

STUDIES ON THE DECOMPOSITION OF THE ORGANOPHOSPHORUS
INSECTICIDE "PHORATE" IN THE SOIL

by

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ABSTRACT

The effect of moisture, pH, and temperature on the decomposition of phorate to non-toxic hydrolysis products and its oxidation to the sulphoxide and sulphone derivatives in a local soil were investigated. The influence of microbial activity in the soil on the kinetics of metabolism, and of inhibitors of autoxidation reactions on the rate of phorate oxidation in the soil were also studied. The rate of decomposition of phorate sulphoxide and sulphone in the soil was determined. The observed patterns of phorate metabolism in the soil are interpreted in terms of a simple first-order kinetic model.

An enrichment technique was used to isolate micro-organisms in the soil which were effective in degrading phorate. The rates of phorate accumulation and metabolism in aqueous cultures of an isolated strain of *Bacillus megaterium* were examined and compared with rates of phorate metabolism in soil artificially enriched with cultures of the bacterium and with the control rates of hydrolysis of phorate, its sulphoxide and its sulphone derivatives at soil pH. Adsorption isotherms for phorate and its oxidised metabolites on soil, sand and clay were plotted. The effect of sorptive interactions between soil particles and bacteria on the metabolism of phorate in the soil is discussed.

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INTRODUCTION

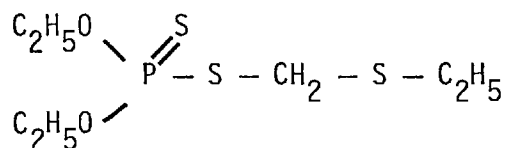
The organophosphorus compounds today account for approximately 30% of the registered synthetic insecticides and acaricides, and the number is steadily increasing (Matsumura, 1975). The term 'organophosphate' is used generically to cover all insecticidal organic compounds containing phosphorus. The majority of organophosphorus insecticides are neutral esters or amides derived from phosphoric acid, its anhydride, or sulphur analogues. Certain fluorides have been used commercially and several phosphonic acid derivatives have also been developed as insecticides. Because the number of currently used organophosphorus insecticides is so large, they are classified into different groups according to the phosphorus acid from which they are derived. The majority of commercial organophosphorus insecticides are derivatives of phosphoric, phosphorothionic, and phosphorothionoic (phosphorodithioic) acids. The organophosphorus compounds are inhibitors of a variety of esterases and it is generally agreed that the toxicity of organophosphorus insecticides is associated with the inhibition of acetylcholine esterase (3.1.1.7) which catalyses the hydrolysis of the neurotransmitter acetylcholine at the post-synaptic membrane of cholinergic synapses. The inhibition of acetylcholine esterase by organophosphorus compounds is 'irreversible' and is due to phosphorylation of the esteratic site of the enzyme by the organophosphate acting as a phosphoryl donor (Corbett, 1974).

1. METABOLISM OF ORGANOPHOSPHORUS INSECTICIDES

Many organophosphorus insecticides are highly lipophilic molecules and are able to penetrate readily through the integument of animals. Biotransformation within the organism generally proceeds in a direction in which the molecule becomes more polar and more water-soluble. Such biological modifications of organophosphorus insecticides result in great changes in the toxicity of the compounds. Many of the common phosphorodithioate triester insecticides are poor inhibitors of acetylcholine esterase in the pure state; their activity as effective anticholine esterase in vivo is the net result of the competing biochemical processes of activation and detoxication. The term 'activation' is used to describe the metabolic conversion of compounds with little or no intrinsic biological activity into one or more active or toxic metabolites. Detoxication, or degradation, is far the major route of organophosphate ester metabolism, and activation itself can be regarded as a process resulting finally in degradation (Lykken and Casida, 1969). Even if biotransformation occurs to a very small extent, it can be important from the toxicological point of view. The principal routes and mechanisms by which foreign compounds, including the synthetic insecticides, are metabolised in animals and micro-organisms have been reviewed in detail (Hathaway, 1975; Wilkinson and Brattsten, 1973; Matsumura, 1975).

1.1 The Metabolism of Phosphorodithioate Insecticides

Phorate (diethyl S-(ethylthiomethyl) phosphorothiolothioate) (I), the insecticide used in the present study, is a plant-systemic insecticide which is widely used as a soil-applied granular formulation.



(I)

It is useful not only as a general systemic insecticide, but also as a pre-planting treatment for seeds of alfalfa, cotton, many vegetables, peanuts, and potatoes (Gunther and Jeppson, 1960). Phorate is slightly soluble in water (19 ppm at 25°C) (Lord and Burt, 1964), and completely miscible with most organic solvents.

Phorate belongs to a group of phosphorothiolothiate insecticides including disulfoton, demeton, demeton-s-methyl, and terbuphos, which have short alkyl side chains, one of which contains a thioether linkage. These compounds are all plant-systemic insecticides, due partly to their facile metabolic conversion to a range of more water-soluble toxic metabolites, and they all have uses as soil insecticides. Since the parent compound is, in each case, a poor inhibitor of acetylcholine esterase, the persistence of toxicity in the soil after application depends as much on the stability of the metabolites to hydrolysis or other chemical alterations and to leaching through the soil, as on the stability of the applied insecticide. Phorate, and structurally related compounds, are susceptible to oxidative desulphuration, thioether oxidation and hydrolysis.

1.1.1 Oxidative desulphuration: Phosphorothioate and phosphorodithioate triesters are poor inhibitors of choline esterases, unless the compounds are oxidatively desulphurated (Heath, 1961; O'Brien, 1967). Phorate is susceptible to oxidative desulphuration of the thiophosphoryl sulphur atom to form the oxygen analogue of phorate, phoratoxon. Phoratoxon, and its sulfoxide and sulphone, have been reported to be more susceptible to hydrolysis at or near neutral pH than the corresponding phorate derivatives (Faust and Gomaa, 1972). They are more potent inhibitors of acetylcholine esterase derived from a number of sources (Bowman and Casida, 1957; Metcalf et al, 1957; Archer et al, 1963; Wright, 1976) and are much more water-soluble (le Patourel and Wright, 1976; Bowman, 1973). Oxidative desulphuration in living tissue may be catalysed, in part, by microsomal oxidases (Nakatsugawa et al, 1968) but the reaction has also been reported to occur in the absence of biological catalysts (Dauterman, 1971). Dauterman et al (1960) concluded that the formation of the oxygen analogue of dimethoate on the surfaces of potato, cotton and corn was non-enzymic, as it could be duplicated by atmospheric exposure of thin films of the compounds on glass plates.

1.1.2 Thioether oxidation: Both phorate and its oxygen analogue (phoratoxon) are susceptible to thioether

oxidation to their corresponding sulfoxide and sulphone (Bowman and Casida, 1957; Metcalf et al, 1957). Although microsomal oxidases may catalyse this metabolic conversion in mammals (Hutson, 1975), there is much evidence to suggest that the reaction may have a non-biological mechanism in other situations (Crosby, 1969). Thioether groups in insecticidal organophosphorus compounds were photochemically oxidised to sulfoxides and sulphones by ultra-violet irradiation (Mitchell et al, 1968) and gamma radiation converted phorate and disulphoton to more stable and potent inhibitors of acetylcholine esterase (Grant et al, 1969). Light and heat both increased the rate of oxidation of 0,0 diethyl 0-p-methyl sulphinyl phenyl phosphorothionate to its sulphone, when the compound was exposed on glass surfaces or on cotton leaves (Benjamini et al, 1959).

- 1.1.3 Hydrolysis: Organophosphorus triesters are all susceptible to hydrolysis. The hydrolysis rates of organophosphorus insecticides and their metabolites are of great importance, because this reaction results in the detoxication of insecticides. The hydrolysis rate is dependent upon the chemical structure of the organophosphorus compound and upon the reaction conditions, such as pH, temperature and the presence of certain catalysts (Eto, 1974; Fest and Schmidt, 1973; Heath, 1961). In general, the rate of

hydrolysis is lowest between pH 2 and pH 5, and increases under more acidic or alkaline conditions. Some metal ions, either free or complexed, and a number of low molecular weight organic compounds are known to catalyse hydrolytic reactions of this type. Ketelaar et al (1956) reported that at pH 7.8-8.6 the catalysis of hydrolysis by Cu(II) is more effective on phosphorothionate triesters than on their oxygen analogues. Mortland and Raman (1967) reported that at pH 5-6 Cu(II) ions catalysed the hydrolysis of dursban, diazinon, and ronnel. They concluded that the catalytic effect of Cu(II) in these reactions was dependent upon the concentration of the free metal ion; it was relatively inactive when present on ion exchange resins, which bind it tightly.

In general, oxidation of the thioether side chain of compounds similar to phorate tends to increase the stability of the product to hydrolysis (Eto, 1974). The half-life of dementon-s-methyl at 20°C (pH 1-5) was found to be 88 days, while that of its sulfoxide and sulphone was 236 days (Muhlmann and Schrader, 1957).

- 1.1.4 Oxidised metabolites of phorate: Phorate is a highly lipophilic molecule which partitions from aqueous solutions into living organisms and organic solvents. Oxidised metabolites of phorate differ widely in their polarity but all are more polar than phorate.

Oxygen analogues are, in general, more polar than the corresponding thiophosphates, and sulfoxides are more polar than sulphones which are more polar than the parent sulphides (Bowman, 1973). The differences between the polarities of phorate and its metabolites govern their relative mobilities in thin-layer and liquid chromatographic systems. Several such systems have been developed (Bowman et al, 1969; Grant et al, 1969) which affect complete or partial separation of mixtures of phorate and its metabolites. The differences in the polarities are also important toxicologically in terms of their effect on the rate of metabolism and persistence of phorate in the soils. Table 1 gives the ρ values (fraction of compound partitioning into the non-polar phase of an equivolume two phase system) for phorate and its oxidised metabolites in different two phase systems: chloroform-water (Bowman and Casida, 1957), hexane-water, benzene-water (Bowman et al, 1969) and olive oil-water (le Patourel and Wright, 1976).

Table 1 : ρ Values for Phorate and its Metabolites

Compound	Chloroform/water	Hexane/water	Benzene/water	Olive oil/water
P = S, S	1.00	1.00	-	0.98
P = S, SO	1.00	0.54	-	0.39
P = S, SO ₂	1.00	0.98	-	0.88
P = O, S	1.00	0.89	-	-
P = O, SO	1.00	0.00	0.21	0.02
P = O, SO ₂	0.99	0.01	0.78	0.18

2. THE METABOLISM OF PESTICIDES IN THE SOIL

Pesticides may reach the soil in a variety of ways. They may be applied to the surface or mixed with or injected into the soil. Moreover, pesticides even when not applied directly to soil eventually find their way into soil through 'fall out' during and after application to crops (Lichtenstein, 1960).

The metabolism of insecticides in the soil is of importance not only with respect to their biological effectiveness, but also because of long term hazards associated with the accumulation of persistent residues in the soil and the danger of leaching of toxic materials into natural waters. The subject has been reviewed both mechanistically (Hamaker, 1972) and in terms of the biological implications of the many processes occurring in the soil (Edwards, 1964, 1972, 1973; Alexander, 1969; Matsumura and Bush, 1972; Kaufman, 1974; Wright, 1974).

Since the work described in this thesis concerns phorate, an organophosphorus insecticide, particular emphasis will be given to this group of compounds, other pesticides being mentioned only when appropriate.

2.1 Factors Affecting the Metabolism of Pesticides in the Soil

Soil is a complex of diverse mineral materials, intimately combined with organic matter and humic compounds. It contains a vast range of living organisms all competing with one another for food and living space. The rate of insecticide metabolism in the soil and the metabolic pathways involved are dependent upon the chemical and physical properties of the insecticide itself. Characteristics such as water solubility and polarity, which in turn influence the lipophilic nature of the compound as well as volatility and chemical reactivity, are especially important. The physical, chemical and microbiological

properties of the soil also affect the persistence and metabolism of insecticides in the soil.

Micro-organisms have been reported to play an important part in the breakdown of pesticides in the soil (Edwards, 1973). The comparison of the rate of disappearance of the compound from sterilised and non-sterilised soils is the most common way of studying the role of micro-organisms in pesticide breakdown. The sterility of the soil thus must be considered carefully. In laboratory studies, soil can be completely sterilised by steam or dry heat and by gamma irradiation. A number of chemical compounds have been used to partially sterilise soil, so that there is selective destruction of microbial populations. These chemicals differ in their action on the microbial population in the soil. Some of them are volatile compounds, e.g. carbon disulphide, formaldehyde, halogenated hydrocarbons and ethylene oxide; these compounds act as soil fumigants (Allison, 1967). Non-volatile compounds, e.g. sodium and potassium azide, calcium hypochlorite, mercuric chloride and sodium propionate, have found uses as partial sterilising agents (Elkan and Moore, 1960). Antibiotics have been reported to have an inhibitory effect upon micro-organisms, mainly fungi and bacteria, in the soil (Wain and Carter, 1972).

The effect of different methods of sterilisation on the chemical and physical properties of soil may influence the degradation of pesticides in the sterilised soil. Waksman and Starky (1923) reported that steam and chemical sterilisation made soil organic matter more available for decomposition by micro-organisms. Malowany and Newton (1947) found that steam sterilised soils often had a lower water-holding capacity than unsterilised soils. Van Bavel (1950) reported that steam sterilisation increased the soil aggregation. Aldrich and Martin (1952)

concluded that steam sterilisation and fumigation with D-D, chloropicrin and carbon disulphide produced substantial increases in the amounts of Ca^{++} , Mg^{++} , K^+ and Mn^{++} in extracts of treated soil. Peterson (1962) reported that sterilisation by either electron beam or X-rays did not affect soil respiration, and he suggested that the respiratory enzymes are contained within dead microbial cells, and that inhibition of enzyme synthesis is a more important effect of radiation than inactivation of enzymes already present in the cells. Lichtenstein and Schulz (1964) reported that soil sterilisation by autoclaving for three hour periods for five consecutive days was incomplete, and that while microbial activity was kept to a minimum, it could still be demonstrated.

Lichtenstein et al (1968) reported that sodium azide used as a sterilising agent retarded the degradation of parathion, but caused a complete disappearance of diazinon during 2 weeks of incubation. The effect of sodium azide on the stability of diazinon in soil apparently was not directly related to the microbial activity, since the reagent catalysed the degradation of diazinon in water. Getzin and Rosefield (1968) studied the persistence of several organophosphorus insecticides in non-sterilised soil and in sterilised soils by autoclaving or irradiation. They found that malathion was rapidly decomposed in non-sterile and irradiated soils, while it persisted in autoclaved soils. Zinophos also degraded more rapidly in irradiated soils than in autoclaved soils, while diazinon showed no differences in the rate of degradation between treatments. They attributed the difference between the amount of each insecticide decomposed in irradiated soil and in autoclaved soil to heat-labile substances which they were able to extract with 0.2 M sodium hydroxide. The heat-labile water-soluble material accelerated the degradation of malathion from non-autoclaved and irradiated

soils. They suggested that autoclaving may cause the destruction of water-soluble heat-labile materials in the soil. The same authors (1971) reported the isolation of a soil enzyme responsible for degrading malathion. The enzyme was extracted from non-irradiated and irradiated soils, but four to five times more activity was recovered from non-irradiated soil than from irradiated soil. The heat-labile enzyme catalysed the hydrolysis of malathion to its mono-acid. This malathion esterase isolated from chehalis clay loam was unusually stable and possessed many of the characteristics necessary for prolonged activity in a cell-free state in soil.

2.2 The Behaviour and Metabolism of Phorate in the Soil

The metabolites produced in the soil after application of phorate under field conditions have been studied by a number of workers. Getzin and Chapman (1960) reported that both under laboratory and field conditions, the only phorate metabolites formed in a number of soils were the sulphoxide and sulphone derivatives. In quartz sand, phorate was rapidly hydrolysed and no oxidised metabolites were formed. These authors suggested that phorate oxidation was due to air oxidation, microbial and possibly soil-catalysed processes. Bache and Lisk (1966), Lichtenstein (1966) and Suetz (1971, 1974, 1975) all reported finding only the sulphoxide and sulphone metabolites in soil treated with phorate in the laboratory or in the field. These findings do not preclude the formation of phorate oxygen analogue metabolites in the soil, either below the detection limits of the analytical procedure or as transitory intermediates, rapidly hydrolysed in the soil. In a recent study, Getzin and Shanks (1970) reported the presence of all five oxidised metabolites of phorate in a silt loam soil, but while the oxygen analogue

metabolites were no longer detectable after eight days, phorate and its sulphoxide and sulphone persisted in the soil for over sixteen weeks. These authors also reported a reduction of phorate sulphoxide back to phorate in a muck soil, the only example to date of this metabolic route.

There is some evidence to suggest that the rates and routes of phorate metabolism in the soil are dependent on the formulation and application techniques. This is partly due to the high vapour pressure of phorate (8.4×10^{-3} mm Hg at 20°C) making evaporation a major route of phorate loss from the soil under field conditions. Patterson and Rawlins (1966) reported that the rate of phorate disappearance from soil in the field increased with soil temperature, although soil moisture was not a major factor affecting phorate degradation when phorate was applied in granular form. Similar results were obtained by Etheridge and Graham Bryce (1970). Lichtenstein et al (1973) reported that when phorate was applied as a granular formulation at 10 lb acre^{-1} , it was least persistent when applied solely to the soil surface, while when it was mixed with 4-5 inches of top soil, it persisted considerably longer. The highest amounts of phorate sulphoxide were recovered in both cases six days after treatment, after which the level of this metabolite declined rapidly; phorate sulphone levels increased until the unhydrolysed residues consisted entirely of phorate sulphone two months after initial phorate application.

Schulz et al (1973) reported that the amount of phorate applied to the field affected the rate of its metabolism in the soil. A 10% granular formulation was more rapidly metabolised when applied at 5 lb acre^{-1} than at 10 lb acre^{-1} .

Since the rate of leaching of unionised organic chemicals through the soil is largely a function of their water solubility or

water/soil organic matter partition coefficient (Briggs, 1973), it is to be expected that the extent to which the toxicity associated with a phorate application to the soil persists at any depth will be associated with both the rate of its metabolism and the nature of the metabolites produced. This aspect of phorate metabolism in the soil has been investigated by Lichtenstein et al (1974). Working under simulated field conditions with continual leaching of the soil, these workers found only the more water-soluble phorate sulfoxide and sulphone in the lower soil layers after application of phorate to the soil surface. Phorate itself only moved appreciably in non-adsorptive quartz sand.

2.3 Metabolism of Other Organophosphorus Pesticides in the Soil

Organophosphorus pesticides are known to be degraded by both biologically-catalysed and by purely chemical processes in the environment. Several approaches have been used to distinguish the nature of the degradation of these pesticides in the soil. Most interest has been focussed on micro-organisms (algae, fungi and bacteria) although small arthropods (nematoda, insecta, etc.), may contribute a significant fraction to the total biomass in the soil.

As a pesticide is typically an unnatural substrate, only a few species of micro-organisms, if any, will be able to utilise it as a growth substrate. However, chemical alteration of pesticides by micro-organisms may occur by non-specific routes incidental to the main pathways of metabolism, when the micro-organisms are growing under normal conditions in the presence of adequate energy sources.

Since Audus (1949, 1950, 1951, 1952) demonstrated a lag period preceding the metabolism of phenoxyacetic acids in the soil and correlated it with the build-up of effective populations of micro-

organisms competent to degrade the herbicides, the presence of such induction periods prior to pesticide degradation in the soil has been taken as evidence of microbial involvement in the degradation process. In many cases, such a lag period is not found; Konrad et al (1967) concluded that the degradation of diazinon in the soil was catalysed by adsorption onto clays. Due to the rapid rate of the chemically-catalysed hydrolysis and the apparently slow adaption of diazinon-degrading micro-organisms, it was concluded that chemical hydrolysis would be complete in many soils before microbial degradation could occur to any significant extent. Similarly, Konrad et al (1969) found that the rate of malathion degradation in the soil followed first-order kinetics with no lag period under controlled temperature and humidity, and concluded that degradation was chemical rather than biological. However, in aqueous extracts of the same soil, the pattern of degradation included an initial seven day lag period followed by rapid malathion hydrolysis due to microbial metabolism. Yaron et al (1974) reported that azinphos-methyl losses followed first-order kinetics, but included an initial lag period in both sterile and unsterile soil. The lag period phenomenon cannot always therefore be used to distinguish between purely chemical processes and biological metabolism in the soil, and the metabolism of organophosphorus compounds by microbial populations in the soil is likely to be greatly influenced by the interaction between the micro-organisms and the soil complex.

The difficulty of interpreting differences in rate of degradation of pesticides in non-sterile and in chemically or physically sterilised soils has already been discussed. Walker and Stojanovic (1973) reported that malathion disappeared from natural soil more rapidly than from soil sterilised by a number of techniques and concluded that the

degradation was accelerated by the microbial population in the soil, to an extent related to the initial population level and to the rate of growth during the incubation period. Konrad and Chesters (1969) reported that the rate of ciodrin degradation in soils followed first-order kinetics and was related to the extent of initial adsorption by the soil. While the rate of degradation was slowed in soil sterilised by gamma irradiation, this decrease was concluded to be due to both the decrease in the extent of ciodrin adsorption resulting from irradiation treatment and to retardation of microbial processes.

If production of certain metabolites of chemicals is characteristic of microbial rather than chemical degradation, then their production in unsterile but not in sterilised soils may be taken as evidence for microbial involvement in the degradation process. This approach has been adopted by Lichtenstein and Schulz (1964), who studied the metabolism of parathion in a Carrington silt loam. Parathion metabolism was found to proceed either by reduction to amino-parathion or by hydrolysis. In steam-sterilised soil, parathion persisted for long periods and no amino-parathion was produced. In non-sterile soils, amino-parathion accumulated and its production could be associated with metabolism by a yeast species in the soil. Similar results were obtained by Sacher et al (1972) using methyl bromide as a sterilant. These authors also suggested that the effect of soil moisture on parathion degradation was secondary, and that the moisture level controlled the activity of the microbial populations degrading the parathion.

An alternative approach to the problem is to investigate the effect of varying the activity of specific micro-organisms in a soil on the rate of pesticide metabolism. The difference in the nature of the microbial populations in flooded and unflooded conditions has been used

in this context. Sethunathan and Yoshida (1973) reported that amino-parathion was only produced in submerged soils because anaerobic organisms and reducing conditions favoured the reduction of the nitro group on parathion. Whether this reduction also took place in sterilised flooded soils was not reported. Sethunathan (1973) showed that hydrolysis of parathion occurred more rapidly in soil pre-treated with parathion under flooded conditions and concluded that the effect was due to enrichment of the soil with the appropriate microbial populations. Sethunathan and Pathak (1972) obtained similar results using diazinon.

3. METABOLISM OF ORGANOPHOSPHORUS INSECTICIDES BY PURE CULTURES OF MICRO-ORGANISMS

Although the role of micro-organisms in metabolising organophosphorus insecticides in the soil may vary greatly depending on soil conditions and the stability of the chemical, many workers have shown that under near optimum growth conditions in aqueous cultures many species of micro-organisms may catalyse the chemical processes of hydrolysis and oxidation of these compounds.

The metabolism of phorate emulsions by a yeast (*Torulopsis utilis*), bacteria (*Pseudomonas fluoresceus* and *Tiobacillus thiooxidans*) and an alga (*Chlorella pyrenoidosa*) was studied by Ahmed and Casida (1958). Both the yeast and alga catalysed phorate hydrolysis and oxidation to its sulfoxide, sulphone and oxon sulfoxide. The bacteria catalysed phorate hydrolysis but not oxidation. le Patourel and Wright (1974a) observed increased rates of phorate sulfoxide production from phorate due to proliferation of *Bacillus megaterium* populations in nematode cultures.

In most instances, while micro-organisms isolated from the soil may be shown to degrade organophosphorus compounds in vitro, the relevance of these findings to the degradation of the compounds in the soil remains to be demonstrated. A summary table of micro-organisms which have been shown to catalyse organophosphate metabolism in pure cultures is given (Table 2). The metabolism of organophosphorus insecticides by pure cultures of micro-organisms has been reviewed by Ware and Roan (1970).

Table 2 : Microbial Degradation of Some Organophosphorus Insecticides by Pure Cultures

Reference	Species	Type*	Compound	Nature of Reaction Catalysed	Isolation by Enrichment Procedures	Growth Media pH
Mick and Dahm (1970)	<i>Rhizobium japonicum</i> <i>Rhizobium melitoti</i>	F F	{Parathion	{Nitro reduction, hydrolysis	No No	7.0 7.0
Siddrapmma <u>et al</u> (1973)	<i>Bacillus</i> Sp. <i>Pseudomonas</i> Sp.	B B	{Parathion	{Hydrolysis, then nitrite liberation	Yes Yes	7.1 7.1
Munnecke and Hsieh (1974)	Mixed microbial culture <i>Pseudomonas</i> Sp.	- B	Parathion P-nitrophenol	Hydrolysis {Production of nitrite and hydroquinone	Yes Yes	7.2 7.2
Malesjewska (1974)	<i>Bacillus cereus</i>	B	Methyl parathion	-	No	-
Matsumura and Boush (1966)	<i>Trichoderma viride</i> <i>Pseudomonas</i> Sp.	F B	{Malathion	{Side chain hydrolysis and demethylation	Yes Yes	7.0 7.0
Mostafa <u>et al</u> (1972)	<i>Pencillium notalum</i> <i>Asperigillus niger</i> <i>Rhizoctonia solani</i>	F F B	{Malathion	Hydrolysis {Hydrolysis and oxon formation Oxon formation	No No No	5.0 5.0 5.0
Paris <u>et al</u> (1975)	Mixed bacterial population	B	Malation	Side chain hydrolysis	Yes	6.8

* B = bacterium; F = fungus

4. ADSORPTION OF ORGANOPHOSPHORUS INSECTICIDES ONTO SOIL

The adsorption of pesticides onto natural soils has been reviewed by Bailey and White (1964, 1970), Hance (1970), Osgerby (1970) and Hamaker and Thompson (1972).

4.1 Adsorption Isotherms

The heterogeneity of soil as an adsorptive material and the ill-defined nature of the adsorptive sites on its surface makes the use of theoretically-based treatment of the relationship between surface concentration of a pesticide on the soil and its solution concentration in the soil-water inappropriate. The Langmuir adsorption isotherm (Langmuir, 1916) is based on a theoretical treatment of the adsorption of gases onto solids, and is not generally applicable to adsorption from solution, nor onto adsorptive surfaces as complex as that of soil. The empirical approach of Freundlich (Freundlich, 1926) in which the surface concentration of adsorbate is related to the solution concentration at equilibrium by a simple power law relationship, has found some use in soil adsorption studies (Hamaker and Thompson, 1972). The Freundlich isotherm is written:

$$x/m = K c_{eq}^{1/n}$$

where x/m represents the mass of adsorbed material (x) in μg per unit mass of soil (m) in grams; C_{eq} represents the equilibrium concentration of material in solution (ppm); and K and n are empirical constants at a defined temperature.

Tables of experimental values for the constants K and n have been collated for a variety of different pesticidal chemicals on various soil types (Hamaker and Thompson, 1972).

A summary of the available data on soil adsorption by organophosphorus compounds is shown in Table 3 . It can be seen that for these organophosphorus triesters, which are unionised and have low water solubilities, the constant 'n' lies very close to unity in a variety of soil types, so that a nearly linear relationship between soil surface concentration and soil solution concentration exists over the concentration ranges tested. No simple correlation has been noted between the physical or chemical properties of this type of compound and the adsorption constant 'K', for a single soil, but Hamaker and Thompson (1972) suggest that hydrophobic forces play an important part in determining the extent to which such molecules are excluded from water and forced into association with each other and with hydrophobic components of soil such as humins. It is therefore of interest that in a study of the adsorption of a number of organophosphorus compounds onto four natural soils, King and McCarty (1968) found that the less water-soluble compound of two pairs of structural analogues, phorate/disulphoton and parathion/methyl parathion, had a consistently higher K value for each soil tested. However, there are many differing types of adsorption site within the soil complex, varying from strongly acidic cationic exchange sites on the clay minerals through to highly lipophilic centres within the soil organic matter. Specific interactions between the pesticide molecule and these sites may play an active part in determining the extent of the adsorption process. Organophosphorus pesticides contain 'polar' phosphoric ester groups and relatively non-polar side chains which will have affinities for differing types of adsorption site. The work of Yaron and Saltzman (1972) suggests that parathion may compete with water for some adsorption sites in soil. In a dry soil/parathion/hexane system, the 'slightly polar' parathion molecules competed efficiently with non-polar hexane molecules

Table 3 : Adsorption of Some Organophosphorus Insecticides
by Soils with Different Organic Carbon Content

Soil	Organic Carbon %	Compound	k*	n*	k' _{oc} [†]	Reference
Hugo	0.22	Phorate	3.18	0.93	1445.5	King and McCarty (1968)
Elkhorn	0.15	"	5.97	0.94	3980.0	
Sweeney	1.12	"	12.8	1.01	1142.9	
Tierra	0.58	"	15.5	1.00	2672.4	
Hugo	0.22	Disulphoton	4.92	0.93	2336.4	
Elkhorn	0.15	"	7.59	0.94	5060.0	
Sweeney	1.12	"	13.2	0.98	1178.6	
Tierra	0.58	"	26.1	1.05	4500.0	
Hugo	0.22	Methyl	6.73	1.04	3059.1	
Elkhorn	0.15	Parathion	10.4	1.04	6933.3	
Sweeney	1.12	"	15.9	1.03	693.0	
Tierra	0.58	"	20.3	1.04	3500.0	
Hugo	0.22	Parathion	7.4	0.99	3363.6	
Elkhorn	0.15	"	13.8	1.04	9200.0	
Sweeney	1.12	"	19.3	1.02	1723.2	
Tierra	0.58	"	26.8	1.06	4620.6	
Hugo	0.22	Ethion	18.8	0.98	8545.4	Graham-Bryce (1967)
Elkhorn	0.15	"	30.6	0.99	20400.0	
Sweeney	1.12	"	57.2	0.91	5107.1	
Tierra	0.58	"	76.2	0.9	13137.9	
Hugo	0.22	Trithion	62.8	1.00	28545.4	
Elkhorn	0.15	"	82.3	1.00	54866.0	
Sweeney	1.12	"	125.0	0.88	11160.7	
Tierra	0.58	"	226.0	0.88	38965.5	
Isleham I	7.6	Disulphoton	49.1	0.87	646.03	
Bottisham	8.8	"	58.6	0.97	646.0	
Spinney	12.0	"	95.7	0.8	797.5	
Prickwillow	15.0	"	100.5	1.00	670.0	
Peacock	11.0	"	70.3	0.94	639.1	
Adventurers	31.0	"	134.6	0.95	434.2	

* Freundlich adsorption isotherm constants

[†] $k'_{oc} = (k \times 100)/\%$ organic carbon

for adsorption sites, but in hydrated soil/parathion/hexane systems there was no soil adsorption of parathion. These workers concluded that increasing the water content of the soil decreased the soil adsorption constant for parathion, but this conclusion is at variance with other work using herbicides (Grover and Hance, 1970; Green and Obien, 1969; Nearpass, 1967) in which no change, or an increase in K with increasing soil water content, was observed.

4.2 Effects of Soil Components on Adsorption

The important variables of soil structure which determine the extent to which a soil adsorbs an organic molecule are the organic matter content and, especially if the molecule contains ionizable groups, the clay content and its ion exchange capacity (Bailey and White, 1964, 1970).

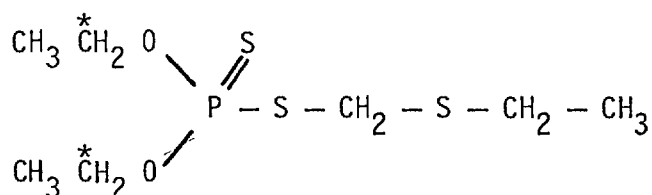
It is evident that although soil organic matter has a much greater adsorptive capacity for organophosphorus compounds than clay due to its hydrophobic nature and high specific surface area (Saltzman et al, 1972), in some soils a high clay content and low organic matter content may make clay the dominant adsorptive component. The nature of the clay minerals in the clay fraction of a soil may strongly influence its physical and chemical properties which, in turn, influence its interactions with pesticides. Saltzman and Yaron (1971) showed that adsorption of parathion from aqueous solution was 25 times greater onto sodium montmorillonite than onto sodium kaolinite, roughly in proportion to the cation exchange capacity and also the specific surface area of the two minerals. Working with very dilute aqueous solutions (< 1 ppm), King and McCarty (1966, 1968) showed that the adsorption constant 'K' of six organophosphorus pesticides on four soils was correlated with the clay

content rather than with the organic matter of the soils (Table 3). The four soils chosen all had low organic matter content, but had clay contents of up to 37% by weight. MacNamara and Toth (1970) studied the adsorption of malathion onto pure clay minerals and onto humic acid. Adsorption onto clay was correlated with the cation exchange capacity of the clay, but the extent of adsorption onto humic acid was several times greater than that onto any of the clay systems studied. Similarly, Graham-Bryce (1967) studied a single organophosphorus compound, disulphoton, in 17 different natural soils with a wide range of organic matter and clay content, and found the value of K to be closely related to the amount of organic matter in the soil. This latter conclusion has also been drawn by other investigators working with a variety of different chemicals (Furmidge and Osgerby, 1967; Lambert, 1968; MacNamara and Toth, 1970) and has lead to attempts to calculate a soil adsorption constant (K_{oc}) on the basis of organic carbon content ($K_{oc} = (K \times 100)/\%$ organic carbon). The data in Table 3 show that K_{oc} is too variable for organophosphorus compounds in soils of different organic matter content for it to be a useful index of 'absorbability onto organic matter'. The organic component of soil may vary in nature and is not the only adsorptive component of soil for different chemicals (Shin et al, 1970; MacNamara and Toth, 1970; Hilton and Yuen, 1963). The biologically-active organophosphorus pesticides are unionised and are not therefore strongly bound at ion-exchange sites within the soil structure. However, Leenheer and Ahlrichs (1971) investigated the effect of varying the cationic component of soil organic matter on its adsorptive capacity for parathion. They found that soil organic matter with H^+ on the exchange sites adsorbed significantly larger amounts of parathion than with Ca^{++} on the exchange sites. A similar phenomenon

was observed using synthetic organic cation exchange resins. It was suggested that the H^+ form of soil organic matter was more hydrophobic than the Ca^{++} form, accounting for the observed differences in parathion adsorption. Parathion was found to be more strongly adsorbed by acid than by neutral or alkaline soils owing to the decreasing competition of water for the adsorption sites as the pH drops.

MATERIAL AND METHODS

1. INSECTICIDE



Phorate "Thimet" (I)

(Diethyl S-(ethylthiomethyl) phosphorothiolothioate)

(Asterisks indicate positions of labelling with ^{14}C)

Ethoxy 1- ^{14}C phorate (I) was obtained from the Radiochemical Centre, Amersham, at a specific activity of 1.9 mCi/mM. Aliquots of the stock solution of the radioactive chemical in benzene were diluted with non-radioactive phorate (Analytical Grade, > 99.7% pure, supplied by Cyanamid Company of Great Britain), to a final specific activity of 11.7 $\mu\text{Ci}/\text{mM}$ (0.045 $\mu\text{Ci}/\text{mg}$). The diluted radioactive phorate was analysed by thin-layer chromatography (tlc) on silica gel plates using chloroform:methanol (39:1 v/v), and found to be free of detectable levels of the five oxidised metabolites.

