# *In Vitro* Conjugation of Chloroacetanilide Herbicides and Atrazine with Thiols and Contribution of Nonenzymatic Conjugation to Their Glutathione-Mediated Metabolism in Corn†

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The contribution of nonenzymatic conjugation of acetochlor, alachlor, metolachlor, pretilachlor, and atrazine with glutathione (GSH) in the metabolism of these herbicides by roots and shoots of the Cargill 7567 and Northrup-King 9283 hybrids of corn (*Zea mays* L.) was investigated. Treatment with 50 $\mu$M of each herbicide revealed the following order of chloroacetanilide phytotoxicity: acetochlor > alachlor > metolachlor > pretilachlor. Atrazine did not injure any of the corn hybrids. Cargill 7567 was more tolerant than Northrup-King 9283 to metolachlor and pretilachlor, but the two corn lines did not differ in their response to acetochlor and alachlor. Total nonprotein thiol, GSH, and cysteine contents were similar in the two corn hybrids. Glutathione *S*-transferase (GST) activities were comparable in the two corn hybrids. Chloroacetanilide-specific GST activities were 2–4-fold greater in roots than in shoots of either hybrid, and enzymatic conjugation with GSH was more efficient with acetochlor and alachlor than with metolachlor and pretilachlor as substrates. Assays on the *in vitro* chemical reactivities of acetochlor, alachlor, metolachlor, pretilachlor, and atrazine with GSH and cysteine revealed that the higher the ratio of thiol to herbicidal substrate concentrations, the higher the amount of conjugate formed. Nonenzymatic conjugation with GSH may contribute significantly to the overall glutathione-mediated metabolism of chloroacetanilide herbicides and atrazine in corn and seems to be dependent on the concentration and molecular structure of the herbicidal substrate as well as on the GSH content of plant tissues (root vs shoot).

## INTRODUCTION

Chloroacetanilide and chloro-*s*-triazine herbicides are used to control grass and broadleaf weeds in a variety of crops. Pretilachlor is a chloroacetanilide herbicide used exclusively in rice (*Oryza sativa* L.), whereas atrazine and the chloroacetanilides acetochlor, alachlor, and metolachlor are commercially important herbicides used for weed control in corn (Esser et al., 1975; LeBaron et al., 1988; Sharp, 1988). The chemical structures of these five herbicides are shown in Figure 1.

Because of the presence of a reactive chlorine in their molecules, chloroacetanilides are known to act as potent alkylating agents interacting with biologically active nucleophiles (Hamm, 1972; Jablonkai and Dutka, 1989; Jaworski, 1969; Sirois, 1972). Nevertheless, the exact mechanism of action of these herbicides is not known at the present time (Hamm, 1974; LeBaron et al., 1988; Sharp, 1988).

Conjugation with glutathione (GSH) and/or homoglutathione (hGSH) has long been established as the major metabolic reaction by which chloroacetanilide herbicides and atrazine are detoxified in higher plants (Frear and Swanson, 1970; Gronwald, 1989; Lamoureux et al., 1991). A positive correlation between plant tolerance to chloroacetanilide herbicides and GSH or hGSH levels has been demonstrated in studies with tolerant and sensitive plant species (Breaux, 1987; Breaux et al., 1987; Jablonkai and Dutka, 1985; Jablonkai and Hatzios, 1991). In addition to GSH conjugation, hydrolysis of atrazine to its hydroxy derivative is known to contribute appreciably to the crop selectivity of this herbicide (Esser et al., 1975).

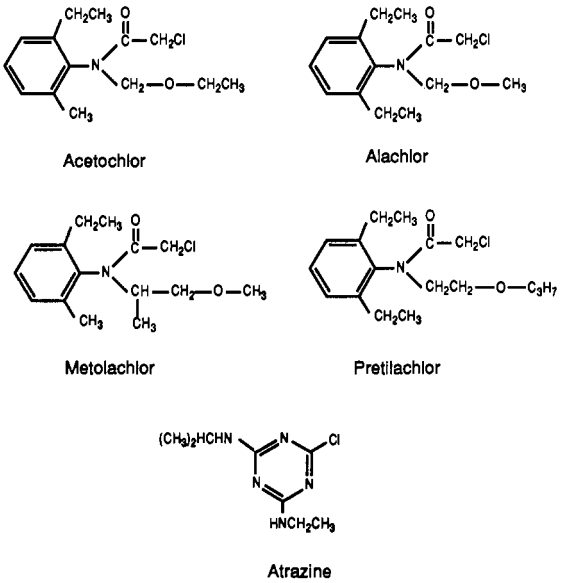


**Figure 1.** Chemical structures of chloroacetanilide herbicides and atrazine.

Conjugation of chloroacetanilides and atrazine may proceed either enzymatically, mediated by glutathione *S*-transferase (GST) enzymes, or nonenzymatically (Frear and Swanson, 1970; Gronwald, 1989; Lamoureux et al., 1991; Leavitt and Penner, 1979; Mozer et al., 1983; Timmerman, 1989). After their formation, the GSH

