The extracted plant tissue retained radioactivity to the extent of 0.02 p.p.m. Not only was this activity retained after initial 80% methanol extraction, but it did not yield to an additional 80% methanol extraction, a 16-hour 10% acetic acid extraction, nor a 2-hour trituration in boiling 5% trichloroacetic acid. Finally, after refluxing with 6*N* hydrochloric acid for 17 hours, radioactive 4-amino-3,5,6-trichloropicolinic acid was isolated; no other radioactive entity down to 0.01 p.p.m. on a fresh plant weight basis was detected. Treatment of plant residues with trichloroacetic acid reagent is reported (*2*) to be a good procedure for removal of most tissue constituents, including polysaccharides, from protein. Since this treatment removed no radioactivity from the solid, extracted residue, it is suggested that the Tordon ultimately isolated from this residue by hydrolysis was combined as an amide with terminal amino groups of protein. The quantity is small but may be significant enough to require a hydrolysis step in any scheme for residue determination.

On a total plant weight basis, the leaf and stem tissue of the cotton plant contained 95% of the total radioactivity present and, of this, 3% was associated with insoluble protein and could not be removed by extraction procedures. The roots contained the remaining 5% of the radioactivity.

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METABOLISM IN PLANTS

Fate of Radioactive *O,O*-Diethyl O-(2-Isopropyl-4-methylpyrimidin-6-yl) Phosphorothioate on Field-Grown Experimental Crops

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Plants grown in a fenced, controlled, and monitored agricultural plot were sprayed with Diazinon labeled with ^{35}S. The residue level of Diazinon fell rapidly below tolerance levels (0.75 p.p.m.) on all crops studied. There was no evidence of predicted sulfur-containing metabolites at levels above 0.1 p.p.m. on crops treated at recommended dosage. The only metabolite identified from the field samples was oxo-Diazinon at an estimated level of 0.01 to 0.05 p.p.m. Radioactive 2-isopropyl-4-methylpyrimidin-6-ol was isolated from tomatoes 5 days after spraying with pyrimidine ring labeled Diazinon-^{14}C. The present evidence suggests that Diazinon is oxidized rapidly to oxo-Diazinon which is, in turn, hydrolyzed to 2-isopropyl-4-methylpyrimidin-6-ol. The latter compound is metabolized, in part, to carbon dioxide by a pathway which does not appear to involve acetoacetic acid (or its amide).

There are still many unsolved problems of potential public health concern resulting from the use of agricultural chemicals to protect food and fiber crops from ravage by pests. One important problem, as yet not completely understood, is the possible conversion of applied pesticides to other products of potentially increased hazard to food consumers. There are several examples of the identification of transformation products of pesticides which are potentially more toxic than the parent compound. An early example was the demonstration of the conversion of schradan to a *N*-oxide with a 10^5 increase in cholinesterase inhibiting activity (*3*).

This paper reports results of a study of the transformation products formed from Diazinon after spraying on spinach, tomato, and snap bean plants.

Very little information is available on the products formed from Diazinon after application to food crops. One important basic study on the hydrolysis of Diazinon is available from the work of Margot and Gysin (*21*).

The general approach taken in this investigation was to predict the most probable transformation products of Diazinon, prepare these compounds, work out methods for their isolation from the crops of interest, and then learn how to purify, separate, and detect them in small amounts. Once the methodology had been developed, crops were sprayed with radioactive forms of Diazinon and the harvested crops analyzed for the predicted transformation products. Some of the most probable transformation products of Diazinon are shown in Figures 1 to 3.

The cholinesterase inhibiting activity of some of the potential transformation products of Diazinon are shown in Table I. While the cholinesterase inhibiting activity of a compound is not a direct measure of the toxicity of a compound to humans, it is an indication of probable hazard (*21*).

Experimental

Synthesis of Potential Transformation Products of Diazinon. Thiophosphoryl Trichloride, b.p. 119–21° C. (748 torr). Prepared in 74% yield (*15*).

O,O-Diethyl Thiophosphorochloridate, b.p. 54–57° C. (1 torr). Prepared in 76% yield (*10*).

Sodium Diethylphosphate. Prepared by the partial saponification of triethyl phosphate (*19*). Reaction of sodium diethylphosphate with *O,O*-diethyl thiophosphorochloridate (*19*) gave a mixture of tetraethyl pyrophosphate (TEPP) (VI), tetraethyl monothionopyrophosphate (S-TEPP) (VII), and tetraethyl dithionopyrophosphate (SS-TEPP) (VIII).

O,O-diethyl phosphorochloridate, b.p. 57–59° C. (1–2 torr) was prepared in 74% yield (*20*).

