Interaction of three Iowa soils with the organophosphorus insecticide phorate and its metabolites, and the effect of these compounds on southern corn rootworm larvae

John Bryan Waller

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Interaction of three Iowa soils with the organophosphorus insecticide phorate and its metabolites, and the effect of these compounds on southern corn rootworm larvae

by

John Bryan Waller

A Dissertation Submitted to the
Graduate Faculty in Partial Fulfillment of
The Requirements for the Degree of
DOCTOR OF PHILOSOPHY

Department: Zoology and Entomology
Major: Entomology (Insect Toxicology)

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INTRODUCTION

The fate of insecticides in soil has received increased attention in recent years (Harris 1970, Kearney and Helling 1969, Lichtenstein 1970). Wherever they are applied, insecticides normally end up in the soil. Here they are subject to attack by the abundant soil microorganisms. In addition to microbial action, insecticides are subject to chemical attack, photolysis, and soil adsorption. A knowledge of the extent to which these various factors contribute to the loss of the insecticide after its application to the soil, is of importance in understanding such factors as environmental contamination and the long-term effectiveness of the compound.

The state of Iowa uses about eight million pounds of insecticides each year for control of soil insects. About one million pounds of phorate, an organophosphorus insecticide, and one million pounds of carbaryl, a carbamate insecticide, are used for control of corn insects (personal communication: H. J. Stockdale, Department of Zoology and Entomology, Iowa State University, Ames, Iowa, 50010). The effect of Iowa soils on phorate has not been studied, although the effect of soils from other states on this compound is known (Getzin and Chapman 1960, Getzin and Shanks 1970).
The choice of a suitable sterilization procedure is of the utmost importance in obtaining adequate controls. Some procedures alter the soil structure so much that the sterilized soil does not represent a true control (Eno and Popenoe 1964).

Corn rootworms are a major economic factor in corn production. There are three species of rootworms that damage corn. These are: the southern corn rootworm, *Diabrotica undecimpunctata;* northern corn rootworm, *D. longicornis;* and western corn rootworm *D. virgifera.* These insects represent an increasing problem to corn growers, because of the development of resistance to chlorinated hydrocarbon insecticides and an increase in monoculture of corn (Ortman and Gerloff 1970). Although phorate has been used 10 years for successful corn rootworm control, there have been only two reports of its LD$_{50}$ value for these insects (Ball 1968, Ball 1969).

Northern and western corn rootworms are the major pest species in Iowa. However, the southern corn rootworm was used as the test insect in this investigation, since it does not undergo diapause and can be reared throughout the year.

This study was concerned with the effects of time, temperature, soil type, and state of soil on the fate of phorate. In addition, the process of phorate adsorption into the soil was studied, since this has an important bearing on the quantitative recovery of this compound. Also, the ED$_{50}$
values and ED95 values for phorate and its metabolites were determined.
ABBREVIATIONS AND DEFINITIONS

Azinphosmethyl: 0,0-dimethyl S-4-oxo-1,2,3-benzotriazin-3-(4H)-ylmethyl phosphorodithioate
Bay 37289: 0-ethyl 0-2,4,5-trichlorophenyl ethyl phosphonothionate
Carbaryl: 1-naphthyl methylcarbamate
Carbofuran: S- [(p-chlorophenylthio) methyl] 0,-diethyl phosphorodithioate
Chlordane: 1,2,4,5,6,7,8,8-octachloro-3a,4,7,7a-tetrahydro-4,7-methanoindane
Chlorpyrifos: 0,0-diethyl O-(3,5,6-trichloro-2-pyridyl) phosphorothioate
DDA: bis (p-chlorophenyl) acetic acid
DDD: 2,2-bis (p-chlorophenyl)-1,1-dichloroethane
DDE: 2,2-bis (p-chlorophenyl)-1,1-dichloroethylene
DDT: 2,2-bis (p-chlorophenyl)-1,1,1-trichloroethane
Demeton: mixture of 0,0-diethyl S-(and O)-2(ethylthio) ethyl phosphorothioates
Diazinon: 0,0-diethyl O-(2-isopropyl-6-methyl-4-pyrimidinyl) phosphorothioate
Dichlorvos: 2,2-dichlorovinyl dimethyl phosphate
Dieldrin: 1,2,3,4,10,10-hexachloro-6,7-epoxy-1,4,4a,5,6,7,8,8a-octahydro-1,4-endo-exo-5,8-dimethanonaphthalene
Dimethoate: 0,0-dimethyl S-(methylcarbamoylmethyl) phosphorodithioate
Disulfoton: $\text{O,0-diethyl S-2-(ethylthio)ethyl phosphorodithioate}$

$ED_{50}$: the amount of a chemical that causes an observable effect in 50% of the population tested

$ED_{95}$: the amount of a chemical that causes an observable effect in 95% of the population tested

GLC: Gas-liquid chromatography

Heptachlor: $1,4,5,6,7,8,8$-heptachloro-$3\text{a},4\text{a},7\text{a}$-tetrahydro-$4\text{,7}$-methanoindene

Imidan: $\text{O,0-dimethyl S-phthalimidomethyl phosphorodithioate}$

Kelthane: $4\text{,4'}$-dichloro-$\alpha$-(trichloromethyl) benzhydrol

$LD_{50}$: the amount of a chemical that causes a lethal response in 50% of the population tested

Malathion: diethyl mercaptosuccinate, S-ester with O,O-dimethyl phosphorodithioate

Methidathion: $\text{S-[}2\text{-methoxy-5-oxo-}\Delta^2\text{-1,3,4-thiadiazolin-4-yl)-methyl]}\text{O,0-dimethyl phosphorodithioate}$

Methoxychlor: $1,1,1$-trichloro-$2,2$-bis(p-methoxyphenyl) ethane

Parathion: $\text{O,0-diethyl O-p-nitrophenyl phosphorothioate}$

Phorate: $\text{O,0-diethyl S-ethylthiomethyl phosphorodithioate}$

Phorate sulfoxide: $\text{O,0-diethyl S-ethylsulfinylmethyl phosphorodithioate}$

Phorate sulfone: $\text{O,0-diethyl S-ethylsulfonyl methyl phosphorodithioate}$

Phoratoxon: $\text{O,0-diethyl S-ethylthiomethyl phosphorothiolate}$
Phoratoxon sulfoxide: \(\text{O,O-diethyl S-ethysulfinylmethyl phosphorothiolate}\)

Phoratoxon sulfone: \(\text{O,O-diethyl S-ethysulfonylmethyl phosphorothiolate}\)

PO: phoratoxon

POSO: phoratoxon sulfoxide

POSO: phoratoxon sulfone

PS: phorate

PSO: phorate sulfoxide

PSO: phorate sulfone

rad: 100 ergs of energy imparted by any ionizing radiation that is dissipated in 1 g of irradiated material

rep: amount of energy derived from any ionizing radiation that is dissipated in 1 g of biological tissue (= 93 ergs)

TLC: Thin-layer chromatography

SKF-525A: 2-diethylaminoethyl 2,2-diphenylvalerate

Zinophos\textsuperscript{\(\circ\)}: \(\text{O,O-diethyl O-2-pyrazinyl phosphorothioate}\)
REVIEW OF LITERATURE

Phorate Metabolism

A schematic of the known pathways of phorate metabolism, with appropriate references, is given in Figure 1.

The first report of phorate metabolism was that of Bowman and Casida (1957). They found that phorate was oxidized by cotton plants. The four major metabolites identified were the sulfoxide and sulfone of phorate, and the sulfoxide and sulfone of phoratoxon. Some water soluble metabolites were formed but not identified. Metcalf et al. (1957), using cotton and lemon plants, repeated this work and confirmed the earlier results of Bowman and Casida. In 1958, Bowman and Casida extended their phorate metabolism studies to insects and mammals. Rats, cows, armyworms, and bean plants were used in a comparative study. The major differences between plants and animals were the rapidity with which the animals hydrolyzed phorate, and the very rapid oxidation of phorate by the plants, to its sulfoxide and sulfone. However, their results indicated a very minor role for the oxygen analogue pathway in bean plants. Essentially the same results were obtained by Bull (1965) with disulfoton. Menn and Hoskins (1962) studied the metabolism of phorate in three insects, Blattella germanica, Oncopeltus fasciatus, and Rhodnius prolixus. They found that after topical application
Figure 1. Metabolic pathways of phorate known to occur in animals, plants, and microorganisms.

Phoratoxon oxidation

Phoratoxon sulfoxide

Phoratoxon sulfone
of phorate, the chief products extracted from inside the insects were the sulfoxide and (or) sulfone. No evidence for the oxygen analogue pathway was found. This was in contrast to the earlier work of Bowman and Casida (1958), who demonstrated that the oxygen analogue pathway was the major one in Prodenia larvae. However, this may simply represent a difference in the metabolic capabilities of different orders of insects.

