Metabolism of Atrazine by *Spartina alterniflora*. 2. Water-Soluble Metabolites

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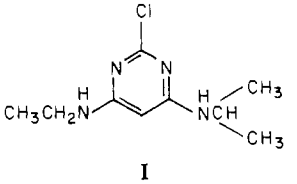
Roots of *S. alterniflora* plants were exposed to ring-labeled [^{14}C]atrazine for 2 days and then transferred to an atrazine-free nutrient solution and sampled 3, 8, 18, and 28 days after transfer. Radioactivity in the aqueous fraction of 80% methanol extracts of shoots from these plants ranged from ca. 58 to 60% of that in the total extract during 8–28 days transfer. Fourteen water-soluble metabolites of atrazine were isolated using cation- and anion-exchange and Sephadex column chromatography and thin-layer chromatography. About half the substances contained fully N-alkylated triazine rings and most of the others contained the 4-amino-6-isopropylamino derivative. Only one metabolite contained the 4-amino-6-ethylamino derivative, and no 4,6-diamino forms were detected. 2-Hydroxyatrazine and 2-hydroxy-4-amino-6-(isopropylamino)-*s*-triazine were also identified. Acid hydrolysates of the metabolites contained low amounts of amino acids such as glutamic acid, glycine, and valine, suggesting a conjugation pathway of atrazine metabolism may be operative in *Spartina*, such as glutathione conjugation that occurs in *Sorghum*.

The increased use of herbicides has resulted in widespread concern that they may be affecting nontarget areas. Because of degradation, dilution, and adsorption to soil particles, herbicides are rarely transported to such areas in toxic levels. However, this raises the question of whether herbicides transported to nontarget areas can accumulate to toxic levels with time and pose a potential long-range threat to the balance of organisms in the ecosystem in which they accumulate. In order to predict how a particular ecosystem would respond to introduction of a herbicide, it is necessary to determine the sensitivity of the system components to the compound and to determine the fate of the herbicide in that particular system. The salt marsh at Sapelo Island, Georgia, was selected as an area having the potential as a nontarget recipient of herbicides used in inland agricultural areas. A simulated salt marsh microecosystem has been constructed (Everest, 1978) using components from this salt marsh and is being used to study the fate of atrazine [I, 2-chloro-4-(ethylamino)-6-(isopropylamino)-*s*-triazine]. Initially, the effects



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of atrazine on each component of the system are being studied separately.

This report concerns the second half of a study on the metabolism of atrazine by the dominant species and primary autotroph of the salt marsh, *Spartina alterniflora* (smooth cordgrass). We previously reported the sensitivity of *Spartina* to atrazine and the chloroform-soluble metabolites of this herbicide in this grass (Pillai et al., 1977) as well as in box crabs (Pillai et al., 1979). We now report the metabolism of atrazine to water-soluble substances in the smooth cordgrass.

MATERIALS AND METHODS

Growth and Treatment of Plants. *S. alterniflora* plants were collected from the salt marsh at Sapelo Island, GA, and maintained at Auburn University as described previously (Pillai et al., 1977). In time course experiments, a uniform lot of vigorously growing plants was divided into ten groups of four plants each. The roots of each group of plants were placed in 300 mL of Hoagland's solution with a 1.23 μM concentration of ring-labeled [^{14}C]atrazine (2.0 μCi, sp act. 24.9 μCi/mg). After 2 days, the plants were removed from the [^{14}C]atrazine solution, the roots were rinsed, and all but two groups were placed in atrazine-free Hoagland's solution for various time periods up to 28 days. The two groups not transferred to the atrazine-free solution and two additional groups taken from the atrazine-free solution after 3, 8, 18, and 28 days, respectively, were extracted as described previously (Pillai et al., 1977). The plants were separated into roots and shoots which were extracted separately by grinding with 80% methanol. The extract was filtered and the filtrate was concentrated using a rotary flash evaporator. The plant material remaining after filtration is referred to as the 80% methanol-insoluble fraction. The concentrated extract was diluted with distilled water and washed three times with chloroform. The combined chloroform fractions and the remaining aqueous fraction were concentrated separately and the radioactive components of the latter fraction were isolated as described below.