2. ASHURST SOIL

The soil for laboratory studies was obtained from random sites representative of an uncultivated plot (Ashurst field, Imperial College Field Station, Sunninghill, Ascot, Berkshire). This field was known to have been uncultivated for six years before this work started. During that period, no pesticides were applied or sprayed onto the soil. The soil was removed from the surface by trowel at depths of 4-10 inches. Lumps were broken up into smaller pieces, and mixed thoroughly by shaking. The soil was air-dried on aluminium trays and then ground in a mortar. It was sieved to a mesh size of 2 mm or smaller, and stored in an air-tight jar for the period of the study.

2.1 Classification of the Soil

2.1.1 pH determination: Air-dried soil (20 g) was placed in a 250 cm³ round-bottomed flask and distilled water (CO₂ free, 100 cm³) was added. The mixture was shaken (5 minutes) and transferred to a beaker. The pH was measured with a glass electrode standardised against hydrogen potassium phthalate (Piper, 1947). Soil pH was found to be 6.44 ± 0.14 . This pH value is the mean of five replicates $\pm$ S.D.

2.1.2 Water content of air-dried soil: Samples of air-dried soil (approximately 5 g) were weighed into weighing bottles. The soil was dried in an oven at 105°C to constant weight. The water content was calculated from the difference between the weights of the soil before and after drying and the mean of five replicates was taken. The results were expressed as the percentage of the weight of oven-dried soil (Piper,

1947). Water content of the air-dried soil was found to be $2.15 \pm 0.09\%$

2.1.3 Field capacity: The field capacity of a soil is usually defined as "the amount of water held in the soil after the excess gravitational water has drained away and after the rate of downward movement of water has materially decreased". Thus, field capacity represents the water content of soil at a certain location in the profile at a certain time after wetting. The determination of field capacity therefore must be made in the field (Veihmeyer and Hendrickson, 1931).

Three different sites at Ashurst field (A, B and C) were chosen for field capacity determination. These sites were covered with polyethylene sheets to prevent evaporation or further wetting immediately after the soil was saturated with rainfall. The soil was allowed to drain for 2 days, then samples of soil (50-70 g) were taken at various depths (3-18 inches) and the moisture content determined in each sample by drying in an oven at 105°C to constant weight. Field capacity was expressed as moisture content of oven-dried soil (Peters, 1965).

Table 4 summarises the moisture content percentage at various depths for the three sites (A, B and C) chosen for field capacity determination.

Table 4: Moisture content (% w/w) of Ashurst
soil at various depths

Depth (inches)	Moisture Content Percentage At Site		
	A	B	C
3- 6	15.55	14.23	19.50
6- 9	27.02	23.64	22.00
9-12	26.44	25.47	24.01
12-15	26.86	22.03	21.43
15-18	19.25	18.78	14.01

Field capacity = $21.35 \pm 4.4\%$. Field capacity was calculated as the mean of 15 samples $\pm$ S.D.

2.1.4 Particle size analysis: Particle size analysis for a sample of Ashurst soil was carried out by the Soil Science Department, Reading University. The analysis was accomplished by the pipette method, and sieving method for sand fractions, without eliminating CaCO_3 . Sodium hexametaphosphate was used as the dispersion agent (Day, 1965). The scheme adopted by the International Society of Soil Science (1929) was used in classifying and naming the different fractions.

Table 5 summarises the results of particle analysis - each fraction being expressed as the percentage of the weight of oven-dried soil.

Table 5: Particle size analysis of
Ashurst soil

Constituent	% by weight
Sand fraction ($>1000\mu$)	1.7
Sand fraction ($> 500\mu$)	3.3
Sand fraction ($> 250\mu$)	19.3
Sand fraction ($> 100\mu$)	34.0
Sand fraction ($> 50\mu$)	17.0
Silt fraction ($50-2\mu$)	8.9
Clay fraction ($< 2\mu$)	15.8

These results indicate that Ashurst field soil can be classified as a loamy-sand soil.

2.1.5 Organic matter determination: The organic matter content of a sample of Ashurst soil was determined by the Soil Science Department, Reading University, using the Walkley-Black wet oxidation procedure described by Piper (1947). This method gives a recovery of 81.2% organic carbon with a standard deviation of 1.5%. To obtain a value for organic matter, the correction factor 1.232 was first applied and then the value was multiplied by the conventional factor 1.724 for mineral soil as suggested by Waksman and Hutchings (1935). The organic carbon, according to the Walkley-Black method, was found to be 2.22%. The organic matter (calculated) was 4.725%.

2.2 Soil pH Adjustments

Soil pH was artificially adjusted to pH 3 and pH 9 for experiments on the effect of pH on phorate metabolism. Dilute solutions of hydrochloric acid (0.1 N) and sodium hydroxide (0.1 N) were used to alter the soil pH (Lavy, 1975). To adjust the soil pH, a soil sample (10 g) was placed in a 100 cm³ round-bottomed flask and distilled water (50 cm³) was added. The mixture was shaken and transferred to a beaker. The pH was measured with a glass electrode and the pH was adjusted to pH 3 by adding increasing amounts of dilute hydrochloric acid solution.

2.3 Soil Sterilisation

Samples of soil (10 g) were placed in Erlenmeyer flasks (150 cm³) and moistened to near field capacity (20% w/w). The flasks were plugged with non-absorbent cotton wool and sterilised by autoclaving at 120°C (15 lb pressure) for 2 hours on each of three consecutive days. The sterility was tested by taking a sample of the soil (1 g) aseptically and mixing it with sterilised tap water (9 cm³). Aliquots (0.1 cm³) were mixed thoroughly and inoculated into nutrient agar medium (Oxoid) in petri-dishes (Fey, 1966).

2.4 Soil Inoculation with *Bacillus megaterium*

A sterilised nutrient broth (Oxoid) (100 cm³) in an Erlenmeyer flask (250 cm³) was inoculated with a pure culture of *B. megaterium* from an agar slant (isolated from Ashurst soil by an enrichment technique as described later), and incubated at 25°C on an orbital shaker for 48 hours. Sterile soils were inoculated with 0.1 cm³ of bacteria suspension (approximately 3.72×10^8 cells/cm³) per 10 g of soil aseptically (Rovira and Bowen, 1966).

3. EXPERIMENTAL PROCEDURES FOR THE STUDIES ON PHORATE METABOLISM IN THE SOIL

The general procedures described below were followed in the experiments. Ashurst soil (loamy-sand) was used in all soil experiments. All studies were conducted under laboratory conditions in 150 cm³ Erlenmeyer flasks. Soil moisture content was adjusted to 20% w/w of air-dried soil immediately before treatment (field capacity, 21.35%).

3.1 ¹⁴C Phorate Application to the Soil

Samples of soil (10 g) were placed in Erlenmeyer flasks (150 cm³). The samples of soil were moistened to near field capacity (20% w/w) with distilled water in the case of non-sterilised soils or with sterilised distilled water under aseptic conditions in the case of sterilised soils. Radioactive phorate in acetone solution was applied to the soil in the flasks (using a microlitre syringe) at the rate of 10 ppm of air-dried soil to give an initial soil solution concentration of 50 ppm. The soil was mixed thoroughly with a spatula to obtain an even distribution of phorate and then plugged with non-absorbent cotton wool. Radioactive phorate was applied to sterilised soils in the same way but under aseptic conditions. The flasks were incubated at $25 \pm 1^{\circ}\text{C}$ in the dark until analysed.

3.2 Phorate Extraction from Soil

Mixtures of chloroform:methanol (Suett, 1971) and hexane:acetone (Getzin and Shanks, 1970) have both been used to extract phorate and its oxidised metabolites from various types of soil and plants. In this study the effect of using either a high or a low percentage of the water-miscible solvent in the mixture on the

convenience and efficiency of extraction was investigated. The solvent mixtures used were:

1. Chloroform:methanol (1:1 v/v)
2. Chloroform:methanol (9:1 v/v)
3. Hexane:acetone (1:1 v/v)
4. Hexane:acetone (4:1 v/v)

Two methods were tried for the extraction of phorate and its oxidised metabolites from the soil.

3.2.1 Soxhlet method: Samples of treated soil (10 g) were transferred from the incubation flasks to Soxhlet extraction thimbles (Whatman , 19 × 90 mm). The incubation flask was rinsed with extraction solvent (40 cm³) and the washings added to the extraction apparatus through the thimble. A further 10 cm³ of extraction solvent was added through the condenser, and the soil samples were extracted continuously for 5 hours.

3.2.2 Mechanical shaker method: Solvent mixtures (30 cm³) were added to the phorate-treated soils in their incubation flasks. The flasks were stoppered with rubber bungs covered with aluminium foil and shaken for one hour on a mechanical shaker. The mixtures were filtered through glass wool plugs in glass funnels (10 cm diameter), and the filtrates were collected in conical flasks (100 cm³). The soil samples in the funnels were washed with two successive

portions of extraction solvent (50 cm³) which were collected in the flasks.

Phase separation occurred in solvent mixtures (1) and (3) during both the Soxhlet and mechanical shaker extraction methods, which made clean-up and radioassay more difficult, while the mixtures (2) and (4) gave adequate recovery of phorate from the soil. Since the percentage recovery was approximately the same using both systems, the simpler shaking method was adopted, so that a large number of samples could be extracted simultaneously. The hexane:acetone (4:1 v/v) mixture was used as the extraction solvent in these studies.

Table 6 summarises the effect of different methods and different solvent mixtures on phorate recovery from Ashurst soil.

Table 6: The recovery of phorate from the soil

Method	Solvent mixtures	Recovery $\pm$ S.D.%	n
Soxhlet	Hexane:acetone (8:2 v/v)	76.0 $\pm$ 2.6	5
	Chloroform:methanol (9:1 v/v)	82.4 $\pm$ 3.5	5
Shaking	Hexane:acetone (8:2 v/v)	81.3 $\pm$ 1.7	5
	Chloroform:methanol (9:1 v/v)	82.5 $\pm$ 4.5	5

3.3 Clean-Up

The efficiency of liquid scintillation counting is sensitive to the presence of coloured materials in the sample (Turner, 1971). Since coloured compounds were present in the soil extract, and these compounds were not removed during subsequent chromatographic separation of phorate metabolites, three clean-up procedures were tried.

3.3.1 Vacuum filtration clean-up: The extracts were filtered through a Buchner funnel (9 cm diameter) containing filter paper (Whatman No. 1) on which was spread an even layer of a mixture of activated charcoal (Norit-OL, 3 g) and celite (2 g). The filtrate was dried over anhydrous sodium sulphate (5 g).

3.3.2 Column clean-up: A clean-up procedure similar to that described by Getz (1962) was used. The soil extract was dried over anhydrous sodium sulphate (5 g) and evaporated under reduced pressure to about 0.5 cm³. The residue containing phorate and its metabolites was added to the top of the glass column (30 × 1.2 cm) containing a one inch layer of silica gel followed by a middle layer of a mixture of activated charcoal (3 g) thoroughly mixed with celite (2 g), and then a one inch layer of silicic acid. The column was eluted with acetone under reduced pressure.

3.3.3 Filtration clean-up: A modification of the method described by Krueger et al (1973) was used. The soil extract was filtered through glass wool in a flask containing anhydrous

sodium sulphate (5 g) and activated charcoal (Norit-OL 0.25 g). Preliminary experiments indicated that the total recovery of phorate decreased if the amount of charcoal added was above 0.25 g without any improvement in the removal of coloured material from the extract. The flask was gently swirled for 2 minutes, and then filtered through Whatman No. 1 filter paper. The filtrate was collected in a pear-shaped flask (100 cm³). The filter paper was washed with 5-10 cm³ of solvent mixture, and the washings collected in the same flask.

The procedures described all gave clear, almost colourless, solutions which were suitable for analysis and radioassay. However, the third method of filtration was adopted in this study because of its simplicity.

3.4 Recovery of ¹⁴C Phorate, Phorate Sulphoxide and Phorate Sulphone from the Soil

Samples of soil, with moisture content adjusted to near field capacity (20% w/w), were treated with ¹⁴C phorate, ¹⁴C phorate sulphoxide or ¹⁴C phorate sulphone at room temperature. The soils were immediately extracted with hexane:acetone (4:1 v/v) and the extracts prepared for detection of radioactivity using the standard methods described before. In the case of recovery at low temperature, the soil's moisture content was adjusted to near field capacity (20% w/w) at 25°C and then kept at 5°C for 24 hours before treatment. The soils were treated and worked-up as above.

Table 7 summarises the percentage of recoveries of phorate and its sulfoxide and sulphone at room temperature using the shaking method and hexane:acetone (8:2 v/v) as the extracting solvent mixture.

Compound	Recovery $\pm$ S.D. %	n
Phorate	81.3 $\pm$ 1.7	5
Phorate sulfoxide	61.7 $\pm$ 5.4	5
Phorate sulphone	96.8 $\pm$ 4.6	5

Table 8 summarises the percentage of recoveries of phorate and its sulfoxide and sulphone at $5 \pm 1^{\circ}\text{C}$ using the shaking method and hexane:acetone (8:2 v/v) as the extracting solvent mixture.

Compound	Recovery $\pm$ S.D. %	n
Phorate	89.9 $\pm$ 1.5	5
Phorate sulfoxide	71.8 $\pm$ 2.1	5
Phorate sulphone	92.1 $\pm$ 4.1	5

4. SEPARATION OF PHORATE AND ITS METABOLITES

The column chromatographic method described by le Patourel and Wright (1974b) was used for the separation of phorate, and its sulfoxide and sulphone. The soil extract was evaporated down to about 0.5 cm³ using a rotary film evaporator, and after the addition of benzene (2 cm³), was applied to the top of a chromatographic column (30 × 1.2 cm) containing 6 g of silicic acid (Mallinkdrodt Silicar CC7, 100-200 mesh). The column was successively eluted with benzene (40 cm³) to elute unchanged phorate, benzene:acetone (40 cm³, 19:1 v/v) to elute phorate sulphone, and acetone (40 cm³) to elute phorate sulfoxide. Each eluate was collected in a 50 cm³ pear-shaped flask, concentrated nearly to dryness using a rotary film evaporator, and then transferred to a scintillation vial with 10 cm³ of a solution of butyl PBD (8 g/litre toluene). Separation of the metabolites by this sequence of solvents was tested using mixtures of radioactive phorate, phorate sulfoxide and phorate sulphone. The column was found to give complete separation of phorate (fraction I), phorate sulphone (fraction II) and phorate sulfoxide (fraction III). This separation is shown in Figure 1.

% of radioactivity of phorate or metabolite applied to column

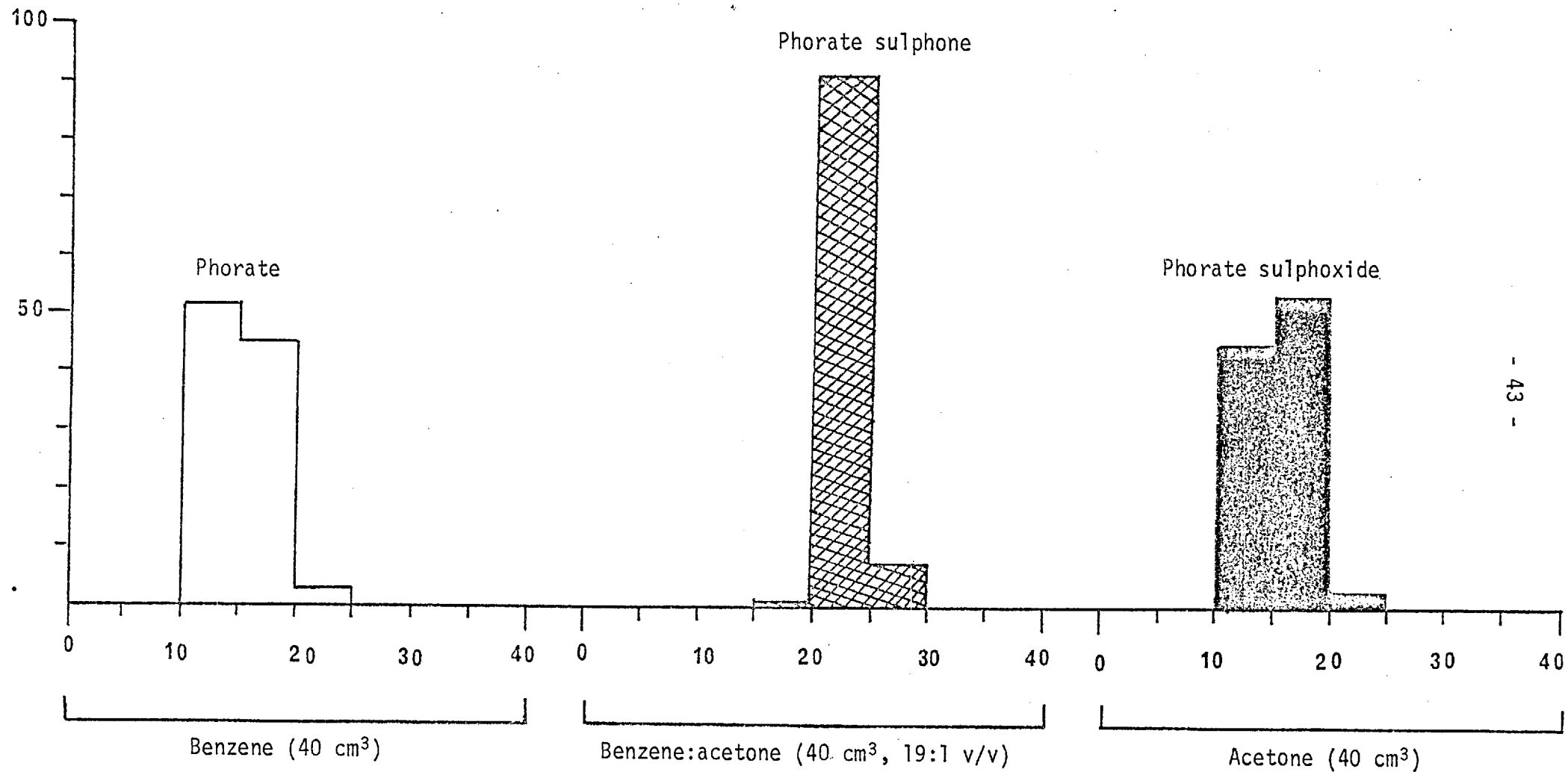


Figure 1: The separation of a mixture of phorate and its sulphoxide and sulphone on silicic acid column chromatography

5. LIQUID SCINTILLATION COUNTING (LSC) OF ^{14}C COMPOUNDS

Radioactive compounds were counted in solution using a Beckman LS250 liquid scintillation counter. The scintillation solutions used were:

1. A toluene based phosphor (10 cm³) consisting of 2-(4'-t-Butyl phenyl)-5-(4''-Biphenyl)-1,3,4-Oxadiazole (Butyl PBD) 8 g/litre (Turner, 1971).
2. A dioxan based phosphor (10 cm³) consisting of 2,5-Diphenyl oxazole (PPO) (4.0 g), 1-4,Bis-(4-Methyl-5-Phenyloxazole-2-Yl) benzene (DM-POPOP) (0.2 g), naphthalene (60 g), methanol (100 cm³), and ethane-1,2-diol (20 cm³) made up to 1 litre with dioxane (Bray's solution) (Bray, 1960).

Standard glass scintillation vials of 22 cm³ capacity with low potassium content were used for counting.

5.1 Efficiency Determination and Quench Correction

The method of the external standard channels ratio was used for correcting efficiency variations caused by sample quenching. This method of standardisation uses the automatic external standard of the instrument, consisting of a compound gamma source of ^{137}Cs which bombards the scintillator solution with gamma rays to generate compton electrons. The only appreciable change in the energy distribution of the spectrum of these electrons for a given scintillator solution is due to the 'quenching' effect of the sample, either because of its colour or chemicals dissolved in it (Neary and Budd, 1970). Samples containing ^{14}C -n-hexadecane of accurately known radioactivity were quenched with increasing amounts of chloroform. The samples were counted and the ratio (recorded pulses

(counts)/calculated radioactive disintegration) for each sample was determined. Calibration curves of external standard channels ratio (s number) versus percentage counting efficiency were plotted, from which the absolute activities of samples could be determined. Background radioactivity was estimated with a blank sample, and was subtracted from all radioactive sample counts.

4.3 Statistics of Radioactivity Measurements

The decay of a radioactive source is a random process. The time at which any one atom will decay cannot be predicted. Hence, there is no 'true' counting rate, and it is only possible to refer to an average or mean counting rate. Statistics may be used in the estimation of errors due to the random nature of radioactivity and the number of counts recorded, in detecting non-random errors, and providing a means of minimising errors. In the measurement of radioactivity, the random nature of radioactivity decay is often the greatest source of error. In practice, the distribution of the data obtained from liquid scintillation counting generate a normal distribution of count values around the true count value or true mean. The precision or reproducibility of individual measurements of the activity of a radioactive sample may be described by a standard deviation of the measurements. The standard deviation σ is:

$$\sigma = \sqrt{\bar{n}}$$

where $\bar{n}$ is the true mean (Thomas, 1967; Wylde, 1970). In a normal distribution, 68.3% of all measured values fall within limits of one standard deviation on either side of the mean. Within two standard deviations, 95.5% of all observations are enclosed.

In this study, all radioactive samples were counted to a 2σ error of 0.5% of the estimated radioactivity of the sample, or for 20 minutes (for high active samples), and for 50 minutes (for low active samples), whichever was the shorter.

6. ANALYSIS OF PHORATE AND ITS METABOLITES BY THIN-LAYER
CHROMATOGRAPHY

Thin-layer chromatographic plates used at the early stages of this work were 20 × 20 cm, 0.25 mm, prepared from a slurry of silica gel G (25 g) and distilled water (50 cm³) using a plate spreader. The plates were allowed to stand for 15 minutes and then activated by heating for one hour at 110°C. The activated plates were stored in a dessicator until used. In later stages of this work, silica gel G plates 20 × 20 cm, 0.25 mm (Merck Darmstadt) were used. The plates were activated before use, and were developed using either chloroform:methanol (39:1 v/v) (Blinn, 1963) or benzene:methanol (19:1 v/v) (Grant et al., 1969). Both these systems resolved the five major oxidised metabolites of phorate but the latter gave more even separation and was used in preference to the former system. The developed plates were sprayed with palladium chloride in hydrochloric acid (1% w/v) (Blinn, 1963). Phorate and its oxidised metabolites could be located as their yellow palladium complexes

7. OXIDATION OF PHORATE TO ITS SULPHOXIDE AND SULPHONE

Two oxidising agents were used to prepare samples of phorate sulphoxide and sulphone.

7.1 Perbenzoic Acid Oxidation of Phorate

The method described by Manuel (1968) using m-chloro-perbenzoic acid was tried. Phorate (10 mg) was dissolved in chloroform (25 cm³) and a 10% solution of m-chloro-perbenzoic acid in chloroform (2 ml) was added. The mixture was allowed to stand for 15 minutes, and then transferred to a separating funnel. It was shaken with a saturated sodium sulphite solution (25 cm³) for 1 minute, and shaken again with a saturated sodium bicarbonate solution for 1 minute. The chloroform layer was separated, dried over anhydrous sodium sulphate, and evaporated to a small volume. Thin-layer chromatographic analysis indicated the presence of large amounts of phoratoxon sulphone and traces of phoratoxon, phoratoxon sulphoxide, and unoxidised phorate.

7.2 Potassium Permanganate Oxidation of Phorate

Potassium permanganate has been used to prepare a series of oxidised metabolites of systox isomers by Fukuto et al (1955). Acid potassium permanganate was used as an oxidising agent in this study. To a solution of ¹⁴C phorate (5 mg) (specific activity of 0.0477 μ Ci/mg) in glacial acetic acid (1 cm³), a solution of potassium permanganate in water (3% w/w) was added dropwise, with shaking, until the colour of the permanganate ions persisted for one minute. The solution was decolourised by dropwise addition of an aqueous solution of sodium bisulphite (5% w/w) and extracted twice with chloroform (5 cm³). The extract was dried over anhydrous sodium sulphate (2 g), evaporated under reduced pressure to a

small volume, and analysed by tlc. The reaction mixture contained phorate sulphoxide (27.2%), phorate sulphone (70.6%), unchanged phorate (0.7%), and unidentified products (1.5%). Phorate sulphoxide and phorate sulphone were separated by column chromatography. The purity of phorate sulphoxide and phorate sulphone were checked quantitatively by tlc and were found to be pure (> 98%).

8. PHORATE, PHORATE SULPHOXIDE AND PHORATE SULPHONE HYDROLYSIS IN AQUEOUS SOLUTIONS

8.1 The Effect of pH on Phorate Hydrolysis

^{14}C phorate was added to buffered water (250 cm³) in Erlenmeyer flasks to a final concentration of 10 ppm (0.038 mM) at three different pH values. The buffers used were $\text{KH}_2\text{PO}_4/\text{Na}_2\text{HPO}_4$, pH 6.4 (ionic strength 0.01), glycine-HCl, pH 3 (0.2 M) and glycine-NaOH, pH 9 (0.2 M) (Dawson et al, 1972). The Erlenmeyer flasks were stoppered and incubated at 25°C.

Three replicates of each aqueous solution of phorate were extracted at 24 hour intervals three times with equal volumes of chloroform, then chloroform extracts were dried over anhydrous sodium sulphate, evaporated and assayed for radioactivity.

8.2 The Effect of Temperature on Phorate Hydrolysis

A similar set of flasks to those above using only phosphate buffer, pH 6.4, were incubated at 5°C. Phorate was extracted every 7 days and assayed for radioactivity as described above.

8.3 The Hydrolysis of Phorate Sulphoxide and Phorate Sulphone

Another experiment was conducted using a phosphate buffer solution at pH 6.4 and purified ^{14}C phorate sulphoxide and phorate sulphone. They were added to the buffer solution to give a final concentration of 10 ppm (0.036 mM) phorate sulphoxide and 10 ppm (0.034 mM) phorate sulphone, then incubated at 25°C. They were extracted every 7 days and assayed for radioactivity as described above.

9. STUDIES ON THE DEGRADATION OF PHORATE IN PURE CULTURES OF SOIL BACTERIA

Ethoxy-1-¹⁴C phorate was diluted with non-radioactive analytical grade phorate, to a specific activity of 27.6 $\mu\text{Ci}/\text{mM}$ (0.1 $\mu\text{Ci}/\text{mg}$) for this series of experiments.

9.1 Culture Media

Media used in these studies were as follows:

- (a) Screening medium (Fred and Waksman, 1928) consisting of 10.0 g mannitol, 0.5 g K_2HPO_4 , 0.2 g $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.1 g NaCl , 10.0 g yeast extract and trace of CaCl_2 in 1.0 litre distilled water (pH 7).
- (b) Basal medium (Shimahara and Yamashita, 1967) consisting of 5.0 g NH_4NO_3 , 2.5 g K_2HPO_4 , 1.0 g $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ and 0.02 g $\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$ in 1.0 litre of distilled water (pH 7.0-7.2).

All media were autoclaved at 120°C (15 lbs) for 15 minutes.

9.2 Isolation of Micro-Organisms

A modification of the enrichment technique, described by Anderson and Horsgood (1971), was used to isolate soil micro-organisms. Dried soil (10 g) was placed in an Erlenmeyer flask and the soil water content adjusted to field capacity. The soil was treated with phorate in acetone (50 μl) to give 50 ppm. Preliminary experiments indicated that this concentration slightly increased the total micro-organism population in the treated soil after 7 days incubation at 25°C. The

flask was plugged with non-absorbent cotton wool and incubated at 25°C for 10 weeks, the water content being maintained at field capacity by weekly addition of distilled water. A sample of the soil (1 g) was then transferred to a mineral basal medium (100 cm³) containing phorate (2 mg) in a 500 cm³ conical flask and the flask incubated at 25°C ± 1°C for a further three weeks with continual agitation. Similar experiments using radioactive phorate indicated that less than 1% phorate remained in the medium after three weeks.

Isolation of effective organisms from the enriched culture solution was accomplished via a serial dilution plate technique. Bacteria were grown on nutrient agar and fungi were grown on potato-dextrose agar. Isolation of pure cultures was accomplished by examining and selecting the individual colonies from the plates. Single colonies were re-streaked on the plates of nutrient agar or potato-dextrose agar and the process repeated until microscopic examination indicated that apparently pure cultures had been obtained. These cultures were finally streaked on slants of nutrient agar and potato-dextrose agar for identification by the Commonwealth Mycological Institute. Isolates were identified as follows:

1. *Penicillium canescens* Sopp.
2. *Penicillium* Sp.
3. *Bacillus cereus* Frankland and Frankland
4. *Bacillus circulana* Jordan
5. *Corynebacterium* Sp.
6. *Bacillus cereus* var. *mycoides*
7. Mixture: (a) *Bacillus megaterium*; (b) *Brevibacterium* Sp.
8. An actinomycete, probably *Streptomyces* Sp.
9. *Achromobacter* Sp.
10. *Bacillus megaterium*
11. *Bacillus pumilus* Gotteil

9.3 Screening of Bacteria for Capacity to Degrade Phorate

From the micro-organisms isolated from the soil, four strains were selected for further examination. They were: *Bacillus megaterium*, *Bacillus cereus* var. *mycoides*, *Corynebacterium* Sp., and *Achromobacter* Sp. Screening medium (100 cm³) was inoculated with pure cultures of the bacteria and incubated for 48 hours on an orbital shaker at 25°C ± 1°C. Cell suspension (1 cm³) was diluted with a further 9 cm³ of screening medium in 20 cm³ test tubes plugged with cotton wool. ¹⁴C phorate in 10 µl acetone was added to a final concentration of 10 ppm and the culture incubated at 25°C ± 1°C. A control incubation of phorate with sterile screening medium was carried out simultaneously.

9.4 Extraction and Analysis of ¹⁴C Phorate and its Metabolites from Micro-Organism Culture Media

Cultures which appeared turbid to the eye were selected, and were extracted at 0, 1, 2, 4, 8 and 16 days with 3 × 10 cm³ of chloroform. The chloroform layers were separated, combined, dried over anhydrous sodium sulphate, and evaporated nearly to dryness. Six replicate incubations were extracted at each time, three being assayed for total radioactivity, and the remaining three being mixed and analysed quantitatively for chloroform-soluble metabolites by thin-layer chromatographic analysis. Aliquots (20 µl) of the extracts were spotted onto silica gel plates together with authentic samples of non-radioactive phorate and five of its metabolites. The plates were developed over 17.5 cm with benzene:methanol (19:1 v/v) and the position of the pure metabolites located with palladium chloride solution (1% w/v). Areas of silica gel corresponding to the positions of the metabolites were scraped from the plate and their radioactivity determined quantitatively by

placing them in counting vials with butyl-PBD scintillant (10 cm³) and assaying by LSC. Duplicate plates were developed in the same solvent system and analysed by autoradiography to confirm that no radioactive metabolites had been overlooked. The plates were kept in contact with Kodak "Kodarex" X-ray film for 100 days and then developed in Phen-X^R (Ilford Limited) and fixed in Amfix (Ilford Limited).

10. ADSORPTION STUDIES

Ethoxy-1- ^{14}C phorate, diluted with unlabelled analytical grade phorate, to a specific activity of $0.1 \mu\text{Ci/mg}$ ($27.6 \mu\text{Ci/mM}$) was used in all adsorption experiments.

10.1 Adsorbents

Ashurst loamy-sand soil was used as the soil adsorbent. Soil preparation and soil properties have been described in section I. The clay sample was donated by Laporte Industries Limited, Redhill, Surrey. It is a naturally-occurring montmorillonite with a particle size < 100 mesh and a cation exchange capacity of $70 \text{ m.eq/100 g dry weight}$. This sample is designated "Surrey Powder No. 1". Quartz sand with a particle size $2-0.05 \text{ mm}$ was washed with hydrochloric acid (12 N) to remove heavy metal impurities, and then washed with distilled water to near neutrality.

The aqueous phase was a $\text{KH}_2\text{PO}_4/\text{Na}_2\text{HPO}_4$ buffer, pH 7.0 ($I = 0.01$) (Dawson et al, 1972).

10.2 Determination of Empirical Freundlich Isotherms

Equilibration of ^{14}C phorate or of its sulfoxide or sulphone derivatives between 10 cm^3 aqueous buffer and 0.5 g soil enabled accurate estimates of radioactivity in each phase to be made in the case of soil. In the case of sand, the small absorbtive capacity of sand made it necessary to increase the weight of this adsorbent to 1 g , while the greater absorbtive capacity of the clay sample made it necessary to reduce the weight of this adsorbent to 0.25 g . Preliminary experiments indicated that the rate of adsorption was independent of the initial phorate concentration in the aqueous phase and that equilibrium was

apparently reached within 3 hours. Longer periods of mixing, up to 5 hours, did not result in any significant increase in adsorption. A 3 hour equilibration time was chosen both for phorate and its metabolites in these studies. Concentrations of phorate between 2 and 12 ppm (initial concentration in aqueous phase) were investigated.

Six concentrations of ^{14}C phorate in buffer (10 cm^3) were prepared at 25°C , each in a 50 cm^3 Pyrex glass centrifuge tube. The weighed adsorbent was added to the buffered phorate solutions and the stoppered tubes hand-shaken vigorously for 5 minutes. The tubes were transferred to a mechanical shaker and shaken for 3 hours in a controlled temperature room at $25^\circ\text{C} \pm 1^\circ\text{C}$. They were then centrifuged at 4,500 rpm on an MSE Minor centrifuge for 15 minutes (soil samples) or 30 minutes (clay samples). Centrifugation was unnecessary for the sand sample. Phorate and metabolites were extracted from the buffer and the adsorbent separately.

10.3 Extraction of Phorate, Phorate Sulphoxide and Phorate Sulphone from Supernatant and Pellet

Using a Pasteur pipette, the supernatant was removed as completely as possible from the pellet, transferred to a separating funnel and extracted with $3 \times 10\text{ cm}^3$ of chloroform. The combined extracts were dried over anhydrous sodium sulphate, evaporated together with washings to a small volume, and assayed for radioactivity in butyl-PBD scintillation medium (10 cm^3).

The pellet remaining after removal of the supernatant contained the following amounts of residual buffer, determined as the difference between the dry weight of adsorbent and the wet weight after supernatant removal:

0.24 ± 0.017 g (soil), 0.33 ± 0.06 g (clay), and 0.18 ± 0.067 g (sand)

Knowing the concentration of phorate or metabolite in the supernatant, a correction could be applied to allow for the radioactivity in the aqueous phase which was assayed as being adsorbed onto the solid phase.

The pellet was thoroughly mixed with anhydrous sodium sulphate (5.0 g) in the centrifuge tube, using a metal spatula, to give an apparently dry powder. Hexane:acetone (4:1 v/v) (15 cm³) was added, and the powder was extracted for 1 hour in the stoppered tube on a mechanical shaker. The extracts and washings were decanted through glass wool into 50 cm³ flasks, evaporated, and assayed for radioactivity by LSC.

The extent of phorate oxidation over the three hour equilibrium period was checked by column chromatographic analysis on silicic acid of two extracts from the supernatant and the pellet for each adsorbent. The extent of phorate hydrolysis was estimated by detecting the radioactivity of an aliquot (1 cm³) of the extracted aqueous solution using Bray's scintillant (10 cm³).

11. STUDIES ON THE UPTAKE AND RETENTION OF PHORATE BY BACTERIA

11.1 Cultivation of Bacteria

Of the bacteria isolated from Ashurst soil by the enrichment technique (as described previously) and screened for their ability to degrade phorate, *Bacillus megaterium* was chosen for further study because it catalysed the oxidation of phorate in pure cultures solely to the sulphoxide and sulphone derivatives, and these metabolites could be rapidly and completely separated using the column chromatographic methods described in section 4.

