In Vitro Conjugation of Glutathione and Other Thiols with Acetanilide Herbicides and EPTC Sulfoxide and the Action of the Herbicide Antidote R-25788

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Nonenzymatic reaction in vitro of ^{3}H-labeled glutathione (GSH) with [^{14}C]alachlor, [^{14}C]metolachlor, [^{14}C]H-22234, and [^{14}C]EPTC sulfoxide formed dual-labeled GSH–herbicide conjugates. GSH did not conjugate in this system with the herbicides buthidazole, atrazine, EPTC, or the herbicide antidote R-25788. Alachlor also conjugated with the thiol-containing compounds cysteine, dithiothreitol, and coenzyme A but not with methionine, acetyl-CoA, mercaptoethanol, or ethanethiol. The alachlor–GSH conjugation reaction yielded more product with increased pH (over pH 6.0), indicating that the in vitro reaction proceeds by way of the GS^- ion. Although the GSH–acetanilide conjugation reaction had a low yield at physiological pH, it could be the basis for the protection of corn from acetanilide herbicide injury by R-25788. It is suggested that R-25788 may protect corn from EPTC injury by increasing the rate of EPTC sulfoxidation, followed by subsequent EPTC sulfoxide–GSH conjugation.

In vitro, nonenzymatic conjugation of glutathione (GSH) with three fungicides was reported by Siegel (1970). Atrazine (2-chloro-4-(ethylamino)-6-(isopropylamino)-*s*-triazine)–GSH conjugates have been isolated from sorghum (*Sorghum vulgare* Pers.) leaf pieces by Lamoureux et al. (1970), and a glutathione-*S*-transferase that catalyzes GSH–atrazine conjugation has been identified in corn (*Zea mays* L.), sorghum, and sugarcane (*Saccharum officianarum* L.) by Frear and Swanson (1970). GSH-*S*-transferases that catalyze the conjugation of GSH with fluorodifen (*p*-nitrophenyl α,α,α-trifluoro-2-nitro-*p*-tolyl ether) have also been isolated from peas (*Pisum sativum* L.) (Frear and Swanson, 1973) and peanuts (*Arachis hypogaea* L.) (Shimabukuro et al., 1973). Although Lay and Casida (1976) reported a GSH-*S*-transferase from corn roots that catalyzed the conjugation of GSH with EPTC (*S*-ethyl dipropylthiocarbamate) sulfoxide, Carringer et al. (1978a) disputed the existence of this enzyme and reported that the GSH–EPTC sulfoxide conjugation proceeded in vitro nonenzymatically. Both Lay and Casida (1976), Lay et al. (1975), and Carringer et al. (1978a) reported that the herbicide antidote R-25788 (*N,N*-diallyl-2,2-dichloroacetamide) increased the GSH content of corn and hypothesized that this GSH increase could cause an increased rate of EPTC detoxification by forming increased GSH–EPTC sulfoxide conjugation after initial EPTC sulfoxidation and thereby explain the mode of action of this antidote. Leavitt and Penner (1978) have recently reported that R-25788 also protects corn from five acetanilide herbicides as effectively as it does from EPTC. The acetanilide herbicide analogue, chloroacetamide, readily conjugates nonenzymatically with certain thiol compounds, including GSH (Lindley, 1960, 1962). The GSH conjugate of propachlor (2-chloro-*N*-isopropylacetanilide) has also been isolated from corn and a nonenzymatic GSH–propachlor conjugation reaction described by Lamoureux et al. (1971). Preliminary experiments failed to find a GSH-*S*-transferase responsible for GSH–acetanilide herbicide conjugation; therefore, the objectives of this study were to (a) characterize the nonenzymatic conjugation of GSH and other thiols with three acetanilide herbicides; alachlor (2-chloro-2′,6′-diethyl-*N*-(methoxymethyl)acetanilide), metolachlor (2-chloro-*N*-(2-ethyl-6-methylphenyl)-*N*-(2-methoxy-1-methylethyl)acetamide), and H-22234 (*N*-chloroacetyl-*N*-(2,6-diethylphenyl)glycine ethyl ester), and the herbicide derivative EPTC sulfoxide and (b) to determine whether the mechanism for the protective action of R-25788 was the same for thiocarbamate and acetanilide herbicides by using a GSH-*S*-transferase deficient inbred corn line.