Isolation of Water-Soluble Atrazine Metabolites. Plants used specifically for the isolation of water-soluble metabolites of atrazine were selected and placed in 1-L plastic cups, five plants per cup, with their roots in 400 mL of Hoagland's solution. Each cup had 2.0 μCi [^{14}C]-atrazine and sufficient nonlabeled atrazine to give a 50 μM concentration and a final specific activity of 0.1 μCi/μmol. The plants were maintained in these cups for 2 days after which the roots were rinsed with nonlabeled atrazine solution and then placed in a fresh atrazine-free Hoagland's solution for 18 days.

Approximately 1 kg of fresh shoot tissue from these plants was ground, in 10-g portions, for 3-min in a Waring blender containing 80% aqueous methanol (10 mL of solvent/g of tissue). After each grinding step, the extract was filtered and the residue was placed in the extracting solvent for 1–2 days at 4 °C until all of the material had been ground. The grinding, soaking, and filtering steps for all the tissue was repeated twice. The combined extracts were concentrated by flash evaporation at 37 °C, dissolved in 400 mL of water, and washed with four 500-mL volumes of chloroform. Portions of the aqueous fraction containing about 10^7 dpm were concentrated as

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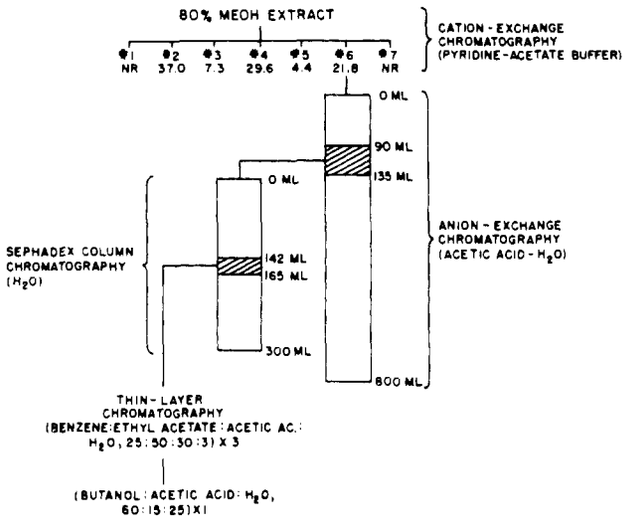

Figure 1. Outline of procedures used in the isolation and purification of water-soluble metabolites of atrazine from *S. alterniflora*. The example is given for fraction 6 from the cation-exchange column but each fraction was treated in a similar manner.

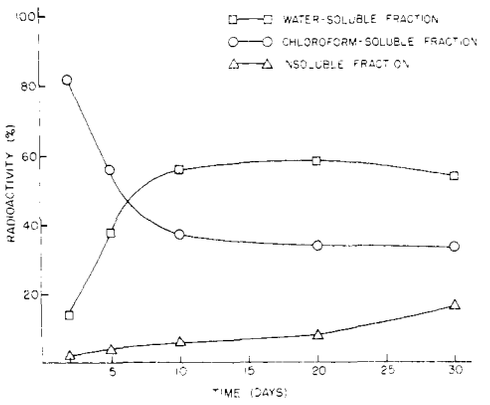

Figure 2. Change in radioactivity with time in the aqueous and chloroform fractions of an 80% methanol extract of shoots and an 80% methanol-insoluble fraction from *S. alterniflora* plants grown in [^{14}C]atrazine for 2 days and then transferred to an atrazine-free nutrient solution for 28 days. [This figure was previously published (Pillai et al., 1977) but is included here to facilitate the discussion.]

