding rates in terms of honeydew excretion between plant-fed and synthetic diet-fed aphids. No difference was found in the rates of honeydew production but the diet-fed insects were noticeably smaller than the plant-fed ones.

6. The metabolism of phorate in artificial diet as a function of time was studied in detail, and the concentration of toxic metabolites recovered from the diet formed the basis of the dose units used in toxicity tests.

7. Thin-layer chromatography (TLC) was used for the quantitative radio-

assay of various metabolites. The limitations of this technique are outlined.

8. Bromine water was used to prepare a mixture of phorate metabolites from phorate, but only phorate sulfone with small amounts of phorate sulfoxide and phoratoxon sulfoxide could be extracted from this mixture.

9. The relative toxicities of phorate and the above metabolites to aphids were assessed. Phorate was found to be the most toxic, being about 127 times more potent than its sulfone. Extremely low mortality levels were associated with phorate sulfoxide and phoratoxon sulfoxide. Such low mortalities and the wandering behaviour at the highest phoratoxon sulfone concentration tested indicated an altered acceptability of the diet due to the presence of these metabolites.

10. The effect of various concentrations of phorate and its metabolites on diet uptake by the aphids was investigated. A general decline in feeding with increasing doses of these compounds was demonstrated. This decline at sublethal doses was attributed to the lowered acceptance of the diet by the insects, while at lethal concentrations, reduction in feeding was thought to be mainly a result of increasing mortality.

ACKNOWLEDGEMENTS

I owe my gratitude to my supervisor, Dr. D.J. Galley, for suggesting this project and for his guidance and encouragement throughout this study especially his helpful criticism of the manuscript.

My thanks also go to Dr. G.N.J. le Patourel for his valuable suggestions, advice and discussions.

I wish to thank Prof. T.R.E. Southwood for granting me permission to undertake the research at the Field Station and providing the necessary facilities.

Thanks are also due to the following members of the Field Station: Drs. G. Murdie and S. Young for their advice on statistical and computer analyses of my data,
Mr. A.R. Ludlow for the use of his computer program,
Mr. H.A. Devitt, Mr. S.C. Bunce, Mr. R.H. Hartley and Mr. J.P. Nicholas for their assistance with the photography.

This work was carried out while I was in receipt of a Commonwealth Scholarship awarded by the Association of Commonwealth Universities, to which I am grateful.

I wish to thank Miss C.M. Collins for the arduous task of typing this thesis.

Finally, I appreciate the varied assistance offered by many friends here without whose unfailing support research would not have been so challenging.

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Appendix 1 (Fig. 3.1)

Toxic ^{14}C-labelled metabolites found in bean leaves, aphids and honeydew 48 hours after foliar application of ^{14}C-phorate.

Bean leaves

Phorate metabolites	% occurrence (Mean ± s.e.)
P = S, S	4.0 ± 1.0
P = S, SO_2	17.1 ± 1.9
P = O, S	3.0 ± 0.6
P = S, SO	60.7 ± 1.9
P = O, SO_2	2.8 ± 0.4
P = O, SO	12.2 ± 1.4

Aphids and honeydew

Phorate metabolites	Aphids		Honeydew	
	Dpm/colony	% occurrence	Dpm/colony	% occurrence
P = S, S	100	40.2	40	19.8
P = S, SO_2	85	34.0	30	15.2
P = O, S	20	7.9	80	39.9
P = S, SO	18	7.2	39	19.3
P = O, SO_2	11	4.4	4	2.2
P = O, SO	16	6.3	7	3.6

Appendix 2 (Fig. 3.2)

Toxic ^{14}C-labelled metabolites found in bean leaves 18 hours after foliar application of ^{14}C-phorate.

Phorate metabolites	% occurrence (Mean ± s.e.)
P = S, S	12.0 ± 7.7
P = S, SO_2	11.6 ± 0.8
P = O, S	7.2 ± 4.9
P = S, SO	64.4 ± 4.6
P = O, SO_2	1.6 ± 0.5
P = O, SO	3.2 ± 0.6

Appendix 3 (Fig. 3.6)

Feeding rate of aphids on bean plants.

Replicates	µg honeydew/aphid/day					
	Age of aphids (days)					
	3	4	5	6	7	8
1	13.2	32.7	42.1	53.0	75.3	90.6
2	7.9	24.2	26.3	41.7	46.4	55.2
3	11.1	14.0	18.0	40.2	54.7	66.9
4	10.3	37.6	43.6	68.7	63.3	72.1
5	9.1	25.9	41.5	57.1	75.7	99.8
Mean	10.3	26.9	34.3	52.1	63.1	76.9
± s.e.	0.9	4.0	5.1	5.2	5.7	8.1

Appendix 4 (Fig. 3.7)

Feeding rate of aphids on artificial diet.