B. megaterium was cultured in 500 cm³ Erlenmeyer flasks using the basic screening medium (150 cm³). After inoculation of the medium under aseptic conditions, the flask was agitated on an orbital shaker for 48 hours at 25°C ± 1°C. Bacterial dry weights were determined by the method of Johnson and Kennedy (1973). A sample of the bacterial culture (20 cm³) was Millipore-filtered using a 0.22 µ Millipore weighed-filter paper under vacuum. The filter pad was removed and dried at 90°C to constant weight. A standard culture contained 1.74 ± 0.04 mg cm³ (n = 6 ± S.D.) after 48 hours.

11.2 Uptake and Retention of Phorate by *Bacillus megaterium*

Aliquots of the culture medium (20 cm³) equivalent to 34.8 ± 0.9 mg dry weight of bacteria were removed from the batch of bacterial cultures and centrifuged at 4,500 rpm in a MSE Minor centrifuge for 15 minutes. The supernatant was decanted and the bacterial cells re-suspended in KH₂PO₄/Na₂HPO₄ buffer (10 cm³, pH 7.0, ionic strength 0.01). ¹⁴C phorate in an acetone solution was injected into centrifugal tubes. The tubes were stoppered and shaken on a mechanical shaker for 3 hours in a controlled temperature room at

25°C $\pm$ 1°C. The bacterial suspensions were separated by centrifugation at 4,500 rpm for 15 minutes, and the supernatant decanted into a separating funnel and extracted with chloroform (3 $\times$ 10 cm³). Subsequent analysis of the supernatant extracts was identical to that previously described for the soil and adsorption studies. The bacterial pellet was mixed with anhydrous sodium sulphate (2.0 g) and extracted with hexane:acetone (4:1 v/v) (10 cm³) by shaking for one hour. Chloroform or hexane:acetone extracts were analysed for phorate and its metabolites on columns of silicic acid.

11.3 Phorate Adsorption and Metabolism in the Soil/Bacteria System

Experiments identical to those described for the uptake and retention of phorate by *Bacillus megaterium* were set up, but to each tube a sample of acid-washed quartz sand, natural Ashurst soil, or Surrey powder No. 1 (0.5 g) was added. Incubation was at 25°C for 3 hours; work up and analysis was the same as in the previous section.

11.4 Micro-Organism Counting

The number of organisms in the soil or in suspension media were assessed by a serial dilutions method (Pawsey, 1974).

RESULTS

1. KINETICS OF PHORATE METABOLISM IN ASHURST SOIL

1.1 Metabolism of Phorate in the Soil

Phorate was incubated with Ashurst soil in the dark at 25°C and at a soil moisture content of 20% w/w. The initial concentration in the soil was 10 ppm. At time intervals of 1, 2, 4, 8, 16, 32, 64 and 128 days, flasks containing samples of the incubate were extracted with a mixture of hexane:acetone (4:1 v/v), the extracts being analysed qualitatively by tlc for oxidised phorate metabolites. The only metabolites detected over the period of incubation were phorate sulfoxide and phorate sulphone.

The above experiment was then repeated using ^{14}C phorate in the place of the non-radioactive phorate. The soil incubates were extracted and analysed quantitatively for phorate, phorate sulfoxide and phorate sulphone, using the methods described in the experimental section. The decline of phorate and the formation of phorate sulfoxide and sulphone in Ashurst soil at 25°C are shown as functions of time in Figures 2a, 2b and 2c. Results are expressed as the total radioactivity of phorate, phorate sulfoxide, or phorate sulphone, corrected for incomplete recovery from the soil, and calculated as a percentage of the original phorate radioactivity added to the soil. Each value is the mean of three replicates. Phorate and its oxidised metabolites persisted in the treated soil under the conditions of this study at detectable levels (> 0.1 ppm) for over 128 days.

The patterns of phorate loss and metabolite accumulation over the period of study can best be rationalised if it is assumed that the oxidative and hydrolytic reactions which give rise to these changes

follow first order kinetics. The assumption is made that the rate of hydrolysis and oxidation of each metabolite in the soil/water system takes place at a rate proportional to the concentration of the metabolite in the system. Since the volume of the system remains constant, under first order kinetics the proportional quantity (total radioactivity of metabolites in the system) can be substituted for its 'concentration'.

The metabolism of phorate and its oxidised metabolites in the soil can be represented diagrammatically by Figures 3a and 3b, depending on whether it is assumed that phorate sulphone is formed via the sulphoxide as an intermediate, or directly from phorate. The former model is considered more probable, and is treated in more detail in this study. The rate of adsorption of phorate onto Ashurst soil was shown to be rapid compared with the rate of phorate hydrolysis or oxidation. It is assumed that the rates of adsorption and desorption of phorate sulphoxide and sulphone are similarly rapid, so that the total amount of phorate or its oxidised metabolite in the soil, whether adsorbed or in solution, could be treated as a single compartment in the kinetic analysis (Robinson, 1973).

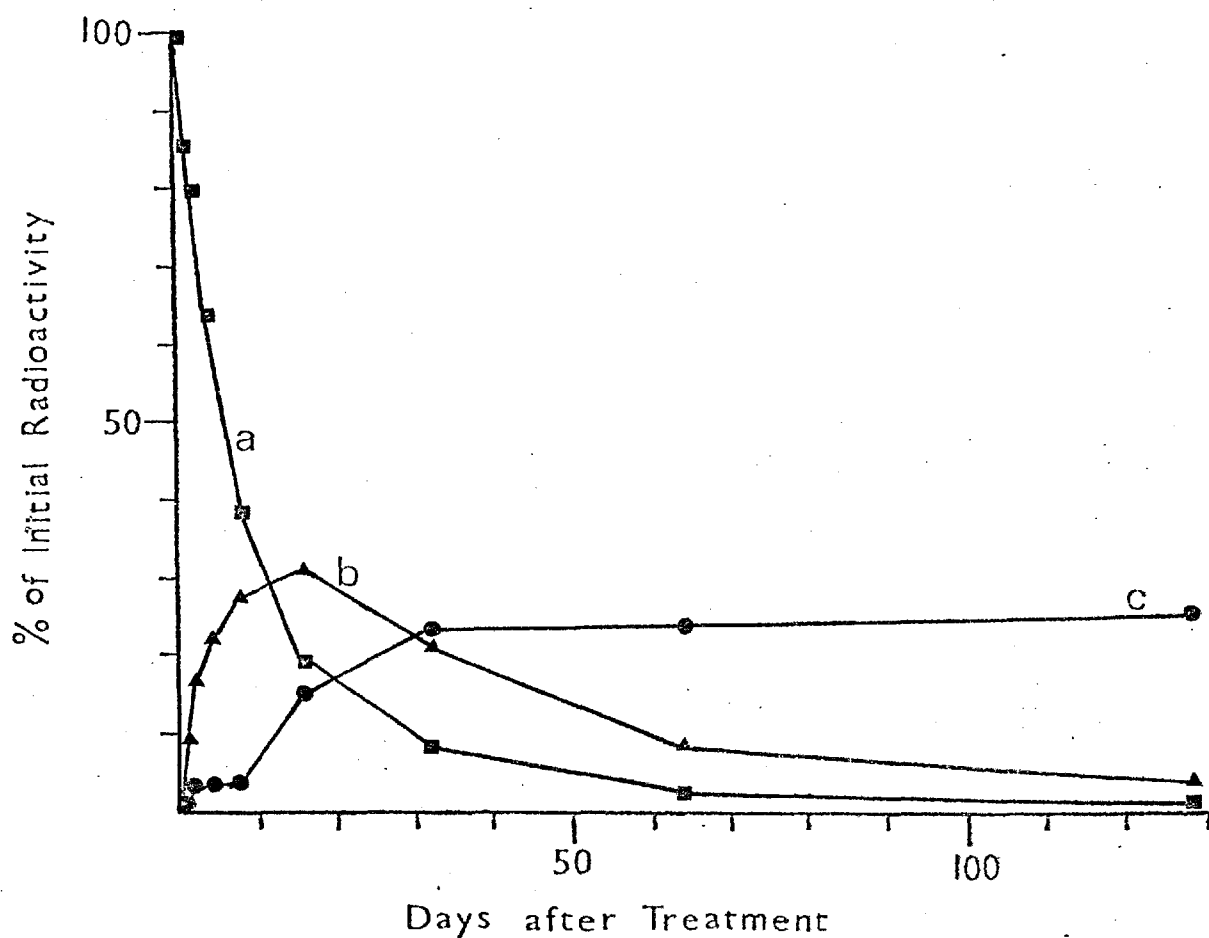


Figure 2: The metabolism of phorate in Ashurst soil at 25°C and 20% moisture content

Key: Figures 2-21: Phorate, ■—■, (a)
Phorate sulfoxide, ▲—▲, (b)
Phorate sulphone, ●—●, (c)

Each value is the mean of three replicates. Standard deviation was calculated and shown in the appendices.

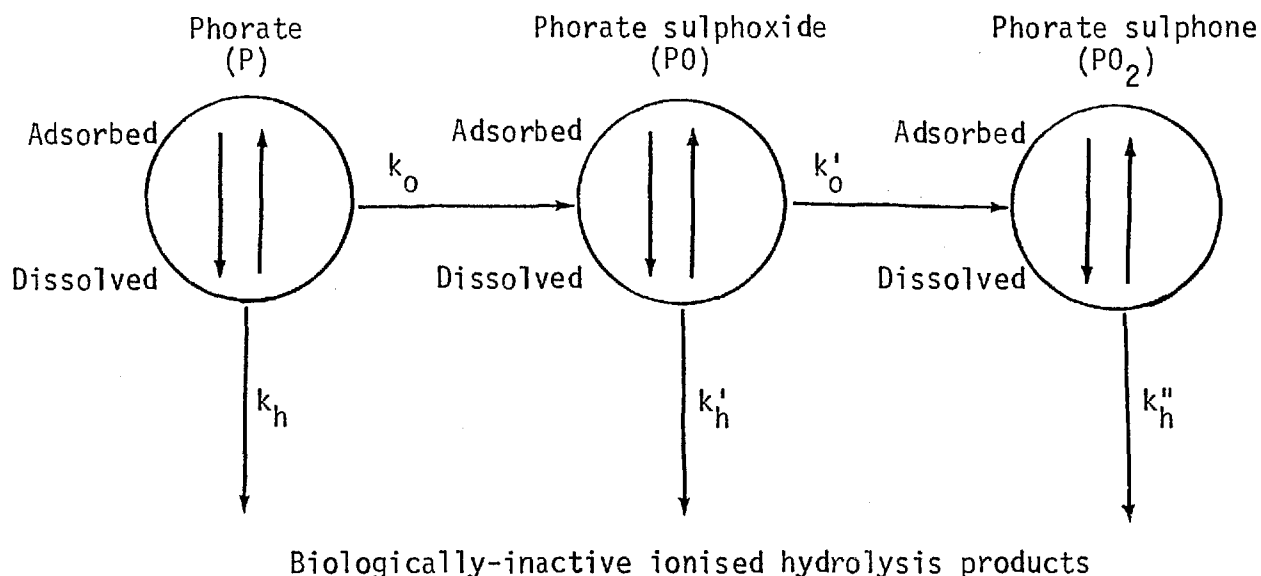


Figure 3a : Model (a) for metabolism of phorate in soil

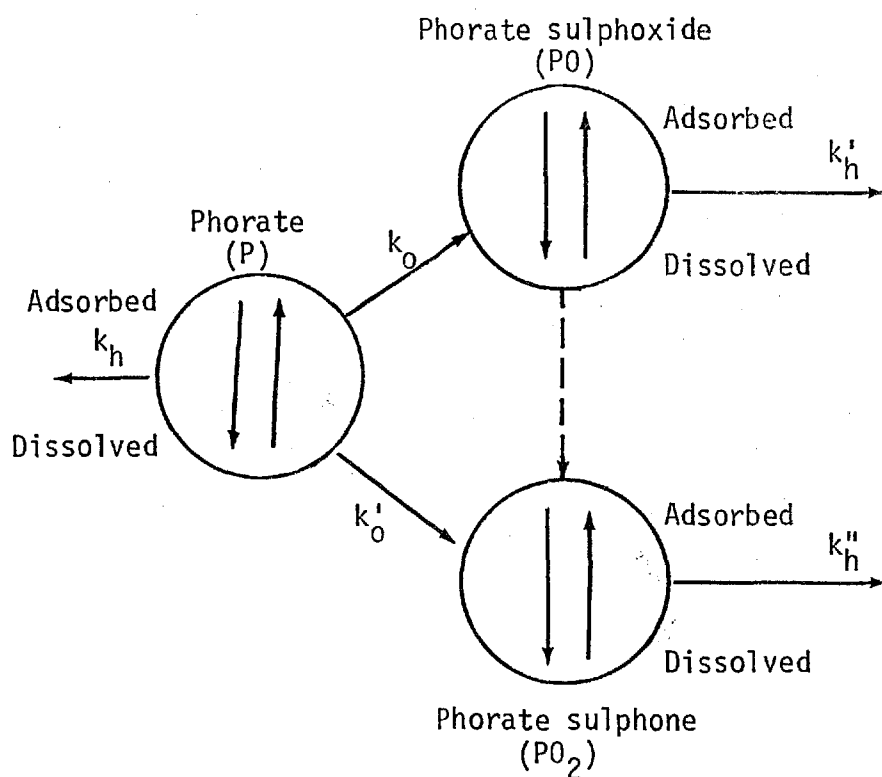


Figure 3b : Model (b) for metabolism of phorate in soil

- k_o : First-order rate constant for phorate oxidation
- k'_o : First-order rate constant for phorate sulfoxide oxidation
- k_h : First-order rate constant for phorate hydrolysis
- k'_h : First-order rate constant for phorate sulfoxide hydrolysis
- k''_h : First-order rate constant for phorate sulphone hydrolysis

Using the nomenclature and model of Figure 3a , the following differential equations govern the rates of metabolism of phorate and its metabolites:

$$d(P)/dt = - (k_o + k_h) (P) \quad (i)$$

$$d(P0)/dt = - (k'_o + k'_h) (P0) + k_o(P) \quad (ii)$$

$$d(P0_2)/dt = - k''_h (P0_2) + k'_o (P0) \quad (iii)$$

The solutions of these simultaneous differential equations can be obtained by a number of standard methods (Rescigno and Segre, 1966; Atkins, 1969). They are:

$$(P) = (P)_o e^{-at} \quad (iv)$$

$$(P0) = \frac{k_o (P)_o}{(a - b)} (e^{-bt} - e^{-at}) \quad (v)$$

$$(P0_2) = k_o k'_o (P)_o \left\{ \frac{e^{-at}}{(a-b)(c-b)} + \frac{e^{-bt}}{(a-b)(a-c)} + \frac{e^{-ct}}{(a-c)(b-c)} \right\} \quad (vi)$$

where $a = k_o + k_h$; $b = k'_o + k'_h$; and $c = k''_h$, and $(P)_o$ represents the total radioactivity of phorate added to the system at zero time.

A similar treatment of the model of Figure 3b leads to the following solutions:

$$(P) = (P)_o e^{-a't} \quad (vii)$$

$$(P0) = \frac{(P)_o k_o}{(a' - b')} (e^{-b't} - e^{-a't}) \quad (viii)$$

$$(PO_2) = \frac{(P)_0 k'_0}{(a' - c')} (e^{-c't} - e^{-a't}) \quad (ix)$$

where $a' = k_0 + k'_0$; $b' = k'_h$; and $c' = k''_h$.

The graphs of the functions (iv), (v) and (vi) are plotted in Figures 4a , 4b , and 4c , and the graphs of functions (vii), (viii) and (ix) are plotted in Figures 5a , 5b , and 5c , for arbitrarily chosen values of the kinetic constants.

It can be seen that the families of curves generated by either of the two models are similar to the experimental curves for phorate metabolism in the soil, and while this shows that the assumption of first order kinetics for phorate metabolism is consistent with the experiment, no choice can be made between the models on this basis.

1.2 Metabolism of Phorate Sulphoxide in the Soil

The decline in the level of phorate sulphoxide and the formation of phorate sulphone in Ashurst soil at 25°C when pure phorate sulphoxide was added to the soil, is shown in Figures 6b and 6c . A similar pattern of approximately exponential decay of phorate sulphoxide to that obtained for phorate was observed, and the accumulation and decay of phorate sulphone under these conditions was similar in form to that of phorate sulphoxide when phorate was initially added to the soil. These relationships can be interpreted in terms of model 3a , but is only consistent with that of 3b if it includes an oxidative pathway between phorate sulphoxide and phorate sulphone (dotted line, 3b). Since the data can be adequately explained using the simpler model of Figure 3a , this is an unnecessarily complicated interpretation.

Using the same kinetic assumptions, and the model and

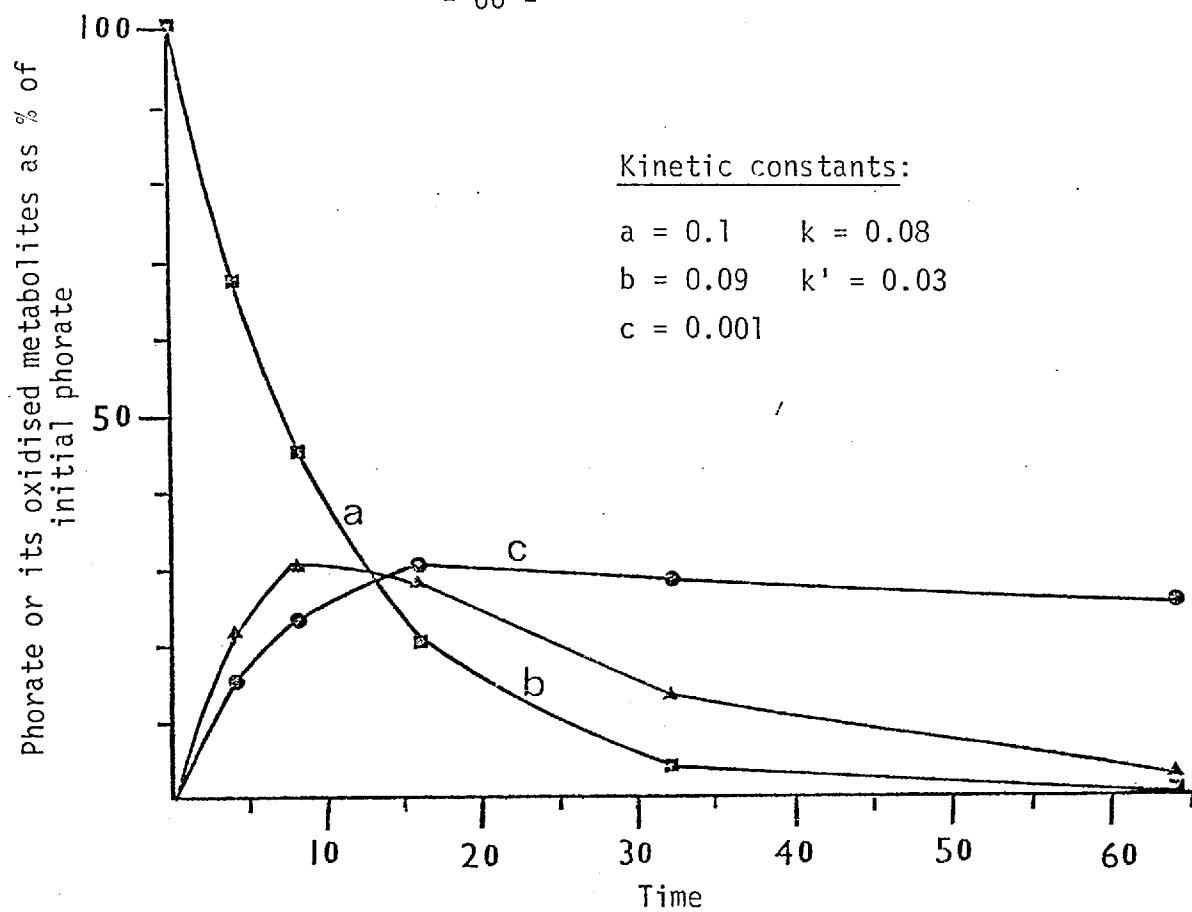


Figure 4: Model 3a

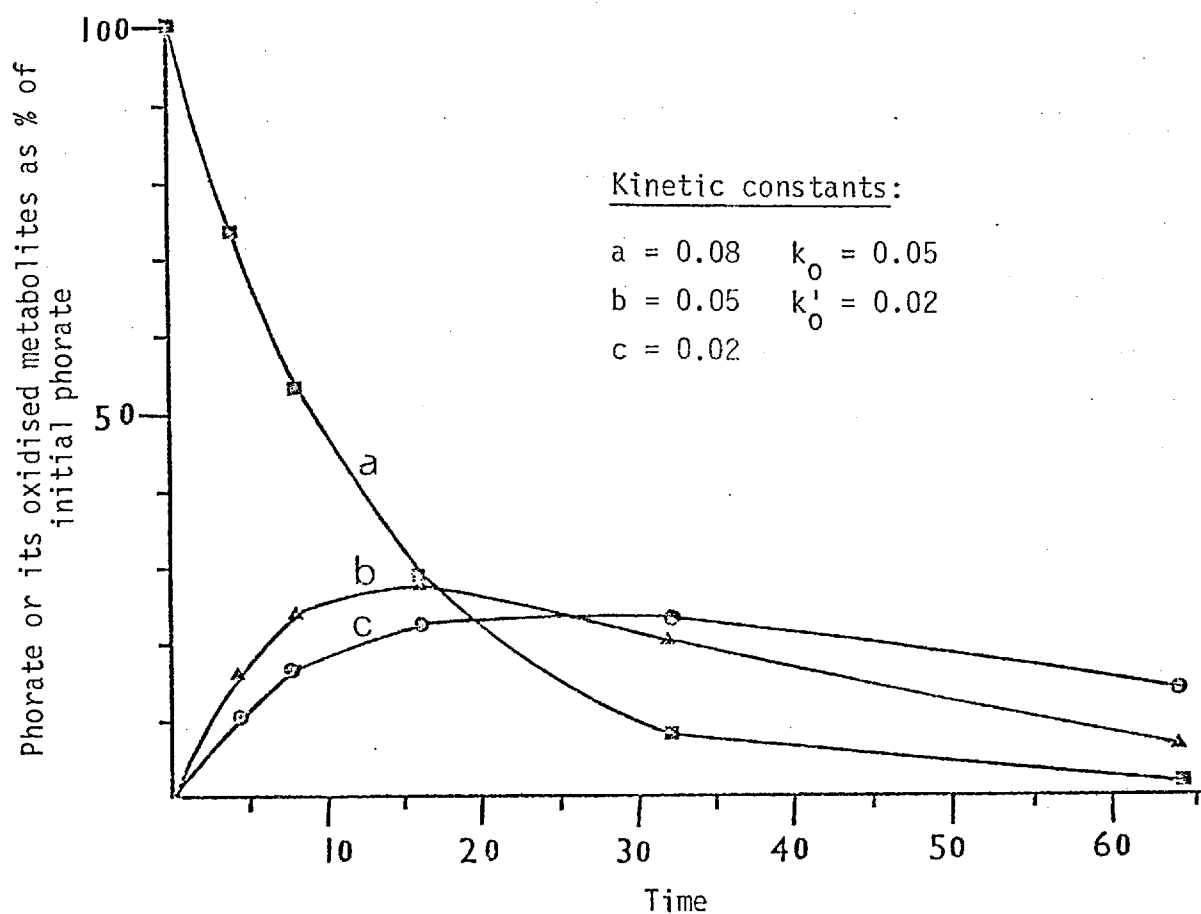


Figure 5: Model 3b

nomenclature of Figure 3a , the following relationships may be derived for the levels of phorate sulfoxide and phorate sulphone as functions of time:

$$(P_0) = (P_0) e^{-bt} \quad (x)$$

$$(P_{O_2}) = \frac{(P_0)_o k'_o}{(b - c)} (e^{-ct} - e^{-bt}) \quad (xi)$$

Again, the graphs of these functions show a qualitative similarity to the experimental curves of Figures 6b and 6c.

1.3 Kinetics of Phorate Sulphone Decay in the Soil

The loss of phorate sulphone from Ashurst soil at 25°C was followed for a period of 64 days. The variation in the level of the compound in the soil with time is shown in Figure 7 . Again, an approximately exponential decay curve was found, consistent with a first-order hydrolysis of phorate sulphone to ionised products.

In terms of models (a) and (b) of Figure 3 , the expected relationship between the phorate sulphone level in the soil and time is:

$$(P_{O_2}) = (P_{O_2})_o e^{-k''_h t} \quad (xii)$$

1.4 Estimation of Rate Constants

From equation (iv) the linear regression of $\ln P/P_o$ on time should give a straight line with a slope corresponding to $-(k_o + k_h)$ and an intercept on the ordinate axis of zero when $t = 0$. The data of Figure 2a was used to test this prediction. A standard computer

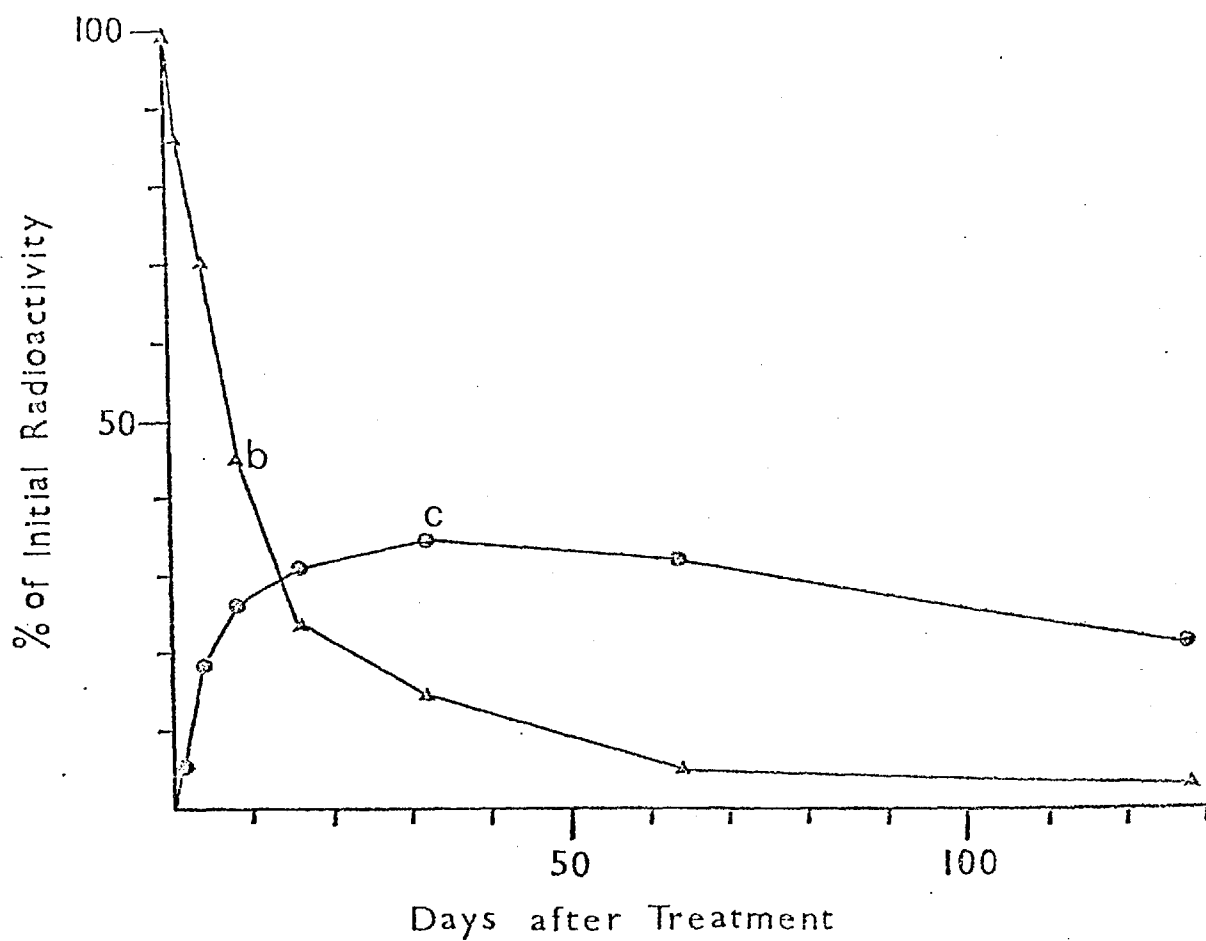


Figure 6: The metabolism of phorate sulfoxide in soil

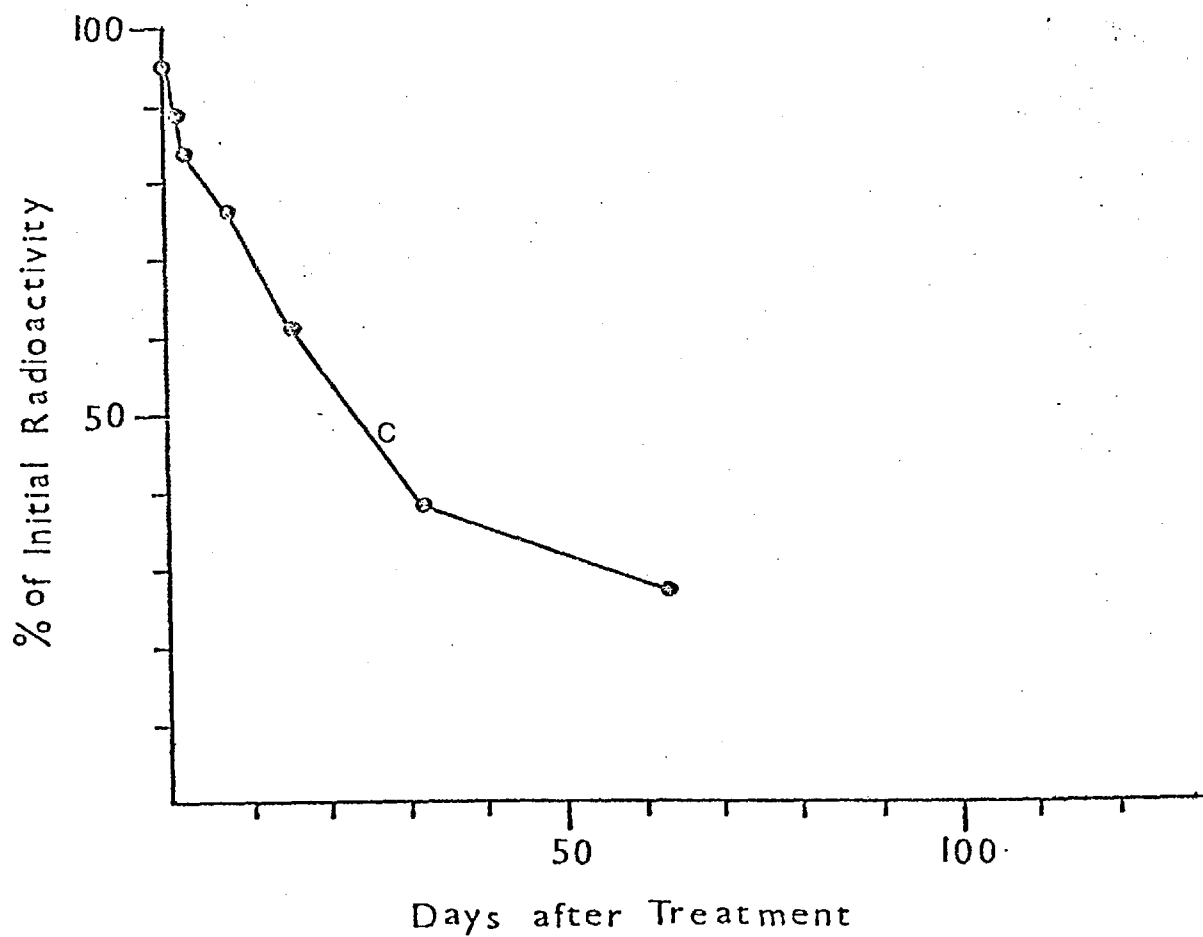


Figure 7: Phorate sulphone decay in soil

programme for unweighted least-squares regression analysis was used to obtain estimates ($\hat{e}$ and $\hat{f}$) of the regression coefficients e and f when the natural logarithm of the fraction of added radioactivity remaining as phorate ($\ln P/P_0$) on the time (t) were fitted to the equation:

$$\ln (P/P_0) = \hat{f} + \hat{e} t$$

Confidence limits ($2\sigma \approx 95\%$) for the estimated regression coefficient $\hat{e}$ were obtained by a standard computer program, on the assumption that the deviations of the data points from the regression line were normally distributed. Confidence limits for the estimate of the intercept $\hat{f}$ on the $\ln P/P_0$ axis at zero time were also obtained.

The correlation coefficient (r) between $\ln P/P_0$ and time and a significance test on the null hypothesis that the data points did not lie on a straight line (Snedecor and Cochran, 1968) were applied to check the assumption of a linear relationship between the two variables over five time intervals, for $t = 0$ to $t = 8, 16, 32, 64$ and 128 days.

Table 9 : Regression Analysis of $\ln P/P_0$

Time (days)	Estimated slope at 95% confidence limits	r	Significance test of linearity	Estimated intercept	Zero* Intercept
0 - 8	0.115 ± 0.013	-0.983	$P < 0.001$	-0.01	$P > 0.6$
0 - 16	0.102 ± 0.006	-0.992	$P < 0.001$	-0.04	$P > 0.5$
0 - 32	0.078 ± 0.007	-0.983	$P < 0.001$	-0.15	$P > 0.01$
0 - 64	0.063 ± 0.006	-0.985	$P < 0.001$	-0.24	$P > 0.001$
0 - 128	0.038 ± 0.006	-0.920	$P < 0.001$	-0.55	$P > 0.001$

* Student's "t" test

The estimates $\hat{e}$ and $\hat{f}$ and the associated statistical data are presented in Table 9. It can be seen that the regression of $\ln P/P_0$ and time was adequately represented by a straight line equation up to 128 days after soil treatment with correlation coefficient > 0.92 . Although the regression analysis showed that the relation between $\ln P/P_0$ and time could be fitted to a linear relationship up to 128 days, the intercept on the ordinate axis deviated significantly from the origin. The statistical analysis indicated that the loss of phorate from the soil was adequately represented by a straight line equation up to 16 days after soil treatment and that up to this time the probability of zero intercept was > 0.5 Student's "t" test. This implies greater than 50% probability that the intercept on the ordinate axis coincided with the origin. This corresponded to the metabolism of about 80% of the original phorate. Thereafter, the rate of loss of phorate showed a progressive deviation away from first-order kinetics as indicated both by a drop in correlation coefficient and an intercept which fell progressively lower than the zero.

Equations (x) and (xii), which follow from the assumption that phorate sulfoxide and phorate sulphone loss from the soil also followed first-order kinetics, similarly predict a linear relationship between $\ln (PO)/(PO)_0$ or $\ln (PO_2)/(PO_2)_0$ and time, for experiments in which pure phorate sulfoxide or pure phorate sulphone were added to the soil. Statistical treatment of the data shown in Figures 6b and 7 gave estimates of the rate constants $(k'_o + k'_h)$ and k''_h . Phorate sulfoxide and phorate sulphone loss from treated soil were adequately represented by first-order kinetics up to 16 days also (Tables 10 and 11). This corresponded to the loss of 77% of the applied radioactivity of phorate sulfoxide and 45% of phorate sulphone.