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0021-8561/93/1441-1736$04.00/0 

conjugates of several herbicides undergo further catabolism resulting in the formation of cysteine conjugates, which are commonly acylated with malonic acid (Lamoureux and Rusness, 1983; Lamoureux et al., 1991). Nevertheless, cysteine conjugates of chloroacetanilide herbicides may also result from their direct conjugation with cysteine (Jablonkai and Dutka, 1992; Leavitt and Penner, 1979).

The relative contribution of nonenzymatic conjugation in the overall formation of herbicidal conjugates with thiols such as GSH and cysteine remains unclear. Nonenzymatic conjugation of chloroacetanilides with thiol compounds has been demonstrated *in vitro* (Gronwald et al., 1987; Jablonkai and Dutka, 1992; Lamoureux et al., 1971; Zama and Hatzios, 1986) and appears to be strongly dependent on the pH of the reaction mixture (Leavitt and Penner, 1979). The potential contribution of the rate of nonenzymatic conjugation in the overall metabolic conjugation of chloroacetanilide herbicides with GSH in tolerant and susceptible plants has been examined recently by Scarponi et al. (1991), but only in shoot tissues of these plant species. Information on the potential contribution of nonenzymatic conjugation in the overall GSH-mediated metabolism of chloroacetanilides and atrazine in root tissues of tolerant or susceptible plants is not presently available.

The objectives of the present study were to determine (a) the *in vitro* reactivities of the nonenzymatic conjugation of acetochlor, alachlor, metolachlor, pretilachlor, and atrazine with the thiols GSH and cysteine and (b) the potential contribution of nonenzymatic GSH conjugation in the overall glutathione-mediated metabolism of these herbicides in root and shoot extracts of the corn hybrids Cargill 7567 and Northrup-King 9283.

## MATERIALS AND METHODS

**Chemicals.** Analytical grade standard of acetochlor [2-chloro-*N*-(ethoxymethyl)-6′-ethylacet-*o*-toluidide] was supplied by Nitrokemia (Fuzfogyartelep, Hungary); alachlor [2-chloro-2′,6′-diethyl-*N*-(methoxymethyl)acetanilide] was supplied by Monsanto Co. (St. Louis, MO); metolachlor [2-chloro-6′-ethyl-*N*-(2-methoxy-1-methylethyl) acet-o-toluidide], pretilachlor [2-chloro-2′,6′-diethyl-*N*-(2-propoxyethyl) acetanilide], and atrazine [6-chloro-*N*-ethyl-*N*′-(1-methylethyl)-1,3,5-triazine-2,4-diamine] were supplied by CIBA Plant Protection (Greensboro, NC). [*carbonyl*-$^{14}$C]Acetochlor (specific activity 37 MBq/mmol) was synthesized from barium [$^{14}$C]carbonate as previously described (Jablonkai et al., 1982). [*carbonyl*-$^{14}$C]Alachlor (specific activity 70.7 MBq/mmol), [*carbonyl*-$^{14}$C]metolachlor (specific activity 2.2 GBq/mmol), [U-*ring*-$^{14}$C]pretilachlor (specific activity 446 MBq/mmol), and [U-*ring*-$^{14}$C]atrazine (specific activity 402 MBq/mmol) were obtained from Monsanto and CIBA Plant Protection. The purity of all $^{14}$C-labeled herbicides ranged from 95 to 99%. Reduced glutathione, cysteine, and all other reagents were purchased from Sigma Chemical Co. (St. Louis, MO) or from Fisher Scientific (Pittsburgh, PA).

**Herbicide Effects on Corn Hybrids.** Three seeds of either corn hybrid (Cargill 7567 and Northrup-King 9283) were placed in 250-mL polystyrene cups filled with vermiculite. After planting, the cups were watered with 50 mL of tap water or an aqueous solution containing 50 μM of each herbicide. The cups were then placed in a greenhouse at 25 °C with 14-h photoperiod and 500 mmol $m^{-2}$ $s^{-1}$ photon flux density. Cups containing vermiculite and the germinating corn seedlings were watered every 2 days from the top, and after 14 days, they were supplied with 50 mL of half-strength Hoagland's solution. After 3 weeks, corn seedlings were harvested and separated into shoot and root sections. Length and fresh weight of these tissues were then measured to evaluate the comparative phytotoxicity of the four chloroacetanilides and atrazine on the growth of the two corn hybrids. The experiment was repeated twice, and all treatments within each experiment were replicated three times.

