Different Rates of Metabolism of Two Chloroacetanilide Herbicides in Pioneer 3320 Corn

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ABSTRACT

The in vitro rates of uptake and detoxification of alachlor and metolachlor were determined using Pioneer corn 3320 seedlings. Equal amounts of the radiolabeled herbicides were applied to etiolated coleoptiles and, at various intervals after treatment, the unabsorbed radioactivity was removed and quantified. Analysis of 80% methanol extracts by reverse phase liquid chromatography showed no significant differences in the rate of uptake of metolachlor and alachlor. However, the rate of glutathione conjugation of alachlor in vivo was two- to threefold greater than the rate for metolachlor at 2 and 4 hours after herbicide application. Since the initial step in detoxification is conjugation of the chloroacetanilide to glutathione, the activities of the enzymes responsible for conjugation, the glutathione-S-transferases (GSTs) were also analyzed in vitro, using crude extracts and the purified GST enzymes. The specific activities of the extracts were consistent with the results in vivo. Using alachlor as a substrate, the specific activity for glutathione conjugation was almost threefold higher than that for metolachlor. Kinetic analysis of purified GST III indicates that the enzyme has a higher affinity for alachlor (Km = 1.69 millimolar) than for metolachlor (Km = 8.9 millimolar).

The basis for the selectivity of particular classes of herbicides such as the triazines (25–27), the thiocarbamates (10, 16, 17), and the chloroacetanilides (15, 18) resides in the specific enzymes responsible for their detoxification. The GSTs2 plants are a family of multifunctional enzymes that conjugate herbicides, toxins, and other xenobiotics to the tripeptide glutathione (1–3, 11) (Fig. 1). The phytotoxin is thus rendered innocuous and water soluble for further degradation and removal. GST activity has been detected in at least 17 plant species (14) and exists in both soluble and membrane-associated forms (5, 27). The soluble GSTs that conjugate alachlor to glutathione in corn or to homoglutathione in soybean have been purified and partially characterized (21) (manuscript in preparation). The resistance of corn to atrazine has been shown to be due to the activity of a soluble GST that is efficient at detoxifying s-triazines (26, 27), although the enzyme itself has not been thoroughly characterized. The importance of the GSTs and GSH levels in herbicide tolerance is also suggested by the reduction in chloroacetanilide and thiocarbamate herbicide induced stress by herbicide antidotes that increase GST activity and GSH levels (6, 21, 28). Detection of microsomal GST activity in plants has been more recent (5) and initial data suggests that, like the cytosolic enzymes, microsomal activity can be modulated by herbicide antidotes.

Corn has at least two enzymes (GST I and GST III) that catalyze the conjugation-detoxification of acetanilide herbicides (21). Recently, complementary DNA clones corresponding to GST I (23) and GST III (20) have been isolated and characterized. Expression of the GST III cDNA in Escherichia coli resulted in demonstrable activity with alachlor as a substrate (20). Although acetanilide herbicides such as alachlor and metolachlor are very effective and provide selective weed control in both corn and soybean, growth chamber and field experiments have indicated that injury to corn seedlings as a result of the application of the two major acetanilide herbicides, alachlor and metolachlor, differs (19, 29). For example, Leavitt and Penner (19) reported that, although alachlor did cause visible injury to corn at a high rate (13.44 kg/ha), it did not lead to the reduction in dry weight and corn height that metolachlor caused when applied at much lower rates (19). Zawierucha (29) similarly observed that injury to alachlor was less than metolachlor across all corn genotypes tested. An interesting physiological question, addressed in the present study, is whether the differential injury caused by the two widely used acetanilide herbicides could be related to possible differences in their conjugation rates by specific GST enzymes.

MATERIALS AND METHODS

Materials. Pioneer 3320 and 3780A corn seed were obtained directly from Pioneer Seed Company. The U-14-C radiolabeled herbicides, alachlor (18.65 mCi/mmol) and metolachlor, (15.2 mCi/mmol) were purchased from Pathfinder Labs, St. Louis.

