Atrazine Metabolism in Sorghum: Chloroform-Soluble Intermediates in the N-Dealkylation and Glutathione Conjugation Pathways

Richard H. Shimabukuro,* Wendy C. Walsh, Gerald L. Lamoureux, and Lester E. Stafford

Two unrelated metabolites of atrazine were isolated from sorghum. Complete N-dealkylation yielded 2-chloro-4,6-diamino-*s*-triazine, which no longer inhibited the Hill reaction and cyclic and noncyclic photophosphorylation in isolated pea chloroplasts. The isolation and identification of the metabolite *N,N'*-bis(4-ethylamino-6-isopropylamino-*s*-triazinyl-2)cystine gave further support to a previous report that atrazine was metabolized by the glutathione conjugation pathway to its lanthionine conjugate. The isolation of the dimer does not necessarily indicate that this is the form of the metabolite in plants. The *N*-cysteine monomer may dimerize *in vivo* during the sequence of reactions leading to the lanthionine conjugate.

The metabolism and detoxication of atrazine (2-chloro-4-ethylamino-6-isopropylamino-*s*-triazine) in higher plants occurs *via* 2-hydroxylation, N-dealkylation, and glutathione conjugation, as discussed in a recent review (Shimabukuro *et al.*, 1971b). Glutathione conjugation seems to be the key pathway responsible for the mechanism of resistance in plants such as corn (*Zea mays* L.), sorghum [*Sorghum bicolor* (L.) Moench], and sugarcane (*Saccharum officinarum* L.) (Lamoureux *et al.*, 1970, 1972; Shimabukuro and Swanson, 1969; Shimabukuro *et al.*, 1971a). N-Dealkylation is significantly less active than glutathione conjugation in corn and sorghum (Shimabukuro, 1967; Shimabukuro *et al.*, 1970). In sorghum, N-dealkylation yielded the metabolites 2-chloro-4-amino-6-isopropylamino-*s*-triazine and 2-chloro-4-amino-6-ethylamino-*s*-triazine (Shimabukuro, 1967). Chloroform-soluble metabolites other than the two mono-N-dealkylated derivatives were also present in sorghum (Shimabukuro, 1967). Plimmer *et al.* (1971) isolated and identified 2-chloro-4,6-diamino-*s*-triazine as one of the reaction products resulting from a free-radical oxidation of atrazine. This metabolite has not been isolated and identified in higher plants, although a metabolite with chromatographic properties similar to those of 2-chloro-4,6-diamino-*s*-triazine was reported to be present in resistant grasses (Hurter, 1967). Another unknown chloroform-soluble metabolite, amounting to 4.1% of the total radioactivity in sorghum shoots, was detected after a 4-day treating period with atrazine-^{14}C (Shimabukuro *et al.*, 1971b).

This paper describes the isolation and identification of two chloroform-soluble metabolites of atrazine in sorghum. No product-precursor relationship existed between the two metabolites. The identification of the unknown metabolites indicated that one was an intermediate in the glutathione conjugation pathway, but the second was an intermediate in the N-dealkylation pathway. The structures of the metabolites, designated as XI and XIII, are shown in Figure 1.

MATERIALS AND METHODS

Plant Material. For small-scale herbicide treatments, sorghum seeds [*Sorghum bicolor* (L.) Moench var. North Dakota 104] were germinated in vermiculite for 11 days before transfer to continuously aerated Hoagland's solution. The plants in nutrient solution were grown in a controlled-environment room with a 12-hr photoperiod, 27° day temperature, 20° night temperature, 40 ± 5% relative humidity, and a light intensity of 1600 ft-candles.

U. S. Department of Agriculture, Agricultural Research Service, Metabolism and Radiation Research Laboratory, State University Station, Fargo, North Dakota 58102.

Plants used for the generation and isolation of larger quantities of metabolites were grown in modified stainless steel trays as previously described (Bakke *et al.*, 1972). These plants were grown and treated with atrazine in the greenhouse.

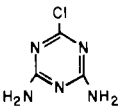
Plant Treatments. Sorghum plants, grown in nutrient solution for 14 days, were selected. Three plants each were treated with 250 ml of 1×10^{-5} *M* solutions (0.23 μCi) of uniformly ring-labeled atrazine-^{14}C, ethylamino-*1*-^{14}C side-chain-labeled atrazine, and ^{36}Cl-labeled atrazine. After treatment for 5 days, the shoots were harvested for subsequent extraction and analysis.

To generate larger amounts of the unknown metabolite designated as compound XI, six trays of sorghum plants, grown for 22 days as described (Bakke *et al.*, 1972), were treated with 1.5 l./tray of 1×10^{-4} *M* ring-labeled atrazine-^{14}C (sp act., 0.01 μCi/μmol). The plants were treated for 5 days and the shoots were extracted for isolation of the metabolite.