before and dried by lyophilization. The dried sample was dissolved in 50 mL of a pyridine–acetate buffer (pH 2.15) and applied to a 2.5 × 95 cm column of AG 50W-X2 (200–400 mesh) cation-exchange resin. The resin and buffers were prepared as described by Schroeder et al. (1962). The column was maintained at 15 °C while being washed with a pyridine–acetate buffer gradient at a rate of 0.6 mL/min. The first chamber of the gradient device contained 400 mL of 0.2 N buffer (pH 3.1) and the second and third chambers each contained 400 mL of the same buffer (2 N) at pH 5.0. Five-milliliter fractions were collected during each column chromatography step. Adjacent fractions containing no radioactivity were combined and discarded. Five major radioactive fractions were obtained from the cation-exchange column, and each was evaporated to dryness by flash evaporation at 37 °C, dissolved in a minimum volume of water, and applied to a separate 2.5 × 95 cm column of AG 1 × 2 (200–400 mesh, acetate form) anion-exchange resin, also maintained at 15 °C. The column was eluted with an acetic acid concentration gradient at a flow rate of 0.4 mL/min. The gradient device contained 275 mL of water in each of the first two chambers, 275 mL of 0.5 N acetic acid in chamber 3, and 275 mL of 0.38 N acetic acid in chamber 4. The column was subsequently washed with 150 mL of 0.5 N acetic acid, followed by 150-mL fractions each of 1–6 N acetic acid. Radioactive fractions from this column were obtained as before and evaporated to dryness by flash evaporation. The residue was washed from the flask with one 5-mL portion and two 1-mL portions of absolute methanol which was then evaporated to dryness under N_2. The residue was then dissolved in a few milliliters of water and placed on a 1.5 × 95 cm column of Sephadex LH-20 which was washed with water at the rate of 0.5 mL/min at 15 °C. Each radioactive fraction from this column was purified by TLC using silica gel G (250-μm thickness) on 20 × 20 cm glass plates. The TLC plates were developed three times in benzene–ethyl acetate–acetic acid–water (25:50:30:3, v/v/v/v) (solvent system I) and radioactive areas were located with a Berthold TLC scanner. The silica gel spots containing radioactivity were removed from the plates and washed five times with methanol. The radioactive substances were then applied to silica gel plates which were developed once in *n*-butanol–acetic acid–water (60:15:25, v/v/v) (solvent system II). In most cases, further purification of the radioactive substance was by dialysis followed by TLC using solvent system II. A schematic outline of the column and thin-layer chromatographic procedures is given in Figure 1.

Characterization of Metabolites. For amino acid analysis, a portion of each purified metabolite was hydrolyzed under nitrogen in 200 μL of 6 N HCl in a sealed ampule under nitrogen at 110 °C for 16 h. Each hydrolysate was diluted with distilled water and evaporated to dryness several times under a vacuum to remove the HCl and then treated for 2 h at room temperature with a mixture of 200 μL of formic acid and 20 μL of 30% hydrogen peroxide (Lamoureux et al., 1973). Amino acids were analyzed using a Beckman amino acid analyzer.

For analysis of the triazine portion of the purified substances from the aqueous fraction of the 80% methanol extract, a portion of each substance was hydrolyzed under nitrogen in 200 μL of 6 N HCl at 50 °C for 8 h. Hydroxy derivatives of atrazine and its metabolites were obtained. The hydrolysates were authentic hydroxyatrazine and hydroxy derivatives of N-dealkylated products of atrazine using solvent system II and 2-propanol–28% ammonium hydroxide–water (80:10:10, v/v/v) (solvent system III).

