Table III. Limits of Detection (LOD)[a] for Compounds with Variable Parameter

compound	$\Delta\nu$, cm^{-1}	band-passes[b]	LOD, ppb
carbaryl	2650	1.5, 1.5	24
	2650	4.0, 1.5	10
	2650	1.5, 4.0	13
naphthol	1400	1.5, 1.5	6
	2650	1.5, 1.5	7
	2650	4.0, 1.5	2
	2650	1.5, 4.0	3
carbofuran	1400	1.5, 1.5	20
	2650	1.5, 1.5	45
	2650	4.0, 1.5	13
	2650	1.5, 4.0	18

[a] LOD defined as concentration (ppb) giving a signal to noise ratio of 3. [b] Band-passes for excitation, emission monochromators in nanometers. *Note*: Linear dynamic ranges were approximately 3.5 orders of magnitude extending from the limits of detection for all conditions and compounds.

ranges were approximately 3.5 orders of magnitude for the three compounds under all conditions.

Figure 4 shows the effect of varying band-passes on the excitation and emission monochromators for the three-component mixture. For scan (a) both monochromator band-passes are 1.5 nm; for (b) the excitation band-pass is 2.5 nm and the emission band-pass is 1.5 nm; and for scan (c) the excitation band-pass is 1.5 nm and the emission band-pass is 2.5 nm. It can be seen that, for these variations in band-passes, signal intensities are increased while resolution is maintained for identification and quantitation purposes. If the discrepancy between band-passes becomes larger than 1.5–2.5 nm, then a *slight* resolution loss can occur particularly for narrow excitation peaks when a wide excitation band-pass is used or for narrow emission peaks when a wide emission band-pass is used; larger band-passes will, of course, increase optical throughput and thus signal levels. Optimum parameter selection will be determined by the complexity of the mixture being studied, the specific components being determined, and the concentration of the analytes. Depending on the application, one may trade resolution for increased sensitivity or vice versa.

In conclusion, we have demonstrated the sensitivity and selectivity of CESLS, an inexpensive and reliable method, for measurement of pesticides and hope that in the future this technique will find wide applicability to studies involving pesticides as well as other complex mixtures where physical separations may be avoided.

Registry No. Carbaryl, 63-25-2; naphthol, 90-15-3; carbofuran, 1563-66-2.

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Chloroacetanilide Herbicide Selectivity: Analysis of Glutathione and Homoglutathione in Tolerant, Susceptible, and Safened Seedlings

E. J. Breaux,*[1] James E. Patanella, and Ernest F. Sanders

The basis for selective phytotoxicity is often the lack of metabolic deactivation in susceptible plants. For example, the selective chloroacetanilide herbicides alachlor, acetochlor, and metolachlor are metabolized less readily by susceptible weeds such as barnyardgrass than by tolerant corn seedlings. Chloroacetanilide herbicide tolerance is due to conjugation with glutathione (GSH; glutamylcysteinylglycine) or homoglutathione (hGSH; glutamylcysteinyl-β-alanine). New analytical methods were developed and used to analyze these tripeptide thiols in plants. These methods are based on the selective derivatization of these detoxification thiols with radiochemically labeled maleimides such as *N*-ethylmaleimide. The maleimide adduct derivatives were then separated by reversed-phase high-performance liquid chromatography (RP HPLC) and quantitated with the aid of a radiochemical HPLC detector. By these new methods it was found that chloroacetanilide herbicide tolerance was related to the seedling detoxification thiol content. It was also found that the herbicide safener flurazole caused the level of GSH to increase in the shoots of treated corn and sorghum.

Chloroacetanilide herbicides are widely used for the control of grass and some problem broad-leafed weeds in a variety of major crops such as corn and soybeans (Beste, 1983). It has been reported that the biochemical basis for selectivity is the metabolic detoxification of these herbicides by conjugation with either glutathione (Lamoureux et al., 1971) or homoglutathione (Breaux, 1986) in tolerant plants. Glutathione or homoglutathione conjugation is also involved in the detoxification of the chloroacetanilide herbicide acetochlor in susceptible crops and weeds (Breaux and Patanella, 1985). This herbicide was found to be metabolized more rapidly in the tolerant plants to

Monsanto Agricultural Company, St. Louis, Missouri 63167.
[1] Present address: Rhone-Poulenc Agrochimie, 14-20 Rue Pierre Boizet, BP 9163, Lyon Cedex 1, France.

