Metabolism of Dimethoate in Bean Plants in Relation to Its Mode of Application

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The metabolism of dimethoate-^{32}P and dimethoate-carbonyl-^{14}C was studied in bean plants using four modes of application. Degradation of dimethoate occurred most rapidly in excised leaves, followed by steam-injected plants, root-treated plants, and least rapidly in plants treated via a foliar application. The oxygen analog, a cholinesterase-inhibiting metabolite of dimethoate, was consistently present in moderate amounts in plants treated by all four modes of application. Des-*N*-methyl dimethoate, a metabolite not previously reported as a product of dimethoate degradation in plants, was detected in trace amounts, while two unknown metabolites, possibly the *N*-hydroxymethyl derivatives of dimethoate and its oxygen analog, were found only in foliar-treated plants. Oxidation of dimethoate took place to a lesser degree than hydrolysis, although it seemed more important in the foliar-treated plants than in the other modes of application. The major hydrolytic metabolites formed were dimethoate carboxylic acid, dimethyl phosphorodithioic acid, and dimethyl phosphorothioic acid. Des-*O*-methyl dimethoate, found in very high concentrations in previous studies of dimethoate metabolism in plants, was detectable only in trace quantities.

Dimethoate, *O,O*-dimethyl *S*-(methylcarbamoylmethyl) phosphorodithioate, introduced as an experimental insecticide in 1956 by Società Montecatini, Milan, Italy, and American Cyanamid Co., Princeton, N. J., is an effective plant and animal systemic insecticide. It is particularly valuable, since cumulative and chronic toxicity studies conducted by Montecatini (1961) and Edson *et al.* (1967) have demonstrated that it is a low hazard insecticide.

The conversion of dimethoate to its oxygen analog by an oxidative process is vital to its toxicity, since it is in this form that the compound inhibits cholinesterase activity (O'Brien, 1960). Selective toxicity of dimethoate to insects can be explained by different levels of oxygen analog at the target area owing to inherent differences in the balance of oxidative and hydrolytic enzymes in insects and mammals (Brady *et al.*, 1960; Krueger *et al.*, 1960; Uchida *et al.*, 1964). Brady *et al.* (1960) found dimethoate to be 326 times more toxic to houseflies than to rats.

Although the oxygen analog is the only toxic metabolite of dimethoate that has been definitely characterized, Sanderson and Edson (1964) isolated additional cholinesterase-inhibiting metabolites which were theorized to be *N*-hydroxymethyl derivatives. Metabolites resulting from the oxidative *N*-demethylation of dimethoate might exhibit mammalian toxicity, since they could be converted to P=O compounds which would be cholinesterase inhibitors. In any metabolic study of dimethoate, it is necessary to evaluate the relation between the amounts of dimethoate and its cholinesterase-inhibiting metabolites and hydrolytic products which are not effective cholinesterase inhibitors.

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The early work on the metabolism of dimethoate in plants was concentrated on establishing the relationship of the oxygen analog to the parent compound. Allesandrini (1962) and Sampaolo (1961) detected more than 0.1 p.p.m. of dimethoate in olive oil, while Sampaolo (1962) and Santi and Giacomelli (1962) reported less than 0.1 p.p.m. of the oxygen analog in olives and no toxic materials in olive oil. They found that olives contained degradation products such as dimethoate carboxylic acid, methyl phosphoric, and phosphoric acid in essentially the same amounts. Allesandrini (1961) indicated that the concentration of the oxygen analog in unwashed olives rose to a maximum of 1 p.p.m. in 11 days in a summer experiment and 20 days in an autumn experiment. Primarily water-soluble metabolites existed from 4 to 5 weeks after treatment, with only trace amounts of dimethoate and oxygen analog detectable at that time. Metabolism of dimethoate-^{32}P followed a similar pathway in peaches and cherries (Santi, 1961a). The nature of degradation of dimethoate in plants was further elucidated by Santi and Giacomelli (1962) working with sugar and fodder beets and by dePietri-Tonelli *et al.* (1961) using several different plants. Hydrolysis of the methylamide group predominated over all other hydrolytic activity, while oxidation occurred to a limited extent in all cases.

However, when dimethoate was applied to lemon tree trunks (Santi, 1961b) the des-*O*-methyl derivative of dimethoate was formed in large amounts. No dimethoate carboxylic acid was detected. Dauterman *et al.* (1960) found des-*O*-methyl dimethoate to be the principal metabolite inside the leaf tissue when dimethoate-^{32}P was applied to the leaf surface of cotton, potato, and corn plants. The carboxylic acid derivative of the oxygen analog was the major metabolite on the leaf surface, but only traces were found inside the leaf. However, when the

same mode of application was used in pea plants, phosphoric acid was the predominant metabolite formed both inside the leaf tissue and on the surface at all harvest times. Dimethoate oxygen analog was formed in moderate amounts, but both Santi (1961b) and Dauterman *et al.* (1960) were unable to detect dimethoate carboxylic acid. The existence of the des-*O*-methyl metabolite was also reported in wheat and sorghum grains (Rowlands, 1966). Dimethoate carboxylic acid predominated in wheat, while the des-*O*-methyl derivative was formed most readily from dimethoate in sorghum grains. The conflicting evidence indicates that different species of plants vary greatly in their metabolic attack on dimethoate.

Hacskaylo and Bull (1963), treating excised cotton leaves with dimethoate-^{32}P, identified 11 metabolites using paper chromatography. In contrast to Dauterman's foliar study with cotton plants, large quantities of dimethoate carboxylic acid were detected, but no des-*O*-methyl dimethoate was isolated. The oxygen analog was formed in the excised leaves, but in smaller quantities than in whole cotton plants.

A comparison of the results of the dimethoate metabolism studies in plants reported by Dauterman *et al.* (1960) and Hacskaylo and Bull (1963) indicates that metabolism of the compound may be influenced by the mode of application. These researchers, both groups working with the cotton plant, obtained different results when dimethoate was taken into excised leaves from a water solution and when a solubilized formulation of dimethoate was applied to the surface of the leaves. A direct comparison of dimethoate metabolism in plants using several different modes of application would probably be a valuable contribution. Furthermore, earlier workers used ^{32}P-labeled dimethoate, so the use of dimethoate labeled in the side chain with ^{14}C would yield additional information about the metabolism of the compound.

