Conjugation of 2-Chloro-4,6-bis(alkylamino)-*s*-triazines in Higher Plants

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Atrazine and four closely related 2-chloro-*s*-triazines were rapidly metabolized to water-soluble compounds in the leaves of tolerant corn, sorghum, and sugarcane. The primary route of metabolism appeared to be the displacement of the 2-chloro group with glutathione or γ-glutamylcysteine. The resulting sulfide conjugates were among the most abundant water-soluble metabolites present 6 hr after treatment. The primary route of metabolism of the 2-chloro-*s*-triazines in susceptible barley was similar to that found in the tolerant species; however, the overall rate of metabolism in susceptible barley was much slower than in the tolerant species.

Three basic reactions have been identified in the metabolism of 2-chloro-4,6-bis(alkylamino)-*s*-triazines in various species of higher plants: hydrolysis of the 2-chloro group; *N*-dealkylation of the side chains; and displacement of the 2-chloro group with glutathione or γ-glutamylcysteine. *N*-Dealkylation of the 4- and/or 6-alkylamino side chains appears to be a general route of metabolism in higher plants (Shimabukuro *et al.*, 1966; Shimabukuro, 1967b; Funderburk and Davis, 1963). Hydrolysis of the 2-chloro group appears to be of primary importance only in those species of higher plants such as corn which contain 2,4-dihydroxy-7-methoxy-1,4-benzoxazin-3-one (benzoxazinone) (Hamilton, 1964; Shimabukuro, 1967b). Conjugation with glutathione or γ-glutamylcysteine is the primary route of metabolism of atrazine in sorghum (Lamoureux *et al.*, 1970). These conjugates also appear to be primary products of atrazine metabolism in corn if the herbicide is absorbed through the leaves. However, when atrazine is absorbed through the roots of corn, *N*-dealkylation, hydroxylation, and conjugation all play important roles (Shimabukuro *et al.*, 1970; Shimabukuro *et al.*, 1971). *In vitro* studies with plant homogenates have indicated that corn, sorghum, sugarcane, Johnsongrass, and Sudangrass all contain a glutathione *S*-transferase, an enzyme which catalyzes the formation of the glutathione conjugate of atrazine (Frear and Swanson, 1970). The glutathione *S*-transferase from corn catalyzed the conjugation of glutathione with atrazine, simazine, 2-chloro-4,6-bis(isopropylamino)-*s*-triazine (propazine), 2-chloro-4-(cyclopropylamino)-6-(isopropylamino)-*s*-triazine (cyprazine), and 2-chloro-4-(ethylamino-6-(*tert*-butylamino)-*s*-triazine (GS-13529). 2-Chloro-4-amino-6-(isopropylamino)-*s*-triazine and 2-hydroxy-4-(ethylamino)-6-(isopropylamino)-*s*-triazine also functioned as substrates with this enzyme, but they were much less active. 2-Methylthio-4,6-bis(isopropylamino)-*s*-triazine (prometryne), 2-methylthio-4-(ethylamino)-6-(isopropylamino)-*s*-triazine (ametryne), and 4,6-bis(isopropylamino)-2-methoxy-*s*-triazine (prometone) did not function as substrates. The enzyme was not detected in species which are susceptible to atrazine.

This paper presents direct evidence that conjugates with glutathione and γ-glutamylcysteine are formed from the first five above-mentioned 2-chloro-4,6-bis(alkylamino)-*s*-triazines in the leaves of corn, sorghum, sugarcane, and barley.

MATERIALS AND METHODS

Plant Material. Sorghum (*Sorghum vulgare* Pers., var. North Dakota 104), corn (*Zea mays* L., hybrid, KE 449), sugarcane (*Saccharum officinarum* L., hybrid C.P. 61-37 X C.P. 56-59), barley (*Hordeum vulgare* L., var. Dickson), oats (*Avena sativa* L., var. Rodney), pea (*Pisum sativum* L., var. Little Marvel), wheat (*Triticum aestivum* L., var. Chris), soybean (*Glycine max* Merr., var. Hawkeye), carrot (*Daucus carota* L., var. Sativa DC.), and lettuce (*Lactuca sativa* L., var. Great Lakes 659) were grown in vermiculite which was intermittently subirrigated with half-strength Hoagland's solution. The plants were maintained in a greenhouse under supplementary fluorescent light to ensure a minimum photoperiod of 12 hr. Sorghum plants which were to be root-treated were grown in aerated one-half strength Hoagland's solution in a controlled environment chamber with a relative humidity of 40%, a 12-hr photoperiod (15,000 lm/m²) at 27°C, and a nyctoperiod at 23°C. Plants were used in experiments after they reached the following ages: sorghum, 30 days; corn, 18 days; sugarcane (grown from cuttings), 6 months; and barley, 28 days. Other species were used as seedlings from 15 to 45 days old.

General Methods. The ^{14}C content of insoluble plant residues was determined by liquid scintillation techniques after the samples were combusted in oxygen (Shimabukuro, 1967b). All other quantitative measurements of radioactivity were made directly by liquid scintillation spectrometry. The effluent from column chromatograms was continuously monitored for radioactivity with a radioactive flow monitor. Radioactive zones on thin-layer chromatograms were qualitatively detected with a radiochromatogram scanner, or by autoradiography. Mass spectra were obtained with a Varian M-66 spectrometer equipped with a V-5500 console; samples were introduced *via* a solid sample probe which was heated to 25–50°C.