**Total Nonprotein Thiol, GSH, and Cysteine Content of Corn Seedlings.** Total nonprotein thiol contents in root and shoot extracts from 4-day-old etiolated seedlings of both corn hybrids were determined spectrophotometrically by using the method described by Fedtke (1981) with slight modifications. Tissues (0.5 g) were dried with liquid nitrogen and then homogenized in 2 mL of ice-cold aqueous ethanol (70%) with a mortar and pestle. The homogenates were centrifuged at 20000*g* for 20 min. A 1-mL portion of the supernatant was then added to the assay cuvettes containing 2 mL of 0.2 M Tris-HCl buffer (pH 7.0) and 1.5 μM 5,5′-dithiobis(2-nitrobenzoic) acid (DTNB) in 0.1 mL of 96% ethanol. The mixture was shaken, and the yellow color was read at 412 nm in Beckman DU-6 spectrophotometer. Blank cuvettes contained the assay mixture without the plant extract. The experiment was repeated twice, and all treatments within each experiment were replicated three times.

Cysteine content of root and shoot tissues was determined according to the method of Gaitonde (1967), which determines cysteine directly in the presence of other naturally occurring amino acids and peptides. The reaction mixture contained 0.5 mL of shoot or root extracts obtained as described earlier, 0.5 mL of glacial acetic acid, and 0.5 mL of acid/ninhydrin reagent prepared with 250 mg of ninhydrin, 6 mL of glacial acetic acid, and 4 mL of 37% hydrochloric acid. In an acetic acid–hydrochloric acid solution, ninhydrin reacts only with cysteine and not with GSH or homocysteine. The assay tubes were covered with parafilm and heated at 100 °C for 10 min. Then the tubes were cooled with tap water and diluted to 5 mL with 95% ethanol. The pink color formed was then read spectrophotometrically at 560 nm. The experiment was repeated twice, and all treatments within each experiment were replicated three times.

**Preparation of Enzyme Extracts.** Seeds of Cargill 7567 and Northrup-King 9283 corn were soaked for 2 h in aerated tap water and germinated on moistened filter paper in a dark incubator set at 28 °C. After 4 days, etiolated seedlings of corn were harvested and divided into root and shoot (stems plus leaves) sections. The tissues were frozen in liquid nitrogen, pulverized with a mortar and pestle, and then homogenized by further grinding after the addition (5 mL/g of fresh tissue) of ice-cold phosphate buffer (0.1 M, pH 6.8) containing 5% v/v polyvinylpolypyrrolidone (PVPP) and 1 mM sodium sulfate to the mortar. The homogenate was filtered through two layers of Miracloth (Calbiochem, LaJolla, CA), and the filtrate was centrifuged at 20000*g* for 20 min at 4 °C. The supernatant was used as crude enzyme extract for GST assays.

Total protein content in the crude enzyme extracts was determined spectrophotometrically by the Coomassie blue G-250 dye-binding assay described by Bradford (1976) using bovine serum albumin (BSA) as a standard.

**Assays for Conjugation of GSH with Chloroacetanilides and Atrazine.** The assays used for the enzymatic (GST-mediated) and nonenzymatic conjugation of the five herbicides with GSH were as follows. GST activities in root and shoot extracts from the two corn hybrids were assayed using radiolabeled samples of all herbicides. The reaction mixture contained 0.375 mL of potassium phosphate buffer (0.1 M, pH 7.6), 0.125 mL of crude enzyme extract, 5 μL of $^{14}$C-labeled herbicidal substrate at two concentrations (0.5 and 50 μM), and 0.05 mL of GSH (0.9 mM). The herbicidal solutions were made with aqueous ethanol (70%), and the reaction was initiated by the addition of GSH to the reaction mixture. The optimum pH for crude GST enzymes from corn or other plants has been reported to range from 6.7 to 9.4 (Lamoureux et al., 1991). Most GST assays with crude plant extracts are conducted in media with pH of 7.4–7.8 (Mozer et al., 1983). After 60 min of incubation at 25 °C, the reaction was stopped by the addition of 100 μL of 5% trichloroacetic acid (TCA). The reaction mixture was then extracted with ethyl acetate (4 × 0.5 mL), and radioactivity corresponding to the $^{14}$C-labeled herbicide–GSH conjugate (aqueous phase) and nonconjugated $^{14}$C-labeled herbicides (organic phase) was determined by liquid scintillation counting (Beckman LS 5800TA Model, Fullerton, CA). The rates of enzymatic conjugation of the herbicides with GSH [nmol (mg of protein)$^{-1}$ h$^{-1}$] were calculated from data obtained with crude GST extracts after the nonenzymatic conjugation rates were subtracted.

