Metabolism of 2-Chloro-4-Ethylamino-6-Isopropylamino-*s*-Triazine (Atrazine) in Excised Sorghum Leaf Sections

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S - (4 - Ethylamino - 6 - isopropylamino - 2 - *s*-triazino)glutathione and γ-L-glutamyl-*S*-(4-ethylamino - 6 - isopropylamino - 2 - *s* - triazino) - L-cysteine were isolated from atrazine treated sorghum leaf sections by ion-exchange chromatography and identified by a combination of spectroscopic, chemical, and chromatographic procedures. These compounds represent a new class of herbicide metabolites in plants and demonstrate the existence of a new pathway for the metabolism of atrazine.

The metabolism and detoxication of atrazine in higher plants has been reported to occur *via* 2-hydroxylation and *N*-dealkylation pathways (Shimabukuro, 1967). Both pathways are found in corn (*Zea mays* L.) (Shimabukuro, 1967). However, resistance of corn to atrazine and 2-chloro-4,6-bis(ethylamino)-*s*-triazine (simazine) was attributed primarily to the ability of corn to convert the herbicides to derivatives hydroxylated in the 2-position of the *s*-triazine ring (hydroxyatrazine and hydroxysimazine) (Castelfranco *et al.*, 1961; Gysin and Knüsli, 1960; Hamilton and Moreland, 1962; and Roth, 1957). In resistant sorghum, only the *N*-dealkylation pathway was thought to operate and 2-chloro-4-amino-6-isopropylamino-*s*-triazine (compound I) and 2-chloro-4-amino-6-ethylamino-*s*-triazine (compound II) were identified as metabolic products (Shimabukuro, 1967; Shimabukuro, 1968).

What appeared to be a single unidentified water-soluble metabolite was among the products produced in the shoots of both corn and sorghum which were root-treated for 48 hours with atrazine (Shimabukuro, 1967). This metabolite was the primary product from the shoots of root-treated sorghum and leaf-treated corn (Shimabukuro and Swanson, 1969b). Hydroxyatrazine formation was significant only when corn was treated with atrazine *via* the roots (Shimabukuro and Swanson, 1969b). Transient inhibition of photosynthesis occurred in sorghum leaf disks incubated in atrazine; rapid recovery of photosynthetic activity was correlated with formation of the unidentified metabolite (Shimabukuro and Swanson, 1969a).

This paper describes the isolation and identification of two closely related water-soluble metabolites from extracts of sorghum leaf sections incubated with atrazine. The results indicate the presence of a third major detoxication pathway of atrazine in higher plants.

EXPERIMENTAL

General Analytical Procedures. Quantitative measurements of ^{14}C were made on a liquid scintillation spectrometer. All samples were corrected for quenching. Solutions were concentrated by rotary vacuum evaporation at 37° C. unless otherwise stated. Column effluents were monitored qualitatively for ^{14}C with a continuous flow scintillation spectrometer. Radioactive components on thin-layer and paper chromatograms were detected by autoradiography or by scanning with a radiochromatogram scanner. Thin-layer chromatography plates were coated with a 250-μ layer of silica gel HF_{254} (Brinkmann Instruments, Inc.) and activated at 110° C. for 45 minutes. Thin-layer chromatograms were developed to 15 cm. Ascending paper chromatograms were developed to 20 cm. on Whatman No. 1 paper. Quantitative amino acid analyses were performed on a Technicon Amino Acid Analyzer with a 140-cm. column of Chromobeads Type B (Technicon Chromatography Corp., 1962). Ultraviolet spectra were obtained in 95% ethanol at 25° C. Mass spectral analyses were performed with a Varian M 66 spectrometer using a solid sample inlet.

Plant Treatment and Extraction. Sorghum (*Sorghum vulgare* Pers. var. North Dakota 104) was grown in the greenhouse in vermiculite subirrigated intermittently with half-strength Hoagland's solution. When the plants reached an age of 50–60 days, fully expanded leaves were excised from the plants and cut into 1-cm. × 1-cm. sections. The leaf sections were divided into two 420-gram lots. Each lot was incubated under fluorescent lights for 20 hours at 24° C. in 2500 ml. of an aqueous solution of $9.11 \times 10^{-5} M$ uniformly ^{14}C ring-labeled atrazine (specific activity 2.41×10^{-2} μCi. per μmole). After incubation, the treating solution was removed by decanting; each lot of leaf sections was homogenized with 2000 ml. of cold 80% methanol in a water-cooled blender for 3 minutes. The homogenate was filtered and the residue was reextracted with two 1250-ml. portions of 80% methanol. The combined filtrates from each lot were concentrated to near dryness, dissolved in 1250 ml. of water, and partitioned three times with 2800-ml. portions of chloroform. Aliquots from the incubating solution and from the chloroform and aqueous phases of the partitioned extract were chromatographed on a thin-layer plate with *n*-butanol–acetic acid–water (12:3:5) (solvent system A).