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the GHS or hGSH thioether conjugates than in susceptible plants. Previous workers have also reported that chloroacetanilide herbicides are metabolized more readily by tolerant than by susceptible seedlings (Dixon and Stoller, 1982; Jaworkski, 1969).

The use of safeners to enhance the tolerance of crops such as sorghum and corn to chloroacetanilide herbicides has recently been reviewed (Hatzios, 1983; Stephenson and Ezra, 1983a). The actual safening mechanism is unclear at present, but enhancement of metabolic detoxification of the herbicide is involved (Gronwald et al., 1986). The detoxification metabolites identified in safened seedlings are also GHS congutates (Ezra et al., 1986; Gronwald et al., 1986; Breaux et al., 1986a). The explanation proposed for the enhanced ability of safened and tolerant plants to form the GSH or hGSH conjugates is that these seedlings contain more GSH or hGSH available for detoxification and an increased level of the enzymes that conjugate GSH with these herbicides, the GSH transferases. Mozer and co-workers previously reported that the chloroacetanilide safeners cause an induction of GST activity (1983). The objective of this study was to identify and quantify the detoxification thiols in susceptible, safened, and tolerant seedlings. In order to accomplish this, a new selective method was developed to analyze plant thiols. The results of this study are described below.

EXPERIMENTAL SECTION

Chemicals. [*ethyl*-^{14}C]-*N*-Ethylmaleimide (20.4 mCi/mmol) was purchased from New England Nuclear Co. (Boston, MA). Ellman's reagent, 5,5'-dithiobis(2-nitrobenzoic acid), unlabeled NEM, and thiol standards such as GSH and cysteine were purchased from Sigma Chemical Co. (St. Louis, MO). Homoglutatione was synthesized and generously donated by by Shad Eubands of the Monsanto Corporate Research Laboratories (St. Louis, MO) in the manner of Neish and Rylett (1963).

***N*-Ethylmaleimide Thiol Adduct Synthesis.** Glutathione or homoglutathione (0.5 mL of a 1 mg/mL aqueous solution) was placed in a graduated centrifuge tube. To this solution was added 0.5 mL of a 0.1 mg/mL solution of [^{14}C]NEM (sp act. 10 000 dpm/μg) in acetonitrile, and the mixture was heated at 50 °C for 30 min. The desired maleimide adducts were purified by HPLC and used as chromatography standards. The same general procedure was used to synthesize the cysteine and other thiol NEM adduct standards.

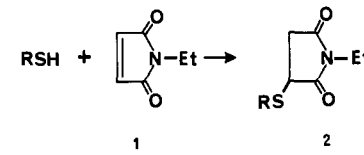
Crop and weed seeds were obtained form commercial sources. The commercial crop varieties used were as follows: corn, Pioneer 3320 and 3382; wheat, Arthur; sorghum, DeKalb 64Y; soybeans, Williams. The safened seeds (0.13% flurazole by weight for sorghum and 0.25% by weight for corn) were obtained from Ron Brinker of the Monsanto Herbicide Evaluation Group. The Pioneer 3320 seedlings were used to study the effect of flurazole on GSH levels while Pioneer 3382 seedlings were used for the other studies.

HPLC Separation of Thiol Adducts. An HPLC instrument assembled from the following modular components was used: two Waters Model M6000A pumps, a Waters Model U6K injector, a Waters Model 680 gradient controller, and a Waters Model 440 absorbance detector. All of the analyses were performed on an Altex (Beckman) ODS Ultrasphere C-18 reversed-phase column (10 mm × 25 cm). Linear gradients were used, and the normal solvent flow rate was 4.0 mL/min. For the quantitative analysis in this report the solvent composition and gradient period used were as follows: 0–100% acetonitrile in 1% acetic acid; linear gradient, 10 min.