![GST Reactions](image)

**Fig. 1.** Structures and reactions analyzed in this report. In maize, alachlor and metolachlor are detoxified via conjugation to the tripeptide glutathione (8, 18, 21). Conjugation to the GSH is by nucleophilic attack of the sulfur atom on the α-chloro-carbon of the hydrophobic herbicide.

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†Abbreviations: GSH, glutathione-S-transferase; GST, glutathione; BSP, bromosulfophthalein.

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were alachlor Elution from oítes. MO. Elution determined prepared by dissolving NaOH nation, affinity and metabolism shown in (Fig. 2).

Methods. The effluent from the affinity column was, of course, the most interesting part of the experiment. The effluent was collected and subjected to HPLC analysis to determine the purity of the alachlor-glutathione conjugate.

Fig. 2. HPLC analysis of the purity of the 14C-labeled alachlor and alachlor used in the metabolism study. Samples of the herbicides were separated on the ODS C-18 column used in the analysis of metabolites. Elution was as described in “Materials and Methods” using a linear gradient to 100% acetonitrile.

Fig. 3. Experimental protocol for the isolation and quantitation of 14-C herbicides and their metabolites. The compounds are extracted in 80% methanol which is then chromatographed on the ODS C-18 column. Elution from the column is achieved through a gradient of acetonitrile from 10 to 100% over 30 min.

MO. The purity of the chemicals was greater than 98% as determined by HPLC analysis in several solvents using a C-18 ODS reverse-phase column (Fig. 2). Mass spectral data indicated that the two compounds yield fragment patterns identical to known standards. Synthetic GST-herbicide conjugate standards were prepared by dissolving 14-C herbicide in 200 µl of ethanol, followed by the addition of 380 µl of 250 mg/ml GSH in 1.75 N NaOH (24). Incubation was carried out at room temperature and reaction was complete after 2 h. Other reagents for extraction, affinity column preparation, and HPLC analysis were purchased from Fisher Scientific Co. and Sigma Chemical Co.

Methods. The steps followed in the study of chloroacetanilide metabolism are shown in Figure 3. Corn seeds were germinated on moistened paper towels and grown in the dark at room temperature. Radiolabeled herbicide (5 µg of alachlor or meto- lachlor in 4 µl hexane) was applied directly to the coleoptiles of 4- to 5-d-old seedlings, using a Hamilton syringe model 701 (Hamilton Corporation, Reno, NV). Following treatment, the seedlings were returned to the dark for 2 or 4 h. The treated coleoptiles were then rinsed with methanol, which was collected for the quantitation of unabsorbed herbicide, and the entire seedling was then frozen in liquid N2. Control experiments in which the herbicide was removed immediately (3–5 s) after application revealed that greater than 90% of the labeled herbicide could be removed by this procedure. For extraction, three seedlings were pooled and pulverized under liquid N2 and homogenized in 80% methanol in water using a Polytron homogenizer (Brinkmann Instruments, Westbury, NY). Each data point represents 10 independent extractions of three pooled sets of seedlings. The tissue was extracted three times with a total volume of 11 ml. The tissue debris removed by centrifugation at 8000 g in a model TJ-6 refrigerated centrifuge (Beckman Corporation, Fullerton, CA) for 15 min at 4°C and the supernatants were combined. After measuring the total volumes, an aliquot of the extract was taken for counting of radioactivity. The methanol extracts were diluted with an equal volume of water and filtered through Millex HV filters (Millipore Corporation, Bedford, MA), without loss of radioactivity. The samples were then injected onto an ODS C-18 column (Altex Corporation, Berkeley, CA) for separation of the radioactive herbicide and its metabolites using a linear gradient of acetonitrile from 5 to 100% over 30 min. The amount of radioactivity eluting in each peak was quantitated either by the collection of fractions and liquid scintillation counting or with a Radiomatic Flo-1 radiodetector (Radiomatic Corporation, Tampa, FL).

