Conjugation of 2-Chloroacetanilide Herbicides with Glutathione: Role of Molecular Structures and of Glutathione S-Transferase Enzymes

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The conjugation of acetochlor, alachlor, butachlor, dimethachlor, metolachlor, pretilachlor, and propachlor with glutathione nonenzymatically and enzymatically by action of the glutathione S-transferase (GST) was investigated. Shoots of corn (*Zea mays*), soybean (*Glycine max*), wheat (*Triticum aestivum*), sorghum (*Sorghum bicolor*), redroot pigweed (*Amaranthus retroflexus*), and lambsquarter (*Chenopodium album*) were employed as enzyme sources for enzymatic tests. The nonenzymatic conjugation rates varied to the following order: propachlor > pretilachlor > alachlor > acetochlor > dimethachlor > metolachlor > butachlor. The presence of enzyme extracts induced significant conjugation increases that differed according to the enzyme source. Furthermore, within each enzyme source a different decreasing order of the conjugation rate, compared to the nonenzymatic reaction, was observed. Therefore, interferences of the molecular structure of the 2-chloroacetanilides also in the enzymatic mechanism of the reaction have been deduced. These findings and the kinetic parameters (K_M and V_{max}), determined for the GST enzymes of each plant, show that the molecular structure of the 2-chloroacetanilides, the catalytic efficiency of GST enzymes, their concentration in the protein bulk, and the protein content in plants are crucial factors in determining plant tolerance.

INTRODUCTION

Besides the entity of herbicide uptake, the degree of plant tolerance to 2-chloroacetanilide herbicides is dependent on the rate of herbicide metabolism. Therefore, a determining factor appears to be the capacity of the plant to detoxify these chemicals at sufficient rates to prevent their accumulation at phytotoxic concentrations (Le Baron et al., 1988). It is recognized that the major detoxification pathway in plants is the conjugation of these herbicides with glutathione (GSH), or homoglutathione (hGSH), by means of a nucleophilic displacement of chlorine by the thiol group of GSH or hGSH. This reaction transforms the parent molecule into an inactive derivative and may occur both nonenzymatically and enzymatically through the action of glutathione S-transferase (GST, EC 2.5.1.18). This inactivation mechanism has been shown, with more or less efficiency, in a large spectrum of tolerant and susceptible plants for acetochlor, alachlor, metolachlor, and propachlor (Lamoureaux et al., 1971; Leavitt and Penner, 1979; Breaux, 1987).

Factors deserving attention for their role in the conjugation rate may be the following: (i) the endogenous content of available thiols; (ii) the efficiency of the GST enzymes; and (iii) the effect of ring substituents and N side chains in the molecular structures. As far as the content of thiols is concerned, sufficient evidence has been given by the study of Breaux et al. (1987), which demonstrated that there is higher level of thiols in tolerant than in susceptible plants. This finding was confirmed by the safeners fluorazole and dichlormid, which seem to protect treated corn and sorghum against 2-chloroacetanilide injury by increasing the level of endogenous GSH (Breaux et al., 1987; Gronwald et al., 1987).

Less experimental evidence exists about the effect exerted on the conjugation rate by the 2-chloroacetanilide structures and by the efficiency of native GST enzymes. The aim of this research was to ascertain the role of these factors in the GSH conjugation of seven diffused 2-chloroacetanilide herbicides, utilizing in the enzymatic reactions tolerant and moderately susceptible crops and susceptible and less susceptible weeds as enzyme sources.

MATERIALS AND METHODS

Chemicals. Analytical master standards of acetochlor [2-chloro-*N*-(ethoxymethyl)-6'-ethylacet-*o*-toluidide], alachlor [2-chloro-2',6'-diethyl-*N*-(methoxymethyl)acetanilide], butachlor [*N*-(butoxymethyl)-2-chloro-2',6'-diethylacetanilide], and propachlor (2-chloro-*N*-isopropylacetanilide) were supplied by Monsanto Co. (St. Louis, MO); dimethachlor [2-chloro-*N*-(2-methoxyethyl)-acet-2',6'-xylidide], metolachlor [2-chloro-6'-ethyl-*N*-(2-methoxy-1-methylethyl)acet-*o*-toluidide], and pretilachlor [2-chloro-2',6'-diethyl-*N*-(2-propoxyethyl)acetanilide] were supplied by Ciba-Geigy Corp. (Greensboro, NC). Reduced glutathione (GSH), 1-chloro-2,4-dinitrobenzene (CDNB), and brilliant blue G were obtained from Aldrich Chemie (Steinheim, Germany). Acetonitrile (HPLC grade) and water (HPLC grade) were purchased from BDH (Poole, England). All other reagents employed were of ACS grade.