Ion-Exchange Chromatography. A 3 × 40 cm. water-jacketed column of 50- to 100-mesh AG 50W-X8 resin (hydrogen ion form) was prepared and washed with distilled water to an effluent pH of 4.5. The column was cooled to 10° C. and the aqueous extract in 500 ml. was applied to the column. The resin bed was eluted with 3600 ml. of 80% methanol, 825 ml. of water, and 2700 ml. of 2*N* ammonia. The ammonia eluate was examined by thin-layer chromatography with solvent system A.

A 2.4 × 100 cm. water-jacketed column (38° C.) of AG 50W-X2 resin (200–400 mesh) and pyridine–acetate buffers were prepared as described by Schroeder *et al.* (1962). The concentrated ammonia eluate was made to 10 ml. with 0.2*N* buffer, pH 2.0–2.5, and applied to the column which was buffered to pH 3.1 with 0.2*N* buffer. The column was eluted stepwise at 1 ml. per minute with 1230 ml. of 0.2*N* pH 3.1

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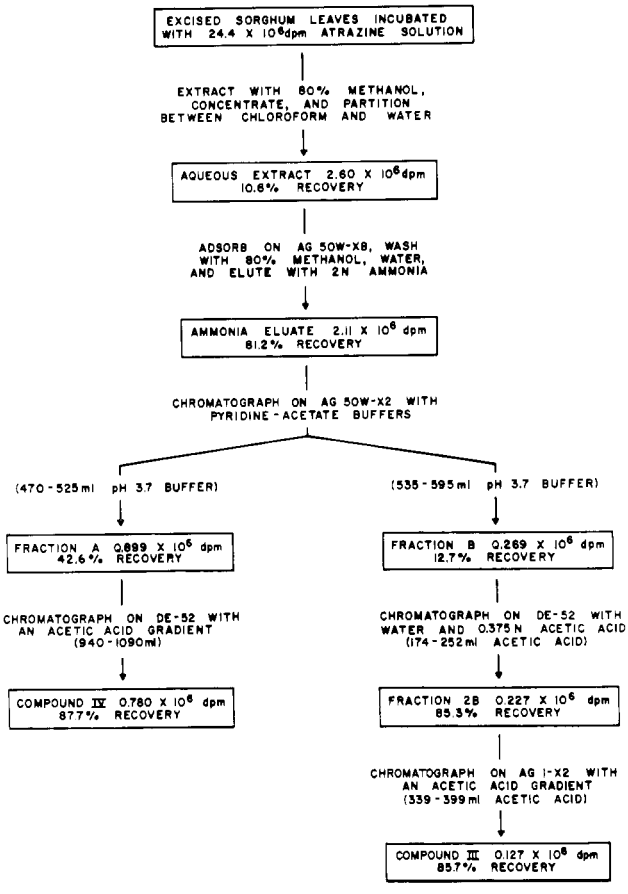

Figure 1. Fractionation and isolation of water-soluble metabolites from sorghum leaf sections treated with ^{14}C-atrazine

Per cent values indicate recovery of ^{14}C from each step and are not cumulative

buffer, 600 ml. of pH 3.7 buffer, and 800 ml. of 2.0*N* pH 5.0 buffer. The pH 3.7 buffer was prepared by mixing 20 parts of 0.2*N* pH 3.1 buffer with 80 parts of 2.0*N* pH 5.0 buffer. Radioactive fractions A and B were eluted with pH 3.7 buffer.

Fraction A was reduced to a volume of 4 ml. and applied to a 2.4 × 80 cm. water-jacketed column (38° C.) of DE-52 anion-exchange cellulose (Whatman) which had been equilibrated with 3*N* acetic acid and washed with water to an effluent pH of 4. The sample was eluted with an acetic acid gradient at a flow rate of 0.7 ml. per minute. The gradient device (Varigrad, Buchler Instruments) was filled with 350 ml. of water in chambers 1 and 2, 350 ml. of 0.50*N* acetic acid in chamber 3, and 350 ml. of 0.350*N* acetic acid in chamber 4. The product eluted from this column was designated compound IV, (Figure 1).

A 2 × 56 cm. column of DE-52 was prepared as above and fraction B was applied in a volume of 4 ml. The column was washed with water and eluted with 0.375*N* acetic acid at a flow rate of 0.38 ml. per minute. The radioactive fraction eluted from this column was designated 2B.