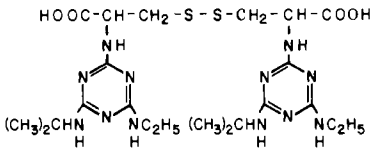
Sorghum plants grown as described (Bakke *et al.*, 1972) for 15 days were treated with 1×10^{-4} *M* ring-labeled atrazine-^{14}C (sp act., 0.11 μCi/μmol) for 20 days. Six trays of plants were exposed to atrazine and harvested as described above for isolation of the unknown metabolite designated as compound XIII.

General Methods. All radioactivity was quantitatively measured by liquid scintillation spectrometry. The ^{14}C activity in insoluble plant residue was assayed by liquid scintillation spectrometry (Shimabukuro, 1967) after dry combustion in oxygen in Schöniger flasks. The effluent from the column chromatograms was continuously monitored with a radioactive flow monitor. The flow cell was packed with anthracene for aqueous effluent and cerium-activated lithium glass beads when methanol was the effluent solvent. Radioactivity on thin-layer chromatograms was detected with a radiochromatogram scanner. Mass spectra were obtained with a Varian M-66 spectrometer equipped with a V-5500 console. Samples were introduced *via* a solid sample probe. Mass spectra were obtained at 70 eV, probe temperature of approximately 85° for compound XI and 100° for compound XIII, and source at 180° for both compounds. Infrared spectra were obtained on a Perkin-Elmer 337 infrared spectrophotometer equipped with a beam condenser. Samples were pressed into a 1.5-mm KBr micropellet for infrared analysis.

Thin-layer plates were coated with a 250-μ layer of silica gel HF and activated at 110° for 1 hr. The chromatograms were developed to a 15-cm solvent front with the following solvent systems: (A) benzene–acetic acid–water (60:40:3, v/v/v); (B) chloroform–ethanol (90:10, v/v); (C) 1-butanol–acetic acid–water (12:3:5, v/v/v); and (D) benzene–ethanol–acetic acid (10:10:1, v/v/v). Radioauto-



2-chloro-4,6-diamino-s-triazine (XI)



N,N'-bis(4-ethylamino-6-isopropylamino-s-triazinyl-2)-cystine (XIII)

Figure 1. Chemical structure of compounds XI and XIII.

graphs of thin-layer plates were made on Kodak no-screen X-ray film.

Determination of Free Chloride Ion. Radioactivity present in the aqueous extracts from atrazine-^{36}Cl treated plants was precipitated with silver nitrate. A 1-ml sample of the aqueous extract was mixed with 1 ml of 0.01 *M* NaCl and 2 ml of 0.016 *M* $AgNO_3$. The solution was heated gently for 5 min, cooled, and centrifuged. The supernatant was separated from the precipitate and the ^{36}Cl activity remaining in solution was determined. A blank consisting of an aqueous solution of atrazine-^{36}Cl was treated similarly for comparison.

Inhibition of Photochemical Activity. Inhibition of the Hill reaction and cyclic and noncyclic photophosphorylation in isolated pea chloroplasts by 1×10^{-6} *M*, 1×10^{-5} *M*, and 1×10^{-4} *M* 2-chloro-4-6-diamino-*s*-triazine was measured as previously described (Shimabukuro and Swanson, 1969). Ferricyanide was used as the electron acceptor for the Hill reaction. Esterification of inorganic phosphate was determined by the molybdenum blue method of Fiske and Subarrow (1925).

Phytotoxicity of Atrazine and Metabolites. The inhibition of shoot growth of extremely sensitive oats (*Avena sativa* L.) was used as a bioassay of phytotoxicity. Oat seedlings were treated in nutrient solution with 1×10^{-5} *M* solutions of atrazine, 2-chloro-4-amino-6-ethylamino-*s*-triazine, and 2-chloro-4,6-diamino-*s*-triazine as previously reported (Shimabukuro, 1967). Shoots were harvested after 8 days and fresh and dry weights were determined.

Synthesis of 2-Amino-4-ethylamino-*s*-triazine and 2-Amino-4-isopropylamino-*s*-triazine. Cyanoguanidine was fused with ethylamine hydrochloride and isopropylamine hydrochloride to produce 1-ethylbiguanide hydrochloride and 1-isopropylbiguanide hydrochloride by the method of May (1947). Ring closure was accomplished by reacting the substituted biguanides with sodium ethoxide and ethylformate (Geigy, 1950). The structures of the resulting *s*-triazines were confirmed by mass spectral and infrared analyses.

Atrazine Metabolism in Plants. The shoots from three sets of plants treated with ring-^{14}C-labeled, side-chain ^{14}C-labeled, and ^{36}Cl-labeled atrazine were extracted and filtered three times with a 4:1 (v/w) ratio of 80% methanol to original fresh weight. The methanol in the extract was removed under vacuum to give an aqueous solution. The aqueous extract was washed with equal volumes of chloroform and the radioactivity present in the aqueous and chloroform fractions was determined as described (Shimabukuro *et al.*, 1966).