Apparatus. The HPLC instrument was assembled from the following modular components: two Perkin-Elmer Series 410 LC pumps, a Rheodyne Model 7125-075 injector, a Perkin-Elmer Model LC 235 diode array detector, an LC column 25 cm × 4.6 mm i.d. C_{18} reverse phase with a Supelguard LC18 guard column (Supelco Inc., Bellefonte, PA), and a Varian Model Cary 210 double-beam grating spectrophotometer.

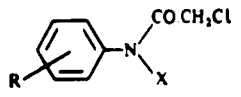
Plant Materials. Seeds of corn (*Zea mays*, hybrid Orfeo), soybean (*Glycine max*, Hodgson 78), wheat (*Triticum aestivum*, hybrid Centauro), sorghum (*Sorghum bicolor*, hybrid Urbino), redroot pigweed (*Amaranthus retroflexus*), and lambsquarter (*Chenopodium album*) were employed. The seeds (50 g) were placed on a filter paper moistened with water in Petri dishes in the dark at 25 °C for 5 days.

Preparation of Enzyme Extract. Five-day-old shoots, obtained as described above, were collected, cut into pieces, and homogenized by sonication with chilled acetone (50 mL) for 1 min. The homogenate was filtered through a Buchner funnel, washed twice with chilled acetone (25 mL), spread out on filter paper, and allowed to dry at room temperature. For the complete protein extraction, aliquots of the obtained powders (0.5 g) were covered with buffer solution (20 mL of phosphate buffer, 0.1 M, pH 9.0) and allowed to stand for 10 min at 4 °C. The mixture was then centrifuged at 48200*g* for 20 min, and the supernatant was used as crude enzyme extract.

The total protein content in the enzyme extract was determined spectrophotometrically by reaction with brilliant blue G according to the Bradford procedure (1976).

0021-8561/91/1439-2010$02.50/0

Table I. Nonenzymatic and Enzymatic Reaction Rates of Seven 2-Chloroacetanilide Herbicides[a]



R	X	herbicide	no enzyme	+enzyme extract from soybean	corn	sorghum	wheat	lambsquarter	redroot pigweed
H	$CH(CH_3)_2$	propachlor	40.2 a	88.1 a (2.2)	90.0 a (2.2)	95.1 a (2.4)	94.8 a (2.3)	98.0 a (2.4)	95.1 a (2.4)
2,6-(diC_2H_5)	$(CH_2)_2OC_3H_7$	pretilachlor	30.1 b	46.6 bc (1.5)	70.5 b (2.3)	63.6 c (2.1)	63.8 b (2.1)	49.2 c (1.3)	62.3 b (2.1)
2,6-(diC_2H_5)	CH_2OCH_3	alachlor	27.9 bc	52.2 b (1.9)	45.8 c (1.6)	50.0 d (1.8)	55.0 c (2.0)	49.4 c (1.8)	59.1 b (2.1)
2-CH_3; 6-C_2H_5	$CH_2OC_2H_5$	acetochlor	26.5 c	31.8 d (1.2)	38.5 d (1.4)	40.1 e (1.5)	43.1 d (1.6)	41.8 d (1.6)	38.9 c (1.5)
2,6-($diCH_3$)	$(CH_2)_2OCH_3$	dimethachlor	21.6 d	25.2 e (1.2)	39.4 cd (1.8)	35.5 e (1.6)	34.9 e (1.6)	40.1 d (1.8)	27.5 e (1.3)
2-CH_3; 6-C_2H_5	$CH(CH_3)$-CH_2OCH_3	metolachlor	16.2 e	17.5 f (1.1)	21.8 e (1.3)	20.6 f (1.3)	18.1 f (1.1)	19.4 e (1.2)	18.3 f (1.1)
2,6-(diC_2H_5)	$CH_2OC_4H_9$	butachlor	15.2 e	39.1 c (2.6)	45.1 c (3.0)	73.0 b (4.8)	59.3 bc (3.9)	77.3 b (5.1)	32.0 de (2.1)