Rao and McKinley (1969) studied the rate of disappearance of eight organophosphorus insecticides, including phorate, from liver homogenates of four animal species. The rates for phorate and disulfoton were considerably higher than for the other compounds. No esterase-inhibiting metabolites of phorate or disulfoton were found. The authors concluded that the oxygen analogues, if formed, were rapidly hydrolyzed or that the oxygen analogue pathway was not operative. A study of the hydrolysis products, which would have elucidated this difficulty, was not undertaken.

Phorate metabolism by microorganisms will be discussed under microorganism-insecticide interaction.

The metabolism of organophosphorus insecticides by plants, insects, and mammals is now well understood and has been the subject of several recent reviews (Dauterman 1971, Fukuto and Metcalf 1969, Lykken and Casida 1969, Menzer and Dauterman 1970).
Microorganism-Insecticide Interaction

The study of the interaction of insecticides with microorganisms has involved organisms taken from such diverse environments as the soil, bovine rumen, and insect gut (Boush and Matsumura 1967, Bro-Rasmussen et al. 1968, Cook 1957, Martin 1966). A wide range of interactions has been observed, ranging from absorption through hydrolysis to oxidation. The three major groups of insecticides will be dealt with separately.

Organochlorine insecticides

Although the organochlorine insecticides are highly stable and persist for a long time in soil (Clore et al. 1961; Nash and Woolson 1967), there is mounting evidence that soil microorganisms effect a gradual breakdown of these compounds (Chacko et al. 1966, Guenzi and Beard 1967, Matsumura and Boush 1967, Matsumura et al. 1971). Naturally, the first compound to receive attention was DDT. It is now known that DDT can be degraded anaerobically in soil and by soil microorganisms. Wedemeyer (1967) found that Aerobacter aerogenes, incubated with DDT under anaerobic conditions, produced DDA as the final product. However, some of the intermediate steps in the proposed pathway were able to operate under aerobic conditions. Guenzi and Beard (1967), using anaerobically incubated soils, obtained similar results. DDD was the
major metabolite, with lesser amounts of DDA and DDE. Chacko et al. (1966) tested the ability of various soil fungi and actinomycetes to degrade DDT. Only the actinomycetes had any effect on the DDT, dechlorinating it to DDD. Burge (1971) found that anaerobic conditions were necessary for the decomposition of DDT, and this process was accelerated by volatile components of alfalfa. DDD was the final product recovered, but accounted for only 26% of the added DDT. Therefore, Burge suggested that the remaining 74% disappears by some route other than via DDA or DDE.

DDT, DDD, methoxychlor, and heptachlor degraded readily in flooded soils, whereas chlordane and dieldrin were more persistent (Castro and Yoshida 1971). DDD was the only metabolite of DDT found, which agrees with the results of Burge (1971). However, he found DDD to be stable, whereas Castro and Yoshida observed a loss of DDD from their soils.

In 1963, Kallman and Andrews showed that commercial yeast converted DDT to DDD by reductive dechlorination. The organism was without effect on DDE. Barker et al. (1965) showed that a bacterium, *Proteus vulgaris*, from mouse intestine also dechlorinated DDT to DDD and the DDD was further metabolized to some unidentified compound. The same picture was observed by Mendel and Walton (1966) with two organisms, *Aerobacter aerogenes* and *Escherichia coli*, isolated from rat intestine. Braunberg and Beck (1968) studied the interaction
of rat intestinal fauna with DDT and found all the organisms tested, with the exception of Gram-positive cocci, degraded DDT to DDD. Anderson and Lichtenstein (1971) showed that *Mucor alternans*, a fungus, degraded DDT to two unidentified metabolites. This degradative ability was coupled with the nitrogen and carbon content of the medium. The largest quantities of metabolites were formed in the presence of glucose and ammonium nitrate.

Minely and San Clemente (1970) found that chlordane, heptachlor, lindane, and DDD prevented growth of *Nitrobacter agilis*, when added to the medium at 10 micrograms/ml. Chacko and Lockwood (1966) reported that dieldrin was resistant to degradation by the actinomycetes and filamentous fungi tested. However, Matsumura and Boush (1967) found that several soil microbes did possess dieldrin degrading ability. Matsumura et al. (1970) extended this work and showed that of 650 isolated soil microorganisms, 12 were able to degrade dieldrin. The metabolite common to all species was photodieldrin. Matsumura et al. (1971) also studied endrin degradation by soil microorganisms and found that of 150 isolates, 25 were active. At least seven metabolites were isolated from a *Pseudomonas* culture. Tu et al. (1968) screened 92 cultures of microorganisms for aldrin-metabolizing ability. They found that although most organisms showed some capacity to convert aldrin to dieldrin, the most
active were species of *Trichoderma*, *Fusarium*, and *Penicillium*.

**Organophosphorus insecticides**

Menn et al. (1960) studied the persistence of carbophenothion in three types of soil. This compound was more stable in silty clay loam than in loamy sand or loam soil. Autoclaving increased the persistence of carbophenothion by a factor of three, indicating that soil microorganisms were responsible for the observed loss.

Ahmed and Casida (1958) investigated the metabolism of several organophosphate insecticides, including phorate, by soil microorganisms. A yeast, *Toralopsis utilis*, two bacteria, *Pseudomonas fluorescens* and *Thiobacillus thiooxidans*, and a green alga, *Chlorella pyrenoidosa*, were the organisms used. The insecticides were absorbed by the microorganisms only to be released later by both living and dead cells in the culture. Both hydrolysis and oxidation occurred, but the type of reaction varied with the organism used. The bacteria and algae could hydrolyze phorate but not oxidize it. The other organisms produced mainly oxidation products with the sulfoxides predominating. Lichtenstein and Schulz (1964) investigated the effects of moisture and microorganisms on the stability of three organophosphorus insecticides in silt, loam soil. After 3 days only 15% of the applied malathion could be recovered. Parathion, unlike some chlorinated hy-
drocarbons, was not lost by volatilization. Degradation was either by hydrolysis or reduction to aminoparathion, and was more rapid in moist soils and in soils containing large numbers of microorganisms. The major factor in the degradation of Imidan® in sandy loam and loam soils was spontaneous hydrolysis (Menn et al. 1965). The evidence was that the degradation curves in nonsterile soil, autoclaved soil, and buffered water were the same. Gunner et al. (1966) studied the interaction between diazinon and soil microflora. Diazinon produced a profound effect on the microflora. An unidentified coccoidal rod became the predominant organism about 30 days after treatment with diazinon at 3 lb/acre. This organism was able to utilize the sulfur, phosphorus, carbon, and nitrogen atoms of the diazinon molecule. Utilization of the diazinon molecule was greatly enhanced by the addition of trace amounts of yeast extract.

The common soil fungus, *Trichoderma viride*, and a bacterium, *Pseudomonas* sp., were found to degrade malathion readily (Matsumura and Boush 1966). After 24 hr at 30 C only 14% of the added malathion remained. Several metabolic pathways appeared to be involved because a variety of end products were observed, i.e. desmethyl malathion 19%, other products 16%, and carboxyesterase products 51%. Autoclaving the soil completely prevented any metabolism of malathion.

Matsumura and Boush (1968) extended their work with *T. viride*
to include other organophosphates. This fungus was very active in metabolizing diazinon, dichlorvos, and parathion. Mick and Dahm (1970) found that parathion incubated with cultures of *Rhizobium* sp. was metabolized primarily to amino-parathion (85%). About 10% of the parathion was hydrolyzed to O,O₂-diethylphosphorothioic acid.

