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Metabolism of parathion by two species of Rhizobium

David Lee Mick
Iowa State University

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METABOLISM OF PARATHION BY TWO SPECIES OF RHIZOBIUM

by

David Lee Mick

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

Major Subject: Insect Toxicology

Approved:

Signature was redacted for privacy.

In Charge of Major Work

Signature was redacted for privacy.

Head of Major Department

Signature was redacted for privacy.

Dean of Graduate School

Iowa State University Of Science and Technology Ames, Iowa

1969
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INTRODUCTION

The role of microorganisms in metabolizing pesticides has received increased attention in recent years. Soil microorganisms are of particular significance in light of the frequency and the quantity of pesticides directly or indirectly applied to the soil.

Due to the many variables of naturally occurring soil systems, only generalities can be made concerning the metabolic activities of any specific soil microorganism. Isolation and laboratory study of selected microorganisms commonly found in soils eliminates much of this variability. However, care should be exercised in application of the results back to the natural soil system.

Among the insecticides that have found soil usage are organic phosphorothionates such as parathion (\(\text{O,0-diethyl O-}p\text{-nitrophenyl phosphorothioate}\); see Figure 11). The metabolism of parathion has been extensively studied in insects and mammals, and more recently, in microorganisms. Reductive and hydrolytic detoxication systems appear to predominate in microorganisms while insects and mammals are able to form the activation metabolite, paraoxon (\(\text{O,0-diethyl O-}p\text{-nitrophenyl phosphate}\)), in an oxidative reaction. Activation of parathion to paraoxon involves replacing sulfur with an oxygen on the parathion molecule (see Fig. 11). Paraoxon is a potent inhibitor of cholinesterase (O'Brien 1960) which results in toxic action by interference with the normal mechanism of

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1 The common or trivial names and proprietary and trademarked names agree generally with those in lists of the Committee on Insecticide Terminology, Entomological Society of America (Billings 1965).

*Rhizobium japonicum* and *R. meliloti*, nitrogen-fixing bacteria of soybeans and alfalfa, respectively, were selected for studies relating to the metabolism of parathion. This investigation was concerned with the ability of these microorganisms to alter the parathion molecule and what affect the parathion might have on them in the process.
REVIEW OF LITERATURE

Reductive, Hydrolytic, and Oxidative Reactions

The soil ultimately receives the majority of agriculturally applied insecticides either by direct application or by indirect routes. The pathways of activation or degradation of these chemicals in the soil are of interest as they relate to the overall problem of environmental pollution.

Various bacteria that utilized aromatic pesticidal compounds such as phenol, naphthalene, and meta-cresol as carbon and other energy sources were isolated from greenhouse soils by Gray and Thornton (1928). Later research efforts were directed toward microbial degradation of the chlorinated hydrocarbon insecticides which have been shown to be highly resistant to attack (Jones 1952, Fleming and Maines 1953, Kigemagi et al. 1958, Lichtenstein et al. 1960, Lichtenstein and Schulz 1960, Randolph et al. 1960, Lichtenstein et al. 1962). However, it has been demonstrated that microorganisms possess the capability of altering chlorinated hydrocarbon insecticide molecules. For example, Kallman and Andrews (1963) and Chacko et al. (1966) found that DDT was dechlorinated to DDD by yeast and actinomycetes, respectively.

The conversion of aldrin to dieldrin in soils has been shown by Edwards et al. (1957), Bollen et al. (1958), and Lichtenstein and Schulz (1959a). Heptachlor is likewise converted in soil to its epoxide, heptachlor epoxide (Gannon and Bigger 1958, Lichtenstein and Polivka 1959). The influence of soil microorganisms on this process was demonstrated by Lichtenstein and Schulz (1960) when an autoclaved soil with a low number of microorganisms or a dry soil low in microorganisms retained aldrin
longer with no or only a small amount of dieldrin being formed. Heptachlor reacted similarly although the rate of epoxidation was less than for aldrin. The persistence of aldrin or heptachlor is greatly increased by the conversion to the epoxide form (Gannon and Bigger 1958, Lichtenstein and Schulz 1960). None of the microorganisms used by Chacko and Lockwood (1966) was able to degrade dieldrin.

The generally less persistent organophosphate and carbamate insecticides have come under recent scrutiny as they have become more popular in agricultural usage. Since parathion was the first organophosphate insecticide to gain widespread use, it probably has been studied more than any other insecticide of the organophosphate group. Averell and Norris (1948) published a colorimetric method of parathion determination, sensitive to at least 20 μg of parathion in 50 ml of benzene, which gave impetus to the detection of this insecticide.

Ginsburg et al. (1949) showed that no parathion remained in a New Jersey soil 7 months after being initially treated with 5 lb parathion per acre. When 20 lb of parathion per acre were applied to the soil, about 12 per cent of the total was detected 7 months after application. Microorganisms were not directly alluded to but were, no doubt, involved in the decrease of parathion recovered from the soil.

Soil microorganisms were implicated by Lichtenstein and Schulz (1964) as causing hydrolytic or reductive degradation of parathion in field collected soil samples. Aminoparathion (O,O-diethyl O-p-aminophenyl phosphorothioate), or reduced parathion, was found in autoclaved soils initially containing approximately 550,000 yeast and 750,000 bacteria per g of dry soil.
Yeast was primarily responsible for the reduction while "bacteria apparently had no effect". Unfortunately, the bacteria were not identified. Dry soils which retarded microorganism activity also were slow to degrade parathion. The more toxic oxidative activation analog of parathion, paraoxon, which is nearly always formed when parathion is administered to a mammal or an insect, (Hazleton 1955, Casida 1956, Krueger et al. 1960, Nakatsugawa and Dahm 1965, Nakatsugawa and Dahm 1967a, Nakatsugawa and Dahm 1967b, Neal 1967, Nakatsugawa et al. 1968, Nakatsugawa et al. 1969) was not detected in this investigation.

Ahmed and Casida (1958) found "little if any oxidation" of parathion, Dow ET-57, dimefox, and schradan by pure cultures of the green alga Chlorella pyrenoidosa (Emerson's strain). However, this same microorganism was able to oxidize phorate to the phosphorodithioate sulfoxide. Two soil bacteria, Pseudomonas fluorescens and Thiobacillus thiooxidans, were incapable of oxidizing phorate. Oxidation of sulfides to sulfoxides was accomplished by Chlorella and the yeast, Torulopsis utilis. Depending upon which insecticides (or derivatives) and microorganisms were incubated, hydrolysis occurred at different rates.

A fungus, Trichoderma viride, and a bacterium, Pseudomonas species, were isolated from an insecticide treated soil by Matsumura and Boush (1966). Both soil microorganisms were able to enzymatically degrade malathion through powerful carboxyesterases. Attempts to detect the oxidative metabolite, malaoxon, were unsuccessful. These same authors (Matsumura and Boush 1968) concluded that degradation of chlorinated...
hydrocarbon (DDT and dieldrin), carbamate (carbaryl), and organophosphate
diazinon, dichlorvos, and parathion) insecticides was probably through an
oxidative system. DDT and dieldrin were slow to degrade while carbaryl
was only slightly susceptible to the action of **T. viride**.

Parathion, and four other organophosphate insecticides were hydrolyzed
by **Pseudomonas melophthora**, the bacterial symbiote of the apple maggot,
through strong esterases, according to Boush and Matsumura (1967).
An oxidative method of attack was apparently lacking in this situation.