[a] The reaction rates are expressed as percent of conjugated herbicide after 3 h of incubation in a reaction mixture containing in 6 mL of phosphate buffer (0.1 M; pH 7.0) 1.0 nmol of herbicides, 5.0 nmol of GSH, and 1 mL of enzyme extract in case of enzymatic reaction. The values are the means of triplicate determinations, and data within a column followed by the same letter are not significantly different at 5% level using Duncan's multiple range test (ratios of enzymatic/nonenzymatic reaction rate are reported in parentheses).

Conjugation of GSH with 2-Chloroacetanilides. Two series of reactions between GSH and the 2-chloroacetanilide herbicides were performed according to the Feng and Patanella procedure (1983) modified as follows. In the first reaction series, to 3.5 mL of phosphate buffer (0.1 M, pH 7.0), which had been deaerated by bubbling N_2 through it, were added in sequence 1.0 mL of enzyme extract, 5.0 nmol of GSH in 1.5 mL of phosphate buffer (0.1 M, pH 7.0), and 1.0 nmol of herbicide (0.5 nmol for butachlor and pretilachlor because of their smaller solubility) dissolved in 40 μL of aqueous methanol solution. The second reaction series was performed likewise but 2.0 nmol of herbicide and a suitable volume of enzyme extract so as to have 1 mg of protein in the reaction mixture were used and the final volume was adjusted to 6 mL with phosphate buffer solution (0.1 M, pH 7.0).

The reaction was stopped by freezing in a dry ice–acetone bath, and the reaction mixtures were then lyophilized.

The nonenzymatic reactions were performed by substituting the enzyme extract with equivalent aliquots of phosphate buffer (0.1 M, pH 9.0). To check eventual herbicide losses by nonconjugating reactions, control tests were carried out in which GSH with 1.5 mL of phosphate buffer (0.1 M, pH 7.0) was substituted.

The lyophilized material was extracted with 10 mL of methanol and centrifuged at 34800*g* for 10 min at 4 °C. The supernatant was utilized to determine the residual nonconjugated herbicide by HPLC procedure. The following isocratic system was employed: mobil phase, water/acetonitrile (10:90); flow rate, 1 mL/min; detection, 220 nm.

GST Activity Assays. Activities of GST enzymes from the plant species were evaluated by determining kinetic parameters with respect to an artificial substrate (CDNB) according to the procedure of Moron et al. (1979), modified by Ando et al. (1988). In the reaction mixture the final concentrations were 1 mM GSH and CDNB ranging from 0.2 to 1.0 mM. A suitable volume of enzyme extract equivalent to 1 g of fresh weight was added to the substrates and the volume adjusted at 6 mL with phosphate buffer (0.1 M, pH 6.5) containing 2.5% ethyl alcohol to dissolve CDNB. The reaction mixtures were incubated for 1 h at 35 °C. The reaction was stopped by addition of 6 mL of 0.33 N HCl. GST activity (v) was calculated by measuring the absorbance at 340 nm and utilizing 9.6 mM cm^{-1} as extinction coefficient (Askelof et al., 1975). K_M and V_{max} values were obtained from linear regression analysis of $1/v$ vs $1/S$ of the linear transformation of the Michaelis–Menten equation according to the Lineweaver–Burk $[1/v = 1/V_{max} + (K_M/V_{max})1/S]$ method.

RESULTS AND DISCUSSION

The GSH conjugation rates of the 2-chloroacetanilides performed in vitro at GSH saturating concentration are reported in Table I. Data show significant interferences of the aryl substituents and of the N side chains in both nonenzymatic and enzymatic conjugation reactions. After 3 h of incubation time, the nonenzymatic conjugations ranged from 40.2% to 15.2% of the added herbicide according to the following order: propachlor > pretilachlor > alachlor > acetochlor > dimethachlor > metolachlor > butachlor.