The compounds present in 5000-dpm aliquots of the chloroform-soluble fractions were separated by thin-layer chromatography with solvent system A. Radioactive zones were detected and quantitated as described (Shimabukuro, 1967). The methods used for detecting and counting ^{36}Cl activity were the same as those used for ^{14}C activity.

The shoots from the 5- and 20-day large-scale treatment with ring-labeled atrazine-^{14}C were extracted as described (Bakke *et al.*, 1972). Each extract was fractionated and the radioactivity was determined as above. Aliquots (5000 dpm) of the chloroform-soluble fractions were spotted on thin-layer plates and developed for quantitation as described above. The remainder of these chloroform-soluble fractions were further purified for isolation and identification of the unknown metabolites.

Purification Procedure. *Purification of Compound XI.* The chloroform-soluble fraction from the 5-day large-scale treatment was evaporated to dryness under vacuum at 40° and dissolved in a minimum volume of methanol. The methanol solution was streaked on 20 × 20 cm thin-layer plates and developed in solvent A. The zone at R_f 0.58 was visualized under uv light, the silica gel was carefully removed and washed five times with 5-ml portions of methanol, and the methanol eluate was concentrated to 10 ml. Recovery of metabolite was about 65%.

The 10-ml methanol eluate was applied to a 1 × 48 cm column of Sephadex LH-20 (methanol equilibrated) and eluted with methanol at a rate of 0.4 ml/min. The total recovery of radioactivity was 82%. The major radioactive fraction (63.7–74.7 ml) accounted for 67% of the radioactivity recovered. This fraction was chromatographed on thin-layer plates in solvent B and the zone at R_f 0.3 was recovered as above. The methanol eluate was concentrated and a portion of it was used to prepare a 1.5-mm KBr pellet for infrared analysis.

Purification of Compound XIII. Purification procedures for compound XIII from 20-day large-scale treatment are outlined in Figure 2. Compound XIII can be partitioned into chloroform with exhaustive washing of the aqueous solution, although its solubility in chloroform is less than that of atrazine. The chloroform-soluble fraction was taken to dryness and dissolved in methanol. A small amount of water was added and methanol was removed under vacuum. Compound XIII was much more soluble in water than atrazine. The resulting aqueous solution was concentrated to about 5 ml and applied to a 1.5 × 90 cm column 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 DE-52 column was eluted stepwise at a rate of 0.46 ml/min with water (fraction A), 1.0 *N* acetic acid (fraction B), and 3.0 *N* acetic acid (fraction C). Fraction B, which contained the major portion of the radioactivity (77.4%), eluted as three poorly resolved radioactive peaks with most of compound XIII present in fraction B2 (59%).

Fraction B2 was concentrated and applied to a 1.5 × 90 cm DE-52 column prepared as described above. The column was eluted at 0.46 ml/min with an acetic acid gradient. The gradient device (Varigrad, Büchler Instruments) contained 400 ml of water in chambers 1, 2, and 3, and 400 ml of 0.1 *N* acetic acid in chamber 4. Most of the radioactivity (compound XIII) was found in fraction D.

Fraction D was concentrated to dryness under vacuum, dissolved in several milliliters of methanol, and chromatographed on a column of Sephadex LH-20 as described in the purification of XI. The radioactivity eluted as an unsymmetrical peak which was divided into four fractions (D1–D4). Compound XIII was isolated as a single radioactive component in fraction D1. Fractions D2 and D3, containing some impurities, were combined, concentrated, and rechromatographed on the Sephadex LH-20 column. The recovered radioactive peak was again divided as above. The D1 fractions from the two Sephadex LH-20 chromatography were combined for further characterization of compound XIII.