Microorganisms in bovine rumen fluid reduced parathion to aminopa­
parathion whereas boiled rumen fluid showed no activity thereby suggest­
ing an enzymatic system for the microorganisms therein (Cook 1957).
Ahmed et al. (1958) found the same reduction to be of primary importance
in a similar study of parathion metabolism by bovine rumen fluid. The
conversion to aminoparathion was dependent upon the concentration of
parathion with a greater yield being obtained at lower levels of para­
thion. Hydrolysis also occurred, with parathion being more easily
cleaved than aminoparathion. Oxidation reactions were of little signif­
icance. None of the authors attempted to isolate the rumen microorgan­
isms(s) responsible for these conversions.

Acid hydrolysis of urine from parathion-fed dairy cows yielded **p**-
aminophenol, probably from the conjugated excretory product, **p**-aminophenyl-
glucuronide. The parathion was evidently hydrolyzed **in vivo** to **p**-nitro-
phenol with subsequent reduction and conjugation, according to Pankaskie
et al. (1952). They did not rule out the possibility that aminoparathion, if present, was hydrolyzed to $p$-aminophenol. In contrast, parathion-fed nonruminating mammals excreted free $p$-nitrophenol (Hazleton 1955, O'Brien 1960). The ability of rumen organisms to reduce parathion to aminoparathion and $p$-nitrophenol to $p$-aminophenol would seem to account for the metabolic differences.

Addition of detergents alkyl benzene sulfonate (ABS) and linear alkyl benzene sulfonate (LAS) to the soil increased the number of bacteria with concomitant increase in persistence of parathion and diazinon (Lichtenstein 1966). Two theories were advanced as possible explanations for this anomaly. First, the build-up in bacteria may have been at the expense of those which normally metabolize the insecticides. Second, perhaps the insecticides were bound more tightly to the soil particles as a result of the detergents. Autoclaving soil treated with parathion and diazinon decreased the rate of metabolism of parathion but had no effect on the persistence of diazinon. Microorganisms were evidently involved in the metabolism of parathion whereas the hydrolysis of diazinon involved nonbiological factors. Getzin (1968) also showed that degradation of diazinon was not affected by autoclaving the soil and increasing soil acidity resulted in an increase of nonbiological degradation. Margot and Gysin (1957) had similar results. Catalytic hydrolysis of diazinon may be facilitated by CuCl$_2$ solutions and Cu-montmorillonite suspensions according to Mortland and Raman (1967). Although degradation of diazinon appears to be primarily nonbiological, Gunner et al. (1966) isolated a soil bacterium capable of
utilizing the diazinon molecule as a source of sulfur, phosphorus, carbon, and nitrogen.

Lichtenstein et al. (1968) performed experiments using sodium azide as an inhibitor of certain microorganisms. Sodium azide increased the persistence of parathion but no diazinon could be detected two weeks after incubation. The latter probably was not a result of soil microorganism activity since sodium azide catalyzed the hydrolysis of diazinon in distilled water.

Along with his work involving diazinon, Getzin (1968) concluded that microorganisms were important in the degradation of the organophosphate insecticide Zinophos®. Neutral and alkaline soil was conducive to the growth of the microorganisms and subsequent degradation of the insecticide.

Miyamoto et al. (1966) found that the microorganism, Bacillus subtilis, hydrolytically formed desmethyl parathion and dimethylphosphorothioic acid from methyl parathion and desmethyl aminoparathion from aminomethyl parathion. Desmethyl parathion was not converted to desmethyl aminoparathion nor was aminomethyl parathion converted to dimethylphosphorothioic acid. Diethylphosphorothioic acid (DEPTA) was a major metabolite of both house flies and rat microsomes in studies by Matsumura and Hogendijk (1964) and Nakatsugawa et al. (1969), respectively.

Influence of Pesticides on Microorganisms

During the era in which DDT was gaining widespread use, Wilson and Choudhri (1946) concluded that application rates ranging from 0.5 to 2 per cent of the dry weight of the soil were not injurious to microorganisms. Ammonification, the accumulation of nitrate, the normal soil
salt concentration, and nodulation of selected legumes, including alfalfa and soybeans, remained normal. Legume bacteria, including those from alfalfa but not soybeans, were grown in pure culture and subjected to a "wire loop" of DDT. After 10 days, growth appeared normal. Jones (1952) and Smith and Wenzel (1948) had similar results with DDT at rates as high as 200 and 400 lb per acre, respectively. In fact the former author noted a growth stimulation. Jones (1956) found that 200 lb per acre of aldrin, dieldrin, and chlordane were considerably more toxic to nitrifying microorganisms than ammonifiers. Abnormally high concentrations of the insecticides did not significantly affect the non-symbiotic nitrogen-fixing bacteria. Chlordane and benzene hexachloride were definitely toxic to nitrifying organisms in the soil at 500 lb per acre as reported by Smith and Wenzel (1948). Martin et al. (1959), using five annual field applications, found no effect on the soil organisms when eight chlorinated hydrocarbon insecticides were applied at rates ranging from 1 to 20 lb per acre.

Nitrogen fixation by black locust seedlings was not affected by 10 lb of chlordane per acre according to Simkover and Shenefelt (1951). Nodulation of soybeans was not reduced until the concentration of DDT reached 1,000 lb per acre according to Appleman and Sears (1946). They showed that this negative effect was a result of nodule bacteria injury.

Schradan (octamethyl pyrophosphoramide), an organophosphate insecticide, applied to the soil at rates as high as 3,000 ppm greatly increased the numbers of *Azotobacter* (Verona and Picci 1952). The growth of other bacteria and fungi also was stimulated when subjected to schradan at 100
to 500 ppm *in vitro*.

Parathion, applied at 10 lb of active ingredient per acre, was not detrimental to the growth of bacteria, streptomycetes, or molds 10 to 20 days after application (Bollen et al. 1954). Kasting and Woodward (1951) had similar results in the greenhouse, with applications of parathion up to 50 ppm having no effect on nitrifiers, cellulose-decomposers, or other groups of soil microorganisms.

Naumann (1958) observed increases in microbial numbers at approximately 7, 12, and 22 days after treating a chernozemic soil with 1 percent parathion powder. In 1959 he discovered that parathion applied at the same rate increased nitrogen fixing and nitrifying microorganisms.

Soil bacterial decomposition of 2,4-D went through a lag phase followed by a rapid decomposition phase (Audus 1952). The lag phase was eliminated by previous addition of 2,4-D to the soil. Whiteside and Alexander (1960) also demonstrated the adaptation of soil microorganisms involved in the decomposition of the herbicide 2,4-D. Decomposition was initially slow but became more rapid as time progressed. Additional 2,4-D was more rapidly attacked with a marked decrease in the lag period. This phenomenon has not been demonstrated with microbial attack on insecticides although nitrate reductase has been shown to be an adaptive enzyme in microorganisms (Kotah, 1963).

The majority of the material in this section is summarized in Table 1 to facilitate comparison of the data. The general consensus is that under normal application rates pesticidal and several other foreign compounds have little effect on the soil microorganisms that have been
studied. Additional information relating to the interactions between microorganisms and pesticides is summarized in a paper by Bollen (1961) and a literature review by Martin (1963).

**Rhizobium**

Breed et al. (1957), Waksman (1952), and Rangaswami (1966) have done an excellent job of presenting information pertaining to the genus *Rhizobium*. The following information is taken from their works.