[^{14}C]NEM Analytical Procedure. Approximately 0.5 g of harvested plant material was sliced into 1–2-mm sections and placed in a 16 × 125 mm test tube. Then, 2.5 mL of a acetonitrile–water (3:1) extraction solvent was added and the mixture homogenized on a Polytron homogenizer (Brinkman Instruments) with a 16-cm probe having a diameter of 12 mm. Homogenization time was 1 min at a setting of 6, and the samples were kept at ice bath temperatures (critical step). The homogenate was centrifuged for 5 min on a benchtop centrifuge (Dynac; setting 90; ca. 3000 rpm). The supernatant was then removed and placed in a 5-mL graduated centrifuge tube. The volume was recorded, and either 0.2- or 0.4-mL aliquots were removed and placed in a 1-mL Reacti-vial. To this was added 50 or 100 μL of [^{14}C]NEM (0.1 μg/μL; 1000 dpm/μL; sp act. 10 000 dpm/μg) dissolved in acetonitrile. The Reacti-vial was sealed, vortexed, and heated at 50 °C for 30 min. The acetonitrile was then removed under a nitrogen stream, leaving approximately 100 μL of an aqueous solution. This solution was then analyzed by HPLC using the conditions described above. The HPLC effluent was passed into a Berthod radioactivity monitor (Model 503 or 504) for detection. Output was to a linear strip chart recorder interfaced to an in-house chromatography data system. The amount of GSH or hGSH was determined by comparison to a standard curve obtained for the ^{14}C-labeled synthetic adducts. Each assay contained four sample sets. Two of the samples were spiked with a known amount of GSH or hGSH and served as fortified checks for recovery purposes. Furthermore, each of the four sample sets was analyzed in duplicate. Therefore, for each assay, eight measurements were made. The results of the NEM method were checked by the Ellman procedure as described by Stephenson and coworkers (1983b).

RESULTS AND DISCUSSION

Thiol Analytical Method. As noted previously, the objectives of the present study were to identify and quantify the detoxification thiols in tolerant and susceptible plant seedlings. In order to do this we developed the use of a new selective HPLC method based on the derivatization of plant thiols with radiochemically labeled maleimides such as *N*-ethylmaleimide (1, eq 1). The main



1 2 (1)

advantages of this method are as follows: (1) It is a selective method, and GSH, hGSH, and cysteine can be quantified independently. (2) The radiochemical probe allows the detection of the thiols in the presence of many light-absorbing and fluorescing plant metabolites. (3) The method is applicable to the study of thiol biosynthesis. This new method is based upon the selective derivatization of the plant thiols with the ^{14}C-labeled maleimide followed by the separation of the resulting maleimide adducts by reversed-phase HPLC. The thiol maleimide adducts (2, eq 1) are quantitated on a radiochemical chromatography detector.

Identification was based on the comparison of chromatographic properties of the plant extract thiol maleimide adducts with synthetic standards. In addition, a second ^{14}C-labeled maleimide was used to confirm the identity of the thiols by mass spectrometry. In this case *N*-(*p*-bromophenyl)maleimide was used to derivatize the plant thiols. The maleimide adducts were then purified and

Table I. Chloroacetanilide-Tolerant and -Susceptible Plants

A. narrow-leafed plants	B. broad-leafed plants
I. Tolerant	
corn (*Zea mays*)	soybean (*Glycine max*)
II. Moderately Susceptible	
sorghum (*Sorghum bicolor*)	morningglory (*Ipomoea purpurea*)
shattercane (*Sorghum bicolor*)	velvetleaf (*Arbutilon theoprasti*)
	mung beam (*Phaseolus aureus*)
wheat (*Triticum aestivum*)	
III. Very Susceptible	
barnyardgrass (*Echinochloa crus-galli*)	redroot pigweed (*Amaranthus retroflexus*)
giant foxtail (*Setaria faberi*)	purslane (*portulaca oleracea*)

Table II. Narrow-Leafed Seedling Glutathione Content

seedling	μg GSH/g fresh wt[a]	μg GSH/shoot
corn	182.4 ± 10.2 (a)	46.8 ± 2.6 (a)
sorghum	102.1 ± 11.4 (b)	5.4 ± 0.7 (b)
wheat	32.6 ± 0.6 (c)	1.6 ± 0.1 (c)
shattercane	59.3 ± 5.6 (c)	1.9 ± 0.1 (c)
giant foxtail	52.1 ± 2.4 (d)	0.3 ± 0.1 (d)
barnyardgrass	57.3 ± 3.0 (d)	0.3 ± 0.1 (d)

[a] Means within columns followed by the same letter are not significantly different at the 95% confidence level.

analyzed by mass spectrometry. The details of the mass spectral analyses will be reported separately (Breaux et al., 1986a).