The reaction of *O,O*-diethyl phosphorochloridate with 2-isopropyl-4-methylpyrimidin-6-ol (see below for preparation) in refluxing benzene using

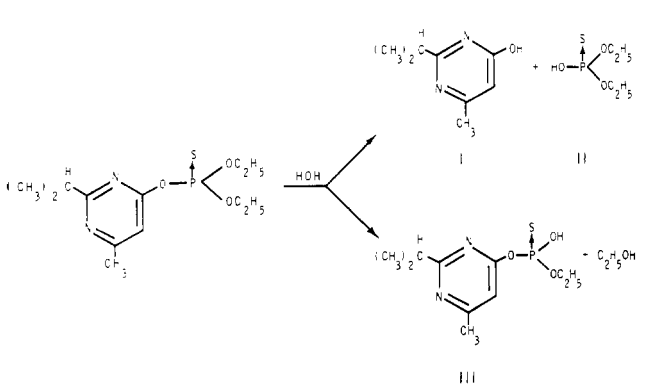

Figure 1. Probable hydrolysis products of Diazinon

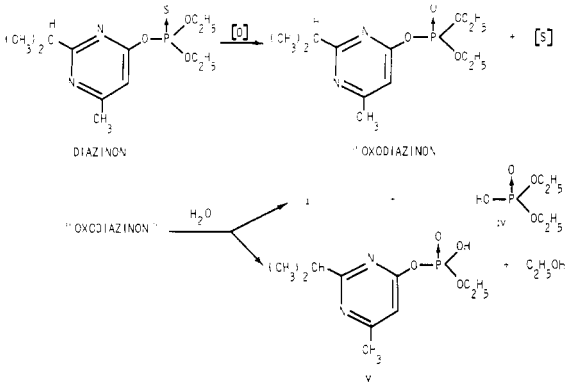

Figure 2. Probable oxidation-hydrolysis products of Diazinon

potassium carbonate as a condensing agent (*18*) gave *O,O*-diethyl *O*-(2-isopropyl-4-methylpyrimidin-6-yl) phosphate (oxo-diazinon) in 27% yield.

Treatment of 2-isopropyl-4-methylpyrimidin-6-ol with phosphorus oxychloride (*21*) gave 6-chloro-2-isopropyl-4-methylpyrimidine, b.p. 57.5–9.5° C. (2 to 3 torr) in 82% yield.

Reaction of the latter compound with sodium ethoxide in ethanol (*21*) gave 6 - ethoxy - 2 - isopropyl - 4 - methylpyrimidine. Excessive foaming of the impure product (traces of benzene used for extraction) on attempted distillation at reduced pressure made it necessary to purify by preparative gas chromatography. Use of a 20% silicone rubber on Chromosorb P column at 122° C. with a helium flow rate of 36 ml. per minute gave the desired compound with a retention time of 7.4 minutes.

Treatment of 3.4 grams of 6-chloro-2-isopropyl-4-methylpyrimidine with 1.52 grams of thiourea in 25 ml. of acetone at reflux for 1 hour gave 4.89 grams of 2 - isopropyl - 4 - methylpyrimidin - 6-ylisothiouronium hydrochloride, m.p. 150–1° C. (with decomposition).

Treatment of 4.20 grams of the latter compound with 21 ml. of 10% sodium hydroxide and 9 ml. of water under reflux for 2 hours gave, after acidification and filtration, 2.66 grams (92%) of 2 - isopropyl - 6 - mercapto - 4 - methylpyrimidine, m.p. 160–1° C. (*21*).

The latter compound was converted to 6-ethylthio-2-isopropyl-4-methylpyrimidine to bis-(2-isopropyl-4-methylpyrimidin-6-yl) sulfide by published procedures (*21*).

Preparation of Pyrimidine Ring-Labeled Diazinon-^{14}C. The literature descriptions for the preparation of intermediates required for the ring labeling process for Diazinon gave poor yields. The preparation of isobutyrylamidine hydrochloride by the method described by Gysin and Margot (*14*) gave yield of only 10 to 27%. The compound is hydrolyzed readily to isobutyrylamide; therefore, aqueous systems should be avoided.

A method using dry conditions (*8*), but modified to omit isolation of the intermediate iminoester, gave the desired product in yields of 40 to 50%.

Isobutyrylamidine Hydrochloride. A three-necked round-bottomed flask was charged with 14.2 grams (0.2 mole) of isobutyronitrile, 9 grams of ethanol, and 8 ml. of ethyl ether. The mixture was cooled in an ice bath and 7.2 grams of anhydrous hydrogen chloride was added over 1 hour from a gas generator (*9*) (from 18 ml. of 37% aqueous hydrochloric acid in excess concentrated sulfuric acid). The mixture was stored at 4° C. for 16 hours. A solution of 3.4 grams (0.2 mole) of anhydrous ammonia (from a lecture bottle) in 50 grams of ethanol was added slowly to the cooled ethyl isobutyrimidine hydrochloride solution. After 1 hour, the ammonium chloride was removed by filtration. After standing at 4° C. for 16 hours, the volatile components of the mixture were removed in a rotary evaporator at reduced pressure. The residue was then taken up into 20 to 25 ml. of ethanol and a small amount of solid removed by filtration. The filtrate was diluted to a volume of 50 ml. and heated to 50° to 60° C. Acetone was added until the turbidity point was reached. Cooling gave 11 grams (46%) of crystalline solid, m.p. 162–3° C. The infrared spectrum, run as a KBr pellet, of the solid was in agreement with that expected for isobutyrylamidine hydrochloride.