Nodule bacteria were observed in the roots of legumes by Lachmann in 1858. Further studies of the bacteria by Woronin in 1866 led to the conclusion that they were rod-shaped and motile. The name *Rhizobium* was used in 1879 by Frank to describe the organism that stimulated plant production of root nodules. Although other workers had associated nodules with nitrogen fixation, the bacterium was isolated into pure culture by Beijerinck in 1888.

In 1932, Fred, Baldwin, and McCoy classified the genus *Rhizobium* as follows: (1) Alfalfa group, *R. meliloti*; (2) clover group, *R. trifolii*; (3) pea group, *R. leguminosarum*; (4) bean group, *R. phaseoli*; (5) lupine group, *R. lupini*; (6) soybean group, *R. japonicum*; and (7) cowpea group, *Rhizobium* sp. There are various strains within each species with differing degrees of specificity between the bacteria and legumes.

The root-nodule bacteria are thought to have five stages in the life cycle: (1) A preswarmer stage (nonmotile) observed in a neutral soil solution, (2) a larger coccus (nonmotile) observed with certain carbohydrates and phosphates, (3) a swarmer stage (motile) in which the cells
become ellipsoidal, (4) a rod form (some motility) developing from elongation of swarmer, and (5) a vacuolated stage (nonmotile) with the chromatin separating into bands in the absence of carbohydrates. The preswarmer stage is produced as these bands become rounded and leave the rod.

The various strains of *Rhizobium* are listed as aerobic and heterotrophic by Breed et al. (1957). They are rod-shaped measuring 0.5 to 0.9 by 1.2 to 3μ. They are motile when young, commonly changing to bacteroidal forms (1) upon artificial culture media containing alkaloids or glucosides or in which acidity is increased, or (2) during symbiosis within the nodule. Optimum conditions for growth are a pH from 5.5 to 7.0 and a temperature from 18 to 28 C. The thermal death point is at 60 to 62 C.
MATERIALS AND METHODS

Chemicals

Technical samples of parathion (M.W. 291.3) were obtained from the Nutritional Biochemicals Corp., Cleveland, Ohio, and the American Cyanamid Co., Princeton, N. J. Preliminary purification of parathion was done according to the method of Edwards and Hall (1949). The chemical was washed with Skellysolve A, dissolved in ether, washed with 10 per cent Na_2CO_3, dried over anhydrous Na_2SO_4, passed through a column of Attapulgus clay and Hyflo Super-Cel (2:1) and the ether was removed by evaporation. The partially purified sample was dissolved in absolute ethanol and crystals were obtained by cooling with a dry ice-acetone mixture. Further purification was accomplished by silica gel column chromatography with chloroform as eluent. The parathion was stored at approximately -15 C.

The parathion was dissolved at 10^{-1} M in absolute ethanol containing 1 per cent Triton X-100 (obtained from the Rohm and Haas Co., Philadelphia, Pa.). Aqueous dilutions of this stock solution were used in the experiments.

^{35}S-Parathion was prepared at the Radiochemical Centre, Amersham, Bucks., England, and purchased from the Nuclear-Chicago Corp., Des Plaines, Ill. Specific activity of ^{35}S-parathion was originally 7.8 mc/mmole. Silica gel column chromatography with chloroform as eluent was used for purification. The eluate was monitored continually for radioactivity. The fractions containing the majority of the activity were pooled,
evaporated, and the residue dissolved in absolute ethanol to make a 10⁻¹M solution. The purity was checked by thin-layer chromatography on silica gel F-254 precoated plates (Brinkmann Instruments Inc., Westbury, N. Y.) using two solvent systems: (1) hexane:ethyl acetate (2:1); (2) hexane: dioxane (2:1). ³⁵S-Parathion on the chromatograms was detected as a single spot by ultraviolet light. Only these spots showed radioactivity when detected through a 2-mm slit in aluminum foil by using an end-window Geiger counter.

Concentrations of parathion and ³⁵S-parathion were determined by gas chromatography. A 20-µl portion of the 10⁻¹M stock solution was diluted in n-hexane (Nanograde, Mallinckrodt Chemical Works, St. Louis, Mo.) to a parathion concentration of 10⁻⁶M. The sample was dried over anhydrous Na₂SO₄. A 3-µl portion of the 10⁻⁶M parathion served as the standard when injected into a Packard dual-column gas chromatograph equipped with electron capture detectors. Peak height ratios (sample/standard) were used for computation of parathion concentrations after establishing a standard curve using known concentrations of parathion (Fig. 1). Samples containing high concentrations of parathion were diluted so that the sample peak height was less than twice that of the standard.

Operational conditions for the gas chromatograph were: carrier gas, 50 ml/min of N₂; injector temperature, 210 C; column temperature, 200 C; and detector temperature, 210 C. Glass Columns, 2 mm (i.d.) x 120 cm were packed with 60- to 80-mesh, silylated Chromosorb G (Johns-Manville Products Corp., New York, N. Y.) coated with approximately 3 per cent Apiezon N. Prior to coating, the Chromosorb was washed with concentrated
HCl and then refluxed for more than 6 hr with a toluene solution containing 5 per cent hexamethyldisilazane, 5 per cent trimethylchlorosilane and 20 per cent pyridine. The silylated Chromosorb G was washed with toluene, methanol, toluene and n-hexane in that order.

Technical samples of aminoparathion hydrochloride and DEPTA were obtained from the American Cyanamid Co., Princeton, N. J. Purified paraoxon was obtained from the same source.

Cultivation of Rhizobium

*Rhizobium japonicum* and *R. meliloti*, obtained as pure cultures from Dr. Lloyd R. Frederick, Department of Agronomy, Iowa State University, were grown on the following medium: mannitol, 10.0 g; K$_2$HPO$_4$, 0.5 g; NaCl, 0.1 g; MgSO$_4$·7H$_2$O, 0.2 g; yeast extract, 1.0 g; agar, 14.0 g; and distilled water, 1.0 l.

About 25 ml of the above medium was autoclaved (121 C, 15 lb/inch$^2$ pressure for 20 min) in 125 ml French square bottles. The bottles were placed horizontally for cooling and stored in a refrigerator for future use.

Inoculation was accomplished by spreading three loopsful of the bacteria over the surface of the medium. Flaming of the loop and bottle mouths was practiced to maintain sterile conditions.

Suspensions of each *Rhizobium* species were obtained by harvesting the bacteria grown in three of the French square bottles. A stock saline solution (0.125 M) was sterilized by autoclaving at 121 C, 15 lb/inch$^2$ pressure for 20 min. Seven ml of stock saline solution.
was put into each of two of the bottles to aid in dislodging the organisms from the surface of the medium. Rubbing the inoculation loop over the surface of the medium assisted in removing the organisms. The contents of each of these two bottles were poured into the third bottle and the dislodging procedure was repeated. This bottle was thoroughly agitated to insure an even distribution of the rhizobial cells for addition to the incubation mixtures.

Incubation Mixture

Incubation mixtures with each Rhizobium species was carried out in a series of 35 ml centrifuge tubes with the same total volume of reactants. Each tube contained the following: 1 ml suspension of R. japonicum or R. meliloti; 1 ml parathion solution or other substrate; 1 ml phosphate buffer; 1 ml stock saline solution. The incubations were terminated at the desired time intervals by extracting with n-hexane or chloroform. An explanation of the extraction procedure will be found in the next section. The $10^{-1} M$ ethanol stock solution of parathion was diluted with stock saline solution to the desired molarity for incorporation of 1 ml into the incubation mixtures.

