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THE METABOLISM OF DIAZINON IN ALFALFA

BY

LARRY LEONARD NELSON

A thesis submitted
in partial fulfillment of the requirements for the
degree Doctor of Philosophy, Major in
Entomology, South Dakota
State University

1968

THE METABOLISM OF DIAZINON IN ALFALFA

THE METABOLISM OF DIAZINON IN ALFALFA

by

LARRY D. HARRIS

Submitted to the Graduate School of Arts and Sciences

This is a report of the results of a study of the metabolism of diazinon in alfalfa. The study was conducted in the laboratory of the Department of Entomology and Zoology, University of Maryland, College Park, Maryland. The results of the study are presented in the following chapters.

This thesis is approved as a creditable and independent investigation by a candidate for the degree, Doctor of Philosophy, and is acceptable as meeting the thesis requirements for this degree, but without implying that the conclusions reached by the candidate are necessarily the conclusions of the major department.

Thesis Adviser

Date

Head, Entomology-Zoology Department

Date

2661-25

THE METABOLISM OF DIAZINON IN ALFALFA
Abstract

LARRY LEONARD NELSON

Under the supervision of Dr. Eugene W. Hamilton

Data on the rate of uptake, translocation, expiration, and metabolism of diazinon in alfalfa were obtained. Non-radioactive, C¹⁴ ring labeled or C¹⁴ ethyl labeled diazinon was applied at the equivalent rate of 1 lb/acre calculated at a 6 in. depth.

Absorption was rapid with detectable quantities of diazinon appearing in the plant within the first 24 hours after treatment. Residue levels in the plant remained relatively constant during the period from 9 to 15 days after treatment. Loss of diazinon from the soil was rapid with an average of 15% of the applied material remaining 15 days after treatment.

No metabolites of diazinon, including diazoxon, were found in any of the soil or alfalfa samples analysed by gas chromatography, thin layer chromatography, or radioautography during the period from time of treatment through 15 days posttreatment. No radioactive CO₂ was detected as a metabolite of C¹⁴ diazinon treatments. Diazinon was expired by alfalfa plants after being absorbed into the plant.

ACKNOWLEDGEMENTS

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INTRODUCTION

The use of organic phosphate insecticides for control of insects resistant to chlorinated hydrocarbon insecticides has rapidly increased in recent years. A number of studies involving soil, plants and animals have been conducted to determine the residual properties of the various organic phosphate insecticides but the literature is far less extensive concerning their metabolism in organisms.

The organic phosphate insecticide diazinon is widely used for control of various plant pests. The present study was undertaken to investigate the metabolism of diazinon in alfalfa.

LITERATURE REVIEW

Diazinon is an organic phosphate insecticide (O,O-diethyl-O-[2-isopropyl-4-methyl-6-pyrimidinyl]-phosphorothioate) with a characteristic short residual life.

A summary of the steps involved in the formation of diazinon are as follows. Isobutyronitrile, hydrochloric acid, ethanol, ammonia, and methyl acetoacetate are combined in a series of reactions to yield 2-isopropyl-4-methyl-6-hydroxypyrimidine (I). The alkaline salt of I is then reacted with diethyl thiophosphoryl chloride (II) to give diazinon (III) (Gysin and Margot 1958).

The formation of technical diazinon by these reactions results in a product of about 90% purity. The diazinon formed is very stable in the absence of water or in the presence of water at a pH of 6.0 to 8.0. Three impurities found in the resulting technical diazinon are 2-isopropyl-4-methyl-6-hydroxypyrimidine (I), triethylthiophosphate (IV), and the monoethyl ester (V).

Possible pathways for the degradation or metabolism of diazinon were presented by Margot and Gysin (1957). The oxidation of diazinon resulted in the formation of the more toxic oxygen analog diazoxon (VI), the usual metabolic product produced when diazinon is metabolized by living organisms. The oxidation of the parent compound in metabolism is known as the "activation reaction". Hydrolysis of technical diazinon with excess water resulted in practically non-toxic products.

Margot and Gysin (1957) examined technical diazinon samples decomposed by heat and found the following breakdown products.

2-isopropyl-4-methyl-6-hydroxypyrimidine
 2-isopropyl-4-methyl-6-ethoxypyrimidine
 2-isopropyl-4-methyl-6-ethylmercaptopyrimidine
 bis-[2-isopropyl-4-methyl-6-pyrimidinyl]-sulfide
 monothiono tetraethylpyrophosphate
 dithiono tetraethylpyrophosphate

Should oxidation and hydrolysis occur simultaneously, the following series of reactions may occur with the formation of tetraethylpyrophosphate (TEPP) (VII).

Other hypothetically possible metabolites of diazinon are triethyl thiophosphate, tetraethyl dithiopyrophosphate, and O-ethyl-O, O-bis(2-isopropyl-4-methyl-6-pyrimidinyl) thiophosphate.