MATERIALS AND METHODS

Synthesis of Dimethoate-Carbonyl-^{14}C. To prepare dimethoate-carbonyl-^{14}C, *N*-methyl-α-chloroacetamide-1-^{14}C of specific activity 1.5 mc. per mmole was obtained from Nuclear Research Chemicals, Orlando, Fla. The ^{14}C-chloroacetamide (255.5 mg., 2.34 mmoles) was dissolved in 50 ml. of chloroform in a 250-ml. round-bottomed flask and potassium *O,O*-dimethyl phosphorodithioate (421.2 mg., 2.34 mmoles) dissolved in 50 ml. of water was added dropwise from a separatory funnel over a period of 30 minutes. The mixture was then refluxed for 90 minutes at 56° C. The chloroform and water fractions were separated and the water fraction was extracted three times with equal volumes of chloroform. The chloroform fractions were combined and evaporated under vacuum. The product was purified on a Celite (Johns Manville's analytical filter aid) partitioning column.

The peak whose infrared spectrum corresponded with that of unlabeled dimethoate contained only 20.1 mg. Another peak representing 45.1 mg. of the unreacted chloroacetamide reacted in the same manner as the initial synthesis with 148.6 mg. (0.82 mmole) of potassium *O,O*-dimethyl phosphorodithioate. The total yield from both runs of the synthesis was 86.5 mg. (18.8%) and the final product had a specific activity of 0.416 mc. per mmole. Thin-layer chromatography of the synthetic dimethoate carbonyl-^{14}C preparation indicated that two materials were present, approximately 75% dimethoate and 25% an unidentified impurity closely related to dimethoate. Since the column chromatographic procedure was not adequate to resolve the two materials completely, the synthetic dimethoate-carbonyl-^{14}C was purified by silica gel thin-layer chromatography before use.

Other Chemicals. Dimethoate-^{32}P of specific activity 5.15 mc. per mmole was obtained from the Radiochemical Center, Amersham, England. When purified on a Celite partitioning column, two peaks were obtained from the labeled dimethoate, the larger being dimethoate as determined by silica gel thin-layer chromatography with unlabeled dimethoate. The dimethoate-^{32}P was stored in chloroform at 4° C. All experiments conducted with the dimethoate-^{32}P were corrected for the half life of the isotope.

Unlabeled dimethoate and five of its potential metabolites were obtained from American Cyanamid Co., Princeton, N. J. The metabolites were: *O,O*-dimethyl *S*-(methylcarbamoylmethyl) phosphorothiolate (dimethoate oxygen analog); *O,O*-dimethyl *S*-(carbamoylmethyl) phosphorodithioate (des-*N*-methyl dimethoate); *O,O*-dimethyl *S*-carboxymethyl phosphorothioate (dimethoate carboxylic acid); *O*-methyl-*O*-hydrogen *S*-carboxymethyl phosphorodithioate (des-*O*-methyl carboxylic acid); and *O*-methyl *O*-hydrogen *S*-(methylcarbamoylmethyl) phosphorodithioate (des-*O*-methyl dimethoate). Other potential metabolites were obtained from commerical sources.

Treatment of Plants. The metabolism of dimethoate was investigated in bean plants (*Phaseolus vulgaris* L.) using four modes of application: injection into the stems of the plant, foliar treatment, uptake through excised leaves, and uptake through the roots of the plants. In preliminary tests on the phytotoxicity of dimethoate to beans, using the stem injection technique, 30 and 100 p.p.m. gave no effect while 300 and 1000 p.p.m. caused chlorosis of the leaves and a general lack of vigor after 2 days.

For the stem injection and foliar applications beans were planted in the greenhouse on April 13, 1966, and treated after 18 days when they were in the two-leaf stage. The stem injection method consisted of first making a puncture at the base of the stem with a microsyringe, then injecting 50 μl. of a water solution of labeled dimethoate into the stem just above the first nodule. The foliar application consisted of spreading 25 μl. of dimethoate-^{14}C dissolved in water over the surface of each of two leaves, giving an approximate dosage of 5.15 p.p.m. of ^{14}C-dimethoate in each plant. Samples were taken at 0, 2, 4, 6, 8, and 10 days. Three replications of two plants each (whole plants, including roots) were taken for each day. For the foliar treatment with dimethoate-^{32}P, plants were grown in vermiculite for 14 days in a Percival plant growth chamber in which the daylight temperature was 72° F. and the night temperature, 62° F. Plants were held under a daily photoperiod of 14 hours light and 10 hours dark. Plants were treated and harvested in the same manner as in the foliar experiment with dimethoate-^{14}C with samples taken at 2 and 4 days.

For the excised leaf application, beans were planted in

the greenhouse and grown for 8 weeks. The third and fourth leaves from the terminal were removed by cutting the petioles under water. The basal ends of the petioles were inserted into separate small vials containing 44 μg. of dimethoate-carbonyl-^{14}C dissolved in 0.4 ml. of water. Each vial was washed twice with 0.2-ml. portions of water after the original solution was taken up, and the leaves were allowed to take up the washings. During and after dimethoate-^{14}C uptake the leaves were placed in a plant growth chamber, in which the daylight temperature was 72° F. and the night temperature, 62° F. Plants were held under a daily photoperiod of 14 hours light and 10 hours dark. After the second washing each leaf was placed in a large vial containing water and returned to the plant growth chamber. Samples were taken at 1, 3, 6, 10, and 14 days after treatment. Three replications consisting of two leaves each were taken on each sampling day.

For the root treatment, beans were planted October 1, 1966, and grown in the green house for 18 days in vermiculite. The whole plants were then carefully removed and the roots immersed in 20-ml. vials containing 64 μg. of dimethoate-^{14}C dissolved in 3 ml. of water. Each vial was washed twice with 1-ml. portions of water after the original solution was taken up, and the plants were allowed to take up the washings. The plants were then potted in soil and placed in a plant growth chamber set for a daylight temperature of 72° F. and a night temperature of 62° F., with 14 hours of light and 10 hours of dark, identical to the excised leaf procedure. Samples were taken at 0, 2, 4, 6, 8, and 10 days after treatment. Three replications were taken for each day, each replication consisting of two plants. Plants treated with dimethoate-^{32}P via the root uptake method were grown in vermiculite for 14 days in the plant growth chamber using the same daylight and night temperatures as prior experiments. Plants were treated and harvested in the same manner for the ^{32}P studies as for the ^{14}C studies.