Combinations of $Na_2HPO_4$ and $KH_2PO_4$ (both 0.2 M) were used to determine optimum pH values for the incubation mixtures. The extent of metabolism under these conditions was determined in 40-hr, time-course studies utilizing the disappearance of parathion, as detected by gas chromatography. The phosphate salt solutions were mixed in different ratios to obtain nine pH values ranging from 5.0 to 8.3. For example,
to obtain a pH of 7.0 the ratio was 2:1 \((\text{Na}_2\text{HPO}_4 : \text{KH}_2\text{PO}_4)\). It was determined experimentally that each pH value used in these experiments remained stable during the incubation period.

One ml of the pH 7.0 buffer was used in the incubation mixtures of another 40-hr incubation study with temperature as the variable. The seven incubation temperatures ranged from 0 to 60 °C (Fig. 3).

The initial number of rhizobia in the incubation mixtures used to obtain the data in Figs. 5 and 7 was estimated by the plate-count technique using 100 x 15 mm sterile plastic Falcon petri dishes. A series of dilution blanks of 99 and 999 ml, with 1 ml being transferred in all cases, were used to obtain plate counts between 30 and 300 colonies. The medium used in the petri dishes was the same as previously described for cultivation of the organisms. Each plate was replicated three times with an average used for the actual count. When studying the affect of parathion on the rhizobia (Figs. 12 and 13) population counts were made at intervals during the 100-hr incubation period.

Two different types of control samples (i.e., curves labeled "check" in Figs. 2 through 5, 12, and 13) were used. One type involved control samples used in conjunction with parathion incubations for metabolism studies. These consisted of replacing the rhizobia in the incubation mixtures with sterile saline solution to determine if non-rhizobial factors were important in the metabolism studies. A second type of control was used when studying the effect of parathion on the rhizobia (Figs. 12 and 13). These control samples included all components of the incubation mixture except parathion which was replaced with
sterile saline solution. Therefore, population changes of rhizobia were determined under the experimental conditions in the absence of parathion.

**Determination of Metabolites**

For gas chromatographic analysis, parathion was extracted by shaking (15 min on a Fisher-Kahn shaker, 275-285 oscillations/min) the incubation mixture with 4 ml n-hexane, centrifuging (5 min at 2500 rpm in an international, Model K, with a no. 279 rotor), and drying approximately 3 ml of the extract over Na$_2$SO$_4$.

For other analyses, the incubation mixture was extracted three times with 4 ml of chloroform. Identification of the metabolites in the chloroform layer was accomplished by thin-layer chromatography after evaporation of the chloroform to approximately 2 ml. Aliquots were spotted 1.5 cm from the bottom of 5 x 20 cm plates spread to a thickness of 0.25 mm with silica gel G containing 13 per cent CaSO$_4$ as binder (Brinkmann Instruments, Inc.). The chromatogram was developed with a 20 ml mixture of n-hexane:chloroform:anhydrous methyl alcohol (7:2:1). The solvent front was allowed to rise 12.5 cm from the bottom of the plate. A palladium chloride solution (0.5 g palladium chloride and 2 ml concentrated HCl diluted to 100 ml with water) was sprayed on the plates to affect a color reaction with parathion and the chloroform-soluble metabolites. When $^{35}$S-parathion was used, portions of the silica gel, 5 mm in width, were scraped into vials containing 20 ml scintillation solution and the radioactivity was counted by a Packard, Model 3003, TriCarb Scintillation
Spectrometer. The scintillation solution was a mixture of 50 g of PPO (2,5-diphenyloxazole), 2 g of dimethyl POPOP (1,2-bis-2-(4-methyl-5-phenyloxazolyl)-benzene), 65 ml of ethanolamine, 5 l of toluene, and methanol to make 10 l. Quenching was checked by internal standard.

A modification by Lausen (1962) of the Averell and Norris (1948) colorimetric method was performed as further identification of the chloroform soluble metabolites. The chloroform was evaporated slowly until only a slight, moist residue remained, then Lausen's procedure was initiated. The reduction step of the procedure (conversion of NO\textsubscript{2} to NH\textsubscript{3}) was omitted so that aminoparathion produced by the rhizobia could be measured. The color was formed by a diazotization reaction of the amino portion of the aminoparathion molecule with N-(1-naphthyl)-ethylenediamine dihydrochloride. A stable purple color developed rapidly with an absorption peak at 560 nm.

Analyses for water-soluble metabolites utilized ion-exchange column chromatography as described by Plapp and Casida (1958). The 1 cm (i.d.) x 21 cm column contained Dowex 1-X8, an anion exchange resin. After the chloroform extractions, the aqueous layer from 10 incubation mixtures of each organism was loaded on the column for the column chromatography runs. Eluting solutions were (capital letters correspond to those shown in Figs. 8 and 9): (A) 0.01 N HCl to 0.1 N HCl, each 50 ml; (B) 0.1 N HCl, 33.3 ml; (C) 0.1 N HCl:methanol (1:3) to 1 N HCl:methanol (1:3), each 50 ml; (D) 1 N HCl:methanol (1:3) to 6 N HCl:methanol (1:3), each 50 ml. The eluate was collected in 3 to 4 ml quantities with a LKB Ultrorac, Type 7000, Fraction Collector. Radioactive samples were
counted by the Packard Scintillation Spectrometer.

Non-radioactive eluates of phosphorus containing compounds were analyzed for phosphorus. If an eluate sample contained an organic solvent, a drop of phenolphthalein solution was added to 1 ml of the sample, the mixture was neutralized with NaOH and the solvent evaporated by blowing with nitrogen. Digestion was accomplished with 2 ml of 60 percent perchloric acid for 1 to 1.5 hr at 150 to 160°C. If a brownish color persisted in the sample, it was cooled, a few drops of H₂O₂ were added and the sample reheated until clear. The samples were cooled after digestion.

After perchloric acid digestion, the samples were diluted with 5 ml of 1 N NaOH and phosphorus determined by the method of Rockstein and Herron (1951). Each sample was mixed with 1 ml of 0.05 M ammonium molybdate. The blue color was developed with 1 ml of 10 percent FeSO₄·7H₂O in 0.15 N H₂SO₄. The absorbancy was read on a Beckman DB Spectrophotometer at 720 nm between 20 min and 1 hr after addition of the FeSO₄ solution. The color remained stable over this time interval. A standard curve was run with known concentrations of Na₂HPO₄ and it was identical to the one by Rockstein and Herron (1951).

In p-nitrophenol metabolism studies, the parathion in the incubation mixture was replaced with 1 ml of 4 X 10⁻⁴ M p-nitrophenol. Analysis for p-nitrophenol was conducted by acidifying 4 ml of the incubation mixture with trichloroacetic acid followed by extraction with 10 ml n-hexane to remove parathion and aminoparathion. After the residual n-hexane was removed by blowing lightly with nitrogen, the solution was
extracted with chloroform:ether (5:1). Four ml of the chloroform:ether layer were shaken with 3 ml of 0.05 M Na₂CO₃ in 25 per cent ethanol. The top layer was removed and the absorbancy read on a Beckman DB spectrophotometer at 400 mµ. A linear p-nitrophenol standard curve was obtained using concentrations of p-nitrophenol from 10⁻⁵ to 5 x 10⁻⁴ M giving absorbance values from 0.25 to 1.37, respectively. The method was capable of detecting accurately as little as 0.1 µg/ml p-nitrophenol.