Thin-Layer and Paper Chromatography. Thin-layer chromatograms were developed to 15 cm on glass plates coated with a 250 μ-layer of silica gel HF_{254} (Brinkmann Instruments, Inc.). Ascending paper chromatograms were developed to 20 cm on Whatman no. 1 paper. The following solvents were used for developing thin-layer chromatograms: (A) 1-butanol–acetic acid–water (12:3:5, v/v/v); (B) benzene–ethyl acetate–acetic acid–water (25:50:30:3, v/v/v/v); (C) ethanol–water (7:3, v/v); (D) benzene–acetic acid–water (60:40:3, v/v/v); (E) ethyl acetate–xylene–formic acid–water (36:1:2:2, v/v/v/v); (F) benzene–acetic acid (25:2, v/v); (G) benzene–methanol (9:1, v/v). Paper chromatograms were developed with solvent system H, 88% phenol–water–ammonia (100:30:0.3, v/v/v).

s-Triazine Substrates. Radioactive and nonradioactive atrazine, 2-hydroxy-4-(ethylamino)-6-(isopropylamino)-*s*-triazine, propazine, simazine, GS-13529, ametryne, prometryne,

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prometone, and nonradioactive 2-chloro-4-amino-6-(ethylamino)-*s*-triazine, 2-hydroxy-4,6-bis(ethylamino)-*s*-triazine, and 2-hydroxy-4,6-bis(isopropylamino)-*s*-triazine were donated by CIBA–Geigy Corporation. Radioactive and nonradioactive cyprazine was donated by Gulf Research and Development Company. R. E. Kadunce provided the nonradioactive 2-chloro-4-amino-6-(isopropylamino)-*s*-triazine. The nonradioactive 2-hydroxy analogs of cyprazine and GS-13529 were prepared by stirring 5 mg of the 2-chloro-*s*-triazine in 3 ml of 6 *N* HCl at 50°C until the samples were completely dissolved. Thin-layer chromatography with solvent A indicated that conversion of the 2-chloro-*s*-triazines to the 2-hydroxy-*s*-triazines was nearly quantitative. The structures of the products were confirmed by infrared spectroscopy.

All radioactive *s*-triazine substrates were uniformly ring-labeled with ^{14}C and ranged in specific activity from 0.504 to 1.64 μCi/μmol. The radioactive 2-chloro-4,6-bis(alkylamino)-*s*-triazines were purified prior to use as previously described for atrazine by Shimabukuro (1967a). Prometryne and ametryne were purified in a similar manner by thin-layer chromatography with solvent G. Prometone required no further purification. Aqueous solutions of the radioactive substrates used for treating excised leaves or shoots in quantitative studies were prepared at a concentration of 1.5×10^{-5} *M*. These solutions were also used for *in vitro* enzymatic preparation of the glutathione conjugate standards. Similar solutions were prepared in half-strength Hoagland's solution for root-treating whole sorghum plants. Radioactive propazine, simazine, cyprazine, and GS-13529 were diluted with cold substrate to a final specific activity of 250,000 dpm/μmole. Aqueous solutions of these substrates (5×10^{-5} *M*) were used in large-scale treatments of excised sorghum leaves for isolation and identification of metabolites.

In Vitro Preparation of the Glutathione Conjugates of 2-Chloro-4,6-bis(alkylamino)-*s*-triazines. Glutathione conjugates of the radioactive *s*-triazines were prepared enzymatically and purified by thin-layer chromatography as described by Frear and Swanson (1970). The structures of these compounds can be derived from Figure 1.

In Vivo Preparation of the γ-Glutamylcysteine Conjugate of Atrazine. The standard γ-glutamylcysteine conjugate of atrazine was isolated from sorghum leaves as previously described by Lamoureux *et al.* (1970).

Quantitative Comparison of Metabolism. The leaves or shoots of various species were excised under water and 1.5 g of excised leaf or shoot tissue was treated by immersing the cut ends in 4 ml of solution contained in 35-ml conical centrifuge tubes. Each combination of tissue and radioactive substrate was replicated twice, except for sugarcane where four replicates were used. Water was added during treatment as necessary. All treatments were made in the controlled environment chamber under the daytime environment described. Excised leaves from sugarcane, corn, and sorghum were treated for 6 hr. Excised tissue from other species was treated for 20 hr. After treatment, each sample of tissue was rinsed with water and homogenized for 3 min with 50 ml of cold 80% methanol in a refrigerated blender. The homogenates were filtered and the residues were extracted two additional times in the same manner. The combined filtrates from each sample were concentrated to near dryness under vacuum at 37°C, dissolved in 25 ml of water, and partitioned three times with 25-ml portions of chloroform. Pairs of intact sorghum plants (total fresh weight 19 ± 1 g) were root-treated with 90 ml of each of the 2-chloro-4,6-bis(alkylamino)-*s*-triazines in a controlled environment chamber. The foliar tissue was harvested after

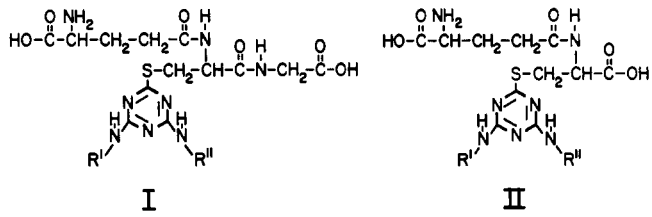

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Figure 1. General structures of the glutathione conjugate (I) and the γ-glutamylcysteine conjugate (II) of the five 2-chloro-4,6-bis-(alkylamino)-*s*-triazines. R′ and R″ are equal to the following alkyl groups in the various conjugates: atrazine, R′ = ethyl, R″ = isopropyl; GS-13529, R′ = ethyl, R″ = *tert*-butyl; cyprazine, R′ = isopropyl, R″ = cyclopropyl; simazine, R′ = R″ = ethyl; propazine, R′ = R″ = isopropyl

48 hr and extracted in the manner described for excised leaf tissue.