The *in vitro* reactivities of the nonenzymatic conjugation of the herbicides with GSH and cysteine were performed as

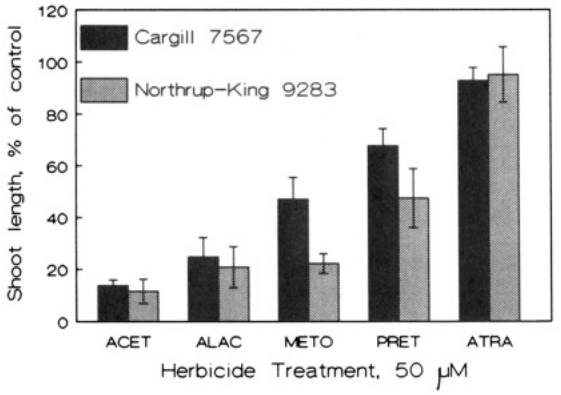


**Figure 2.** Growth response of Cargill 7567 and Northrup-King 9283 corn seedlings at 3 weeks after treatment with 50 μM of the herbicides acetochlor (ACET), alachlor (ALAC), metolachlor (METO), pretilachlor (PRET), and atrazine (ATRA). Data presented are the means of six replications ± standard deviation of each mean. Mean shoot length of untreated seedlings was 23.5 ± 2.7 cm

described in the previous paragraph after the enzyme extract was substituted with an equivalent aliquot of potassium phosphate buffer (0.1 M, pH 7.6). In addition, the reactions were performed with two different concentrations (0.9 and 9 mM) of GSH and cysteine and a broader range of herbicidal substrate concentrations (0.5, 1, 5, 10, 25, and 50 μM). The rates of nonenzymatic conjugation of the herbicides with GSH and cysteine (nanomoles of conjugate formed) were plotted against the concentrations of the herbicidal substrates, and the reactivities were characterized from the slopes of the obtained curves at each concentration of GSH or cysteine. All experiments were repeated twice, and all treatments within each experiment were replicated three times.

Rennenberg (1982) has reported that plant cells contain millimolar concentrations of GSH ranging from 0.1 to 0.7 mM in the cytosol and from 1.0 to 3.5 mM in the chloroplast. Thus, the thiol concentrations used in the present study represent a physiologically practical (0.9 mM) and an excessive concentration (9 mM). Similarly, the range of herbicidal concentrations used includes physiologically practical (0.5–10 μM) and high concentrations (25 and 50 μM) of each herbicide tested.

## RESULTS AND DISCUSSION

**Herbicide Effects on Corn Seedlings.** Data in Figure 2 show the shoot lengths of Cargill 7567 and Northrup-King 9283 corn seedlings at 3 weeks after treatment with 50 μM of the four chloroacetanilide herbicides and atrazine. Overall, the growth responses of the two corn hybrids treated with the four chloroacetanilide derivatives revealed the following order of phytotoxicity: acetochlor > alachlor > metolachlor > pretilachlor. Shoot lengths of corn seedlings treated with acetochlor were significantly reduced (20% of control), whereas shoot lengths of seedlings treated with atrazine were comparable to those of control seedlings (Figure 2).

The differential response of the two corn hybrids to the chloroacetanilide herbicides and atrazine was not unexpected, since atrazine acts as a photosynthetic inhibitor causing chlorosis rather than reducing shoot growth in treated plant seedlings (Esser et al., 1975). Cargill 7567 was more tolerant to metolachlor and pretilachlor than Northrup-King 9283, but the two corn lines did not differ in their response to acetochlor, alachlor, and atrazine. These results are consistent with previous reports of Cargill 7567 tolerance and Northrup-King 9283 susceptibility to the herbicide metolachlor (Cottingham and Hatzios, 1992; Cottingham et al., 1993; Rowe and Penner, 1990; Rowe et al., 1991).

**Total Nonprotein Thiol, GSH, and Cysteine Content of Corn Seedlings.** The selective phytotoxicity of chloroacetanilide herbicides may be affected by factors influencing the GSH-mediated metabolism of these herbicides in higher plants. The conjugation of chloroacetanilides with GSH in plants treated with these herbicides may be influenced by (a) the endogenous content of GSH and other nonprotein thiols, (b) the catalytic efficiency and substrate specificity of GST enzymes, and (c) the interference effects of substituents present in the aryl and *N*-alkyl chain portions of the molecular structures of these herbicides (Scarponi et al., 1991).

**Table I. Content of Nonprotein Thiols of 4-Day-Old Etiolated Seedlings of Cargill 7567 and Northrup-King 9283 Corn[a]**

| | | nmol/g of fresh wt | | |
|---|---|---|---|---|
| hybrid | tissue | total nonprotein thiols | glutathione | cysteine |
| Cargill 7567 | roots | 407 ± 32 | 304 ± 24 | 103 ± 14 |
| | shoots | 613 ± 41 | 522 ± 35 | 91 ± 9 |
| Northrup-King 9283 | roots | 395 ± 27 | 301 ± 21 | 94 ± 10 |
| | shoots | 648 ± 47 | 582 ± 42 | 66 ± 8 |

[a] Data represent mean thiol values from six replications ± standard deviation of each mean.