Studies on plants (species not specified) by Margot and Gysin (1957) indicated loss of insecticidal activity due to evaporation of the insecticide and through hydrolysis according to the following equation:

They concluded that the formation of thiophosphoric acid and phosphoric could also possibly occur as metabolites. No metabolites of diazinon were found in these plants.

Coffin and McKinley (1964) indicated that no metabolism of diazinon to diazoxon occurred in lettuce. Only the parent compound was recovered from sprayed lettuce. No attempt was made to identify hydrolytic degradation products.

The metabolism of diazinon in spinach, snap beans, and tomatoes was investigated by Ralls et al. (1966). The oxygen analog diazoxon was recovered from spinach and snap beans. Examination of tomatoes treated with C^{14} labeled diazinon revealed radioactive carbon dioxide and 2-isopropyl-4-methyl-6-hydroxypyrimidine as metabolic breakdown products.

In studies using beans (Phaseolus vulgaris var. Resistant Asgrow Valentine), Gunner et al. (1966) found that diazinon was passed through the plant unchanged with rapid translocation and emergence in bean root exudates after application of diazinon to aerial portions of the plant. Diazinon has been reported to exhibit a localized systemic action and not translocated in toxic amounts (Gasser 1953). Systemic activity in sugar-beet seedlings was also observed by Onsager and Rusk (1967) in studies conducted with diazinon applied to the soil. Diazinon was translocated in quantities which rendered the plant insecticidal.

Thus, it appears that diazinon may or may not act as a systemic insecticide. It is evident that conflicting results have been obtained in studies investigating metabolism of diazinon in various plants.

Similarly conflicting results have been reported on the fate of diazinon in the soil. Data obtained by Getzin and Rosefield (1966) indicated that metabolism of diazinon in the soil does occur. Radioactive carbon dioxide and unidentifiable water soluble products were detected in soil at a pH of 5.0 to 5.4. Similar results were obtained by Getzin (1967). Radioactive carbon dioxide and 2-isopropyl-4-methyl-6-hydroxypyrimidine were recovered from soil with a pH of 6.7.

Results obtained from diazinon treated soil in studies conducted by Konrad et al. (1967) indicated breakdown of diazinon to 2-isopropyl-4-methyl-6-hydroxypyrimidine and diethyl thiophosphoric acid at soil pH of 1.0, 2.0, and 4.0. At pH 6.0, however, diazinon was quite stable with no breakdown occurring through a two week period.

METHODS AND MATERIALS

Mature alfalfa plants (Medicago sativa L. variety Vernal), selected at random from a field which had no previous history of insecticide use, were used in these studies. The individual plants, with about 18 in. of root system attached, were planted in 5 x 7 in. peat pots in soil obtained from the alfalfa field (Lamoure silty-clay, initial pH of 7.1 determined by the Soil Testing Laboratory of South Dakota State University). The soil pH in each pot was measured at each sampling period thereafter with a Beckman Zeromatic ^(h) pH meter. The plants were held in a greenhouse having a relatively constant average relative humidity of 50% and average temperature of 80° F.

Immediately after potting and transfer to the greenhouse, the plants were cut to a 4 in. height and then allowed to grow to the bloom stage and again cut back to a 4 in. height. This procedure was repeated three times to insure that the actively growing plants had a well developed root system for these studies.

Plants were watered daily to maintain the moisture content of the soil at about 25% on a dry weight basis. The amount of water required to maintain this approximate moisture content was determined by experimentation. A 50 gm sample of soil from each individual pot was taken for moisture determination at the time samples for diazinon and metabolite analysis were taken. Each sample for moisture content determination was weighed, oven dried at 110° C for 24 hours, and reweighed. The percent moisture was calculated from the loss in weight due to drying.

Sampling Procedures

Soil sampling procedures consisted of thoroughly mixing the soil of individual pots and taking a sample of approximately 500 gm from which 50 gm subsamples were taken for separate pH, moisture content and diazinon and metabolite analysis.

Alfalfa samples for insecticide analysis were cut at a height 3 in. from the soil to eliminate aerial portions of the plant which may have become contaminated through the splash effect of application of the insecticide. The remaining plant portions, including the roots were removed from the soil and discarded to insure that plant parts in direct contact with the diazinon treated soil were not used. Alfalfa and soil samples were placed in polyethylene bags and frozen for further use.

Insecticides Used

Geigy Chemical Company supplied the non-radioactive and radioactive diazinon used in this study. The non-radioactive diazinon had a purity of 95.8%. Gas chromatographic analysis of the non-radioactive sample indicated two impurities, neither of which could be identified. Radioactive material consisted of diazinon labeled on the C¹ of the ethyl esters with a specific activity of 3.21 uc/mg and C¹⁴ diazinon labeled on the C² of the pyrimidine ring with a specific activity of 4.00 uc/mg. The purities of the materials were 98.9% and 98.6% respectively. Gas chromatographic analysis of the samples indicated two unidentifiable impurities.