Fraction 2B was dissolved in several ml. of pH 8.5 buffer and applied to a 1 × 93 cm. water-jacketed column (38° C.) of AG 1-X2 resin (200–400 mesh) which had been equilibrated with pH 8.0 pyridine–α-picoline buffer. The column was eluted at a flow rate of 0.35 ml. per minute with an acetic acid gradient. The gradient device, resin preparation, and buffers were described by Schroeder *et al.* (1962). The constant volume mixing chamber of the gradient device was initially filled with 135 ml. of 0.1*N* acetic acid and the reservoir with 0.50*N* acetic acid. After 164 ml. of solvent had been developed through the column, the contents of the reservoir were replaced with 1.0*N* acetic acid. The radioactive product eluted from this column was designated compound III (Figure 1).

Table I. Thin-Layer and Paper Chromatography of III and IV

Chromatographic System	R_f III	R_f IV
A[a]	0.31	0.28
B[b]	0.75	0.64
C[c]	0.32	0.30

[a] TLC developed with solvent system A. [b] Paper chromatogram developed with 88% phenol–water–ammonia (100:20:0.3). [c] TLC developed three times with benzene–ethyl acetate–acetic acid–water (25:50:20:3) and one time with solvent system A; one-dimensional developments.

Table II. Hydrolysis and Amino Acid Analysis of III and IV

Amino acid	Residues per Molecule of Compound[a] III[b]	IV[c]
½-Cystine	0.902	0.913
Glutamic acid	1.03	1.04
Glycine	0.961	0.021

[a] Values are not corrected for hydrolytic decomposition. [b] 0.374 μmole of hydrolysate analyzed. [c] 0.518 μmole of hydrolysate analyzed.

Criteria of Purity. A 0.390-μmole sample of IV was chromatographed on the amino acid analyzer with norvaline as the internal standard. Both III and IV (0.0875 μmole) were examined by paper and thin-layer chromatography. The paper chromatogram was developed in 88% liquid phenol–water–ammonia (100:20:0.3) (solvent system B). One thin-layer plate was developed in solvent system A. A second one-dimensional thin-layer plate was developed three times in benzene–ethyl acetate–acetic acid–water (25:50:20:3) and one time in solvent system A. The plate was air-dried between each step. The thin-layer chromatograms were then examined under ultraviolet light, treated with ninhydrin and placed on X-ray film. The paper chromatogram was treated in a similar manner, but was not examined under ultraviolet light.

Hydrolysis and Analysis of III and IV. Compounds III and IV (0.480 μmole and 0.602 μmole, respectively) were hydrolyzed under an argon atmosphere at 110° C. ± 1° for 20 hours in 6.0*N* glass-redistilled hydrochloric acid. After adding an internal standard of 0.375 μmole of norleucine, the samples were concentrated to dryness and dissolved in 0.50 ml. of 0.1*M* sodium phosphate buffer pH 6.5. They were incubated at 25° C. for 4 hours to oxidize any cysteine to cystine (Colowick and Kaplan, 1963). The hydrolysates were subjected to amino acid analysis. Smaller samples of III and IV were hydrolyzed in 6*N* hydrochloric acid at 45° C. for 20 hours and chromatographed on thin-layer plates with solvent system A. The chromatogram was examined under ultraviolet light, treated with ninhydrin spray reagent, and placed on X-ray film.

Compounds III and IV were treated with performic acid (20 μl. of 30% hydrogen peroxide in 200 μl. of formic acid) at room temperature for 2 hours and concentrated to dryness. The reaction products were sampled for chromatography on thin-layer plates with solvent system A. The chromatogram

was examined under ultraviolet light, treated with ninhydrin, and placed on X-ray film. The reaction products remaining were hydrolyzed in the manner used for amino acid analysis, concentrated, and chromatographed on paper with solvent system B. This chromatogram was treated with ninhydrin.

Amino-Terminal Analysis. The DNP-derivatives of III and IV were prepared by modification of the method of Sanger and Thompson (1953). Solutions of III and IV (0.22 μmole each) in 0.20 ml. of aqueous 0.1% trimethylamine were reacted separately with shaking for 2 hours in the dark with 10 μl. of 2,4-dinitrofluorobenzene in 0.20 ml. of ethanol. The reaction mixtures were partitioned with six portions of ether and the aqueous phases were concentrated to dryness. The resulting residues were dissolved in 6*N* hydrochloric acid and hydrolyzed for 4 hours under nitrogen at 110° C. ± 1°. The hydrolysates were concentrated to dryness, dissolved in 1 ml. of water, and partitioned with five 1-ml. portions of ether. The ether and aqueous phases were compared with standard DNP-amino acids by thin-layer chromatography using two solvent systems described by Stahl (1965): chloroform–*tert*-amyl alcohol–acetic acid (70:30:2) and chloroform–benzyl alcohol–acetic acid (70:30:3).