Most of the insecticide-microorganism studies have been with single, pure cultures. However, Gunner and Zuckerman (1968) reported that *Arthrobacter* sp. and *Streptomyces* sp. acted synergistically on diazinon. Only 6% of the diazinon remained after 7 days incubation. Separately these organisms had no effect on the insecticide. Bro-Rasmussen et al. (1968) investigated the effects of sterilization, soil type, and water content on the breakdown of diazinon. They found the half life of diazinon was greatest in dry, sterilized soil. If the soil was moist, even though it was sterile, the half life of diazinon was about half that in dry soil. This showed that degradation was not solely owing to microorganisms. Methidathion was rapidly degraded in four types of soil (Getzin 1970). After 16 weeks, 90% of the insecticide had disappeared and 66% was expired as carbon dioxide. Some water-soluble metabolites were found. Fumigated soils were used as controls. Since only 3% of the added methidathion was expired as carbon dioxide, it was suggested that microorganisms were primarily responsible for methidathion loss.
(1970) investigated the effect of Bay 37289, diazinon, chlorpyrifos, and Zinophos on soil microbial activities. Initially there was a reduction in the number of bacteria and fungi, which returned to the original level after 2 weeks. All the insecticides increased ammonium production, and in most cases sulfur oxidation was equal to or better than the control. Oxygen consumption increased with increasing concentration of the insecticide, suggesting oxidative degradation of the chemicals or possibly an uncoupling of oxidative phosphorylation. Getzin and Chapman (1960) found that phorate, incubated with a sandy soil, clay loam, or muck soil was oxidized to its sulfoxide and sulfone. Only a very small amount of the phorate was hydrolyzed. In a later study, the fate of phorate and phoratoxon in soil was investigated (Getzin and Shanks 1970). Both these compounds were rapidly oxidized to their sulfoxides and sulfones. However, the oxidation products of phorate persisted for 16 weeks, whereas those of phoratoxon degraded to low levels within 2-8 days. They also found some conversion of the phorate sulfoxide back to phorate. An unusual finding, in another study of phorate and disulfoton degradation in soil, was that large amounts of the sulfones of the oxygen analogues were present (Menzer et al. 1970). These authors suggested that soil type has a greater influence on rate of decomposition than does temperature.
In addition to soil microorganisms, bovine rumen microorganisms have been studied to determine their effect on ingested insecticides. Parathion was reduced to aminoparathion and also hydrolyzed to some extent by bovine rumen fluid (Cook, 1957). Boiled fluid showed no activity, demonstrating the biological nature of the process. Ahmed et al. (1958) confirmed the earlier work of Cook (1957) with parathion, and in addition, studied several other organophosphates. Kutches et al. (1970) showed that although DDT, carbaryl, and toxaphene caused a significant decrease in dry matter disappearance from sheep rumen liquor, malathion did not. The authors suggested that rapid hydrolysis of the malathion prevented its having any effect. With the exception of DDT, all the insecticides caused a significant reduction in the numbers of ciliates.

Carbamate insecticides

One of the first studies involving carbamate insecticides and microorganisms was that of Boush and Matsumura (1967). They showed that carbaryl and five other insecticides were readily degraded by intestinal symbiotic bacteria, Pseudomonas melophthora, of the apple maggot, Rhagoletis pomonella. Only about 50% of the added insecticide was recovered as carbaryl, the remainder was recovered mainly as solvent-soluble metabolites. The soil fungus, Trichoderma viride, produced 9% solvent soluble and 2% water soluble me-
tabolites after 24-hr incubation at 30 C with tritiated carbaryl (Hatsumura and Boush 1968). Cowley and Lichtenstein (1970) tested the effect of five insecticides on the growth of 17 species of soil fungi. Most of the insecticides had some fungicidal effect. Carbaryl inhibited the growth of Fusarium oxysporum by about 40%, but this inhibitory effect was completely suppressed by the addition of yeast extract. The only study in which metabolites of carbamates, produced by incubation with soil microorganisms, have been identified is that of Liu and Bollag (1971). After incubation of carbaryl with the fungus, Gliocladium roseum, for one week, 17% of the original material was metabolized to more polar compounds. These metabolites were identified as 1-naphthyl N-hydroxymethyl carbamate, 4-hydroxy-1-naphthyl methyl carbamate, and 5-hydroxy-1-naphthyl methyl carbamate. The authors also observed that changing the growth conditions of the fungus influenced the type of metabolite formed.

Soil Sterilization

A wide range of physical and chemical methods of sterilization have been employed to produce a soil free of microorganisms so that the properties of the soil itself may be studied. McLaren et al. (1957) showed that soil which received 2.2 Mrep from an electron beam was free from microorganisms (bacteria, actinomycetes, and fungi). This soil
possessed a urease-like activity towards urea, but no
trypsin-like activity towards benzoylarginineamide. However,
autoclaved soil was without any enzymic activity.
Peterson (1962) found that soil, sterilized by electron beam
or X-rays, respired at a rate approaching that of nonsterile
soil. The explanation offered was that although the microor-
ganisms were killed by the radiation, the respiratory enzymes
they contained were not affected. McLaren et al. (1962)
arrived at the same conclusions as the earlier workers, i.e.
that a 2-4 Mrad dose of radiation was necessary to ensure
complete sterilization, and soil so sterilized still
manifested enzymic activity. They concluded that irradiation
of soil provides a means of eliminating fungi, bacteria,
viruses, and enzymes if the dose was high enough, without
drastically altering the soil structure. Popenoe and Eno
(1962) found that doses of gamma radiation up to 2 Mrad, did
not produce completely sterile soil. The effects of gamma-
radiation, steam, and methyl bromide on soil were investigat-
ed by Eno and Popenoe (1964). They found nutrient release
was much greater after steam treatment than after the other
two treatments. However, they concluded that irradiation
does increase the availability of nitrogen, phosphorus, and
sulfur from some soils, and that fumigation with methyl
bromide or ethylene oxide alters the soil structure less than
irradiation. Stotzky and Mortensen (1959) found that gamma
radiation of soil did not affect bacterial development, pH, mineralization of phosphorus, and evolution of carbon dioxide. Stanovick et al. (1961) irradiated soil with neutrons and gamma rays, and found that although the bacteria and actinomycetes were reduced by about 66%, the fungus population increased by a factor of two. Lichtenstein et al. (1968) investigated the effect of various sterilizing agents on the persistence of parathion and diazinon in soil and water. Autoclaving caused a drastic reduction in the numbers of bacteria; sodium azide treatment was less effective. On the other hand, linear alkyl benzene sulfonate caused as much as a 50-fold increase in the bacterial count. Although soil treated with linear alkyl benzene sulfonate contained more microorganisms, the persistence of parathion and diazinon increased as a result of this treatment. An interesting result was that although sodium azide reduced the number of microorganisms, the persistence of parathion was increased, whereas diazinon was decreased. The authors suggested that sodium azide caused the disappearance of diazinon by catalyzing its hydrolysis.

Nonbiological Degradation

In addition to the many ways insecticides are altered in biological systems, there are several nonbiological factors that play an important role in the degradation of these com-
pounds. Light is probably the most important. Several studies involving the use of ultraviolet light and parathion have been conducted (Cook and Pugh 1957, Koivistoinen and Merilainen 1962, Payton 1953). These authors all concluded that exposure to ultraviolet light caused an increase of in vitro anticholinesterase activity owing to paroxon production. An interesting result was that ultraviolet light also caused isomerization, parathion being converted to its S-phenyl isomer. Mitchell et al. (1968) found a wide variety of oxidation products were formed from organophosphate insecticides exposed to ultraviolet light (254 nm). Dursban was hydrolyzed in the presence of water, when exposed to ultraviolet light; the hydrolysis product was then photodechlorinated (Smith 1968).

On exposure to ultraviolet light or sunlight, five of the six carbamate insecticides tested were converted to a number of decomposition products (Crosby et al. 1965). Mosier et al. (1969) showed that DDT, both as a solid and in n-hexane solution decomposed when exposed to ultraviolet light. A wide variety of products were formed and a free radical mechanism for their formation was suggested. Plimmer et al. (1970) drew the same conclusions from their results obtained by irradiating methanolic solutions of DDT with ultraviolet light. Ivie and Casida (1970) found an interesting interaction between rotenone and various cyclodiene insecti-
cides. Rotenone catalyzed the photoisomerization of dieldrin when both were applied to plant surfaces. Thus, light can bring about oxidation, isomerization, hydrolysis, and dehalogenation. An excellent review of photodecomposition of pesticides is given by Crosby (1969a).

Elevated temperatures cause decomposition of organophosphate compounds. This particular nonbiological factor is of little importance in the context of soil insecticides, since the temperatures required are usually over 100 C.

Several studies involving oxidation of insecticides in the absence of light have been undertaken. Dauterman et al. (1960) showed that dimethoxon was formed when dimethoate, on various plant leaves and glass plates, was exposed to air. Paraoxon was formed in trace amounts when thin films of parathion were exposed to air (Koivistoinen and Merilainen 1962). Oxygen is known to react with sulfoxides to form sulfones (Benjamini et al. 1959).

Water, like light and air, is virtually ubiquitous in the environment. The most widely recognized reaction of water with pesticides is that of hydrolysis (Crosby 1969a, Dauterman 1971). Under acid or neutral conditions the alkyl-oxygen bond is attacked, while under alkaline conditions the phosphorus-oxygen bond is cleaved. In addition to hydrolysis, water can initiate other reactions. One such reaction is a transalkylation in which, for example, one molecule of
demeton donates a methyl group which is used to methylate the terminal sulfur of another molecule (Heath and Vandekar 1957). Kelthane, an acaricide, reacts rapidly with water at alkaline pH to produce chloroform and 4,4-dichlorobenzophenone (Crosby 1969b).
MATERIALS AND METHODS

Soils

Clarion, Webster, and Harps soils were chosen for this study, since they make up 20% of Iowa's total soil area. The remaining 80% is made up of many soil types none of which represents more than 10% of the total. The soils were dug from an area with no previous history of insecticide use and stored in plastic bags at 4 C until needed. The properties of these soils are given in Table 1.