The insecticides were applied to the soil of individually potted plants, 12 to 18 in. in height, at the equivalent rate of 1 lb actual insecticide/acre calculated at a 6 in. depth. Materials were

added as 50 ml of water emulsion in a surface drench and washed into the soil with an additional 100 ml of water.

Extraction and Purification

The dual solvent technique described by Thornburg (1963) was used to extract and purify the soil and alfalfa samples. In this process each sample is homogenized with a blender in Nanograde ^(R) isopropyl alcohol: benzene (2:1). Homogenates were poured into a separatory funnel, and, by a series of partitionings with 17% salt solution, the insecticide and non-polar metabolites partitioned into the upper benzene fraction. The isopropyl alcohol:salt fractions from these partitionings were combined and stored under refrigeration for later analysis for polar metabolites. The benzene fraction was purified by adding activated nuchar-attaclay in a ratio of 10:1 (sample:nuchar-attaclay), shaking, and filtering immediately through Whatman #1 filter paper. Samples were then stored under refrigeration.

Known quantities of analytical standards of diazinon and five possible metabolites supplied by Geigy Chemical Company were added to untreated homogenized soil and alfalfa samples. Extracts were purified using the above described method. Percent recoveries are shown in Table 1.

Gas Chromatographic Methods

Gas-liquid chromatography was used to determine diazinon and metabolites in soil and alfalfa benzene extracts. The Varian Aerograph ^(R) model 600-C electron capture gas chromatograph used was equipped with a 5 ft by 1/8 in. glass column packed with Chromosorb W coated with 10% Dow 11. Operating parameters were: oven temperature 190° C, injector temperature

Table 1.-Percent recoveries, sensitivities, and retention times for diazinon and five possible metabolites using gas chromatography.

	<u>Percent recovery</u>		<u>Sensitivity</u> (picograms)	<u>Retention</u> <u>Time</u> ^{a/}
	Soil	Alfalfa		
diazinon	84	93	20	.50
diazoxen	86	71	200	1.20
monothiono-TEPP	98	95	80	.20
dithiono-TEPP	95	96	230	.44
2-isopropyl-4-methyl- 6-mercaptopyrimidine	99	94	100	2.27
2-isopropyl-4-methyl- 6-hydroxypyrimidine	88	85	40	1.82

^{a/} Retention times calculated using aldrin as unity (1.00) for reference.

200° C, voltage EC-10, range EC-10, attenuation 1x, nitrogen carrier at a flow rate of 50 ml/min, recording chart speed of $\frac{1}{2}$ in/min (Sargent ^R recorder model SR), sample injection size 2 ul. Sample peak areas obtained by gas chromatography were calculated by triangulation. Quantitative results were obtained by comparison of sample peak areas with peak areas of known concentrations of standards. Sensitivity of detection and retention times (relative to aldrin as unity for diazinon and five possible metabolites) are shown in Table 1.

Thin Layer Chromatographic Methods

Benzene extracts of soil and alfalfa were analyzed for diazinon and metabolites by thin layer chromatography. The TLC plates were coated with a 250 u layer of Silica Gel and activated at 110° C for $\frac{1}{2}$ hour. The developing tank was lined with filter paper, mobile solvent added and the tank atmosphere allowed to equilibrate for at least 1 hour at 24° C prior to developing the TLC plates to insure a saturated atmosphere. Approximately 25 ul of each purified soil or alfalfa benzene extract was spotted at intervals of $\frac{3}{4}$ in. and 1 in. from the bottom of 8 x 10 in. glass thin layer plates with 25 ul micro pipettes. There were thus 9 spots applied/TLC plate. The plates were developed 15 to 20 minutes at 24° C in benzene:chloroform:ethyl acetate (2:2:1) mobile solvent. Since R_f values normally vary slightly from plate to plate, 10 ug standards of diazinon and possible metabolites were included on each plate.

Since samples were not visible after development of thin layer plates, it was necessary to use spray reagents to detect samples. Diazinon and any possible metabolites were visualized by spraying TLC

plates with flourescein spray (Walker and Beroza 1963) and then immediately examining the plate under ultraviolet light. The plates were next sprayed with methyl orange (Dutt and Seow 1963) to visualize other metabolites. Color development of standards after spraying with methyl orange required 15 to 20 minutes. A tracing of a developed thin layer plate showing separation of diazinon and five possible metabolites is found in Fig. 1. R_f values for diazinon and five possible metabolites included in Table 2 were computed using diazinon as unity (1.00).

Radioautography Methods

Translocation of C^{14} diazinon in the plant was determined by radioautography. In addition, thin layer plates were examined for radioactive metabolites of diazinon in soil and alfalfa benzene extracts by the same method.