Carboxy-Terminal Analysis. Carboxy-terminal analyses were performed on III and IV using a modification of the methods of Akabori *et al.* (1952, 1956). The metabolites and standards (0.5–1.0 μmole) to be analyzed were heated separately in sealed tubes with 0.50 ml. of hydrazine at 100° C. Compound IV, oxidized glutathione and γ-glutamylglutamic acid (Cyclo Chemical Corp.) were allowed to react for 20 min. α-Glutamylglutamic acid (Cyclo Chemical Corp.) and III were allowed to react for 2 hours and for 50 minutes, respectively. After cooling, excess hydrazine was removed under vacuum at 45° C. The crude reaction products were dissolved in 0.50 ml. of water and allowed to react with 40 μl. of benzaldehyde for 10 minutes with vigorous stirring. The suspensions were partitioned with four 0.50-ml. portions of ethyl ether and the aqueous phases were concentrated to dryness and dissolved in 50% aqueous methanol for chromatography.

The mono-benzal derivative of L-glutamic acid–γ-hydrazide was prepared by slowly adding 3 mmoles of freshly redistilled benzaldehyde dissolved in 20 ml. of methanol with rapid stirring to 3 mmoles of L-glutamic acid–γ-hydrazide in 50 ml. of water. The reaction was kept under nitrogen for 2 hours and then concentrated. The white crystalline product was removed and recrystallized from water (m.p. 173–173.5° C.. *Anal.* Calcd. for $C_{12}H_{15}N_3O_3$: C, 57.82; H, 6.06; N, 16.85. Found: C, 57.82; H, 6.02; N, 16.63).

Glycine, alanine, glutamic acid, the mono-benzal derivative of glutamic acid–γ-hydrazide, and the various reaction mixtures were spotted for chromatographic analysis (0.03–0.25 μmole). One-dimensional paper chromatograms were developed with solvent system B, solvent system A, and lutidine–aniline–water (9:1:4). Thin-layer chromatograms were developed with *n*-butanol–acetic acid–water (90:10:25) and isopropanol–ammonia–water (10:1:1). The thin-layer chromatograms were examined under ultraviolet light and then the thin-layer and paper chromatograms were treated with ninhydrin.

Hydrogenolysis. Samples of 2-ethylamino-4-isopropylamino-6-methylmercapto-*s*-triazine (ametryne), III, and IV were suspended in 0.50 ml. of absolute ethanol containing an excess of Raney nickel and heated at 85° C. in sealed tubes for 20 hours. The Raney nickel was removed by filtration and washed with three 0.5-ml. portions of ethanol. The combined filtrate and washings from each reaction mixture were concentrated and streaked onto thin-layer plates. The thin-layer plates were developed in ethanol–water (70 to 30) and the products were detected under ultraviolet light or by scanning for radioactivity. The silica gel zones containing the product were removed and washed three times with 0.50-ml. portions of ethanol and filtered. The filtrates from each sample were concentrated to dryness and analyzed by mass spectroscopy.

RESULTS AND DISCUSSION

The metabolites of atrazine produced by short term treatment of sorghum leaf disks are the same as those produced by root-treated sorghum plants (Shimabukuro and Swanson, 1969a), indicating that atrazine metabolism in sorghum leaf sections qualitatively approximates atrazine metabolism in intact plants. However, quantitative differences in the metabolites produced in leaf sections should not be related to intact plants until additional studies are conducted.

Water-soluble compounds III and IV were isolated from atrazine-^{14}C-treated leaf sections as shown in Figure 1. The radioactivity present in the incubating solution and the chloroform phase of the partitioned extract was predominantly atrazine (R_f 0.73). Thin-layer chromatography of the aqueous phase of the partitioned extract showed that most of the radioactivity was present as unresolved metabolites at R_f 0.32. No radioactivity was present in the 80% methanol and the water eluates from the AG 50W-X8 column and the R_f of the radioactive metabolites in the 2*N* ammonia eluate was unchanged. The metabolites in the ammonia eluate were resolved into radioactive fractions A and B by chromatography on AG 50W-X2. Purification of these fractions to compounds III and IV was completed as shown in Figure 1.

