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.

Acknowledgment

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HERBICIDE METABOLISM

Dealkylation of Atrazine in Mature Pea Plants

R. H. SHIMABUKURO, R. E. KADUNCE, and D. S. FREAR

Crop Research Division, Agricultural Research Service, U. S. Department of Agriculture, Metabolism and Radiation Research Laboratory, Fargo, N. D.

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.

In this investigation, a major metabolite of atrazine was detected in the shoots of mature pea plants, *Pisum sativum*, L., var. Little Marvel. This degradation product, which has not been demonstrated previously in higher plants, was not identical with hydroxyatrazine. The purpose of this investigation was to isolate and identify the metabolite. The significance of this metabolite to the present concepts of tolerance or resistance to atrazine by different species will be discussed.

Materials and Methods

Plant Material. Pea plants were grown to maturity in vermiculite under normal greenhouse conditions. Seven days after anthesis the roots were washed free of vermiculite and exposed for 48 hours to 150 ml. of aerated Hoagland's solution containing 0.180 μc. of uniformly ring-labeled atrazine-C^{14} (specific activity 7.8 μc. per mg.). Nonlabeled atrazine was added to make the final concentration a 5-p.p.m. solution of labeled and nonlabeled atrazine. Plants absorbed and translocated atrazine-C^{14} at room temperature in a ventilated laboratory hood with a fixed fluorescent-light bank giving a total illumination of 900-foot candles at the level of the shoot apex during a 12-hour photoperiod.

For the purpose of isolating larger amounts of the metabolite, mature plants (about 500 grams fresh weight) were similarly treated in the greenhouse with a 5 p.p.m. solution of nonradioactive atrazine.

Extraction and Purification Procedure. At the end of the 48-hour treatment period, the shoots were extracted by homogenizing the fresh material with 80 ml. of 95% methanol per 25 grams fresh weight. The homogenate was filtered, and the residue was reextracted twice by resuspending in 95% methanol. The mixture was boiled at 70° C. for 15 minutes and then filtered. Methanol was evaporated under vacuum at 25° C. from the combined filtrate, and the remaining aqueous portion was further concentrated to a small volume. All evaporation and concentration operations were conducted under the same conditions. In radioactive atrazine experiments, chlorophyll and other residues were removed from the concentrated aqueous portion by centrifugation at 13,300 G for 15 minutes at 0° C. After removal of the supernatant, the residue was extracted three times by resuspending it in water, followed by centrifugation. The aqueous supernatants were combined and concentrated to 10 ml., and assayed for C^{14} activity by liquid-scintillation counting (0.5-ml. aliquot of supernatant in 20 ml. scintillation liquid; 5.0 grams PPO and 0.5 gram of dimethyl POPOP per liter of 30% absolute ethanol in toluene). All samples were corrected for quenching, and counts were expressed as disintegrations per minute (d.p.m.) for direct comparisons. In nonradioactive atrazine experiments, the aqueous portion containing chlorophyll and other residues was filtered through glass wool and Whatman No. 4 filter paper before concentration.

The aqueous extract was washed five times with equal volumes of chloroform to remove atrazine and chloroform-soluble metabolites. The chloroform fraction was evaporated to dryness and redissolved in methanol. In plants treated with radioactive atrazine, the resulting chloroform-washed aqueous and methanol solutions from the above procedure were assayed for C^{14} activity by liquid-scintillation counting. The methanol solutions from radioactive and nonradioactive plant extracts were spotted on silica gel HF-coated plates, 250 microns in thickness, and the thin-layer chromatograms were developed in benzene–acetic acid (50:4). The silica gel was scraped off between R_f 0.37 and R_f 0.26, extracted with methanol, and filtered through a filter of 1.5-micron porosity. Portions of the concentrated radioactive and nonradioactive methanol filtrates derived from this purification procedure were mixed and used in determining the distribution of C^{14} activity by gas-liquid chromatographic separation. The nonradioactive filtrate was also used for the isolation of the metabolite by gas chromatography and identification by infrared spectrophotometry. All R_f values calculated from thin-layer chromatographic separation are based on a 15-cm. solvent migration.

Detection of labeled atrazine and its metabolite was by autoradiography of thin-layer plates on Kodak no-screen x-ray film. In nonlabeled samples, atrazine and the metabolite were detected by viewing the chromatogram under ultraviolet light. However, care must be exercised in using this method of detection, for other natural fluorescence quenching substances were present in the plant extract. Positive detection was achieved by flame ionization and electron affinity, gas-liquid chromatography [5% Carbowax 20M (*15*) on Chromosorb W, 60/80 mesh, acid washed; column temperature, 220° C.; injector temperature, 300° C.; carrier gas, nitrogen for ionization detector and helium for dual detector operation].