Table 10 : Regression Analysis of $\ln (P_0)/(P_0)_0$ on Time

Time (days)	Estimated slope at 95% confidence limits	r	Significance test of linearity	Estimated intercept	Zero* Intercept
0 - 8	0.095 ± 0.017	-0.968	$P < 0.001$	-0.02	$P > 0.5$
0 - 16	0.089 ± 0.009	-0.986	$P < 0.001$	-0.03	$P > 0.4$
0 - 32	0.062 ± 0.009	-0.963	$P < 0.001$	-0.16	$P > 0.05$
0 - 64	0.045 ± 0.006	-0.966	$P < 0.001$	-0.28	$P > 0.001$
0 - 128	0.028 ± 0.005	-0.928	$P < 0.001$	-0.52	$P > 0.001$

* Student's "t" test

Table 11 : Regression Analysis of $\ln (P_{O_2})/(P_{O_2})_0$ on Time

Time (days)	Estimated slope at 95% confidence limits	r	Significance test of linearity	Estimated intercept	Zero* Intercept
0 - 8	0.034 ± 0.008	-0.935	$P < 0.001$	-0.07	$P > 0.1$
0 - 16	0.032 ± 0.005	-0.962	$P < 0.001$	-0.08	$P > 0.1$
0 - 32	0.028 ± 0.002	-0.982	$P < 0.001$	-0.10	$P > 0.001$
0 - 64	0.019 ± 0.003	-0.964	$P < 0.001$	-0.16	$P > 0.001$

* Student's "t" test

The two rate constant functions $(k_o + k_h)$ ($= a$) and $(k_o' + k_h')$ ($= b$), and the rate constant k_h'' ($= c$) estimated by the techniques described can be substituted into equations (v) and (xi) to obtain estimates of k_o and k_o' . k_h and k_h' can then be obtained by difference from a and b , respectively.

As these relationships are not linear with time, it was

necessary to use a non-linear curve fitting method to fit the data to the predicted equations. For this purpose, to make least-squares estimates of non-linear parameters, a computer program due to Marquardt (Marquardt, 1963) was used. The estimated values of k_o , k_o' and the derived values of k_h , k_h' are presented in Table 12.

The wide confidence intervals on the estimates of k_o , k_o' , k_h and k_h' are a reflection of limitations of this quantitative approach to phorate metabolism in the soil, but the method does ideally provide a basis on which comparisons between different soil treatments on the rates of the various metabolic processes can be made.

Table 12: Rate Constants for Phorate Metabolism in Ashurst Soil at 25°C

Rate Constant	Days ⁻¹ at 95% Confidence Limits
$k_o + k_h (= a)$	0.102 ± 0.006
$k_o' + k_h' (= b)$	0.089 ± 0.009
$k_h'' (= c)$	0.032 ± 0.005
k_o^*	$\begin{cases} 0.082 \\ 0.073 < k_o < 0.091 \end{cases}$
$k_h = a - k_o$	0.020 ± 0.0108
$k_o'^*$	$\begin{cases} 0.052 \\ 0.048 < k < 0.056 \end{cases}$
$k_h' = b - k_o'$	0.037 ± 0.0098

* Values of k_o and k_o' estimated from mean values of a, b and c calculated by linear regression from 0-16 days. Confidence limits of k_o and k_o' reflect error in fitting experimental data to equations (v) and (xi), and do not take into account variation in a, b and c.

Reductive Metabolism of Oxidised Phorate Metabolites

Reductive metabolism of phorate sulphoxide, which has been reported under conditions of high moisture content (Getzin and Shanks, 1970) was not observed to any significant extent in this study. Levels of phorate formed from phorate sulphoxide were consistently below 1% of added radioactivity through out 128 days incubation, and this is within the limits of error of the analytical technique. Metabolism of phorate sulphone was only followed up to 64 days, but over this period the levels of phorate sulphoxide and phorate radioactivity were similarly both below 1% of added phorate sulphone radioactivity.

2. THE KINETICS OF HYDROLYSIS OF PHORATE, PHORATE SULPHOXIDE AND PHORATE SULPHONE IN BUFFERED AQUEOUS SOLUTIONS

Kinetic studies on the rates of hydrolysis of phorate and its oxidised metabolites in aqueous solutions gives information about the half-life of these compounds in solution. The rate constants calculated for hydrolysis of phorate and its oxidised metabolites in solution can be compared with the rate constants for hydrolysis of phorate and its metabolites in the soil, to obtain information about the effect of adsorption on hydrolysis. The following experiments were conducted, as described in the experimental section, to obtain these hydrolytic rate constants at a pH of 6.4 (phosphate buffer I = 0.01) (the natural pH of Ashurst soil) for phorate at 5°C and 25°C, phorate sulphoxide and phorate sulphone at 25°C. The hydrolytic rate constants of phorate at pH 3 (glycine-HCl buffer 0.2M) and at pH 9 (glycine-NaOH buffer 0.2M) were also obtained.

Preliminary experiments indicated that the rate of phorate hydrolysis was greater at 25°C than 5°C, and was much greater than its oxidised metabolites at 25°C. Therefore, the experiments of phorate hydrolysis at 25°C were conducted only for 2 weeks. The analysis of phorate hydrolysis was carried out at 24 hour intervals for the first week, then once more on the 14th day. The experiment of phorate hydrolysis at 5°C was conducted for 4 weeks and the analysis was carried out at 7 day intervals. The experiments of phorate sulphoxide and phorate sulphone hydrolysis were conducted for 5 weeks and the analysis was also carried out at 7 day intervals.

The linear regression analysis of C/C_0 on time, where C is the concentration of phorate, phorate sulphoxide or phorate sulphone and C_0 is the concentration of phorate, phorate sulphoxide or phorate sulphone

at zero time, indicated that phorate, phorate sulphoxide and phorate sulphone can be approximately represented by first-order kinetics over the time interval of the experiments under the above experimental conditions.

The integrated rate equation of a first-order reaction is:

$$\ln C/C_0 = -k t$$

$$T_{\frac{1}{2}} = \frac{\ln 2}{k}$$

Table 13 shows the hydrolytic rate constants of phorate and its oxidised metabolites under different experimental conditions associated with statistical analysis.

Table 13 : The Hydrolysis of Phorate and its Oxidised Metabolites in Buffered Aqueous Solutions

Compound	pH	Temperature ($^{\circ}\text{C}$)	k (day^{-1}) at 95% confidence limits	r	Significance test of linearity	$T_{\frac{1}{2}}^*$
Phorate	6.4	25	0.23 $\pm$ 0.01	-0.997	P > 0.001	2.9
Phorate	3.0	25	0.22 $\pm$ 0.02	-0.996	P > 0.001	3.1
Phorate	9.0	25	0.25 $\pm$ 0.02	-0.996	P > 0.001	2.7
Phorate	6.4	5	0.011 $\pm$ 0.002	-0.997	P > 0.001	60.0
Phorate sulphoxide	6.4	25	0.0085 $\pm$ 0.002	-0.966	P > 0.01	81.1
Phorate sulphone	6.4	25	0.015 $\pm$ 0.009	-0.925	P > 0.01	46.2

* Mean half lives (days)

3. THE EFFECT OF PHYSICAL CONDITIONS ON THE RATE OF PHORATE
METABOLISM IN ASHURST SOIL

Results obtained from the first section indicated that phorate was metabolised in Ashurst moist soil at 25°C by two routes - oxidation of phorate thioether sulphur to the sulfoxide and sulphone metabolites, and hydrolysis to non-toxic hydrolytic products. The results also showed that phorate and its metabolites were hydrolysed at differing rates in buffered aqueous solution. Since phorate is at equilibrium between the soil solution and the adsorbed phase, a reduction in soil moisture content would be expected to reduce the rate of hydrolysis of phorate in the soil. The effect of such a reduction in soil moisture content on the rate of oxidation would depend on whether oxidation took place primarily in aqueous solution or in the adsorbed phase.

The following experiments attempt to explore the effects of moisture content, inhibitors of oxidation process, temperature and pH on the rate of phorate metabolism in Ashurst soil. In all experiments, phorate, phorate sulfoxide and phorate sulphone were quantitatively determined by radioassay. The assumption was made that the difference between the total radioactivity initially added and total radioactivity recovered as phorate and its oxidised metabolites after correction for the recovery efficiencies of these compounds from soil was due to the hydrolysis in the soil/water system.

The rate of phorate metabolism in Ashurst soil indicated that the loss of phorate in moist soil at 25°C followed first-order kinetics up to 16 days. All experiments designed to study the effect of physical and biological conditions on the rate of phorate metabolism were followed up to 128 days, but to obtain comparable results with the control experiment the kinetics of metabolism were only followed up to 16 days in all

experiments. In order to compare the influence of different factors on phorate metabolism in the soil, it is useful to determine the half-life ($T_{\frac{1}{2}}$). This is the time required for one-half of initial phorate added to the soil to be lost during the metabolic process. This can be calculated from the equation:

$$T_{\frac{1}{2}} = \frac{0.693}{k} \quad (\text{days})$$

where k is the rate constant of phorate decay in soil and was calculated as the slope of regression lines of $\ln P/P_0$ on time up to 16 days. The half-life of phorate under different conditions was estimated from the decay constants, and this $T_{\frac{1}{2}}$, rather than the experimental half-life, was compared with that of the control.

3.1 The Effect of Autoxidation Inhibitors on the Rate of Phorate Metabolism in the Soil

This experiment was designed to control phorate oxidation in the soil by the addition of autoxidation inhibitors. Phorate sulfoxide and phorate sulphone are known to be more susceptible to movement (and consequently to leaching) in the soil than phorate itself (Lichtenstein et al, 1974) and thus the addition of autoxidation inhibitors may have the practical consequence of prolonging the bioactivity of phorate in the soil.

Two inhibitors were chosen according to their water solubility. They were hydroquinone (HQ, water soluble) and butylated hydroxy toluene (2,6-di-tert-butyl-4-methyl phenol, BHT, water insoluble). The soil was treated with an aqueous solution of HQ to give a 0.1% concentration of HQ per soil air-dried weight, while BHT was added at the same concentration in acetone (10 μ l acetone for each treatment) and

thoroughly mixed into the soil immediately before treatment with phorate. The metabolism of phorate in the soil treated with BHT was followed only up to 64 days. The moisture content was maintained at 20% (w/w) throughout the experiment.

The rates and patterns of phorate metabolism, and phorate sulphoxide and sulphone formation in both treated soils are shown in Figures 8 and 9. The effect of HQ is seen as a retardation of the rate of phorate decay in the soil. The amount of phorate remaining unmetabolised after 128 days in the soil was 6% of the phorate initially added. Phorate sulphoxide was shown to be the major oxidised metabolite which accumulated in the soil to a maximum of 24% of initial radioactivity added after 32 days and then persisted in the soil at a 20% level after 128 days. Phorate sulphone accumulated only to a maximum level of 5.5% of the initial radioactivity added after 128 days. The estimated rate constant of phorate decay under these experimental conditions was 0.029, $0.018 \leq k \leq 0.039 \text{ day}^{-1}$ with confidence limits at 95%. This gave a mean estimated half-life of 23.7 days.

BHT did not affect phorate metabolism in the soil up to 8 days, but then phorate metabolism slowed down compared with the control. The amount of phorate remaining unmetabolised after 64 days in the soil was 4.0% of the phorate initially added. Phorate sulphoxide was shown to be the major metabolite in the soil treated with BHT. It accumulated to a maximum level of 23% of the initial radioactivity added after 16 days and then declined to 10% level after 64 days. Phorate sulphone accumulated progressively to a maximum level of 7% of the initial radioactivity added after 64 days. The estimated rate constant of phorate decay in the soil under these experimental conditions was 0.079, $0.062 \leq k \leq 0.097 \text{ day}^{-1}$ with confidence limits at 95%. This gave an

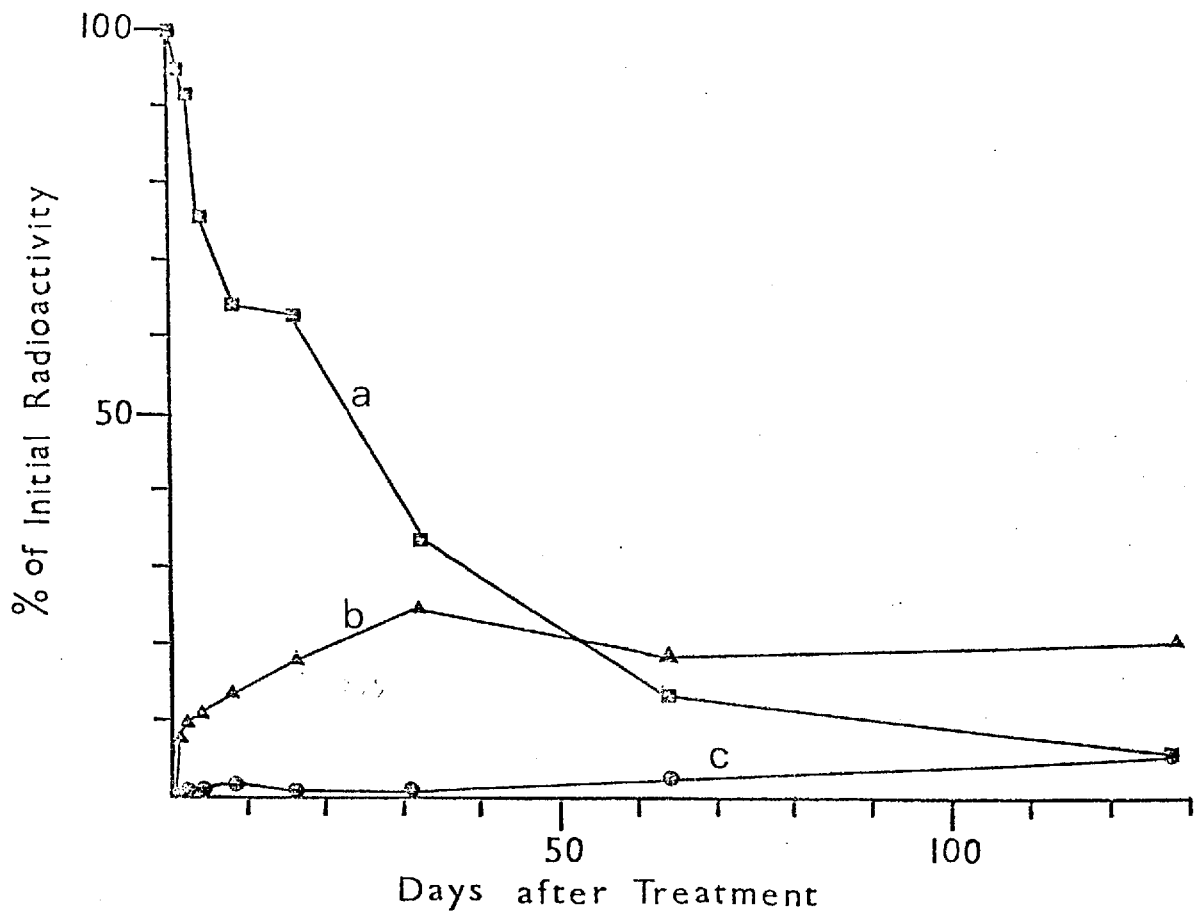


Figure 8: The metabolism of phorate in soil treated with HQ

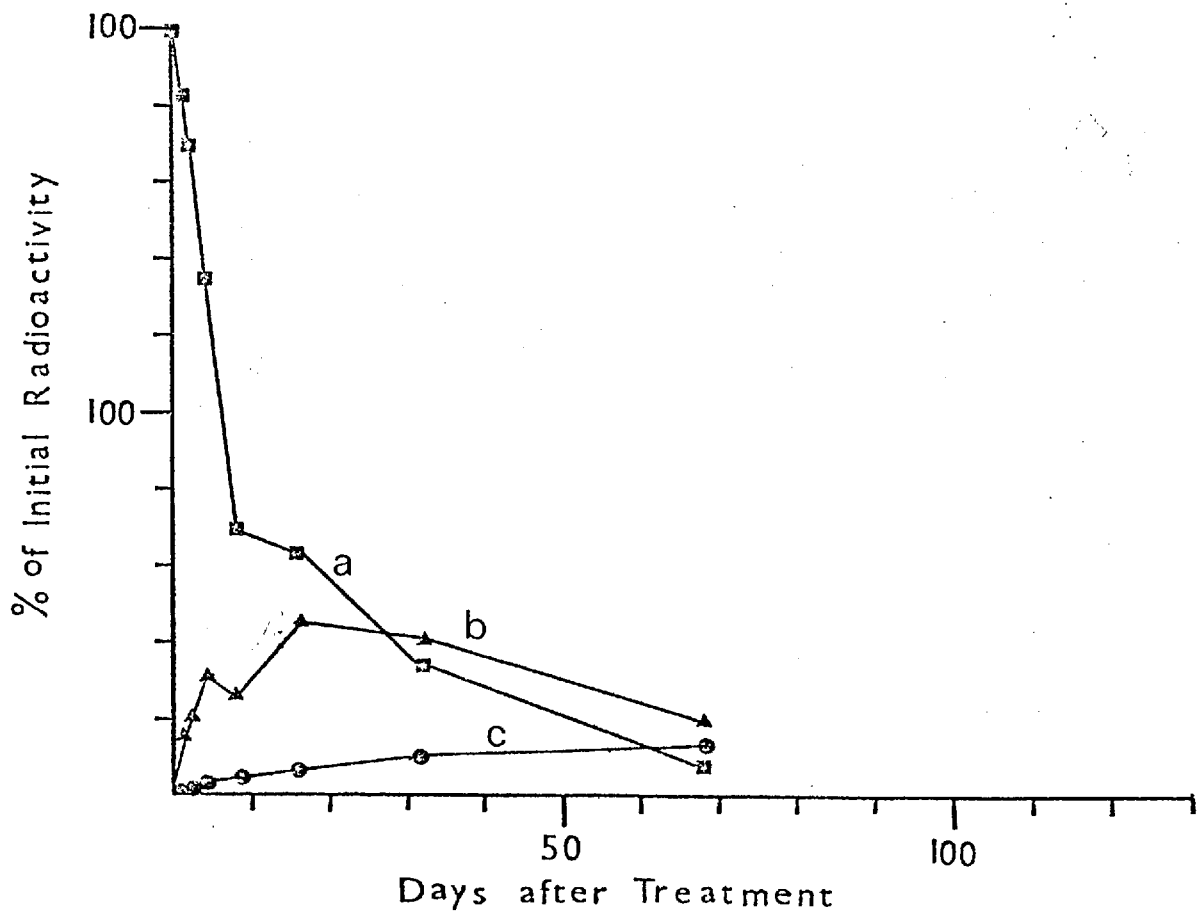


Figure 9: The metabolism of phorate in soil treated with BHT

estimated mean half-life of 8.6 days. The phorate mean half-life in the control experiment was found to be 6.7 days.

These results indicate clearly that the addition of HQ (water soluble) to the soil prolonged the persistence of phorate in the soil considerably and inhibited the formation or accumulation of phorate sulphone, which appears to be the most persistent metabolite in the control experiment. HQ affected the rate of phorate sulfoxide oxidation, and therefore it persisted in the soil at a higher level than in the control experiment. On the other hand, the addition of BHT (water insoluble) did not affect the rate of phorate metabolism, but affected the accumulation of its oxidised metabolites. The rate of phorate sulphone accumulation was slightly greater than in the soil treated with HQ, but much less than in the control experiment. These results indicate that the inhibitory effect of these compounds on phorate oxidation is related to their water solubility, which in turn suggests that much of the oxidation is taking place in the soil solution, rather than on the soil surface.

3.2 The Effect of Soil Moisture on Rate of Phorate Metabolism in the Soil

This experiment was designed to investigate the effect of soil moisture content on the rate of phorate metabolism in Ashurst soil. The soil moisture content was initially adjusted to 20% (w/w) and the soil was incubated at 25°C. The soil was allowed to dry out. It was found that 76.5% of the water added was evaporated after 1 week (Figure 10) and the soil completely dried out to the air-dried soil moisture content after 2 weeks. The rates and the patterns of phorate metabolism and phorate sulfoxide and sulphone formation in the soil which was

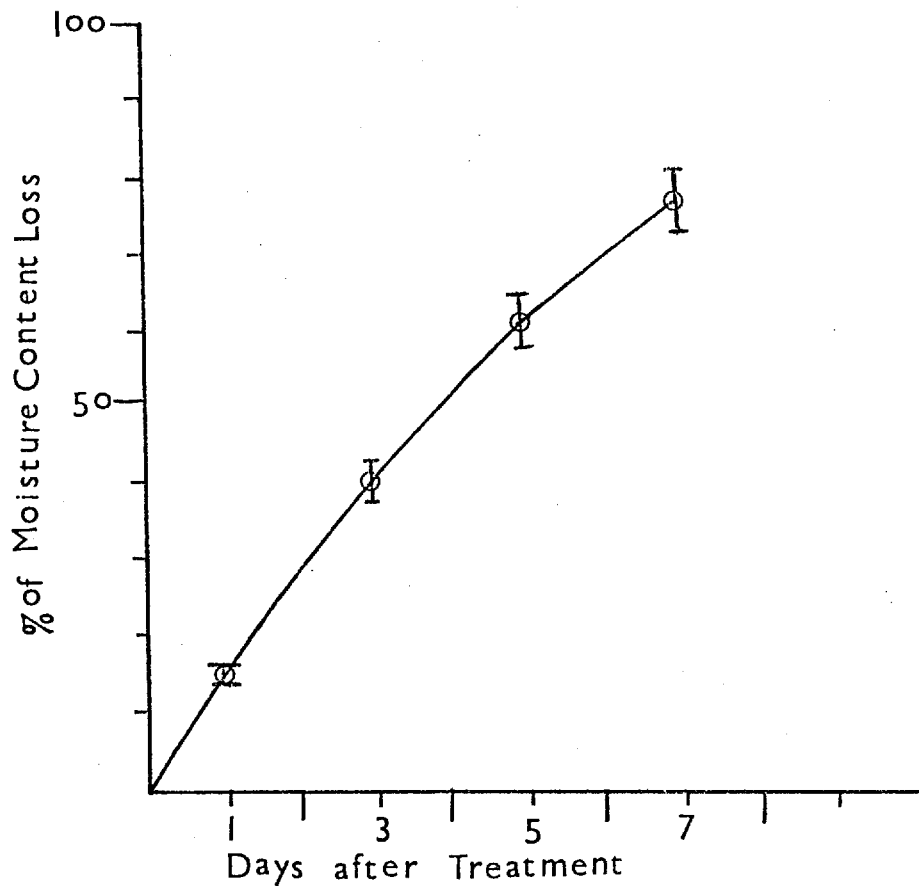


Figure 10: The loss of moisture from the soil incubated at 25°C and 20% initial moisture content

allowed to dry out over the first two weeks of incubation are shown in Figure 11. The rate of phorate metabolism was similar to that rate of phorate metabolism in the control experiment for 8 days, but then slowed down. The amount of phorate remaining unmetabolised after 128 days in the soil was 10% of the initial phorate added. Phorate sulphoxide was the major metabolite produced. It accumulated to a maximum level of 32% of applied radioactivity at 8 days and then declined. Phorate sulphone accumulated to a maximum level of 6% of the radioactivity initially added to the soil at 8 days and then also declined. The estimated rate constant of phorate decay in the soil under these conditions was 0.084 , $0.075 < k < 0.093 \text{ day}^{-1}$ with confidence limits at 95%. This gave a mean estimated half-life of 8.2 days.

These results indicate that drying out the soil affected both the rate of phorate hydrolysis and oxidation. Drying the soil suppressed the formation of phorate sulphone considerably, showing that the oxidation of phorate sulphoxide is dependent upon the soil moisture content; this may also be true for phorate oxidation but this is not apparent from the results obtained in this experiment.

3.3 The Effect of the Temperature on the Rate of Phorate Metabolism in the Soil

This experiment was designed to investigate the effect of low temperature (5°C) on the rate of phorate metabolism in Ashurst soil. The soil water content was initially adjusted to 20% (w/w) and then the soil was incubated at 5°C with no water being added throughout the experiment. The rates and the patterns of phorate metabolism and phorate sulphoxide and sulphone formation in the soil incubated at 5°C are shown in Figure 12 and can be compared with the data of Figure 11

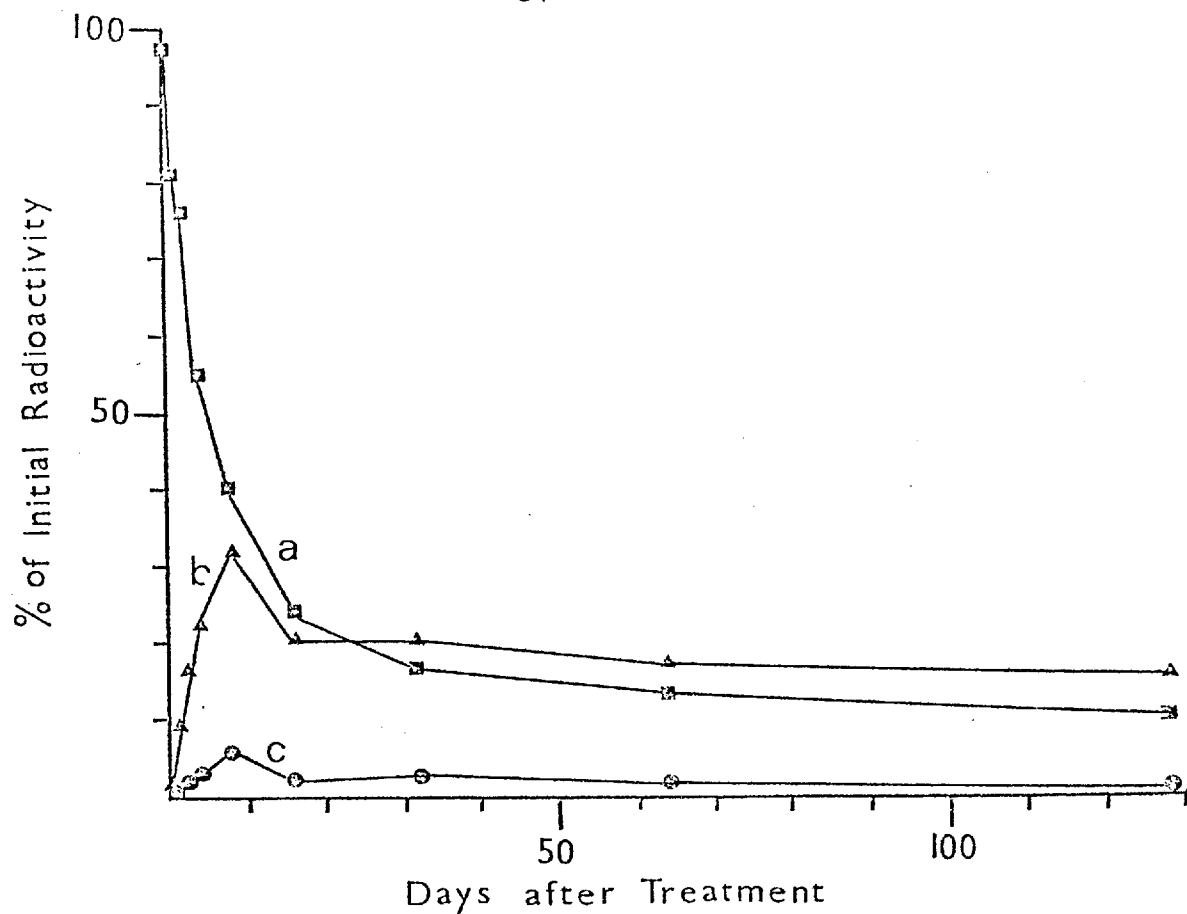


Figure 11: The metabolism of phorate in dry soil

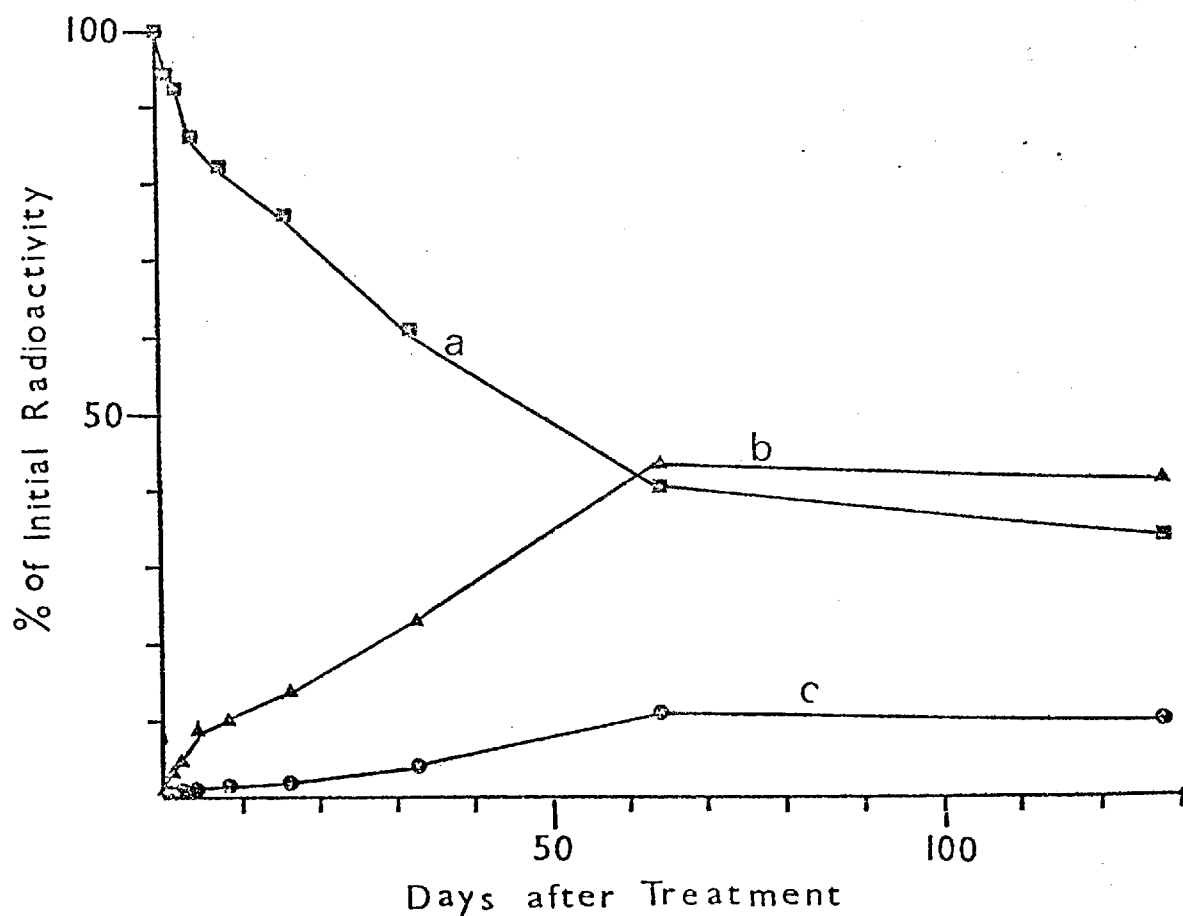


Figure 12: The metabolism of phorate in soil at 5°C

obtained under identical conditions at 25°C. As expected, the rate of phorate metabolism was retarded considerably at the lower temperature. The amount of phorate unmetabolised after 128 days in the soil was 33% of the phorate initially added. Phorate sulfoxide was the major metabolite and accumulated progressively to about 42% of the initial radioactivity added up to 128 days (the highest phorate sulfoxide recovery under all experimental conditions). Phorate sulphone accumulated in the soil progressively to a maximum level of 10% of the initial radioactivity added up to 128 days. The estimated rate constant of phorate decay in the soil under these experimental conditions was 0.014, $0.011 \leq k \leq 0.017$ day⁻¹ with confidence limits at 95%. This gave an estimated mean half-life of 47.8 days.

These findings show that, while the rate of phorate hydrolysis in the soil was greatly decreased by incubation at 5°C, the rate of phorate oxidation was much less. The ratio of the first-order rate constants for phorate hydrolysis in buffered aqueous solutions at 25°C and at 5°C was 20.2:1 (Table 13) and a similar ratio may be presumed to apply to the rates of hydrolysis of phorate sulfoxide at the two temperatures. Phorate sulphone formation was again suppressed by incubation at 5°C under soil drying conditions.

3.4 The Effect of Soil pH on the Rate of Phorate Metabolism in the Soil

This experiment was performed to investigate the effect of soil pH on the rate of phorate metabolism. In this experiment the soil was initially adjusted to pH 3 and pH 9 as described previously. The soil moisture content was initially adjusted to 20% (w/w), then the soil was incubated at 25°C, no more water being added during the experiment.