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MATERIALS AND METHODS

Reagents and Equipment. L-[*glycine*-2-^{3}H]Glutathione (sp act., 2500 mCi/mM) was purchased from New England Nuclear. Nonlabeled GSH, L-cysteine, L-methionine, DL-dithiothreitol, and 2-mercaptoethanol were purchased from Sigma Chemical Co. Oxidized glutathione was prepared by bubbling O_2 through a solution of reduced GSH for 30 min. Coenzyme A (lypholized) was purchased from Nutritional Biochemicals Co., acetyl-CoA from Schwarz/Mann, and ethanethiol from Eastman Organics. Formulated, technical, and uniformly ^{14}C-ring-labeled alachlor (sp act., 1.7 and 1.4 mCi/mM) were donated by Monsanto Corp. Technical and uniformly ^{14}C-ring-labeled metolachlor (sp act., 4.5 mCi/mM) as well as formulated and uniformly ^{14}C-ring-labeled atrazine (sp act., 2.1 mCi/mM) were donated by CIBA-GEIGY Corp. Technical and carbonyl ^{14}C-labeled H-22234 (sp act., 1.2 mCi/mM) were donated by Hercules Corp. Formulated butylate (*S*-ethyl diisobutylthiocarbamate), formulated and carbonyl-labeled [^{14}C]EPTC (sp act., 1.33 mCi/mM), and formulated and technical R-25788 were donated by Stauffer Chemical Co. Uniformly labeled buthidazole [3-(5-(1,1-dimethylethyl)-1,3,4-thiadiazol-2-yl)-4-hydroxy-1-methyl-2-imidazolinone] (sp act., 12.7 mCi/mM) was donated by Velsicol Chemical Corp. ^{14}C-labeled EPTC sulfoxide (sp act., 1.33 mCi/mM) was synthesized from the ^{14}C-carbonyl-labeled EPTC by the method of Lay and Casida (1976). All other chemicals used were reagent grade. Buffers were made in sterilized, deaerated, distilled water to an ionic strength of 0.1 M by the method of Cherry (1973). Liquid scintillation spectrometry (LSC) was done by a Packard Tri-Carb Model 3320 liquid scintillation spectrometer with separate channels for ^{3}H, ^{14}C, and 233 Ra external standard. The scintillation cocktail used was Aqueous Counting Scintillant from Amersham. Mixtures were lypholized on a Virtis model lypholizer. The thin-layer chromatography (TLC) system used was: silica gel 60 or 60 F precoated TLC plates (E. Merck) developed in butanol/acetic acid/water, 6:2:3, and visualized with either autoradiography (Kodak No-Screen X-ray film), ninhydrin spray reagent (Patton and Chism, 1951), nitroprusside (sodium) spray reagent (Toennies and

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Table I. Reaction of GSH with Three Acetanilide Herbicides

reactants[a]	TLC[b] peaks, R_f	dpm[c] ^3H	dpm[c] ^{14}C	reaction with ninhydrin[d]	reaction with nitroprusside[e]	cochromatographed with	identification	% of extractable[f] ^{14}C in conjugate
[^{14}C]alachlor + [^{3}H]GSH	0.10	3200		+	–	GSSG		
	0.30	19100		+	+	GSH		
	0.47	1700	1700	+	–		GS-alachlor	19[a]
	0.76		9400	–	–	alachlor		
[^{14}C]metolachlor + [^{3}H]GSH	0.10	2200		+	–	GSSG		
	0.30	18800		+	+	GSH		
	0.47	1100	800	+	–		GS-metolachlor	7[a]
	0.76		17700	–	–	metolachlor		
[^{14}C]H-22234 + [^{3}H]GSH	0.10	2600		+	–	GSSG		
	0.30	21000		+	+	GSH		
	0.47	1200	1100	+	–		GS-H-22234	6[a]
	0.76		21500	–	–	H-22234		