The chloroform fractions were examined by thin-layer chromatography with solvents D and F. The aqueous fractions were examined by ion exchange and thin-layer chromatography. The thin-layer chromatograms of the aqueous fractions were developed unidirectionally three times with solvent B and once with solvent A. For ion-exchange chromatography, aliquots of the aqueous fractions (approximately 120,000 dpm) were concentrated to dryness, dissolved in 0.5 to 1.0 ml of 0.2 *N* pH 2.1 buffer, and applied to a 1 × 105 cm water-jacketed column of AG 50W-X2 Aminex resin (200–325 mesh) (Bio-Rad Laboratories) at 15°C. The column was eluted at 0.3 ml/min with a pyridine–acetate buffer gradient developed from three chambers. The radioactivity in each peak was determined quantitatively. Preparation of the buffers and regeneration of the ion exchange resin has been described by Schroeder *et al.* (1962). Initially the first chamber of the gradient device contained 350 ml of 0.2 *N* pH 3.1 buffer and the second and third chambers each contained 350 ml of 2.0 *N* pH 5.0 buffer. Large volume changes which occurred with AG 50W-X2 resin during cycling made it necessary to remove the resin from the column before it was regenerated for the next analysis. The average deviation of elution volumes was ±4 ml.

Nonenzymatic rates of reaction of 2-chloro-4,6-bis(alkylamino)-*s*-triazines with glutathione were measured. The reactions were run in duplicate for 24 hr under nitrogen at 30°C with the following concentrations of ingredients: 0.2 *M* pH 6.9 phosphate buffer, 1×10^{-2} *M* reduced glutathione, and 4×10^{-5} *M* triazine substrate. The reactions were stopped and the products were analyzed by the method of Frear and Swanson (1970).

Isolation of Metabolites. Excised sorghum leaves (60 g/lot/300 ml of treating solution) were placed in the controlled environment chamber and treated with a 5×10^{-5} *M* solution of the appropriate herbicide. To obtain absorption of approximately 50% of the radioactivity, the leaves were incubated for 20 hr with GS-13529, cyprazine, and simazine, and for 40 hr with propazine. The tissue was extracted and partitioned as described above, except adjustments were made for the larger quantities of tissue. A 5:1 (v/w) ratio of solvent to tissue was used in extraction, and 2 ml each of chloroform and water per gram of tissue were used in partitioning. The number of lots treated and the total yield of water-soluble metabolites were as follows: four lots of propazine yielded 5.88 μmol; three lots of simazine yielded 3.26 μmol; one lot of GS-13529 produced 2.32 μmol; and one-half lot of cyprazine yielded 2.33 μmol.

The water-soluble metabolites were concentrated to dryness and suspended in 6 to 10 ml of 0.2 *N* pH 2.15 buffer. The

Table I. A Comparison of the Conversion of 2-Chloro-4,6-bis(alkylamino)-*s*-triazines to Water-Soluble Metabolites in Excised Leaves or Shoots of Sugarcane, Corn, Sorghum, and Barley

Compound	% water-soluble	% Chloroform-soluble	% Insoluble in 80% methanol	% Recovery of ^{14}C
		Sugarcane leaves[a]		
GS-13529	75	24	1	87
Atrazine	70	29	1	92
Propazine	67	31	2	86
Cyprazine	54	44	2	96
Simazine	35	64	1	96
		Corn leaves[a]		
Atrazine	75	20	5	96
GS-13529	70	25	5	98
Cyprazine	69	28	3	100
Propazine	68	28	4	92
Simazine	46	51	3	103
		Sorghum leaves[a]		
Atrazine	72	26	2	99
Propazine	65	33	2	100
GS-13529	64	34	2	98
Cyprazine	60	38	2	102
Simazine	48	50	2	104
		Barley shoots[b]		
Atrazine	37	62	1	90
Propazine	35	64	1	92
Simazine	32	67	1	92
Cyprazine	22	77	1	97
GS-13529	15	84	1	78
		in vitro Nonenzymatic[c,d]		
Cyprazine	17	83		
Simazine	10	90		
Propazine	10	90		
Atrazine	6	94		
GS-13529	4	96		

[a] Excised leaves of sugarcane, corn, and sorghum were analyzed 6 hr after treatment. [b] Excised barley shoots were analyzed 20 hr after introduction of the substrates. [c] The nonenzymatic reaction mixtures were analyzed 24 hr after introduction of the substrates. [d] The percent water-soluble and chloroform-soluble metabolites were determined by thin-layer chromatography instead of by partitioning.