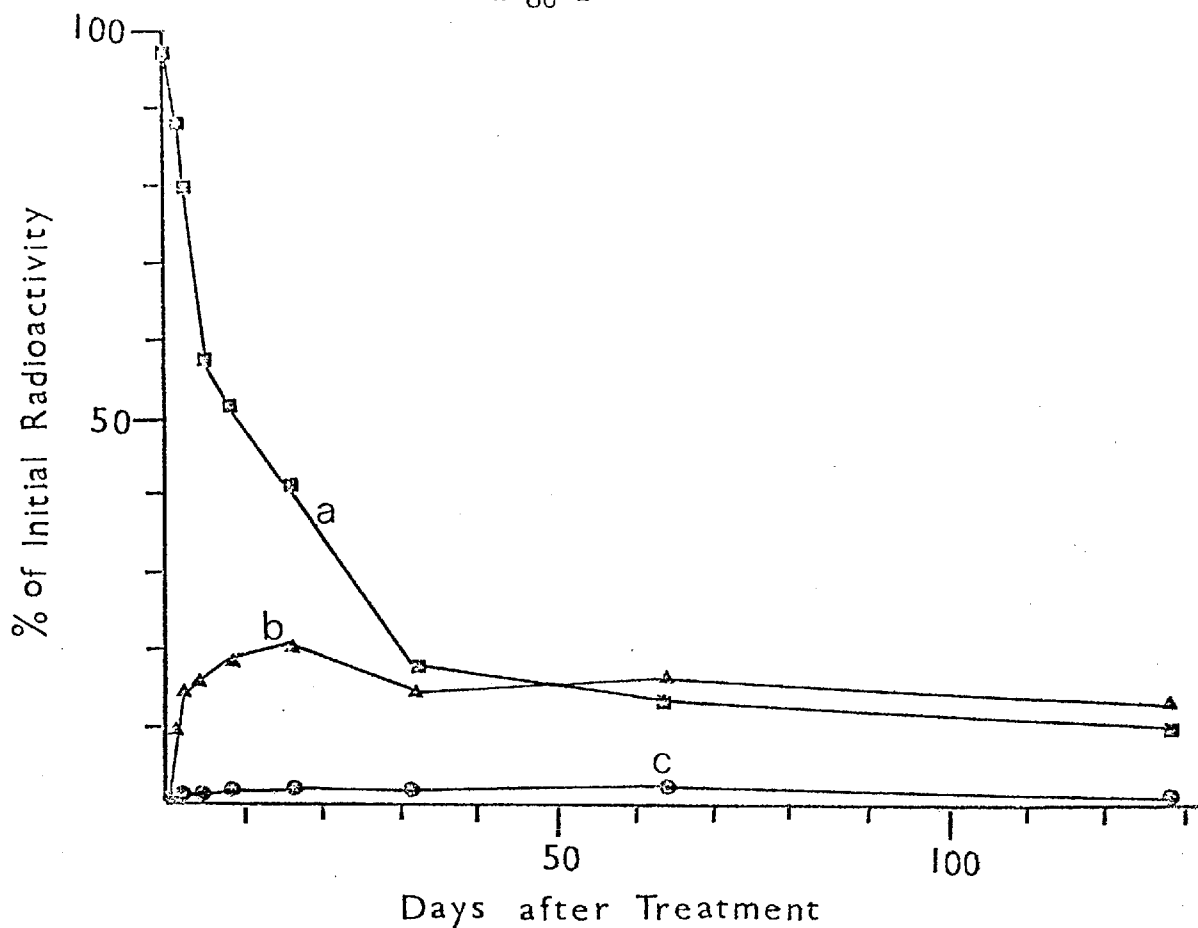


Figure 13: The metabolism of phorate in soil with pH artificially adjusted to pH 3

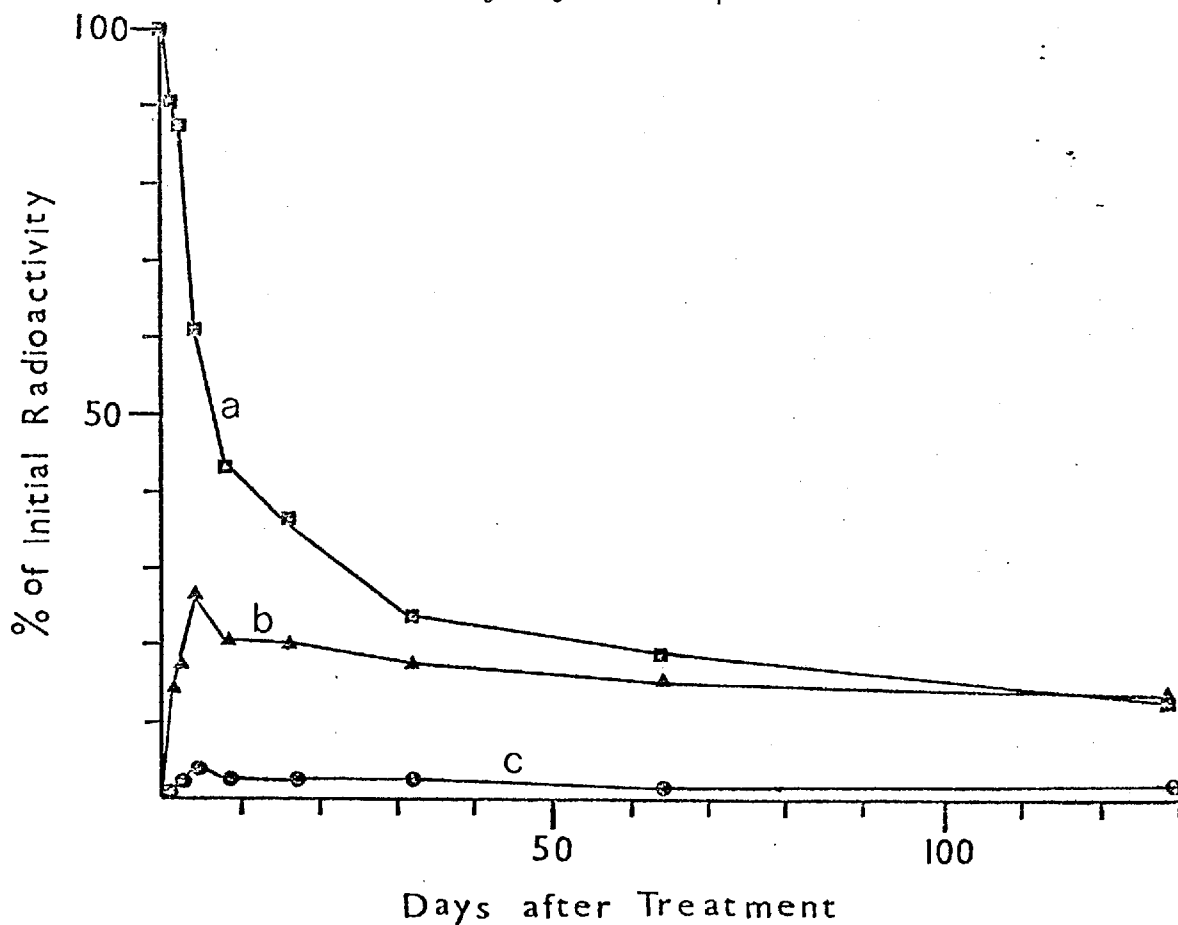


Figure 14: The metabolism of phorate in soil with pH artificially adjusted to pH 9

The rates and patterns of phorate metabolism and phorate sulfoxide and sulphone formation in soil with the pH altered to pH 3 and pH 9 are shown in Figures 13 and 14.

The rate of phorate metabolism in soil with pH 3 was similar to that of the control (soil allowed to dry out) up to 4 days, then the rate slowed down. The amount of phorate remaining unmetabolised after 128 days in the soil under these conditions was 10% of the initial phorate added. Phorate sulfoxide was the major oxidised metabolite with a maximum accumulation level of 20% of the initial radioactivity added at 16 days. This indicated that phorate sulfoxide formation under these conditions was slower than in the control, or that its metabolism was more rapid. Phorate sulphone accumulated to a maximum 2% level of the initial radioactivity added at 64 days, and then also declined, due to hydrolysis. The estimated rate constant of phorate decay in the soil under these experimental conditions was 0.053 , $0.040 \leq k \leq 0.066$ day⁻¹ with confidence limits at 95%. This gave an estimated half-life of 13.1 days.

The rate of phorate metabolism in the soil at pH 9 was faster than at pH 3, and was similar to that of the control. Phorate sulfoxide was the major oxidised metabolite with a maximum accumulation of 26% at 4 days. Phorate sulphone accumulated to a maximum of 4% of the initial radioactivity added at 4 days, and it then declined. The estimated rate constant of phorate decay in the soil under these experimental conditions was 0.066 , $0.052 \leq k \leq 0.079$ day⁻¹ with confidence limits at 95%. This gave an estimated half-life of 10.5 days.

These results indicate that the rate of phorate metabolism in soil with the pH artificially raised or lowered (pH 3 or 9) was only slightly different from that of the control (pH 6.4) but that phorate

decay at pH 9 was marginally the fastest. The results obtained from the experiment on phorate hydrolysis in buffered aqueous solutions similarly showed very small differences in the rate of phorate hydrolysis over the pH range 3 - 9 (Table 13).

4. THE EFFECT OF BIOLOGICAL FACTORS ON THE RATE OF PHORATE METABOLISM IN ASHURST SOIL

There is considerable literature concerning the role of micro-organisms in the metabolism of pesticides in the soil. Although it is difficult to differentiate precisely between the chemical or biological nature of pesticide metabolic reactions in the soil, there are a number of different possible approaches to the study of the role of micro-organisms on pesticide degradation in the soil. The following series of experiments attempt to investigate the effect of biological degradation on the soil metabolism of phorate. The comparison of the relative metabolism of phorate in steam sterilised and non-sterilised soil under different moisture content, the effect of streptomycin sulphate as a partial sterilising agent, the inoculation of steam-sterilised soil with a pure culture of *Bacillus megaterium*, the soil pre-incubation before treatment, and the addition of additional carbon source to the soil, were all investigated.

4.1 The Effect of Steam-Sterilisation of Soil on the Rate of Phorate Metabolism in the Soil

This experiment was designed to investigate the effect of steam-sterilisation of the soil on subsequent phorate metabolism. The soil was sterilised as described previously, and the soil moisture content was maintained at 20% (w/w) throughout the experiment. The rates and patterns of phorate metabolism, and phorate sulfoxide and sulphone formation in steam-sterilised soil are shown in Figure 15. Steam sterilisation retarded subsequent phorate metabolism in the soil, and phorate sulfoxide was the only major oxidised metabolite produced. Phorate sulphone accumulated to a maximum level of 1% of the initial

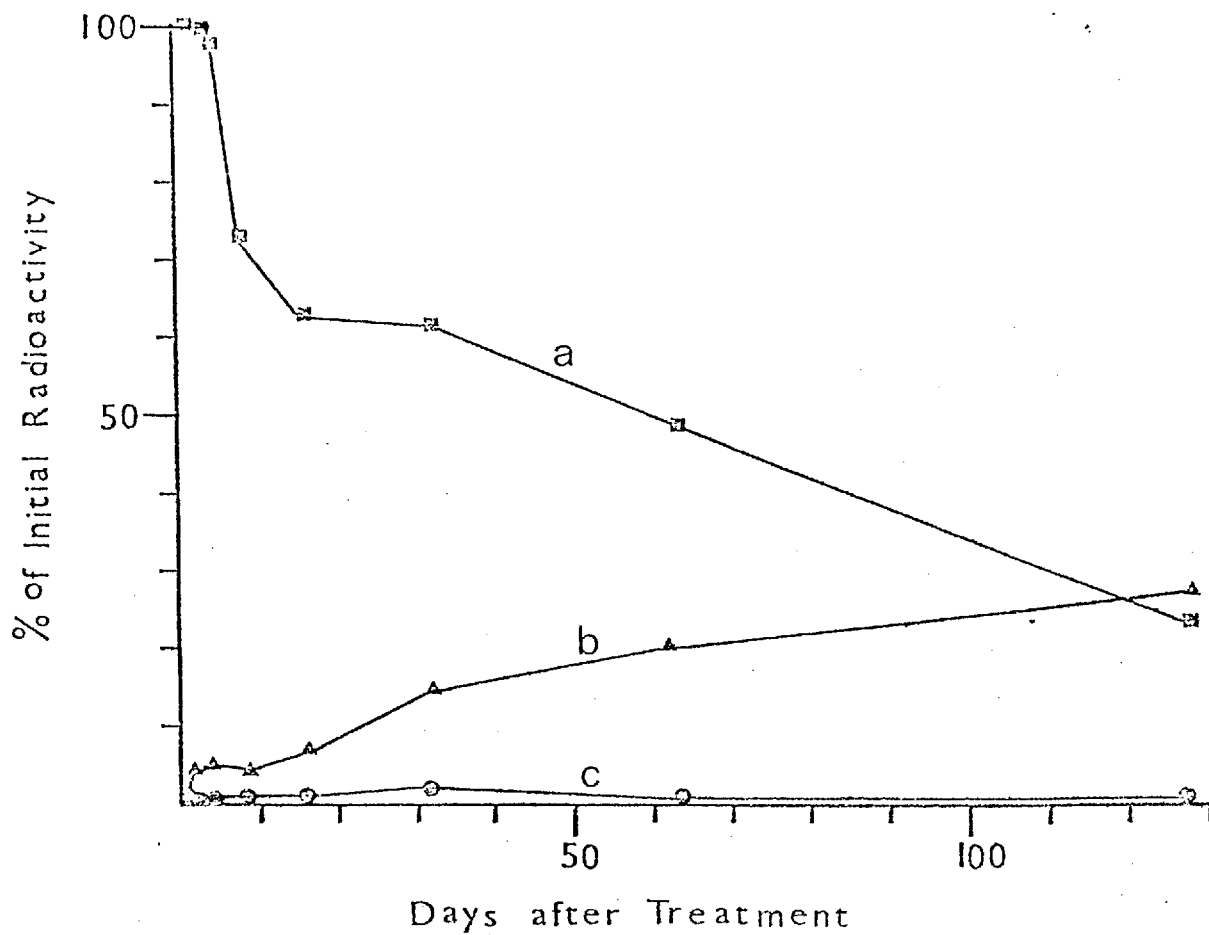


Figure 15: The metabolism of phorate in steam sterilised soil

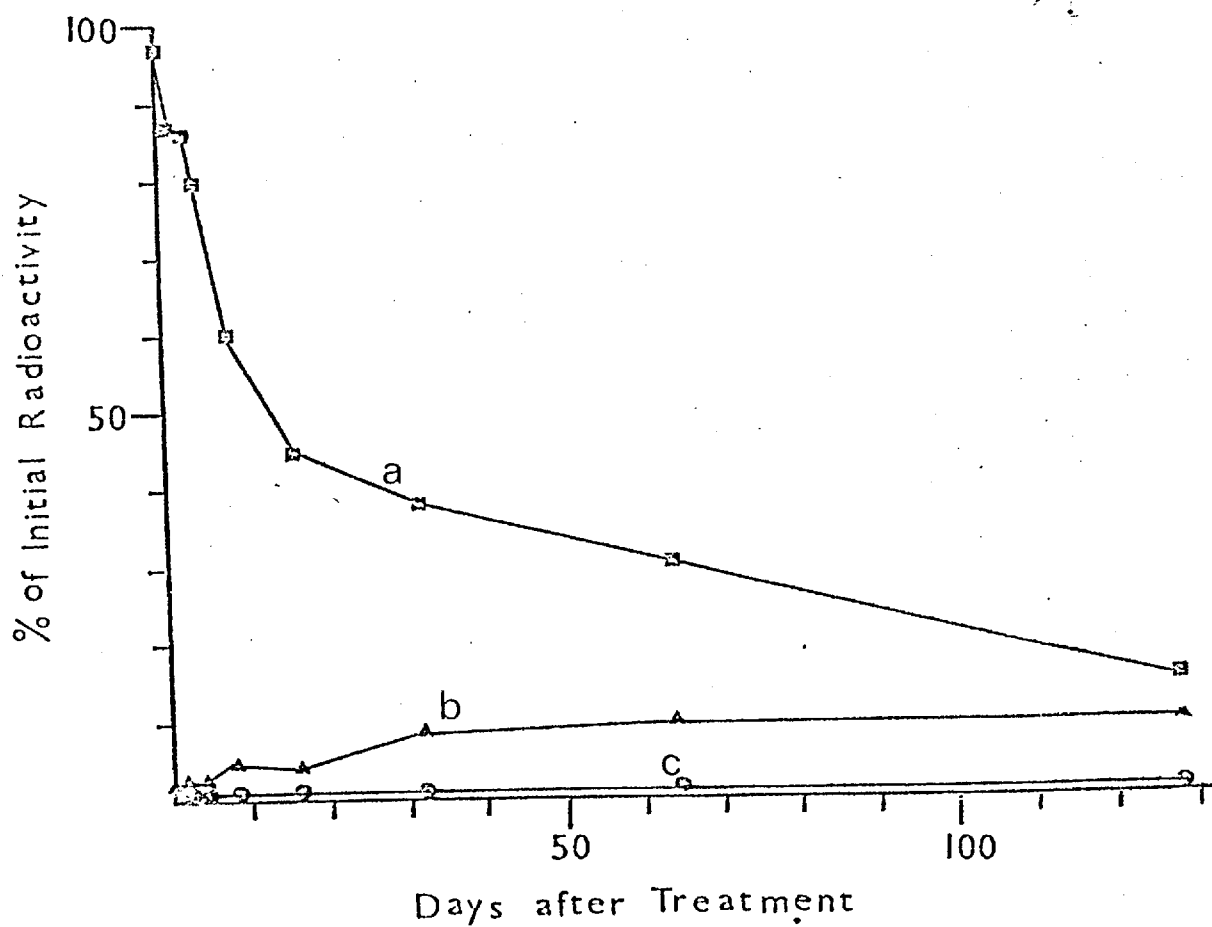


Figure 16: The metabolism of phorate in steam sterilised dry soil

radioactivity added. The amount of phorate remaining unmetabolised after 128 days in the soil was 24% of phorate added. The estimated rate constant of phorate decay under these conditions was 0.033, $0.025 \leq k \leq 0.041 \text{ day}^{-1}$ with confidence limits at 95%. This gave an estimated mean half-life of 21.3 days, so that the discrepancy between the residual amount, assuming first-order kinetics, was approximately 24% against 1% under normal conditions. These results indicate that steam sterilisation of the soil suppressed the formation of phorate sulphone completely. Although the amount of phorate available for hydrolysis was greater than in the control, steam sterilisation retarded the hydrolysis of phorate. This experiment suggested that steam-sterilisation affected the rate of phorate metabolism, but it is difficult to establish whether this was due to the destruction of micro-organisms in the soil or due to the changes in chemical and physical properties of the soil.

4.2 The Effect of Sterilisation of Soil on the Rate of Phorate Metabolism in the Dry Soil

This experiment was designed to investigate the role of microbial metabolism in sterile soil, when the soil was allowed to dry out. The purpose of this experiment was to eliminate any possible microbial contamination during the addition of sterile water to the incubated soil, and rule out the possibility of building up microbial population which may play a role in phorate metabolism (Lichtenstein and Schulz, 1964). The soil was sterilised as described previously, and moisture content was adjusted to 20% (w/w). It was then allowed to dry out. The rates and patterns of phorate metabolism, and phorate sulphoxide and sulphone formation under these conditions are shown in Figure 16. The steam-sterilisation retarded subsequent phorate

metabolism in the soil, and phorate sulphoxide was the only major oxidised metabolite, which accumulated at a maximum level of 9% at 128 days. When sterile soil was allowed to dry out, the rate of phorate sulphoxide formation decreased considerably compared with the sterile soil under moist conditions, and also non-sterile soil allowed to dry out. Phorate sulphone accumulated to a maximum level of < 1.0% of the phorate initially added to the soil. The amount of phorate remaining unmetabolised after 128 days in the soil was 15% of the phorate initially added. The estimated rate constant of phorate decay under these conditions was $0.048, 0.041 \leq k \leq 0.056 \text{ day}^{-1}$. This gave an estimated mean half-life of 14.2 days. The discrepancy between the residual phorate remaining after 128 days and that expected, assuming first-order kinetics, was approximately 15% against 10% in the control experiment. These results indicate that the micro-organisms may not be a very important factor in phorate metabolism since phorate hydrolysed faster under these conditions than under sterile moist conditions.

4.3 The Effect of Inoculation of Sterile Soil with Pure Cultures of *Bacillus megaterium* on the Rate of Phorate Metabolism in the Soil

This experiment was designed to investigate the effect of *Bacillus megaterium* inoculation into steam-sterilised soil on the rate of phorate metabolism in comparison with the rate of phorate metabolism in sterile soil. The moisture content was maintained at 20% (w/w) throughout the experiment and then the soil incubated at 25°C. The rates and patterns of phorate metabolism and phorate sulphoxide and sulphone formation in steam-sterilised inoculated soil are shown in Figure 17.

The sterile soil inoculation with *B. megaterium* increased the

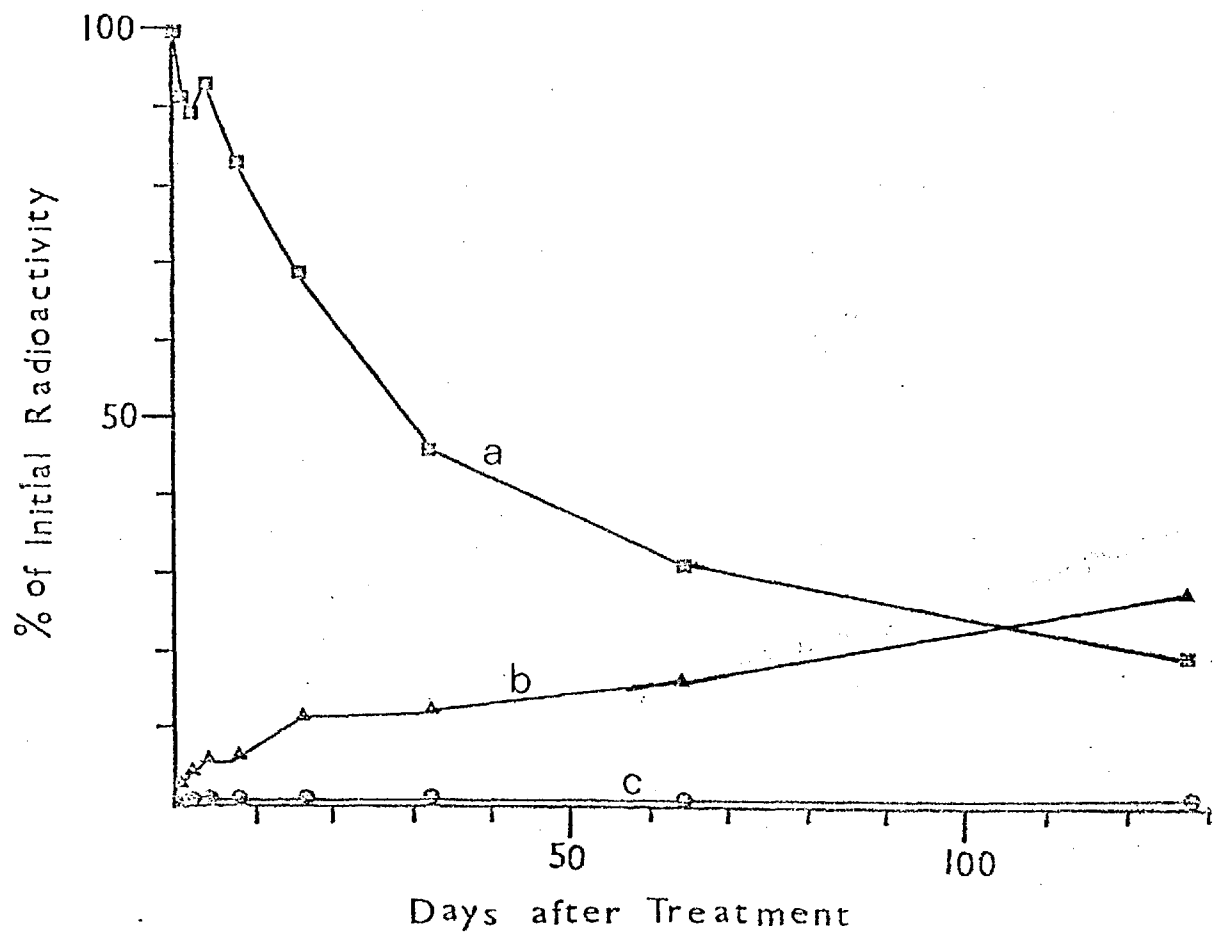


Figure 17: The metabolism of phorate in steam-sterilised soil inoculated with *Bacillus megaterium*

metabolism of phorate for 32 days comparing with sterile soil, but was slower than the control. Phorate sulphoxide was the only major oxidised metabolite produced. It was produced in the same pattern as in steam sterile soil but persisted at a higher level after 128 days (25% of the initial radioactivity added). The amount of phorate sulphone accumulated up to 128 days was < 1.0% of the initial radioactivity added. The estimated rate constant of phorate decay under these conditions was 0.021, $0.014 \leq k \leq 0.026 \text{ day}^{-1}$. This gave an estimated mean half-life of 33.1 days, so that the discrepancy between the residual phorate remaining after 128 days and the expected amount according to first-order kinetics was 20% against 24% under sterile conditions.

These results indicate that the inoculation of sterile soil with *Bacillus megaterium* cells did not increase phorate metabolism considerably, and *B. megaterium* was not an important factor in metabolising phorate in the soil under these conditions. No test was made to test whether *B. megaterium* would have survived in soil or not.

4.4 The Effect of Partial Sterilisation by Streptomycin Sulphate on Rate of Phorate Metabolism in the Soil

The soil was partially sterilised using an aqueous solution of streptomycin sulphate. It was chosen as sterilising agent because of its effectiveness as a bacteriostatic antibiotic. It is extremely water soluble, relatively stable in aqueous solution of pH 3 - 7 and to heat (Goodman, 1962). The soil was treated with two different concentrations of streptomycin sulphate at 0.05% and 0.1% of streptomycin to soil air-dried weight. The soil moisture content was initially adjusted at 20% (w/w) and then the soil was incubated at 25°C. No water was added during incubation and the soil was left to dry out. The rates and patterns of

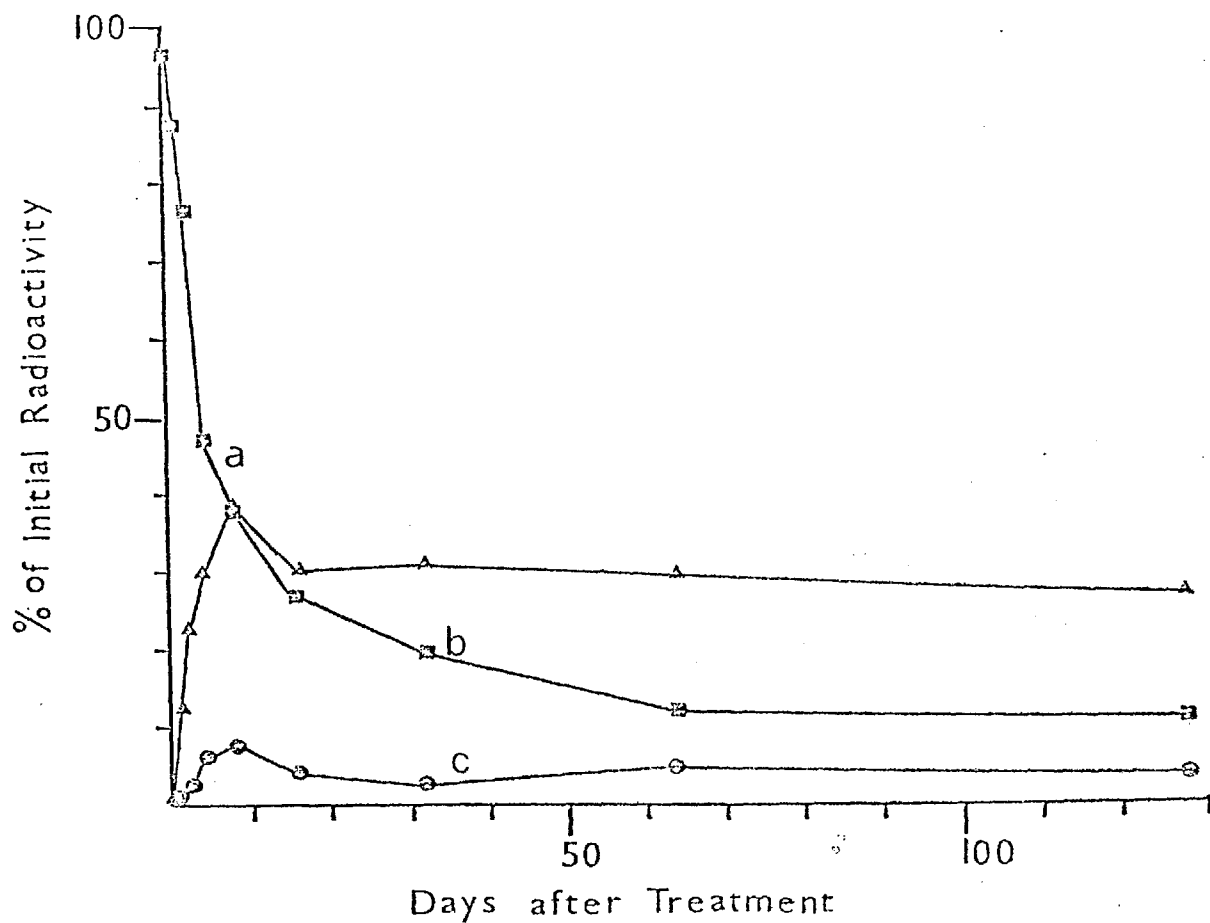


Figure 18: The metabolism of phorate in soil treated with (0.05%) streptomycin sulphate

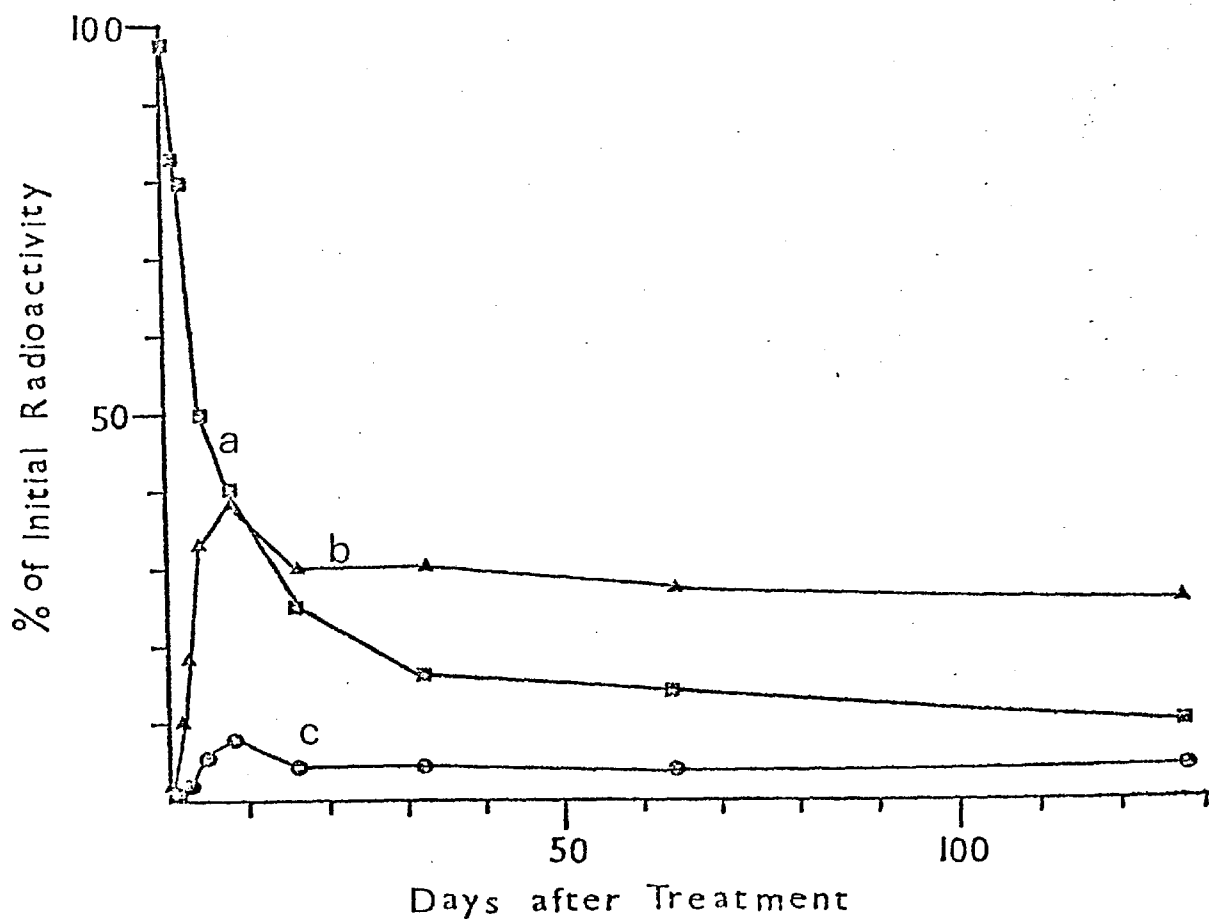


Figure 19: The metabolism of phorate in soil treated with (0.1%) streptomycin sulphate

phorate metabolism and phorate sulfoxide and sulphone formation are shown in Figures 18 and 19.

The rate of phorate metabolism and phorate sulfoxide and phorate sulphone formation were similar at both concentrations of streptomycin. The partial chemical sterilisation did not retard phorate metabolism in the soil significantly, although phorate sulfoxide was formed in greater amounts than in the control experiment. The phorate sulfoxide concentration reached a maximum after 8 days and then persisted in the soil at a level of 26% of total radioactivity added. The addition of streptomycin sulphate increased the formation of phorate sulphone slightly compared with the control, although this effect is probably not statistically significant. The rate constant of phorate decay in the soil treated with 0.05% streptomycin sulphate was 0.083, $0.069 \leq k \leq 0.097 \text{ day}^{-1}$ with confidence limits at 95%. This gave an estimated mean half-life of 7.6 days. The rate constant of phorate decay in the soil treated with 0.1% streptomycin sulphate was 0.079, $0.064 \leq k \leq 0.095 \text{ day}^{-1}$. This gave an estimated mean half-life of 8.7 days.

These results could be interpreted as suggesting that the addition of streptomycin sulphate inhibited the hydrolysis of oxidised metabolites, resulting in greater persistence and accumulation of phorate sulfoxide and sulphone in the soil treated with streptomycin sulphate than in the control soil under the same moisture conditions, although the difference was small.

4.5 The Effect of Pre-Incubation of the Soil before Treatment on the Rate of Phorate Metabolism in the Soil

It has been reported that the metabolism of some pesticides

in the soil showed a lag period before the metabolism of the pesticide in question started. The explanation for this lag period was that this time was required to build up an effective microbial population to metabolise the pesticide (Audus, 1949,1951). The present experiment was designed to investigate the effect of soil pre-incubation on the rate of phorate metabolism in the soil.

The soil moisture content was adjusted at 20% (w/w) and then the soil was incubated for 7 days before treatment with radioactive phorate. Following treatment with radioactive phorate, the moisture content was maintained at a constant level throughout the experiment.

The rates and patterns of phorate metabolism and phorate sulphoxide and sulphone formation are shown in Figure 20. Soil pre-incubation for 7 days before treatment increased the rate of phorate metabolism compared with the control. Phorate sulphoxide and phorate sulphone were both formed in considerable amounts. Phorate sulphoxide accumulated to a maximum 27% of the radioactivity added at 8 days, and then declined rapidly, persisting in the soil at 4% of the initial radioactivity added at 128 days. Phorate sulphone accumulated progressively to 25% level of the initial radioactivity added at 128 days. The rate constant of phorate decay in the soil under these experimental conditions was 0.12, $0.10 \leq k \leq 0.134 \text{ day}^{-1}$ with confidence limits at 95%. This gave an estimated mean half-life of 5.8 days.

These results indicate that the soil pre-incubation for 7 days before treatment did not affect the rate of phorate metabolism significantly.