Extraction and Assay of GST. Etiolated whole seedlings were pulverized under liquid N2 to a fine powder and volume extracted with 200 mM Tris-HCl buffer (pH 8.3) containing 1 mM DTT, 1 mM EDTA, and 5% (w/v) polyvinylpyrrolidone. Following centrifugation at 10,000 g for 15 min, 1/10 volume of freshly prepared 1.4% protamine sulfate in water was added to the extract, and the precipitated nucleic acids were removed by centrifugation before the supernatant was adjusted to 70% saturation by the addition of solid ammonium sulfate. The precipitated protein was collected by centrifugation. Following resuspension in 50 mM potassium phosphate-1 mM DTT (pH 7.5), the samples were dialyzed overnight at 4°C against 20 volumes of the same phosphate buffer. This was the crude extract used for determining total soluble GST activity.

Purification of the individual isozymes was achieved by affinity chromatography and reverse-phase liquid chromatography (21). Purification of GST I, the 30,000-Da enzyme, was achieved as previously described using the bromosulphophthalein-glutathione agarose affinity resin (4, 21). The gene encoding the maize GST I enzyme has been cloned and expressed in transgenic petunia tissue (23). Purification of the 26,000 Da GST III, was carried out by the use of the alachlor-glutathione affinity column in which the synthetic conjugate is covalently attached to the column resin using 6-aminohexanoic acid activated Sepharose (manuscript in preparation). The effluent from the BSP affinity column was applied to the alachlor affinity column. Bound material was eluted as with the BSP affinity column, using 10 mM GSH in 50 mM potassium phosphate-1 mM DTT (pH 7.5) (column buffer). The bound fraction was dialyzed against 15 mM ammonium acetate at 4°C overnight. The protein was then applied to a Vydac C-18 column (Hesperia, CA) equilibrated with 0.1% trifluoroacetic acid. Fractions eluted in an acetonitrile gradient to 100% were immediately diluted fivefold with buffer containing 50 mM potassium phosphate-1 mM DTT (pH 7.5) and placed on ice. The fraction with activity was concentrated using an Amicon Diaflo miniconcentrator (Danvers, MA). The
reactions were carried out in 20% dimethylformamide, without affecting enzyme activity. However, the use of higher concentrations resulted in the precipitation of GSH. Reactions were carried out for thirty min over which time the incorporation was in the linear range.

RESULTS AND DISCUSSION

Uptake of Alachlor and Metolachlor by Corn Coleoptiles. It is known that the absorption of the chloracanilide herbicides by corn seedlings in soil is primarily through the coleoptile (9, 22). However, since soil uptake of the radiolabeled herbicide is subject to greater error because of problems associated with microbial metabolism as well as obtaining uniform dispersal of herbicides, the herbicides were directly applied to coleoptiles by application with syringe. Since differences in the rate of uptake of alachlor and metolachlor would significantly influence interpretation of

Table 1. Recoveries of Radioactive Herbicides and Metabolites from Pioneer 3320 Corn Seedlings

<table>
<thead>
<tr>
<th>Time Point</th>
<th>Fraction</th>
<th>[%] Metolachlor</th>
<th>[%] Alachlor</th>
</tr>
</thead>
<tbody>
<tr>
<td>h</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>Total % in methanol rinse</td>
<td>11.95 ± 1.89</td>
<td>11.48 ± 0.92</td>
</tr>
<tr>
<td></td>
<td>Total % in extract</td>
<td>70.71 ± 4.86</td>
<td>63.84 ± 3.17</td>
</tr>
<tr>
<td></td>
<td>Total % recovery</td>
<td>82.66 ± 3.10</td>
<td>75.32 ± 4.90</td>
</tr>
<tr>
<td>4</td>
<td>Total % in methanol rinse</td>
<td>7.89 ± 0.74</td>
<td>5.74 ± 0.74</td>
</tr>
<tr>
<td></td>
<td>Total % in extract</td>
<td>55.12 ± 2.47</td>
<td>50.84 ± 4.90</td>
</tr>
<tr>
<td></td>
<td>Total % recovery</td>
<td>63.01 ± 2.20</td>
<td>56.58 ± 3.20</td>
</tr>
</tbody>
</table>

FIG. 4. Radioactivity profile of extract of alachlor sample (c) from Table I after separation by reverse-phase liquid chromatography as compared to the elution profile of herbicide (a) and synthetic glutathione conjugate standard (b). The parent herbicides elute at 100% acetonitrile. Conjugates elute at 47% acetonitrile.