Kodak [®] 8 x 10 in. non-screen industrial, type KK, x-ray film was placed in contact with the thin layer plate or alfalfa sample. Alfalfa samples were taped to a 8 x 10 in. sheet of cardboard. The sample and x-ray film was then sandwiched between plywood sheets. This packet was placed in a metal light-tight telescoping box and the lid weighted to insure direct contact between the film and sample. Exposure time was 30 days. Exposed films were processed with Kodak X-ray Developer and Replenisher for 6 minutes, fixed in Kodak X-ray Fixer and Replenisher for 9 minutes and rinsed in tap water for a minimum of 1 hour. After drying, contact prints were made from the exposed film. A Brown [®] Portable Copy Exposure Unit, fitted with an opaque glass to reduce light intensity, was used to make the prints. A rheostat was attached to the exposure unit

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Figure 1.-Tracing of a thin layer plate indicating separation of diazinon and five possible metabolites standards.

1. 2-isopropyl-4-methyl-6-hydroxypyrimidine
2. diazoxen
3. 2-isopropyl-4-methyl-6-mercaptopyrimidine
4. monothiono tetraethylpyrophosphate
5. diazinon
6. dithiono tetraethylpyrophosphate

FRONT

BASELINE

Table 2.-Sensitivities, R_f values, and spot color of diazinon and five possible metabolites using thin layer chromatography.

	Sensitivity (ug)	R_f^a	Spot Color	
			Flouroscein and UV	Methyl orange
diazinon	2	1.00	Blue	Yellow
diazoxon	2	$.39 \pm .05$	Blue	
monothione-TEPP	2	$.75 \pm .02$		Orange
dithione-TEPP	3	$1.34 \pm .04$		Yellow
2-isopropyl-4-methyl- 6-mercaptopyrimidine	2	$.66 \pm .01$	Blue	Yellow
2-isopropyl-4-methyl- 6-hydroxypyrimidine	2	$.06 \pm .01$	Blue	

^a R_f values determined using diazinon with an R_f value of $.59 \pm .04$ as unity.

for greater control of light to insure identical exposure time and light intensity from one film to the next. Prints were made using Kodak 8 x 10 in. single weight Ektamic Sc-F[®] photographic paper.

The relative distribution of C¹⁴ ring and ethyl labeled diazinon in the leaves of plants analyzed by radioautography was determined by densitometry. Contact negatives for this purpose were made from radioautographs using 8 x 10 in. Kodak Kodalith LR[®] photographic film. Overexposed contact prints were made from the contact negatives to give varying shades of white to radioactive areas enabling more accurate measurements of densitities to be made. Exposure time and intensity were controlled as stated above.

Densitometry Methods

Relative densities of radioactivity in prints of radioautographs were measured using a Joyce, Loebble, and Co.[®] double beam recording and integrating model 5-4060 densitometer set for reflectance operation with a slit width of 0.5 mm, a path length of 1.0 mm, and a specimen scanning drive gear ratio of 1:3. Areas representing individual leaves were cut out of the developed print obtained by radioautography. A border of approximately $\frac{1}{2}$ in. around the leaf print was included to be measured as background. "Leaves" for ring or ethyl labeled C¹⁴ diazinon treated plants were selected at random from the top, middle, and lower $\frac{1}{3}$ of each spear of alfalfa taken on each of the sampling dates. Only "leaves" from the areas designated were analyzed since the purpose of the procedure was to determine relative distribution of radioactivity which should be the same in all leaves of any given plant. Individual "leaves" were

mounted on thin cardboard for analysis by densitometry. Each was scanned transversely mid-way between the base and tip and longitudinally from the base to tip, just to the right of the midrib. The spectrum obtained from each leaf was then divided into three equal parts and the maximum peak heights in each third measured in millimeters. Maximum peak heights were used as an indicator of C^{14} content.

Liquid Scintillation Methods

The C^{14} activity in soil or alfalfa benzene or water:isopropyl alcohol fractions taken from C^{14} diazinon treatments was measured by liquid scintillation counting. Scintillation counts were made using a Packard Tri-Carb ^(R) automatic liquid scintillation counter with a counting efficiency of 83.9%. Samples were counted for 20 minutes. Scintillation fluid was prepared as follows: 4 mg PPO (2,5-diphenyl-oxazolyl) and 0.1 gm dimethyl POPOP (1,4-Bis-2-[5-phenyloxazolyl]-1-benzene) dissolved in 1 liter of toluene.

Rate of Absorption Studies

Non-radioactive diazinon was applied to three replicates consisting of six individually potted plants/replicate. Plants and soil samples from each replicate were collected at 1, 3, 6, 9, 12, and 15 days post-treatment. A fourth replicate of six plants served as an untreated control. Three 50 gm soil samples from each pot were analyzed by gas chromatography for diazinon and metabolites. Additional samples were taken for pH and dry weight determination. The entire plant sample taken from each pot was weighed and analyzed for diazinon and metabolites by gas chromatography.

Translocation Studies

Either C^{14} ring labeled diazinon or C^{14} ethyl labeled diazinon was applied to the soil of individually potted alfalfa plants. One spear of alfalfa from each of the replicates was selected at random for determination of translocation of C^{14} by radioautography. The relative radioactivity in developed prints of radioautographs were determined by densitometry. Three replicates of each type of labeled material were sampled at 1, 3, 6, 9, 12, and 15 days posttreatment.