Analytical Procedures. EXTRACTION PROCEDURES. All samples in the four modes of application were frozen for at least 30 minutes immediately after harvesting. The samples treated by foliar application were washed three times with acetone to remove surface residues prior to maceration. Foliar and stem injection samples were macerated in acetone in a Waring Blendor for 5 minutes. The excised leaf and ^{14}C-root uptake samples were macerated in acetone in a Lourdes Multi-Mix for 3 minutes. The Lourdes mixer gave a more finely ground plant pulp than the Waring Blendor and also lessened the amount of acetone necessary in the grinding procedure. A modification of the Potter-Elvehjem homogenizer (Scientific Glass Apparatus Co., No. JT-5000, Disintegrinder) was used for plants treated by foliar and root-uptake methods with dimethoate-^{32}P. The Disintegrinder was more effective in breaking down the cellular structure of the plant material, enabling a higher recovery of dimethoate and its metabolites.

After the plants were macerated, each sample was suction-filtered in a Büchner funnel, leaving a plant-pulp fraction and a water-acetone solution. The acetone was evaporated under a stream of air, with heating by a 100-watt bulb. The remaining water solution was extracted three times with an equal volume of hexane to remove the plant pigments. Subsequent Celite column chromatography demonstrated that 66.5% of the radioactivity present in the hexane extract was unaltered dimethoate. The remainder of the radioactivity was eluted from the column in the methanol wash and not further fractionated. The data reported in the tables for dimethoate recovery include the material extracted by hexane. The water solution was then extracted three times with an equal volume of chloroform, leaving a chloroform fraction and a final water fraction.

The plant pulp and the hexane extracts were assayed for radioactivity in a thin-window, gas-flow counter. The chloroform and final water fractions were assayed for radioactivity by liquid scintillation counting and subjected to further purification by column, thin-layer, and paper chromatography.

The extraction and purification procedures outlined above are summarized in Figure 1.

CHROMATOGRAPHY. A two-dimensional silica gel G thin-layer chromatographic system was used to resolve both chloroform- and water-extractable metabolites. First, the chloroform-extractable and water portions were evaporated nearly to dryness with a gentle stream of air. The chloroform-extractable metabolites were dissolved in a small amount of chloroform and spotted on thin-layer plates. Acteone was used for spotting the residue from the water fractions. Thin-layer plates of 0.5-mm. thickness were prepared, dried at 105° C. for 1 hour, and stored in a desiccator until use. The plates were developed in 5 to 1 acetone-water in the first direction. After the plates had dried, they were turned on their sides and run in the second direction with 3.3% methanol in chloroform.

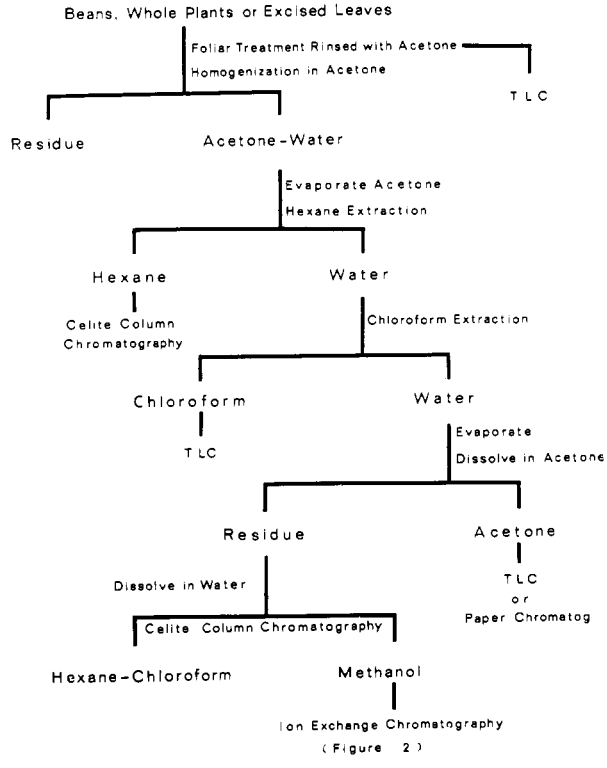

Figure 1. General extraction, fractionation, and purification procedures

After the water fraction was evaporated and the acetone-soluble material was spotted on thin-layer plates, a viscous, residual, acetone-insoluble substance remained, which contained considerable radioactivity. In studies with plants treated with dimethoate-^{32}P and -^{14}C by root uptake and dimethoate-^{32}P by foliar application, the viscous substance was dissolved in a small amount of water and placed on a Celite partioning column (Clemons and Menzer, 1968). After the sample was placed on the column, hexane and chloroform mixtures were used for elution. The system began with pure hexane; then increasing amounts of chloroform were mixed with the hexane until pure chloroform was used. Twenty-milliliter fractions were collected. Three peaks were obtained: dimethoate, dimethoate oxygen analog, and a material designated as unknown III. The Celite was then removed from the column and extracted with methanol. The methanol was evaporated to a small volume in a rotary evaporator, transferred to a liquid scintillation vial, and evaporated to dryness under a stream of air while being warmed with a 100-watt bulb.

After evaporation of the methanol extract of the Celite column packing material, a viscous, water-soluble material remained which contained considerable radioactivity. Metabolites in this extract were fractionated on an ion-exchange column (Plapp and Casida, 1958). Ten-milliliter fractions were collected.

With plants that were treated with dimethoate-^{32}P a paper chromatographic procedure was employed (Hacskaylo and Bull, 1963). The water fraction, after extraction with chloroform, was evaporated with a stream of air and spotted with a small portion of acetone on Whatman No. 1 filter paper. The paper strips were eluted in an ascending direction with acetonitrile, water, and ammonium hydroxide (40:9:1). The solvent ran 30 cm., taking approximately 9 hours.

Detection and Identification of Metabolites. To detect unlabeled dimethoate and its metabolites added to thin-layer and paper chromatograms for co-chromatography with radioactive spots, a spray reagent was prepared by diluting 5 ml. of a 5% palladium chloride stock solution with 1 ml. of concentrated HCl and 94 ml. of 95% ethanol. Dimethoate and its metabolites gave brown or brownish yellow spots on a white background. The spray reagent was made up just before use each time.