A 9 to 1 stream splitter and gas chromatographic fraction collector were used to collect C^{14}-labeled effluents for liquid scintillation counting and nonlabeled effluents for infrared analysis. Labeled effluents were collected on silicone-coated terphenyl crystals, which were subsequently added to 15 ml. of scintillation counting liquid (5.0 grams of PPO and 0.3 gram of dimethyl POPOP per liter of toluene) and assayed for C^{14} activity. For infrared analysis, the effluents were collected on KBr, pressed into 1.5-mm. diameter pellets and analyzed on a Perkin-Elmer 337 infrared spectrophotometer equipped with a beam condenser.

The aqueous fraction, containing water-soluble radioactive substances, was further purified by ion exchange on a 1.5-cm., jacketed, tap water–cooled column of AG 50W-X8 (H+) resin followed by elution with 6*N* NH_4OH. Ammonia was removed under vacuum at 25° C. before the eluate was spotted on a thin-layer plate and developed in *n*-butanol–acetic acid–water (120:30:50).

Results

In two experiments with radioactive atrazine, between 88 and 90% of the total C^{14} activity extracted from the shoot was chloroform-soluble. Separation of the chloroform-soluble components by thin-layer chromatography indicated that radioactivity was predominantly found in two spots (Figure 1) with R_f values of 0.37 and 0.26, when developed in solvent B (Table I).

Although very little activity was present in the water-soluble fraction, separation of the radioactive components

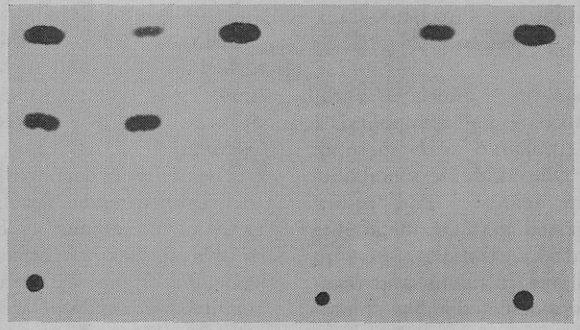

Figure 1. Autoradiogram of thin-layer chromatogram developed in benzene–acetic acid (50:4)

Left to right: ring-labeled atrazine and hydroxyatrazine plus plant extract (treated), plant extract (treated), ring-labeled atrazine, ring-labeled hydroxyatrazine, plant extract (control) (ring-labeled atrazine added to plant material just prior to homogenization in methanol), ring-labeled atrazine and hydroxyatrazine plus plant extract (control)

indicated the presence of two spots with higher R_f values (0.75, 0.63) and one with lower R_f (0.44) than hydroxyatrazine (0.57). However, no detectable amount of hydroxyatrazine was present.

The degradation of simazine to hydroxysimazine is known to occur nonenzymatically (*1*, *7*, *13*) and is catalyzed by the cyclic hydroxamate, 2,4-dihydroxy - 3 - keto - 7 - methoxy - 1,4-benzoxazine (benzoxazinone). Plants lacking in benzoxazinone failed to produce hydroxysimazine when treated with simazine (*6*). Similar results can be expected from atrazine treatment. An attempt to identify benzoxazinone from the shoots of mature pea plants (53 grams fresh weight) was made by a method used by Hamilton (*6*). The extract was spotted on silica gel-HF thin-layer plates (300 microns in thickness) and developed in solvent A (Table I). Benzoxazinone (R_f 0.54) and its glucoside (R_f 0.14) extracted from corn-root tips plus authentic benzoxazinone were used as references for detection by spraying thin-layer chromatograms with 2% $FeCl_3$ in acetone (*14*). No detectable amounts of the catalyst were found in the shoots of mature pea plants. The lack of any appreciable amount of benzoxazinone may account for the absence of hydroxyatrazine in the tissue.

The slightly more polar nature of the major metabolite as compared with atrazine, and the absence of hydroxyatrazine, suggested that the unknown compound may be a dealkylated product similar to that reported in the soil fungus treated with simazine (*8*). However, in contrast to simazine, the dealkylation of atrazine can result in two possible compounds: 2-chloro-4-amino - 6 - isopropylamino - *s* - triazine (I) and 2-chloro-4-amino-6-ethylamino-*s*-triazine (II). To identify the metabolite, authentic atrazine and compound II were obtained from commercial sources, and compound I was prepared by the method of Thurston *et al.* for 2 - chloro - 4 - amino - 6 - alkylamino-*s*-triazines (*16*) [2 - chloro - 4 - amino-6 - isopropylamino - *s* - triazine, m.p. 135–137° C.; reported m.p. (*5*) 134.5–136.5° C.].