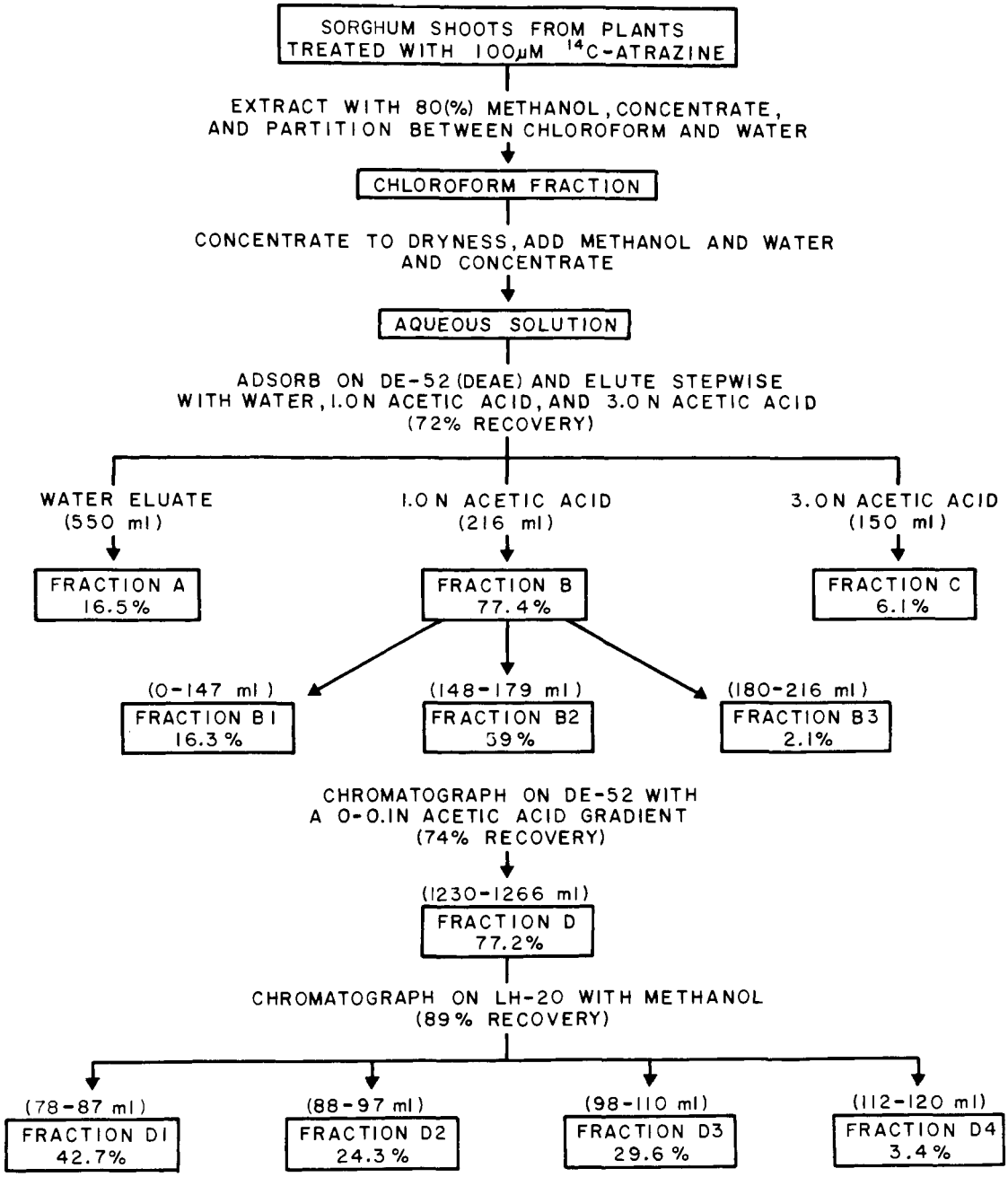


Figure 2. Purification procedure for *N,N'*-bis(4-ethylamino-6-isopropylamino-*s*-triazinyl-2)cystine extracted from the shoots of sorghum plants treated with ring-labeled atrazine-^{14}C for a 20-day period. The recoveries indicate the percent ^{14}C recovered at each step during chromatography.

RESULTS AND DISCUSSION

Metabolism of Atrazine and Distribution of ^{14}C and ^{36}Cl in Plants. A radioautograph of a thin-layer chromatographic separation of the chloroform-soluble fractions from ring-atrazine-^{14}C and atrazine-^{36}Cl treated plants is shown in Figure 3. The chloroform-soluble fraction derived from ethyl side chain atrazine-^{14}C is not shown on the same thin-layer plate. However, a radioautograph from a separate plate indicated that compound XI (spot B) was missing and compound XIII (spot A) was present. All three differentially labeled atrazines showed spots at D (unchanged atrazine) and C. Spot C was a mixture of the two mono-N-dealkylated derivatives from ring-atrazine-^{14}C and atrazine-^{36}Cl; only 2-chloro-4-amino-6-ethylamino-*s*-triazine was present from ethyl side chain atrazine-^{14}C. No radioactive products of atrazine were produced by incubating the treating solutions of atrazine in the absence of the plants, indicating that the above metabolites must have been formed by the plants and not by bacterial action or some other process (Lamoureux *et al.*, 1973).

The results indicated that compound XI had not been dechlorinated and at least one of the side chains (ethylamine) had been dealkylated. The ^{36}Cl from atrazine-^{36}Cl and ^{14}C from ring atrazine-^{14}C were present in compound XI. Compound XI also appeared to be more polar than the monodealkylated metabolites. The absence of compound XI from ethyl side chain atrazine-^{14}C supported the conclusion that N-dealkylation had occurred to remove the ethyl side chain.