Alfalfa Respiration Studies

Expiration of Diazinon By Alfalfa

One set of three replicates was treated with C^{14} ring labeled diazinon. A second set of three replicates was treated with C^{14} ethyl labeled diazinon. A third set served as the untreated control. Single spears of this alfalfa, less roots, were selected at random from each replicate at 14 days posttreatment for determination of C^{14} by radioautography. Fifteen days posttreatment the remaining portion of individual plants were removed from the soil and the roots rinsed clean in tap water. These plants from the C^{14} ring labeled diazinon treatments were placed in a single 2000 ml flask fitted with a 2-holed rubber stopper and containing 1000 ml of nutrient solution consisting of 0.25% Ra-Pid-Gro[®] in water (Fig. 2). The flask was connected to an 800 ml wide mouth bottle fitted with a 2-holed rubber stopper and containing 250 ml Nanograde benzene. The benzene trap was in turn connected to a General Electric A-C rotor type vacuum pressure pump, enabling air samples to be washed of diazinon as they passed through the benzene.

Figure 2.-Apparatus used to collect diazinon expired by alfalfa plants.

Air samples were collected during the light period (photosynthesis) for 1 hour each day over a period of 5 days. Each collection was added to the previous one so the final collection period represented a composite of all 5 sampling periods. After the final sampling period, each 250 ml benzene trap was evaporated to 1 ml using a gentle air stream. Diazinon content was determined by gas chromatography. After the last samples were taken, a single spear of alfalfa from each replicate was selected at random for determination of the presence of radioactive material by radioautography. A similar procedure was followed for C^{14} ethyl labeled diazinon treated plants. The radioautographs of the alfalfa spear obtained in this experiment were subjected to densitometry to determine distribution of C^{14} in leaves of plants grown 14 days in C^{14} diazinon treated soil and also after removal of plants from the soil and grown 5 days in nutrient solution.

Expiration of CO_2 by Alfalfa

Twenty-two micrograms of C^{14} ring labeled diazinon were fortified with 1400 ug non-radioactive diazinon to obtain a total diazinon content in the soil of each pot equivalent to 1 lb/acre at a 6 in. depth. The above concentrations were the same for C^{14} ethyl labeled treatments. The concentration of C^{14} ring or ethyl labeled diazinon added to each pot was calculated to give an average activity of 250 cpm/ul of plant extract. The amount used was based on concentrations taken up by the plant one day posttreatment as determined in the study on the rate of absorption.

A series of three replicates of five plants each, and an untreated control, were used to determine if radioactive carbon dioxide was evolved

Figure 3.- Apparatus used to collect CO_2 expired by alfalfa plants.

Figure 4.-Saran Wrap pot cover used to minimize volatilization of insecticide from soil during respiration studies.

the suction to simultaneously draw air from four separate replicates.

The sampling interval was once again 1, 3, 6, 9, 12, and 15 days posttreatment. On each sampling day, expired air from each replicate was collected for 1 hour during the dark period (respiration) and 1 hour during the light period (photosynthesis). The relative CO_2 was then compared.

Each 2 ml Hydroxide of Hyamine sample containing expired CO_2 was collected and placed in 12 ml of scintillation fluid in a standard counting vial and stored under refrigeration for determination of radioactivity by liquid scintillation.

On the 15th day, a pH determination was made from each pot. The five individually potted plants constituting a single replicate were collected and combined into one sample. The soil in these pots was thoroughly mixed and a sample of approximately 500 gm taken. Each replicate was handled similarly. These soil and alfalfa samples were saved for later analysis.

Metabolism Studies

Alfalfa and soil samples, containing either ring or ethyl C^{14} labeled diazinon obtained in the CO_2 studies, were analyzed for the presence of diazinon and metabolites. Four 50 gm soil samples were taken from four replicates for ethyl C^{14} labeled diazinon treatments. One 50 gm sample was used for a dry weight determination. The remaining three samples were analyzed by thin layer chromatography and radioautography for diazinon and metabolites. The entire alfalfa sample from each replicate of ethyl C^{14} labeled diazinon was weighed. A similar procedure was

followed for alfalfa and soil samples treated with C^{14} ring labeled diazinon.

All soil and alfalfa samples were extracted and purified as indicated previously. The water-isopropyl alcohol fractions obtained from each extraction were individually centrifuged at 8,000 RPM for 15 minutes in a Servall ^(R) centrifuge to isolate any remaining non-polar solvent from the polar water-isopropyl alcohol fraction. The centrifuged water-isopropyl alcohol fraction was removed by pipette. Water was added to the centrifuged samples to bring them to an even volume of 400 ml. Two milliliters of each soil or alfalfa water-isopropyl alcohol fraction was placed in 12 ml scintillation fluid and analyzed for C^{14} by liquid scintillation.