Analyses for both free and conjugated p-aminophenol were conducted on the p-nitrophenol incubations. The determinations were carried out by the method of Brodie and Axelrod (1948). The 4 ml incubation mixture was added to 50 ml of isoamyl alcohol:ether (1.5:100) in a glass-stoppered bottle. One ml of distilled water was added, the mixture was agitated for 10 min on a Fisher-Kahn shaker, and centrifuged. Forty ml of the alcohol:ether phase plus 6 ml of 0.01 N HCl were shaken for 5 min followed by centrifugation and removal of the ether phase. Different volumes of the acid phase were diluted to 5 ml with 0.01 N HCl. One ml each of freshly prepared 1 per cent phenol solution and sodium hypobromite solution were added. The absorbancy of the indophenol dye was read on a Beckman DB spectrophotometer at 620 mµ. The conjugated p-aminophenol determination consisted of adding 4 ml incubation mixture to 1 ml of concentrated HCl in a 15 ml graduated conical tube. Two ml of distilled water were added and the mixture was autoclaved at 15 lb pressure for 1.5 hr. After cooling, the volume was adjusted to 6 ml with distilled water. A 5 ml aliquot was thoroughly mixed with
5 g of \( K_2HPO_4 \). With the addition of 50 ml of isoamyl ether the determination of \( p \)-aminophenol followed the procedure described earlier in the paragraph.

The method of Jungreis and Soriano (1961) also was used in an attempt to detect \( p \)-aminophenol. One, 2, 3, and 4 ml of the incubation mixture were added to the same volumes of ethanol. Two ml of a borax-sodium hydroxide buffer solution were used to adjust the solution to pH 10. One ml of a 10 per cent solution of phenol in water was added plus water to make a total volume of 10 ml. After 2 hr the absorbancy was read on a Beckman DB Spectrophotometer at 620 \( \mu m \).

Both methods of \( p \)-aminophenol analyses were sensitive to about 1 \( \mu g/ml \) \( p \)-aminophenol. Thus, anything greater than 10 per cent conversion of \( p \)-nitrophenol \( (1 \times 10^{-4} M \) in the incubation mixture) to \( p \)-aminophenol could be detected as \( p \)-aminophenol.
RESULTS

Temperature and pH Optima

Two major factors in establishing optimum growth conditions for bacteria are temperature and pH. Four-day-old cultures of *Rhizobium japonicum* and *R. meliloti* were incubated with $10^{-6}$ M parathion for 40 hr at the pH values and temperatures shown in Figs. 2 and 3.

Gas chromatographic analyses showed that a pH between 7.0 and 7.3 gave optimum metabolism of parathion by both *Rhizobium* species at 25°C (Fig. 2). The retention time for parathion was 8.0 min. A pH of 7.0 was selected for future incubations.

A temperature of 40°C provided for maximum parathion metabolism while the pH was held at 7.0 (Fig. 3). A temperature of 25°C was selected for future incubations because the organisms behaved relatively the same as at 40°C. Degradation (disappearance) of parathion was significant at 25°C, it was easier to maintain, and soil temperatures probably would average closer to 25°C than 40°C.

Age of *Rhizobium* for Optimum Parathion Metabolism

To determine the most active age, the microorganisms were harvested from triplicate cultures at one through eight days of age for incubation with $10^{-6}$ M parathion. Metabolism of parathion was again measured by its disappearance as detected by gas chromatography. Metabolism of parathion by *R. japonicum* was optimum when the microorganisms were four-days old at initiation of incubation while *R. meliloti* required only two days growth prior to incubation for optimum metabolism (Fig. 4). The microorganisms were harvested at these two ages for future incubations.
Metabolism of $10^{-6}$ M Parathion by Rhizobium

Time-course studies showed that about 95 per cent of the $10^{-6}$ M parathion was metabolized in 50 hr by both Rhizobium species (Fig. 5). The curves in Fig. 5 are based on gas chromatographic injections of n-hexane extractions of the incubation mixtures. To aid in the determination of metabolites, $^{35}$S-parathion was used in 10 incubation mixtures with each organism. Incubation of $10^{-6}$ M $^{35}$S-parathion with each Rhizobium species was stopped after 50 hr by extracting with chloroform. Three chloroform extractions, each with 4 ml of chloroform, showed that about 90 per cent of the radioactivity was in the chloroform layer and the remaining 10 per cent was in the aqueous portion (Table 2).

For qualitative determination of parathion and other chloroform-soluble products, aliquots of the chloroform layer were spotted on silica gel G thin-layer chromatography plates which were developed with n-hexane:chloroform:anhydrous methyl alcohol (7:2:1). After spraying with palladium chloride solution, two spots were evident with $R_f$ values of 0.64 (parathion) and 0.23 (aminoparathion). A repeat of this procedure produced identical results. Also, authentic samples of parathion and aminoparathion, treated as above, produced spots with $R_f$ values of 0.64 and 0.25, respectively. These data compare favorably with the $R_f$ values reported by Lichtenstein and Schulz (1964) of 0.64 for parathion and 0.27 for aminoparathion.

To determine, quantitatively, the amount of parathion and aminoparathion in the chloroform extract, $^{35}$S-parathion was used in the incubation mixtures. After three extractions, each with 4 ml chloroform, and
evaporation to approximately 1 ml by blowing lightly with nitrogen, 
25 μl were spotted and developed on a thin-layer plate along with au­
thentic samples of parathion and aminoparathion. Spraying with palla­
dium chloride solution showed the location of the authentic samples 
as described in the preceding paragraph. The silica gel on the thin-
layer plates was scraped off in 5 mm increments and these were individually 
counted in a scintillation counter. The areas of high count density were 
identical with the parathion and aminoparathion spots delineated by 
palladium chloride. Approximately 85 per cent of the radioactivity was 
located at the aminoparathion position for both *Rhizobium* incubations 
(Fig. 6).

Rhizobial conversion of parathion to aminoparathion was determined 
in a time-course study (Fig. 7). Identification of the extracts in the 
chloroform layer was accomplished by colorimetric analyses as described 
by Lausen (1962). The reduction step was omitted in the procedure. 
Therefore, a positive color test measured the ability of the microorganisms 
to reduce parathion to aminoparathion.

The water-soluble metabolites of 35S-parathion incubations were 
separated by ion-exchange chromatography as described by Plapp and 
Casida (1958) using Dowex 1-X8 anion exchange resin (Fig. 8). Peak IV 
represents O,O-diethyl phosphorothioic acid (DEPTA). An authentic 
sample of DEPTA was run through the same column chromatography procedure. 
Phosphorus determinations of each fraction by the method of Rockstein 
and Herron (1951) showed that DEPTA was eluted coincident with peak IV 
(Fig. 8). Plapp and Casida (1958), Matsumura and Hogendijk (1964),
Miyamoto et al. (1966), and Nakatsugawa et al. (1969) have had similar results.

Peak III was also in evidence from the authentic DEPTA run. Therefore, peak III (Fig. 8) is not a separate metabolite but represents decomposition of DEPTA by the catalytic action of the Dowex 1-X8 resin under acidic conditions (Matsumura and Hogendijk 1964, Nakatsugawa et al. 1969). Peak I was not identified. The letter "X" on the abscissa represents the aqueous portion drained from the column in the loading operation. A peak II will be described later in connection with Fig. 9.

Aminoparathion (10^{-5} M) was incubated with each *Rhizobium* species to determine if DEPTA was being formed. After three extractions of each incubation mixture with 4 ml of chloroform, the aqueous layer was subjected to Dowex 1-X8 column chromatography. Phosphorus determinations by the method of Rockstein and Herron (1951) of the fractions collected from column chromatography did not show any peak in eluate C (Fig. 9). DEPTA was shown previously to be eluted in eluate C (Fig. 8). Incubation of aminoparathion with each organism did not result, therefore, in the formation of DEPTA.