Compound XIII appeared to be unrelated to XI and no product-precursor relationship could be assigned to the formation of compounds XI and XIII. The detection of ^{14}C activity in compound XIII from ring atrazine-^{14}C indicated that the *s*-triazine ring was probably intact, but the absence of compound XIII from atrazine-^{36}Cl indicat-

Table I. Metabolism of Atrazine in Sorghum Plants

Compounds	Distribution of radioactivity in shoots (% of total radioactivity)		
	Ring atrazine-^{14}C	Atrazine-^{36}Cl	Ethyl side-chain atrazine-^{14}C
Unchanged atrazine	4.0	2.8	4.2
Mono-N-dealkylated metabolites	2.9	2.4	1.6[a]
Compound XI	1.6	0.7	0.0
Compound XIII	3.5	0.0	4.0
Other unknowns	2.2	0.2	4.3
Water-soluble metabolites	72.8	93.9[b]	74.1
Methanol-insoluble residue	13.0	[c]	11.8

[a] Includes only 2-chloro-4-amino-6-ethylamino-*s*-triazine. [b] Includes free chloride ions in solution. [c] Not measured.

Table II. Thin-Layer Chromatographic Comparison of Chloroform-Soluble *s*-Triazine Compounds

Compound	R_f in solvent systems		
	A	B	D
Atrazine	0.85	0.75	0.91
2-Chloro-4-amino-6-ethylamino-*s*-triazine	0.73	0.44	0.82
2-Amino-4-ethylamino-*s*-triazine	0.33	0.23	0.64
2-Amino-4-isopropylamino-*s*-triazine	0.44	0.31	0.71
Compound XIII	0.27	0.07	0.45

Table III. Inhibition of Fresh and Dry Weights of Oat Shoots

Compounds[a]	Inhibition, %	
	Fresh weight	Dry weight
Control	0	0
Atrazine	91	77
2-Chloro-4-amino-6-ethylamino-*s*-triazine	79	80
2-Chloro-4,6-diamino-*s*-triazine	12	15

[a] Oat seedlings were treated in nutrient solution with 1×10^{-5} *M* concentration of each *s*-triazine compound.

Table IV. Metabolism of Atrazine in Sorghum Plants

Compounds	Distribution of radioactivity in shoots (% of total radioactivity)	
	5-Day treatment period	20-Day treatment period
Unchanged atrazine		0.3
Mono-N-dealkylated metabolites	9.0[a]	2.0
Compound XI	1.7	1.7
Compound XIII	4.0	5.0
Other unknowns	0.4	3.2
Water-soluble metabolites	74.5	67.5
Methanol-insoluble residue	10.4	20.3

[a] Includes unchanged parent atrazine and mono-N-dealkylated metabolites.

ed that the *s*-triazine ring was no longer substituted with chlorine in the 2 position. Compound XIII was also detected in the ethyl side chain atrazine-^{14}C-treated plants, which indicated that the ethylamine side chain of atrazine was still intact. The presence of one side chain in compound XIII showed conclusively that this metabolite was not a product of the subsequent metabolism of compound XI.

The relative distribution of radioactivity in the shoots of sorghum plants treated with the three labeled forms of atrazine over a 5-day period is shown in Table I. Compounds XI and XIII account for a relatively small percent of the total radioactivity present in sorghum shoots. The water-soluble fraction accounted for most of the radioactivity derived from all three differentially labeled forms of atrazine. In the water-soluble fraction from atrazine-^{36}Cl, 89.5% of the ^{36}Cl activity was precipitated as $Ag^{36}Cl$.

Reductive dechlorination is a possible mechanism leading to the formation of compound XIII. Reductive dechlorination of 2-chloro-4-amino-6-ethylamino-*s*-triazine will yield 2-amino-4-ethylamino-*s*-triazine. However, thin-layer chromatography indicated that compound XIII was not a product of the reductive dechlorination of the mono-N-dealkylated derivative of atrazine (Table II). The compound 2-ethylamino-4-isopropylamino-*s*-triazine was not compared by thin-layer chromatography, but it would be expected to be less polar than 2-amino-4-ethylamino-*s*-triazine. Dechlorination of atrazine in sorghum by nonenzymatic hydrolysis as in corn during a 48-hr period was not detectable (Shimabukuro, 1967, 1968). However, extensive dechlorination occurred by glutathione conjugation within 6 hr in excised sorghum leaves (Lamoureux *et al.*, 1972). Therefore, the results suggested that dechlorination in compound XIII may have occurred *via* glutathione conjugation rather than by some other mechanism. This indicated that compound XIII may be related to intermediates in the glutathione conjugation pathway.

Isolation, Purification, and Identification of Compound XI. A total of 1.5×10^7 dpm of ^{14}C activity was extracted from 3.5 kg fresh weight of sorghum shoots. The unpurified extract contained about 12 μmol of compound XI. Part of this extract was purified as described under the section on methods. The final yield of purified compound XI was about 4 μmol.