The soil and alfalfa benzene fractions were concentrated to 25 ml in a gentle air stream. The process of evaporation was aided by placing the samples in a 40° C water bath. After concentration to 25 ml, a 2 ml sample was placed in 12 ml of scintillation fluid and analyzed for C^{14} by liquid scintillation. Results obtained as counts/20 minutes/2 ml sample were converted to cpm/ml as follows:

$$\begin{array}{r} 1142 \text{ counts/20 min/2 ml} \\ - \quad 497 \text{ background} \\ \hline 645 \text{ c/20 min/2 ml} \\ 645 \text{ c/20 min/2 ml} = 16.15 \end{array}$$

The data obtained was then multiplied by 25 ml to give total activity per sample and divided by the weight of each sample to give cpm/gm of sample.

$$16.15 \text{ cpm/ml} \times 25 \text{ ml} = 403.75 \text{ cpm/25 ml sample}$$

$$403.75 \text{ cpm/25 ml} \div 36.4 \text{ gm dry weight} = 11.09 \text{ cpm/gm}$$

This figure was finally divided by the specific activity per milligram expressed in counts per minute of the C^{14} ring and ethyl groups labeled diazinon to present the final expression of results in terms of ug radioactive materials per gram of soil or alfalfa.

$$11.09 \text{ cpm/gm} \div 888,000 \text{ cpm/mg of } C^{14} \text{ ring labeled diazinon} =$$

$$.000012 \text{ mg } C^{14} \text{ ring labeled diazinon} = .012 \text{ ug/gm}$$

The benzene fraction extracts remaining after sampling for liquid scintillation were concentrated to 0.5 ml and analyzed for diazinon and metabolites by thin layer chromatography. Percent recoveries of known amounts of C^{14} diazinon added to untreated soil and alfalfa was 89% and 85% respectively. After development and visualization of spotted standards and after checks for diazinon and metabolites, individual thin layer plates were examined by radioautography. Plates were sprayed with Spray-O-Namel[®], a colorless nitrocellulose and acrylic spray, to minimize loss of material by volatilization during the developing period.

Since the samples treated with C^{14} diazinon fortified with non-radioactive diazinon did not contain enough radioactivity for conclusive results in the metabolism determinations, the study was repeated with unfortified ring or ethyl labeled C^{14} diazinon. All methods used were the same as those indicated previously. The use of C^{14} diazinon at a higher concentration in this final study gave higher concentrations of radioactivity in the plant to work with after extraction and purification.

RESULTS

In all of the metabolism studies conducted no metabolites of diazinon, including diazoxon, the oxygen analog, were found in either the soil or alfalfa.

Gas chromatographic analysis of benzene fractions of soil and alfalfa samples taken during the absorption determination using non-radioactive diazinon indicated the presence of diazinon. No metabolites were detected.

Thin layer chromatography and subsequent radioautography of the developed plates of soil and alfalfa samples taken from C¹⁴ ring and C¹⁴ ethyl labeled diazinon treatments used in the CO₂ studies and analyzed 15 days posttreatment indicated only one compound present. This compound had an R_f value of $0.59 \pm .04$, identical to that of diazinon. Good separation of analytical standards of diazinon and five possible metabolites was achieved and the slight variation from plate to plate (Table 2) can be attributed to changes in relative saturation of tank atmosphere with mobile solvent, humidity, and temperature.

Thin layer chromatography and radioautography of soil and alfalfa samples treated with 100% C¹⁴ ethyl labeled or 100% C¹⁴ ring labeled diazinon also failed to demonstrate the presence of diazinon metabolites.

Data obtained from 20 minute scintillation counts of Hydroxide of Hyamine samples (used to collect CO₂ expired from alfalfa plants) indicated no radioactive CO₂ was released from either C¹⁴ ring or C¹⁴ ethyl labeled diazinon treatments, either during respiration or photosynthesis.

Liquid scintillation analysis for radioactivity in water-isopropyl

alcohol fractions from soil or alfalfa treated with C^{14} ring or C^{14} ethyl labeled diazinon indicated no radioactivity above background was present. The average amount of C^{14} ring labeled diazinon found in benzene extracts of alfalfa plants was calculated to be 0.016 ug/gm of alfalfa. The average amount of C^{14} ethyl labeled diazinon found was 0.025 ug/gm of alfalfa. Calculations showed that the amount of diazinon or metabolites in benzene extracts of soil to be an average of 0.016 ug/gm for the C^{14} ring labeled diazinon treatments and an average of 0.057 ug/gm in the C^{14} ethyl labeled diazinon treatments. Residues of diazinon in the soil samples herein reported are based on dry weight. All residues of diazinon in alfalfa are calculated on a fresh weight basis.

Determinations of soil pH at the time samples were taken showed the pH varied from 6.9 to 7.7 between individually potted plants. The optimum pH range for alfalfa growth is 6.0 to 7.5.

The rate of absorption of non-radioactive diazinon is shown in Fig. 5 expressed in terms of ug/gm of fresh alfalfa. Each plotted point represents the average of three replicates of treated plants for that sample day posttreatment. Initial absorption was rapid, with detectable quantities appearing in the plant within the first 24 hours after application of the insecticide. Concentrations of diazinon in the plant continued to increase up to 9 days. Residues remained relatively constant from 9 days through the 15th day, the last day of sampling, with concentrations remaining at an average level of 0.310 ug/gm of plant tissue throughout this period.