Data in Table I show that the contents of total nonprotein thiol, GSH, and cysteine in root and shoot tissues of the two corn hybrids were very similar. Therefore, the differential phytotoxicity of the four chloroacetanilides on either of the two corn hybrids (Figure 2) does not appear to result from differences in the endogenous content of nonprotein thiols that may be involved in the metabolic detoxification of these herbicides. Furthermore, it is evident that the differential growth responses of Cargill 7567 and Northrup-King 9283 corn to metolachlor and pretilachlor are not determined by the levels of endogenous thiols.

**GST-Mediated Conjugation of Chloroacetanilides and Atrazine with GSH.** *In vitro* assays with crude extracts of glutathione *S*-transferase (GST) from either of the two hybrids showed that enzymatic conjugation of chloroacetanilide herbicides with GSH was more efficient (3–12-fold) in corn roots than in shoots (Table II). Atrazine-specific GST activities in roots of the two corn hybrids were similar to those obtained from shoots (Table II).

The specificities of GSTs extracted from shoots and roots of the two corn hybrids appeared to be dependent on the concentration of the herbicidal substrate. At 0.5 μM of substrate, small but nonsignificant differences were found among GST activities mediating the conjugation of chloroacetanilides with GSH (Table II). However, GST activity from roots of both corn hybrids exhibited relatively low specificity for mediating the conjugation of pretilachlor with GSH. At 50 μM of the herbicidal substrate, shoot GST activity mediating the conjugation of acetochlor, alachlor, and pretilachlor with GSH was 2-fold greater than that of metolachlor (Table II). Root GST activities mediating the conjugation of acetochlor and alachlor with GSH were 2- or 3-fold greater than that of metolachlor and pretilachlor (Table II). O'Connell et al. (1988) have also reported a 2–3-fold greater GST activity from corn shoots, mediating the conjugation of alachlor with GSH compared to that of metolachlor.

Shoot GST activity specific for atrazine was 2–6-fold greater than that mediating the conjugation of chloroacetanilide herbicides with GSH, in either of the corn hybrids and at both concentrations of the herbicidal substrate

**Table II. GST Activity from Shoot and Root Extracts of Cargill 7567 and Northrup-King 9283 Corn Mediating the Conjugation of Chloroacetanilide Herbicides and Atrazine with GSH[a]**

| | | GST activity, nmol (mg of protein)$^{-1}$ h$^{-1}$ | | | |
|---|---|---|---|---|---|
| | | 0.5 μM concn of herbicidal substrate | | 50 μM concn of herbicidal substrate | |
| hybrid | herbicide | shoots | roots | shoots | roots |
| Cargill 7567 | acetochlor | 0.05 ± 0.005 | 0.6 ± 0.04 | 2.2 ± 0.2 | 11.1 ± 0.9 |
| | alachlor | 0.07 ± 0.005 | 0.8 ± 0.06 | 2.2 ± 0.2 | 11.6 ± 0.8 |
| | metolachlor | 0.07 ± 0.007 | 0.6 ± 0.05 | 0.9 ± 0.07 | 5.4 ± 0.5 |
| | pretilachlor | 0.05 ± 0.005 | 0.2 ± 0.02 | 1.8 ± 0.2 | 6.2 ± 0.6 |
| | atrazine | 0.16 ± 0.01 | 0.2 ± 0.01 | 5.9 ± 0.5 | 5.1 ± 0.4 |
| Northrup-King 9283 | acetochlor | 0.07 ± 0.008 | 0.8 ± 0.07 | 1.8 ± 0.2 | 9.2 ± 0.9 |
| | alachlor | 0.08 ± 0.008 | 0.8 ± 0.08 | 1.5 ± 0.1 | 12.1 ± 0.9 |
| | metolachlor | 0.05 ± 0.006 | 0.5 ± 0.04 | 0.7 ± 0.06 | 4.8 ± 0.4 |
| | pretilachlor | 0.04 ± 0.05 | 0.2 ± 0.01 | 1.7 ± 0.1 | 5.0 ± 0.5 |
| | atrazine | 0.16 ± 0.01 | 0.2 ± 0.02 | 4.6 ± 0.4 | 6.2 ± 0.5 |

[a] Data represent mean GST activities from six replications ± standard deviation of each mean. For reaction mixture and conditions see Materials and Methods.