RESULTS AND DISCUSSION

Translocation and Metabolism of Atrazine. Atrazine was readily absorbed and translocated by *S. alterniflora*. After 2 days of continuous exposure to the radiolabeled herbicide, approximately 90% of the absorbed atrazine was present in the shoots (Pillai et al., 1977). The extracts of *S. alterniflora* shoots were separated into chloroform, aqueous, and 80% methanol-insoluble fractions. The chloroform fraction had approximately 80% of the radioactivity after the initial 2-day exposure to radioactive herbicide (Figure 2). Radioactivity in this fraction declined rapidly during the first 5–6 days, followed by a slower decrease. There was a corresponding increase in radioactivity of the aqueous fraction, suggesting a precursor-product relation between components of the chloroform and aqueous fractions. In smooth cordgrass, atrazine and its N-dealkylation products isolated from the chloroform phase were accompanied by relatively polar metabolites (Pillai et al., 1977). The amount of radioactivity in these polar metabolites increased slowly with time, ranging from approximately 2% in plants treated for 2 days to about 11% in *S. alterniflora* 28 days after transfer

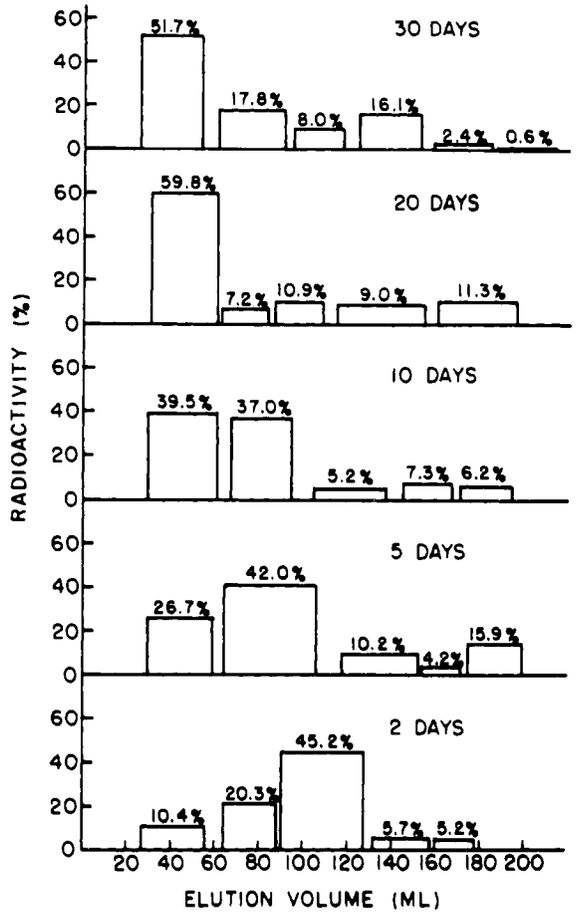

Figure 3. Radioactive fractions of the aqueous fraction of the *S. alterniflora* extract passed through a cation-exchange (50W-X2 Aminex resin, 200–235 mesh) column. Samples were taken at 0, 3, 8, 18, and 28 days after a 2-day exposure to [^{14}C]atrazine. Values at the top of each bar are the percent radioactivity in the particular fraction and is an average of two replications. Each replication consisted of four plants.

to the atrazine-free solution. Similar metabolites were reported in the chloroform fraction from sorghum. The chloroform-soluble atrazine metabolites in both sorghum and *S. alterniflora* appear to be minor metabolites of the herbicide and do not accumulate rapidly with time. Perhaps they are transitory intermediates that are converted to water-soluble atrazine metabolites (Shimabukuro et al., 1973).

Radioactivity in the 80% methanol-insoluble fraction of the *S. alterniflora* extract increased slowly with time, reaching about 18% of the total after 28 days in the atrazine-free nutrient solution (Figure 3). An increase in radioactivity in the insoluble fraction of sorghum extracts also occurred with time, but at a higher rate than in *S. alterniflora* (Lamoureux et al., 1973), reaching ca. 30% of the total by the end of the experimental period. The chemical form of the 80% methanol-insoluble metabolites of atrazine are unknown.