Radioactive spots were detected by exposing the chromatograms to Kodak No-Screen medical x-ray film for at least 3 days before developing, depending on the amount of radioactivity present. The spots on the x-ray film could then be compared with the spots resulting from the palladium chloride spray.

Radioactive metabolites separated on Celite partitioning columns or ion exchange columns were rechromatographed on silica gel thin-layer plates with unlabeled dimethoate and its analogs to evaluate the coincidence of the spots on x-ray film with the spots resulting from the palladium chloride spray.

Counting Procedures. A Packard Tri-Carb liquid scintillation counter (Model 3375), and a Nuclear-Chicago thin-window, gas-flow counter (Model 1402) were used for assaying radioactivity. Three solutions were used for liquid scintillation counting: a standard counting solution for chloroform-extractable compounds, and two other solutions for aqueous samples. The standard counting solution consisted of 5 grams of PPO (2,5-diphenyloxazole) and 0.3 gram of dimethyl POPOP [1,4-bis-2-(4-methyl-5-phenyloxazolyl) benzene] per liter of toluene. In the earlier stages of this work a counting solution for aqueous samples devised by Bray (1960) was used: 60 grams of naphthalene, 4 grams of PPO, 0.2 gram of dimethyl POPOP, 100 ml. of absolute methanol, and 20 ml. of ethylene glycol made up to 1 liter with *p*-dioxane. The authors later found that a counting solution for aqueous samples containing Triton X-100 was more stable and had a greater capacity for water. The Triton X-100 solution contained 5.5 grams of PPO and 20 mg. of dimethyl POPOP per liter of a 2 to 1 mixture of toluene and Triton X-100.

The gas-flow counter was used for all samples where solubility in one of the counting solutions was extremely difficult to achieve or where the counting solution would be drastically quenched by dark colors from plant pigments.

CO_2 Trapping Studies. Plants grown in the greenhouse until they had reached the two-leaf stage were treated with dimethoate-^{14}C to evaluate the degradation of the chemical to $^{14}CO_2$. The plants were treated with 114 μg. of dimethoate-^{14}C by either stem-injection or foliar application to give a concentration of 21.8 p.p.m.

Immediately after treatment the plants were placed in bell jars, which were sealed at the bottom on glass plates. Air was drawn through the system at the rate of approximately 250 ml. per minute. The CO_2 evolved was collected in test tubes containing 60 ml. of 2-aminoethanol. A barium hydroxide solution was used to check the efficiency of the 2-aminoethanol trap in absorbing the CO_2 evolved. The entire apparatus was placed in a plant growth chamber maintained at a daylight temperature of 72° F. and a night temperature of 62° F. on a 14-hour day, 10-hour night schedule. Samples were taken at 2, 4, 6, 8, 12, 16, 20, 24, 30, and 36 hours and every 12 hours thereafter up to 10 days, then once daily for another 7 days.

The radioactivity recovered in the 2-aminoethanol was assayed by removing a 1.0-ml. aliquot from each sample and adding 2 ml. of methyl Cellosolve and 15 ml. of the standard counting solution. No precipitate of $BaCO_3$ was recovered.

RESULTS

Identification of Metabolites of Dimethoate. Eighteen metabolites of dimethoate were isolated from bean plants treated with dimethoate-carbonyl-^{14}C or dimethoate-^{32}P. Seven of the metabolites have been identified (Table I, Figure 2). Dimethoate, des-*N*-methyl dimethoate, dimethoate oxygen analog, dimethoate carboxylic acid, des-*O*-methyl dimethoate, des-*O*-methyl carboxylic acid, *O,O*-dimethyl phosphorodithioic acid, and *O,O*-dimethyl phosphorothioic acid were identified by co-chromatography with known materials on silica gel thin-layer plates. Of the 11 unknowns at least two, unknowns I and II, contained both ^{14}C and ^{32}P, as detected on thin-layer plates. Unknowns III and IV contained only ^{14}C and were detected on thin-layer plates, while unknowns V, VI, and VII also contained ^{14}C and were isolated from the ion exchange column. Unknown III was also isolated from the Celite column. Unknown VIII contained only ^{32}P and was isolated from thin-layer plates, while unknowns IX, X, and

Table I. Common Name, R_f Values, Chemical Name, and Presence of Isotopes of Dimethoate and Its Metabolites Recovered from Bean Plants on Thin-Layer and Paper Chromatograms

Common Name	R_f TLC[a] 1	R_f TLC[a] 2	R_f P.C.[b]	Chemical Name	Isotopes Contained
Dimethoate	0.95	0.40	0.95	*O,O*-Dimethyl *S*-(methylcarbamoylmethyl) phosphorodithioate	^{32}P, ^{14}C
Des-*N*-methyl dimethoate	0.90	0.30	...	*O,O*-Dimethyl *S*-(carbamoylmethyl) phosphorodithioate	^{32}P, ^{14}C
Unknown I	0.80	0.40	...	...	^{32}P, ^{14}C
Unknown II	0.75	0.35	...	...	^{32}P, ^{14}C
Unknown III	0.80	0.30	...	...	^{14}C
Dimethoate oxygen analog	0.80	0.25	0.85	*O,O*-Dimethyl *S*-(methylcarbamoylmethyl) phosphorothiolate	^{32}P, ^{14}C
Des-*O*-methyl dimethoate	0.60	0.10	...	*O*-Methyl *O*-hydrogen *S*-(methylcarbamoylmethyl) phosphorodithioate	^{32}P, ^{14}C
Dimethoate carboxylic acid	0.60	0	0.55	*O,O*-Dimethyl *S*-carboxymethyl phosphorodithioate	^{32}P, ^{14}C
Des-*O*-methyl carboxylic acid	0.50	0	0.15	*O*-Methyl *O*-hydrogen *S*-carboxymethyl phosphorodithioate	^{32}P, ^{14}C
Unknown IV	0.45	0	...	...	^{14}C
Dimethyl phosphorodithioic acid	0.35	0	0.50	*O,O*-Dimethyl phosphorodithioic acid	^{32}P
Dimethyl phosphorothioic acid	0.25	0	0.35	*O,O*-Dimethyl phosphorothioic acid	^{32}P
Unknown VIII	0.70	0	...	...	^{32}P

[a] Thin-layer chromatography on silica gel G using 5 to 1 acetone-water in direction 1 and 3.3% methanol in chloroform in direction 2. [b] Chromatography on Whatman No. 1 paper eluting with 40:9:1 acetonitrile-water-ammonium hydroxide.