2 - Isopropyl - 4 - methylpyrimidin-6-ol-4-^{14}C. A mixture of 5.04 grams (0.04 mole) of isobutyrylamidine hydrochloride, 0.0227 grams of ethyl acetoacetate-3-^{14}C (activity 1 mc.), 4.63 grams (0.04 mole) of methyl acetoacetate, and 15 ml. of ethanol were charged to a 50-ml. flask. The mixture was stirred with a Teflon-coated magnetic stirring bar. A solution of 1.0 gram (0.0492 gram atom) of clean sodium metal in 20 ml. of ethanol was added dropwise over a period of 30 minutes at 25° to 35° C. After being stirred at room temperature for 3 hours, the mixture was heated under reflux for 4 hours. After the mixture was cooled, the ethanol was removed at reduced pressure. (The ethanol fraction recovered had a total activity of 86.5 μc.) The residue was transferred to a separatory funnel with several small portions of water and chloroform. The organic phase was separated and the aqueous phase extracted with a portion of chloroform. The aqueous phase (pH 10) was adjusted to pH 6 with 6*N* sulfuric acid and extracted with three portions of chloroform. The chloroform solution was dried by pouring it through a pad of anhydrous sodium sulfate. Evaporation of the chloroform solution gave a solid. Trituration with cold acetone and collection by filtration gave a crystalline product weighing 5.44 grams (88% yield), m.p. 171–3° C. The specific activity of the product was 0.131 μc. per mg.

Diazinon-4-^{14}C was prepared (Figures 4 and 5) essentially according to a previous description (*18*) for Diazinon-^{32}P, except that the crude product was purified by distillation. The reactants were 3.08 grams (0.020 mole) of 2-isopropyl-4-methylpyrimidin-6-ol-4-^{14}C and 4.0 grams (0.0212 mole) of *O,O*-diethyl

Table I. Cholinesterase-Inhibiting Activity of Some Organophosphate Compounds (*21*)

Compound	Mg. % for 50% Inhibition
Diazinon	8×10^{-1}
SS-TEPP (VIII)	7.9×10^{-4}
Oxo-Diazinon	1.8×10^{-5}
TEPP (VI)	9.7×10^{-5}
S-TEPP (VII)	5.5×10^{-6}

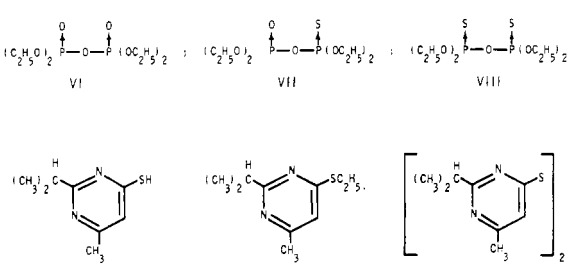

Figure 3. Probable transformation products of Diazinon

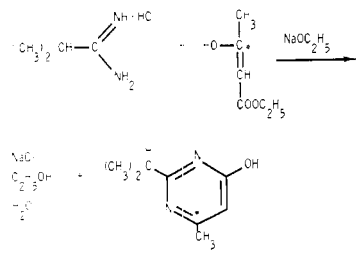

Figure 4. Initial reactions in Diazinon synthesis

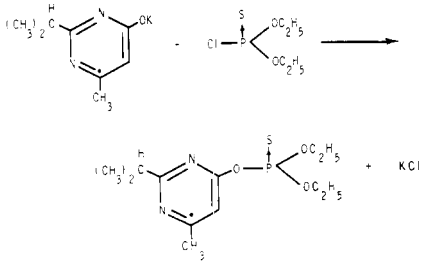

Figure 5. Final step in Diazinon synthesis

thiophosphorochloridate. The crude product weighed 5.82 grams and had a specific activity of 0.0556 μc. per mg. Thin-layer chromatography of the crude Diazinon showed *O,O*-diethyl thiophosphorochloridate as an impurity. Distillation in a microwave short path distillation unit gave 4.20 grams (69%) boiling at 125° C. (0.3 torr). This material showed only a trace of contamination and had a specific activity of 0.060 μc. per mg. An autoradiogram of a thin-layer plate separation of the product showed only one radioactive spot which corresponded in R_f to that of authentic Diazinon.