[a] [^{14}C]Alachlor and [^{14}C]metolachlor uniformly ring labeled, [^{14}C]H-22234 carbonyl labeled, GSH [2-^{3}H]glycine labeled. [b] The system used: silica gel 60, E. Merck developed in butanol/acetic acid/H_2O, 6:2:3. [c] The specific activity of each reactant was approximately equal (0.7 mCi/nmol) so that any conjugation product would have approximately equal number of each labeled isotope. [d] Ninhydrin used to visualize both reacted and nonreacted GSH. [e] Nitroprusside (sodium) reacts with free SH groups. [f] These data from a separate experiment between ^{14}C herbicides and nonlabeled GSH, replicated three times. Means followed by the same letter are not significantly different at the 5% level as judged by Duncan's Multiple Range Test. Because the data are in the percent for they were converted to their arcsines for statistical analysis.

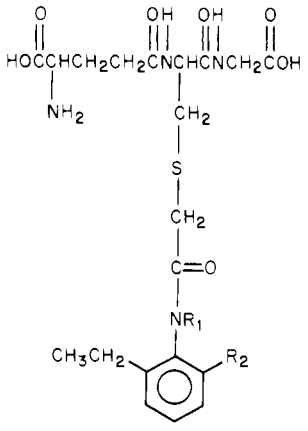
Kolb, 1951), or dividing the plate into 1 × 2 cm blocks and scraping each block into scintillation vials for LSC.

Reaction of Thiols with Herbicides. The reaction between [^{3}H]GSH and [^{14}C]acetanilide herbicide was studied by the addition (in sequence) of 1 mL of phosphate buffer, pH 7.0, 0.1 M, which had been deaerated by bubbling N_2 through it for 30 min, 100 nmol of [^{14}C]-acetanilide herbicide (alachlor, metolachlor, or H-22234 each diluted to 0.07 μCi/100 nm with nonlabeled herbicide) in 10 μL of ethanol, 1300 nmol of GSH in 0.2 mL of phosphate buffer, and 0.4 nmol of [^{3}H]GSH in 50 μL of 0.05 N acetic acid (ca. 1 μCi). The solution was mixed and allowed to react for 3 h at 30 °C under a N_2 atmosphere. The reaction was stopped by freezing the mixtures in a dry ice–acetone bath and then lypholized. The residue was extracted with 0.5 mL of methanol, and 100-μL samples were applied to the TLC plates, developed, and visualized. This method was adapted from Lamoureux et al. (1971). These experiments were also repeated without [^{3}H]GSH. The specific activity of the [^{14}C]acetanilide herbicide and the [^{3}H]GSH in the reaction mixtures was approximately equal so that any conjugate formed containing 1 mol of GSH residue per mole of herbicide residue would have near equal amounts of ^{3}H and ^{14}C dpm. The experiments with pH contained 1 mL of the following buffers: acetate pH 4.6, phosphate pH 6.0, phosphate pH 7.0, phosphate 8.0, or Tris-HCl pH 8.6, plus 100 nmol of [^{14}C]alachlor and 1000 nmol of GSH in one experiment and 10 000 nmol of GSH in another. The reaction mixtures for the alachlor-thiol experiments were identical with the acetanilide–GSH experiment except only nonlabeled thiols were used at a concentration of 1000 nmol per reaction mixture. The reaction between GSH and other herbicides was studied by substituting the following for the acetanilide herbicides in the standard reaction mixture: 100 nmol of technical R-25788, 100 nmol of [^{14}C]buthidazole (1.3 μCi), 38 nmol of [^{14}C]EPTC, 38 nmol of [^{14}C]EPTC sulfoxide, and 23 nmol of [^{14}C]atrazine, all in 10 μL of ethanol (except the buthidazol which was in 20 μL of ethanol). Results for all experiments were expressed as the percent of ^{14}C recovered from the TLC plate as conjugate as compared to the total amount of ^{14}C recovered per spot.