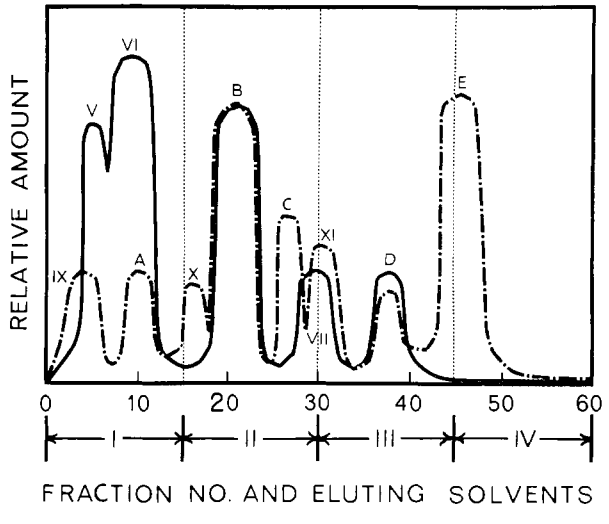

Figure 2. Separation of dimethoate metabolites by Dowex 1-X8 ion exchange column chromatography

Bean plants treated with dimethoate-^{32}P (— — — — —) and dimethoate-carbonyl-^{14}C (————————) in separate experiments. Columns eluted with I, elution gradient pH 2 to pH 1 HCl; II, elution gradient pH 1 HCl plus methanol (1 to 3) to 1*N* HCl plus methanol (1 to 3); III, elution gradient 1*N* HCl plus acetone (1 to 3) to concentrated HCl, water, and acetone (1:1:6); IV, concentrated HCl, water, and acetone (1:1:6). Roman numerals at individual peaks are unknown materials as referred to in the text.

A. Dimethyl phosphoric acid
B. Dimethoate carboxylic acid
C. Dimethyl phosphorothioic acid
D. Des-*O*-methyl dimethoate
E. Dimethyl phosphorodithioic acid

XI also contained ^{32}P and were isolated from the ion exchange columns.

Since unknowns I and II contained both the phosphorus and carbonyl moieties of the parent material, are closely related to each other in polarity as shown by their R_f values on both thin-layer and paper chromatograms, and are closely related to dimethoate and the oxygen analog since their R_f values are similar, it is possible that unknowns I and II are the *N*-hydroxymethyl derivatives of dimethoate and the oxygen analog, respectively. The chloroform-water partition coefficients are 7.0 for unknown I and 6.0 for unknown II, compared to 13.0 for dimethoate. Unknown III did not contain the phosphate portion of the molecule and is presumably a product of cleavage at the the S—C or P—S bond.

Unknown IV, a polar compound, was found only in excised leaves treated with dimethoate-^{14}C and is also likely a product of cleavage at the P—S or S—C bonds. Cleavage must occur at these sites, since the phosphate moieties have been isolated as *O,O*-dimethyl phosphorodithioic acid and *O,O*-dimethyl phosphorothioic acid. Unknowns V and VI, detected on the ion exchange columns, are also probable products of hydrolysis at the above-mentioned sites, since they do not contain phosphorus and occur in quantities similar to the corresponding phosphate portions. Unknowns VII and XI are possibly the same compound (Figure 2), since they are eluted from the ion exchange columns in approximately the same positions. However, since neither migrates on the thin-layer system, it was difficult to compare and equate them.

Unknown VIII is known to contain only ^{32}P, since radioactivity corresponding to unknown VIII was not de-

Table II. Percentages of Administered Dose of Labeled Compounds Recovered[a] from Plants Treated with 5.15 P.P.M. of Dimethoate-carbonyl-^{14}C on Surface of Leaves

Metabolites	Days after Treatment 2	4	6	8	10
Dimethoate	28.90	22.81	18.38	12.99	12.34
Des-*N*-methyl dimethoate	0.72	0.05	0.03	0.05	0.05
Dimethoate oxygen analog	0.69	0.81	0.61	0.53	0.48
Dimethoate carboxylic acid	0.12	0.14	0.07	0.06	0.20
Des-*O*-methyl carboxylic acid	0.21	0.17	0.15	0.05	0.21
Unknown I	8.76	1.26	...	...	...
Unknown II	2.65	...	...	...	...
Hexane extract	2.25	1.86	1.63	1.15	1.11
Plant residue	8.71	8.85	11.70	16.16	16.23
Total recovery	53.01	35.95	32.57	30.99	30.62

[a] Recovery data based on hexane extract, chloroform extract, and final acetone extract (Figure 1). Residue from final acetone extract not included.

tected on thin-layer plates in the ^{14}C studies and two of the three unknowns isolated on the ion exchange column in the ^{32}P studies did not compare in quantity or chromatographic position with any compound found on ion exchange columns in the ^{14}C studies. Methyl phosphorodithioic acid, methyl phosphorothioic acid, methyl phosphoric acid, or the completely demethylated phosphorodithioic acid or phosphorothioic acid are possible compounds representing unknowns IX and X. Unknown XI could be one of the above-mentioned compounds or may also contain the carbonyl function of dimethoate and be identical with unknown VII.

Radioactivity was detected on thin-layer and paper chromatograms at the origin and presumably represents phosphoric acid, which does not migrate in either of the chromatographic systems, and/or other components of plant extracts.

Recovery of Administered Radioactivity. Recovery of metabolites was poor in the foliar, stem-injected, and excised leaf treatments with dimethoate-^{14}C, but when Celite and ion exchange columns were used in the ^{32}P studies and in the root treatment with dimethoate-^{14}C, the results were more satisfactory. The data presented in the tables are not corrected for the low recoveries achieved in some cases. Recoveries of metabolites are tabulated as per cent of administered radioactivity in Tables II to VII.