Diazinon-^{35}S. Potassium 2-isopropyl-4-methylpyrimidin-6-ylate was prepared by heating a mixture of 6.088 grams of 2-isopropyl-4-methylpyrimidin-6-ol, 2.764 grams of potassium carbonate, and 10 ml. of water until gas evolution stopped. On cooling, a solid separated. The mixture was frozen and the water removed by lyophilization. The product was a hygroscopic white powder weighing 8.745 grams. A suspension of 540 mg. of the potassium salt in 9 ml. of toluene was distilled until 6 ml. of distillate had been collected. The cooled suspension was treated with 540 mg. of *O,O*-diethyl thiophosphorochloridate-^{35}S (activity 1 mc.). The radioactive reagent was rinsed into the reaction flask with four 0.5-ml. portions of toluene. The reaction mixture was stirred and heated at 80° to 90° C. for 21 hours. The cooled reaction mixture was transferred to a separatory funnel using several toluene rinses to get complete transfer. The toluene solution was washed with 3-ml. portions of: (a) saturated NaCl solution; (b) 1*N* KOH solution (two washings); (c) repeat (a); (d), 3*N* H_2SO_4 (three washings); (e) repeat (a); and (f) water at pH 8 (three washings). The toluene solution was dried with anhydrous sodium sulfate and evaporated at reduced pressure. The liquid product was heated at 70° to 90° C. for 10 minutes at a pressure of 1 torr to complete the removal of lower volatility components. The Diazinon-^{35}S weighed 640 mg. and had a specific activity of 1.15 μc. per mg. The infrared spectrum was identical to that of the best sample provided to us by the Geigy Chemical Co.

Radioactive Diazinon Sprays. The sprays used in the application of labeled Diazinon to crops consisted of a water suspension (0.1 to 0.2%) of a mixture of Celite–sodium lauryl sulfate–Diazinon (4:1:5). The water was added to the emulsifiable concentrate just prior to application to the crop.

Spraying of Crops with Radioactive Diazinon. Spinach. Viroflay variety spinach which had been planted as seedlings on Feb. 16, 1965, was sprayed on April 21 with Diazinon-^{35}S. The crops were grown near Planada, Calif., in the San Joaquin Valley some 140 miles southeast of San Francisco. Four plants were treated with the equivalent of 16 mg. of Diazinon having a specific activity of 2.81 μc. per mg. Each of a set of four plants was sprayed with commercial Diazinon and with *O,O*-diethyl thiophosphorochloridate which was present as a contaminant in the Diazinon-^{35}S preparation. Two each of the three types of sprayed plants were harvested after 5 and 12 days.

Snap Beans. Eight bean plants (Tendercrop bush beans) were each sprayed with 13.9 mg. of Diazinon-^{35}S having a specific activity of 1.15 μc. per mg. on July 21, 1965. The beans had been planted from seed on May 17. Two plants were harvested within 2 hours after spraying. The remaining six sprayed plants were harvested 5 days after spraying. All the harvested beans (in polyethylene bags) were frozen rapidly between blocks of solid carbon dioxide and transported back to a freezer in Berkeley to be stored prior to analysis.

Tomatoes. Pearson variety tomatoes were grown from seedlings planted on May 17, 1965. Eight plants were sprayed with 5 ml. of a freshly prepared emulsion of Diazinon-^{35}S (366 mg. per 100 ml.). Two plants were sprayed with 10 ml. and two plants with 20 ml. of the emulsion. Two of the minimum plants were picked of fruit within 2 hours after spraying on August 10. The remaining fruit of the harvested plants were picked on August 13. An unusual summer rain (0.08 inch) fell on the tomato plants on August 11 and 12.

Small Chamber Spraying of Tomato Plants with Diazinon-^{14}C. A spray emulsion containing 39.3 mg. of Diazinon-^{14}C (2.40 μc.) was applied to three 100-day old Pearson tomato plants which did not have fruit. The sprayed plants were put in a plastic glove box. Air was aspirated in one tubulation of the box and out through a gas washing bottle containing 250 ml. of 4% $Ba(OH)_2$–8 H_2O solution. The reagent was replaced periodically and the $BaCO_3$ which had precipitated was collected by filtration, washed with a large volume of water, and dried at reduced pressure. The $BaCO_3$ was counted by gel suspension counting using 0.90 gram of Cab-O-Sil, 100 to 200 mg. of $BaCO_3$, and 20 ml. of scintillator solution. The total net counts found in the $BaCO_3$ collected at different time intervals during the first 100 hours after spraying are shown in Figure 6.

Crude Diazinon-^{14}C from a labeling using 1 mc. of ethyl acetoacetate-3-^{14}C in a 0.002-mole scale experiment was purified by preparative gas chromatography. The conditions used were: 10-foot × 1/4-inch 20% diethylene glycol succinate on Chromosorb P at 200° C.; helium flow rate, 83 ml. per minute; filament current, 190 ma. The first injection of 50 μl. gave a Diazinon peak at 22 minutes which was collected. This material weighed 39.7 mg. and had a specific activity of 1.57 μc. per mg. Paper and thin-layer chromatography of the sample did not reveal any contaminating by-products. Subsequent collection of the Diazinon peak from additional injections gave impure samples which were contaminated by long retention time breakdown products.

Three VF-36 variety tomato plants bearing green ripe fruit were sprayed with an emulsion containing 38.1 mg. of the purified Diazinon-^{14}C. The plants were harvested after 5 days to provide 885 grams of fruit and 899 grams of leaves. The fractions were frozen and stored until extraction or steam distillation.