Percent of the total diazinon applied to the soil remaining therein

Figure 5.-Rate of absorption of non-radioactive diazinon by alfalfa.

100% COTTON FIBER

on the sampling days is indicated in Fig. 6. Each plotted point represents the average of 9 samples consisting of 3 samples taken from each of the 3 replicates of treated soil. Loss of diazinon from the soil was initially great with over 50% of the applied amount lost within the first 24 hours after treatment. After the rapid initial loss, diazinon levels in the soil slowly decreased until after 15 days, only 15% of the diazinon originally applied remained in the soil. The 15% represents a residue of 0.112 ug/gm of soil.

Radioautographs taken 1, 3, 6, 9, 12, and 15 days posttreatment (Figures 7, 8, and 9) show that both C^{14} ring and ethyl labeled diazinon are concentrated in the outer portions of the alfalfa leaves during translocation. Light areas in the figures indicate radioactivity. C^{14} concentrations in the plant after one day were too low for radioautography (Fig. 5). Radioautographs of the 9 and 12 day samples could not be distinguished from the 15 day radioautographs. All three series exhibited the same intensity of C^{14} development.

Residue analysis of the soil taken 15 days posttreatment from pots used in the translocation study indicated an average of 0.338 ug diazinon/gm in the C^{14} ring labeled samples and an average of 0.373 ug/gm in the C^{14} ethyl labeled soil samples. These results show diazinon was still available to the plant at the time 15 day radioautographs were made.

Average distributions of C^{14} in the leaves of alfalfa, determined by densitometry, are shown in Figures 10, 11, 12, and 13. The leaf samples scanned longitudinally for radioactivity had more C^{14} in the tip 1/3 than in the center 1/3 with the lowest density in the 1/3 representing

Figure 6.--Rate of disappearance of non-radioactive diazinon
from the soil.

Figure 7.-Radioactivity in the plant after 3 days due to C^{14} ring and ethyl labeled diazinon. Diazinon applied to the soil.

- a. C^{14} ring labeled alfalfa.
- b. C^{14} ethyl labeled alfalfa.

Figure 8.—Radioactivity in the plant after 6 days due to C^{14} ring and ethyl labeled diazinon. Diazinon applied to the soil.

- a. C^{14} ring labeled alfalfa.
- b. C^{14} ethyl labeled alfalfa.

Figure 9.—Radioactivity in the plant after 15 days due to C^{14} ring and ethyl labeled diazinon. Diazinon applied to the soil.

- a. C^{14} ring labeled alfalfa.
- b. C^{14} ethyl labeled alfalfa.

Figure 10.-Ring labeled diazinon. C^{14} distribution in leaves given in terms of maximum peak height. Peak height obtained from spectra of longitudinal scan with a densitometer.

Figure 11.-Ethyl labeled diazinon. C^{14} distribution in leaves given in terms of maximum peak height. Peak height obtained from spectra of longitudinal scan with a densitometer.

Figure 12.-Ring labeled diazinon. C^{14} distribution in leaves given in terms of maximum peak height. Peak height obtained from spectra of transverse scan with a densitometer.

Figure 13.-Ethyl labeled diazinon. C^{14} distribution in leaves given in terms of maximum peak height. Peak height obtained from spectra of transverse scan with a densitometer.

the base of the leaf. In samples scanned transversely, the highest density occurred in the right and left 1/3 of each leaf. The lowest density was found in the center 1/3 of individual leaves. The extreme variation of C^{14} content in the 1, 3, and 6 day leaf samples probably occurred because the low concentration of diazinon (Figures 10, 11, 12, and 13) was difficult to measure accurately by densitometry. Small differences between individual plants with regard to size and vigor may also be a factor.

Results from studies conducted to detect diazinon expired from alfalfa plants grown in C^{14} diazinon treated soil indicated no diazinon in the benzene trap of the untreated control. Quantities of C^{14} ring or C^{14} ethyl labeled diazinon obtained from benzene traps and detected by gas chromatography were calculated to be 0.111 ppm and 0.460 ppm respectively. These results expressed in terms of average amount of diazinon expired per plant per hour were 7.73 ng for the C^{14} ring labeled treatments and 30.6 ng for the ethyl labeled treatments.

No visual differences were evident between radioactivity in alfalfa samples taken 14 days after growth in C^{14} diazinon treated soil and that in alfalfa samples taken from the treated soil and grown for an additional 5 days in nutrient solution. Subsequent examination of alfalfa samples by densitometry showed that concentrations of radioactivity were greater in the tip, right, and left 1/3 of leaves examined and less in the center and basal 1/3 in samples of both C^{14} ring and ethyl labeled diazinon treatments, (Figures 14, 15, 16, and 17). In

Figure 14.--Ring labeled diazinon. Relative C^{14} content in leaves at 14 days and after 5 days additional growth in nutrient solution. Transverse densitometer scan.