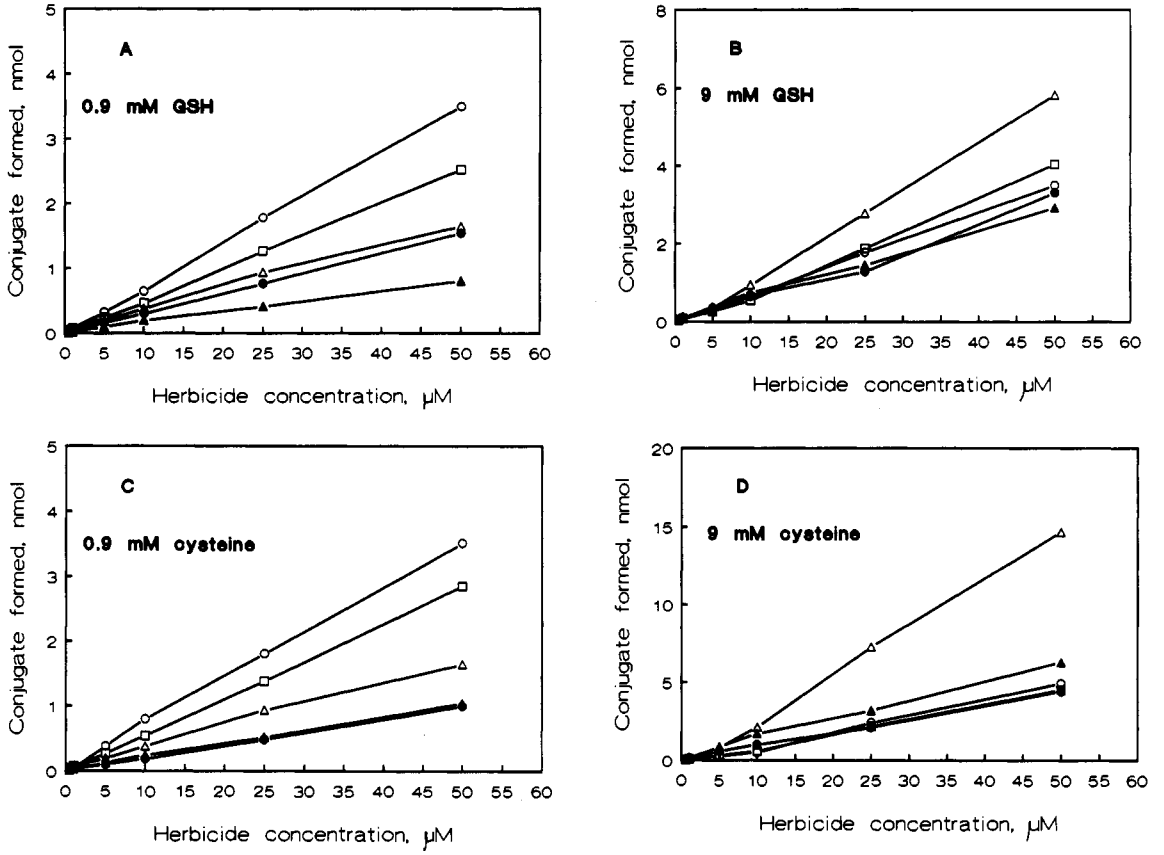


**Figure 3.** Chemical reactivity of the herbicides acetochlor (△), alachlor (▲), metolachlor (○), pretilachlor (●), and atrazine (□) with 0.9 and 9 mM of the thiols GSH (A and B) and cysteine (C and D) as a function of herbicide concentration. Data presented are means of six replications.

(Table II). In corn roots, however, atrazine-specific GST activity was 2-fold lower than that of alachlor and acetochlor but comparable to that of metolachlor and pretilachlor (Table II).

From the results presented in Table II, it is evident that metolachlor-specific GST activity from shoots or roots of Cargill 7567 was comparable to that of Northrup-King 9283. Thus, one could argue that the observed differential response of the two corn hybrids to treatment with this herbicide (Table I) may not be related to differences in GST activity. Nevertheless, earlier studies by Cottingham and Hatzios (1992) have shown that metolachlor-specific GST activity was expressed earlier in the tolerant Cargill 7657 than in the susceptible Northrup-King 9283. Thus, differences in the developmental expression of GST activity as well as the *de novo* synthesis and expression of specific GST isozymes with high affinity for a given herbicidal substrate (Mozer et al., 1983; Timmerman, 1989) may play a significant role in the observed selectivity of chloroacetanilide herbicides that is based on GSH-mediated metabolism. In addition, increased expression of GST activity has been observed in corn and soybean pretreated with alachlor and metolachlor (O'Connell et al., 1988).