Later studies have indicated that chromatography on AG 50W-X8 may be eliminated if the extract is concentrated to a minimum volume and filtered through a plug of glass wool or cotton to remove chloroplasts and cell debris. The filtrate obtained in this manner can be concentrated, buffered, and placed onto a column of AG 50W-X2. Problems encountered in removing the volatile buffers in the last step in the purification of fraction B can probably be avoided by changing the order of use of AG 1-X2 and DE-52.

Compounds III and IV appeared to be pure as indicated by thin-layer and paper chromatography. The R_f values are shown in Table I. Compound IV also appeared to be pure when chromatographed on a Technicon amino acid analyzer where it had a retention time relative to norvaline of 1.389. Both compounds were readily visible under ultraviolet light on thin-layer chromatograms and gave a positive reaction with ninhydrin on thin-layer and paper chromatograms. Mixtures of III and IV were not satisfactorily resolved by the chromatographic systems shown in Table I and a single radioactive zone was detected when a mixture of III, IV, and the aqueous extract was chromatographed on a thin-layer plate with solvent system A.

The key steps used to determine the structure of III are summarized in Figure 2. The structure of IV was determined in a similar manner.

As shown in Table II, approximately one residue each of glutamic acid, ½-cystine and glycine were liberated per molecule of III hydrolyzed, and approximately one residue each of glutamic acid and ½-cystine were liberated per molecule of IV hydrolyzed. Qualitative identification of these amino acids was confirmed by paper chromatography after performic acid oxidation and hydrolysis of III and IV;

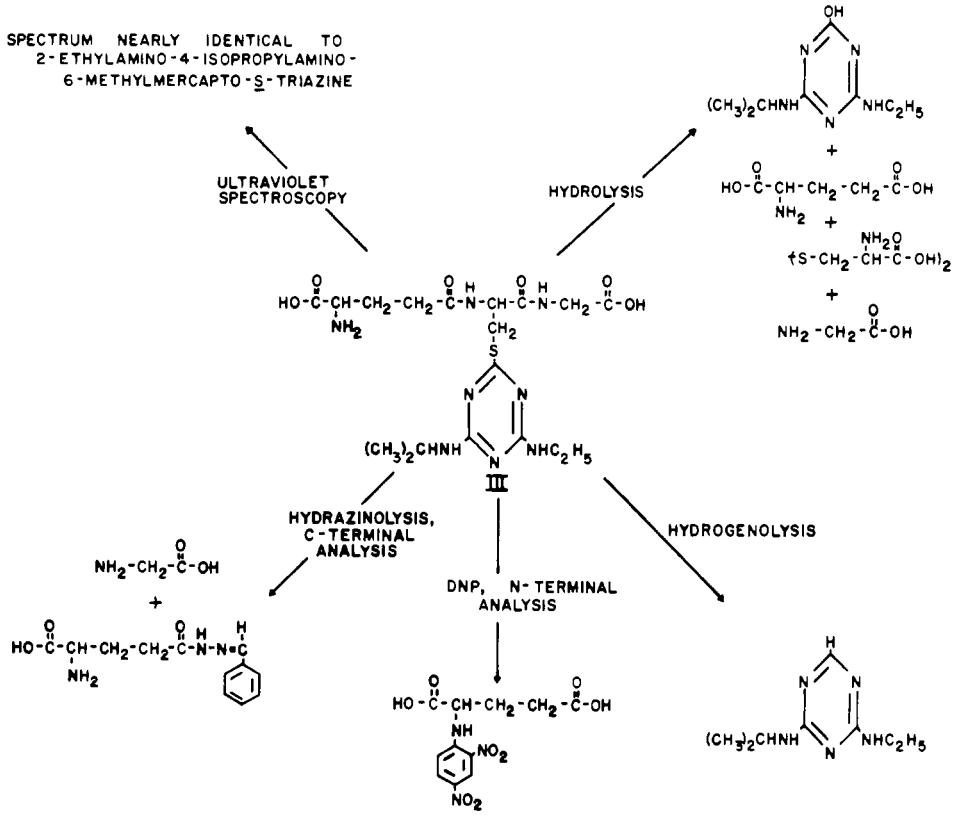

Figure 2. Summary of steps used in identification of III

Results were similar for IV except glycine was not liberated by hydrolysis or hydrazinolysis