Dimethoate is most rapidly degraded in the excised leaves of bean plants, followed by stem injection, root, and foliar-treated samples in that order (Figure 3). To compare the different modes of application in Figure 2, the data for stem-injection and excised-leaf treatments are corrected for poor recoveries. Oxygen analog, a cholinesterase-inhibiting metabolite of dimethoate, was recovered from all treatments, although in varying amounts. Oxidation occurred most rapidly in the excised-leaf, stem-injected, and root-treated samples and more slowly in plants treated by the foliar method. Amounts of the oxygen analog recovered were fairly consistent at all harvest times, the maximum being 10.65% of administered radioactivity at 10 days in plants treated with dimethoate-^{14}C by the root-uptake method. Trace quantities of des-*N*-methyl dimethoate, a previously unreported metabolite, were recovered in all treatments. Unknowns I and II are the most important unknowns recovered; although they were detected only in plants treated by the foliar application, they were present in significant quantities. These metabolites may be anticholinestereases, since they are similar in polarity to dimethoate. Fortunately, they are rapidly degraded and are not detectable after 4 days. Dimethoate oxygen analog was the known metabolite occurring in the highest concentrations, followed by several water-soluble metabolites: dimethoate carboxylic acid, *O,O*-dimethyl phosphorodithioic acid, *O,O*-dimethyl phosphorothioic acid, and unknowns V and VI. Largest quantities of dimethoate carboxylic acid were formed in the stem injection, followed by excised leaf, root, and foliar samples in that order. *O,O*-Dimethyl phosphorodithioic acid was recovered in higher

Table III. Percentages of Administered Dose of Labeled Compounds Recovered[a] from Plants Treated with 1.78 P.P.M. of Dimethoate-^{32}P Applied to Surface of Leaves

Metabolites	Days after Treatment 2	4
Dimethoate	65.65	44.11
Dimethoate oxygen analog	2.24	4.45
Dimethoate carboxylic acid	1.60	2.37
Des-*O*-methyl carboxylic acid	0.08	0.09
Des-*O*-methyl dimethoate	0.39	0.49
Dimethyl phosphorodithioic acid	2.48	4.19
Dimethyl phosphorothioic acid	0.43	0.76
Dimethyl phosphoric acid	0.38	0.74
Unknown I	3.18	0.16
Unknown II	1.16	0.20
Unknown VIII	0.06	0.05
Unknown IX	0.12	1.02
Unknown X	0.11	0.54
Unknown XI	0.42	1.17
Origin	0.02	0.02
Hexane extract	1.86	1.24
Plant residue	2.39	6.97
Total recovery	82.57	68.57

[a] Recovery based on hexane extract, chloroform extract, final acetone extract, and fractionation of final residue (Figure 1).

Table IV. Percentages of Administered Dose of Labeled Compounds Recovered[a] from Bean Plants Treated with 5.15 P.P.M. of Dimethoate-carbonyl-^{14}C by Injection into Stem

Metabolites	Days after Treatment				
	2	4	6	8	10
Dimethoate	23.88	20.40	13.40	10.96	10.78
Des-*N*-methyl dimethoate	0.11	0.02	0.03	0.03	0.06
Dimethoate oxygen analog	1.17	1.81	1.68	2.43	1.07
Dimethoate carboxylic acid	1.54	0.25	0.20	0.07	0.13
Des-*O*-methyl carboxylic acid	0.06	0.01	0.07	0.04	0.11
Unknown III	...	...	0.13	...	...
Hexane extract	2.11	1.68	1.20	0.98	0.95
Plant residue	8.23	10.95	13.21	14.18	15.37
Total recovery	37.10	35.12	29.92	28.69	28.47

[a] Recovery data based on hexane extract, chloroform extract, and final acetone extract (Figure 1). Residue from final acetone extract, not included.

Table V. Percentages of Administered Dose of Labeled Compounds Recovered[a] from Excised Leaves of Bean Plants Treated with 44.00 μg. of Dimethoate-carbonyl-^{14}C

Metabolites	Days after Treatment				
	1	3	6	10	14
Dimethoate	38.46	24.02	16.39	12.25	9.38
Des-*N*-methyl dimethoate	0.03	0.03	0.06	0.02	...
Dimethoate oxygen analog	1.92	1.60	4.56	1.81	4.10
Dimethoate carboxylic acid	0.39	0.85	0.89	0.57	0.06
Des-*O*-methyl carboxylic acid	0.13	0.11	0.50	0.16	...
Unknown III	0.32	0.19	0.25	...	...
Unknown IV	0.78	0.21	...	...	...
Hexane extract	2.94	2.05	1.31	1.14	0.85
Plant residue	7.09	14.63	16.39	17.88	25.21
Total recovery	52.06	43.69	40.35	33.83	39.60

[a] Recovery based on hexane extract, chloroform extract, and final acetone extract (Figure 1). Residue from final acetone extract, not included.

Table VI. Percentages of Administered Dose of Labeled Compounds Recovered[a] from Plants Treated with 12.36 P.P.M. of Dimethoate-carbonyl-^{14}C Applied to Roots

Metabolites	Days after Treatment					
	0	2	4[b]	6	8	10
Dimethoate	55.46	30.35	26.83	21.06	19.45	12.05
Des-*N*-methyl dimethoate	0.10	0.08	0.05	0.03	0.03	0.02
Dimethoate oxygen analog	1.74	3.02	6.49	6.00	9.20	10.65
Dimethoate carboxylic acid	0.56	0.76	3.02[b]	0.20	0.18	0.17
Des-*O*-methyl carboxylic acid	0.17	0.26	0.11	0.11	0.11	0.10
Des-*O*-methyl dimethoate	0.13	0.16	0.91[b]	0.28	0.14	0.15
Unknown III	0.11	0.53	0.34	0.43	0.44	0.56
Unknown IV	...	...	2.14[b]	...	...	...
Unknown VI	...	...	3.46[b]	...	...	...
Unknown VII	...	...	1.35[b]	...	...	...
Hexane extract	2.63	1.90	1.71	1.52	1.39	0.94
Plant residue	4.80	4.30	5.80	7.20	8.10	12.60
Methanol fraction	9.62[b]	18.95[b]	...	18.61[b]	15.40[b]	15.39[b]
Total recovery	75.32	60.31	52.21	55.44	54.44	52.63

[a] Recovery data based on hexane extract, chloroform extract, final acetone extract, and fractionation of final residue (Figure 1).
[b] Ion exchange columns run on methanol fraction in 4-day sample and recoveries added to data from thin-layer and Celite columns.