extracts were chromatographed at 15°C on water-jacketed 1.75 × 105 cm columns of AG 50W-X2 resin (200–400 mesh). The columns were eluted with the same pyridine–acetate buffer gradient described in the previous section; however, the column flow rate was increased to approximately 0.6 ml/min. The elution volume of the major radioactive peak and the percent of the eluted radioactivity present in that peak for each of the preparations were as follows: propazine, 420 ml, 86%; simazine, 400 ml, 61.3%; GS-13529, 384 ml, 73%; cyprazine, 376 ml, 82%. Each major fraction was concentrated to dryness, dissolved in a minimum volume of water, and chromatographed with an acetic acid gradient at 15°C on a 1 × 95 cm water-jacketed column of AG 1-X2 (acetate) 200–400 mesh resin (Bio-Rad Laboratories) at a flow rate of 0.30 ml/min. The acetic acid gradient was varied slightly for each sample. However, a satisfactory gradient was produced by having 120 ml of water in each of the first two chambers and 120 ml of 3.0 *N* acetic acid in the third chamber. The gradient elution was followed by a final elution with 150 ml of 3.0 *N* acetic acid. The concentrations of minor metabolites eluted from this column were very low. In each case the recovery of radioactivity in the form of the major metabolites varied from 62 to 74%. Final purification of each of the major metabolites was achieved by chromatography at 15°C on a 1 × 100 cm water-jacketed column of Sephadex LH-20 (Pharmacia Fine Chemicals, Inc.) eluted with water at 0.10 ml/min. Each of the metabolites had an elution peak of 62 to 75 ml. Recoveries varied from 82 to 92%.

CHARACTERIZATION OF METABOLITES. A 0.10-μmol sample (25,000 dpm) of each of the major metabolites prepared from propazine, simazine, cyprazine, and GS-13529 was hydrolyzed under nitrogen in 200 μl of 6 *N* HCl in a sealed 0.500-ml Microflex tube (Kontes, Inc.) at 110°C for 16 hr. Each hydrolysate was evaporated to dryness several times and then treated for 2 hr at room temperature with a mixture of 200 μl of formic acid and 20 μl of 30% hydrogen peroxide. The reaction products were compared by paper chromatography to glutamic acid, glycine, and cysteic acid. Products were detected with ninhydrin. A 0.012-μmol sample of each of the above metabolites was also hydrolyzed in 6 *N* HCl at 50°C for 8 hr. These hydrolysates were compared by thin-layer chromatography in solvent A to the 2-hydroxy analogs of atrazine, propazine, simazine, cyprazine, and GS-13529. The nonradioactive standards were detected under ultraviolet light and the radioactive products were detected by autoradiography.

Individual samples of prometryne, ametryne, GS-13529, simazine, cyprazine, and the major metabolites isolated from sorghum were treated with Raney nickel (Lamoureux *et al.*, 1970). The reaction mixtures obtained from cyprazine and the major metabolite of cyprazine were purified by thin-layer chromatography with solvent E, while the other products were purified by thin-layer chromatography with solvent C. The reaction products were detected under ultraviolet light or with a radioactive strip scanner.

Samples (0.5 μmol) of prometryne and the major metabolite of simazine and propazine were individually refluxed with 2.5 ml of 0.5 *N* sodium methoxide in absolute methanol in 25-ml pear-shaped flasks protected from the atmosphere with drying tubes filled with calcium chloride. After 16 hr the samples were concentrated to dryness under vacuum and partitioned between 5 ml of water and four 5-ml portions of ethyl ether. The product in each of the combined ethyl ether fractions was purified by thin-layer chromatography with solvent G.

The purified products obtained from the reactions with Raney nickel and sodium methoxide were analyzed by mass spectrometry.

RESULTS

Quantitative Comparison of Metabolism. The herbicidal 2-chloro-4,6-bis(alkylamino)-*s*-triazines, atrazine, propazine, simazine, GS-13529, and cyprazine were rapidly metabolized to water-soluble metabolites in excised leaves of tolerant corn, sorghum, and sugarcane; however, the rate of conversion of these herbicides to water-soluble metabolites was much slower in excised shoots of susceptible barley (Table I). After 6 hr the average percent conversion of the 2-chloro-4,6-bis(alkylamino)-*s*-triazines to water-soluble metabolites in excised leaves of the tolerant species was 66% in corn, 62% in sorghum, and 60% in sugarcane, as compared to 28% after 20 hr in susceptible barley. The conversion of atrazine to water-soluble metabolites in excised leaves of other susceptible species during a 20-hr treatment was 17.0% in oats, 15.2% in peas, 14.4% in wheat, 3.83% in soybeans, 1.2% in carrots, and 1.2% in lettuce. The higher concentrations of water-soluble metabolites in the leaves of tolerant species as compared to susceptible species are significant in terms of selectivity. It confirms a previous observation (Shimabukuro *et al.*, 1970) that tolerance to 2-chloro-4,6-bis(alkylamino)-*s*-triazine is

dependent upon the plants' ability to metabolize these herbicides rapidly to nontoxic compounds, primarily water-soluble products.

The methylthio-*s*-triazines, ametryne and prometryne, and the methoxy-*s*-triazine, prometone, were not readily metabolized to water-soluble metabolites in excised sugarcane leaves during the 6-hr treatment period. The percent of water-soluble metabolites produced from these compounds was 8, 12, and 9%, respectively. This is an agreement with the *in vitro* studies of Frear and Swanson (1970) which showed that the methylthio- and methoxy-*s*-triazines were not substrates for glutathione *S*-transferase isolated from corn. Because of the slow rate of conversion of prometryne, prometone, and ametryne to water-soluble metabolites, these compounds were not studied further.