The analyses for organic carbon were carried out according to Mebius (1960). Soil water capacity was measured by flooding a known amount of air-dried soil with water, and allowing it to drain to constant weight. The difference in weight, owing to the water, was expressed as a percentage of the dry weight of the soil. All analyses, except organic carbon and water-holding capacity, were carried out by the Iowa State University Soil Testing Laboratory.

Soil Sterilization

Soils were sterilized by both chemical and physical methods. Chemical: 5-g soil samples were treated with 0.1 ml of 2.5% sodium azide or with 0.05 ml of diethyl oxydiformate for 24 hr before addition of the insecticide. Physical: 5-g soil samples were autoclaved for 30 min at 15 psi (120
Table 1. Some properties of the soil types used

<table>
<thead>
<tr>
<th>Properties</th>
<th>Clarion</th>
<th>Webster</th>
<th>Harps</th>
</tr>
</thead>
<tbody>
<tr>
<td>pH</td>
<td>6.2</td>
<td>6.9</td>
<td>7.4</td>
</tr>
<tr>
<td>Organic matter (%)</td>
<td>3.1</td>
<td>2.2</td>
<td>4.0</td>
</tr>
<tr>
<td>Water holding (%)</td>
<td>35</td>
<td>45</td>
<td>53</td>
</tr>
<tr>
<td>Available potassium (lb/acre)</td>
<td>235</td>
<td>215</td>
<td>170</td>
</tr>
<tr>
<td>Available phosphorus (lb/acre)</td>
<td>40</td>
<td>56</td>
<td>5</td>
</tr>
<tr>
<td>Sand &gt;50μ (%)</td>
<td>40</td>
<td>29</td>
<td>16</td>
</tr>
<tr>
<td>Silt 50-2μ (%)</td>
<td>37</td>
<td>42</td>
<td>53</td>
</tr>
<tr>
<td>Clay &lt;2μ (%)</td>
<td>23</td>
<td>29</td>
<td>31</td>
</tr>
</tbody>
</table>

*a* Soil analyses performed by the Department of Agronomy, Iowa State University.

*b* Determined by the author.
C), or soil in 50-g batches was exposed to gamma radiation. The dose received by the soil was 5-6 Mrad. The irradiation was carried out at the Ames Laboratory of the United States Atomic Energy Commission gamma facility (60Co source). The effectiveness of these sterilization procedures was checked by incubating 5 g of sterilized soil in 100 ml of Brain Heart Infusion (BHI) broth (Difco Laboratories, Detroit, Mich.) for 48 hr at 30 C. Streaks from the cultures were made on BHI agar plates. These were incubated for 24 hr, and observed for growth of microorganisms.

Chemicals

Reagent grade chemicals were used throughout, except that solvents for GLC were Nanograde® (Mallinckrodt Chemical Works, St. Louis, Mo.) quality. Unlabeled phorate and its metabolites, supplied gratis by the American Cyanamid Company, Princeton, N. J., were purified on a 60-100 mesh Florisil® column. Since the sulfoxide and sulfone of phorate were not soluble in n-hexane, they were dissolved in a mixture of n-hexane and chloroform (9:1 v/v). The subsequent elution procedure followed that of Patchett and Batchelder (1961). Phoratoxon, its sulfoxide, and sulfone decomposed on the Florisil® column. Since GLC and TLC of these compounds showed them to be >99.5% pure they were used in the dosage-mortality studies, without prior cleanup. Ethoxy-1-14C-
labeled phorate was purchased from Amersham/Searle, Arlington Heights, Ill. The radiochemical purity was checked at inter-
vals by TLC, autoradiography, and scintillation spectrometry. The phorate always was found to be better than 99.8% pure.

The 10^{-*M} aqueous phorate solution, used for soil treat-
ment, was prepared by taking 100 microliters of 10^{-2M} phorate
in n-hexane and evaporating the solvent in a stream of nitro-
gen. One hundred microliters of 1% Triton\textsuperscript{®} X-100, supplied
gratis by the Rohm and Haas Co., Philadelphia, Pa., in
ethanol were added to the phorate, the flask shaken and made
up to 10 ml with glass-distilled water. The same procedure
was followed for the $^{14}$C-labeled phorate except 10 microli-
ters of a stock solution in benzene were used. The aqueous
phorate solution was sterilized by forcing it through a 0.2
micron Metrical\textsuperscript{®} filter (Gelman Instrument Co., Ann Arbor,
Mich.), and used within 2 hr after preparation. This solu-
tion was not stored, because experiments showed a gradual
loss of phorate with time. At 0 C the phorate concentration
fell to 90% of its original value. It stayed at this level
until the end of the experiment (6 days). At 20 C the pho-
rate concentration fell steadily to 59% of the original
value, at the end of 6 days.
Incubation and Extraction Procedures

Five grams of soil were weighed into sterile, 30-ml screw-capped centrifuge tubes, and 0.5 ml sterile, aqueous $10^{-4}$M phorate added. The tubes were sealed with Teflon®-lined caps and placed in the dark in constant temperature cabinets. The soil was incubated at 10, 20, and 30 C for different periods of time up to a maximum of 16 days.

Soil samples were extracted in one of two ways.

1. Four and a half ml of water and 10 ml of chloroform were added to the soil sample. This was shaken for 15 min on a Fisher-Kahn reciprocal shaker and centrifuged at 700 g for 5 min. The tube now contained three layers: a top layer of water, a middle layer of chloroform, and a bottom layer of soil. A 50-microliter sample of the aqueous layer was taken and the remainder of this layer removed by suction. A 50-microliter sample of the chloroform layer was taken for analysis of phorate and its metabolites.

2. Ten ml of a n-hexane:acetone mixture (2:1 v/v) and 5 g anhydrous sodium sulfate were added to the soil sample, and centrifuged as described in (1). A 5-8 ml sample was removed and stored over anhydrous sodium sulfate at 4 C in a screw-capped vial until required for analysis.
Thin-Layer Chromatography and Autoradiography

Phorate and its metabolites were chromatographed on precoated silica gel, 250 micron, thin-layer plates (Brinkmann Instruments, Inc., Westbury, N.Y.). The 20 x 20 cm plates were divided into 2 cm lanes by scraping away the layer with a sharp pencil. Since distortion occurred in the two outside lanes, they were not used. Usually 50 microliters of the soil extracts were spotted on a line drawn 2 cm from the bottom edge of the plate. The chromatogram was developed for 15 cm in a benzene:methanol mixture (9:1 v/v) (Grant et al. 1969). The spots were located by spraying with a 0.25% solution of palladium chloride in ethanol (Blinn 1963). The limit of detection with this method was about 0.5 micrograms for phorate, phorate sulfoxide, phorate sulfone, 2 micrograms for phoratoxon, and 4 micrograms for phoratoxon sulfoxide and phoratoxon sulfone. A typical chromatogram is shown in Figure 2.

If 14C-labeled phorate was used, an autoradiograph was prepared after the plate was developed and dried. The thin-layer plate was placed in contact with a sheet of Gaf Non-Screen medical X-ray film (GAF Corporation, New York, N.Y.) for 12 days. The film was developed in Kodak X-ray developer and fixed in Kodak general purpose fixer. The autoradiograph was used to locate the spots on the thin-layer plates. The
Figure 2. Thin-layer chromatogram of phorate and its five oxidative metabolites. Developed in benzene:methanol (9:1 v/v) and sprayed with 0.25% palladium chloride in methanol.
spots were scraped into 20 ml of scintillation fluid and the quantity of radioactivity determined by liquid scintillation spectrometry.

**Gas-Liquid Chromatography**

A Packard gas chromatograph, model 7821, equipped with an alkali-flame ionization detector was used. The platinum helix in this detector was coated with potassium chloride. The helix, after heating to redness in a flame, was dipped into crystalline potassium chloride. Further heating melted the salt causing it to flow evenly over the helix. Coiled glass columns (120 cm long x 2 mm ID) were packed with 3% Apiezon® N on acid-washed, dimethyl dichorosilane-treated, 60-80 mesh Chromosorb® G. The liquid phase was applied to the Chromosorb® G as follows: 1 g of Apiezon® N was dissolved in 100 ml of chloroform and added to 33 g of Chromosorb® G contained in a rotary evaporator flask. The chloroform was evaporated under reduced pressure (15-20 inches of mercury) with a rotary evaporator (A.S. Aloe Co., St. Louis, Mo.). Final traces of the solvent were removed from the coated support by drying overnight at ca. 90 C. Columns were conditioned at 220 C with a helium gas-flow rate of 20 ml/min, for 2-3 days.