In the enzymatic conjugation tests, the enzyme extracts were added at constant and suitable amounts to minimize changes in the reaction milieu, with respect to that of the nonenzymatic reaction.

The addition provoked significant increases in conjugation, ranging from 98.0% to 17.5% following a decreasing order which was variable for each enzyme source but quite different from that of the nonenzymatic reactions. The enzymatic/nonenzymatic reaction ratios were considered so that a comparison between enzymatic and nonenzymatic herbicide conjugation could be made for each enzyme source. In particular, the highest activation levels were found for butachlor, propachlor, pretilachlor, and alachlor. In fact, the conjugation rate was more than triple for butachlor, more than double for propachlor, and almost double for pretilachlor and alachlor. The variability of the ratios obtained for each enzyme source demonstrates that the aryl substituents and N side chains, besides exerting their effects on nonenzymatic conjugation, were also able to interfere with the enzymatic mechanism of the reaction. Consequently, interferences on the capacity of the GST enzymes to bind the 2-chloroacetanilide derivatives could be supposed.

The lack of systematic changes in the herbicide structure makes it impossible to attribute the observed effects and interferences to some specific aryl substituents or groups in the N side chains. The relationships between molecular structure and herbicidal activity are reported in earlier studies. It was ascertained that maximum herbicidal action is obtained when the *N*-alkyl chain is three carbons long and that heteroatom breaks, such as oxygen, could permit longer aliphatic substituents on the nitrogen atom without loss of activity (Hamm, 1974). An interpretation for such effects is given in terms of the lipophilic character of the structure which could assist the herbicide parent molecule in uptake and translocation to the site of action (Sirois, 1972). Nevertheless, the existence of structure interactions in the conjugation reaction suggests that the ring substituents and *N*-alkyl chains may be determinant in the phytotoxic character. In fact, the phytotoxicity of 2-chloroacetanilide herbicides has been found to be related to their reduced capacity to conjugate with GSH (Leavitt and Penner, 1979; O'Connell et al., 1984). Also, the in

Table II. 2-Chloroacetanilide Herbicides Conjugated (nmol) per Unit (mg) of Protein from Six Plant Sources[a]

enzyme source	protein content, mg/g of fw + ES	acetochlor	alachlor	butachlor	dimetachlor	metolachlor	pretilachlor	propachlor
soybean	4.65 ± 0.51	0.19 c	0.29 d	0.23 d	0.15 d	0.10 bc	0.23 c	0.52 d
corn	3.25 ± 0.28	0.23 c	0.27 d	0.28 c	0.23 c	0.13 ab	0.43 c	0.54 d
sorghum	2.66 ± 0.15	0.38 b	0.48 b	0.70 a	0.34 b	0.08 c	0.61 b	0.91 b
wheat	3.64 ± 0.22	0.23 c	0.29 d	0.32 c	0.19 cd	0.09 c	0.34 d	0.51 d
lambsquarter	2.72 ± 0.21	0.32 b	0.38 c	0.60 b	0.31 b	0.15 a	0.38 cd	0.76 c
redroot pigweed	0.70 ± 0.05	0.71 a	1.07 a	0.58 b	0.51 a	0.13 ab	1.16 a	1.76 a

[a] The data represent the amount of conjugated herbicide after 3 h of incubation in a reaction mixture containing in 6 mL of phosphate buffer (0.1 M; pH 7.0) 2.0 nmol of herbicide, 5.0 nmol of GSH, and 1 mg of protein extracted from plant sources. The values are the means of triplicate determinations, and data within a column followed by the same letter are not significantly different at 5% level using Duncan's multiple range test.