Methods of Extraction and Cleanup. Spinach plants were extracted according to the procedure of Laws and Webley (*17*). This was changed for tomatoes and snap beans since it was felt that the separation into petroleum ether–soluble and water-soluble fractions was not necessary. After the plant material had been extracted with dichloromethane, the extract was filtered through anhydrous sodium sulfate, and adjusted to the desired volume. An aliquot representing approximately 50 grams of raw product was thoroughly mixed with 2 to 3 grams of Darco G-60 charcoal in a fine sintered glass funnel. This mixture was then rinsed 5 times with 15-ml. portions of acetone, each time filtering the acetone into a 125-ml. suction flask. The acetone fraction (after evaporation) was generally sufficiently clean for its radioactivity to be measured without a significant amount of quenching. For either paper or thin-layer chromatography, it was necessary to do further cleanup if the sample represented more than 5 grams of plant material.

Although several cleanup procedures have been described (*4*, *24*), these were not entirely satisfactory for present purposes. The wood-cellulose Darco G-60 charcoal column cleanup (*23*) was the most effective for the isolation of Diazinon, SS-TEPP, and oxo-Diazinon from spinach extract. However, this procedure did not give satisfactory cleanup for optimum separations by paper or thin-layer chromatography. A new cleanup method was developed (*13*) which was similar to that recently described by Abbott and Thomson (*1*).

Paper and Thin-Layer Chromatography. The reversed phase system

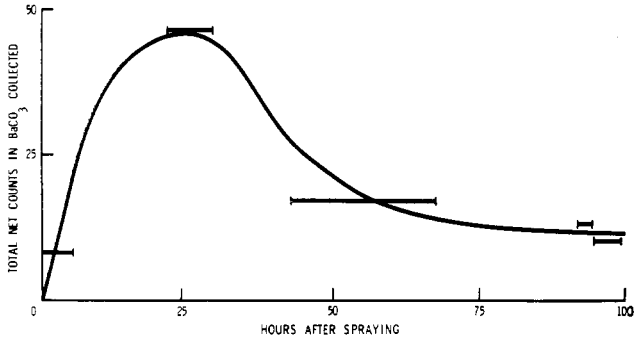

Figure 6. Rate of production of $^{14}CO_2$ from Diazinon-4-^{14}C sprayed tomato plants

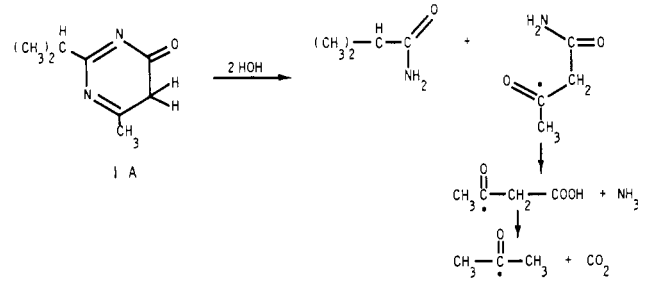

Figure 7. Probable path of metabolism of 2-isopropyl-4-methylpyrimidin-6-ol by tomato plants

for paper chromatography described by Getz (*12*) was used in the initial part of this project. The coating systems used were: (a) 10% mineral oil and 35% acetone in water and, (b) 10% Epon resin 828 and 35% acetone in water. Neither of these systems gave complete separations of all the compounds of interest in this project. A method combining the two systems in a single sheet of paper was devised (*5*). A solvent system described by Kovacs (*16*) for use in thin-layer chromatography was adapted to paper chromatography and excellent separations were obtained. With this system, the sulfur-containing organophosphate compounds can be detected readily using the spray reagent described by Kovacs; this is not true for the systems described above.

Reverse phase cellulose thin-layer chromatography was used for the detection of the cholinesterase inhibiting organophosphates. The stationary phases and solvent systems used were the same as those used for paper chromatography. The cellulose plates were prepared by blending 30 grams of cellulose (Cellex-N-1, Bio-Rad Laboratories, Richmond, Calif.) with 150 ml. of distilled water at high speed in an Omni-Mixer for 2 to 3 minutes. The glass plates were coated in a conventional manner and allowed to dry overnight; no activation was necessary. The results were approximately the same as for paper chromatography. Development time was somewhat shorter and the spots were better defined, resulting in some increase in sensitivity over paper chromatography.

For the detection of sulfur-containing organophosphates, the absorbent used was Alumina G (E. Merck A.G.). Plates 250 microns thick were prepared by slurrying 30 grams of adsorbent with about 60 ml. of distilled water. After allowing the plates to air dry, they were stored in a desiccator until ready for use.

To separate Diazinon from SS-TEPP, 10% chloroform in petroleum ether (b.p. 30° to 60° C.) was used. The 2,6-dibromo - *N* - chloro - *p* - benzoquinoneimine detection system (*2*) was used except that 1*N* hydrochloric acid in water was sprayed on the plates after development (rather than incorporated in the adsorbent). Compounds containing a P—S linkage show up as a bright red against a white background with a sensitivity of approximately 0.1 μg.