The purified metabolite was added to KBr and its infrared spectrum was obtained from a 1.5-mm KBr pellet. The spectrum of the compound (XI) and that of authentic 2-chloro-4,6-diamino-*s*-triazine were similar. The same KBr pellet was used to obtain the mass spectrum of the metabolite. The mass spectrum was similar to the published spectrum of authentic 2-chloro-4,6-diamino-*s*-triazine (Ross and Tweedy, 1970). The spectrum had a molecular ion which was the base peak at m/e 145. The ratio of intensities of m/e 145:m/e 147 confirmed the substitution of one chlorine in the molecular ion. Loss of chlorine yielded a peak at m/e 110 (32). Other peaks at m/e 68 (44) and 43 (68), caused by fragmentation of the *s*-triazine ring, were present in the spectrum. The structure of compound XI is shown in Figure 1.

Inhibition of Hill Reaction and Photophosphorylation. The compound 2-chloro-4,6-diamino-*s*-triazine did not inhibit the photochemical reactions in isolated pea chloroplasts. No significant inhibition of the Hill reaction and noncyclic and cyclic photophosphorylation occurred even at a concentration of 1×10^{-4} *M*. The half-maximal inhibitor concentration (I_{50}) for the Hill reaction in isolated pea chloroplasts was reported to be 2.0×10^{-6} *M* for atrazine and 4.6×10^{-5} *M* for the mono-N-dealkylated derivatives. Ferricyanide-dependent noncyclic photophosphorylation was reduced 100 and 37% of the control by a

1.0×10^{-6} *M* concentration of atrazine and 2-chloro-4-amino-6-isopropylamino-*s*-triazine, respectively (Shimabukuro and Swanson, 1969). Therefore, the removal of the one alkyl side chain followed by the removal of the second is a stepwise detoxication of atrazine to yield a nonphytotoxic derivative. The bioassay on highly susceptible oats showed very clearly that 2-chloro-4,6-diamino-*s*-triazine had very little biological activity (Table III).

Isolation, Purification, and Identification of Compound XIII. A total of 9.1×10^7 dpm of ^{14}C activity was extracted from 1.9 kg of fresh sorghum shoots after a 20-day treatment period. Another 2.3×10^7 dpm of ^{14}C activity was found in the methanol-insoluble residue. A distribution of radioactivity from sorghum plants treated with atrazine-^{14}C for the isolation of compound XI (5-day treatment period) and for the 20-day period is given in Table IV.

The concentration of compound XIII from 5- to 20-day treatment periods did not show any appreciable increase. Compound XIII accounted for 5.0% of the total ^{14}C activity after 20 days. A reduction in water-soluble metabolites occurred after 5 days and the methanol-insoluble residue doubled between 5 to 20 days.

The unpurified extract contained about 2.6 μmol of compound XIII. Part of the extract was purified as indicated in Figure 2. The final purified compound XIII amounted to 0.33 μmol. The radiochemical purity of compound XIII was confirmed by thin-layer chromatography with solvent system A (R_f 0.27).

A single radioactive product was obtained when compound XIII was derivatized with methanol–HCl by method I of Greenstein and Winitz (1961). Hydrolysis of the derivatized product with 6 *N* HCl, 100°, over a 24-hr period yielded the parent compound XIII and several more polar hydrolytic products. The results indicated that compound XIII was probably a complex acidic conjugate which was readily converted to a methyl ester. The mass spectrum of this derivative was qualitatively identical to the mass spectrum of the dimethyl ester of *N,N'*-bis(4-ethylamino-6-isopropylamino-*s*-triazinyl-2)cystine prepared *in vitro* (Lamoureux *et al.*, 1973) (Figure 1). The mass spectrum and probable nature of the major ion fragments of the dimethyl ester of compound XIII are shown in Figure 4. The ion at *m/e* 313 was probably derived from the monomer formed by ionization and cleavage of the disulfide bond, while the cluster of ions at *m/e* 279, 280, and 281 is probably derived from cleavage α to the sulfur atom. The peak at *m/e* 280 was the base peak in the spectrum. α Cleavage was found to be the predominant fragmentation pattern for *N,N'*-bis(trifluoroacetyl)-cystine dimethyl ester (Harp and Gleason, 1971). The ions at *m/e* 249 and 220 would result from the loss of OCH_3 and $COOCH_3$ from the ion at 280. The doubly charged ions at *m/e* 132.5 and 102.5 no doubt result from the loss of $\cdot CH_3$ from the most abundant ions at *m/e* 280 and 220, respectively. The formation of doubly charged ions by loss of $\cdot CH_3$ is commonly observed with 2,4-bis(alkylamino)-*s*-triazines. The presence of these doubly charged ions supports the structures given for the ions at *m/e* 220 and 280. These mass spectral fragmentation data support the proposed structure of compound XIII (Figure 1). Further, compound XIII cochromatographed with the *in vitro* prepared *N,N'*-bis(4-ethylamino-6-isopropylamino-*s*-triazinyl-2)cystine on a two-dimensional thin-layer chromatogram developed in solvent C in the first direction and solvent A in the second direction.

The identification of compound XIII as *N,N'*-bis(4-ethylamino-6-isopropylamino-*s*-triazinyl-2)cystine (Figure 1) is consistent with the discussed results. Previous information indicated that the ethyl side chain was probably intact and the chlorine in the 2 position had been displaced.