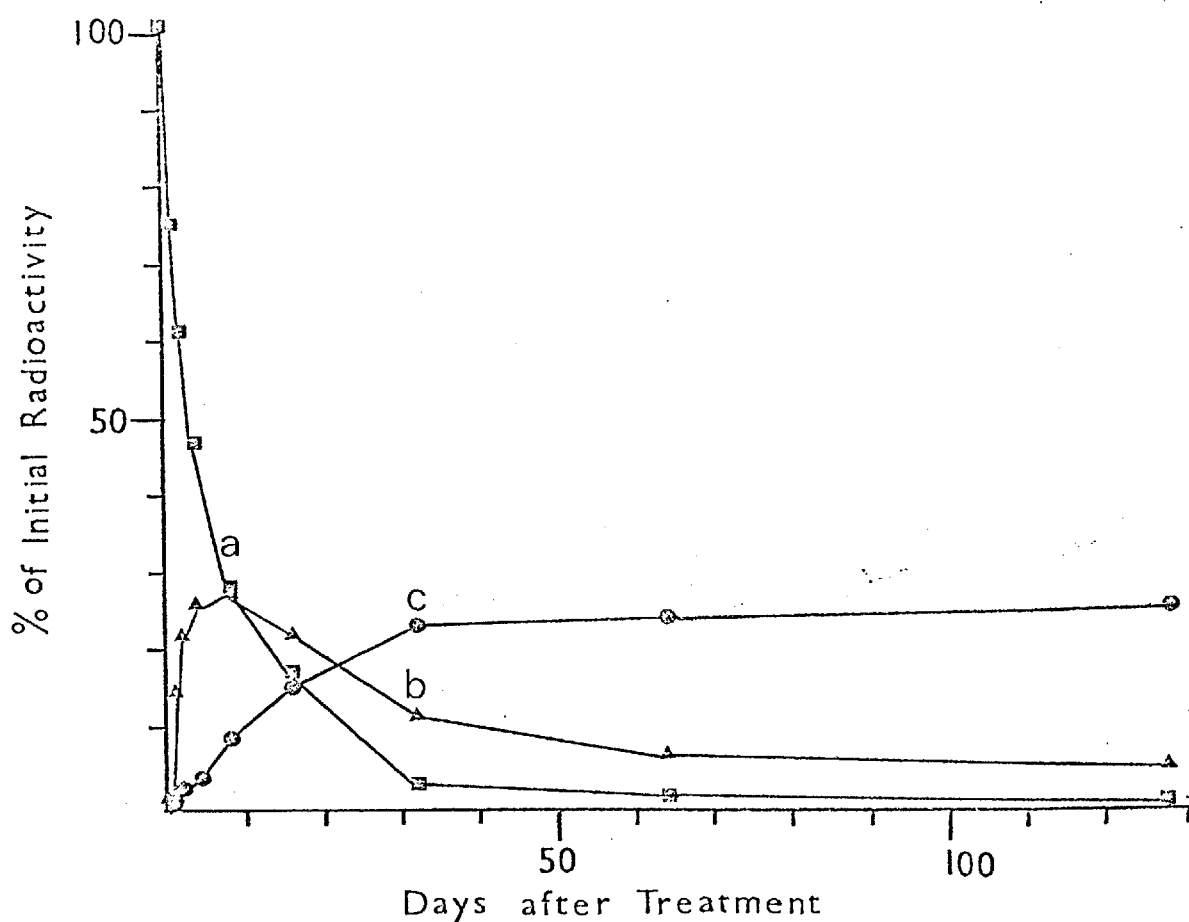


Figure 20: The metabolism of phorate in soil pre-incubated for 7 days before treatment with phorate

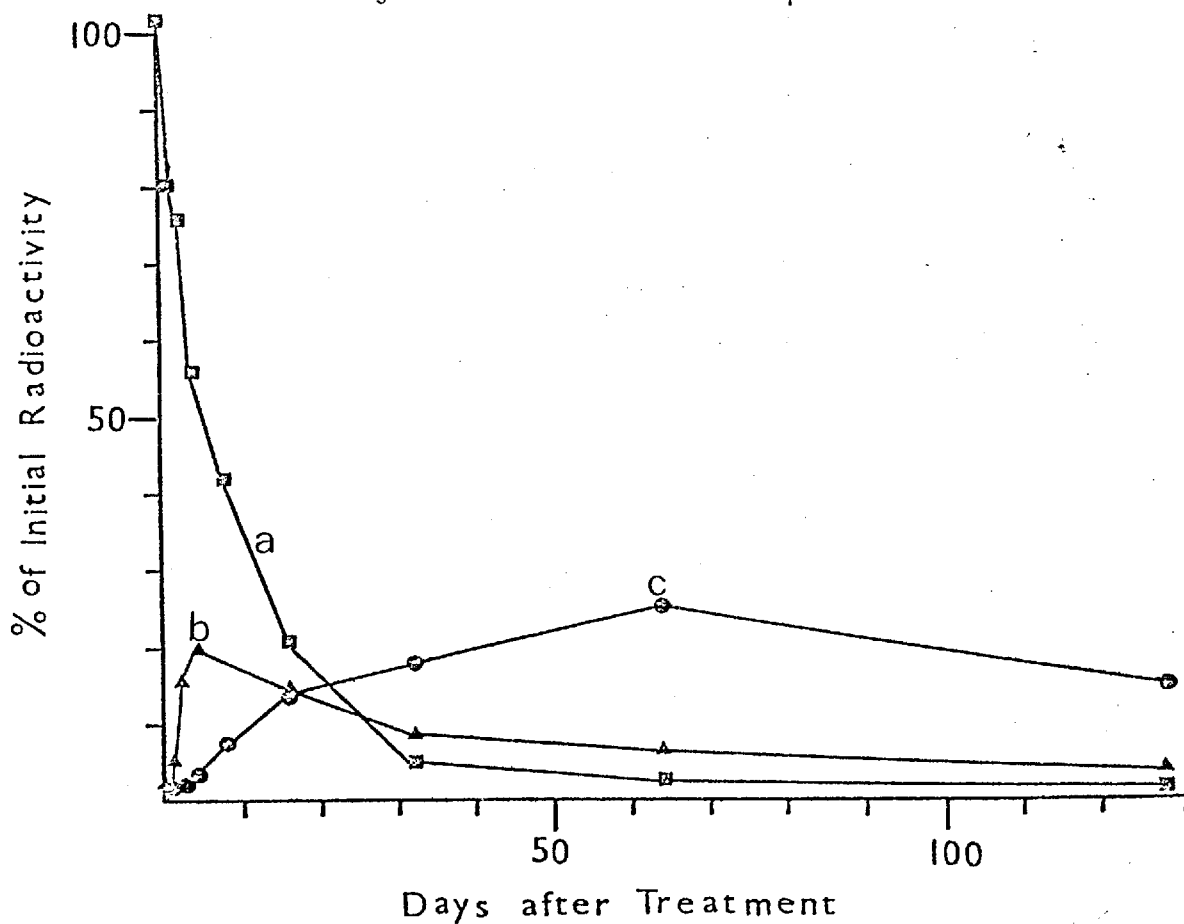


Figure 21: The metabolism of phorate in soil pre-incubated for 7 days before treatment with glucose solution

4.6 The Effect of Addition of Glucose to the Soil on the Rate of Phorate Metabolism in the Soil

The present experiment was conducted to investigate the effect of adding an additional source of carbon to the soil on the rate of phorate metabolism. Soil moisture content was adjusted to 20% (w/w) using a 0.1 M glucose solution instead of distilled water and the soil was incubated for 7 days before treatment with radioactive phorate at 25°C. The glucose solution (0.1 M) was used to maintain the moisture content constant throughout the experiment. The rates and the patterns of phorate, phorate sulfoxide and phorate sulphone decay or accumulation in the soil pre-incubated and treated with glucose solution are shown in Figure 21. The rate of phorate metabolism was similar to that of the control as well as in water pre-incubated soil. However, the rates of oxidised metabolite production and hydrolysis were different. Phorate sulfoxide accumulated to a maximum 19% of the initial radioactivity added at 4 days, then declined and persisted at 4% at 128 days. Phorate sulphone accumulated at the same rate as the control or water pre-incubated soil up to 64 days, then declined to only 15% of the initial radioactivity added. The rate constant of phorate decay in the soil under these experimental conditions was 0.095, $0.105 \leq k \leq 0.133 \text{ day}^{-1}$. This gave an estimated mean half-life of 7.2 days.

These results indicate that the addition of glucose considerably increased the rate of phorate sulfoxide and sulphone hydrolysis in the soil.

5. METABOLISM OF PHORATE IN PURE CULTURES OF BACTERIA

Four of the bacterial strains isolated from the soil by the enrichment technique described earlier were screened for their ability to catalyse the oxidation and hydrolysis of phorate in aqueous culture media. It was hoped that a strain could be found with a simple pattern of oxidative phorate metabolism so that the effect of soil absorption of the bacterial population on the subsequent metabolism of phorate in the soil could be readily interpreted. The strains used were: *Bacillus megaterium*, *Bacillus cereus* var. *mycoides*, *Corynebacterium* Sp., and *Achromobacter* Sp. The techniques of bacterial culture and incubation with radio-labelled phorate have been described in the experimental section.

The results of analysis of the incubation medium and bacteria for phorate and its oxidised and hydrolysed metabolites showed that recovery of radioactivity from all incubations, including controls, ranged from 82-89% (see Appendices). This loss was traced to the transfer and filtration operations during work-up of the chloroform extract of the incubation medium. Since this loss affected both phorate and oxidised products equally, it was considered legitimate to apply a correction by distributing 14.1% of the applied activity (the difference between the mean recovery of all analyses, and 100%) between phorate and its oxidised metabolites in proportion to their determined levels in the incubation medium, and adding these percentages to the determined values. Analysis of the variance of the recovery figures over the five experiments gives no statistical support to the view that the lower recovery figures from the *B. megaterium* and *B. cereus* incubations belong to a different population than the recovery figures of the other three incubations. Figures 22 to 26 show the variation in the corrected radioactivity

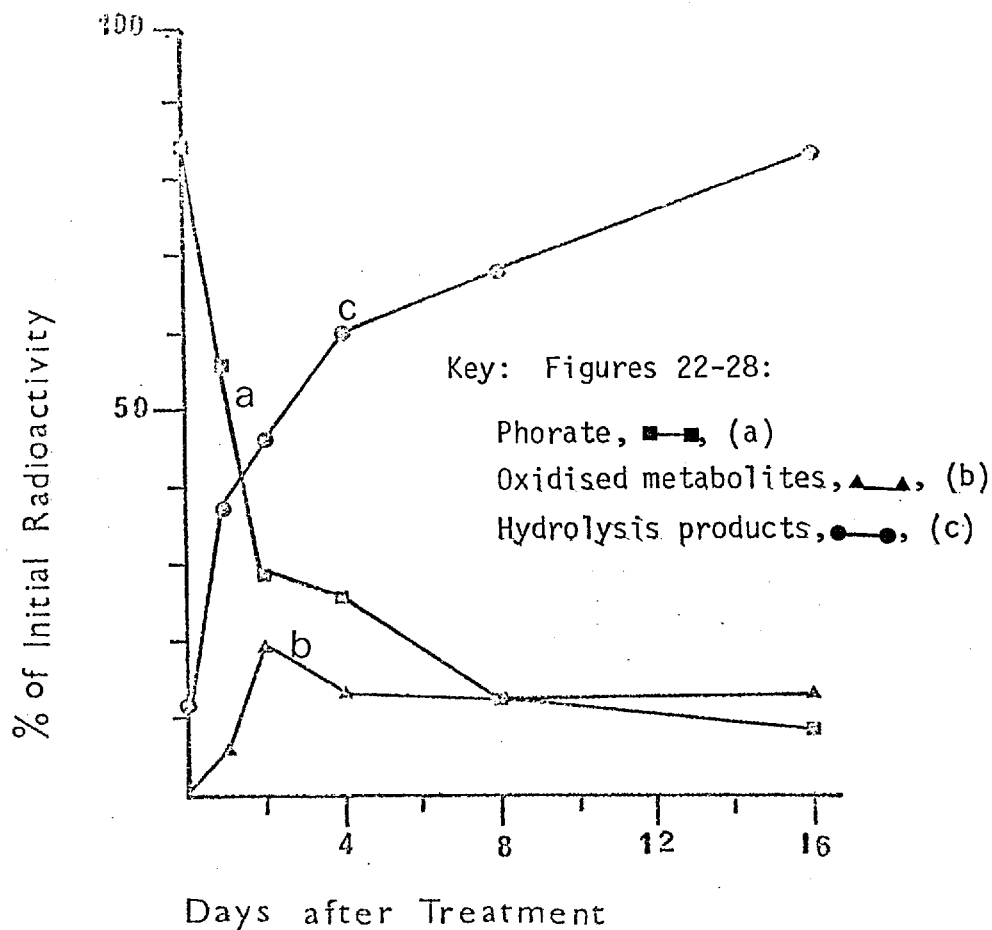


Figure 22: The metabolism of phorate in a pure culture of *Bacillus megaterium*

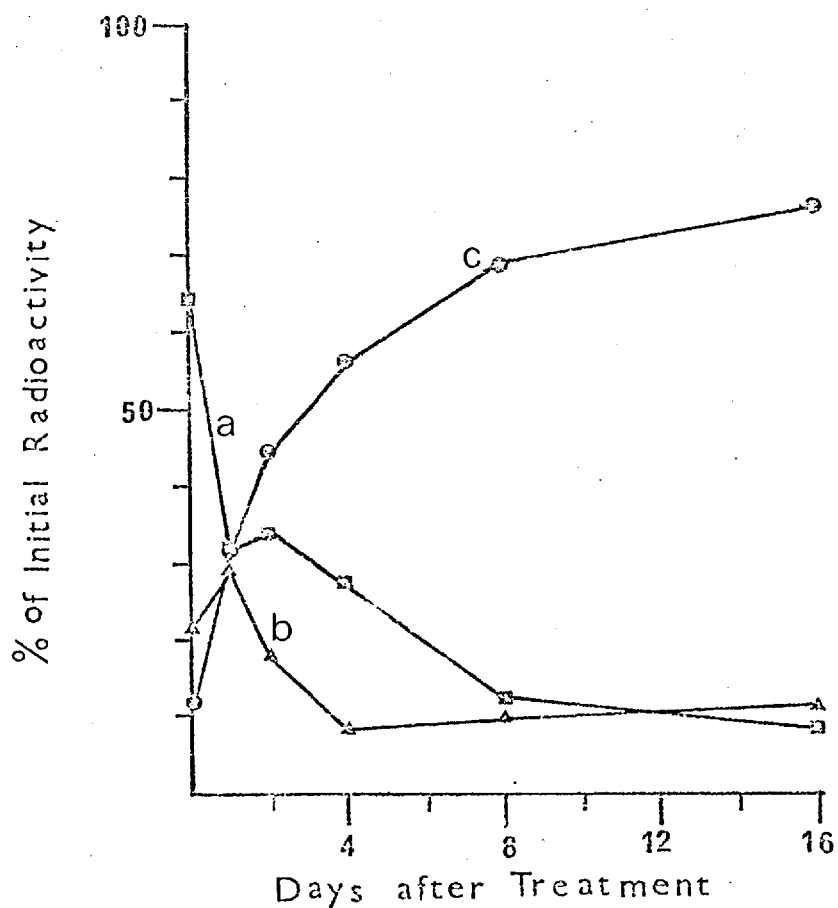


Figure 23: The metabolism of phorate in a pure culture of *Bacillus cereus* var. *mycoides*

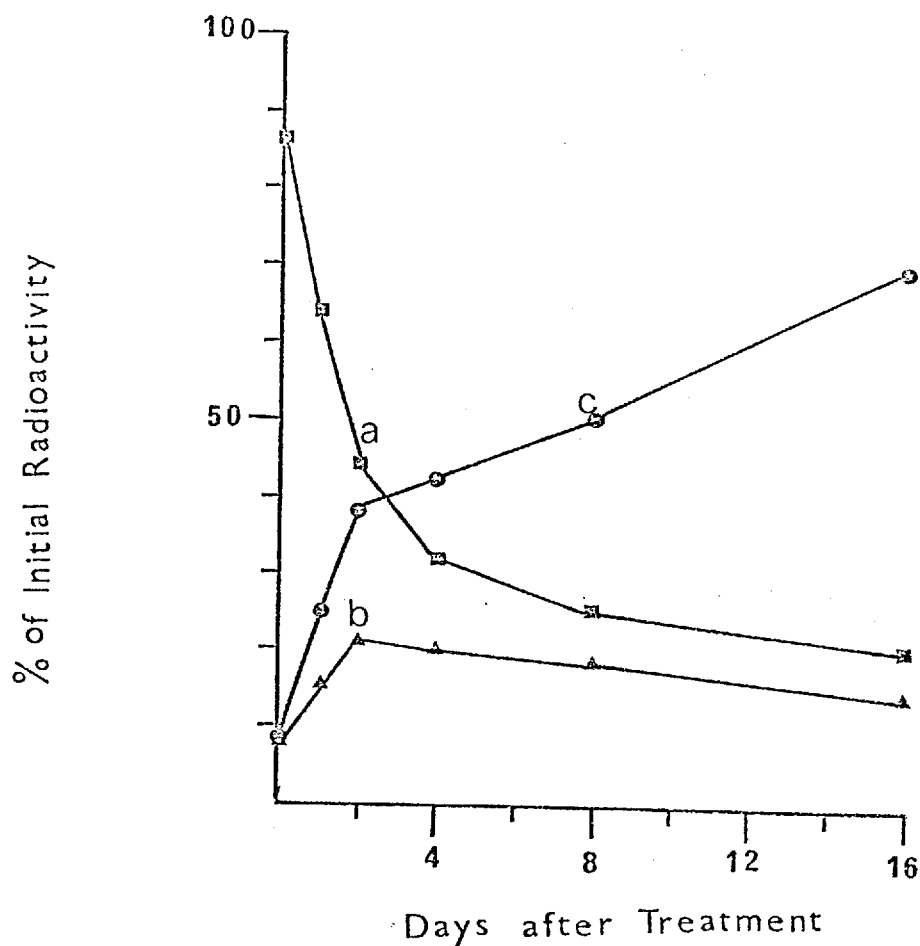


Figure 24: The metabolism of phorate in a pure culture of *Corynebacterium* Sp.

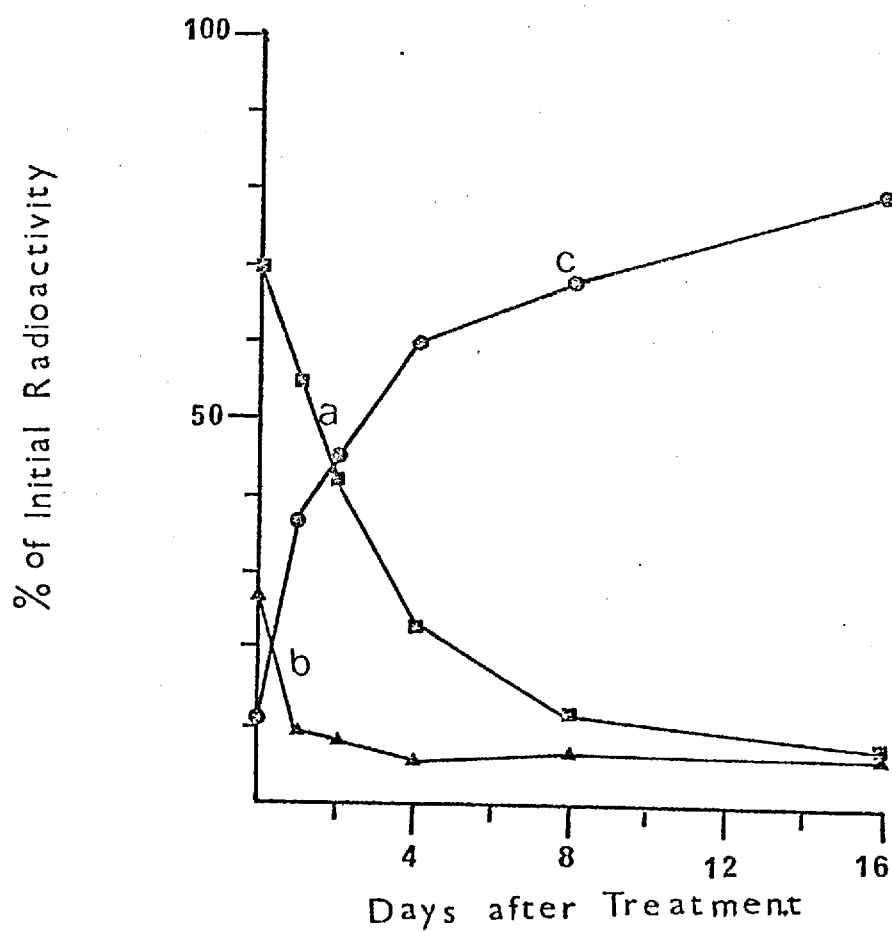


Figure 25: The metabolism of phorate in a pure culture of *Achromobacter* Sp.

levels of phorate, total oxidised products and total hydrolysis products, for the control and bacterial incubations, expressed as percentages of the radioactivity of phorate added to the medium.

Bacterial populations potentially may accelerate the rate of hydrolysis of phorate and/or the rate of hydrolysis of any or all of its oxidised products. They may also accelerate the rate of oxidation of phorate to its various oxidised products. These possible effects would give rise to different patterns of variation of phorate metabolism from the control. The results are best interpreted by considering the simplest first-order kinetic model of the system and investigating the effect of these possible accelerations in the rate of different metabolic routes in the presence of the bacteria:

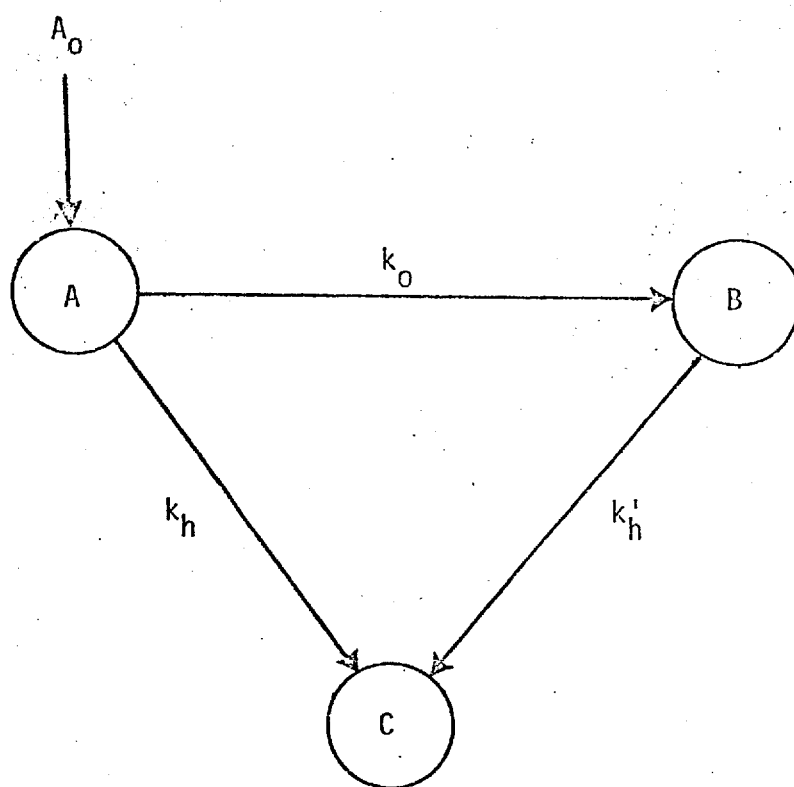


Figure 27 : A model of metabolism of phorate in pure culture

In the model (Figure 27), A represents the total amount of phorate

in the system 'bacteria plus culture medium', equal to A_0 at zero time; B represents the total amount of oxidised products in the system, and C represents the total amount of radioactive hydrolytic products.

Metabolism is assumed to follow first-order kinetics, and k_h and k'_h are respectively the first-order rate constants for phorate hydrolysis and a composite of the rate constants for hydrolysis of the various oxidised products which have been assumed in the model to be represented by a single compartment since, experimentally, oxidative metabolism of phorate consisted predominantly of phorate sulfoxide formation. k_o is the sum of the rate constants for oxidation of phorate to the range of oxidised products. A standard treatment of this system (le Patourel, 1975) gives:

$$A = A_0 e^{-(k_h + k_o) t}$$

$$B = A_0 \frac{k_o}{(k_o + k_h - k'_h)} (e^{-k'_h t} - e^{-(k_o + k_h) t})$$

$$C = A_0 \left\{ 1 - \frac{k_o}{(k_h + k_o - k'_h)} e^{-k'_h t} - \frac{(k_h - k'_h)}{(k_h + k_o - k'_h)} e^{-(k_o + k_h) t} \right\}$$

Graphs showing the general form of these equations for arbitrarily chosen values of k_h , k'_h and k_o are shown in Figures 29a, 29b and 29c. It is clear that the general form of the curves are similar to the experimental data. An increase in the rate of either phorate oxidation or hydrolysis would lead to a more rapid initial decline in phorate levels in the incubation medium; an increase in the rate of phorate oxidation would lead to a higher maximum value of oxidation products in the medium and also to a decrease in the length of time before the maximum was reached; an increase in the rate of phorate hydrolysis or oxidised products hydrolysis would lead to a more rapid accumulation of total hydrolysis

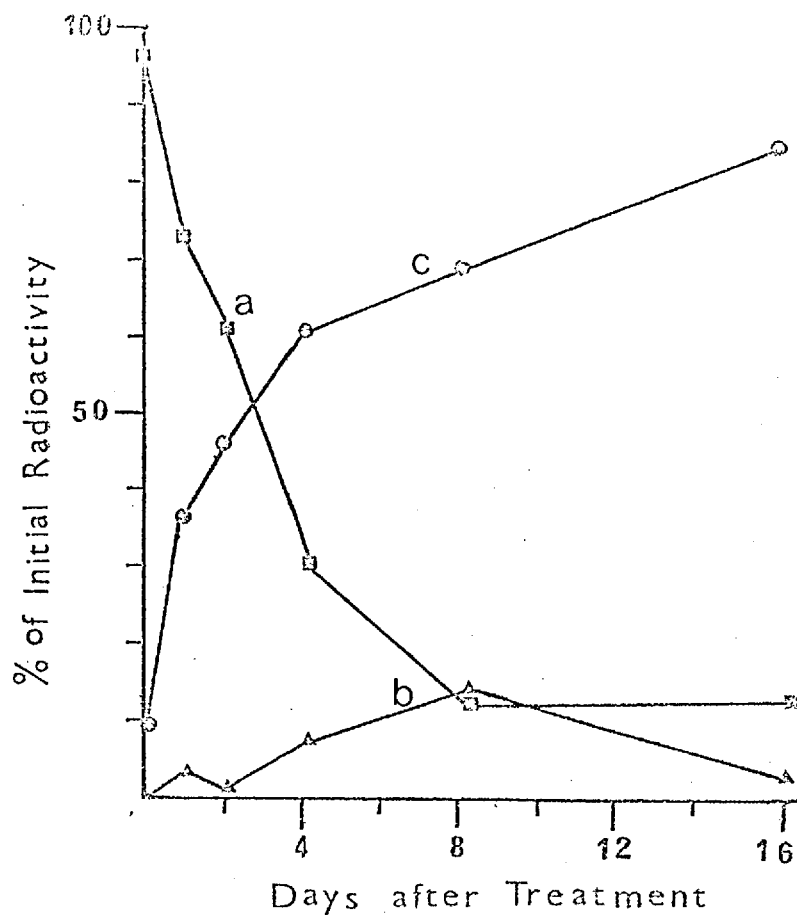


Figure 28: The metabolism of phorate in media (control)

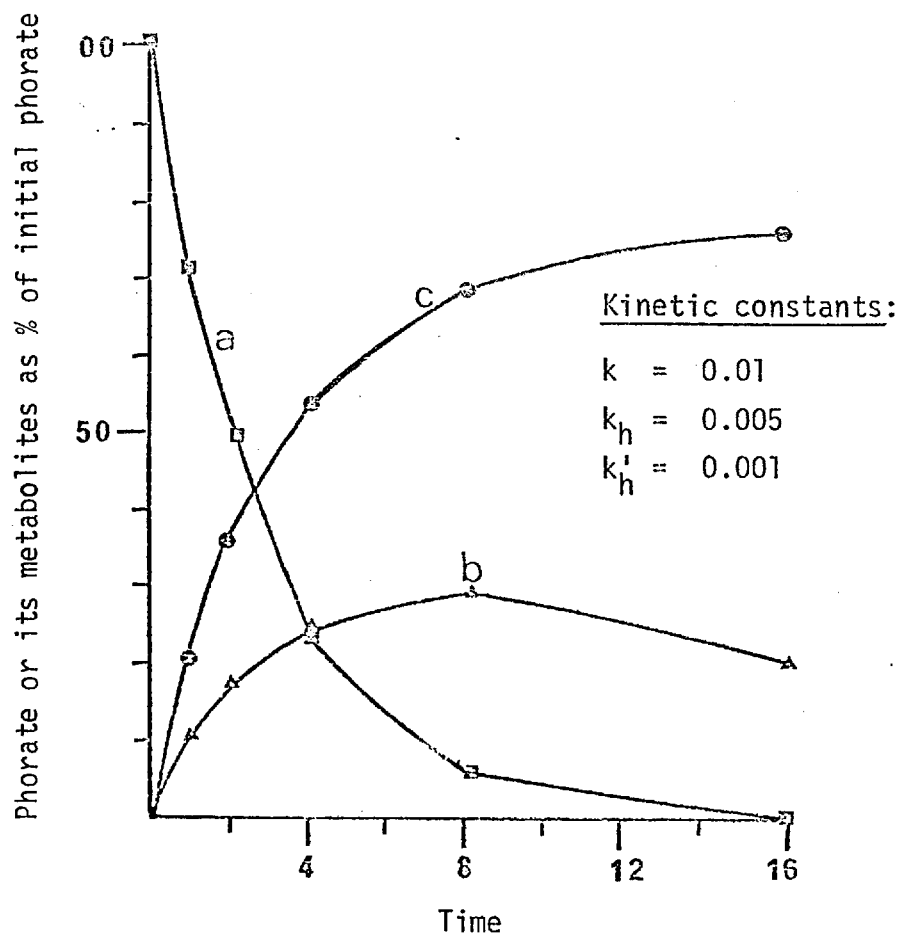


Figure 29: The metabolism of phorate in pure cultures represented as first-order kinetics

products.

It can be seen from Figures 22 to 26 that all four bacterial cultures catalyse phorate metabolism (hydrolysis and/or oxidation) since the slopes of the graph of phorate loss from the medium are steeper over the first four days than that of the control. However, the most prominent feature of these curves is the differing extents to which loss of phorate from the system occurred during the brief equilibration period (about 10 minutes) prior to analysis at zero time in three of the bacterial populations. The reason for this phenomenon is not clear, but since it was almost exclusively associated with oxidation of phorate to its sulfoxide rather than to hydrolysis, a rapid and complete reduction of some oxidised species liberated by the bacteria into the incubation medium, followed by a slower process of oxidation in the bacteria themselves, is suggested. *Corynebacterium* Sp. showed a rate of phorate metabolism which was, at least after four days, slower than that of the control, an effect most probably due to inactivation of the phorate at lipophilic sites within the bacteria.

The principal oxidation product of phorate accumulating in all four bacterial systems was the sulfoxide metabolite. As indicated earlier, phorate sulfoxide is more stable hydrolytically than either phorate or phorate sulphone at pH 6.4 in phosphate buffer, and since the 'oxon' metabolites of phosphorodithioate triesters are usually less stable hydrolytically than their parent compound (Heath, 1961; Eto, 1974), it is probable that it is the most stable metabolite of phorate to hydrolysis. Catalysis of phorate oxidation by the bacterial populations can be seen both by the criterion of increased accumulation of oxidised products and by the time taken to reach the maximum level in the system, relative to the control. There is insufficient evidence to decide

whether the slight difference in rate of production of hydrolysis products by the bacterial systems was due to catalysis of phorate hydrolysis or whether it was due to catalysed oxidation to some hydrolytically unstable metabolite such as phoratoxon. Detailed analysis of the hydrolysis products might answer this question, but was not attempted in this study. Oxidative pathways to products other than phorate sulfoxide were insufficiently rapid to lead to detectable accumulations in the control medium, while phorate sulphone was detected at low concentrations in all bacterial systems, and 'oxon' metabolites showed low accumulation in the *Corynebacterium* Sp., *Achromobacter* Sp., and *Bacillus cereus* incubations.

As a result of these analyses, *B. megaterium* appeared to be the most suitable organism for incorporation into a soil/bacterium/pesticide system since although not having the capacity to catalyse phorate oxidation as efficiently as *B. cereus* oxidation was almost exclusive to the sulfoxide metabolite.

6. ADSORPTION OF PHORATE BY ASHURST SOIL, CLAY (SURREY POWDER) AND SAND

Adsorption studies were carried out to determine the distribution of phorate between a buffered aqueous phase and either Ashurst soil, clay (Surrey powder No. 1) or quartz sand. Similarly, the distribution of phorate sulfoxide and phorate sulphone between aqueous buffer and Ashurst soil was investigated.

The extent of oxidation of phorate by soil, clay or sand was checked by column chromatographic analysis for the first concentration in the adsorption isotherm determination (2 ppm). The analysis of the radioactive material extracted from soil, clay or sand and from the aqueous buffer following a three hour equilibrium period after the addition of radioactive phorate indicated the presence of small amounts of phorate sulfoxide in the extracts from both adsorbents and the aqueous phase, but most of the radioactivity was recovered in the form of phorate. Because of the small amounts of oxidised metabolites recovered from both extracts, it was felt unnecessary to perform chromatographic separation of metabolites prior to the radioactivity assay, and the radioactivity was determined in extracts directly. The results of chromatographic analysis are summarised in Table 14.

Table 14 : The Effect of Adsorption on Phorate Oxidation (3 Hours)

Adsorbent	Composition of Adsorbent Extract (%)			Composition of Aqueous Extract (%)		
	P = S,S	P = S,S0	P = S,S0 ₂	P = S,S	P = S,S0	P = S,S0 ₂
Ashurst Soil	97.9	0.7	1.4	98.0	0.4	1.4
Clay (Surrey Powder No.1)	94.1	2.5	3.5	93.9	2.5	3.6
Sand	98.9	0.0	0.0	98.7	0.3	0.9

The effect of adsorbents on the hydrolysis of phorate was determined throughout the experiments for all concentrations and all adsorbents. Table 15 shows the effect of adsorption on phorate hydrolysis over a three hour equilibration period.

Table 15 : The Effect of Adsorption on Phorate Hydrolysis (3 Hours)

Adsorbate	Adsorbent	% Hydrolysis Products*
Phorate	Ashurst Soil	5.3 + 0.63
Phorate	Clay (Surrey Powder No.1)	9.5 ± 0.76
Phorate	Sand	9.5 ± 0.10
Phorate	Control (no adsorbent)	2.2
Phorate sulphoxide	Ashurst Soil	0.0
Phorate sulphoxide	Control (no adsorbent)	0.07
Phorate sulphone	Ashurst Soil	1.1
Phorate sulphone	Control (no adsorbent)	0.1

* Hydrolysis products were calculated from the mean of the hydrolysis products produced in the six concentrations used for adsorption determination, while control with no adsorbents was calculated from the results obtained in section 2.

In separate experiments using all three adsorbents, the extent of adsorption of the mixture of phosphorothioic acids formed by hydrolysis was determined. In each case the acid mixture remained almost completely (> 98%) in the aqueous phase.

In most pesticide adsorption studies on the soil, the adsorption isotherm is usually measured by shaking an aqueous solution of pesticide

of known concentration with a known mass of soil until equilibrium is reached. The concentration in the water is then determined by analysis, while that on soil particles is inferred from the difference between the initial and final concentrations. In this study, an attempt was made to check that this inference is correct by measuring the radioactivity in the soil or other adsorbent extracts. The results obtained indicated that the sum of the radioactivity in the centrifuged pellet and in the aqueous phase at equilibrium was lower than the amount of phorate or its metabolites added. The loss was therefore attributed to irreversible adsorption on the adsorbents and to a lesser extent on the glass walls of the centrifuge tubes. Since the acid hydrolysis products of phorate are adsorbed only to a very small extent by any of the adsorbents, it seems most probable that the irreversibly adsorbed radioactivity was in the form of phorate or unhydrolysed metabolites.

In order to characterise the adsorptive capacity of different adsorbents for phorate, the results in this study are represented as isotherms. An adsorption isotherm is a mathematical expression relating the quantity of material (X , μg) adsorbed per unit weight of adsorbent (m , g) to the concentration of the solute remaining in the solution at equilibrium at a given temperature (C , $\mu\text{g cm}^{-3}$). Thus an adsorption defines a distribution ratio between the solid and liquid phases for the solute in question.

In this study the empirical Freundlich equation was used to express the distribution ratio. This equation is expressed as:

$$X/m = K C^{1/n}$$

Regression analysis was carried out to test the linearity of the

relationship between the amount of adsorbate per unit of adsorbent and the concentration at equilibrium, and it showed that the data could be fitted to a simple linear relationship with a correlation coefficient > 0.95 over the range of initial phorate concentrations studied. The regression analysis gave an estimated value of the slope of the regression line and its associated 95% confidence limits. Calculation of the value of the estimated intercept of the regression line on the y-axis showed that in each case the intercept deviated from the origin, but lay within the 95% confidence intervals of the intercept.

Table 16 gives the estimated slope (K) calculated from the amount of phorate extracted from the adsorbents at equilibrium (A) and also the estimated slope (K) calculated from the amount of phorate lost from the aqueous phase, taking into account the amount hydrolysed during the equilibration process (B) assuming $n = 1$.

The data was plotted in the standard manner as its log transform:

$$\log \frac{X}{m} = \log K + \frac{1}{n} \log C$$

and $1/n$ and $\log K$ determined as, respectively, the slope and the intercept on the ordinate axis. Regression analysis was used to obtain values and 95% confidence limits for both K and n simultaneously. Tables 17 and 18 summarise the values of K and n with their respective confidence limits at 95% obtained by these two methods of calculation.