Replicates	µg honeydew/aphid/day				
	Age of aphids (days)				
	3	4	5	6	7
1	10.8	24.7	31.3	52.9	58.9
2	5.6	20.3	24.7	46.3	51.3
3	18.2	33.4	37.7	69.5	73.2
4	12.8	26.9	26.5	56.6	55.0
5	15.1	30.5	35.8	62.1	57.8
6	15.0	30.8	33.2	65.1	53.3
Mean	12.9	27.8	31.5	58.8	58.3
± s.e.	1.8	2.0	2.1	3.5	3.2

Appendix 5 (Fig. 3.8)

Metabolism of phorate to its toxic metabolites in artificial diet.

Time (Hr.)	% of total radioactivity in diet					
	PSS	$PSSO_2$	POS	PSSO	$POSO_2$	POSO
0	82	1.3	0.4	13.2	0.0	2.7
1	67	4.0	2.0	15.0	10.0	2.1
2	67	3.2	4.4	16.2	6.1	2.8
6	84	1.9	2.6	5.9	3.2	2.2
9	66	7.7	2.0	17.6	4.0	2.5
24	76	6.1	1.2	11.6	5.1	0.4
30	73	16.2	1.1	6.3	2.5	1.1
48	74	5.2	3.3	13.3	2.4	2.2
72	41	5.5	4.7	32.1	7.3	9.5
96	73	3.2	0.0	19.0	5.3	0.0

Appendix 6 (Fig. 3.9)

Oxidation and hydrolysis of phorate in artificial diet.

Time (Hr.)	Oxidised metabolites		Hydrolysed metabolites	
	nmol l^{-1}	% recovery	nmol l^{-1}	% recovery
0	2638	97.0	81	3.0
1	1711	77.2	506	22.8
2	1132	70.8	468	29.2
6	1458	83.8	283	16.2
9	1268	78.3	352	21.7
24	935	66.3	474	33.7
30	766	56.8	582	43.2
48	427	39.3	659	60.7
72	392	29.7	929	70.3
96	278	22.8	940	77.2

Appendix 7a (Fig. 3.10)

Dose-response relationship between phorate and *A. fabae*.

Dose (nmol l^{-1} phorate)	No. of aphids	No. affected
65.3	95	95
46.8	115	113
36.1	119	116
21.6	398	238
17.6	172	88
15.0	224	92
13.1	244	22
6.7	120	7
3.2	126	5
2.7	207	10
2.4	217	8
0.0	314	12

Appendix 7b (Fig. 3.10)

Dose-response relationship between phorate sulfone and *A. fabae*.

Dose (nmol l^{-1} phorate equivalent)	No. of aphids	No. affected
6351	80	78
5851	53	48
5709	102	86
5297	48	46
3837	56	38
3778	98	45
3246	73	51
3069	73	59
3064	91	62
2898	72	52
1662	178	43
1455	156	28
1356	135	35
880	83	3
866	86	6
808	71	3
97	64	2
93	59	6
92	80	0
0	104	0

Appendix 8 (Fig. 3.11)

Relationship between initial phorate concentration and final toxic metabolite concentration after 48 hours.

Initial phorate concentration (nmol l^{-1})	Final toxic metabolite concentration (nmol l^{-1} phorate equivalent)	% initial phorate
452	6.0	1.3
988	8.5	0.9
1110	18.6	1.7
1562	21.5	1.4
1977	17.4	0.9
3463	33.9	1.0
4564	47.3	1.0
6454	66.1	1.0
	Mean	1.1
	± s.e.	0.1

Regression analysis:

Slope ± s.e. = 0.00975 ± 0.000677

Appendix 9 (Fig. 3.12)

Relationship between initial phorate sulfone concentration and final concentration of toxic metabolites after 48 hours.

Initial phorate sulfone concentration (nmol l^{-1} phorate equivalent)	Final toxic metabolite concentration (nmol l^{-1} phorate equivalent)	% initial phorate sulfone
100	92	92
100	93	93
100	97	97
1000	808	81
1000	866	87
1000	880	88
1650	1356	82
1650	1455	88
1650	1662	>100
3920	2898	74
3920	3064	78
3920	3069	78
7850	5297	67
7850	5709	73
7850	5851	75
7850	6351	81
	Mean	83.4
	± s.e.	2.3

Regression analysis:

Slope ± s.e. = 0.725 ± 0.0198

Appendix 10 (Fig. 3.13)

Effect of phorate concentration on diet uptake.

Concentration of toxic metabolites in diet		Diet uptake (n 1 aphid^{-1})
nmol 1^{-1} phorate equivalent	Log concentration	Mean ± s.e.
0	-	627 ± 30
2.4	0.38	607 ± 60
2.7	0.44	582 ± 29
6.7	0.83	553 ± 5
13.1	1.12	396 ± 72
15.0	1.18	405 ± 50
17.6	1.24	402 ± 21
36.1	1.56	136
65.3	1.81	138