Chloroform-Soluble Metabolites. The chloroform fractions of the extracts obtained from excised leaves or shoots of sugarcane, corn, sorghum, and barley were examined by thin-layer chromatography. The predominant radioactive component in each chloroform fraction cochromatographed with the appropriate parent herbicide. However, detectable levels of metabolites were present in most extracts. The minor chloroform-soluble metabolites from excised sugarcane leaves and barley shoots cochromatographed with the mono-*N*-dealkylated standards. The chloroform fractions from corn and sorghum contained significant concentrations of metabolites which had R_f values lower than those of the mono-*N*-dealkylated *s*-triazines. Although mono-*N* dealkylation of atrazine occurs in both corn and sorghum (Shimabukuro, 1967b), failure to observe these metabolites in excised corn and sorghum leaves in this study may have been due to the short treatment period employed.

Insoluble Metabolites. The level of incorporation of radioactivity from 2-chloro-4,6-bis(alkylamino)-*s*-triazines into insoluble products was quite low in sugarcane, sorghum, corn, and barley during the treatment periods employed (Table I). This is consistent with a previous finding which showed that atrazine was slowly converted to 80% methanol-insoluble products in several species of higher plants (Shimabukuro, 1967b).

Water-Soluble Metabolites. The aqueous fractions from the leaves of whole sorghum plants and from excised leaves or shoots of sorghum, corn, sugarcane, and barley were examined by thin-layer chromatography. The aqueous fraction from each plant sample, except those treated with simazine, contained a principal component which chromatographed at R_f 0.38 ± 0.04. This R_f corresponded to within ±0.02 of the corresponding standard glutathione conjugates. Simazine-treated plant samples had a primary zone of radioactivity at R_f 0.28 which was within ±0.02 of the standard glutathione conjugate of simazine. The autoradiogram of the thin-layer chromatogram of the aqueous extracts obtained from excised sugarcane leaves was typical of the results obtained with the other species, and is shown in Figure 2.

The aqueous fractions from excised leaves of corn, sorghum, and sugarcane were subsequently chromatographed on columns of AG 50W-X2. The aqueous fractions from the leaves of sorghum plants root-treated with atrazine and excised barley shoots treated with atrazine were also chromatographed in a similar manner. Each extract contained a radioactive metabolite which cochromatographed with the appropriate standard glutathione conjugate, except for barley. A radioactive metabolite with the expected elution volume for a γ-glutamylcysteine conjugate was present in each extract. The chromatographic results (Figures 3 and 4) indicate that

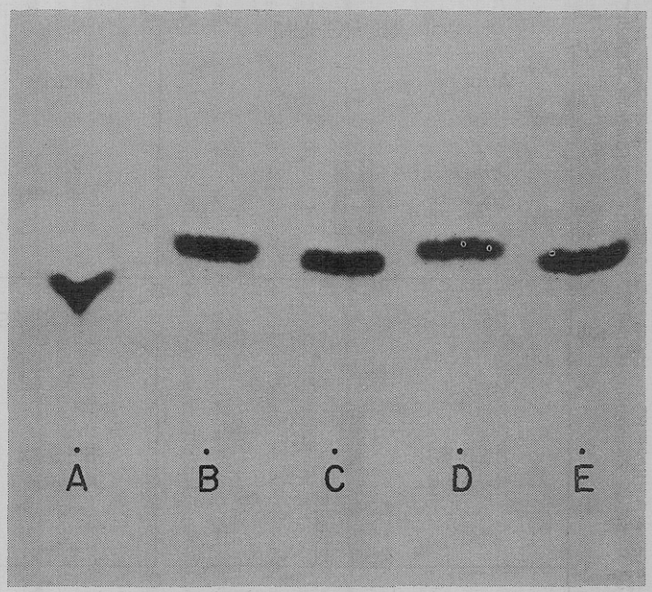

Figure 2. Thin-layer chromatogram of the aqueous phases of the extracts obtained from excised sugarcane leaves treated with: A, simazine; B, propazine; C, atrazine; D, cyprazine; E, GS-13529. Chromatogram developed unidirectionally, three times with solvent B and one time with solvent A. The parent triazine herbicides migrate to the solvent front with this system

conjugates of glutathione and/or γ-glutamylcysteine were major metabolites of the 2-chloro-4,6-bis(alkylamino)-*s*-triazines in the leaves of root-treated sorghum, and in the excised leaves of corn, sorghum, sugarcane, and barley.

A product–precursor relationship appears to exist between the γ-glutamylcysteine and glutathione conjugates of atrazine. The γ-glutamylcysteine conjugate was the principal product found in sorghum leaves after stem injection of seedlings with the glutathione conjugate of atrazine (Shimabukuro *et al.*, 1972). The crude preparations of glutathione *S*-transferase from corn used in this study contained a peptidase enzyme which converted the glutathione conjugate of atrazine to the γ-glutamylcysteine conjugate. These results suggest that γ-glutamylcysteine conjugates of atrazine and other 2-chloro-4,6-bis(alkylamino)-*s*-triazines are formed from the corresponding glutathione conjugates.

The water-soluble metabolites which appeared to be the γ-glutamylcysteine conjugates of propazine, simazine, GS-13529, and cyprazine were isolated from excised sorghum leaves. In each case the isolated metabolite had an elution volume of approximately 175 ml on an analytical column of AG 50W-X2 resin. This compared favorably with the elution volume of the γ-glutamylcysteine conjugate of atrazine (Figure 3). Each of these metabolites, except for that derived from simazine, had an R_f of 0.33 when chromatographed on a thin-layer plate in solvent A. The metabolite of simazine had an R_f of 0.28. Each metabolite produced a single spot which absorbed uv light, produced a positive reaction with ninhydrin, and was radioactive.