Plant Culture for Herbicide–Antidote Response Study. Corn inbred GT112 (Shimabukuro et al., 1971) which was atrazine susceptible, glutathione-*S*-transferase deficient was grown in a greenhouse mix soil (1:1:1 sand/peat/soil) in 946-mL waxed cups in a greenhouse supplemented with artificial lighting (16-h day) with a maximum temperature of 38 °C and a minimum of 30 °C. The response of this inbred to the herbicides EPTC, butylate, alachlor, and atrazine, alone or in combination with R-25788, was measured in three experiments. All herbicides and R-25788 were applied preplant-incorporated with 2.1 kg/cm^2 pressure in 935 L/ha spray volume with a link belt sprayer. When the herbicides were applied in combination with R-25788, the herbicides were applied and incorporated first and the R-25788 applied and incorporated 15 min later. After 4 weeks, corn heights and dry weights were measured. Only plant heights are reported. The dry weight results were similar.

RESULTS AND DISCUSSION

The GSH conjugates of alachlor, metolachlor, and H-22234 were identified on TLC plates as spots with both ^{3}H and ^{14}C cochromatographing in near equal relative abundance (Table I). These spots reacted with ninhydrin (which reacts with the free amino group of the GSH and therefore visualizes both conjugated and nonconjugated GSH) did not react with nitroprusside (which reacts with free thiol groups), and did not cochromatograph with any of the original reactants (Table I). Failure of the dual-labeled conjugate to react with nitroprusside indicates that the site of conjugation was the sulfur of the GSH. The presence of ^{14}C in both the alachlor and the metolachlor reaction products indicates that the phenyl ring of the herbicides was maintained in the conjugate since they were both phenyl labeled. The presence of ^{14}C in the H-22234 reaction product indicates that the carbonyl carbon was also maintained in the conjugate since the H-22234 was carbonyl labeled. Based on this evidence the proposed structure of the GSH–acetanilide herbicide conjugate was formulated (Figure 1). Although there were no significant differences between the amounts of conjugate formed by the three acetanilide herbicides (Table I), the trend in



GS-alachlor conjugate, $R_1 = CH_3OCH_2$, $R_2 = CH_3CH_2$

GS-metolachlor conjugate, $R_1 = CH_3OCH_2CH(CH_3)$, $R_2 = CH_3$

GS-H-22234 conjugate, $R_1 = CH_3CH_2OC(=O)CH_2$, $R_2 = CH_3CH_2$

Figure 1. Structure of GS–acetanilide herbicide conjugate. Adapted from Lamoureux et al. (1971) and Carringer et al. (1978b).

Table II. pH Dependence of the GSH-Alachlor Conjugation Reaction

pH of reaction	initial concn of GSH in reaction mixture: 1000 nmol, % [^{14}C]-alachlor in conjugate	initial concn of GSH in reaction mixture: 10 000 nmol, % [^{14}C]-alachlor in conjugate
4.6	18.5 b[a]	43.6 b
6.0	7.4 a	8.9 a
7.0	17.0 ab	38.5 b
8.0	26.4 b	85.5 c
8.6	76.5 c	99.1 d

[a] Means within columns followed by a common letter or letters are not significantly different at the 5% level as judged by the Duncan's Multiple Range Test. Because the data are in the percent form they were converted to their arcsines for statistical analysis.

amounts formed has a negative relationship to their relative toxicity to corn (Leavitt and Penner, 1978) (i.e., alachlor < metolachlor < H-22234).