Monocot Seedling Thiol Analysis. The site of herbicide uptake in narrow-leafed seedlings that leads to phytoxicity has been reported to be the shoot (Narsaiah and Harvey, 1977). The initial grass thiol analyses were therefore conducted on the shoots of grasses and subsequently on the other plant parts and intact plants. Six narrow-leafed weed and crop plants were chosen for study (Table I). Corn was chosen as the tolerant seedling while barnyardgrass and giant foxtail seedlings were chosen as the susceptible seedlings. Sorghum, shattercane, and wheat were chosen as examples of moderately susceptible seedlings. In order to approximate the field use situation the shoots of 3–5-day-old etiolated seedlings were used for the thiol analyses. The shoots were analyzed before the first leaf had emerged through the coleoptile. This is the development stage in which injury is usually observed for grasses. As can be seen in Figure 1, glutathione is the major soluble thiol in the shoots of the narrow-leafed seedlings. Other unidentified thiols were detected in the plant extracts. However, in most cases these thiols were minor components. Wheat was the major exception. As can be seen in Figure 2, a more polar thiol adduct was detected, chromatographically similar to the cysteine adduct. The homoglutathione adduct was not detected in any of the narrow-leafed seedling extracts. Glutathione was confirmed as the major thiol in corn and sorghum extracts by mass spectrometry as reported separately (Breaux et al. 1986a).

The results of the quantitative analysis of the narrow-leafed seedling shoots are shown in Table II. The more tolerant seedling shoots such as corn shoots have more GSH when expressed on a per shoot or per gram basis. These results were verified by the DTNB method (Stephenson et al. 1983b). The only significant discrepancy noted between the Ellman and [^{14}C]NEM methods was with wheat, which was not unexpected since wheat contained significant levels of non-GSH thiol activity (Figure 2).

Effect of Flurazole Safener on GSH Content. The use of safeners is a common practice in the corn and

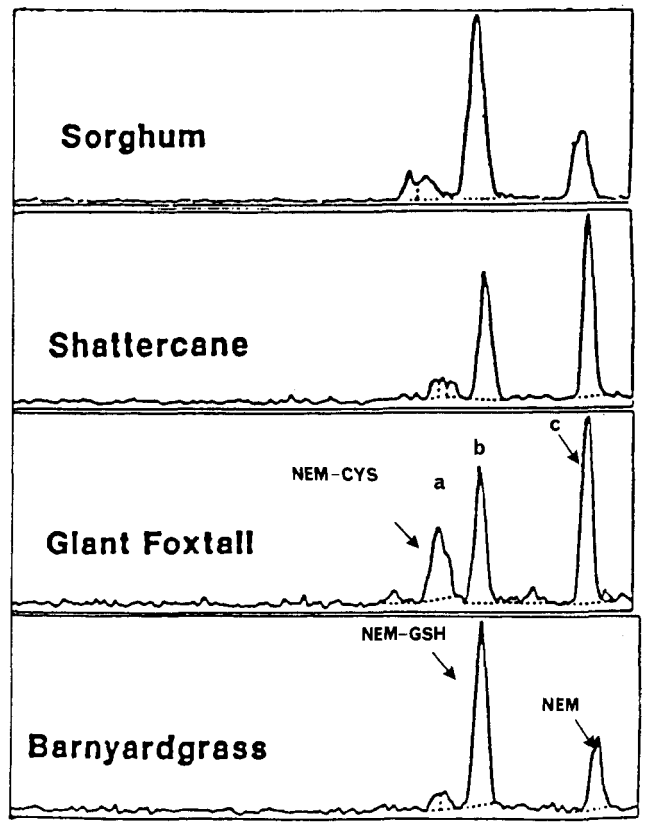


Figure 1. HPLC analysis of narrow-leafed seedling shoot thiols. HPLC conditions: column, Altex Ultrasphere C-18, 10 mm × 25 cm. Eluents: A, 1% acetic acid; B, acetonitrile. Gradient conditions: A to B in 10 min; linear gradient (Waters 680 solvent programmer, curve 6); flow rate, 4 mL/min; detector, Berthold Model 503 radioactivity chromatography detector. Peaks: (a) NEM–cysteine conjugate, 7.3 min; (b) NEM–glutathione conjugate, 7.6 min; (c) underivatized NEM, 9.2 min.