Table VII. Percentages of Administered Dose of Labeled Compounds Recovered[a] from Plants Treated with 2.11 P.P.M. of Dimethoate-^{32}P Applied to Roots

Metabolites	Days after Treatment					
	0	2	4	6	8	10
Dimethoate	77.74	41.87	32.87	17.37	10.56	6.18
Des-*N*-methyl dimethoate	0.07	0.06	0.04	0.06	0.02	0.01
Dimethoate oxygen analog	2.51	5.08	5.22	3.88	4.34	4.40
Dimethoate carboxylic acid	1.00	2.78	2.58	2.59	2.86	2.59
Des-*O*-methyl carboxylic acid	0.10	0.12	0.37	0.32	0.26	0.21
Des-*O*-methyl dimethoate	0.19	0.61	0.51	0.36	0.15	0.19
Dimethyl phosphorodithioic acid	0.74	3.20	2.49	3.47	2.52	1.62
Dimethyl phosphorothioic acid	0.22	1.30	1.80	3.05	1.91	2.00
Dimethyl phosphoric acid	0.09	0.38	0.49	0.44	0.21	0.29
Unknown VIII	0.02	...	...	...	...	...
Unknown IX	0.01	1.17	0.72	1.34	0.66	0.47
Unknown X	0.17	0.22	0.21	0.81	1.04	0.77
Unknown XI	0.01	0.48	0.54	0.74	0.66	0.30
Origin	0.05	0.15	0.40	0.89	0.46	0.15
Hexane extract	2.02	1.17	1.20	0.89	0.69	0.22
Plant residue	4.98	9.98	12.63	17.36	28.87	25.07
Total recovery	89.96	68.57	62.07	53.57	55.21	44.47

[a] Recovery data based on hexane extract, chloroform extract, final acetone extract, and fractionation of final residue (Figure 1).

quantities in the plants treated by allowing them to take up dimethoate-^{32}P through the roots than in the plants treated with dimethoate-^{32}P on the surface of the leaves. However, the opposite was found for *O,O*-dimethyl phosphorothioic acid. Corresponding ^{14}C-fragments of the dithioic acid and thioic acid are speculated to be unknowns V and VI recovered in 4-day plants treated with dimethoate-^{14}C by the root uptake method, since they correspond approximately to phosphate moieties in the per cent recovered. Trace amounts of the hydrolysis products, des-*O*-methyl dimethoate, des-*O*-methyl carboxylic acid, dimethyl phosphoric acid, phosphoric acid, and unknowns IV, VII, IX, X, and XI were isolated. Unknown III was detected in trace quantities in plants from all modes of application except the foliar. It is a fairly stable compound, since it was still detectable after 10 days in the root-treated plants, although it does not contain the phosphate moiety of dimethoate and would, therefore, not be a cholinesterase inhibitor.

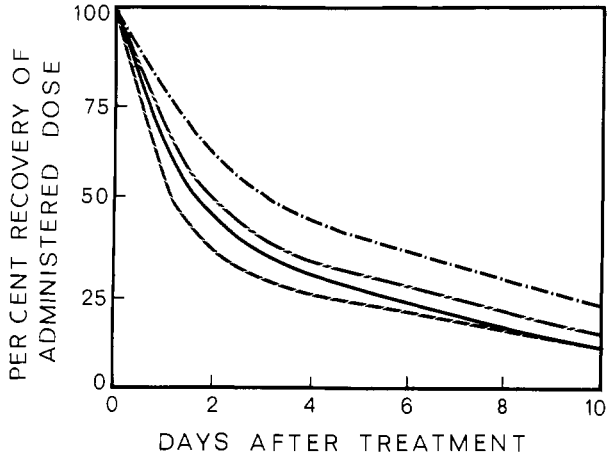

Figure 3. Per cent of administered dose represented by dimethoate after application to bean plants using dimethoate-^{32}P and dimethoate-carbonyl-^{14}C

Degradation after:
- —·—·— Foliar treatment
- — — — Root-uptake
- ——— Stem-injection
- - - - - Excised leaf

Incorporation of breakdown products of dimethoate into the plant pulp was significant in both ^{14}C and ^{32}P studies, although levels of radioactivity were nearly twice as high in the ^{32}P samples. Radioactivity assayed in the hexane fraction was primarily the result of materials extractable from plant water rather than incorporation of radioactivity into the plant pigments. Since the partition coefficient of dimethoate itself between hexane and water is 1.38, the recovery of dimethoate in the hexane after three extractions of the water would be 93%. Levels of radioactivity in the hexane progressively diminished with time in all modes of application and with both isotopes.

Degradation of dimethoate-^{14}C to $^{14}CO_2$ was not rapid and after 17 days only 15.1% of administered radioactivity was recovered as labeled carbon dioxide. The figure may be higher than would be expected in the field, since the high humidity inside the bell jar rapidly accelerated plant metabolism and growth.

DISCUSSION

Variations in metabolism of dimethoate carbonyl-^{14}C and -^{32}P related to the mode of application of the insecticide were insignificant in most cases. A difference in rate of metabolism was observed especially in conversion to the oxygen analog among the four routes of administration. The rate of oxidation of the labeled dimethoate was directly correlated with the ease of translocation to the leaf

tissue, where the majority of oxidation enzymes are apparently located. Thus, dimethoate treated via the excised-leaf method of application would be most rapidly available for oxidation, followed by the stem-injection method, and root treatment. The foiliar application, where the dimethoate molecule must penetrate into the leaf tissue, unlike the other methods where the insecticide is rapidly absorbed and translocated into the leaves, should result in the lowest oxidation rates. The data are in agreement with this hypothesis.

The formation of hydrolysis products occurred at similar rates related to the mode of application, except that the highest recoveries of the dimethoate carboxylic acid at the early harvest time were in the stem-injection samples. However, at later harvest times, the pattern of dimethoate carboxylic acid formation related to the mode of application was similar to that of conversion to the oxygen analog. Therefore, it is concluded that the varying rates of accessibility to degrading enzymes owing to the mode of application of the insecticide causes variation in recoveries of dimethoate and its metabolites (Figure 3).