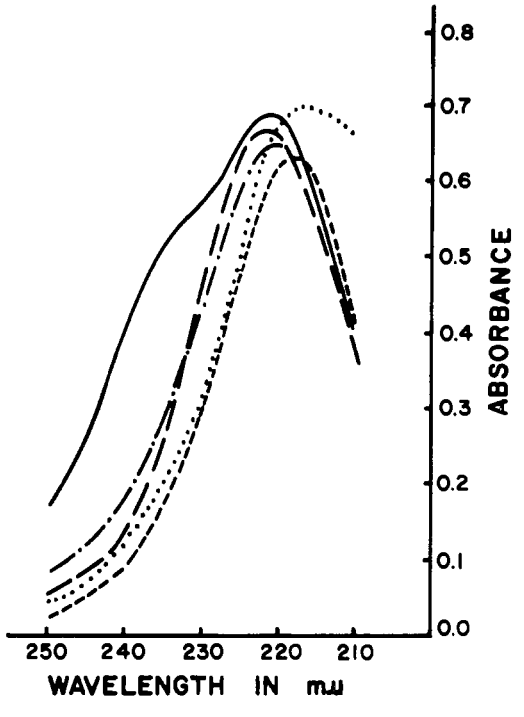

Figure 3. Effect of different functional groups on ultraviolet absorption spectra of bis(alkylamino)-*s*-triazines

Spectra of the following compounds were obtained in 95% ethanol at $2 \times 10^{-5} M$: —, ametryne (CH_3S—) — — —, atrazine (Cl); · · ·, 2-amino-4,6-bis(ethyl amino)-*s*-triazine (H_2N—); - - - -, 2-methoxy-4,6-bis(isopropylamino)-*s*-triazine (CH_3O—); and —·—· 2-ethylamino-4-isopropylamino-*s*-triazine (H—)

cysteine and cystine are converted to cysteic acid under these conditions.

DNP–Glutamic acid was produced during amino-terminal analysis of III and IV. Other DNP–amino acids were not detected. Glycine was liberated in good yield by hydrazinolysis of III, and the mono-benzal derivative of glutamic acid–γ-hydrazide was produced from both III and IV. The mono-benzal derivative of glutamic acid–γ-hydrazide prepared from glutamic acid–γ-hydrazide was identical to that obtained by carboxy-terminal analysis of γ-glutamylglutamic acid and glutathione. The mono-benzal derivatives of glutamic acid–γ-hydrazide and glutamic acid–α-hydrazide produced by carboxy-terminal analysis of model peptides were readily distinguishable from each other by paper and thin-layer chromatography.

Hydroxyatrazine (R_f 0.49) was identified by thin-layer chromatography as the only radioactive product produced by acid hydrolysis of III or IV at 45° C., but hydrolysis at 110° C. caused side reactions and three additional radioactive products were detected during quantitative amino acid analysis. Hydroxyatrazine was also produced when III and IV were treated with performic acid at room temperature.

The ultraviolet spectra of atrazine, simazine, and 2-chloro-4,6-bis(isopropylamino)-*s*-triazine (propazine) are nearly identical, indicating that minor variations in the alkylamino side chains do not cause a significant change in the ultraviolet spectrum. The ultraviolet spectra of some model bis(alkylamino)-*s*-triazines with different substituents (H—, CH_3S—, CH_3O—, Cl—, and H_2N—) in the 2-position of the triazine ring are shown in Figure 3. The absorption of ametryne in the region from 235 to 250 mμ is significantly greater than that of the other model compounds. The ultraviolet spec-

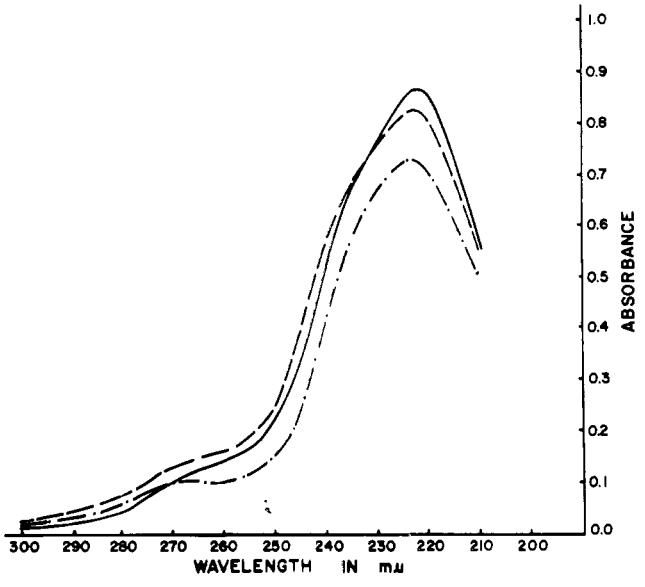

Figure 4. Ultraviolet absorption spectra of ametryne, III, and IV

Concentrations: —, 2.18 × $10^{-5}M$ ametryne; —·—·, 1.97 × $10^{-5}M$ Compound III; and — — —, 2.31 × $10^{-5}M$ Compound IV

trum of ametryne is nearly identical to that of III and IV as shown in Figure 4.