**Chemical Reactivity of Chloroacetanilides and Atrazine with GSH and Cysteine.** Data in Figure 3 show the reaction rates of the nonenzymatic conjugation of chloroacetanilides and atrazine with GSH and cysteine as a function of the concentrations of the herbicidal substrates and of the two thiols. Plots of the amount (nanomoles) of thiol–herbicide conjugates formed against the herbicidal substrate concentrations used (0.5, 1, 5, 10,

**Table III. Contribution of Nonenzymatic Conjugation of GSH with Chloroacetanilide Herbicides and Atrazine to Their Overall GSH-Mediated Metabolism in Shoot and Root Extracts of Cargill 7567 and Northrup-King 9283 Corn[a]**

| | | nonenzymatic GSH conjugation,[b] % of total | | | |
|---|---|---|---|---|---|
| | | 0.5 μM concn of herbicidal substrate | | 50 μM concn of herbicidal substrate | |
| hybrid | herbicide | shoots | roots | shoots | roots |
| Cargill 7567 | acetochlor | 47.5 ± 5.4 | 17.9 ± 2.2 | 70.1 ± 5.8 | 37.2 ± 4.7 |
| | alachlor | 48.1 ± 3.9 | 7.6 ± 0.6 | 75.4 ± 4.9 | 40.0 ± 3.7 |
| | metolachlor | 57.5 ± 4.8 | 39.4 ± 2.9 | 96.2 ± 7.1 | 75.3 ± 6.2 |
| | pretilachlor | 21.9 ± 2.6 | 10.0 ± 0.9 | 72.6 ± 5.3 | 64.7 ± 4.4 |
| | atrazine | 50.8 ± 3.7 | 52.9 ± 4.6 | 75.2 ± 4.8 | 78.7 ± 4.7 |
| Northrup-King 9283 | acetochlor | 22.7 ± 1.7 | 17.8 ± 1.2 | 64.8 ± 3.8 | 40.1 ± 3.2 |
| | alachlor | 25.0 ± 2.0 | 8.6 ± 0.6 | 77.3 ± 4.1 | 33.8 ± 2.9 |
| | metolachlor | 68.8 ± 3.7 | 54.5 ± 3.4 | 97.0 ± 3.6 | 93.5 ± 5.0 |
| | pretilachlor | 28.2 ± 1.6 | 9.1 ± 0.7 | 85.3 ± 3.9 | 73.2 ± 3.9 |
| | atrazine | 47.6 ± 2.8 | 46.8 ± 2.5 | 65.6 ± 3.1 | 69.9 ± 3.6 |

[a] Data represent mean percent values from six replications ± standard deviation of each mean. For reaction mixture and conditions see Materials and Methods. [b] Calculated as percent of the total conjugation of the herbicides with GSH (nonenzymatic plus enzymatic).

25, and 50 μM), at each of the two thiol concentrations (0.9 and 9 mM), revealed linear relationships (Figure 3A–D). Because of the high ratios of the concentrations of the thiols to herbicidal substrates used, the rates of these nonenzymatic conjugation reactions appear to follow pseudo-first-order kinetics.

With the exception of metolachlor, it is apparent that the higher the ratio of thiol to herbicidal substrate concentrations, the higher the amount of conjugate formed (Figure 3). Metolachlor was the most reactive herbicide at the low (0.9 mM) thiol concentration, but the amount of metolachlor–thiol conjugates formed did not change significantly when the concentration of GSH or cysteine increased to 9 mM.

At the low thiol concentration (0.9 mM), the rates of nonenzymatic conjugation of the five herbicides with GSH were comparable to those obtained with cysteine (Figure 3A,C). At the higher thiol concentration (9 mM), acetochlor and alachlor conjugated more efficiently with cysteine than with GSH (Figure 3B,D). The greater chemical reactivity of cysteine toward selected chloroacetanilide derivatives, in comparison to that of GSH, has been reported also by Friedman (1973).

The rates of the nonenzymatic conjugation of atrazine with the two thiols tested appeared to be comparable to those of the chloroacetanilides (Figure 3). The potential for a direct chemical reaction of chloro-*s*-triazine herbicides with GSH has been reported (Frear and Swanson, 1970; Lamoureux et al., 1973), and it appears to be dependent on the pH of the reaction (Leavitt and Penner, 1979).

The observed differences in the reactivity of the chloroacetanilide herbicides with GSH and cysteine are most likely due to the influence of the different substituents present in the aryl and *N*-alkyl chain of the molecules of these chloroacetanilide herbicides (Figure 1). Several investigators have shown previously that ring and *N*-alkyl chain substituents influence the lipophilicity and herbicidal activity of chloroacetanilide herbicides (Hamm, 1972, 1974; Jablonkai and Dutka, 1989; Scarponi et al., 1991; Sirois, 1972). Moreover, 2-chloroacetanilide herbicides are known to exist in two isomeric/rotameric forms, due to steric hindrance of the ortho substituent on the ring (Chupp et al., 1967; LeBaron et al., 1988; Moser et al., 1985). Interestingly, the least favored rotamer, at equilibrium, is also the herbicidally active rotamer and the form that conjugates with thiols such as GSH, by means of a nucleophilic displacement of its chlorine by the SH group of the thiols (Chupp et al., 1967; Moser et al., 1985).