The pH dependence of the alachlor–GSH conjugation reaction can be seen in Table II. Except for acetate buffer at pH 4.6, the amount of conjugate formed in vitro increased with increased pH to almost 100% when 10 000 nmol of GSH were used in Tris-HCl buffer, pH 8.6. However, it should be noted that Frear and Swanson (1970) reported that Tris buffer catalyzed a nonenzymatic reaction between atrazine and GSH which tricine buffer, at the same pH, did not. The amount of conjugate formed this way was small, between 10 and 20% of added atrazine. The pK of the sulfhydryl group of GSH has been reported as 8.66 (Boyland and Chasseaud, 1969). This means that the reactive species of GSH is probably the GS^- ion as previously reported for GSH–chloroacetamide conjugation (Lindley, 1962). The anomalous behavior in acetate buffer at pH 4.6 could be the result of a different reaction mechanism, or the alachlor may be unstable at the low pH.

Alachlor also conjugated with cysteine, dithiothreitol, and coenzyme A (Table III). No appreciable conjugate formation was detected with methionine, acetyl-CoA,

Table III. Reaction of Alachlor with Other Thiols

reactants	R_f of conjugate[a]	% of extract. ^{14}C in conjugate
[^{14}C]alachlor + cysteine	0.51	11.7a[b]
+ dithiothreitol	0.35	9.1a
+ coenzyme A	0.36	3.0a
+ methionine	NR[c]	
+ acetyl-CoA	NR	
+ mercaptoethanol	NR	
+ ethanethiol	NR	

[a] TLC systems used: silica gel 60F, E. Merck, in butanol/acetic acid/H_2O, 6:2:3. [b] Means followed by the same letter or letters are not significantly different at the 5% level as judged by Duncan's Multiple Range Test. [c] NR = no reaction.

Table IV. Reaction of GSH with Various Herbicides

reactants	R_f of conjugation[a]	% of ^{14}C in conjugate
[^{3}H]GSH + R-25788	NR[b]	
+ [^{14}C]buthidazole	NR	
+ [^{14}C]EPTC	NR	
+ [^{14}C]EPTC, S=O	0.38	60.3
+ [^{14}C]atrazine	NR	

[a] TLC system: silica gel 60F, E. Merck: butanol/acetic acid/ H_2O, 6:2:3. [b] NR = no reaction.

mercaptoethanol, or ethanethiol. Although R-25788 has been reported to increase GSH content of corn (Carringer et al., 1978a; Lay and Casida, 1976; Lay et al., 1975), the authors are unaware of any reports on the effect of R-25788 on the concentration in corn of other thiols such as cysteine or coenzyme A.

No conjugation product of GSH with R-25788 could be detected (Table IV). GSH also did not conjugate in vitro with other chemicals, buthidazole, EPTC, or atrazine. However, GSH conjugated with the EPTC sulfoxide with 60% of the recoverable ^{14}C found in the conjugate (Table IV). These results support the conclusion of Carringer et al. (1978b) that EPTC sulfoxide conjugates nonenzymatically with GSH.

Although the physiological significance of the GSH–acetanilide herbicide conjugation is unknown in vivo, it occurs at physiological pH. The reported stimulation of GSH synthesis by R-25788 (Carringer et al., 1978b; Lay and Casida, 1976; Lay et al., 1975), coupled with GSH–acetanilide conjugation, could explain the protective action of R-25788 against the acetanilide herbicides in corn. Similar rationale has been used to explain the protection of corn from EPTC injury (Carringer et al., 1978a). The possibility that R-25788 could have the same mode of action in preventing acetanilide and thiocarbamate herbicide injury was investigated by examining the response of inbred corn line, GT112, to both herbicide classes. This inbred is glutathione-*S*-transferase deficient and atrazine susceptible (Shimabakuro et al., 1971). As shown in Table V, the two thiocarbamate herbicides EPTC and butylate caused no inhibition of growth in this corn genotype, whereas alachlor and atrazine did. The growth inhibition by alachlor was prevented by R-25788 but the inhibition by atrazine was not. In two other genotypes of corn, normal (DeKalb XL 316) and thiocarbamate susceptible (DeKalb XL 306), atrazine at 6.72 kg/ha did not inhibit growth (data not presented). Butylate at 3.36 kg/ha inhibited the growth of the thiocarbamate susceptible corn. EPTC at 6.76 kg/ha and alachlor at 4.48 and 6.72 kg/ha