Phosphate compounds used in the buffer solution and incubation medium (Na₂HPO₄, KH₂PO₄, and K₂HPO₄; 20 mg each) were dissolved in 10 ml of distilled water and eluted on a Dowex 1-X8 as previously described. Phosphorus determinations by the method of Rockstein and Herron (1951) showed a definite blue color reaction from 60 through 130 ml of the eluate. This is the same area in which the aqueous layer of the amino-
parathion incubations produced a color reaction as shown by peak II of Fig. 9. The buffer solution ($Na_2HPO_4$ and $KH_2PO_4$) and probably the $K_2HPO_4$ from the medium represent the majority of peak II.

Fig. 10 shows the time course degradation of parathion by both *Rhizobium* species along with the metabolite production. The DEPTA values were figured as the percentage of radioactivity in the aqueous layer after the three chloroform extractions of $^{35}S$-parathion time-course incubations (Table 3). The small percentages at zero hour of incubation probably are a result of residual parathion and aminoparathion remaining in the aqueous layer after the chloroform extractions.

An active oxidative enzyme system was evidently lacking or inoperative since paraoxon, the oxidative activation product, was not detected by either thin-layer or gas chromatography. Three µl of authentic $10^{-6}$M paraoxon samples in n-hexane injected into the gas chromatograph produced peaks about half the height of the $10^{-6}$M parathion peaks. Thus, the instrument was capable of detecting paraoxon. The retention time for paraoxon was 5.3 min. Glassware was silylated by treating for about 10 min with a toluene solution containing 5 per cent dimethyldichlorosilane and 20 per cent pyridine to prevent the loss of paraoxon due to adsorption onto the glass surface.

Metabolism of $10^{-4}$ and $10^{-5}$M Parathion by *Rhizobium*

Increasing the parathion concentration resulted in different rates of metabolism for both *Rhizobium* species (Fig. 5). *R. japonicum* metabolized less parathion than *R. meliloti*, the difference being
greatest at the higher concentration of parathion. This difference is of even greater magnitude if one considers that *R. meliloti* was about 25 times fewer in number than *R. japonicum* (see next section).

**Effect of Parathion on Rhizobium**

The 4 ml incubation mixtures initially contained about $1.0 \times 10^8$ *R. meliloti* (Fig. 12) and $2.5 \times 10^9$ *R. japonicum* (Fig. 13) as determined by the plate-count method. These figures are typical of the results obtained in three other attempts to count the *Rhizobium* in the incubation mixtures.

The *R. meliloti* in the control showed a three-fold increase in numbers during the first 10 to 20 hr of incubation with a gradual decline thereafter to about $1.6 \times 10^8$ at the termination of the experiment at 100 hr (Fig. 12). Fig. 12 also shows that incubation of *R. meliloti* with $10^{-4}$, $10^{-5}$, and $10^{-6}$M parathion resulted in an overall reduction of the number of bacteria. The highest concentration of parathion ($10^{-6}$M) depressed the bacterial population the most during the first 18 hr. An increase above initial count occurred between 20 and 30 hr, and a decline for $10^{-4}$ and $10^{-5}$M incubations only at 55 hr. The $10^{-6}$M concentration produced a gradual decline in populations after 20 hr. The organisms under the influence of $10^{-4}$ and $10^{-5}$M parathion increased in the time span from 55 to 100 hr.

The *R. japonicum* in the control sample increased from $2.5 \times 10^9$ to $9 \times 10^{-9}$ during the first 30 hr of incubation, decreased to $3.3 \times 10^9$ in the next 10 hr followed by a linear reduction to $2.7 \times 10^9$ at 100
hr. The incorporation of $10^{-4}$, $10^{-5}$, and $10^{-6}$M parathion in the incubation mixtures produced an initial decrease in *R. japonicum* during the first 15 hr of incubation. Thereafter, the same trend as for the control was followed with the exception that the bacterial numbers were decreased by the presence of parathion.
Preliminary studies, not reported in this dissertation, utilizing a commercial yeast produced evidence of parathion metabolism. However, upon isolating bacteria associated with the yeast, it was found that two species of bacteria were the active metabolic agents rather than the yeast. Attention was then focused on the myriads of soil bacteria that might be instrumental in altering the parathion molecule since most insecticides eventually end up in or on the soil. \textit{R. japonicum} and \textit{R. meliloti} were chosen to determine if they possess the ability to metabolize parathion in addition to their nitrogen fixing capabilities.

Parathion was metabolized by incubating it with the two nitrogen-fixing \textit{Rhizobium} species. The major metabolite was aminoparathion which accounted for about 85 per cent of the initial parathion. Miyamoto et al. (1966) found \textit{Bacillus subtilus} reduced Sumithion\textsuperscript{®} to aminosumithion and methyl parathion to the amino homolog of methyl parathion by 65 and 70 per cent, respectively. Reduction of parathion to aminoparathion was the primary route of degradation by yeast used in the experiments of Lichtenstein and Schulz (1964). Bacteria were stated to have no effect on this reduction but the bacteria were not identified. Cook (1957), Ahmed et al. (1958), and others showed that rumen microorganisms also are active in reducing parathion to aminoparathion.

Hydrolytic cleavage of parathion yielded O,O-diethyl phosphorothioic acid (DEPTA), which represented about 10 per cent of the initial parathion. However, DEPTA was not a degradative product of aminoparathion metabolism. Lichtenstein and Schulz (1964) noted that
hydrolytic or reductive degradation of parathion was dependent on the soil microorganism population. The major route of parathion degradation by *Pseudomonas melophthora*, the bacterial symbiote of the apple maggot, was apparently hydrolytic by strong esterases (Boush and Matsumura, 1967) and Ahmed and Casida (1958) clearly showed hydrolysis to be important in the degradation of organophosphorus insecticides.

This study has proven that rhizobia reduced the nitro group of parathion to an amino group in the formation of aminoparathion. Therefore, it seemed logical that p-nitrophenol, the remaining portion of the parathion molecule after hydrolysis to DEPTA, would be reduced to p-aminophenol (Fig. 11). Conjugation of the latter compound was possible based on the results by Pankaskie et al. (1952) whereby dairy cows hydrolyzed parathion to p-nitrophenol and reduced the latter to p-aminophenol. The p-aminophenol was conjugated with glucuronic acid and excreted in the urine as p-aminophenylglucuronide.

To determine if the rhizobia were capable of metabolizing p-nitrophenol, a 40-hr time-course was run with incubation mixtures containing 1 x 10^-4 M p-nitrophenol. The *R. japonicum* metabolized half the p-nitrophenol during the 40 hr whereas *R. meliloti* metabolized only one-fourth the initial amount. The control samples containing no microorganisms maintained the initial concentration of p-nitrophenol during the 40 hr. The probable metabolic route was reduction although the p-aminophenol was not detected. Its susceptibility to the action of air and light may have hindered its detection. Also, either p-nitrophenol or p-aminophenol may have been altered to another structure not detectable by the color-
imetric techniques used in this experiment.

Ahmed and Casida (1958), Miyamoto et al. (1966), Lichtenstein and Schulz (1964) have shown that a bacterial oxidative system is often lacking or inoperative since parathion was not oxidized to paraoxon. Similar results were obtained with both Rhizobium species of these experiments since paraoxon was not detected by either thin-layer or gas chromatography.