The migration on a thin-layer chromatogram of atrazine and compounds I and II were compared with those of radioactive atrazine and the metabolite extracted from plants. The results (Table I) suggested that the metabolite was compound I. Compounds with R_f values similar to atrazine and compound I were also detected in the chloroform-soluble fraction of nonradioactive, atrazine-treated plants. Gas chromatographic separation of the nonradioactive, chloroform-soluble fraction gave two peaks, atrazine (relative t_R 1.0) and a metabolite (relative t_R 2.0), which agreed with the relative retention times of authentic atrazine and compound I (Table I). Both peaks were also found to be positive to the electron-affinity detector, strongly indicating the presence of chlorine in the metabolite. Compound II was not detectable by thin-layer or gas-liquid chromatography.

The relative amounts of metabolite and unchanged atrazine were determined by gas-liquid cochromatography of the radioactive and nonradioactive chloroform-soluble fractions. Since the quantity of labeled atrazine and metabolite was not sufficient to give a positive response with the flame ionization detector, the radioactive sample was diluted with nonradioactive sample prior to separation and collection of C^{14}-labeled compounds. Collection and assay of the effluent at the peaks and at the zones between the peaks indicated that C^{14} activity was predominantly present in the peaks at relative retention times of 1.0 and 2.0 (Table II). The ratio of metabolite to atrazine, calculated from the results in Table II, indicated that the metabolite concentration was 1.2 to 2.0 times greater than unchanged atrazine. Compound I can occur as an impurity in samples of atrazine, but it was reported to be present in small amounts (*15*). In shoots of peas, the detection of high concentrations of the metabolite ruled out the possibility of artifacts from impurities. Such impurities were negligible upon thin-layer chromatography of the authentic atrazine-C^{14} used in the experiments (Figure 1).

Identification was further confirmed by comparison of infrared spectra of compound I and the metabolite. The spectra were superimposable. The spectrum of the metabolite isolated from the shoots of mature pea plants is given in Figure 2. The results confirmed the identity of the metabolite as 2-chloro-4-amino-6-isopropylamino-*s*-triazine.

Table I. R_f Values and Relative Retention Times of Substituted *s*-Triazines and Metabolite

Compound	Solvent Systems A[a]	Solvent Systems B[b]	Relative t_R
Atrazine	0.87	0.37	1.0
I[c]	0.80	0.26	2.0
II[d]	0.80	0.22	2.7
Metabolite	0.80	0.26	2.0
OH-atrazine	0.27	0.00	0.0[e]

[a] Benzene–acetic acid–water (50:50:3).
[b] Benzene–acetic acid (50:4).
[c] (2-Chloro-4-amino-6-isopropylamino-*s*-triazine).
[d] (2-Chloro-4-amino-6-ethylamino-*s*-triazine).
[e] Hydroxyatrazine did not chromatograph under the conditions used.

Table II. Distribution of C^{14} Activity Separated by Gas-Liquid Chromatography

Zone	Per Cent of Total C^{14} Activity Chromatographed[a] Experiment 1	Experiment 2
A[b]	1.2	1.0
B[c]	31.4	40.8
C[d]	1.5	4.7
D[e]	62.2	50.1
E[f]	3.7	3.4

[a] The values are the average of two separations based on 15-microliter injections of concentrated chloroform-soluble fractions.
[b] Solvent front and interval before atrazine peak.
[c] Atrazine peak (relative retention time 1.0).
[d] Interval between B and metabolite peak.
[e] Metabolite peak (relative retention time 2.0).
[f] Interval between D and relative retention time 3.0.

Discussion

A major product in the degradation reaction of atrazine in the shoots of mature pea plants was identified as the dealkylated compound, 2-chloro-4-amino-6-isopropylamino-*s*-triazine. Approximately 10% of the atrazine absorbed by roots and translocated to the shoots was water-soluble, but no hydroxyatrazine was detectable in this fraction.

The ability of corn to degrade atrazine to hydroxyatrazine has been attributed as the factor responsible for the tolerance of this species to atrazine. However, some doubt has been raised as to the relationship between degradation and tolerance in several species (*6*). Sorghum, which has considerable resistance, was found to be lacking in benzoxazinone and no conversion of the chlorotriazine to the hydroxy derivative occurred, although other susceptible species, containing benzoxazinone, were capable of effecting such a conversion (*6*). A situation similar to that of sorghum occurred in peas, except that the mature pea plant was moderately susceptible to atrazine. These plants were severely injured or killed within 12 days in a 1-p.p.m. culture solution of atrazine.