Water-Soluble Metabolites of Atrazine. Five–eight days after exposure of *S. alterniflora* to [^{14}C]atrazine, all but about 3% of the herbicide was metabolized to either chloroform or water-soluble substances which appear to persist for a considerable length of time (Figure 2). For example, after 28 days in atrazine-free nutrient solution, radioactivity in the aqueous extract of [^{14}C]atrazine-treated *S. alterniflora* represented ca. 58% of the total. The resistance of this marsh grass to atrazine is attributed to the rapid metabolism of the herbicide to nontoxic derivatives

Table I. Summary of Results of the Isolation of Some Water-Soluble Metabolites of Atrazine from *S. alterniflora*

no. and letter desig[a]	fractions from the anion exchange column[b]	total aqueous fraction[c]	compd no.
2A	30.7	11.9	1
2B[d]	5.3	2.0	
2C	24.6	9.5	2, 3, 3a
2D	8.6	3.4	10
2E	15.1	5.8	4[e]
2F[d]	6.8	2.6	
2G[d]	8.7	3.4	
3A	100	7.6	12
4A	28	8.6	13[f]
4B	72	22.3	14, 14a, 15, 15a, 16
5A	100	4.7	17
6A	100	22.8	8, 8a

[a] The numeral indicates the cation-exchange column fraction number. Fraction 1 contained no radioactivity. The letter designates the fraction(s) resulting when the cation-exchange column fraction was placed on the anion-exchange column. [b] Values are percentages of the radioactivity in the fractions from the anion-exchange column. [c] Values are percentages of the aqueous fraction (ca. 3.5 × 10^6 dpm) of the 80% methanol extract. [d] These fractions not processed further. [e] 2-Hydroxyatrazine. [f] 2-Hydroxy-4-amino-6-(isopropylamino)-*s*-triazine.

(Pillai et al., 1977). Atrazine metabolism in *Spartina* follows a pattern similar to that in sorghum, which is also very resistant to the herbicide, but damage is more rapid than in *S. alterniflora* (Lamoureux et al., 1973). For example, extracts of sorghum plants treated as described for *S. alterniflora* contained over 60% of the radioactivity in the aqueous fraction at the end of the 2-day exposure to the herbicide. Radioactivity increased slightly thereafter but decreased again after 20 days in the atrazine-free solution.

After passing the aqueous fraction of the *S. alterniflora* extract through a series of cation- and anion-exchange and Sephadex columns, it was apparent that there are numerous water-soluble metabolites of atrazine, most of which appear to have a high turnover rate during a 28-day period after exposure to the herbicide. Five radioactive fractions were obtained by passing the aqueous fraction through a cation-exchange column (Figure 3) and 79% of the radioactivity placed on the column was recovered. Fifty-two to sixty percent of the radioactivity accumulated in fraction 2 (fraction 1 contained no radioactivity) after the first 8 days and changes in the relative proportions of radioactivity in other fractions occurred during the final 20 days of the experiment.

Chromatographic treatment of *Spartina* extract fractions from the cation-exchange column resulted in the isolation of 16 individual water-soluble radioactive substances. To simplify discussion of the isolation and purification of the atrazine metabolites, each radioactive fraction from the cation-exchange column was assigned a number 2–6 as indicated above and each radioactive fraction from the anion-exchange column was given a letter. For example, fraction 2C represents the third radioactive fraction from the anion-exchange column when fraction 2 of the cation-exchange column was placed on the former column. Upon isolation, individual atrazine metabolites were arbitrarily given a number (Table I).