The gas chromatograph was operated under the following conditions. Temperature: injector 225 C, detector 220 C,
Figure 3. Typical GLC traces obtained with the Apiezon® N column.
Figure 4. Peak height ratio standard curve. The standard was $5 \times 10^{-6}$M phorate.
and column 190 C; gases: helium (carrier) 35 ml/min, air 250 ml/min, hydrogen 20-25 ml/min. The retention times of phorate and phorate sulfone were 3.6 and 7.5 min respectively. Typical gas chromatograms, obtained under these conditions, are shown in Figure 3. The n-hexane:acetone soil extract was kept over anhydrous sodium sulfate. Two microliters of the extract were injected into the gas chromatograph for quantitation by the peak-height ratio method. Standard peak-height ratio curves were prepared from a series of phorate stock solutions (1-7 X 10^-6M) by comparison with the standard (5 X 10^-6M) concentration (Figure 4). Two microliters of the standard solution were injected every sixth sample. The ratio of the peak heights (sample/standard) was calculated and used to read the concentration of the compound from the standard curve. The same procedure was used for determination of phorate sulfone.

Zero-time extraction efficiency values, for phorate sulfoxide and phorate sulfone, were measured with a diethylene glycol succinate (DEGS) column (5% DEGS on Gas Chrom Q, 60-80 mesh). The detector and operating parameters were the same as used for phorate, with the following exceptions: hydrogen 30 ml/min, helium 30 ml/min, column temperature 195 C. The retention times were, phorate sulfoxide 3.9 min and phorate sulfone 5.7 min.
Liquid Scintillation Spectrometry

Quantitative measurements of 14C-labeled phorate and its metabolites were made by a Packard Tri-Carb Liquid Scintillation Spectrometer (Model 3003, Packard Instrument Co., Downers Grove, Ill.). The settings used for all measurements were, sample channel: gain 25%, window 50-600; external standard channel: gain 5%, window 700-infinity. Background was determined and the instrument operated in the background subtraction mode. To obtain maximum counting accuracy each sample was counted for 10,000 counts or, for samples of very low activity, for 100 min. In most cases 50-microliter samples were removed from the soil extracts for counting. To monitor possible quenching of the activity in the samples, the automatic external standard mode of operation was used. Each sample was added to 20 ml of scintillation fluid contained in a low-potassium-1 vial (Packard Instrument Co.). Samples were cooled to 4° C before counting.

The scintillation counting fluid consisted of 50 g of scintillation grade PPO (2,5-diphenyloxazole), and 2 g of scintillation grade dimethyl POPOP (1,4-bis-2-{4-methyl-5-phenyloxazolyl}-benzene) dissolved in five liters of reagent grade toluene. To this mixture was added 500 ml of methanol:aminoethanol (40:6 v/v) and made up to 10 liters with methanol.
Rootworms

Initially southern corn rootworms were cultured in the laboratory according to the method of Howe and George (1966). Adults were kept at 25 C, exposed to a 15-hr daylight period and fed either broad bean foliage or lettuce. Petri dishes containing 10 layers of gauze, 4 layers of filter paper, and 4 layers of cotton gauze moistened with water were provided as oviposition sites. The eggs were collected from the gauze and filter paper by swirling in a beaker of distilled water. When the eggs had settled to the bottom of the beaker most of the water was decanted. The eggs were filtered off; and either stored for 5 days on damp filter paper and cotton in plastic sandwich boxes, or were put onto the soil surface of trays of 3-4 inch tall corn plants.

Subsequently a less time-consuming method of rearing the southern corn rootworm was employed (D. J. Wright, Agricultural Div., American Cyanamid Co., Princeton, N. J., Mass rearing the southern corn rootworm, 2 pp., unpublished. Nov., 1965). Corn was spread evenly at about one kernel per 3 cm² over damp paper toweling which covered the bottom of a 5 x 20 x 30 cm enameled-steel tray. Another layer of damp paper toweling covered the kernels and the tray was sealed with plastic sheet (Saran® wrap) to maintain high humidity. A good mat of roots developed in about 3-4 days, after which
eggs or newly hatched larvae were added. After about 12 days, 30 larvae were allowed to pupate. They were placed in an 8 X 8 X 2 inch cake pan containing a 2:1 mixture of moist soil and vermiculite with about 20 kernels of germinated corn. Adults were kept and eggs were collected as described above. This rearing procedure was carried out at 25 C and 50% R.H. with a 15-hr daylight period. The corn, variety 3773, used for rearing the rootworms was donated by Mr. F. Dicke of Pioneer Hi-Bred International Inc., Des Moines, Iowa. This corn, which was treated with the fungicide Captan®, had no deleterious effect on the larvae. In fact, the fungicide allowed vigorous root hair growth which most likely helped in the establishment of the first-instar larvae.

Syringe Calibration

The determination of ED$_{50}$ and ED$_{95}$ values requires that the larvae be treated with a precisely known quantity of insecticide. Therefore, the dose delivered by the syringe (no. 725, 0.25 ml, Hamilton Co., Inc., Whittier, Calif.) was determined by two methods.

(1) The syringe was filled with mercury and 10 deliveries made into a small, weighed aluminum cup. The cup and mercury were weighed accurately and the weight of mercury calculated. This weight was divided by the specific gravity of mercury, then by 10, to obtain the volume of one delivery. Ten repli-
cations were made. Because of the high surface tension of mercury, less than 10 deliveries could not be made accurately.

(2) The syringe was filled with an acetone solution of $^{14}$C-labeled carbaryl. Single and multiple deliveries were made onto 1-cm squares of Kleenex tissue which were dropped into scintillation vials containing 20 ml of scintillation fluid. The vial was thoroughly shaken by hand to extract the radioactivity from the tissue. The counting procedure is described under "Liquid Scintillation Spectrometry." Five replications of 1, 2, 5, and 10 deliveries were made. At least 10,000 counts were collected and correction made for background. The corrected count was divided by the number of deliveries to obtain a counts/delivery value. The activity in the $^{14}$C-labeled carbaryl solution was determined by counting five separate samples delivered from a 50-microliter±0.5% glass micropipet (Corning Glass Works, Corning, N.Y.). The activity was expressed as counts/microliter and the volume of the delivery from the syringe obtained by dividing this value into the counts/delivery figure.
The standard deviations of the mean values, for both the methods described, were calculated according to the formula:

$$SD = \sqrt{\frac{\sum (x - \overline{x})^2}{n-1}}$$

where:  
- $n =$ the number of observations  
- $x =$ one observation  
- $\overline{x} =$ the mean of $n$ observations

Table 2 shows the results obtained by the two methods, and the close agreement between them. The mean value of the radiometric calibration was 0.52 microliter and was used in the calculation of the $ED_{50}$ and the $ED_{95}$ values.

The coefficient of variation (4.3%) which I obtained with the radiometric method, agreed closely with the value of 4.6% obtained by Hewlett and Lloyd (1960). Both these values were obtained from the measurements of single deliveries. As the number of deliveries increased, so the coefficient of variation decreased. The coefficient of variation is the standard deviation expressed as a percentage of the mean.

Dosage-Mortality Measurements

Individual third-instar southern corn rootworm larvae were treated topically with 0.52 microliter of n-hexane:acetone (2:1 v/v) solution of phorate, or one of its metabolites. The five concentrations used were 0.5, 1, 2, 4,
Table 2. Syringe calibration

<table>
<thead>
<tr>
<th>Number of deliveries</th>
<th>Mean volume (microliter)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Radiometric a</td>
</tr>
<tr>
<td>1</td>
<td>0.516±0.022</td>
</tr>
<tr>
<td>2</td>
<td>0.524±0.0001</td>
</tr>
<tr>
<td>5</td>
<td>0.526±0.01</td>
</tr>
<tr>
<td>10</td>
<td>0.514±0.0001</td>
</tr>
</tbody>
</table>

a Five replicates.
b Ten replicates.
c Standard deviation.
and \(8 \times 10^{-4}\) M. The insecticide was applied from a 250 micro-liter syringe fitted with a 27-gauge needle, by means of a foot-operated, electrically powered microapplicator. Each larva was held by a vacuum pencil placed laterally on the thorax, and the insecticide solution applied to the abdominal dorsum. The larva was held for about 10 s while the solvent evaporated, then placed in a plastic sandwich box containing damp filter paper and a few kernels of germinated corn. Twenty larvae were treated at each dose level. Five dose levels were used; a control group was treated only with the solvent. After 48-hr exposure to the insecticide, the batches of larvae were placed on soil in plastic boxes. Larvae unaffected by the treatment were able to burrow into the soil, whereas affected ones were not. The number of larvae remaining on the soil surface, after 30 min, was recorded and used in the dosage-mortality calculations. In addition to the method just described, the effects of the chemicals were assessed at 72 hr by observing larvae for signs of vigorous movement, when touched with forceps. A program for regression analysis, supplied with the IBM 360/65 computer and modified to accommodate dosage-mortality data, was used to calculate both the \(ED_{50}\) and \(ED_{95}\) values and their confidence limits.
RESULTS AND DISCUSSION

Soil

Properties

Some of the properties of the soils used in this study are shown in Table 1. The most important factors related to recovery of insecticides from soil are pH and organic matter content. Changes in these factors bring about the greatest changes in the recovery of insecticides. Crosby (1970) stated that organic matter is exceptionally important in the nonbiological breakdown of insecticides. Organophosphorus esters could alkylate lignins, and basic amino acids could catalyze the hydrolysis of organophosphorus compounds, dehydrochlorination of DDT, and lindane. Insecticides are absorbed into crops most readily from sandy soils (low organic content) and least readily from silt soils (high organic content) (Getzin and Chapman 1960, Lichtenstein 1966). However, the organic matter content of the soils used in this study shows little variation between one soil type and the next.