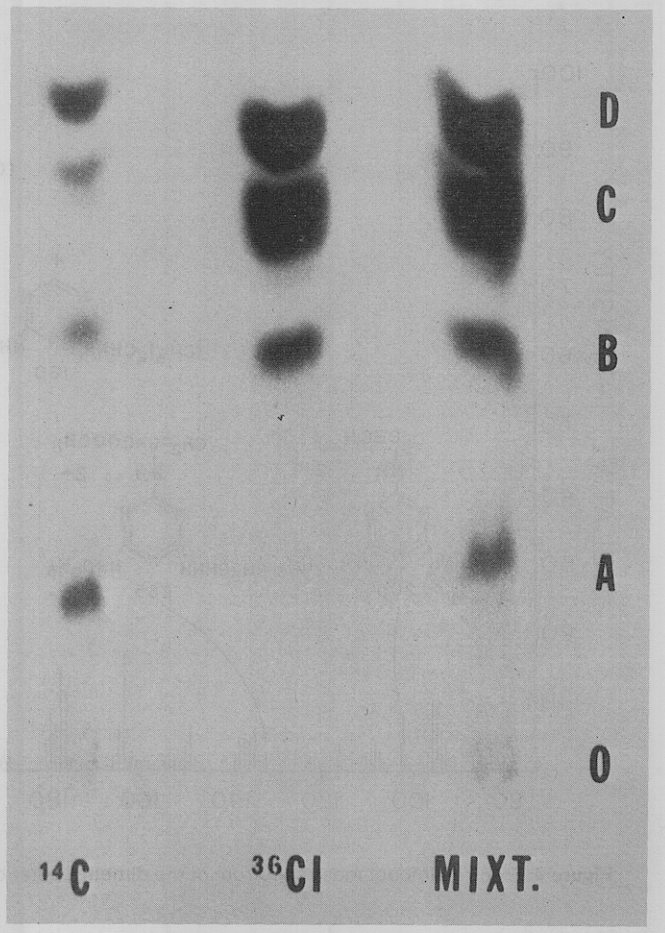


Figure 3. Thin-layer chromatographic separation of the chloroform-soluble fraction from sorghum plants treated with uniformly ring-labeled atrazine-^{14}C (^{14}C) and atrazine-^{36}Cl (^{36}Cl). Aliquots from the two plant samples were cochromatographed (MIXT.). O, origin; A, compound XIII; B, compound XI; C, mixture of mono-N-dealkylated derivatives; D, atrazine. Thin-layer chromatogram developed in solvent A.

CONCLUSION

The isolation and identification of 2-chloro-4,6-diamino-*s*-triazine indicate that complete N-dealkylation of atrazine occurs in higher plants such as sorghum. The removal of both *N*-alkyl side chains inactivated atrazine. The dealkylated metabolite showed no inhibition of the Hill reaction and cyclic and noncyclic photophosphorylation in pea chloroplasts. N-Dealkylation is not a major degradation pathway in plants and the relatively low concentration of 2-chloro-4,6-diamino-*s*-triazine in plants over a 5- to 20-day period suggests that the turnover rate of this metabolite is very slow. The subsequent metabolism of this metabolite is still unknown.

The isolation and identification of *N,N'*-bis(4-ethylamino-6-isopropylamino-*s*-triazinyl-2)cystine give further support to the theory of metabolism of atrazine to its lanthionine conjugate *via* the glutathione conjugation pathway (Lamoureux *et al.*, 1973). Atrazine was initially conjugated to glutathione and subsequently converted to γ-glutamyl-*S*-(4-ethylamino-6-isopropylamino-*s*-triazinyl-2)-cysteine. The metabolism of the dipeptide conjugate to its lanthionine conjugate involves the *S*-cysteine and *N*-cysteine conjugates as intermediates (Lamoureux *et al.*, 1973). The *S*-cysteine conjugate was detected in plant extracts and produced *in vitro* by the action of γ-glutamyl transpeptidase on the γ-glutamylcysteine conjugate. The *in vitro* synthesized *S*-cysteine conjugate was highly unstable and a rearrangement occurred to give *N,N'*-bis(4-