Hydrogenolysis of III, IV, and ametryne under conditions known to cleave sulfide bonds (Sondheimer and Rosenthal, 1958) yielded a predominant product with an R_f of 0.76. In the case of III and IV, this product was radioactive and free amino acids or peptides were not produced. A substantial loss of amino acids under these conditions was reported by Klosterman *et al.* (1967). In each case the mass spectrum of the product at R_f 0.76 was identical. It was characterized by a large molecular ion at *m/e* 181, a base peak at *m/e* 166 which corresponds to loss of CH_3, peaks at *m/e* 153 and *m/e* 139 which correspond to loss of ethylene and propylene *via* McLafferty rearrangements and a peak at *m/2e* 75.5 which corresponds to loss of two CH_3 groups. This spectrum is consistent with the structure 2-ethylamino-4-isopropylamino-*s*-triazine.

The amino acid and end-terminal analyses show that III is a tripeptide conjugate containing a γ-glutamylcysteinylglycine (glutathione) side chain in which the carboxyl group of the glycine residue and the α-carboxyl group of the glutamic acid residue are free. In a similar manner, IV was shown to be a dipeptide conjugate containing a γ-glutamylcysteine side chain in which the α-carboxyl group on the glutamic acid residue is free. The *s*-triazine ring and the alkylamino side chains at the 4- and 6-positions of the ring are intact and unaltered in III and IV as evidenced by liberation of hydroxyatrazine during hydrolysis and liberation of 2-ethylamino-4-isopropylamino-*s*-triazine during hydrogenolysis. The presence of a sulfide bond in III and IV between the cysteine residue in the peptide side chain and the 2-position of the *s*-triazine ring was indicated by the liberation of 2-ethylamino-4-isopropylamino-*s*-triazine by hydrogenolysis and the facile formation of hydroxyatrazine upon treatment with performic acid. The similarities of the ultraviolet spectra of ametryne, III, and IV is additional evidence for this assignment.

The optical isomers of the amino acids were not determined. However, the formation of a metabolite from atrazine and glutathione by an enzyme system isolated from sorghum (Lamoureux *et al.*, 1969) and the formation of glutathione conjugates with aromatic compounds by mammals (Booth *et al.*, 1960) indicates that these amino acid residues are probably of the L-configuration.