Hydrolysis is an important reaction of many insecticides, and the type of product formed is governed by the pH of the medium in which the reaction takes place (Crosby 1969b). Azinphosmethyl was relatively stable in water below pH 10 (Liang and Lichtenstein 1972). The pH values of
Clarion, Webster, and Harps soils were similar. Therefore, it was difficult to attribute the greater stability of phorate in Harps soil to a difference in pH between the soils. However, as pointed out by Crosby (1969b) environmental factors seldom influence chemical reactions by themselves; a combination of factors is usually required. Penetration of soil insecticides is greatly influenced by soil moisture content. Carter and Stringer (1970) showed, with one exception, that increasing the soil moisture content increased the penetration of organochlorine insecticides. Clarion soil with a high sand content has a low water-holding capacity, whereas Webster and Harps, with low sand content, have higher water-holding capacities. Henzer et al. (1970) stated that soil type had a greater influence on the rate of decomposition of phorate and disulfoton than did soil temperature. This is contrary to my results which show a marked temperature effect, the higher the temperature the more rapid the degradation of phorate.

Metabolite recovery

The extraction efficiency of phorate and its metabolites at zero time was measured by liquid scintillation spectrometry and GLC. In addition to metabolite recovery, the effect of adsorption was measured by extracting treated soil in a soxhlet apparatus for 6 hr.

Table 3 shows the zero-time recovery values for phorate,
Table 3. Percent recovery of phorate, its sulfoxide, and sulfone from soil at zero time, extracted with hexane:acetone (2:1 v/v) and determined by GLC.

<table>
<thead>
<tr>
<th>Soil</th>
<th>Phorate</th>
<th>Sulfoxide</th>
<th>Sulfone</th>
</tr>
</thead>
<tbody>
<tr>
<td>Clarion</td>
<td>109±2</td>
<td>73±6</td>
<td>103±9</td>
</tr>
<tr>
<td>Webster</td>
<td>108±6</td>
<td>64±5</td>
<td>97±6</td>
</tr>
<tr>
<td>Harps</td>
<td>102±6</td>
<td>61±6</td>
<td>93±6</td>
</tr>
<tr>
<td>Sterile soil</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Clarion</td>
<td>108±3</td>
<td>82±4</td>
<td>104±4</td>
</tr>
<tr>
<td>Webster</td>
<td>110±5</td>
<td>73±3</td>
<td>100±6</td>
</tr>
<tr>
<td>Harps</td>
<td>103±3</td>
<td>61±5</td>
<td>91±7</td>
</tr>
</tbody>
</table>

a Soils fortified to give a concentration in the extract of $10^{-7}$M for each compound. Values are the means of three replicates expressed as a percentage of the phorate added.

b Standard deviation.

c Sterilized by autoclaving.
Table 4. Percent radioactivity recovered from soil, incubated for 48 hr with 0.5 ml $10^{-4}$M, $^{14}$C-labeled phorate, and extracted with chloroform.

<table>
<thead>
<tr>
<th>Soil</th>
<th>Nonsterile</th>
<th>Autoclaved</th>
<th>γ-irradiated</th>
</tr>
</thead>
<tbody>
<tr>
<td>Clarion</td>
<td>$74\pm9$</td>
<td>$57\pm3$</td>
<td>$59\pm8$</td>
</tr>
<tr>
<td>Webster</td>
<td>$68\pm11$</td>
<td>$54\pm2$</td>
<td>$55\pm11$</td>
</tr>
<tr>
<td>Harps</td>
<td>$63\pm10$</td>
<td>$56\pm4$</td>
<td>$54\pm10$</td>
</tr>
</tbody>
</table>

\(a\) Values are means of three replicates expressed as a percentage of the phorate added.

\(b\) Standard deviation.
its sulfoxide, and sulfone from soils fortified with these compounds at 10^{-4} M and extracted with a mixture of hexane:acetone (2:1 v/v). These values were obtained by GLC and agree with those obtained by Getzin and Shanks (1970). Even at zero time some phorate sulfoxide is bound to the soil. However, the hexane:acetone solvent system gives good recovery of phorate and its sulfone. Recovery of ethoxy-1-{^14}C-labeled phorate, at zero time, from the three soils, was determined by TLC and liquid scintillation spectrometry. The values are as follows: Clarion 97.8±5.1%, Webster 95.2±4.3%, Harps 99.6±1.6%. These agree favorably with the GLC values. Table 4 shows the extraction efficiency for the chloroform:water (2:1 v/v) system at 48 hr. The extraction efficiency of this system for the sulfoxide and sulfone was not obtainable because no radiolabeled metabolites were available. The recovery values at 48 hr are much lower than at zero time, suggesting adsorption of the phorate and its metabolites into the soil. Soxhlet extraction for 6 hr with hexane:acetone (2:1 v/v) or with chloroform, showed that phorate and its metabolites were indeed strongly bound to the soil. Only 65% of the added radioactivity could be recovered by either solvent system. Recovery of phorate and its metabolites from soil, by solvent extraction, was poor. Recovery from the thin-layer plates, by scraping and liquid scintillation counting, was excellent. A value of 97±8% was
obtained from the results of 68 experiments.

No phoratoxon, its sulfoxide, or sulfone were found in any of the experiments in which phorate was incubated with soil. Therefore, the oxygen analogue pathway (Figure 1) must be lacking in the soils tested. It might be argued that the oxygen analogue was formed and, as is usual for this metabolite, rapidly hydrolyzed. However, substantially more hydrolysis products than the 2% found should have occurred in the aqueous phase of the chloroform:water extraction system.

**Time-course study**

Recovery of phorate from all three soils varied inversely with temperature and time during the 16-day experiment. The amount of phorate sulfone recovered from the three soils increased with time for the first 12 days. During the last four days the amount of phorate sulfone recovered, decreased at 20 and 30 C, whereas it increased at 10 C. Figure 5 shows the effect of time and temperature on the loss of phorate from Harps soil. Clarion and Webster soils had similar curves but showed an even greater loss of phorate for a given temperature.

Recovery of added phorate from Harps soil after 4 days incubation, was 40% at 10 C, 10% at 20 C, and 5% at 30 C. After 16 days at 10 C, only 10% of the added phorate was recovered, and less than 2% was recovered at 20 C or 30 C. This loss of phorate was accompanied by an increase in pho-
Figure 5. Time course curve for phorate (open symbols) at 10, 20, and 30 °C, in Harps soil. Each point is a mean of three replicates.
rate sulfone. Phorate sulfoxide was not detected by the GLC system used, but as will be discussed later, was formed in substantial amounts. Figure 6 shows the pattern of phorate loss during the first 12 hr of incubation at 30 C. About 50% of the added phorate was lost from Clarion and Webster soils during the first 6 hr of incubation. Only 30% was lost from the Harps soil during the same period. The most likely explanation of this difference is that phorate is more strongly adsorbed to Clarion and Webster soils. Addition of the phorate in 0.5 ml water put the soils close to their maximum water-holding capacity, thus reducing the chances of an effect owing to differences in moisture content. There was a slow loss of phorate from all the autoclaved soils, amounting to 10% after 12 hr. This was probably owing to adsorption, since incubation of these autoclaved soils in BHI broth showed them to be sterile. Subsequent experiments, in which 14C-labeled phorate was incubated with autoclaved soils, showed that very little oxidation to the sulfoxide occurred and none to the sulfone.

**Sterilization effects**

The most effective soil sterilization procedure was autoclaving for 30 min at 15 psi (120 C). Only about 10% of the phorate was lost after 24 hr incubation at 30 C, compared with about 20% for the other three methods (Table 5). The values for autoclaved soil were significantly different from
Figure 6. Time course curve for loss of phorate added to Clarion, Webster, and Harps soil and incubated at 30 C. Each point is a mean of two replicates.
Table 5. Effect of sterilization on recovery of phorate (0.5 ml $10^{-6}$M) from soil after 24 hr incubation at 30 C

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Soil type</th>
<th>Clarion</th>
<th>Webster</th>
<th>Harps</th>
</tr>
</thead>
<tbody>
<tr>
<td>Autoclaving</td>
<td></td>
<td>4.57 a</td>
<td>4.67 a</td>
<td>4.75 a</td>
</tr>
<tr>
<td>Gamma irradiation</td>
<td></td>
<td>2.92 bc</td>
<td>3.35 bc</td>
<td>3.67 bc</td>
</tr>
<tr>
<td>Diethyloxydiformate</td>
<td></td>
<td>3.22 bc</td>
<td>3.68 bcd</td>
<td>4.05 bc</td>
</tr>
<tr>
<td>Sodium azide</td>
<td></td>
<td>4.17 d</td>
<td>3.88 d</td>
<td>4.04 bc</td>
</tr>
</tbody>
</table>

*a* Any means, within a soil type, having the same letter are not significantly different at the 5% level.