Separations of 2,4-dinitrophenylhydrazones by thin-layer chromatography have been described (*3*, *6*, *7*). None of the solvent-adsorbent combinations described gave a satisfactory separation of the 14 reference compounds examined. A solvent system (heptane-acetone-acetonitrile, 100:35:15 v./v./v.) was found which gave somewhat better separation of several of the compounds tested. When spotted on a 250-micron thick silica gel G plate, the 2,4-dinitrophenylhydrazones from the steam distillate of tomato leaves were separated into three distinct spots with this solvent system.

Isolation of Volatile Carbonyl and Tricarboxylic Acid Fractions from Tomato Fruit and Leaves Sprayed with Diazinon-^{14}C in Chamber. Portions of 190 grams of unsprayed control tomato leaves and two 190-gram portions of leaves from Diazinon-^{14}C sprayed plants were blended with 300 ml. of water, and the slurry was steam distilled until 250 ml. of distillate had been collected from each sample. The pH of the distillates was in the range of 5.8 to 6.1. Twenty-five milliliters of a solution of 1 gram of 2,4-dinitrophenylhydrazine hydrochloride in 100 ml. of 12*N* hydrochloric acid was added to each portion of distillate. After standing at 4° C. for 16 hours, the precipitated derivatives were collected, washed, and dried. The weights of 2,4-dinitrophenylhydrazones obtained from each distillate are recorded in Table II.

The volatile carbonyl compounds were regenerated by the method of flash exchange and analyzed by gas chromatography (*22*). No peaks were found which would correspond to any C_1 to C_6 aldehyde or ketone. Two changes from uniformity of baseline of the recorder trace suggested the presence of higher molecular weight aldehydes or ketones.

Tomato fruit pulp, 450 grams, was macerated with an equal weight of water. After filtering through Whatman No. 12 paper, the volume of the filtrate was adjusted so that 2 ml. represented 1 gram of fruit. The fruit acids were separated by allowing 30 ml. of the filtrate to pass through a cation exchange resin (Dowex 50-W, 20 to 50 mesh, activated with 10 ml. of 2*N* hydrochloric acid followed by 50 to 75 ml. of water). The effluent went into an anion exchange resin (Duolite A-4 activated with 10 ml. of 1*N* sodium hydroxide solution, followed by 50 to 75 ml. of water). The ion exchange tubes were approximately

Table II. Weights of Mixed 2,4-Dinitrophenylhydrazones from Volatile Carbonyl Compounds in Tomato Leaf Steam Distillate

Tomato Sample	*Weight of 2,4-DNPh, Mg. per 190 Grams of Leaf*
Control	25.1
Diazinon-^{14}C Sprayed	25.5
Replicate	25.7

6 inches long and 8 mm. o.d. with a 25-ml. solvent reservoir (Wilkens Anderson Co., Chicago, Ill.). After placing the sample in the cation exchange tube, it was washed with approximately 50 ml. of water to remove nonionic substances. The acids were then eluted with two 2-ml. portions of 2*N* ammonium hydroxide, followed by approximately 20 ml. of water. The eluate was placed in a liquid scintillation counting vial and concentrated to a volume of about 1 ml.

Liquid Scintillation Counting of Radioactive Samples. Sulfur-35 or ^{14}C-labeled compounds soluble in organic solvents were counted in a toluene scintillator system. The sample was transferred in solution to a standard 20-ml. capacity counting vial and the solvent removed by evaporation in a stream of air or nitrogen. Ten milliliters of toluene and 10 ml. of scintillator solution—prepared from 75 mg. of 2,5-diphenyloxazole (PPO) and 1.5 mg. of 1,4 - bis - 2 - (5 - phenyloxazolyl)benzene (POPOP) in 10 ml. of toluene—was added to the residue. The prepared vial was cooled to −4° C., placed in the counting chamber of a Packard Tricarb Liquid Scintillation Spectrometer Model No. 3203, and the radioactivity measured. Water-soluble radioactive materials were counted by placing 1 ml. of the material in a counting vial with 10 ml. of 2-ethoxyethanol and 10 ml. of the scintillation solution.

Autoradiography of Paper and Thin-Layer Separations. Alumina and silica gel thin-layer plates were sprayed with Neatan New (E. Merck A.G., Darmstadt, Germany) to prevent damage during handling. Cellulose thin-layer and paper chromatograms required no special treatment. The chromatograms (in a dark room, using Safelight Wratten Series 6 B) were placed on a sheet of

Kodak Industrial X-ray Film, Type KK, sealed in a light-tight envelope and placed in a plywood-foam rubber press to ensure good contact. Exposure of the film was carried out in a desiccated box at 4° C. for 1 to 2 weeks, depending on the amount of radioactivity present. The exposed films were processed in Kodak D-19 developer for 4 minutes, followed by a stop bath. The film was then fixed for 10 to 15 minutes and rinsed in running tap water for at least 1 hour.