The structures of III and IV are concluded to be *S*-(4-ethyl-

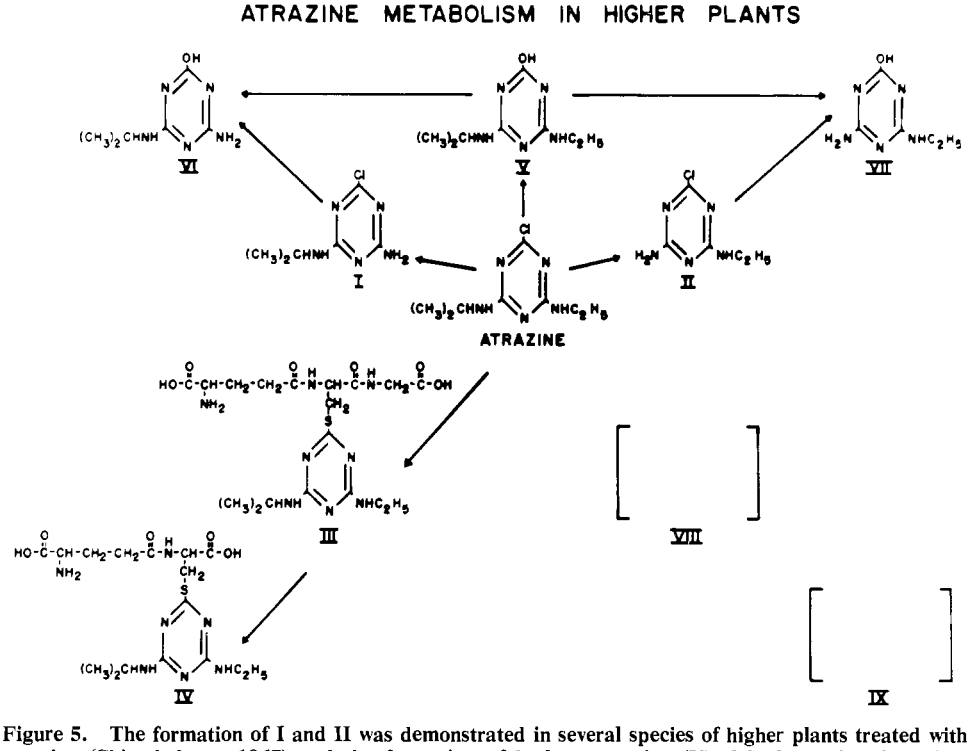

Figure 5. The formation of I and II was demonstrated in several species of higher plants treated with atrazine (Shimabukuro, 1967) and the formation of hydroxyatrazine (V), 2-hydroxy-4-amino-6-isopropylamino-*s*-triazine (VI), and 2-hydroxy-4-amino-6-ethylamino-*s*-triazine (VII) was demonstrated in corn (Shimabukuro, 1968). The presence of III and IV in sorghum was shown. Compounds III and IV also appear to be present in corn (Shimabukuro, 1968). Two unidentified water-soluble metabolites, VIII and IX are formed in sorghum treated for extended periods (Shimabukuro, 1967)

amino-6-isopropylamino-2-*s*-triazino)glutathione and γ-L-glutamyl - *S* - (4 - ethylamino - 6 - isopropylamino - 2 - *s*-triazino)-L-cysteine, respectively, Figure 5. Compound IV accounted for 77% of the radioactivity isolated in the aqueous phase and III accounted for the remaining 23%.

The presence of a third major pathway for the metabolism and detoxication of atrazine in higher plants is shown by the identification of III and IV from excised sorghum leaf sections. This metabolic pathway appears to be the primary mode of detoxication of atrazine in sorghum. As evidenced by the presence of similar metabolites, this pathway also appears to be very active in corn, but not in peas (*Pisum sativum* L.), wheat (*Triticum vulgare* Vill.), or soybeans (*Glycine max* Merril.) (Shimabukuro, 1967). Depletion of sulfhydryl groups in beans (*Phaseolus sp.*) treated with *s*-triazine herbicides can be compensated for by treating the plants with glutathione (Lalova, 1968), but it is not known if this is related to metabolite formation or some other phenomenon.

Compound III appears to be formed in sorghum by an enzymatically catalyzed condensation of glutathione with atrazine (Lamoureux *et al.*, 1969). The formation of IV can be most easily explained by the action of a carboxypeptidase on III or by the enzymatically catalyzed condensation of γ-L-glutamyl-L-cysteine with atrazine. Carboxypeptidase activity has been reported in higher plants (Visuri *et al.*, 1969), and γ-L-glutamyl-L-cysteine is an intermediate in glutathione biosynthesis (Snoke *et al.*, 1952).

Reactions involving the enzymatic formation of glutathione conjugates with halogenated compounds, polycyclic compounds (Booth *et al.*, 1960), arene oxides (Jerina *et al.*, 1968) and aromatic nitro compounds (Al-Kassab *et al.*, 1963) have been demonstrated in mammals by *in vitro* experiments. The formation of glutathione conjugates from halogenated compounds have been demonstrated in insects by *in vivo* experiments (Cohn *et al.*, 1964). In mammals, glutathione conjugates appear to be intermediates of mercapturic or premercapturic acids which can be formed in a stepwise manner by conjugation with glutathione, removal of the glutamyl residue, removal of the glycine residue, and *N*-acetylation (Booth *et al.*, 1961). A corresponding metabolic pathway involving an initial condensation with glutathione does not seem to have been previously demonstrated in higher plants. However, the isolation of III and IV from atrazine treated sorghum leaf sections is evidence that such a pathway may exist in some higher plants. In the metabolism of atrazine in sorghum, the formation of a γ-glutamylcysteine conjugate, IV, is somewhat unique. In the metabolism of mercapturic acid forming compounds in mammals, a cysteinylglycine conjugate, but not a γ-glutamyl-cysteine conjugate, is formed as an intermediate (Booth *et al.*, 1960).

It was previously shown (Shimabukuro, 1967) that the unresolved mixture of III and IV were the only water-soluble metabolites present in sorghum 48 hours after initiating root treatment of the plants with radioactive atrazine. These metabolites accounted for *ca.* 35% of the radioactivity in the plants. It was further shown that after an additional 48 hours, 48% of the radioactivity in the plants was in the aqueous fraction and two additional water-soluble metabolites were present. After 336 hours only these latter two metabolites were present in the aqueous phase and they represented 30.8% of the radioactivity in the plants, indicating that III and IV may be precursors of these compounds. This relationship would be expected if atrazine is metabolized in a manner similar to that of the mercapturic acid forming compounds in mammals.

Reactions involving the metabolism of atrazine in higher plants are summarized in Figure 5.

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