Passage of the cation-exchange column fractions separately through anion-exchange columns achieved further separation of the atrazine metabolites (Figure 4). Each radioactive fraction from the anion-exchange column was

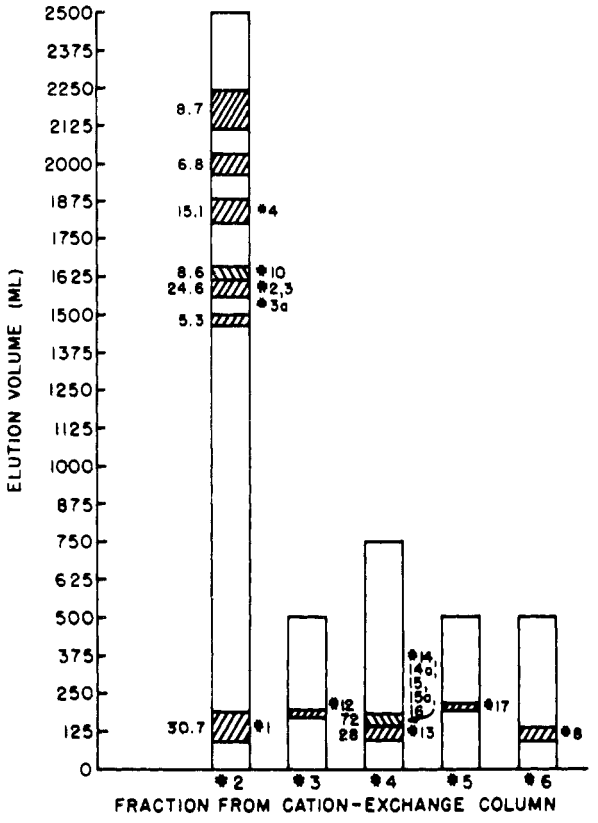

Figure 4. Separation of components in each fraction from the cation-exchange column by anion-exchange chromatography (AG-1 × 2, 200–400 mesh). Values on the left of each bar represent the percent radioactivity in the respective fractions from the column and values on the right designate a particular atrazine metabolite subsequently isolated from that fraction by TLC. The fractions from the cation-exchange column respond to the following elution volumes for "20 days" shown in Figure 3: 2, 30–63 mL; 3, 65–85 mL; 4, 90–109 mL; 5, 115–159 mL; 6, 165–172 mL.

subsequently passed through a Sephadex LH-20 column, but little separation of radioactive substances was achieved. This procedure seemed to only aid in the purification process by removing some nonradioactive substances. Fractions from the anion-exchange column containing relatively small amounts of radioactivity (2B, 2F, and 2G) were not processed further. Each radioactive component was further purified (or separated) by TLC using two solvent systems. A summary of the results obtained by these chromatographic procedures is given in Table I. TLC in two solvent systems indicated that fractions 2A, 2D, 2E, 3A, 4A, and 5A from the anion-exchange columns contained only a single radioactive component. 2-Hydroxyatrazine (4) was identified in fraction 2E and 2-hydroxy-4-amino-6-(isopropylamino)-*s*-triazine (13) was identified in fraction 4A (Table I), these two together comprised about 9% of the total radioactivity extracted from the *S. alterniflora* shoots. Hydrolysis appears to be the principal mode of atrazine detoxification in some resistant plants, but seems to be limited to benzoxazinone-containing species such as corn (Shimabukuro, 1967). Benzoxazinone is a naturally occurring catalyst of atrazine hydrolysis (Hamilton et al., 1962). However, several resistant and intermediately susceptible species do not produce the 2-hydroxy derivatives of atrazine (Shimabukuro et al., 1971). Whether they are formed with the aid of the benzoxazinone catalyst in *S. alterniflora* is not known. The dealkylated 2-hydroxy derivative of atrazine has also been identified in corn and other species (Shimabukuro et al., 1971). Whether dealkylation occurs before or after the hydrolysis of atrazine in *S. alterniflora* is not known, but apparently both alternatives occur in some plants. Additionally the results presented here show that both the hydrolysis and conjugation pathways of atrazine can occur in smooth cordgrass.