Results and Discussion

Analysis of Sprayed Spinach. Spinach samples (200 grams) were extracted (*11*) and cleaned by the preparative thin-layer technique. Extracts representing 1- to 10-gram portions of the spinach were spotted on cellulose thin-layer plates and the separated compounds detected by cholinesterase inhibition. Extracts representing 10 grams of spinach were spotted on alumina thin-layer plates and the thiophosphate compounds detected with the Braithwaite spray (*2*). The radioactivity in the Diazinon spots was measured and the Diazinon content calculated after ^{35}S decay corrections had been made. The residual content of Diazinon of spinach is tabulated in Table III.

Cellulose thin-layer chromatograms of extracts of 5-day harvest spinach showed evidence of increased content of oxo-Diazinon. The evidence of oxo-Diazinon as a metabolite of Diazinon on field-sprayed spinach is based on chromatographic data. The levels of this material as a residue on spinach is so small (estimated at 0.005 to 0.01 p.p.m.) that isolation and identification by infrared or other spectroscopic techniques was not possible. The levels of plant extractives, even with rigorously cleaned samples, was so high compared to oxo-Diazinon concentrations that useful spectra could not be obtained. The chromatographic data from three different solvent systems gave strong evidence for oxo-Diazinon on spinach harvested 5 days after spraying. The R_f values obtained (relative to S-TEPP as 1.00) are given in Table IV.

There was no evidence for any substantial amount of the sulfur containing transformation products shown in Figure 3 or for 2-isopropyl-4-methylpyrimidin-6-ol on spinach sprayed at recommended dosage.

Analysis of Sprayed Snap Beans. Snap beans were blended with chloromethane (1 gram of beans to 4 grams of solvent) and filtered through anhydrous sodium sulfate. A volume of extract corresponding to 30 grams of beans was applied to a charcoal–cellulose–silica gel thin layer and developed. The adsorbent area which contained the purified Diazinon was scraped into a fine porosity sintered glass funnel and washed with 50 ml. of acetone. The evaporated extract was transferred to a counting vial and the radioactivity determined.

Table III. Residual Diazinon on Spinach

Sample	Net D.P.M. per Gram Spinach	Diazinon Content, P.P.M.
After application (estimated)	...	13
5-day harvest	178	0.25
12-day harvest	24	0.03

Table IV. R_f Values for Phosphate and Pyrophosphate Esters

Compound	R_f in System 1	2	3
TEPP	1.8	4.5	0.14
S-TEPP	1.0	1.0	1.0
Oxo-Diazinon	1.2	2.6	1.5

Systems:

(1) S & S 3MM paper coated with 10% mineral oil and 10% Epon resin; developed with 35% acetonitrile in water.
(2) S & S 3MM paper coated with 10% Epon resin; developed with 35% acetonitrile in water.
(3) Prewashed cellulose thin-layer plate coated with 30% dimethylformamide; developed with methylcyclohexane saturated with dimethylformamide.

The results are tabulated in Table V. Paper chromatography of extracts of snap beans harvested 7 days after spraying showed an increase in the amount of a cholinesterase inhibiting compound with an R_f corresponding to that of oxo-Diazinon.

Tomatoes Sprayed with Diazinon-^{35}S. The tomatoes, separated into peel and pulp-seed samples, were extracted twice with dichloromethane. The combined extracts of each sample were filtered through glass wool into a separatory funnel. Water was added to aid in separating the phases and the dichloromethane layer filtered through anhydrous sodium sulfate. The final volume of the extract was measured and aliquot portions equal to 25 grams of peel and 40 grams of pulp-seed were concentrated. These samples were cleaned by preparative thin-layer chromatography. The activity in the tomato sample is tabulated in Table VI.

Tomatoes treated with larger amounts of Diazinon-^{35}S and harvested after 3 days were extracted and cleaned as described above. Experiments are in progress to determine the presence of ^{35}S-labeled breakdown products.

Tomato Plants Sprayed with Diazinon-^{14}C in Small Chambers. The development of radioactive carbon dioxide in the atmosphere around tomato plants sprayed with pyrimidine ring–labeled Diazinon-^{14}C is shown graphically in Figure 6. The formation of radioactive barium carbonate is evidence that the pyrimidine ring of Diazinon is degraded by growing tomato plants.

A hypothetical pathway for pyrimidine ring degradation is shown in Figure 7.