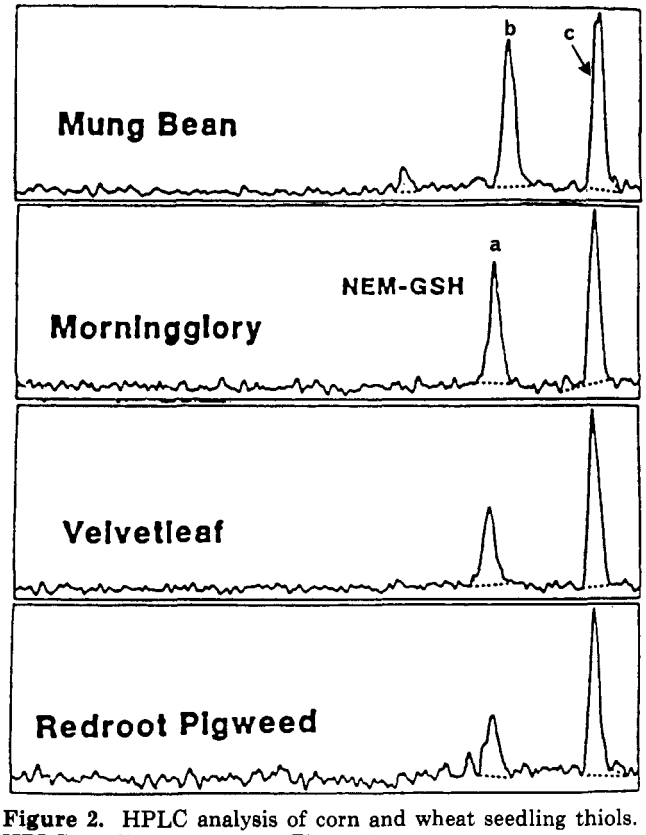


Figure 2. HPLC analysis of corn and wheat seedling thiols. HPLC conditions: same as Figure 1.

Table III. Effect of Flurazole Seed Safener on the Glutathione Content of Corn and Sorghum Shoots

seedlings	μg GSH/g fresh wt[a]	μg GSH/shoot
unsafened corn	293.8 ± 30.8 (a)	53.5 ± 5.6 (a)
safened corn	370.2 ± 23.7 (b)	64.9 ± 4.1 (b)
unsafened sorghum	102.1 ± 11.4 (c)	5.4 ± 0.7 (c)
safened sorghum	161.4 ± 24.1 (d)	8.2 ± 1.1 (d)

[a] Means within columns followed by the same letter are not significantly different at the 95% confidence level.

sorghum growing areas. This is especially true in the case of sorghum since a seed treatment of the safener will protect emerging sorghum seedlings from chloroacetanilide injury, which allows several problem weeds to be controlled in sorghum. The biochemical basis for the safening action is poorly understood at present (Hatzios, 1983; Stephenson and Ezra, 1983a). However, it has recently been reported that the safeners cause the chloroacetanilides to be metabolized more readily to the corresponding GSH conjugate (Gronwald et al., 1986; Ezra et al., 1986; Breaux et al., 1986b). The basis for the enhanced metabolism is not clear at present. Two possible reasons for the enhanced rate of metabolism are that the safened seedlings contain more GSH and/or more GST activity. An increase in GSH transferase activity has been reported for both the chloroacetanilide herbicide safeners (Mozer et al., 1983) and the thiocarbamate herbicide safeners (Lay and Casida, 1976; Mozer et al., 1983). The safeners have also been reported to increase plant GSH levels. For example, flurazole has been reported to cause an increase in the level of GSH in safened sorghum roots (Rubin et al., 1985). Since the chloroacetanilides are shoot active, we have also examined the effect of the safener flurazole on the sorghum and corn shoot levels.

The safener flurazole was found to cause an increase in both corn and sorghum shoot GSH levels as can be seen in Table III. The NEM analytical procedure revealed that GSH was the major thiol in both the safened and unsafened seedlings. The flurazole-safened sorghum shoots were found to contain approximately 50% more GSH than was found in the unsafened sorghum shoots. Safened corn shoots were found to have a 25% higher concontent of GSH than unsafened seedling shoots.