Table II. Chemical Nature of the *s*-Triazine Ring in the Individual Radioactive Components from the Aqueous Fraction of the 80% Methanol Extract of *S. alterniflora* Grown in [^{14}C]Atrazine

s-triazine[a]	water-soluble compounds isolated
2-hydroxyatrazine	1, 2, 4, 8a, 10, 16
2-hydroxy-4-amino-6-(isopropylamino)-*s*-triazine	3, 3a, 8, 14, 14a, 15, 17
2-hydroxy-4-amino-6-(ethylamino)-*s*-triazine	15a
2-hydroxy-4,6-diamino-*s*-triazine	

[a] 2-Hydroxy derivatives were obtained on hydrolysis of the water-soluble atrazine metabolites.

The remaining fractions from the anion-exchange column (2C, 4B, and 6A) contained two–five individual radioactive substances, none of which cochromatographed with the remaining 2-hydroxy derivatives of atrazine, 2-hydroxy-4-amino-6-(ethylamino)-*s*-triazine and 2-hydroxy-4,6-diamino-*s*-triazine (Table I).

On the basis of previous research on atrazine metabolism in sorghum (Shimabukuro et al., 1971), it was suspected that the remaining water-soluble metabolites are intermediates in the glutathione conjugation pathway. With the exception of compounds 4 and 13, each radioactive water-soluble substance isolated was subjected to acid hydrolysis so that the chemical nature of the *s*-triazine ring and possibly the conjugate group might be determined. The results of hydrolysis showed that about one-half the conjugated water-soluble metabolites were derivatives of atrazine and the other half derivatives of 2-chloro-4-amino-6-(isopropylamino)-*s*-triazine (Table II). 2-Hydroxy-4-amino-6-(ethylamino)-*s*-triazine was obtained on hydrolysis of only compound 15a and no 2-hydroxy-4,6-diamino-*s*-triazine was detected in any of the hydrolysates.

Amino acid analyses of the hydrolysates revealed little about the chemical nature of the conjugate portion of the water-soluble atrazine metabolites. In each case, the usual protein amino acids were detected in very low concentrations (<5 nmol), suggesting that the samples may have contained small amounts of protein. However, in some cases a particular amino acid was present in a higher relative amount than the others and was possibly part of the conjugate material. The hydrolysate of compound 2, for example, contained high relative proportions of glutamic acid, glycine, and an unidentified substance. Since glutathione is composed of these two amino acids, and cysteine, the results suggest that glutathione conjugation may occur in *Spartina* as in some other resistant plants (Shimabukuro et al., 1971). Samples of compounds 8, 8a, 14, 16, and possibly 3a contained glycine in higher relative proportions than "background" amino acids, suggesting that they may also be intermediates in glutathione conjugation as in sorghum. A conjugated intermediate containing glycine would be expected to occur early in the pathway (Lamoureux et al., 1973). Compound 14a contained valine, the significance of which is not clear. Amino acids at levels above background were not detected in the other samples.

Fraction 6A appeared to be a single radioactive substance through each of the columns and first thin-layer chromatographic step (Figure 1). However, TLC of frac-