Table V. Residual Content of Diazinon on Snap Beans

Sample	Net D.P.M. per 30 Grams of Beans	Diazinon Content, P.P.M.
Unsprayed control	0	0.00
2-hour harvest	25,153	0.38
7-day harvest	2,566	0.04

Table VI. Residual Content of Diazinon on Tomatoes

Sample	Net D.P.M. per Gram Tomatoes	Diazinon Content, P.P.M.
0 days peel	50.8	0.04
0 days pulp-seed	8.2	0.007
3 days peel	8.8	0.003
3 days pulp-seed	0.0	<0.001

The keto tautomer of 2-isopropyl-4-methylpyrimidin-6-ol could hydrolyze to form acetoacetic acid amide and isobutyrylamide. If this mechanism of hydrolysis were correct, radioactive acetone (from decarboxylated acetoacetic acid) or labeled tricarboxylic acid cycle compounds (from the entry of labeled acetoacetic acid into the cycle) should be formed. Tomato leaves from the Diazinon-^{14}C sprayed plants were examined for the presence of radioactive acetone, and the tricarboxylic acid cycle compounds from tomatoes were assayed for radioactivity. The steam distillate from tomato leaves of both sprayed and unsprayed plants did not contain acetone. The method used would reveal acetone down to a level of 0.1 p.p.m. If radioactive acetoacetic acid or its amide had been present in the tomato leaves, the steam distillation of the acidic mixture would have produced radioactive acetone. The 2,4-dinitrophenylhydrazones of tomato leaf steam distillate were examined by thin-layer chromatography. Of the three spots obtained, only one showed an R_f comparable to any of the 14 reference standards used; this was isobutyraldehyde 2,4-dinitrophenylhydrazone. However, the analysis of the mixture by flash exchange gas chromatography had shown that isobutyraldehyde was not present in tomato leaf steam distillate.

The results of counting the tricarboxylic acid fraction of tomatoes are shown in Table VII. Since the activity found in the acids from tomatoes is the same as background, none of the Diazinon-^{14}C radioactivity was transferred to the acids of the Krebs cycle.

The thin-layer chromatography of compounds in the chloroform extract of the aqueous filtrate from tomatoes gave a single radioactive spot corresponding in R_f to that of 2-isopropyl-4-methylpyrimidin-6-ol. The disintegrations per minute for the compound isolated from 41 grams of tomato pulp was 9017. In a confirming experiment, 2-isopropyl-4-methylpyrimidin-6-ol recovered after

Table VII. Radioactivity of Tricarboxylic Acid Cycle Compounds Isolated from Diazinon-^{14}C Sprayed Tomatoes in Chamber

Sample Number	Weight of Tomato Pulp Used, G.	Counts per Minute
1	10	26
2	10	22
3	15	27

Table VIII. Radioactivity and 2-Isopropyl-4-methylpyrimidin-6-ol Content of Tomatoes Sprayed with Diazinon-^{14}C in Chamber

Sample Number	Purified Compound Recovered, %	D.P.M. per 41 Grams of Tomatoes	Residue Content, P.P.M.
1	72	9080	0.03
2	52	9216	0.03

adding the unlabeled compound to tomato pulp filtrate from sprayed plants had the radioactivity shown in Table VIII. The fact that the 2-isopropyl-4-methylpyrimidin-6-ol isolated (and purified to constant specific activity) had substantial radioactivity is conclusive proof that this compound is a metabolite of Diazinon in tomato plants.

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HERBICIDE METABOLISM

Dealkylation of Atrazine in Mature Pea Plants

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A major metabolite of atrazine was detected in the shoots of mature pea plants. Chromatographic and spectral methods of analysis were used to identify the metabolite as 2-chloro-4-amino-6-isopropylamino-*s*-triazine. Hydroxyatrazine, the major metabolite reported to occur in other higher plants, was not detected. Results indicate that an alternate pathway other than the degradation of 2-chlorotriazine to the 2-hydroxy analog exists in higher plants.

ATRAZINE (2-chloro-4-ethylamino-6-isopropylamino - *s* - triazine) and simazine [2-chloro-4,6-bis(ethylamino)-*s*-triazine] are initially degraded to the 2-hydroxy derivative in several species of higher plants (*1, 2, 6, 7, 9, 12*). The tolerance of corn, *Zea mays*, L., to these herbicides is believed to be due largely to its ability to degrade simazine (*1, 4, 7, 12*) and atrazine (*10*) to hydroxysimazine [2 - hydroxy - 4,6 - bis(ethylamino)-*s*-triazine] and hydroxyatrazine (2 - hydroxy - 4 - ethylamino - 6 - isopropylamino-*s*-triazine). The detection of large amounts of the hydroxy derivative, in both in vivo and in vitro simazine metabolism studies (*1, 7, 12*), established hydroxysimazine as the major degradation product. This reaction may occur with all chlorotriazines (*9*).

More recently, it was reported that in the soil fungus, *Aspergillus fumigatus*, Fres., the major product of simazine degradation was 2-chloro-4-amino-6-ethylamino-*s*-triazine (*8*). Experiments with chain-labeled simazine-C^{14} have shown that the metabolism of the alkyl side chain does occur in higher plants (*3*). However, the results presented (*3*) suggested that side-chain metabolism occurred subsequent to an initial hydroxylation reaction at the 2-chloro position. Dealkylation of a chlorotriazine, as demonstrated with *A. fumigatus*, has not been reported to occur in higher plants.