FIG. 5. Percentage of extractable radioactivity as the glutathione conjugate and as unmetabolized herbicide at the 2 and 4 h time points. Each determination represents the average of 10 replicate samples. The differences observed in the levels of alachlor and metolachlor glutathione conjugates are significant at >95% confidence levels.

FIG. 6. SDS gel profile of corn GST III. The affinity-column isolated proteins and HPLC purified proteins were applied to each lane of the SDS-PAGE gel (13). The acrylamide to bis(acrylamide) ratio was 30:0.174 and the gel concentrations for the separating and the stacking gels were 15 and 5% respectively. Two μg of each were applied and the proteins were visualized using a silver stain procedure (21, 27). Lane A, mol wt standards; lane B, crude extract after GSH-alachlor affinity chromatography; lane C, purified GST III after Vydac C-18 reverse-phase chromatography.

the metabolism data, uptake of both herbicides was measured at 2 and 4 h after application. The unabsorbed herbicide was removed by gently rinsing the tissue with methanol. The data in Table I show that within 2 h after application, less than 12% of either herbicide remains unabsorbed. This indicates that for both herbicides, transport into the coleoptile is very rapid and nearly complete in 2 h for the compounds. No significant difference
alachlor seedlings contained almost three times as much of the recoverable radioactivity in the form of the GSH-conjugate as the metolachlor-treated seedlings (Fig. 5). The difference is significant at 95% confidence levels using the student t-test. There is also a corresponding decrease in the amount of alachlor present relative to metolachlor. The alachlor treated corn seedlings had 23% of the radioactivity as the GSH-conjugate compared to 8% for the metolachlor treated seedlings. At 4 h after application, the differences in the amounts of alachlor and metolachlor conjugates present was again statistically significant at the 5% probability level.

At 4 h, 46% of the recoverable alachlor was conjugated and 42% of the radioactivity was the active parent compound. In contrast, only 20% of the recoverable metolachlor radioactivity was the GSH-conjugate and greater than 60% is the parental compound. The GSH-conjugate is a transient intermediate and, consistent with the findings of Lamoureaux et al. (15). After 4 h, less polar metabolites of the conjugate appear that may represent degradation of the tripeptide moiety. Hydroxylated chloroacetanilide metabolites (12) would also be solubilized by the extraction procedure and would be detectable by the HPLC analysis. Since these metabolites are not observed in the 2- and 4-h timepoints, the GSH-conjugant likely represents the primary metabolite under these conditions, in agreement with previous studies (8, 15).

**In Vitro Conjugation of Alachlor and Metolachlor by GST Enzymes.** To determine whether the large differences observed in rates of alachlor and metolachlor conjugation in the *in vivo* experiments (Fig. 5) could be explained by differences in extractable GST enzyme activity, the rates of conjugation of the herbicides by crude extracts of corn coleoptiles were determined. Pioneer 3320 seedlings were harvested at the same growth stage as employed for the experiments in the analysis of *in vivo* metabolism. Total protein was extracted and GST activity assayed exactly as described previously (21). It was found that the specific activity of the extracts for alachlor conjugation (3.0 µg/mg-h) was threefold higher than for metolachlor conjugation (1.1 µg/mg-h). The difference in specific activity correlated well with the observed two- to threefold difference in rate of *in vivo* metabolism. Since there are two enzymes in corn which catalyze the conjugation of chloroacetanilide herbicides (21), the individual GST species were purified to determine whether the observed differential rates of conjugation of alachlor and metolachlor would be reflected in the kinetic properties of the purified enzyme.