Table 16 : K Values for Phorate and Metabolites on Soil, Clay and Sand

Adsorbate	Adsorbent	K at 95% Confidence Limits (A)	K at 95% Confidence Limits (B)
Phorate	Ashurst Soil	11.45 ± 1.21	15.80 ± 1.75
Phorate	Clay (Surrey Powder No.1)	42.16 ± 8.14	44.54 ± 7.95
Phorate	Sand	0.64 ± 0.27	4.11 ± 0.90
Phorate sulphoxide	Ashurst Soil	0.53 ± 0.11	0.53 ± 0.11
Phorate sulphone	Ashurst Soil	1.53 ± 0.64	2.02 ± 1.12

Table 17 : K and n Values of Adsorption Isotherms

Adsorbate	Adsorbent	1/n at 95% Confidence Limits (A)	K at 95% Confidence Limits (A)
Phorate	Ashurst Soil	1.04 ± 0.10	10.35 ± 1.20
Phorate	Clay (Surrey Powder No.1)	1.22 ± 0.11	28.69 ± 2.67
Phorate	Sand	1.13 ± 0.40	0.42 ± 0.19
Phorate sulphoxide	Ashurst Soil	0.96 ± 0.16	0.57 ± 0.15
Phorate sulphone	Ashurst Soil	1.08 ± 0.40	1.07 ± 0.52

Table 18 : K and n Values of Adsorption Isotherms

Adsorbate	Adsorbent	1/n at 95% Confidence Limits (B)	K at 95% Confidence Limits (B)
Phorate	Ashurst Soil	0.99 ± 0.08	15.67 ± 1.72
Phorate	Clay (Surrey Powder No.1)	1.2 ± 0.11	30.6 ± 2.97
Phorate	Sand	1.59 ± 0.41	1.14 ± 0.55
Phorate sulphoxide	Ashurst Soil	0.96 ± 0.16	0.57 ± 0.15
Phorate sulphone	Ashurst Soil	0.86 ± 0.48	2.77 ± 1.50

The results obtained from these studies suggest that the adsorption process has only a small effect on the oxidation of phorate during the three hour period needed for equilibration. The effect of adsorption on the hydrolysis of phorate was examined, and it was found that the rate of phorate hydrolysis during the three hour period was greater in the case of clay and sand than soil. Phorate sulphoxide was stable to hydrolysis and no hydrolysis products were detected, while only traces of hydrolysis products were detectable in the case of phorate sulphone.

The values of K decrease in the order clay > soil > sand indicating that, as expected, phorate adsorption decreases with decreasing 'clay plus organic matter' content. Results obtained from adsorption isotherms for phorate sulphoxide and phorate sulphone on Ashurst soil showed that the order of increasing adsorption on soil was phorate sulphoxide < phorate sulphone < phorate.

These results indicate that the calculation of adsorption constants needs very careful interpretation because the measurements can be seriously affected by both the decomposition of pesticide and irreversible adsorption. The difference in K values calculated by estimating loss of material from solution or by estimating extent of recovery from adsorbent is attributed to irreversible adsorption and, to a much lesser extent, to adsorption on the glass walls of centrifuge tubes. Preliminary experiments indicated that the extent of adsorption of phorate was the same on sterile and unsterile soils.

7. THE EFFECT OF SORPTIVE INTERACTION BETWEEN *Bacillus megaterium* AND SOIL PARTICLES ON THE DEGRADATION OF PHORATE

The nature of the sorptive interactions between micro-organisms and soil particles and the effect of such interactions on the ability of either material to adsorb or catalyse the metabolism of pesticides have not been investigated (Hance, 1970). Adsorption of micro-organisms onto the surface of soil particles might affect the ability of micro-organisms to catalyse metabolic processes in two conflicting ways. There is likely to be a greater population density of micro-organisms on surfaces than in solution, so metabolic activity could be enhanced by soil adsorption of the pesticide, increasing the concentration of pesticide in regions rich in micro-organisms. Conversely, the adsorption of soil or clay particles onto the surface of bacteria might both reduce the surface area of bacteria available to take up pesticide from solution and also reduce the adsorptive surface area of clay or soil. In this case, the sorptive interaction between soil and bacteria might tend to reduce the total adsorptive capacity of mixture and also reduce the amount of phorate metabolised within a given time.

The experiments in this section were designed to investigate whether or not the adsorption of phorate onto mixtures of clay or soil and the bacterium *Bacillus megaterium* was additive, and also to examine the effect of adsorbing clay or soil particles onto the surface of bacteria upon the ability of bacteria to catalyse the metabolism of phorate. Quartz sand with negligible adsorptive capacity was used as a comparison.

Aliquots of standard cultures of *B. megaterium* (20 cm³, containing 1.74 mg cm⁻³ dry weight) were used in these studies. This volume of culture medium was chosen to give a reasonable bacterial mass with which to study the sorptive interaction between the micro-organisms and soil

particles. A three hour incubation period was chosen in all these experiments in order to obtain comparable results with the previous experiments on adsorption of phorate onto pure adsorbents. Column chromatographic analysis was carried out on bacterial extracts, as well as on the aqueous extracts. The hydrolysis products were determined by measuring the radioactivity in a 1 cm³ sample of extracted aqueous solutions.

7.1 Uptake, Accumulation and Metabolism of Phorate from Solution by *Bacillus megaterium*

B. megaterium cells were vigorously shaken with increasing concentrations (2-12 ppm) of aqueous phorate solutions (10 cm³) for a three hour period. Bacterial cells were separated from aqueous solutions by centrifugation and the supernatant solution was extracted and analysed for metabolites of phorate. The bacterial pellet was also extracted and analysed for phorate metabolites on or in the cells.

The results obtained indicated that phorate sulphoxide was the sole unhydrolysed phorate metabolite in the supernatant buffer and small amounts of phorate remained unadsorbed by the bacteria. The amount of phorate sulphoxide produced after 3 hours was proportional to the initial phorate concentration over the concentration range tested. The extent of phorate sulphone formation was < 2.0 % of added radioactivity (see Appendices). The analysis of bacterial extracts indicated that the radioactivity present on or in the bacteria was mainly in the form of phorate, only small amounts of phorate sulphoxide being detected together with traces of phorate sulphone. The total amount of hydrolysis products formed over the three hour incubation period also increased with increasing initial phorate concentration (see Appendices).

A regression analysis was carried out to test the linearity of the following relationships:

1. The relationship between the amount of phorate adsorbed by bacteria, and the initial phorate concentrations.
2. The total phorate sulphoxide in the bacteria plus supernatant and the initial phorate concentration.
3. The relationship between the amount of phorate adsorbed by bacteria and concentration remaining in the aqueous solution after a three hour incubation period.
4. The relationship between the amount of phorate sulphoxide adsorbed on or in the bacteria and phorate sulphoxide concentration which remained in the solution after a three hour incubation period.

The results obtained by the regression analysis of the above relationships are summarised in Table 19. These results indicated that all these relationships could be fitted to a simple linear relationship. The regression analysis gave an estimated value of the slope of the regression lines associated with 95% confidence limits of this slope (proportionality constant). A Student "t" test was used to estimate the probability that the intercept on the ordinate axis coincided with the origin (Table 19).

Table 19 : Regression Analysis Data for the Above Relationships

Relationship	Proportionality Constant at 95% Confidence Limits	r	Test of linearity	Intercept	Zero* Intercept
1	0.36 ± 0.14	0.960	$P < 0.01$	- 1.33	$P > 0.7$
2	0.42 ± 0.1	0.984	$P < 0.001$	- 9.3	$P > 0.001$
3	6.1 ± 4.6	0.879	$P < 0.05$	- 16.86	$P > 0.4$
4	0.08 ± 0.01	0.993	$P < 0.001$	0.21	$P > 0.1$

* Student's "t" test

These results show that the total uptake of phorate from aqueous solution by *Bacillus megaterium* was proportional to the initial concentration of phorate, that the production of phorate sulphoxide by bacteria was proportional to the initial phorate concentration and that the ratio of phorate or phorate sulphoxide adsorbed by bacteria to phorate or phorate sulphoxide in buffered supernatant was constant. The confidence limits associated with the proportionality constant of the relationship between the amount of phorate adsorbed by bacteria and the concentration which remained in the aqueous solution were wide and this could be due to fluctuations of bacteria activity. All these proportionalities refer to a three hour incubation period.

7.2 Uptake, Accumulation and Metabolism of Phorate from Solution by Mixtures of *Bacillus megaterium* and Soil, Clay or Sand

This experiment was designed to investigate:

1. The assumption that adsorption of phorate was additive

when soil, clay or sand were mixed with *Bacillus megaterium* cells.

2. The effect of adsorption of bacteria on the solid phase on the activity of *B. megaterium* in metabolising phorate.

Bacterial cells were suspended in buffer (10 cm³) with increasing concentrations of phorate (2-12 ppm). Adsorbent (soil, clay or sand, 0.5 g) was added to the solution and shaken vigorously for a three hour period. The adsorbent-bacteria mixture was separated from the supernatant and extracted and analysed for phorate metabolites. The supernatant was similarly extracted and analysed.

The results were plotted in the form of Freundlich isotherms, and the linearity of the isotherms was checked statistically as in section 6 using regression analysis. It was found that the adsorption of phorate from solutions by bacteria plus soil, clay or sand mixtures followed the empirical Freundlich equation:

$$X/m = K C^{1/n}$$

where (X, µg) is the quantity of phorate adsorbed per unit weight of adsorbent (m, g) (the weight of the bacteria was ignored), C is the concentration of phorate in the solution at equilibrium (µg.cm⁻³), and K and n are the isotherm constants.

Table 20 shows the slopes (K) for the relation between X/m on C with 95% confidence limits, assuming n = 1.

Table 20 : K Values for Phorate on Bacteria-Adsorbent Mixtures

Bacteria-Adsorbent	K at 95% Confidence Limits
Bacteria-Soil	124.29 ± 22.67
Bacteria-Clay	115.38 ± 27.98
Bacteria-Sand	30.74 ± 8.5

The data were also plotted in the standard manner as their log transformation:

$$\log \frac{X}{m} = \log K + \frac{1}{n} \log C$$

K and n values were obtained by linear regression of log X/m on log C.

The values of K and n for mixtures of bacteria plus soil, clay or sand with respective 95% confidence limits are summarised in Table 21.

Table 21 : K and n Values for Phorate on Bacteria-Adsorbents Mixtures

Bacteria-Adsorbent	1/n	K
Bacteria-Soil	1.064 ± 0.11	110.7 ± 9.0
Bacteria-Clay	0.9951 ± 0.18	133.7 ± 19.1
Bacteria-Sand	1.079 ± 0.24	95.8 ± 18.40

These results indicated that the presence of bacteria cells in the aqueous phase increased the magnitude of adsorption of phorate on soil, clay or sand. If the adsorption of phorate by adsorbent plus bacteria is additive, the mixture of adsorbent and bacteria should adsorb the same amount of phorate after a three hour incubation period as would be adsorbed by the two components separately. This was checked by calculating proportionality constants (by regression analysis) between the amount of phorate adsorbed by each component separately and the amount of phorate which remained in solution after three hours. The sum of the proportionality constants for bacteria and for each adsorbent were compared in turn with the proportionality constants found experimentally for the mixtures of bacteria and adsorbent. The proportionality constants are shown in Table 22 together with respective 95% confidence limits.

Table 22 : Proportionality Constants for Phorate on Bacteria, Adsorbents and Mixtures of Bacteria-Adsorbents

Adsorbent	Proportionality Constant
Bacteria	6.13 $\pm$ 4.6
Soil	0.61 $\pm$ 0.06
Soil-Bacteria	6.91 $\pm$ 1.10
Clay	2.33 $\pm$ 0.46
Clay-Bacteria	5.77 $\pm$ 1.40
Sand	0.041 $\pm$ 0.016
Sand-Bacteria	5.30 $\pm$ 1.4

Table 23 shows the comparison between the proportionality constants calculated on the basis of additive adsorption by the bacteria and adsorbent and the proportionality constants determined experimentally, together with their respective 95% confidence limits.

Table 23 : Proportionality Constants Calculated and Found Experimentally

Adsorbent Mixture	Proportionality Constant (Calculated)	Proportionality Constant (Found Experimentally)
Soil-Bacteria	6.74 ± 4.62	6.19 ± 1.1
Clay-Bacteria	8.46 ± 4.63	5.77 ± 1.4
Sand-Bacteria	6.16 ± 4.60	5.30 ± 1.4

The results in Table 23 indicate that, although the mean of the proportionality constants of calculated values differed from those obtained experimentally, the respective 95% confidence intervals for the calculated values were very wide. This was due to the wide confidence limits associated with the bacterial adsorption, so that the confidence limits of both experimentally obtained and calculated proportionality constant values overlapped. Therefore, no firm conclusion could be drawn as to whether the adsorption of phorate by bacteria-adsorbent mixtures was additive or not.

7.3 The Effect of Addition of Adsorbents on Phorate Metabolism by *Bacillus megaterium*

Since phorate sulphoxide was the sole oxidised metabolite in

solution or adsorbed onto bacteria cells (or bacteria plus adsorbent), bacterial metabolic activity can be expressed in terms of total phorate sulphoxide produced from phorate, represented by the sum of the amounts of phorate sulphoxide in solution and phorate sulphoxide adsorbed.

Regression analysis was carried out to test the linearity of the relationship between the initial phorate concentration ($\mu\text{g } 10 \text{ cm}^{-3}$) and total amount of phorate sulphoxide produced (μg) and showed that the data could be fitted to a simple linear relationship with a correlation coefficient > 0.933 .

Table 24 shows the proportionality constants obtained by regression analysis between initial phorate concentration and the amount of phorate sulphoxide produced, with respective 95% confidence limits.

Table 24: Proportionality Constant for Phorate Sulphoxide Formation

Adsorbent	Proportionality Constant
Bacteria	0.426 ± 0.1
Bacteria-Soil	0.13 ± 0.07
Bacteria-Clay	0.182 ± 0.06
Bacteria-Sand	0.26 ± 0.02

These results indicated that the addition of adsorbents affected the rate of phorate oxidation by *Bacillus megaterium*. Phorate oxidation to phorate sulphoxide decreased in the order bacteria > bacteria plus sand > bacteria plus clay > bacteria plus soil, showing that increased adsorption

of phorate lowers the availability of the chemical for bacterial metabolism, even when the bacteria are themselves in intimate contact with the particles of adsorbent.

DISCUSSION

The Metabolism of Phorate in Ashurst Soil at 25°C and 20% Moisture . Metabolites and Recovery

Phorate sulphoxide and phorate sulphone were found to be the only oxidised phorate metabolites in the soil when incubations were carried out under controlled laboratory conditions. The analysis of soil extracts by tlc did not reveal the presence of phoratoxon or its sulphoxide or sulphone. These results coincided with those obtained by other investigators in a wide range of soil types (Getzin and Chapman, 1960; Backe and Lisk, 1966; Lichtenstein, 1966; Lichtenstein et al, 1974; and, Suett, 1971, 1974, 1975). Because these were the only metabolites detected in the soil, the only recovery experiments carried out used pure radioactive phorate, phorate sulphoxide and phorate sulphone. It was found that the percentage recovery from the soil at 25°C increased in the order: phorate sulphoxide < phorate < phorate sulphone. The low recovery of phorate sulphoxide from the soil could be due to its polarity and the formation of strong ion-dipole bonds with the ionised hydrophilic components in the soil organic matter. The percentage recovery of phorate and its oxidised metabolites from the soil incubated at 5°C followed the same pattern, but the percentage recovered was higher in the case of phorate and phorate sulphoxide, while no significant difference was found in the percentage of phorate sulphone recovered. This higher percentage recovery at low temperatures might be expected because the rate of irreversible adsorption, dependent on diffusion into micropores in clay and organic matter, and also chemisorption, will be slower at lower temperatures.

Rate of Metabolism

The experiment showed a rapid decline of phorate concentration in the soil at 25°C. This decline was due to oxidation of phorate to phorate sulfoxide and to its hydrolysis to non-toxic metabolites. No lag period was observed in the decay process. The rate of phorate sulfoxide accumulation was rapid during the initial incubation, and the concentration of this metabolite reached its maximum around 16 days after phorate application and then declined due to oxidation to phorate sulphone and hydrolysis to its corresponding non-toxic metabolites. On the other hand, the rate of formation of phorate sulphone was slow but the metabolite accumulated steadily and persisted at a high level in the soil 128 days after phorate application to the soil. The persistence of phorate sulphone in the soil at this high level was due to its relative stability to the hydrolysis, the only route for its dissipation from the soil. Phorate oxidation in the soil may thus be interpreted as a two-stage process consisting of initial oxidation to phorate sulfoxide followed by a slower oxidation to the sulphone. An additional direct route of phorate oxidation to phorate sulphone cannot be ruled out, but seems less likely.

The present results could not confirm precisely the work by Getzin and Chapman (1960), Suett (1971), and Lichtenstein et al (1974), who reported that oxidation of phorate to its sulfoxide and sulphone was the major route of phorate metabolism in the soil, while hydrolysis was less important. They reported that hydrolysis was the only route of phorate metabolism in quartz sand, and thus attributed oxidation of phorate in soil to micro-organisms which were absent in the quartz sand.

In a complex system such as soil which possesses a very high surface area and active biological components, the reactions by which phorate is

metabolised could be catalysed purely chemically or by biological systems, or both. Alexander (1969) reported that if extracellular chemical breakdown occurs, whether uncatalysed, or catalysed by inorganic or extracellular organic chemicals, it begins immediately after application of pesticide to soil and the rate of breakdown either remains constant or declines with time. By contrast, microbial breakdown does not begin until the microbial population build-up, and then the rate of chemical loss increases with time. The results obtained from this experiment did not show clearly whether the metabolism of phorate was chemical or biological in nature and no firm conclusion could be drawn. Therefore, further experiments, under different physical and biological conditions, were conducted.

Kinetics of Phorate Metabolism in the Soil

Because of the complexity of the soil system, it is somewhat unreasonable to expect that there will be a simple rate law governing the kinetics of degradation of pesticides in the soil. However, as studies have indicated that the degradation of many pesticides such as diazinon (Sethunathan and MacRae, 1969), atrazine (Armstrong et al, 1967), malathion (Konrad et al, 1969), cioldrin (Konrad and Chesters, 1969), atrazine, simazine, ametryne, bromacil, and terbacil (Zimdahl et al, 1970), and azinophosmethyl (Yaron et al, 1974) approximately followed first-order kinetics in the soil, it seemed reasonable to test the adequacy of first-order kinetics as a first approximation for the metabolism of phorate in the soil.

The kinetics of intermediate product formation are complicated by simultaneous formation and degradation (Hamaker, 1972). Although it is, in principle, possible to estimate rate constants of formation of

metabolites formed in sequence from a common precursor by analysing their concentrations as a function of time after initial application of the precursor (Atkins, 1968) such methods lead to rapid accumulation of errors (Glass and de Garreta, 1967). Therefore, in the present studies, both phorate and its metabolites were added to the soil and their rates of decline in the soil were examined separately. The results obtained showed that the rates of phorate, phorate sulphoxide and phorate sulphone decline in the soil could be approximated by first-order kinetics only up to 16 days after their application to the soil. It seems that the most probable reason for the deviation from first-order kinetics after 16 days is due to the irreversibly adsorbed fraction of chemical becoming less available for chemical attack by either oxygen or water than the chemical which is reversibly adsorbed on the soil surfaces. Alternatively, there is the possibility of changes in the microbial population with time, caused by depletion of food resources. Another possibility is that the reactive sites may become poisoned by the products of this and other reactions going on in the soil (Hamaker, 1972).

Attempts to separate the composite rate constants for metabolism of phorate or phorate sulphoxide into oxidative and hydrolytic components using non-linear curve fitting techniques demonstrated that variation between replicates was too great in the present experiments to allow accurate estimates to be made. Furthermore, the sensitivity of the estimated value of k_0 and k'_0 to error in the estimates of the decay constants for phorate and its sulphoxide in the soil is at a maximum when, as in the present case, the values of the decay constants are closely similar in magnitude (equations (ix) and (xii)).

The analysis of metabolic kinetics by a first-order rate model showed that although the concepts were useful in gaining understanding of

the patterns of accumulation and decay, there are considerable practical limitations to attempts to fit kinetic data to non-linear theoretical equations. An alternative approach to the problem would have been to investigate anaerobic decay rates of phorate and its sulfoxide and sulphone in the soil and to use this data to separate the rate constants for oxidation and hydrolysis of the compounds under aerobic conditions.

The kinetics of hydrolysis of phorate, its sulfoxide and sulphone, were studied in buffered aqueous solutions at a comparable pH to that of the soil. It was found that the rate of hydrolysis of these compounds followed first-order kinetics in aqueous solutions. The rate constants of hydrolysis of phorate and its oxidised metabolites in the buffered aqueous solutions decreased in the order: phorate > phorate sulphone > phorate sulfoxide. The rate constant of phorate hydrolysis in a buffered aqueous solution was found to be 2.25 times greater than the rate constant of phorate degradation in the soil, while the rate constants of phorate sulfoxide and sulphone degradation in soil were respectively 10.45 and 2.13 times greater than their corresponding rate constants of hydrolysis in buffered soil-free systems. These results indicated that soil adsorption retarded the rate of phorate hydrolysis and at the same time catalysed the rate of phorate sulphone hydrolysis. However, it was difficult to establish the effect of soil adsorption on phorate sulfoxide hydrolysis since this metabolite was simultaneously formed and then both oxidised and hydrolysed in the soil. These findings could be explained by the high adsorptive capacity of the soil for phorate and small adsorptive capacity for its sulfoxide and sulphone (see later discussion), and they also suggest that hydrolysis took place primarily in the soil solution. The adsorption of phorate on the soil surface decreased its concentration in the soil solution, while the oxidised metabolites

remained mainly in the soil solution. Furmidge and Osgerby (1967) studied the kinetics of phosphorylated atrazine degradation in the soil. They reported that the decomposition of the compound took place solely in solution and the sorption decreased the rate of degradation by removing chemical from the solution. However, Armstrong et al (1967) demonstrated that the hydrolysis of atrazine in aqueous solution and in soil perfusion systems followed first-order kinetics at the same pH and reported that the rate of hydrolysis was much faster in the soil than in the soil-free system. The same conclusion has been drawn by other authors who studied the effect of adsorption on the rate of hydrolysis of organophosphorus insecticides. Konrad and Chester (1969) reported that the rate of ciodrin degradation in the soil was much faster than in the soil-free systems. Konrad et al (1969) reported the same findings with respect to the hydrolysis of malathion. The results of the above studies indicate that whether adsorption catalyses or retards hydrolytic reactions depends a great deal on the structure of the compound, the type of bond being hydrolysed, and the nature of the soil surface.

Although it has been reported (see Introduction) that most organophosphorus insecticides are less stable in alkaline solution, the results obtained from the present study showed that the rate of hydrolysis of phorate in buffered aqueous solutions was almost independent of the pH in the range 3-9. However, as expected, the rate of phorate hydrolysis in aqueous solutions at low temperature (5⁰C) was much slower (1:0.05) than the rate at 25⁰C.

The Effect of Physical Factors on the Rate of Phorate Metabolism in the Soil

The rate of phorate decline in the soil treated with hydroquinone was decreased mainly due to the inhibition of its oxidation to phorate

sulphoxide. Similarly, phorate sulphoxide oxidation to phorate sulphone was greatly reduced. The inhibition of phorate and phorate sulphoxide oxidation lead to their persistence at higher levels than in the control. These results may have practical importance relating to the control of persistence and biological activity of phorate in the soil. The rate of decline of phorate in the soil treated with butylated hydroxytoluene was similar to that of the control. Phorate sulphoxide accumulated at lower levels than in the control but persisted almost at the same level as in the control 64 days after phorate application to the soil. Phorate sulphone formation in the soil was greatly reduced under these conditions. These findings suggest that the mechanisms of inhibition of phorate oxidation by HQ was different from that of BHT, and since HQ (a water soluble antioxidant) inhibited the oxidation process to a greater extent than BHT, they could indicate that phorate oxidation takes place primarily in the soil solution. BHT (a lipophilic compound) could have penetrated the soil micro-organisms and interfered with the redox system in the cells. These results are similar to the findings of Lichtenstein et al (1963) who reported that sesamex, a methylenedioxyphenyl synergist, and autoxidation inhibitor, blocked the epoxidation of aldrin and dieldrin and increased its persistence in the soil. They suggested that sesamex may either have inhibited the enzymes responsible for the biological oxidation of aldrin or reduced the number of micro-organisms containing these enzyme systems.

The effect of soil moisture on the rate of phorate metabolism was studied by leaving the soil to dry out after addition of phorate at 20% moisture content. The patterns and the rates of phorate metabolism and

phorate sulphoxide and sulphone accumulation were similar to that of the control up to 8 days after phorate application. The decrease of moisture content after 16 days greatly retarded both the hydrolysis and oxidation processes of phorate and its metabolites, so that the levels of phorate sulphoxide and sulphone metabolites in the soil remained almost constant thereafter. The rate of phorate decline decreased more slowly than the rate of its decline in the control. These results clearly indicate that soil moisture content plays an important role in determining the rate of both oxidation and hydrolysis of phorate and its oxidised metabolites. Lichtenstein (1966) reported that the soil moisture influences pesticide breakdown directly or indirectly by affecting the microbial population in the soil. The increased persistence of phorate and phorate sulphoxide in the dried soil was due to the decrease in the rate of hydrolysis. Evidently, the water content of air-dry soil, while far exceeding the concentration of pesticide in the soil, was unavailable for hydrolytic attack on the pesticide, and also the oxidation of the compounds required the presence of free water in the soil.

When phorate was incubated in the soil at 5°C, its rate of decline was considerably retarded, and the levels of phorate sulphoxide in the soil rose to a higher level than at 25°C. Phorate sulphone levels remained low throughout the experiment. These effects may be attributed to the relative temperature coefficients of the rates of phorate or phorate sulphoxide hydrolysis and oxidation in the soil. In buffered solution, the ratio of the hydrolytic rate constants for phorate hydrolysis was 1:0.05 (25°C:5°C). If the oxidation process is less temperature-dependent than hydrolysis, then the observed pattern of metabolite accumulation would be expected. Suett (1975) observed a more rapid

oxidation (i.e. increased accumulation of oxidised metabolites with time) of phorate and disulphaton under field conditions in warm weather, and supposed that oxidation of these compounds was primarily a micro-biological process, inhibited at temperatures below 6-7°C. It is possible that phorate sulfoxide oxidation is partly or entirely microbially catalysed, since sulphone accumulation was inhibited both at a low temperature and by steam or partial chemical sterilisation (see below).

Soil pH could have an effect on the metabolism of phorate, either directly or indirectly by affecting the degree of adsorption by soil and the activity of the microbial population. When the soil pH was artificially adjusted to two different values (pH 3 and pH 9), the rate of phorate metabolism was slightly slower than in the control. This slower rate of phorate metabolism was accompanied by a slower accumulation of its oxidised metabolites. However, the differences between the patterns of phorate metabolism in the soil at pH 3, pH 9 and natural pH were small and generally confirmed the view that hydrolysis had only a slight pH dependence within this range.

The Effect of Micro-Organisms on the Rate of Metabolism of Phorate in the Soil

When phorate was applied to steam-sterilised soil, with the soil moisture content kept constant, its rate of decline was considerably retarded, and it persisted in the soil at a much higher level than that of the control 128 days after phorate application. Phorate sulfoxide levels rose slowly, and this metabolite persisted in the soil at a much higher level than in the control, while the formation of phorate sulphone almost ceased. These results indicate that steam-sterilisation affected both the rates of oxidation and hydrolysis of phorate and

phorate sulfoxide. In an experiment in which the steam-sterilised soil was allowed to dry out, phorate metabolism and metabolite accumulation followed the same patterns, although unexpectedly phorate declined to a lower level than in the soil with constant moisture content. These results coincided with other work concerning the effect of steam-sterilisation on the rate of pesticide degradation in the soil (see Introduction).

Since phorate sulphone formation was inhibited, it is possible that the effect of steam-sterilisation on phorate metabolism was primarily due to physical and chemical changes in the soil properties which had a greater effect on phorate sulfoxide oxidation than on phorate oxidation, but the possibility of destruction of micro-organisms capable of selectively oxidising phorate sulfoxide to sulphone cannot be ruled out.

In an attempt to sterilise the soil without altering its physical and chemical properties, streptomycin sulphate, an antibiotic, was used as a soil sterilising agent. The results obtained from this experiment indicated a greater accumulation of phorate sulfoxide and sulphone in the soil than in the control under similar moisture conditions, while phorate declined at a similar rate to that of the control. These results indicated that the addition of streptomycin sulphate inhibited the rate of hydrolysis of phorate oxidised metabolites, but no firm conclusion could be drawn as to whether this effect was due to the effect of streptomycin sulphate on the micro-organisms in the soil or not. Hattori (1973) suggested that most antibiotics are likely to be adsorbed by soil colloids or otherwise inactivated fairly quickly by microbes in the soil. Krasil'nikov (1961) reported that basic antibiotics such as streptomycin are strongly adsorbed by a soil.

Talbert et al (1970) reported that *Bacillus* species which are

common in soil were the major factor governing the hydrolysis of the methyl ester of Amiben herbicide in the soil. To test whether *Bacillus* species were dominant in the metabolism of phorate, high concentrations of *Bacillus megaterium* cells were inoculated into steam-sterilised soil. The results obtained indicated that phorate decline and phorate sulphoxide accumulation followed the same pattern as in steam-sterilised soil. The rate of phorate decline was faster than that in steam-sterilised soil, but phorate sulphoxide accumulated at lower levels. No phorate sulphone was formed, as observed in uninoculated steam-sterilised soil. These results could suggest that the soil inoculation with *B. megaterium* cells slightly increased the rate of phorate and phorate sulphoxide hydrolysis, but no significant effect on the rate of oxidation was observed. Flashinski and Lichtenstein (1974) demonstrated that the fungus *Rhizopus arrhizus* lost its capacity to degrade dyfonate when it was inoculated into the soil. It was capable of degrading dyfonate only when a glucose containing medium was added to the soil.

Since phorate metabolism in the soil did not exhibit the presence of a lag period typical of a process dominated by microbial-catalysed metabolism, an experiment was conducted with the soil moisture content adjusted to 20% and incubated at 25°C for 7 days before it was treated with phorate. It was felt that this incubation period would build up an active soil microbial population. The results obtained from this experiment indicated that phorate metabolism, and phorate sulphoxide, and phorate sulphone accumulation patterns in the soil were similar to that of control. However, phorate decline was slightly faster, and phorate sulphoxide accumulated in lower levels, while phorate sulphone accumulated at the same level as in control. The differences obtained in phorate metabolism from this experiment and control were not

significant and did not confirm that the soil pre-incubation contributed to the rate of phorate metabolism in the soil.

Microbial degradation of pesticides in soil can sometimes be accelerated by providing an additional source of energy to the soil. An experiment was performed with glucose which was added as an additional source of carbon. The results obtained indicated that the addition of glucose to the soil did not increase the rate of phorate decline in the soil but phorate sulfoxide and phorate sulphone accumulated at significantly lower levels than in the control. These results suggested that an increase in the rate of phorate sulfoxide and sulphone hydrolysis could be due to a build up of a microbial population capable of catalysing these hydrolysis processes in the soil fortified with glucose as an additional source of carbon. These results are similar to that of Lichtenstein and Schulz (1964) and Rajaram and Sethunathan (1975) who reported that the breakdown of parathion was speeded up when glucose was incorporated in the soil. Guenzi and Beard (1968) similarly reported that when ground alfalfa foligae was added to soil containing DDT, the breakdown of the pesticide was greatly accelerated.

The Effect of Sorptive Interaction Between Soil Micro-Organisms and Soil Particles on the Degradation of Phorate

The results obtained from the studies on the metabolism of phorate in Ashurst soil indicated that phorate was metabolised solely through thioether oxidation and hydrolysis. The rates of both reactions were affected by adsorption on the soil particles and possibly by the microbial population in the soil. Many research workers have investigated the effects of various physical parameters of the soil, such

as pH, moisture content, type of exchangeable ions, nature of the clay mineral, and the organic matter content, on the extent of adsorption of various pesticides. Relatively little has been reported concerning exactly what sort of interactions exist between the adsorbent and adsorbate and no work has been reported on the interaction between the adsorbent, and the micro-organisms, and the effect of this interaction on the rate of metabolism of the adsorbate either by chemical or biologically catalysed routes.

In order to study the interaction between the micro-organisms and the adsorbents on the rate of phorate degradation, several experiments were conducted. The metabolism of phorate by a bacterial strain in aqueous suspensions were investigated together with the effect of the interaction between the soil or other adsorbents and bacterial cells on the rate of phorate uptake and the activity of micro-organisms in metabolising phorate.

The Metabolism of Phorate in Pure Cultures

The results obtained from the experiments in which four bacterial strains were screened for their ability to degrade phorate in pure culture media indicated that the four chosen bacterial strains showed an ability to catalyse phorate hydrolysis and oxidation. Since phorate was oxidised mainly to phorate sulphoxide, the results have been expressed in terms of total oxidative metabolism represented by the sum of oxidised metabolites. The most common feature of phorate metabolism by *Bacillus cereus* var. *mycoide*, *Corynebacterium* Sp., and *Achromobacter* Sp. was that phorate oxidation occurred immediately after it was introduced to the incubation medium. This could be explained by assuming that these strains released extracellular catalysts or oxidants into the culture fluid and

that these chemicals caused immediate phorate oxidation. *Bacillus megaterium* was the only strain in which phorate showed a different pattern of oxidation. No oxidised products were detected at zero time but oxidised products accumulated gradually and then declined. This suggested that phorate, a highly lipophilic chemical, penetrated *B. megaterium* cell membranes and was metabolised inside the cells to more polar oxidised metabolites which then diffused into the medium. The control also demonstrated appreciable rates of phorate hydrolysis and oxidation in the absence of any added catalyst. The results on phorate metabolism by bacteria in aqueous cultures were somewhat disappointingly confused. Nevertheless, the suggestions advanced here must be considered only a first approximations, and re-investigation is needed in order to clarify the role of micro-organisms in phorate metabolism in pure cultures. However, the main purpose of this experiment was to choose a bacterial strain which was able to oxidise phorate with a simple oxidative pattern in order to study the interaction between the micro-organisms and soil components. *B. megaterium* was chosen because it was found to oxidise phorate mainly to phorate sulfoxide and, to a much lesser extent, to phorate sulphone. Also, phorate metabolism took place inside the cells and this facilitated interpretation of the studies on the interaction between the adsorption of bacterial cells on the soil components and their metabolic activity in degrading phorate.

Adsorption Isotherms

The results obtained earlier suggested that adsorption of phorate on the soil particles plays an important role in controlling the rate of the chemical alteration of phorate in the soil. Adsorption of phorate on soil particles reduces the concentrations of phorate in the soil

solution, thus removing part of the pesticide from hydrolytic attack.

Adsorption studies were carried out to investigate the adsorptive capacity of Ashurst soil for phorate and its sulfoxide and sulphone. For comparison, similar adsorption studies of phorate onto pure clay (Surrey Powder No. 1) and quartz sand were performed. The results obtained, with the statistical analysis, indicated that the adsorption isotherms could be adequately fitted to the empirical Freundlich equation. However, as reported by Graham-Bryce (1967) from his studies on disulphoton adsorption by soil, quantities adsorbed calculated from soil extracts gave better fit to Freundlich's equation than those calculated by difference between initial and final solution concentrations of pesticides. The constant 'n' in the isotherms were very close to unity and its confidence limits at 95% spanned unity. However, in the case of adsorption of phorate by sand and clay, the mean value deviated from the unity but with wider confidence limits at 95%. This indicated that adsorption of phorate by sand and clay probably deviated slightly from the Freundlich isotherm. K values for the three adsorbents were used as an indication of their adsorptive capacity. The values of K decreased in the order: clay > soil > sand, indicating that, as expected in view of low clay and organic matter content of Ashurst soil, phorate adsorption decreased in that order.