The biochemical mechanism responsible for enhanced GSH levels is uncertain. It has been proposed that the safeners may interfere with the regulation of glutathione biosynthesis in treated seedlings (Breaux et al., 1986b). The regulation of GSH biosynthesis in plants has been reported to be due to the feedback inhibition of glutamylcysteine synthetase by the product of this biosynthetic pathway, GSH (Rennenberg, 1982). It has recently been reported that chemicals that react with GSH may also cause an increase in GSH levels in other biological systems (Kondo et al., 1984). In this case it was found that the GSH conjugate of 2,4-dinitrochlorobenzene prevented the normal feedback inhibition of glutamylcysteine synthetase caused by GSH. We have recently reported that flurazole forms a GSH conjugate in corn and sorghum (Breaux, 1986b). It is possible that the GSH conjugate may interfere with GSH feedback regulation in safened seedlings. Studies to test this hypothesis are under way.

Dicot Seedling Thiol Analysis. Six broad-leafed seedlings with varying degrees of chloroacetanilide herbicide tolerance were chosen for the thiol analyses. The susceptible plants used were purslane and redroot pigweed. Velvetleaf, morningglory, and mung bean seedlings were examples of moderately susceptible seedlings while soybean was the tolerant plant used in the study. Young etiolated seedlings were used. The seedlings were extracted

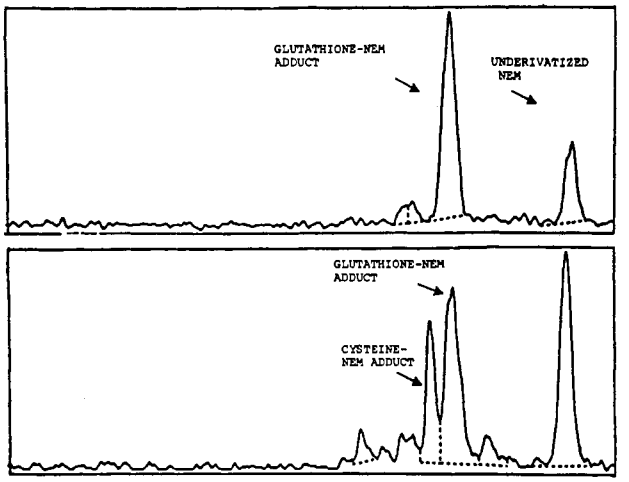


Figure 3. HPLC analysis of broad-leafed seedling thiols. HPLC conditions: same as Figure 1. Peaks: (a) NEM–glutathione conjugate, 7.6 min; (b) NEM–homoglutathione conjugate, 7.9 min; (c) underivatized NEM, 9.2 min.

Table IV. Broad-Leafed Seedling Glutathione Content

seedling	μg GSH/g fresh wt[a]	μg GSH/seedling
soybeans	125.9 ± 6.1 (a)	91.6 ± 2.2 (a)
mung bean	86.1 ± 5.0 (a)	32.2 ± 1.5 (a)
morningglory	108.0 ± 9.9 (a)	16.3 ± 1.7 (a)
velvetleaf	67.3 ± 4.6 (b)	6.9 ± 0.3 (b)
redroot pigweed	37.6 ± 1.4 (b)	0.1 ± 0.1 (b)
purslane	56.9 ± 8.8 (b)	0.2 ± 0.1 (b)

[a] Means within columns followed by the same letter are not significantly different at the 95% confidence level.

Table V. Distribution of Glutathione in Narrow-Leafed Seedlings

seedling	μg GSH/shoot	μg GSH/root	μg GSH/seed
corn	37.1 ± 5.1	33.8 ± 1.8	49.7 ± 3.2
sorghum	3.9 ± 0.1	1.2 ± 0.1	3.5 ± 0.4
wheat	3.6 ± 0.5	1.4 ± 0.1	4.6 ± 0.1
shattercane	1.7 ± 0.2	0.7 ± 0.1	2.2 ± 0.2

and analyzed by the [^{14}C]NEM method to identify the thiols in the broad-leafed seedlings. As can be seen in Figure 3 most of the broad-leafed seedlings also contained GSH as the main soluble thiol. However on the basis of retention times and subsequent mass spectral analysis, the major thiol in soybean and mung bean seedlings was identified as homoglutatione (hGSH). Homoglutathione is a homologue of GSH in which glycine has been replaced by β-alanine (Carnegie, 1963). We also used the NEM method to determine whether soybean seedlings also contain GSH. By using this technique hGSH was found to be the major detectable thiol in soybean seedlings.