Mean of three replicates.

*b* Mean of two replicates.
the values for the other treatments, for all soil types. Sodium azide was a more effective sterilization procedure than diethylloxidiformate or gamma irradiation, for both Clarion and Webster soils (Table 5). These results suggest that autoclaving has a much greater effect on soil structure than do the other treatments. This was possibly an effect on organic matter or surface properties of the soil particles, resulting in less adsorption of the phorate. Autoclaving was the only treatment that caused a large increase in temperature during the sterilization process.

Figure 7 compares the loss of phorate from untreated Webster soil during the first 24 hr of incubation at 30 C, with that from Webster soil sterilized by four separate techniques. The effectiveness of each sterilization technique was tested by incubating samples of the sterilized soil in BHI broth. Soil samples sterilized by the physical methods (autoclaving and gamma irradiation) produced no growth of microorganisms in 48 hr. However, soils sterilized by chemicals (diethylloxidiformate and sodium azide) produced a teeming culture of bacteria. This indicates that some microorganisms in the soil can metabolize phorate, but are destroyed or inhibited by chemical sterilants. It is possible that the microorganisms were metabolizing the sterilants in preference to the insecticide. Both chemical treatments were about as effective as gamma irradiation in preventing phorate loss.
Figure 7. Effect of four methods of sterilization on loss of phorate from Webster soil. Each point is a mean of two replicates.
Therefore, inhibition of microorganism activity seems the most likely explanation of the results.

The nature and quantity of the metabolites produced by autoclaved and gamma-irradiated soil was investigated. Thin-layer chromatography and autoradiography of 50 microliters of a chloroform extract, showed the presence of phorate, phorate sulfoxide, and phorate sulfone. However, there were marked differences between the quantities of compounds produced in the nonsterile, autoclaved, and gamma-irradiated soils. Autoclaved soil completely suppressed sulfone formation and greatly reduced sulfoxide production. Very little sulfone was produced in gamma-irradiated soil, whereas the amount of sulfoxide was about the same as in the nonsterile soil (Table 6). These results suggest that the oxidation of phorate to its sulfoxide is mainly a nonbiological process, and the sulfoxide to sulfone conversion is brought about mainly by microorganisms. The different values for sulfoxide production obtained for autoclaved and gamma-irradiated soil may be owing to destruction, by autoclaving, of extracellular enzymes or soil structure, necessary for this conversion (Eno and Popenoe 1964, McLaren et al. 1957).

**Inhibitor study**

To examine further the nature of the oxidation process of phorate in soil, experiments were performed in which soil was incubated with compounds known to inhibit oxidative reac-
Table 6. Percentages of phorate (PS), phorate sulfoxide (PSO), and phorate sulfone (PSO₂) recovered from TLC of chloroform extracts of soil to which 0.5 ml 10⁻⁴ M ethoxy-l-¹⁴C-labeled phorate was added.

<table>
<thead>
<tr>
<th>Compound</th>
<th>Nonsterile</th>
<th>Autoclaved</th>
<th>Gamma-irradiated</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>C W H</td>
<td>C W H H</td>
<td>C W H</td>
</tr>
<tr>
<td>PS</td>
<td>7.7 4.3 7.3</td>
<td>88.5 87.5 91.2</td>
<td>32.6 28.1 42.7</td>
</tr>
<tr>
<td>PSO</td>
<td>58.2 63.2 71.5</td>
<td>11.5 12.5 8.8</td>
<td>65.2 70.0 55.3</td>
</tr>
<tr>
<td>PSO₂</td>
<td>34.2 32.5 21.3</td>
<td>0.0 0.0 0.0</td>
<td>2.1 1.9 2.0</td>
</tr>
</tbody>
</table>

a Values are means of three replicates expressed as a percentage of the total activity recovered from the thin-layer plate. Samples were incubated for 48 hr.

b Clarion.

c Webster.

d Harps.
tions in mammals.

Five grams of Clarion soil were treated with 0.5 ml 10⁻³ M SKF-525A, or with 0.5 ml 10⁻³ M 8-hydroxyquinoline or air in the tubes was displaced with carbon monoxide once per hour for 4 hr. Each treatment was made 4 hr before adding the phorate. After adding the phorate to the carbon monoxide treated soil, the air in the tube was again displaced to remove oxygen that may have been introduced. The soil samples were incubated for 48 hr with 0.5 ml 10⁻⁴ M phorate, extracted with hexane:acetone (2:1 v/v) and analyzed by GLC. The results are shown in Table 7.

Both SKF-525A and 8-hydroxyquinoline suppressed the formation of phorate sulfone, but were without effect on phorate sulfoxide production. This confirms the biological nature of the sulfoxide to sulfone conversion, and the predominantly nonbiological nature of the phorate to sulfoxide reaction. As in other experiments, autoclaving prevented sulfone formation and greatly reduced sulfoxide production. Recovery of the compounds from carbon monoxide treated soil was about the same as from the control soil. Therefore, carbon monoxide has no effect on the soil's ability to oxidize phorate. This suggests a difference between the cytochromes of mammals and microorganisms.
Table 7. Effect of three inhibitors on the oxidation of phorate (0.5 ml $10^{-4}$M) by Clarion soil

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Phorate</th>
<th>Phorate sulfoxide</th>
<th>Phorate sulfone</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>SKF-525A</td>
<td>42±10</td>
<td>27±3</td>
<td>8±0.3</td>
<td>77</td>
</tr>
<tr>
<td>8-hydroxy quinoline</td>
<td>40±3</td>
<td>25±4</td>
<td>6±1</td>
<td>71</td>
</tr>
<tr>
<td>Carbon monoxide</td>
<td>21±10</td>
<td>39±4</td>
<td>29±6</td>
<td>89</td>
</tr>
<tr>
<td>Autoclave</td>
<td>94±4</td>
<td>5±0.7</td>
<td>0</td>
<td>99</td>
</tr>
<tr>
<td>Control</td>
<td>19±9</td>
<td>32±3</td>
<td>26±8</td>
<td>77</td>
</tr>
</tbody>
</table>

a Values are means of three replicates expressed as a percentage of the phorate added. Determined by GLC.

b Treatments carried out 4 hr before addition of insecticide.

c Standard deviation.
Dosage-mortality measurements

The oxygen analogue pathway is a major one in plant metabolism of phorate (Bowman and Casida 1957, Metcalf et al. 1957). Therefore, rootworms are likely to contact the end products of this pathway from feeding on treated plants. For the sake of completeness, and to add to our knowledge of this group of compounds, phorate, phoratoxon, and their thioether oxidation products were tested against southern corn rootworm larvae.

Initially, the effectiveness of the treatments was assessed by observing larvae for vigorous movement when touched with forceps. This was a rather subjective method, and sometimes showed that all dose levels were equally effective. In fact, it was impossible to get a regression line for phoratoxon sulfone by this method. However, regression lines for phorate and its four other oxidative metabolites were obtained. Table 8 gives the ED$_{50}$ values, ED$_{95}$ values, and the slopes of the lines, when larvae were assessed 72 hr after treatment. The most obvious fact is the very gradual slope of the regression lines, leading to exaggerated ED$_{95}$ values. This is particularly misleading in the case of phoratoxon, as will be seen later.
Table 8. Toxicity values, in mg/kg, of phorate and four of its metabolites for third-instar southern corn rootworm larvae, assessed 72 hr after treatment by observing them for vigorous movement when touched.

<table>
<thead>
<tr>
<th>Compound</th>
<th>( ED_{50} ) with confidence limits</th>
<th>( ED_{95} ) with confidence limits</th>
<th>Slope</th>
</tr>
</thead>
<tbody>
<tr>
<td>Phorate</td>
<td>10.8 (3.9)</td>
<td>1449 (112)</td>
<td>1.067</td>
</tr>
<tr>
<td>Phorate sulfoxide</td>
<td>6.4 (8.0)</td>
<td>58 (37)</td>
<td>1.712</td>
</tr>
<tr>
<td>Phorate sulfone</td>
<td>14.8 (19.5)</td>
<td>166 (386)</td>
<td>1.568</td>
</tr>
<tr>
<td>Phoratoxon</td>
<td>7.9 (11.0)</td>
<td>275 (1475)</td>
<td>1.066</td>
</tr>
<tr>
<td>Phoratoxon sulfoxide</td>
<td>7.0 (8.9)</td>
<td>77 (153)</td>
<td>1.586</td>
</tr>
</tbody>
</table>

\( a \) Mean of three replicates.
Table 9. Toxicity values, in mg/kg, of phorate and five of its metabolites for third-instar southern corn rootworm larvae, assessed 48 hr after treatment by allowing them to burrow in moist soil.