Glutamic acid and cysteic acid were liberated by strong acid hydrolysis and performic acid oxidation of the metabolites of propazine, GS-13529, cyprazine, and simazine. The simazine metabolite also liberated a small amount of glycine. These results indicated that the metabolites in question were γ-glutamylcysteine conjugates. The presence of a small amount of glycine in the hydrolysate of the simazine metabolite was probably due to contamination of the γ-glutamylcysteine conjugate with a small amount of the corresponding glutathione conjugate. Mild acid hydrolysis of each metabolite yielded a

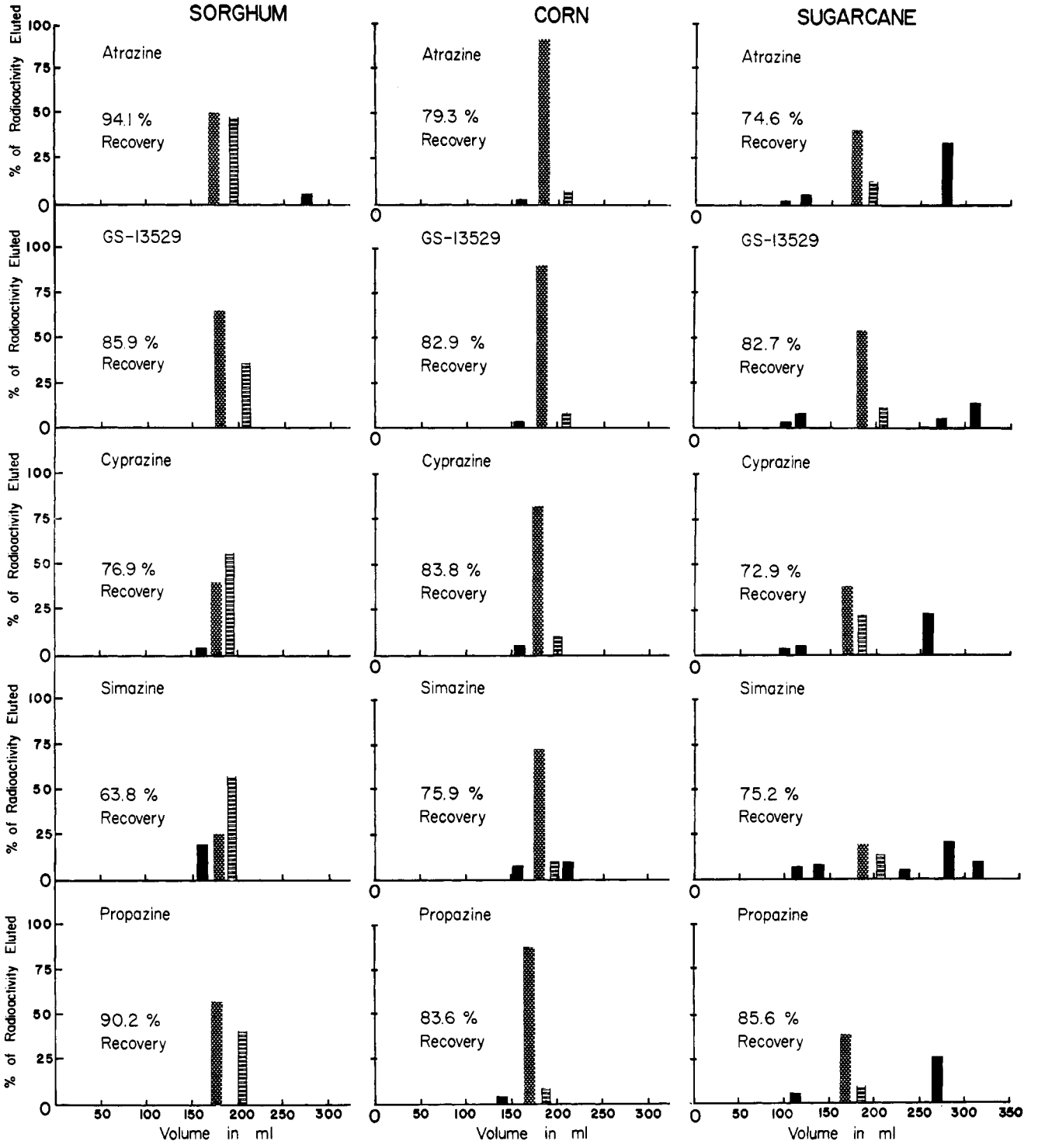

Figure 3. Chromatography of the aqueous phases of the extracts from excised leaves of sorghum, corn, and sugarcane on columns of AG 50W-X2 resin with pyridine–acetic acid buffer gradient. Bars etched with horizontal lines indicate metabolites which cochromatographed with standard glutathione conjugates. Cross-hatched bars indicate metabolites with elution volumes corresponding to γ-glutamylcysteine conjugates. All unidentified metabolites are indicated by solid bars

major radioactive product which cochromatographed on a thin-layer plate with the 2-hydroxy-4,6-bis(alkylamino)-*s*-triazine analog of the respective herbicide from which the metabolite was formed. A minor radioactive product was also liberated in very low yield by each of the metabolites. The minor products had R_f's of 0.06 to 0.16 lower than the major products. The minor products appeared to be 2-hydroxy-4-amino-6-(alkylamino)-*s*-triazines.