A positive relationship between susceptibility and the amount of atrazine absorbed was not present in several species of varying tolerances, but a definite relationship between susceptibility and the amount of unchanged atrazine present in plants was reported to exist (*11*). Such results suggest that susceptibility of a given species to atrazine is primarily related to its inability to metabolize or alter the original molecule. Although it has been postulated that the first step in the metabolism of atrazine involves hydroxylation at the 2-position (*1*, *7*, *12*), this investigation revealed that another initial reaction in the metabolism of the herbicide is present in higher plants. This indicated that at least two pathways are involved in the initial degradation of atrazine. The moderately susceptible pea plant was apparently incapable of converting atrazine to hydroxyatrazine. But it had an active system, capable of metabolizing or

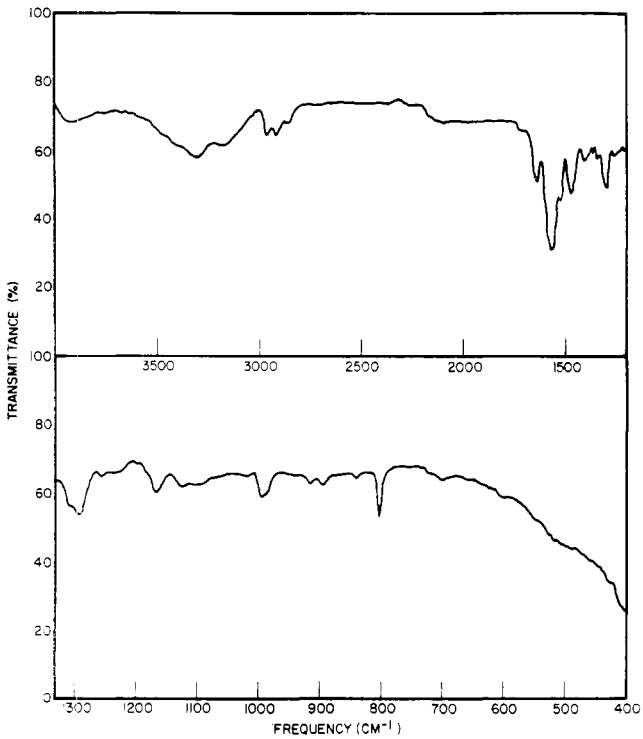

Figure 2. Infrared spectrum of 2-chloro-4-amino-6-isopropylamino-s-triazine isolated from shoots of mature pea plants

The compound was collected on KBr as the effluent from the gas chromatographic column and pressed into 1.5-mm. diameter pellets

altering the original atrazine molecule that resulted in the accumulation of 1.2 to 2.0 times as much dealkylated product as atrazine within 48 hours. Therefore, susceptibility of the pea plant to atrazine was not due to its inability to metabolize atrazine. If this is true, the amount of unchanged atrazine found in some species may not necessarily be correlated with its tolerance. Although some herbicidal activity has been attributed to 2-chloro-4-amino-6-isopropylamino-*s*-triazine (*4*), no tolerance study of peas to this compound has been reported. Apparently, an alternate pathway other than the degradation of 2-chlorotriazine to the 2-hydroxy analog exists in some higher plants. The importance of this pathway with regard to the resistance or susceptibility of a species will require further investigation.

Results indicate that in mature pea plants the principal degradation reaction of atrazine is the dealkylation of the ethyl group at the 4-position of the triazine ring. The second possible product, 2-chloro-4-amino-6-ethylamino-*s*-triazine, was not detected. Subsequent metabolism of the dealkylated product may follow closely the proposed pathway for simazine degradation in the soil fungus *A. fumigatus* (*8*).

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RESIDUES IN SOYBEANS

Insecticide Residues in Soybeans Grown in Soil Containing Various Concentrations of Aldrin, Dieldrin, Heptachlor, and Heptachlor Epoxide

W. N. BRUCE and G. C. DECKER

Illinois Natural History Survey, Urbana, Ill.

SEVERAL investigations have reported on the translocation of aldrin, dieldrin, heptachlor, and heptachlor epoxide in crops grown on treated soil. Lichtenstein (*5–8*), working with vegetable crops, found the highest residues in root crops such as carrots and radishes. Peanuts grown in soil treated with aldrin and heptachlor contained significant amounts of dieldrin, heptachlor, and heptachlor epoxide, as reported by Beck (*1*) and Bruce (*2*). Soybeans grown on heptachlor-treated soil contained heptachlor and heptachlor epoxide according to Eden and Arthur (*4*). Bruce (*2*) established a direct relationship between oil content of seeds and residue content of crops grown on aldrin- and heptachlor-treated soil.

Because of the economic importance of soybeans in Illinois and the large