<table>
<thead>
<tr>
<th>Compound</th>
<th>$ED_{50}$ with confidence limits</th>
<th>$ED_{95}$ with confidence limits</th>
</tr>
</thead>
<tbody>
<tr>
<td>Phorate</td>
<td>3.5 4.3</td>
<td>27.8 51.8</td>
</tr>
<tr>
<td></td>
<td>2.9 2.9</td>
<td>18.3 1.835</td>
</tr>
<tr>
<td>Phorate sulfoxide</td>
<td>3.1 3.6</td>
<td>11.9 16.2</td>
</tr>
<tr>
<td></td>
<td>2.6 2.6</td>
<td>9.4 2.787</td>
</tr>
<tr>
<td>Phorate sulfone</td>
<td>4.2 5.0</td>
<td>20.5 31.2</td>
</tr>
<tr>
<td></td>
<td>3.5 3.5</td>
<td>15.3 2.372</td>
</tr>
<tr>
<td>Phoratoxon</td>
<td>2.1 2.4</td>
<td>5.8 6.8</td>
</tr>
<tr>
<td></td>
<td>1.8 1.8</td>
<td>5.2 3.632</td>
</tr>
<tr>
<td>Phoratoxon sulfoxide</td>
<td>3.3 3.9</td>
<td>12.1 16.7</td>
</tr>
<tr>
<td></td>
<td>2.9 2.9</td>
<td>9.5 2.942</td>
</tr>
<tr>
<td>Phoratoxon sulfone</td>
<td>3.1 3.6</td>
<td>13.0 17.2</td>
</tr>
<tr>
<td></td>
<td>2.6 2.6</td>
<td>10.6 2.650</td>
</tr>
</tbody>
</table>

*a Mean of three replicates.
To overcome this problem a method was devised of assessing the larvae by allowing them to burrow. This technique makes use of the insect's tendency to burrow and to avoid light. Table 9 shows the results obtained when larvae were assessed by this technique, 48 hr after treatment. The regression lines for the six compounds tested are shown in Figures 8-13. Phoratoxon is the most toxic of the compounds tested. The ED<sub>95</sub> value obtained by this method was 5.8 mg/kg compared with 275 mg/kg for the other method. The only other study of the effect of phorate on corn rootworms was that of Ball (1968). However, adult western corn rootworms were used so it was difficult to make a comparison. Ball (1968) showed there was an increase in the LD<sub>50</sub> values for phorate in Nebraska during the years 1963 to 1967. In 1963 the mean value for 18 counties in Nebraska was 15.8 mg/kg. This value had risen to 25.6 mg/kg in 1967. The value of 3.5 mg/kg that I obtained was about one fifth of the lowest value (15.8 mg/kg) given by Ball (1968). This indicates a difference in susceptibility between the two species, or the larval stage is more susceptible than the adult.

As mentioned earlier, the only compounds found in Clarion, Webster, and Harps soils, after treatment with phorate, were phorate, its sulfoxide, and sulfone. The ED<sub>50</sub> values for these compounds are essentially the same ca. 3.5 mg/kg.
Therefore, although phorate is rapidly converted to its sulfoxide and sulfone in the soil, the dosage-mortality data show that these products will afford good control of the rootworms.
Figure 8. Regression line for phorate for third-instar southern corn rootworm larvae. Each point is a mean of three replicates and was obtained by treating larvae with 0.52 microliter of the following dose levels of phorate in hexane: acetone (2:1 v/v): 0.5, 1, 2, 4, and $8 \times 10^{-4}$M.
Figure 9. Regression line for phorate sulfoxide for third-instar southern corn rootworm larvae. Each point is a mean of three replicates and was obtained by treating larvae with 0.52 microliter of the following dose levels of phorate sulfoxide in hexane:acetone (2:1 v/v): 0.5, 1, 2, 4, and $8 \times 10^{-5}$M.
PHORATE SULFOXIDE

EXPECTED PROBIT ○

OBSERVED PROBIT ●

LOG OF DOSE LEVEL
Figure 10. Regression line for phorate sulfone for third-instar southern corn rootworm larvae. Each point is a mean of three replicates and was obtained by treating larvae with 0.52 microliter of the following dose levels of phorate sulfone in hexane:acetone (2:1 v/v): 0.5, 1, 2, 4, and 8 x 10^{-6} M.
PHORATE SULFONE

EXPECTED PROBIT •

OBSERVED PROBIT □

LOG OF DOSE LEVEL
Figure 11. Regression line for phoratoxon for third-instar southern corn rootworm larvae. Each point is a mean of three replicates and was obtained by treating larvae with 0.52 microliter of the following dose levels of phoratoxon in hexane:acetone (2:1 v/v): 0.5, 1, 2, 4, and $8 \times 10^{-4}$M.
PHORATOXON

EXPECTED PROBIT •

OBSERVED PROBIT ○

LOG OF DOSE LEVEL
Figure 12. Regression line for phoratoxon sulfoxide for third-instar southern corn rootworm larvae. Each point is a mean of three replicates and was obtained by treating larvae with 0.52 microliter of the following dose levels of phoratoxon sulfoxide in hexane:acetone (2:1 v/v): 0.5, 1, 2, 4, and $8 \times 10^{-9}$M.
PHORATOXON SULFOXIDE

EXPECTED PROBIT ○

OBSERVED PROBIT ●

LOG OF DOSE LEVEL
Figure 13. Regression line for phoratoxon sulfone for third-instar southern corn rootworm larvae. Each point is a mean of three replicates and was obtained by treating larvae with 0.52 microliter of the following dose levels of phoratoxon sulfone in hexane:acetone (2:1 v/v): 0.5, 1, 2, 4, and $8 \times 10^{-4}$M.
PHORATOXON SULFONE

EXPECTED PROBIT ○

OBSERVED PROBIT ●

LOG OF DOSE LEVEL
SUMMARY

Five grams of soil (Clarion, Webster, or Harps) were incubated with 0.5 ml 10⁻⁴ M phorate, which approximates the current usage rate (2 lb/acre). Both unlabeled and ethoxy-1-¹⁴C-labeled phorate were used in these experiments. Incubations were carried out at 10, 20, and 30 C for periods up to 16 days. The higher the temperature the more rapid the loss of phorate. After 2 days at 30 C only 15% of the added phorate remained, approximately 30% was adsorbed to the soil, 35% oxidized to the sulfoxide, and 20% to the sulfone. Soxhlet extraction of soil incubated with ¹⁴C-labeled phorate for 2 days resulted in a 70% recovery of the added activity, again leaving 30% unextractable and presumably adsorbed to the soil. Phorate sulfoxide and sulfone were the only metabolites of phorate in soil extracts detected by gas-liquid chromatography, and autoradiography of thin-layer chromatography plates. The oxygen analogue pathway does not appear to be operative in this soil system, since phoratoxon and its sulfoxide and sulfone were never detected by any of the procedures used. Hydrolysis constitutes a minor pathway of phorate breakdown. Less than 2% of the added ¹⁴C-labeled phorate was recovered in the aqueous phase from a chloroform:water (2:1 v/v) extract of soil incubated for 48 hr.
Soils were sterilized by autoclaving (15 psi for 30 min), gamma irradiation (5 Mrad), sodium azide (2.5 mg in 0.1 ml water/5 g soil), and diethyl oxymidiformate (50 microliters/5 g soil). These techniques showed that oxidation of phorate to its sulfoxide was mainly nonbiological, whereas the sulfoxide to sulfone conversion was mainly biological. Confirmation of the nature of these conversions was obtained by incubating soils with SKF-525A and 8-hydroxyquinoline. Both these compounds greatly reduced phorate sulfone formation, but had no effect on sulfoxide production. It was assumed that microorganisms were responsible for these conversions in nonsterile soil, but no attempt was made to determine their identity. The results indicate that loss of phorate from three types of Iowa soil is a complex process. Some phorate is adsorbed, thioether oxidation to the sulfoxide and sulfone by soil microorganisms occurs, and some nonbiological conversion to the sulfoxide takes place. It is suggested that long-term protection afforded the growing plant is owing to thioether oxidation products rather than phorate.

The toxicity of phorate and its five oxidative metabolites, against third-instar southern corn rootworm larvae, was determined. Effects of the chemicals were assessed by treating larvae with 0.52 microliter of each compound in a n-hexane:acetone (2:1 v/v) solution. The effect of the treat-
ment was assessed by allowing treated larvae to burrow into moist soil for 30 minutes. The ED$_{50}$ values, ED$_{95}$ values, and regression lines with their coefficients are given. Phorate was the least toxic compound and phoratoxon the most toxic of the metabolites.
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