Table V. Response of Atrazine Susceptible, GSH-*S*-transferase Deficient, Corn Inbred Line GT-112 to Butylate, EPTC, Alachlor, and Atrazine in Three Experiments

experiment no.	herbicide (rate, kg/ha)	corn height, cm/plant	
		R-25788	
		0.0 kg/ha	1.12 kg/ha
1	control	21.7 c[a]	23.7 c
	butylate (3.36)	23.4 c	22.1 c
	alachlor (4.48)	13.9 b	23.5 c
	atrazine (6.72)	6.9 a	8.9 a
2	control	41.0 b	34.5 b
	EPTC (6.72)	39.0 b	36.6 b
	atrazine (6.72)	14.0 a	17.4 a
3	control	26.2 b	24.2 ab
	alachlor (6.72)	20.8 a	26.3 b

[a] Means within experiments followed by a common letter or letters are not significantly different at the 5% level according to Duncan's Multiple Range Test.

inhibited the growth of both genotypes. R-25788 prevented butylate, EPTC, and alachlor injury to both genotypes. Since the inbred corn line, GT112, responded differently to thiocarbamates and alachlor, the metabolism of these two herbicide groups may differ. Whatever rendered this genotype thiocarbamate tolerant did not protect it from alachlor injury, and furthermore the action of R-225788 to prevent alachlor injury was not required for the prevention of EPTC injury. Therefore, not only may the metabolism of the two herbicide classes differ, but the protective effect of R-25788 may have a different basis. Differences in GSH conjugation could result from the requirement that EPTC be converted to its sulfoxide prior to GSH conjugation, which is not required for alachlor. Though they were not compared in the same experiment, EPTC sulfoxide formed three times as much GSH conjugate in vitro in one experiment as alachlor did in the others, indicating that the GSH content in corn could be relatively more important for acetanilide detoxication than for EPTC detoxication. Casida et al. (1974) reported that corn was injured by EPTC at 3.4 kg/ha but could tolerate EPTC sulfoxide applications of 27 kg/ha without damage. EPTC sulfoxide was more toxic to other plants than EPTC, however. Thus corn can readily detoxify EPTC sulfoxide without prior R-25788 treatments to raise the GSH content. R-25788 could therefore protect corn from EPTC injury by increasing the rate of EPTC sulfoxidation. Increased rate of EPTC sulfoxide metabolism by increased GSH conjugation would only be secondary. Since the acetanilide herbicides do not appear to react as readily with GSH as EPTC sulfoxide nor require prior activation in order to react with GSH, R-25788 may prevent acetanilide herbicide injury by simply increasing the GSH content of corn. Jaworski (1956) reported that exogenous applications of GSH could partially overcome phytotoxicity from the acetamide herbicide CDAA (a R-25788 analogue). The differential response of the inbred corn line GT112 to both herbicides could be explained by ease of GT112 sulfoxidation of EPTC, which then reacts with the natural GSH levels; GSH levels, however, not high enough to protect the genotype from alachlor unless R-25788 raises them. Failure of R-25788 to protect this inbred corn line from atrazine injury indicates that R-25788 does not stimulate glutathione-*S*-transferase activity or the rate of atrazine–GSH conjugation in corn.

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