Two notable differences in metabolism of dimethoate independent of the rate of degradation were observed in the foliar-treated samples. Unknowns I and II were detected only in samples from this route of administration, while unknown III was isolated in all samples, except those from the foliar application of dimethoate. Unknowns I and II are important in evaluating the metabolism of dimethoate in bean plants, since they are nonpolar compounds containing both the phosphate and carbonyl moieties of dimethoate, thereby having possible anticholinesterase activity. Also, they were found in significant quantities at the early harvest times. Sanderson and Edson (1964) reported the presence of four unidentified cholinesterase-inhibiting metabolites in rat liver slices and in human and rat urine after dimethoate-^{32}P administration. Although the unknowns reported by Sanderson and Edson (1964) may be similar or identical to unknowns I and II, it was not possible to equate them on the basis of chloroform-water partition coefficients. Unknown III, on the other hand, did not contain the phosphate moiety, is therefore not an anticholinesterase agent, and was detected only in trace amounts.

The fact that plants treated with dimethoate-^{14}C by root uptake were grown in the greenhouse in soil while plants treated with dimethoate-^{32}P were grown in vermiculite in the plant growth chamber prior to treatment may explain the difference in recovered amounts of the oxygen analog. The balance of enzymes in the plants grown under the different environmental conditions may have been different. A similar discrepancy was noted in recoveries of unknowns I and II in the dimethoate-^{14}C and -^{32}P foliar-treated plants and may also have been due to differences in handling the two groups of plants.

Incorporation of ^{14}C and ^{32}P into the structure of the bean plant would be expected. The phosphate moiety may be more readily utilized by the plant in structural material than the carbonyl, since higher levels of ^{32}P were detected in the pulp than ^{14}C. However, this variation could be partially explained by the evolution of $^{14}CO_2$, leaving less ^{14}C available for incorporation into the plant structure than would be possible for the ^{32}P of dimethoate.

A single metabolic pathway for dimethoate in plants has been difficult to elucidate because several groups of workers have reported recovery of different major metabolites. The most significant reports were by Dauterman *et al.* (1960), who treated cotton plants by a foliar application and reported conversion of dimethoate primarily to des-*O*-methyl dimethoate, and Hacskaylo and Bull (1963), who used excised leaves of cotton plants and reported dimethoate carboxylic acid to be the major metabolite. Metabolism in mammals (Chamberlain *et al.*, 1961; Dauterman *et al.*, 1959; Sanderson and Edson, 1964; Uchida *et al.*, 1964) was similar to that reported in plants by Hacskaylo and Bull. However, neither des-*O*-methyl dimethoate nor dimethoate carboxylic acid was isolated in high concentrations in any of the four modes of application in the present study, although dimethoate carboxylic acid consistently predominated over des-*O*-methyl dimethoate which was found only in trace quantities. That the dimethoate carboxylic acid predominates is supported by the preliminary observation that unknown VI chromatographs in the same position on ion exchange columns as mercaptoacetic acid. *O,O*-Dimethyl phosphorodithioate, *O,O*-dimethyl phosphorothioate, and dimethoate carboxylic acid were the hydrolytic products detected in the largest amounts, although dimethoate was possibly initially converted to dimethoate carboxylic acid and subsequently enzymatically hydrolyzed to the dithioate or thioate. The oxygen analog of dimethoate was the metabolite consistently found in the highest concentration in plants treated via all four methods of application, although in the 2-day foliar treatment samples, unknowns I and II were the predominant metabolites. When the recoveries of dimethoate and its metabolites were evaluated, apparently degradation followed several pathways of equal value quantitatively if not of equal importance when toxicity of each metabolite is considered. The metabolic pathways proposed by Dauterman *et al.* (1960) and Hacskaylo and Bull (1963) are generally supported by the present study with the addition of unknowns I and II which could not be accounted for by any of the compounds reported in the pathways. In addition, the exact sequence of reactions involved in the metabolism of the carbonyl function as represented by unknowns III, IV, V, VI, VII, and VIII remains to be worked out.

One can assess metabolism of an insecticide by an evaluation of the balance between intoxicating and detoxicating enzyme systems. The most important enzyme operative in dimethoate metabolism in bean plants is the one responsible for conversion to the oxygen analog, a metabolite several times more toxic to mammals than dimethoate itself (Bazzi, 1963). The toxicity of this metabolite becomes a very important factor in use of dimethoate when its stability and persistence in bean plants are considered. *N*-Hydroxymethyl derivatives of dimethoate and its oxygen analog may be formed, and must be included in an evaluation of the toxic metabolites of dimethoate. The conversion of dimethoate to unknowns I and II must take place on the leaf surface or in the cell wall of the leaf membrane. Unknowns I and II probably do not form inside the leaf tissue, since dimethoate is rapidly translocated into the leaf tissue when treated via the other three modes of application and could be converted in the leaves to the unknowns. Although neither unknown was recovered in

acetone rinses of the leaf surface of plants treated via the foliar application, it is possible that the enzyme acts on dimethoate on the leaf surface, but immediately after conversion the metabolites are absorbed into the leaf tissue. Oxidative enzymes demethylating the methylamide moiety of dimethoate to form the toxic des-*N*-methyl compound must be considered, although only trace amounts of this material were found. The oxidation of dimethoate associated with the leaf surface may be nonenzymatic. Mitchell (1961) has reported that oxidation of the P=S of dimethoate is not necessarily enzymatic, since the oxygen analog has been formed on nonenzymatic surfaces in the presence of ultraviolet light.

Competing with the intoxicating enzymes for active sites on the dimethoate molecule are the detoxicating enzymes, primarily the amidases responsible for cleavage at the C—N and the esterases which split dimethoate and dimethoate oxygen analog at the S—C and P—S bonds. Esterases acting at the S—C site of dimethoate apparently are in the highest concentrations within the leaf tissue, while the enzyme acting on the P—S bond is found predominantly in the structures of the bean plant, which are responsible for translocation of materials from the roots to the leaves.

Dimethoate metabolism varies primarily in the rate of degradative products formed in relation to the mode of application, although all degrading enzymes are not found in equal concentrations in all areas of the plant. However, in the final evaluation there does not appear to be a significant difference in the amounts of toxic metabolites recoverable at the later harvest times in any of the four modes of application.

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