A former parathion recommendation for corn rootworm control in Iowa called for 1 lb per acre of actual insecticide to be spread in a 7-inch band over the corn row. Since an acre of soil 6 inches deep weighs about 2,000,000 lb, the 1 lb of parathion represents 0.5 ppm. However, considering only the 7-inch band to the same 6-inch depth and 42 inch corn rows and 1 lb per acre of parathion, the concentration in the band is increased to 3.0 ppm. The latter situation is more nearly what would be found under field conditions at the time of application of the insecticide.

The $10^{-6}$ M parathion in the 4 ml incubation mixture represents 0.3 ppm. This concentration is probably realistic in a soil situation around the periphery of the zone of application as biological, physical, chemical, and mechanical forces either move or alter the insecticide molecules relative to their initial state. To ascertain what effect Rhizobium might have on parathion at greater concentrations, incubations containing $10^{-5}$ M (3 ppm) and $10^{-4}$ M (30 ppm) parathion were used. These concentrations may more nearly represent conditions as they exist near the zone of application. R. meliloti was able to metabolize more parathion than R. japonicum as the parathion concentration increased. This happened
despite the fact that the higher concentrations of parathion decreased the R. meliloti population more than the R. japonicum. Since there were initially about 25 times fewer R. meliloti than R. japonicum \((1.0 \times 10^8 \text{ compared to } 2.5 \times 10^9)\) and since the increased parathion concentrations were more detrimental to the population of R. meliloti, it seems logical to conclude that it is more efficient than R. japonicum in metabolizing parathion and becomes increasingly so as the parathion concentration is increased. It was not determined at what parathion concentration no metabolism occurs, if such a concentration does exist.

No implication is intended as to the deleterious effects that soil applications of parathion might have on nitrogen fixation of soybeans and alfalfa. However, \(10^{-4}\), \(10^{-5}\), and \(10^{-6}\text{M}\) parathion decreased the population of both Rhizobium species when compared to control incubations which contained no parathion. The \(10^{-4}\text{M}\) parathion had the most adverse effect on R. japonicum resulting in a population decrease of up to 60 per cent when compared to the population of an incubation mixture that contained no parathion. The decrease of R. meliloti numbers when incubated with \(10^{-4}\text{M}\) was even more severe. After 15 hr of incubation, a 90 per cent population reduction was noted when compared to the control. The increase in population of R. meliloti during the 55 to 100 hr incubation period may be indicative of growth stimulation from the degradation products of parathion. This phenomenon also was noted by Naumann (1958).

No lag time was evident in the attack on the parathion molecule by the Rhizobium even though the rhizobial population decreased during the
first 15 hr of incubation. It appears that although both Rhizobium species went through a 15 hr adaptive period, the ability to metabolize parathion was not hindered. Whiteside and Alexander (1960) found that soil microorganisms went through an adaptive period when exposed to 2,4-D as measured by their ability to decompose the herbicide. No mention was made of the microbial population.
SUMMARY

Incubations of $10^{-6}$M parathion with the nitrogen-fixing bacteria of alfalfa and soybeans, *R. meliloti* and *R. japonicum*, respectively, showed that the primary route of degradation was by reduction to aminoparathion. To a lesser extent, O,O-diethyl phosphorothioic acid (DEPTA) was formed as a hydrolysis product from parathion. The reduction process accounted for about 85 per cent of the initial parathion while DEPTA represented about 10 per cent. The 5 per cent remaining parathion was not metabolized. Although DEPTA was a hydrolytic product of parathion degradation, aminoparathion was not hydrolyzed to DEPTA when incubated with the two species of *Rhizobium*.

An oxidative system in the two *Rhizobium* species was either lacking or inoperative for no paraoxon, the oxidative metabolite, was detected by gas chromatographic procedures. The gas chromatograph was sensitive to the compound since 3 μl injections of $10^{-6}$M paraoxon produced peaks about half the height of the $10^{-6}$M parathion peaks.

The corn rootworm control recommendation for Iowa specified 1 lb per acre of parathion in a 7-inch band over the row. This amounts to 3 ppm in a soil zone 6 inches deep. Incubation mixtures containing 3 ppm ($10^{-5}$M parathion) and 30 ppm ($10^{-4}$M parathion) were used to simulate conditions that might exist near the zone of application. The $10^{-6}$M parathion (0.3 ppm) might correspond more to a peripheral zone around the application band where biological, physical, chemical, and mechanical forces alter the original application of parathion. At the increased concentrations of parathion ($10^{-4}$ and $10^{-5}$M), *R. meliloti* was able to
metabolize more parathion than *R. japonicum* even though the higher rates were more detrimental to its population and fewer organisms existed initially.

No conclusions can be drawn from these experiments concerning the interaction of parathion in the soil and nitrogen fixation of alfalfa and soybeans. However, populations of both *Rhizobium* species were adversely affected by the presence of parathion in the incubation mixture. Both species went through an apparent 15-hr lag period during which the numbers decreased before multiplying to maximum populations at 25 to 30 hr of incubation time. Thereafter, a general decline of the populations occurred. The populations of the rhizobia were always less under the influence of parathion when compared to incubations containing no parathion.
Table 1. Effect of insecticides on microorganisms

<table>
<thead>
<tr>
<th>Soil Microorganism</th>
<th>Insecticide</th>
<th>lb/acre&lt;sup&gt;a&lt;/sup&gt;</th>
<th>ppm</th>
<th>Other</th>
<th>Effect on Microorganisms</th>
<th>Reference</th>
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<tbody>
<tr>
<td>Unidentified</td>
<td>DDT</td>
<td>0.5 to 2% of dry wt. of soil&lt;sup&gt;b&lt;/sup&gt;</td>
<td>Ammonification, accumulation of nitrate, soil salt concentration, nodulation of soybeans &amp; alfalfa remained normal</td>
<td>Wilson &amp; Choudhri (1946)</td>
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<td>Rhizobia</td>
<td>DDT</td>
<td>1000&lt;sup&gt;b&lt;/sup&gt;</td>
<td>500</td>
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<td>Reduction of soybean nodulation</td>
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<td>200</td>
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<td>No effect</td>
<td>Smith &amp; Wenzel (1948)</td>
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<td>Growth stimulation</td>
<td>Jones (1956)</td>
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<td>Smith &amp; Wenzel (1948)</td>
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<td></td>
<td>N-fixation of black locust seedlings not effected</td>
<td>Simkover &amp; Shenefelt (1951)</td>
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<sup>a</sup> 2,000,000 lb per acre of soil (6 inches deep) was used to convert lb/acre to ppm or vice versa.

<sup>b</sup> Rate of application reported in paper.
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<tr>
<th>Soil Microorganism</th>
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<th>lb/acre&lt;sup&gt;a&lt;/sup&gt;</th>
<th>ppm</th>
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<td></td>
<td>streptomycyes, or molds</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>10-20 days after</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>application</td>
<td></td>
</tr>
<tr>
<td>Unidentified</td>
<td>Parathion</td>
<td></td>
<td>1% powder&lt;sup&gt;b&lt;/sup&gt;/</td>
<td></td>
<td>Increase of nitrifiers</td>
<td>Naumann (1958)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>and N-fixers</td>
<td></td>
</tr>
</tbody>
</table>
Table 2. Chloroform extractions of *Rhizobium japonicum* and *R. meliloti* incubations with $10^{-6}$ M $^{35}$S-parathion$^a$.