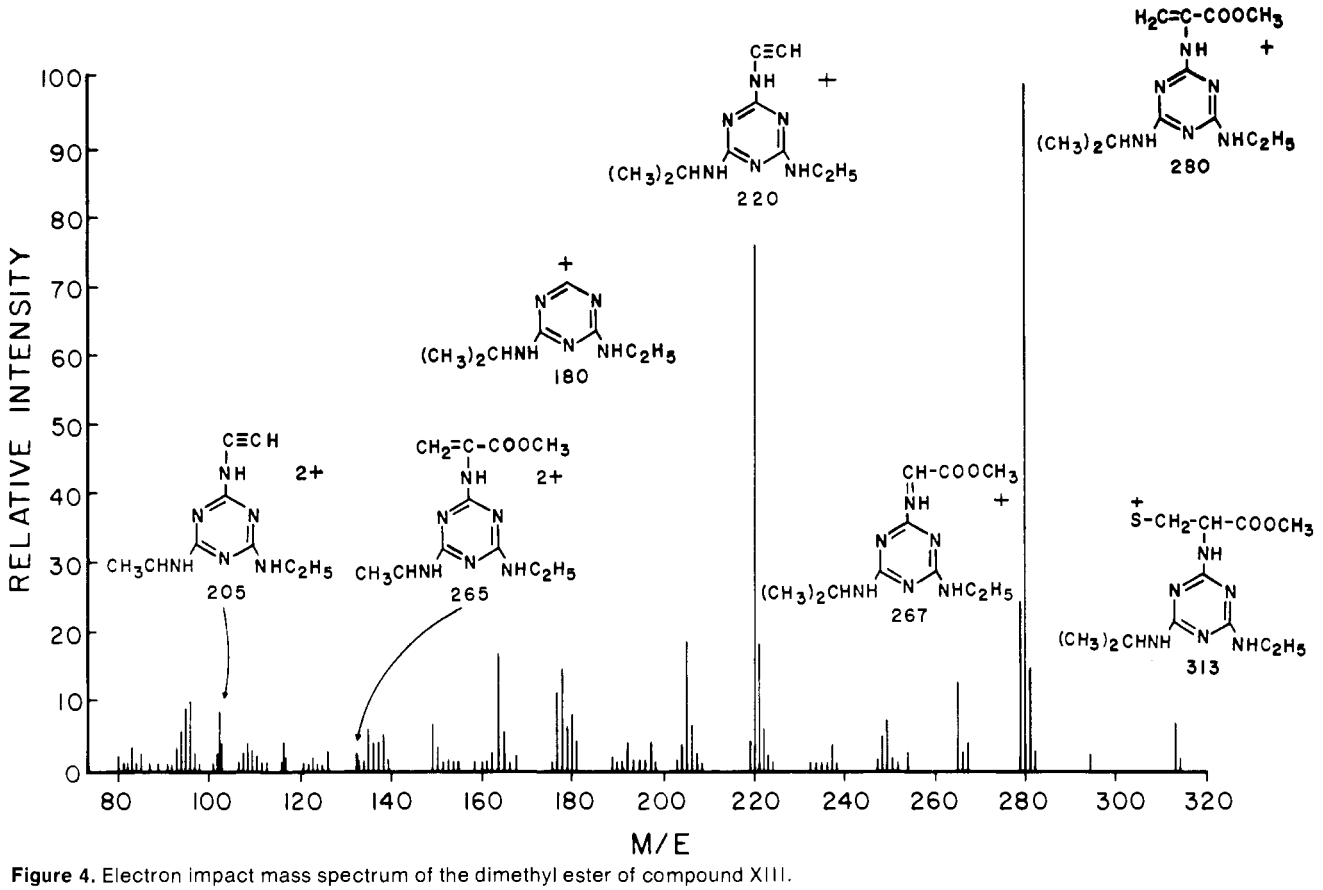


Figure 4. Electron impact mass spectrum of the dimethyl ester of compound XIII.

ethylamino-6-isopropylamino-*s*-triazinyl-2)cystine (Lamoureux *et al.*, 1973). The isolation and identification of this same metabolite from sorghum plants treated with atrazine, as reported in this paper, verify that the rearrangement of the *S*-cysteine conjugate to the *N*-cysteine conjugate does occur *in vivo*. The isolation of the metabolite as the dimer does not necessarily indicate that this is the form of the metabolite in plants. The concentration of *N,N'*-bis(4-ethylamino-6-isopropylamino-*s*-triazinyl-2)cystine was relatively low and remained fairly constant as compared to other intermediates in the glutathione conjugation pathway. The *N*-cysteine conjugate *in vivo* seems to be a transient intermediate of low concentration which is converted to the lanthionine conjugate (Lamoureux *et al.*, 1973). It seems probable that some dimerization may occur during the reaction sequence *in vivo* to produce the symmetrical disulfide which slowly accumulates in the plant. It is known that symmetrical disulfides are thermodynamically stable forms (Greenstein and Winitz, 1961).

ACKNOWLEDGMENT

Uniformly ring-labeled atrazine-^{14}C (sp act., 2.2 μCi/μmol), ethylamino-*1*-^{14}C side-chain-labeled atrazine (sp act., 1.4 μCi/μmol), and ^{36}Cl-labeled atrazine (sp act., 0.08 μCi/μmol) were generously provided by CIBA-Geigy Chemical Corp. The authors wish to thank Mr. Richard Zaylskie for the mass spectra of metabolites. Authentic 2-chloro-4,6-diamino-*s*-triazine and 2-chloro-4-amino-6-ethylamino-*s*-triazine were synthesized by V. J. Feil and F. S. Tanaka, respectively.

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