**Contribution of Nonenzymatic Conjugation in the GSH-Mediated Metabolism of Chloroacetanilides and Atrazine.** Data in Table III present the percent contribution of the nonenzymatic conjugation to the overall GSH-mediated metabolism of the four chloroacetanilide herbicides and atrazine in shoots and roots of the two corn hybrids. Similar to the case of the enzymatic (GST-mediated) conjugation of these herbicides with GSH, nonenzymatic conjugation was found to be dependent on the concentration and molecular structure of the herbicidal substrate.

The contribution of nonenzymatic conjugation to the overall conjugation of all herbicides with GSH in root or shoot extracts of both corn hybrids was much more pronounced at the high (50 μM) than the low concentration (0.5 μM) of herbicidal substrate (Table III). At both concentrations of herbicidal substrate, the contribution of nonenzymatic conjugation to the total conjugation of the four chloroacetanilide herbicides with GSH was greater in shoots rather than in roots of both corn hybrids and ranged from 9 to 97% depending on the herbicidal substrate used (Table III).

At 0.5 μM of herbicidal substrate, the percent contribution of nonenzymatic conjugation in the total conjugation of acetochlor, alachlor, and pretilachlor with GSH in roots of both corn hybrids was relatively small, ranging from 8 to 18%. The respective percentages of the contribution of the nonenzymatic conjugation to the overall glutathione-mediated metabolism of these herbicides ranged from 23 to 28% in shoots of Northrup-King 9283 and from 22 to 48% in shoots of Cargill 7567 corn (Table III). At 50 μM of herbicidal substrate, the percent contribution of nonenzymatic conjugation in the total conjugation of acetochlor, alachlor, and pretilachlor with GSH increased approximately by 2–3-fold in shoots and by 2–8-fold in roots of the two corn hybrids (Table III). Nonenzymatic conjugation appeared to be contributing to a much greater extent in the total conjugation of metolachlor with GSH in both roots and shoots of the two corn hybrids and at both concentrations of herbicidal substrate as compared to that of the other three chloroacetanilide herbicides (Table III).

These results are in agreement with those reported by Scarponi et al. (1991) showing that the contribution of nonenzymatic conjugation to the total conjugation of several chloroacetanilide herbicides with GSH ranged from 40 to 92% depending on plant species. In that study, the reported contribution of nonenzymatic conjugation in the total conjugation of metolachlor with GSH ranged from 60% in corn and sorghum shoots to 90% in soybean, wheat, and redroot pigweed (Scarponi et al., 1991). By contrast, Gronwald et al. (1987) have reported that the contribution

of nonenzymatic conjugation in the total conjugation of metolachlor with GSH in shoots of grain sorghum was rather small (10%).

The percent contribution of nonenzymatic conjugation in the total conjugation of atrazine with GSH was very similar in roots and shoots of either of the two corn hybrids and ranged from 47 to 53% at 0.5 μM and from 66 to 79% at 50 μM of herbicidal substrate (Table III). These percentages are considerably higher than those of Lamoureux et al. (1973), who found that the contribution of nonenzymatic conjugation of atrazine with GSH was only 10–20% of its total conjugation detected in shoot extracts of grain sorghum.

Overall, the results of the present study demonstrate that nonenzymatic conjugation with GSH may contribute significantly to the overall glutathione-mediated metabolism of chloroacetanilide herbicides and atrazine in corn and is dependent on the concentration and molecular structure of the herbicidal substrate as well as on the thiol content of plant tissues (root vs shoot). The observed variability in the percent contribution of nonenzymatic conjugation to the total conjugation of the chloroacetanilides with GSH demonstrates the importance of the particular substituents in the aryl and *N*-alkyl chain in the phytotoxicity and GSH-mediated metabolism of these herbicides.

## ACKNOWLEDGMENT

This work was supported partially by funds allocated to Hatch projects of the Virginia Agricultural Experiment Station, Virginia Polytechnic Institute and State University. We are grateful to the SOROS Foundation—Hungary, New York, and the CIBA Plant Protection Co., Greensboro, NC, for partial financial support of this project. We thank CIBA Plant Protection Co., Monsanto Co., and Nitrokemia for providing the analytical as well as radiolabeled samples of the herbicides and Cargill Seeds and Northrup-King Co. for providing the corn seeds used in this study.

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Received for review March 18, 1993. Accepted July 14, 1993.[•]

[•] Abstract published in *Advance ACS Abstracts*, September 15, 1993.