Mass spectrometry was used to further characterize the *s*-triazine moiety of each of the γ-glutamylcysteine conjugates isolated from sorghum. The conjugates of GS-13529 and cyprazine were converted to the 2,4-bis(alkylamino)-*s*-triazine analogs by hydrogenolysis with Raney nickel. The metabolites of simazine and propazine either gave poor yields with this reaction or underwent side reactions. They were converted to the 2-methoxy-4,6-bis(alkylamino)-*s*-triazines by refluxing the metabolites with sodium methoxide in absolute methanol. The primary radioactive product from each reaction was isolated by thin-layer chromatography and analyzed by mass spectroscopy. Each mass spectrum was

characterized by a large parent ion and a large M −15 ion; one of these ions was the base peak in each spectrum. Other principal peaks included those due to the loss of alkyl and alkylamino side chains by the processes discussed by Ross and Tweedy (1970). In addition, each spectrum contained a relatively abundant ion which corresponded to M − 30/2*e* which occurred at a nonintegral mass. The mass spectra of the derivatized *s*-triazines and the standards agreed. These spectra indicated that except for the peptide substituent on the 2 position of the triazine ring, the structures of the *s*-triazine portions of the conjugates were identical to the structures of the parent herbicides. This agreed with data obtained by mild acid hydrolysis and thin-layer chromatography. It was concluded that each of these metabolites had the general formula γ-L-glutamyl-*S*-[4,6-bis(alkylamino)-*s*-triazinyl-2]-L-cysteine, in which the 4- and 6-alkylamino groups remained unchanged from the parent herbicide.

DISCUSSION

In excised sorghum leaves the five 2-chloro-4,6-bis(alkylamino)-*s*-triazines were metabolized in the same manner during the first 6 hr. The primary route of metabolism appeared to be independent of the point of entry into the plant. The initial metabolic steps appeared to be conjugation with glutathione and conversion of the glutathione conjugate to the corresponding γ-glutamylcysteine conjugate. The general structures of the metabolites are shown in Figure 1. The glutathione and γ-glutamylcysteine conjugates were the two major metabolites present in sorghum leaves 6 hr after treatment. They accounted for an average of 94.5% of the radioactivity that was isolated from the aqueous extracts by column chromatography on AG 50W-X2. A trace of a 2-hydroxy-4-amino-6-(alkylamino)-*s*-triazine appeared to be liberated upon mild acid hydrolysis of each of the four isolated γ-glutamylcysteine conjugates. The presence of these compounds in the hydrolysates suggests that in each case a small amount of the parent herbicide underwent mono-*N*-dealkylation to a 2-chloro-4-amino-6-(alkylamino)-*s*-triazine, which was then converted to a water-soluble conjugate in the same manner as the parent triazine. If this is true, conjugation of the mono-*N*-dealkylated substrates is probably a minor mode of metabolism in excised sorghum leaves.

The primary route of metabolism of 2-chloro-4,6-bis(alkylamino)-*s*-triazines in the leaves of sugarcane, corn, and barley also appears to involve the formation of glutathione and γ-glutamylcysteine conjugates. The conjugates formed in the three species above cochromatographed with the appropriate standards on thin-layer plates and on columns of AG 50W-X2 resin, but they were not identified by chemical degradation and mass spectrometry.

The glutathione conjugate and the γ-glutamylcysteine conjugate are not the final products of atrazine metabolism in sorghum (Lamoureux *et al.*, 1970) or of 2-chloro-*N*-isopropylacetanilide (propachlor) metabolism in corn (Lamoureux *et al.*, 1971). Therefore, it seems likely that corresponding conjugates of *s*-triazines other than atrazine would also be transient intermediates. In excised sugarcane leaves a metabolite was produced from atrazine which constituted 32% of the radioactivity eluted from a column of AG 50W-X2 resin (elution volume 275 ml) (Figure 3). This metabolite was also produced in excised sorghum leaves, excised barley shoots, and in the leaves of root-treated sorghum seedlings (Figures 3 and 4). It was one of the principal products present 2 days after the γ-glutamylcysteine conjugate of atrazine was injected into the stem of a sorghum seedling. In excised

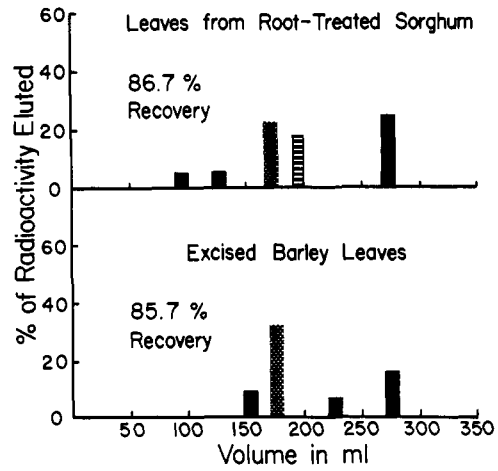

Figure 4. Aqueous phases of extracts from atrazine-treated tissue chromatographed on columns of AG 50W-X2 resin with a pyridine–acetic acid buffer gradient. Bars etched with horizontal lines indicate the metabolite cochromatographed with the standard glutathione conjugate of atrazine. Cross-hatched bars indicate the metabolite cochromatographed with the standard γ-glutamylcysteine conjugate of atrazine. Unidentified metabolites are indicated by solid bars

sugarcane leaves each of the 2-chloro-4,6-bis(alkylamino)-*s*-triazines formed a metabolite with an elution volume of approximately 275 ml (Figure 3). These metabolites appear to be comparable to the atrazine metabolite and are probably formed from their corresponding γ-glutamylcysteine conjugates.