<table>
<thead>
<tr>
<th>SAMPLE</th>
<th>PER CENT OF RADIOACTIVITY</th>
<th>SAMPLE</th>
<th>PER CENT OF RADIOACTIVITY</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td><em>R. japonicum</em></td>
<td><em>R. meliloti</em></td>
<td></td>
</tr>
<tr>
<td>Incubation mixture at 0 hr</td>
<td>100</td>
<td>100</td>
<td></td>
</tr>
<tr>
<td>Chloroform extract after 50 hr incubation</td>
<td>91</td>
<td>92</td>
<td></td>
</tr>
<tr>
<td>Aqueous layer after 50 hr incubation</td>
<td>9</td>
<td>8</td>
<td></td>
</tr>
</tbody>
</table>

$^a$ The percentage values are averages of three separate trials.
Table 3. Time course of the radioactivity in the aqueous layer after chloroform extractions of *Rhizobium japonicum* and *R. meliloti* incubations with $^{35}$S-parathion.

<table>
<thead>
<tr>
<th>Time (Hours)</th>
<th>R. japonicum</th>
<th>R. meliloti</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>0.3</td>
<td>0.2</td>
</tr>
<tr>
<td>2</td>
<td>3.7</td>
<td>2.9</td>
</tr>
<tr>
<td>5</td>
<td>6.6</td>
<td>7.2</td>
</tr>
<tr>
<td>10</td>
<td>9.0</td>
<td>8.0</td>
</tr>
<tr>
<td>17</td>
<td>10.1</td>
<td>10.0</td>
</tr>
<tr>
<td>20</td>
<td>10.5</td>
<td>9.8</td>
</tr>
<tr>
<td>25</td>
<td>10.6</td>
<td>10.0</td>
</tr>
<tr>
<td>30</td>
<td>11.0</td>
<td>10.2</td>
</tr>
</tbody>
</table>
FIGURES
Figure 1. Standard curve relating the ratio of sample and standard peak heights to parathion concentration. The standard was $10^{-6}\text{M}$ parathion. One of 2 sets of similar data obtained.
Figure 2. Parathion remaining after 40-hr incubation with *Rhizobium japonicum* and *R. meliloti* at pH values ranging from 5.0 to 8.3. Different ratios of Na$_2$HPO$_4$ and KH$_2$PO$_4$ (both 0.2 M) were used to obtain the pH values in the incubation mixtures. The temperature was maintained at 25°C.
PARATHION CONCENTRATION (10^{-6} M)

pH

R. meliloti

CHECK

R. japonicum
Figure 3. Parathion remaining after 40-hr incubation with *Rhizobium japonicum* and *R. meliloti* at temperatures ranging from 0 to 60°C. The pH was maintained at 7.0.
Figure 4. Time-course of $10^{-6}$M parathion metabolism by different ages of *Rhizobium japonicum* and *R. meliloti*. The numbers at the right of the curves indicate the age of the organism in days at initiation of incubation. The incubation mixture was buffered to pH 7.0 and the temperature maintained at 25°C. One of three sets of similar data obtained.
Figure 5. Time-course of parathion metabolism by *Rhizobium japonicum* and *R. meliloti* using three concentrations of parathion ($10^{-4}$, $10^{-5}$, and $10^{-6}$ M) in the incubation mixtures. The incubation mixtures were buffered to pH 7.0 and the temperature was maintained at 25 C. The *R. japonicum* cultures were four-days old and the *R. meliloti* two-days old at initiation of incubation. More than one point at certain time intervals represents data from additional experiments run under the same conditions. Only one run of the control samples was plotted because a horizontal line was obtained in all runs. There was an average of $2.6 \times 10^{-9}$ *R. japonicum* and $8.7 \times 10^{-6}$ *R. meliloti* in the incubation mixtures at initiation of incubation.
Figure 6. Thin-layer chromatographic partitioning of chloroform-soluble metabolites from incubations of *Rhizobium japonicum* and *R. meliloti* with $^{35}$S-parathion. The incubation mixture was buffered to pH 7.0 and the temperature was maintained at 25 C. The *R. japonicum* cultures were four-days old and the *R. meliloti* two-days old at initiation of incubation. One of two sets of similar data obtained.
Figure 7. Time-course of aminoparathion production from incubation of $10^{-6}$M parathion with *Rhizobium japonicum* and *R. meliloti*.

The incubation mixture was buffered to pH 7.0 and the temperature was maintained at 25°C. The *R. japonicum* cultures were four-days old and the *R. meliloti* two-days old at initiation of incubation. One of two sets of similar data obtained. There were $2.4 \times 10^{-9}$ *R. japonicum* and $1.2 \times 10^8$ *R. meliloti* in the incubation mixtures at initiation of incubation.
Figure 8. Ion-exchange chromatogram of the water-soluble metabolites from incubations of $10^{-6}$ M $^{35}$S-parathion with *Rhizobium japonicum* and *R. meliloti*. The incubation mixture was buffered to pH 7.0 and the temperature was maintained at 25°C. The *R. japonicum* cultures were four-days old and the *R. meliloti* two-days old at initiation of incubation. One of five sets of similar data obtained. The eluting solutions, designated by capital letters, are given in the text. The letter "X" represents the aqueous portion drained from the column in the loading operation. An authentic sample of DEPTA was subjected to ion-exchange chromatography and colorimetrically analyzed for phosphorus at 720 nm using a Beckman DB spectrophotometer.
**PHOSPHORUS DETERMINATION OF DEPTA**

- **Counts per minute (X 1000)**
- **Absorbance**

Graph showing elution peaks labeled I, II, III, and IV with corresponding absorbance levels.

Labels:
- ΔΔ R. japonicum
- ○○ R. melliloti
Figure 9. Ion-exchange chromatogram of the water-soluble metabolites of $10^{-5}$M aminoparathion incubations with Rhizobium japonicum and R. meliloti. The experimental conditions are the same as given in the legend for Fig. 8.
Figure 10. Time-course production of the major metabolites of $10^{-6}$M parathion and $^{35}S$-parathion incubations with Rhizobium japonicum and R. meliloti. The DEPTA values were calculated as the percentage of radioactivity in the aqueous layer after three chloroform extractions. The aminoparathion curve is reproduced from Fig. 7 and the parathion curve from Fig. 5.
A R. japonicum

A R. meliloti

AMINOPARATHION

DEPTA

PARATHION

CONCENTRATION ($10^{-6} \text{ M}$)

INCUBATION TIME (HOURS)
Figure 11. Parathion degradation pathways by *Rhizobium japonicum* and *R. meliloti*. 
Paraoxon

Parathion

Aminparathion

Diethyl phosphorothioic acid (DEPTA)

(— pathway non-existent)

(----- probable pathway)
Figure 12. Time-course of *Rhizobium meliloti* population in 4 ml incubation mixtures under the influence of $10^{-4}$, $10^{-5}$, and $10^{-6}$ M parathion. The values at the right of the curves indicate the molarity of the parathion. The check samples contained all components of the incubation mixture except parathion. The incubation mixture was buffered to pH 7.0 and the temperature was maintained at 25°C. The organisms were two-days old at initiation of incubation.
Figure 13. Time-course of *Rhizobium japonicum* population in 4 ml incubation mixtures under the influence of $10^{-4}$, $10^{-5}$, and $10^{-6}$ M parathion. The values at the right of the curve indicate the molarity of the parathion. The check samples contained all components of the incubation mixture except parathion. The incubation mixture was buffered to pH 7.0 and the temperature was maintained at 25 C. The organisms were four-days old at initiation of incubation.
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I am indebted to my wife for typing the manuscript.

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