Table III. V_{max} and Apparent K_M Values of Crude Glutathione *S*-Transferases from Six Plant Sources (CDNB Substrate)[a]

plant source	V_{max}, μmol of conjugated herbicide/min (g of fw)$^{-1}$	K_M, mM
soybean	0.38 c	1.09 d
corn	0.66 b	1.79 c
sorghum	1.64 a	7.70 b
wheat	0.42 c	2.03 c
lambsquarter	0.13 d	9.94 a
redroot pigweed	0.02 e	1.25 d

[a] The data represent the means of triplicate determinations. Means within a column followed by the same letter are not significantly different at 5% level using Duncan's multiple range test.

vitro study of Jablonkai and Dutka (1989) on the structure/SH-alkylating reactivity and phytotoxicity relationships of some 2-chloroacetanilides tends to support this hypothesis, although the relative contribution of molecular substituents to the formation of GSH–2-chloroacetanilide conjugates in vivo remains uncertain.

Taking into account the ubiquitous presence of GSH and GST enzymes in plants, an important clue for elucidating the degree of plant tolerance to the 2-chloroacetanilides may be drawn by examining the catalytic efficiency of GST enzymes of susceptible and tolerant plant species. To evaluate and compare the conjugation potential of crude GST enzymes from the studied plant sources, the conjugation rates were also determined in reaction mixtures containing 1 mg of proteic extract (Table II).

Significantly different rates of GSH conjugation were observed: the highest catalytic efficiency was shown by the extract of redroot pigweed for all of the tested herbicides, followed by sorghum and lambsquarter, followed by soybean, corn, and wheat at almost equivalent levels. These findings seem to be in contrast with agronomic practice, which indicates that corn and soybean are tolerant crops, sorghum and wheat are moderately susceptible crops, lambsquarter is susceptible, and redroot pigweed is very susceptible to the more employed 2-chloroacetanilide herbicides. Nevertheless, these results may not be surprising since the conjugation in vivo depends on several crucial factors. Apart from the role of endogenous conjugation thiols, which was not considered in our experiments performed at saturating GSH conditions, the catalytic efficiency of GST enzymes, their concentration in the protein bulk, and the protein content of the plant must be considered. On this account, a characterization of the crude GST enzymes was accomplished by determining the kinetic parameters: V_{max} and apparent K_M (Table III). The tests were performed by employing CDNB as artificial substrate for its high GSH conjugating capacity (Keen et al., 1976).

Even though it should be recognized that apparent K_M does not give a true measure of the affinity, the remarkable differences in the K_M values found for the various GST enzymes confirm the native diversity of the structural factors related to this parameter, such as the substrate binding capacity and isoenzyme distribution.

Significant differences in the V_{max} values were also found. As V_{max} is a factor dependent on the enzyme concentration, data may be considered representative of the amount of the enzyme in the 5-day-old shoots. From the results given in Table III, sorghum was the best enzyme source followed by corn, wheat, and soybean, lambsquarter, and redroot pigweed. These findings help explain the behavior in vivo of susceptible redroot pigweed, less susceptible lambsquarter, moderately susceptible wheat, and tolerant corn. It was surprising to find that sorghum was too high with respect to its degree of susceptibility (Devlin et al., 1983). Based upon results of Breaux et al. (1987), one explanation could be its lower endogenous GSH content with respect to other tolerant plants, which can result in a reduction in the conjugation rate in vivo.

Soybean showed a GST content not significantly different from those of moderately susceptible wheat, although it is tolerant to some widely used 2-chloroacetanilides such as alachlor and metolachlor. Therefore, soybean tolerance seems to be not exclusively dependent on GST activity. In fact, in a recent study, analogue tolerance to alachlor by both corn and soybean seedlings has been noted, in spite of the lesser GST activity in soybean which caused higher alachlor residues in soybean than in corn (Scarponi et al., 1991). In conclusion it can be deduced that crucial factors of plant tolerance to 2-chloroacetanilides are, besides the content of endogenous thiols (Breaux et al., 1987), the catalytic efficiency of GST enzymes, their concentration in the protein bulk, and the protein content in plants.

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Received for review April 23, 1991. Accepted July 29, 1991.

Registry No. GSH, 70-18-8; GST, 50812-37-8; acetochlor, 34256-82-1; alachlor, 15972-60-8; butachlor, 23184-66-9; propachlor, 1918-16-7; dimethachlor, 50563-36-5; metolachlor, 51218-45-2; pretilachlor, 51218-49-6.