GST I is a homodimer of subunit mol wt 29,500 and is easily purified based on its affinity for bromosulphathalein, a large dye molecule that has been used extensively for the purification of mammalian GSTs (21). GST III is also a homodimer of subunit mol wt 26,000, and is purified by passage over the acetanilide affinity column (21, 27). Initial results indicated that greater than 80% (data not shown) of the alachlor or metolachlor conjugating activity resided with GST I, so only this species was studied in further detail. An SDS polyacrylamide gel showing the GST III preparation following purification to homogeneity (Fig. 6). The results of the kinetic analysis with GST III using metolachlor and alachlor as substrates is shown in Figure 7. The results were obtained by using an RS1 program (BBN Research Systems, Cambridge, MA) to fit the data to a hyperbola, although at low substrate concentration the fit is not ideal. The apparent Km of GST III for alachlor is 1.69 mM, fivefold less than the apparent Km for metolachlor, 8.9 mM. The solubility of the herbicide substrates in aqueous solvents limits the direct measurement of Km values at higher concentrations, so the values are reported here as apparent Km values.

Our results indicate that the uptake of alachlor and metolachlor by the corn seedling coleoptiles occurs very rapidly and at

![Figure 7](https://example.com/figure7.png)

**Fig. 7.** Direct plots of conjugation assay results to determine apparent Km values. Assay procedures are described in text. Two µg of purified GST III were used in each assay mix and the amount of product formed was determined by reverse-phase liquid chromatography and scintillation counting of the product peaks. Apparent Km values are 1.65 mM using alachlor as a substrate and 8.5 mM using metolachlor. Concentration of glutathione was 10 mM.

was found as well in the absorbed (the aqueous methanol extractable) material, which represented 63 to 71% of the applied radioactivity for both herbicides. At 4 h following application of the herbicides, the results were similar in that there were no significant differences observed in the uptake of alachlor or metolachlor by corn coleoptiles.

**In Vivo Metabolism of Alachlor and Metolachlor.** Since the uptake experiments revealed no significant differences in the absorption of alachlor and metolachlor by corn coleoptiles, an extensive study of the metabolism of the two compounds was initiated (Fig. 3). It was expected on the basis of previous *in vivo* (15) and *in vitro* (18) studies that the metabolites for alachlor and metolachlor would be very similar if not identical. However, the rates at which the two compounds are metabolized *in vivo* is not known. Therefore the aqueous methanol extracts obtained from samples in Table I were subjected to reverse phase liquid chromatography and the metabolites were quantified by liquid scintillation counting of radioactivity. The aqueous methanol extracts contained both the parent herbicide and the GSH-herbicide conjugates as well as other metabolites.

Figure 4 shows a typical radioactive profile of an extract of an alachlor treated seedling in which the parental herbicide and its GST-conjugate are easily resolved. Two h after treatment, the
similar rates. There is, however, at least a two- to threefold difference in the rates at which the two compounds are detoxified via GSH-conjugation, which is reflected in different affinities of the GST III for the two substrates. The observed difference in alachlor and metolachlor metabolism rates provides a reasonable explanation for the greater phytotoxicity observed following metolachlor treatment (19, 29). Since alachlor is metabolized to the herbicidally inactive GST-conjugate more rapidly than metolachlor, the probability of injury to emerging corn seedlings is reduced. Finally, the above results indicate that GST III is the major GST species in corn involved in chloroacetanilide herbicide conjugation. Isolation of a full length corn GST III cDNA clone and expression of enzyme activity in E. coli extracts has recently been reported (20). Using gene transfer methods, it may be possible to increase GST enzyme levels in plants in order to reduce the extent of herbicidal injury (7).

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LITERATURE CITED


29. ZAWIERUCHA JF 1984 A screening technique for detecting corn genotype sensitivity to selected herbicides. Penn State Thesis, Department of Agronomy. Pennsylvania State University, University Park, PA