Although the tolerant species (corn, sorghum, and sugarcane) were quite similar in their ability to convert the 2-chloro-4,6-bis(alkylamino)-*s*-triazines to water-soluble metabolites (Table I), notable differences were detected in the relative concentrations of the glutathione conjugates, γ-glutamylcysteine conjugates, and other products which were present in these aqueous extracts (Figure 3). In excised sorghum leaves an average of 47% of the radioactivity isolated from the aqueous extracts by column chromatography was in the form of glutathione conjugates and 47% was in the form of γ-glutamylcysteine conjugates. Comparable metabolites in corn leaves accounted for 8% of the radioactivity as glutathione conjugates and 83% as γ-glutamylcysteine conjugates. In excised sugarcane leaves an average of 14 and 39% of the radioactivity was isolated in the form of the glutathione and γ-glutamylcysteine conjugates, respectively, and 22% was in the form of products believed to be derived from γ-glutamylcysteine conjugates. These species differences probably re-

Table II. Comparison of the Relative Substrate Specificity of Excised Corn Leaves with the Substrate Specificity of Glutathione *S*-Transferase from Corn

Substrate	Glutathione *S*-transferase[a]	Excised corn leaves[b]
Atrazine	0.68	1.00
GS-13529	1.00	0.93
Cyprazine	0.52	0.92
Propazine	0.42	0.90
Simazine	0.05	0.62

[a] These data were adapted from Table 3 [*Phytochemistry* **9**, 2127 (1970)] and are presented here with the permission of *Phytochemistry*. The values are based on the mμmol of glutathione conjugate/mg protein/2 hr, but the values have been normalized for comparison. [b] These values are based on the percent conversion of the substrates to water-soluble metabolites in 6 hr, but the results have been normalized for comparison.

flect the relative activities of the various transferase and hydrolyase enzymes which are capable of reacting with the glutathione conjugates once they are formed. The differences among the plant species in the subsequent metabolism of the glutathione conjugates may not affect susceptibility since glutathione conjugation is the initial detoxication reaction. It is not certain, however, whether these differences in the subsequent rate of metabolism of the glutathione conjugates would have any lasting effect on the ultimate disposition of these triazines in the plants.

The substrate specificity of excised corn leaves for the various triazine herbicides is compared with the data of Frear and Swanson (1970) for glutathione *S*-transferase isolated from corn (Table II). The general order of reactivity was the same in both studies, with the exception of atrazine, which appeared to be more reactive than GS-13529 in excised corn leaves. Quantitatively, there was much less difference among these compounds in the amount of water-soluble metabolites formed in excised corn leaves than might have been expected on the basis of the *in vitro* studies. In excised leaves the rate of conjugation was so rapid that other factors such as absorption, translocation, and penetration of the substrate into the site of metabolism may have been limiting factors.

It is not certain whether the formation of glutathione and γ-glutamylcysteine conjugates is an enzymatic reaction in excised barley shoots. The metabolism of atrazine to water-soluble metabolites was faster in barley (36.9% in 20 hr) than in the other susceptible species (1.2 to 17% in 20 hr). The rate of formation of water-soluble metabolites in barley seems to be faster than expected on the basis of a nonenzymatic reaction (Table I). However, a glutathione *S*-transferase was not detected in barley leaves (Frear and Swanson, 1970); the substrate specificity of excised barley leaves is quite different than that observed in the tolerant species, and the rate of conversion of the substrates to water-soluble metabolites was much slower in barley than in the tolerant species.

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The following papers form Part II of a symposium on The Chemistry of Essential Oils and Related Products. For Part I, see J. AGR. FOOD CHEM., pages 1074–1123, November/December 1971.

Oxymercuration of Terpenoid Alcohols

Gottfried Brieger* and Elizabeth P. Burrows

Oxymercuration of geraniol was shown to give two products, a bicyclic ketal and the related alcohol 2-[2-methyl-5-isopropyl tetrahydrofuranyl]ethanol. No normal addition products were found. Oxymercuration of *cis*-linalool oxide gave as single product the related bicyclic ether. Myrcenol also cyclized in high yield on oxymercuration to 1-[2,6,6-trimethyltetrahydropyranyl]ethanol. Oxymercuration with less than one equivalent of mercuric acetate gave the rearranged ether, 2-allyl-6,6-dimethyltetrahydropyran. Ethoxymercuration proceeded normally and selectively at the terminal double bond of both geraniol and farnesol, yielding the corresponding monoethoxy ethers 7-ethoxygeraniol and 11-ethoxyfarnesol.

Intramolecular cyclizations during the addition of mercuric salts to olefins were noted earlier by Sand and Singer (1902, 1903). They observed that 2-allylphenol cyclized during reactions with mercuric salts to give 2-methyl-2,3-dihydrobenzofuran. Similarly, α-terpineol was found to give 1,8-cineole, a report recently confirmed by Coxon *et al.* (1968). Cyclization of β-(7-norbornenyl)ethanol was reported by Bly *et al.* (1967). Cyclization was also observed during the oxymercuration of cyclooctadienes by Bordwell and Douglass (1966) and Moon *et al.* (1969). Moon and Waxman (1969) also noted the formation of bicyclic ethers on oxymercuration of 2-(2-cyclohexenyl)ethanol and 2-(2-cyclopentenyl)ethanol.

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