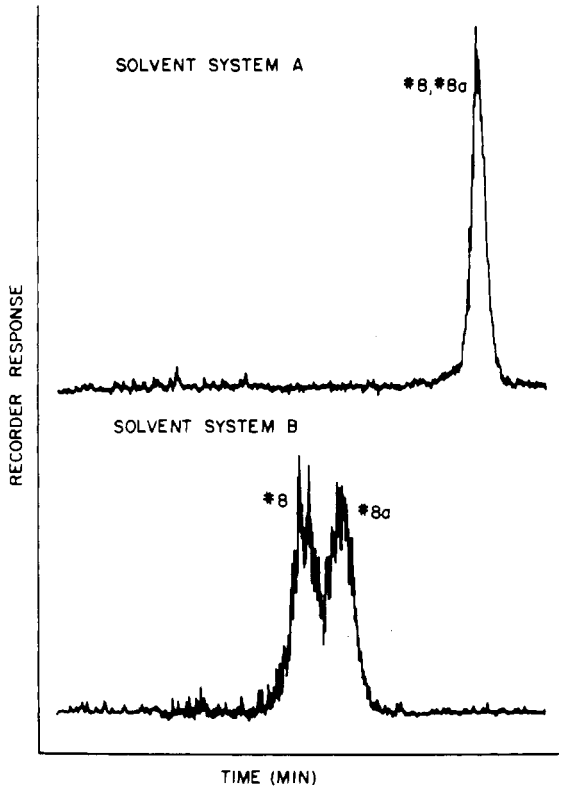

Figure 5. (Upper) Radioactive scan of TLC plate spotted with fraction 6A from the anion-exchange column and developed in solvent system A; (lower) radioactive scan of a TLC plate spotted with the radioactive substance(s) removed from the plate developed in solvent system A. The second plate was developed in solvent system B. 8 and 8a are reference numbers for the two substances resolved.

tion 6A using solvent system II showed that it was an equal mixture of two substances (Figure 5). On the basis of the hydrolysis data, they differ in the chemical nature of the *s*-triazine, 8a being a derivative of atrazine and 8 a derivative of 2-chloro-4-amino-6-(isopropylamino)-*s*-triazine. Compounds 3, 14, and 15 appeared chromatographically pure according to solvent system I. As before, compounds 15 and 15a differed according to the *s*-triazines, being derivatives of 2-chloro-4-amino-6-(isopropylamino)- and 2-chloro-4-amino-6-(ethylamino)-*s*-triazines, respectively. On the other hand, the other two pairs of radioactive substances differed according to the predominant amino acid obtained on hydrolysis rather than the triazine moiety of the molecule. For example, compounds 14 and 14a differed by glycine and valine, respectively. The chemical basis on which compounds 3 and 3a were separated by TLC is not clear (Table II).

Approximately 38% of the radioactivity from [^{14}C]-atrazine supplied to the *Spartina* plants for 2 days was recovered after 18 subsequent days of incubation in the atrazine-free nutrient solution. This represents 6.23 μmol of atrazine, or its metabolites, 87% (5.42 μmol) of which was present in the shoots. The distribution of radioactivity between the chloroform- and water-soluble fractions of shoot extracts was about 40:60, respectively. A relatively large portion of the radioactivity was concentrated in a few atrazine metabolites. Twenty-five percent of the total radioactivity in the shoots was distributed among only the three dealkylated metabolites of atrazine. This, combined with 9% of the total radioactivity in 2-hydroxyatrazine and 2-hydroxy-4-amino-6-(isopropylamino)-*s*-triazine, accounted for 34% of the atrazine absorbed and present in the shoots being distributed between only five metabolites. The remaining 66% of the radioactivity from absorbed atrazine was distributed among at least 12 metabolites representing between 2 and 6% of the herbicide taken up by the plant and translocated to the shoots.

The extent to which these substances are conjugated or otherwise metabolized after 30 days in the plant is not known. The final form of a large portion of the *s*-triazine ring appears to be 80% methanol-insoluble as suggested by extrapolation in Figure 2. This material appears to be tightly linked to the structural components of the cells. Accumulation of atrazine metabolites in this fraction of *S. alterniflora* is considerably slower than in sorghum (Lamoureux et al., 1973). How incorporation of the atrazine metabolites into this insoluble form relates to persistence in the environment is also not known.

ACKNOWLEDGMENT

We thank Paul Melius of the Chemistry Department for the amino acid analyses and Ciba-Geigy Corp., Greensboro, NC, for supplying radiolabeled atrazine and N-dealkylated derivatives of the herbicide.

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Received for review May 23, 1979. Accepted December 13, 1979. This work was supported by a grant from the Environmental Protection Agency, No. R-803835030 BY.