The results obtained from adsorption isotherms for phorate sulfoxide and phorate sulphone on Ashurst soil indicated that both could be adequately represented by Freundlich isotherms with mean 'n' values close to unity but with confidence limits at 95% wider than those associated with phorate n values. The adsorption of phorate and its sulfoxide and sulphone by Ashurst soil increased in the order:

phorate sulfoxide < phorate sulphone < phorate. The differences in phorate adsorption of Ashurst soil and that of its sulfoxide and sulphone could be explained by the differences in their polarities and water:organic solvent partition coefficients. Since phorate sulfoxide and phorate sulphone are more polar than phorate, it would be expected that at equilibrium more phorate sulfoxide and phorate sulphone will remain in the aqueous phase than in the case of phorate, a lipophilic molecule which was strongly adsorbed on the soil. The movement of a chemical in a soil is limited by the extent by which it is adsorbed by the soil. For a non-volatile chemical, this depends on the partitioning of the chemical between soil-water and soil-solids in an equilibrium, the position of equilibrium in any given soil being determined by the physical characteristics of the chemical. The metabolism of phorate in the soil via thioether oxidation and hydrolysis is similar to that of aldicarb (Bromilow, 1973) and the ratio of Q values (Lambert, 1967) for the parent and metabolites are similar in the two cases (1:0.05:0.1 for phorate, phorate sulfoxide, and phorate sulphone; 1:0.1:0.2 for aldicarb, aldicarb sulfoxide, and aldicarb sulphone (Bromilow, 1973)). It may reasonably be assumed that phorate sulfoxide will be the most mobile phorate metabolite in most soil types, and it has been demonstrated to be so in some cases (Lichtenstein et al, 1974). Prediction of phorate or metabolite concentration with depth in soils may be obtained by integration of information on kinetics of metabolism with isotherm data, but this has not been attempted in this study.

The Uptake, Accumulation and Metabolism of Phorate by *Bacillus megaterium* Cells

In the soil, the bacteria may be adsorbed onto the surface of soil

particles and this adsorption may affect their chemical activity. However, little is known whether, and in what manner, the chemical activity of bacterial cells would change when they are transferred from a free state (suspended in a liquid medium) to a liquid-solid interface.

An experiment was designed to investigate the uptake, accumulation and metabolism of phorate by *Bacillus megaterium* cells from solutions. The results obtained indicated clearly that *Bacillus megaterium* initially accumulated large amounts of phorate, and metabolised phorate to phorate sulphoxide. The analysis of the aqueous phase revealed that phorate sulphoxide was the major constituent after a three hour incubation period. The analysis of the bacterial pellet revealed that phorate was the major constituent accumulated in or on the bacterial cells during this period. This supported the earlier findings that phorate penetrated into the bacterial cells, and then diffused into the aqueous phase after it had been converted to phorate sulphoxide. The uptake of phorate from aqueous solution by *Bacillus megaterium* cells was found to increase linearly with the increase of phorate concentration. Similarly, phorate sulphoxide production increased linearly with the increase of the initial concentration of phorate. The relationship between the amount of phorate adsorbed by bacteria and the concentration remaining in the aqueous phase was found to be approximately linear, but confidence limits on the proportionality constant were very wide due to the fluctuation in bacterial activity. Finally, the relationship between the amount of phorate sulphoxide produced in the aqueous phase and that adsorbed on or in the bacteria was found to be linear. All these relationships were derived after three hour incubation periods. This indicated that bacteria could play a major part in determining the distribution and metabolism of phorate in aqueous suspensions.

The Uptake, Accumulation and Metabolism of Phorate by *Bacillus megaterium*
Plus Soil (or its Components)

Marshall (1971) reported that, as natural habitats for microbial growth and metabolism, soil must be evaluated on the basis of both the properties and the organisation of its solid constituents. Since the degree of sorption between micro-organisms and soil particles is broadly related to surface area and the surface charge properties of the particles, the colloidal fraction (clays and organic matter) normally is the most important adsorbing soil component. He reported that three types of interaction might occur between soil particles and micro-organisms. These types are: the sorption of micro-organisms onto the surfaces of large soil particles, sorptive interaction between cells and soil particles of about bacterial size resulting in the formation of aggregates, and the sorption of very small particles onto the surfaces of micro-organisms. The effect of these interactions on the behaviour and metabolic activity of micro-organisms has been reviewed by Marshall (1971) in detail.

In order to investigate the effect of interactions between the soil components and micro-organisms, experiments similar to those performed on the uptake and accumulation of phorate from aqueous solutions of *B. megaterium* were conducted with soil, clay or sand added to the cell suspension. The purpose of these experiments was to test whether the adsorption of phorate on bacteria plus adsorbent was additive and also to investigate the effect of adsorption of bacteria onto the solid phase on the activity of *B. megaterium* in metabolising phorate. The results obtained indicated that the adsorption of phorate by soil, clay or sand was increased significantly by the presence of bacterial cells, indicating that micro-organisms could be an important factor in the

retention of phorate in the soil. The adsorption of phorate by bacteria plus soil, clay or sand was found to follow the Freundlich isotherm. However, no firm conclusion could be drawn as to whether adsorption onto the mixtures was additive or not. This was due to fluctuations in bacterial concentrations, since the proportionality constant for phorate adsorption on bacterial cells was obtained from different batches of medium and some irregularity in population growth pattern was observed in different flasks. Ko and Lockwood (1968) reported similar findings. They found that fungal and actinomycet mycelia added to soil containing dieldrin, DDT and PCNB accumulated these compounds to levels above ambient concentrations. The amount of dieldrin accumulated by *Rhizoctonia solani* increased with increasing amount of mycelium, concentration of dieldrin, and incubation time of mycelium in soil. Addition of living mycelium of *R. solani* to chemically oxidised soil restored its ability to retain PCNB and dieldrin against aqueous leaching.

The addition of soil, clay or sand to *Bacillus megaterium* cell suspensions retarded the rate of accumulation of phorate sulphoxide (the sole oxidised metabolite). The rate of phorate sulphoxide accumulation decreased in the order: bacteria > bacteria/sand > bacteria/clay > bacteria/soil. These results could indicate that the adsorption of phorate onto the adsorbents lowers the availability of the chemical for bacterial oxidation. The soil-bacteria mixture was the least active in supporting phorate oxidation to its sulphoxide. This could be explained by assuming that the presence of organic matter in the soil leads to adsorption of more bacterial cells and these become less active in catalysing the oxidation process. An alternative explanation for the effects obtained by adding adsorbents to the bacterial suspensions is that adsorption affected the capacity of the bacteria to oxidise phorate.

Hattori and Furnsoka (1960) reported that the oxidative activity of *Escherichia coli* for compounds such as formate, succinate, fumarate, glucose, asparagine or alanine, was much slower when the cells were adsorbed on an ion exchange resin (Dowex-1) than when they were suspended in solution. The same authors (1961) reported that *Azotobacter agile* adsorbed on ion exchange resin was less active in oxidising glucose or succinate than when the cells were suspended freely in a solution.

The results obtained in the present study indicate that *Bacillus megaterium*, which is a common soil bacterium, was able to accumulate phorate, and oxidise it to its sulphoxide when it was in aqueous suspension. The adsorption of phorate onto soil increased in the presence of bacterial cells and this indicated that bacteria could play an important part in the adsorption of phorate on the soil particles. On the other hand, the adsorption of bacterial cells onto the soil particles decreased its ability to oxidise phorate.

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APPENDICES

The following abbreviations are used:

P = S,S : phorate
P = S,S0 : phorate sulphoxide
P = S,S0₂ : phorate sulphone
SD : standard deviation
P = 0,S : phoratoxon
P = 0,S0 : phoratoxon sulphoxide
P = 0,S0₂ : phoratoxon sulphone

APPENDIX I

The figures in Tables 1-16 are expressed as the percentage recovery of the initial radioactivity added to the soil. The results are corrected for appropriate soil recovery factors for phorate and its oxidised metabolites. These figures are the mean of three replicates $\pm$ S.D.

Table 1: The Metabolism of Phorate in Ashurst Soil (Control)

Time (days)	P = S,S	P = S,S ₀	P = S,S ₀ ₂
0	99.8 $\pm$ 8.2	0.6 $\pm$ 0.1	0.4 $\pm$ 0.1
1	85.9 $\pm$ 4.0	8.7 $\pm$ 1.8	1.0 $\pm$ 0.2
2	80.0 $\pm$ 8.4	16.5 $\pm$ 1.9	3.1 $\pm$ 1.6
4	64.0 $\pm$ 2.3	21.9 $\pm$ 2.2	2.9 $\pm$ 0.5
8	38.8 $\pm$ 2.3	27.1 $\pm$ 2.2	3.8 $\pm$ 0.3
16	19.7 $\pm$ 0.4	30.7 $\pm$ 1.0	15.5 $\pm$ 0.6
32	8.2 $\pm$ 1.2	21.3 $\pm$ 2.3	23.5 $\pm$ 5.6
64	1.7 $\pm$ 0.05	8.7 $\pm$ 1.0	23.8 $\pm$ 6.3
128	1.0 $\pm$ 0.08	3.7 $\pm$ 0.5	25.7 $\pm$ 0.7

Table 2: The Metabolism of Phorate in the Soil Treated with HQ

Time (days)	P = S,S	P = S,S ₀	P = S,S ₀ ₂
0	99.8 $\pm$ 8.1	0.5 $\pm$ 0.1	0.4 $\pm$ 0.02
1	95.0 $\pm$ 1.8	8.1 $\pm$ 0.6	0.3 $\pm$ 0.1
2	91.8 $\pm$ 3.3	9.5 $\pm$ 2.2	0.5 $\pm$ 0.05
4	75.4 $\pm$ 5.2	10.8 $\pm$ 0.4	0.8 $\pm$ 0.3
8	64.2 $\pm$ 9.3	13.6 $\pm$ 1.6	2.1 $\pm$ 2.0
16	63.9 $\pm$ 3.0	17.8 $\pm$ 0.2	0.5 $\pm$ 0.06
32	33.6 $\pm$ 2.2	24.1 $\pm$ 2.7	0.8 $\pm$ 0.2
64	13.0 $\pm$ 0.5	18.5 $\pm$ 1.5	2.6 $\pm$ 0.5
128	5.8 $\pm$ 1.1	20.5 $\pm$ 1.8	5.5 $\pm$ 0.66

Table 3: The Metabolism of Phorate in the Soil Treated with BHT

Time (days)	P = S,S	P = S,S ₀	P = S,S ₀ ₂
0	100.4 ± 1.3	0.9 ± 0.1	0.3 ± 0.03
1	91.7 ± 2.0	6.5 ± 0.04	0.5 ± 0.18
2	84.7 ± 4.0	10.4 ± 0.1	0.6 ± 0.02
4	67.7 ± 3.8	15.6 ± 0.9	1.3 ± 0.18
8	35.1 ± 1.7	12.9 ± 2.1	2.0 ± 0.8
16	31.7 ± 1.7	22.7 ± 4.9	2.7 ± 1.0
32	16.9 ± 2.5	20.2 ± 5.00	5.5 ± 0.95
64	3.8 ± 0.5	9.6 ± 1.0	6.7 ± 0.9

Table 4: The Metabolism of Phorate in Soil
Following Drying Pattern Shown in Figure

Time (days)	P = S,S	P = S,S ₀	P = S,S ₀ ₂
0	93.3 ± 2.7	1.6 ± 0.3	0.6 ± 0.05
1	80.9 ± 5.4	9.1 ± 0.2	0.1 ± 0.1
2	75.4 ± 0.8	16.8 ± 0.5	1.7 ± 0.04
4	54.9 ± 4.6	22.8 ± 1.6	3.0 ± 0.2
8	40.0 ± 1.0	31.8 ± 3.3	6.1 ± 0.7
16	24.4 ± 0.2	20.2 ± 2.9	2.2 ± 0.2
32	16.1 ± 1.4	20.5 ± 1.5	3.0 ± 0.2
64	13.2 ± 0.3	17.1 ± 1.8	1.4 ± 0.3
128	10.6 ± 1.7	15.8 ± 2.0	1.3 ± 0.7

Table 5: The Metabolism of Phorate in the Soil at 5°C

Time (days)	P = S,S	P = S,S ₀	P = S,S ₀ ₂
0	100.0 ± 1.6	0.7 ± 0.0	0.4 ± 0.1
1	93.7 ± 0.9	3.7 ± 1.2	0.5 ± 0.1
2	92.6 ± 0.6	3.3 ± 0.6	0.4 ± 0.1
4	86.8 ± 4.4	8.2 ± 2.3	0.6 ± 0.2
8	82.7 ± 1.7	9.2 ± 0.7	0.72 ± 0.1
16	76.3 ± 1.3	13.4 ± 0.6	1.4 ± 0.1
32	59.5 ± 3.8	22.6 ± 1.2	3.9 ± 0.34
64	40.7 ± 3.5	43.1 ± 4.0	11.0 ± 1.7
128	33.0 ± 0.1	41.8 ± 3.0	9.6 ± 2.1

Table 6: The Metabolism of Phorate in the Soil
with pH Artificially Adjusted to pH 3

Time (days)	P = S,S	P = S,S,O	P = S,S,O ₂
0	97.2 ± 2.6	0.9 ± 0.5	0.3 ± 0.1
1	88.4 ± 1.3	9.5 ± 0.2	0.3 ± 0.0
2	79.7 ± 4.0	14.4 ± 1.3	0.6 ± 0.1
4	57.3 ± 2.7	16.0 ± 3.0	1.4 ± 0.4
8	52.4 ± 7.5	18.0 ± 1.2	1.7 ± 0.2
16	41.2 ± 7.2	20.1 ± 3.5	1.8 ± 0.9
32	18.1 ± 5.2	14.4 ± 0.6	1.4 ± 0.7
64	13.5 ± 1.9	16.1 ± 2.4	2.1 ± 0.4
128	9.6 ± 0.8	13.2 ± 1.8	0.7 ± 0.3

Table 7: The Metabolism of Phorate in the
Soil with pH Artificially Adjusted to pH 9

Time (days)	P = S,S	P = S,S,O	P = S,S,O ₂
0	101.7 ± 2.8	0.6 ± 0.1	0.2 ± 0.0
1	89.9 ± 2.4	14.2 ± 1.5	1.2 ± 0.5
2	87.0 ± 4.3	17.4 ± 1.9	1.9 ± 0.0
4	61.8 ± 2.0	26.5 ± 1.8	3.6 ± 0.3
8	42.9 ± 0.5	20.1 ± 2.8	2.3 ± 0.1
16	36.8 ± 0.6	19.8 ± 1.2	2.6 ± 0.0
32	24.1 ± 2.5	17.1 ± 1.0	2.3 ± 0.2
64	19.3 ± 0.8	15.5 ± 0.4	1.5 ± 0.8
128	12.6 ± 0.9	13.5 ± 1.0	1.3 ± 0.1

Table 8: The Metabolism of Phorate in Steam-Sterilised Soil

Time (days)	P = S,S	P = S,S,O	P = S,S,O ₂
0	100.0 ± 1.8	0.5 ± 0.3	1.0 ± 0.7
1	102.0 ± 1.8	1.6 ± 0.5	0.6 ± 0.2
2	99.9 ± 1.8	3.9 ± 0.2	0.9 ± 0.4
4	98.5 ± 2.4	4.4 ± 0.8	0.5 ± 0.1
8	73.1 ± 6.0	3.9 ± 1.0	0.3 ± 0.05
16	62.9 ± 11.3	6.6 ± 1.0	0.4 ± 0.1
32	62.1 ± 4.5	14.4 ± 0.5	1.5 ± 0.6
64	49.1 ± 0.9	19.9 ± 1.11	0.7 ± 0.2
128	23.9 ± 5.0	27.3 ± 2.3	0.5 ± 0.2

Table 9: The Metabolism of Phorate in Steam-Sterilised Dry Soil

Time (days)	P = S,S	P = S,S ₀	P = S,S ₀ ₂
0	95.6 ± 5.1	1.0 ± 0.2	0.4 ± 0.1
1	86.7 ± 11.0	1.4 ± 0.1	0.4 ± 0.1
2	86.2 ± 7.4	2.1 ± 0.5	0.3 ± 0.1
4	80.0 ± 4.0	2.1 ± 0.3	0.4 ± 0.1
8	59.5 ± 8.0	3.8 ± 0.5	0.4 ± 0.3
16	43.9 ± 0.5	3.3 ± 0.3	0.3 ± 0.2
32	37.6 ± 4.0	8.6 ± 1.0	0.6 ± 0.4
64	30.3 ± 4.1	9.5 ± 0.3	0.8 ± 0.4
128	15.4 ± 1.6	9.5 ± 0.2	0.5 ± 0.2

Table 10: The Metabolism of Phorate in Steam-Sterilised Soil
Inoculated with *Bacillus megaterium*

Time (days)	P = S,S	P = S,S ₀	P = S,S ₀ ₂
0	100.0 ± 1.8	0.5 ± 0.2	1.0 ± 0.8
1	90.6 ± 9.9	3.0 ± 0.1	0.7 ± 0.2
2	89.8 ± 8.1	3.7 ± 0.7	0.5 ± 0.2
4	93.6 ± 2.5	5.8 ± 0.6	0.4 ± 0.0
8	82.9 ± 3.2	6.0 ± 2.0	0.5 ± 0.0
16	68.8 ± 3.0	11.0 ± 0.9	0.3 ± 0.1
32	48.8 ± 8.0	12.2 ± 1.3	0.8 ± 0.2
64	30.9 ± 1.6	16.1 ± 1.6	0.8 ± 0.4
128	19.2 ± 2.8	25.2 ± 5.7	0.6 ± 0.3

Table 11: The Metabolism of Phorate in Soil Treated with
Streptomycin Sulphate at 0.05% Concentration

Time (days)	P = S,S	P = S,S ₀	P = S,S ₀ ₂
0	96.3 ± 2.6	1.7 ± 0.2	0.6 ± 0.0
1	87.6 ± 4.5	12.3 ± 3.2	1.3 ± 0.8
2	77.0 ± 1.8	21.3 ± 3.2	2.1 ± 0.7
4	46.9 ± 0.8	29.8 ± 4.5	6.0 ± 0.2
8	38.0 ± 1.5	38.2 ± 1.5	7.4 ± 1.6
16	27.0 ± 2.0	30.6 ± 1.3	3.8 ± 1.2
32	19.7 ± 2.2	31.1 ± 1.3	2.6 ± 1.4
64	11.8 ± 1.6	29.8 ± 0.9	4.8 ± 0.8
128	10.7 ± 0.6	27.2 ± 2.3	3.9 ± 0.4

Table 12: The Metabolism of Phorate in Soil Treated with Streptomycin Sulphate at 0.1% Concentration

Time (days)	P = S,S	P = S,S ₀	P = S,S ₀ ₂
0	96.9 ± 2.7	1.7 ± 0.24	0.5 ± 0.1
1	83.0 ± 11.9	9.8 ± 1.0	0.6 ± 0.1
2	79.9 ± 1.6	18.0 ± 0.7	1.6 ± 0.1
4	49.9 ± 1.2	32.9 ± 3.9	5.7 ± 0.3
8	40.11 ± 1.0	37.2 ± 3.8	7.4 ± 2.0
16	25.2 ± 4.4	29.6 ± 1.5	4.4 ± 1.2
32	16.1 ± 1.2	30.2 ± 1.3	4.8 ± 0.5
64	14.3 ± 1.5	27.2 ± 2.0	3.6 ± 0.7
128	10.0 ± 1.2	25.5 ± 2.3	3.8 ± 0.4

Table 13: The Metabolism of Phorate in Soil Pre-Incubated for 7 Days Before Treatment with Phorate

Time (days)	P = S,S	P = S,S ₀	P = S,S ₀ ₂
0	102.3 ± 3.6	1.9 ± 0.1	0.5 ± 0.1
1	75.9 ± 3.2	13.9 ± 0.7	1.7 ± 1.1
2	61.5 ± 3.7	21.5 ± 1.7	3.1 ± 0.6
4	46.8 ± 4.3	25.5 ± 0.7	4.4 ± 3.0
8	28.3 ± 1.2	26.4 ± 5.1	9.8 ± 2.7
16	17.2 ± 9.6	22.0 ± 8.0	15.0 ± 7.0
32	3.4 ± 1.0	11.1 ± 2.3	22.8 ± 4.6
64	1.6 ± 0.3	6.0 ± 0.8	24.0 ± 1.4
128	1.0 ± 0.1	3.7 ± 0.5	25.7 ± 0.7

Table 14: The Metabolism of Phorate in Soil Pre-Incubated for 7 Days Before Treatment with Glucose Solution Added

Time (days)	P = S,S	P = S,S ₂ O	P = S,S ₂ O ₂
0	103.9 ± 4.1	2.0 ± 0.8	0.3 ± 0.0
1	79.9 ± 5.4	5.7 ± 1.2	1.7 ± 0.7
2	76.1 ± 3.6	8.3 ± 1.1	2.0 ± 0.8
4	55.4 ± 4.8	15.4 ± 7.4	3.0 ± 0.5
8	41.4 ± 0.6	19.3 ± 2.2	7.8 ± 1.0
16	20.9 ± 1.9	14.3 ± 2.3	13.8 ± 1.0
32	4.7 ± 0.8	7.8 ± 1.9	17.7 ± 1.1
64	2.5 ± 1.2	6.0 ± 2.1	24.9 ± 4.3
128	1.3 ± 1.8	3.1 ± 0.7	15.4 ± 3.5

Table 15: Metabolism of Phorate Sulphoxide in the Soil

Time (days)	P = S,S ₂ O	P = S,S ₂ O ₂
0	98.9 ± 10.8	0.2 ± 0.1
1	85.9 ± 7.7	5.5 ± 0.2
4	70.7 ± 1.8	18.5 ± 0.8
8	44.9 ± 3.4	26.3 ± 3.4
16	23.4 ± 3.0	31.0 ± 2.1
32	14.5 ± 3.1	34.1 ± 2.1
64	5.0 ± 0.6	32.0 ± 1.9
128	2.7 ± 1.0	21.5 ± 2.1

Table 16: Decline of Phorate Sulphone in the Soil

Time (days)	P = S,S ₂ O ₂
0	95.9 ± 2.9
1	89.7 ± 0.1
2	83.7 ± 1.6
4	77.3 ± 0.6
8	71.9 ± 3.5
16	55.2 ± 6.0
32	37.7 ± 2.9
64	

APPENDIX II

The hydrolysis of phorate and its sulfoxide and sulphone in buffered aqueous solutions. All these figures are the mean of three replicates $\pm$ S.D.

Table 1: The Effect of pH on Phorate Hydrolysis at 25°C

Time (days)	Phorate remaining %		
	pH 3	pH 6.4	pH 9
0	91.7 $\pm$ 2.6	95.4 $\pm$ 1.1	91.2 $\pm$ 1.6
1	74.6 $\pm$ 2.3	71.2 $\pm$ 0.5	65.8 $\pm$ 1.4
2	59.5 $\pm$ 2.7	52.0 $\pm$ 3.0	60.4 $\pm$ 0.5
3	37.0 $\pm$ 0.2	47.1 $\pm$ 0.3	50.4 $\pm$ 2.7
4	35.9 $\pm$ 1.5	31.9 $\pm$ 0.8	32.5 $\pm$ 1.4
5	28.0 $\pm$ 1.0	28.0 $\pm$ 1.4	26.5 $\pm$ 0.1
6	24.3 $\pm$ 2.3	21.9 $\pm$ 0.2	22.7 $\pm$ 1.2
7	19.8 $\pm$ 1.0	19.2 $\pm$ 0.2	18.2 $\pm$ 1.0
14	3.9 $\pm$ 0.2	3.3 $\pm$ 0.1	2.4 $\pm$ 0.2

Table 2: The Effect of Low Temperature (5°C)
on Phorate Hydrolysis at pH 6.4

Time (days)	Phorate remaining %
0	95.4 $\pm$ 1.1
7	86.5 $\pm$ 2.4
14	81.4 $\pm$ 2.1
21	75.7 $\pm$ 3.1
28	68.3 $\pm$ 0.5

Table 3: The Hydrolysis of Phorate Sulphoxide
and Sulphone at pH 6.4 and 25⁰C

Time (days)	Phorate sulphoxide remaining %	Phorate sulphone remaining %
0	93.6 $\pm$ 0.8	94.7 $\pm$ 1.5
7	85.3 $\pm$ 2.5	67.6 $\pm$ 1.3
14	81.6 $\pm$ 1.3	61.1 $\pm$ 1.2
21	78.8 $\pm$ 0.2	60.5 $\pm$ 0.2
28	76.4 $\pm$ 1.2	54.6 $\pm$ 0.7
35	66.4 $\pm$ 0.2	49.7 $\pm$ 1.1

APPENDIX III

THE METABOLISM OF PHORATE IN PURE CULTURES OF BACTERIA*

Table 1: Metabolism of Phorate in Nutrient Medium (Control)

Time (days)	Solvent Soluble Metabolites %		Water Soluble Metabolites %	Total Recovery %
	P = S, S	P = S, SO		
0	80.8		9.4	92.9
1	59.0	2.9	26.4	88.3
2	47.2	0.8	39.2	86.37
4	20.2	4.8	62.0	86.81
8	4.7	6.4	74.9	85.96
16	3.3	0.8	86.3	90.43

Table 2: Metabolism of Phorate in Pure Culture of *Bacillus megaterium*
in vitro

Time (days)	Solvent Soluble Metabolites %			Water Soluble Metabolites %	Total Recovery %
	P = S, S	P = S, SO	P = S, SO ₂		
0	69.6	0.0	0.0	11.2	80.8
1	43.3	4.1	0.0	36.7	84.08
2	20.0	13.3	0.0	45.8	79.2
4	16.3	7.5	0.8	60.4	85.0
8	5.1	4.8	0.4	68.2	78.5
16	2.8	4.0	0.0	83.5	90.3

* All values of metabolites are uncorrected.

Table 3: Metabolism of Phorate in Pure Culture of *Bacillus cereus* var. *mycoides* in vitro

Time (days)	Solvent Soluble Metabolites %						Water Soluble Metabolites %	Total Recovery %
	P = S,S	P = S,S,O	P = S,S,O ₂	P = O,S	P = O,S,O	P = O,S,O ₂		
0	53.9	18.1	1.9	0.0	1.5	1.3	12.1	88.8
1	24.8	20.0	2.4	0.0	0.0	0.0	32.5	79.6
2	25.8	14.1	1.0	0.0	1.0	0.7	43.5	86.1
4	16.8	2.9	0.0	0.0	0.5	0.9	56.1	77.2
8	6.4	3.5	0.0	0.0	0.0	0.0	68.4	78.3
16	3.4	3.9	0.0	0.0	0.0	0.0	76.5	83.8

Table 4: Metabolism of Phorate in Pure Culture of *Corynebacterium* Sp. in vitro

Time (days)	Solvent Soluble Metabolites %						Water Soluble Metabolites %	Total Recovery %
	P = S,S	P = S,S,O	P = S,S,O ₂	P = O,S	P = O,S,O	P = O,S,O ₂		
0	82.3	6.2	0.0	0.0	0.0	0.0	8.8	97.3
1	51.7	10.2	1.8	0.5	0.0	0.0	25.3	89.5
2	34.8	11.6	2.6	1.5	0.5	0.0	37.3	88.3
4	23.2	11.4	2.6	0.8	0.0	0.0	42.5	80.5
8	17.0	9.1	2.8	0.9	0.0	0.0	50.2	80.0
16	14.0	5.4	2.7	0.7	0.0	0.0	69.4	92.2

Table 4: Metabolism of Phorate in Pure Culture of *Achromobacter* Sp. in vitro

Time (days)	Solvent Soluble Metabolites %						Water Soluble Metabolites %	Total Recovery %
	P = S S	P = S,S0	P = S,S0 ₂	P = 0,S	P = 0,S0	P = 0,S0 ₂		
0	60.0	11.3	5.7	1.6	2.5	1.1	15.7	97.9
1	43.6	2.4	1.5	1.1	1.3	0.9	38.6	87.5
2	30.5	1.5	1.1	0.5	0.5	2.0	51.5	87.6
4	12.0	1.9	0.3	0.0	0.5	0.5	67.4	82.6
8	3.0	1.4	0.3	0.0	0.3	0.0	77.3	82.3
16	1.5	1.4	0.0	0.0	0.0	0.0	84.3	87.2

APPENDIX IV

Adsorption isotherm determinations (mean of three replicates).

Table 1: Adsorption of Phorate onto Soil

Initial Concentration (ppm)	Concentration at Equilibrium ($\mu\text{g.cm}^{-3}$)	Adsorbed* ($\mu\text{g.g}^{-1}$)	Adsorbed** ($\mu\text{g.g}^{-1}$)
2	1.07	11.48	16.85
4	2.15	21.45	34.41
6	3.37	36.36	48.97
8	4.32	50.53	65.8
10	5.26	60.17	86.48
12	6.48	70.98	101.01

Table 2: Adsorption of Phorate onto Clay

Initial Concentration (ppm)	Concentration at Equilibrium ($\mu\text{g.cm}^{-3}$)	Adsorbed* ($\mu\text{g.g}^{-1}$)	Adsorbed** ($\mu\text{g.g}^{-1}$)
2	0.76	20.38	21.79
4	1.36	44.08	46.40
6	2.07	65.44	69.60
8	2.65	90.34	95.71
10	3.21	114.26	122.20
12	3.57	145.13	152.16

* The amount adsorbed calculated as the amount extracted from adsorbent

** The amount adsorbed calculated as the difference between the initial and final concentrations in the aqueous solution after subtracting the amount hydrolysed.

Table 3: Adsorption of Phorate onto Sand

Initial Concentration (ppm)	Concentration at Equilibrium ($\mu\text{g.cm}^{-3}$)	Adsorbed* ($\mu\text{g.g}^{-1}$)	Adsorbed** ($\mu\text{g.g}^{-1}$)
2	1.7	0.88	2.33
4	3.33	1.44	8.21
6	4.40	1.84	16.53
8	5.83	3.97	19.21
10	7.57	3.72	25.0
12	8.37	5.27	31.15

Table 4: Adsorption of Phorate Sulphoxide onto Soil

Initial Concentration (ppm)	Concentration at Equilibrium ($\mu\text{g.cm}^{-3}$)	Adsorbed* ($\mu\text{g.g}^{-1}$)	Adsorbed** ($\mu\text{g.g}^{-1}$)
2	1.85	1.12	1.12
4	3.82	1.85	1.85
6	5.85	2.94	2.94
8	7.74	4.4	4.4
10	10.21	5.91	5.91
12	12.1	6.12	6.12

* The amount adsorbed calculated as the amount extracted from adsorbent.

** The amount adsorbed calculated as the difference between the initial and final concentrations in the aqueous solution after subtracting the amount hydrolysed.

Table 5: Adsorption of Phorate Sulphone onto Soil

Initial Concentration (ppm)	Concentration at Equilibrium ($\mu\text{g}.\text{cm}^{-3}$)	Adsorbed* ($\mu\text{g}.\text{g}^{-1}$)	Adsorbed** ($\mu\text{g}.\text{g}^{-1}$)
2	1.70	2.12	4.17
4	3.53	3.64	10.16
6	5.59	5.64	9.76
8	7.35	9.89	14.62
10	9.02	13.37	20.86

* The amount adsorbed calculated as the amount extracted from adsorbent.

** The amount adsorbed calculated as the difference between the initial and final concentrations in the aqueous solution after subtracting the amount hydrolysed.

APPENDIX V

The uptake, accumulation and metabolism of phorate from aqueous solution by *Bacillus megaterium* or *Bacillus megaterium* plus adsorbents. All figures are the mean of three replicates.

Table 1: The Uptake, Accumulation and Metabolism of Phorate from Aqueous Solutions by *Bacillus megaterium*
After 3 Hour Incubation Period

Initial Concentration ($\mu\text{g.10 cm}^{-3}$)	Final Concentration in Solution ($\mu\text{g.10 cm}^{-3}$)			Adsorbed on Bacteria Cells (μg)			Hydrolysis Products ($\mu\text{g.10 cm}^{-3}$)
	P = S,S	P = S,S,O	P = S,S,O ₂	P = S,S	P = S,S,O	P = S,S,O ₂	
20	3.41	2.52	0.26	7.94	0.23	0.05	1.0
40	7.10	5.10	0.76	15.72	0.82	0.03	2.97
60	5.70	13.50	0.82	17.23	1.32	0.20	4.70
80	7.02	18.72	1.11	22.12	2.01	0.20	7.12
100	7.81	31.85	1.84	39.52	2.83	0.66	7.99
120	9.49	38.52	1.83	44.29	3.54	0.53	9.31

Table 2: The Uptake, Accumulation and Metabolism of Phorate from Aqueous Solutions by *Bacillus megaterium*
Plus Soil After 3 Hour Incubation Period

Initial Concentration ($\mu\text{g.10 cm}^{-3}$)	Final Concentration in Solution ($\mu\text{g.10 cm}^{-3}$)			Adsorbed on the Mixture (μg)			Hydrolysis Products ($\mu\text{g.10 cm}^{-3}$)
	P = S,S	P = S,S,O	P = S,S,O ₂	P = S,S	P = S,S,O	P = S,S,O ₂	
20	2.1	2.2	0.16	11.0	1.5	0.3	0.9
40	3.8	4.7	0.22	19.2	2.5	0.5	1.56
60	6.7	5.9	0.44	36.0	1.9	0.2	2.65
80	8.7	6.1	0.38	45.7	1.95	0.3	2.30
100	9.8	13.4	1.00	56.4	3.0	0.4	4.21
120	11.1	14.67	0.75	68.6	3.20	0.8	4.66

Table 3: The Uptake, Accumulation and Metabolism of Phorate from Aqueous Solutions by *Bacillus megaterium* Plus Clay After 3 Hour Incubation Period

Initial Concentration ($\mu\text{g.10 cm}^{-3}$)	Final Concentration in Solution ($\mu\text{g.10 cm}^{-3}$)			Adsorbed on the Mixture (μg)			Hydrolysis Products ($\mu\text{g.10 cm}^{-3}$)
	P = S,S	P = S,S,O	P = S,S,O ₂	P = S,S	P = S,S,O	P = S,S,O ₂	
20	1.92	0.64	0.1	12.87	0.64	0.14	1.5
40	3.76	0.98	0.15	23.50	0.92	0.12	1.2
60	5.00	4.35	0.37	36.86	4.00	0.55	2.3
80	6.61	6.42	0.27	49.28	4.60	0.48	3.0
100	10.4	7.09	0.61	63.19	7.55	0.51	3.8
120	11.86	9.71	0.51	71.06	8.85	0.70	4.9

Table 4: The Uptake, Accumulation and Metabolism of Phorate from Aqueous Solutions by *Bacillus megaterium*
Plus Sand After 3 Hour Incubation Period

Initial Concentration ($\mu\text{g.10 cm}^{-3}$)	Final Concentration in Solution ($\mu\text{g.10 cm}^{-3}$)			Adsorbed on the Mixture (μg)			Hydrolysis Products ($\mu\text{g.10 cm}^{-3}$)
	P = S,S	P = S,S0	P = S,S0 ₂	P = S,S	P = S,S0	P = S,S0 ₂	
20	1.65	5.87	0.35	8.23	0.97	0.1	1.63
40	3.11	10.80	0.55	14.4	1.71	0.27	3.80
60	5.45	15.33	0.98	20.27	1.73	0.38	5.05
80	6.39	19.39	1.14	28.4	1.96	0.32	6.36
100	8.97	23.87	1.43	45.13	3.50	0.52	7.9
120	9.75	28.8	1.23	51.23	4.67	1.11	9.55