Figure 15.--Ethyl labeled diazinon. Relative C^{14} content in leaves at 14 days and after 5 days additional growth in nutrient solution. Transverse densitometer scan.

Figure 16.--Ring labeled diazinon. Relative C^{14} content in leaves at 14 days and after 5 days additional growth in nutrient solution. Longitudinal densitometer scan.

Figure 17.--Ethyl labeled diazinon. Relative C^{14} content in leaves at 14 days and after 5 days additional growth in nutrient solution. Longitudinal densitometer scan.

samples treated with C^{14} ring labeled diazinon, average concentrations of radioactivity decreased in all examined areas of leaves after extraction from the soil and growth in nutrient solution. This indicated a loss of diazinon from the plants. The average maximum density in samples treated with C^{14} ethyl labeled diazinon decreased in the right, left, and basal 1/3 of leaves examined. Density levels did not change in the center 1/3. Concentrations increased in the 1/3 representing the tips, indicating an outward movement of the radioactive materials. Translocation of diazinon to nutrient solution from alfalfa through the root system may occur. Similar results are reported by Gunner et al. (1966) in their work with diazinon in beans. At the time of experimentation, this possibility was not considered. Therefore, the nutrient solution was not examined for translocated diazinon.

DISCUSSION

These studies demonstrated the rapidity with which diazinon is absorbed by the plant through the root system. This indicates that diazinon can act as a systemic in alfalfa, and, once in the plant, an apparent equilibrium between concentrations of diazinon in the soil and plant exists after 9 days. Thus, it appears that the amount of diazinon found in the plant might be dependent to a great extent upon the concentration of diazinon in the soil. That is, as the residues in the soil decrease to concentrations lower than those existing in the plant, one can expect the absorption into the plant to decrease.

After 15 days, the average amount of C^{14} ring labeled diazinon detected by liquid scintillation, 0.016 ug/gm, was the same for both the plant and soil samples indicating apparent equilibrium.

Diazinon was expired from plants grown in diazinon treated soil. Therefore, the stabilization or decrease of diazinon levels in the plant is not due to metabolism but instead, due to the apparent equilibrium between plant, soil and loss of diazinon by expiration. The apparent equilibrium may not be a true equilibrium between plant and soil. Equilibrium may exist because of the inability of the plant roots to come in contact with each soil colloid. Consequently, the diazinon located at such points is not available to the plant unless it moves adjacent to the root system by some process of solubility or the root system expands.

The greater amount of C^{14} ethyl labeled diazinon expired from the plant may indicate that the ethyl esters of the C^{14} ring labeled diazinon are lost in some way during expiration. As a result, less diazinon would

be detected in those treatments.

The initial rapid loss of diazinon from the soil can be attributed to a number of causes. According to Lichtenstein and Schultz (1964), and Gunner et al. (1966), such factors as volatilization, breakdown and utilization by microorganisms, and leaching all play a part in the decrease of insecticide levels in the soil. It was not the purpose of the study to investigate these aspects of diazinon metabolism.

Data indicate that diazinon is not metabolized by alfalfa under the conditions of the experiments. This conclusion was substantiated by several experiments, and agrees with results obtained by Gunner et al. (1966) in beans. The data collected does not exclude the possibility, however, that metabolism could be occurring. It may be occurring at such a slow rate that concentrations of stable metabolites are never enough to detect at any one period with the sensitivities attained in these studies. The overall diffusion of C^{14} indicated by radioautographs of the plant tissues could be due to a very low concentration of diazinon or diazinon metabolites. The data does not exclude the possibility that under different soil or growing conditions metabolism could occur. It appears that occurrence of diazinon metabolism depends upon the plant species (Coffin and McKinley 1964, Ralis et al. 1966, and Gunner et al. 1966).

SUMMARY

Studies were conducted to determine the fate of diazinon when taken up by alfalfa. Data on the rate of absorption, translocation, expiration, and metabolism of diazinon in alfalfa were obtained.

Non-radioactive, C^{14} ring labeled or C^{14} ethyl labeled diazinon were used in the studies. Materials were applied at the equivalent rate of 1 lb/acre calculated at a 6 in. depth. The use of radioactive materials permitted the use of sensitive detection methods such as radioautography and liquid scintillations.

The rate of absorption was rapid with detectable quantities of diazinon appearing in the plant within the first 24 hours after treatment. Residue levels in the plant remained relatively constant 9 to 15 days after treatment. Loss of diazinon from the soil was rapid with an average of 15% of the applied material remaining 15 days after treatment.

No metabolites of diazinon, including diazoxon, were found in any of the soil or alfalfa samples analyzed by gas chromatography, thin layer chromatography, or radioautography during the period from time of treatment through 15 days posttreatment. No radioactive CO_2 was detected as a metabolite of C^{14} diazinon treatments. Diazinon was expired by alfalfa plants after being absorbed into the plant.

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