The NEM method indicated that the broad-leafed seedlings contained mainly one thiol in each case. Since the DTNB method is much more rapid, we used this method for the quantitative analysis of broad-leafed thiols. As can be seen in Table IV the tolerant seedlings contained a much higher level of either GSH or hGSH than did the susceptible seedlings.

The NEM method was also used to compare the location of the thiols in the various part of the plant seedlings. For example, the coleoptile, seed, and roots of corn seedlings were examined for this purpose. In each case, GSH was the major thiol detected. The DTNB method was then used to quantify the thiol content in the seedling parts. In the case of the narrow-leafed seedlings, the thiols were evenly distributed (Table V). This was not the case with the broad-leafed seedlings, however. Most of the thiol activity was located in the cotyledons (Table VI).

Table VI. Distribution of Glutathione and Homoglutathione in Broad-Leafed Seedlings

seedling	μg GSH/hypocotyl	μg GSH/dicotyl
soybean[a]	18.3 ± 1.2	73.3 ± 1.0
mung bean	18.8 ± 1.5	13.4 ± 0.1
morningglory	5.5 ± 0.9	10.7 ± 0.8
velvetleaf	2.7 ± 0.1	4.1 ± 0.2

[a] Soybean and mung bean seedlings contain homoglutathione.

In summary, a new method was developed and used to determine the level of detoxification thiols in chloroacetanilide-tolerant and -susceptible seedlings. This new method was also used to determine the effect of the chloroacetanilide herbicide safener flurazole on corn and sorghum seedlings shoot GSH content. It was found that the tolerant seedlings contained more GSH or hGSH than did the susceptible seedlings. Furthermore, the safener treatment increased the level of GSH in the shoots of the treated corn and sorghum seedlings. Based upon these results, one explanation for the higher level of chloroacetanilide tolerance is that the pool of the detoxification thiols, GSH or hGSH, is higher in tolerant plants than in susceptible plants. A second possibility is that the activity of the enzymes that catalyze the conjugation of the herbicides with GSH, the glutathione-*S*-transferases, is higher in the tolerant plants than in the susceptible plants. This could be due either to a higher level of protein or to the presence of more catalytically efficient GSTs in the tolerant seedlings (Mozer et al., 1983). The primary factor, however, may be the level of the GST or hGST substrate, GSH or hGSH, in seedlings emerging through the layer of chloroacetanilide herbicide in the soil. The more susceptible small seedlings such as foxtails and purslane have only a small amount of GSH available for herbicide detoxification when compared to the more tolerant corn seedlings. Since the biosynthesis of GSH is dependent upon the light-driven reduction of sulfate (Rennenberg, 1982), the seedlings cannot make more GSH while they are still preemerged in the soil–herbicide layer and not receiving sufficient light for sulfate reduction. In summary, our results showed a correlation between the GSH content of seedlings and chloroacetanilide herbicide selectivity.

The NEM thiol method is in the process of being adapted for the analysis of the oxidized form of glutathione, glutathione disulfide. The preliminary results indicate that most of the GSH in seedlings is in the reduced form, which is in agreement with published work (Rennenberg, 1982). A second area in which this analytical methodology is proving useful is the investigation of thiol biosynthesis. Preliminary studies have shown that unlabeled NEM can be used to trap ^{35}S-labeled GSH formed in vivo from [^{35}S]cysteine. In this case the same derivatization procedure, HPLC conditions, and radiochemical HPLC detector could be used as employed in the [^{14}C]-NEM method for analyzing unlabeled thiols.

Registry No. GSH, 70-18-8; hGSH, 18710-27-5; [^{14}C]NEM, 108031-85-2; GSH-NEM adduct, 23559-30-0; hGSH-NEM adduct, 107959-93-3; flurazole, 72850-64-7.

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