INVESTIGATIONS ON THE MECHANISM OF ACTION OF THE OXIME ETHER SAFENERS FOR THE PROTECTION OF GRAIN SORGHUM AGAINST METOLACHLOR

by

Samuel P. Yenne

Dissertation submitted to the Faculty of the

Virginia Polytechnic Institute and State Unviersity

in partial fulfillment of the requirements for the degree of

DOCTOR OF PHILOSOPY

in

Plant Physiology/Weed Science

APPROVED:

K. K. Hatzios, Chairman

J. G. Foster

J. Derr

J. Hess

D. M. Orcutt

JUNE, 1989

Blacksburg, Virginia

INVESTIGATIONS ON THE MECHANISM OF ACTION OF THE OXIME ETHER SAFENERS FOR THE PROTECTION OF GRAIN SORGHUM AGAINST METOLACHLOR

by

SAMUEL P. YENNE

Committee Chairman: Kriton K. Hatzios Department of Plant Pathology, Physiology, and Weed Science

(ABSTRACT)

Herbicide safeners (protectants, antidotes) are used to protect crop plants from herbicide injury. Currently our understanding of the mechanisms involved in the protection of plants by safeners is not well defined; therefore, investigations were conducted to elucidate the mechanism(s) of action of the oxime ether safeners. Molecular comparisons of selected herbicide-safener combinations using computer-aided molecular modeling revealed that the chemical structures of safeners and herbicides are very similar at the molecular level; and, indicate that these compounds could bind at the same active site of the target protein or they may serve as inducers of metabolic enzymes which detoxify herbicides. Metolachlor at 10 μM and seed-applied CGA-133205 had no effect on germination while treatment with seed-applied oxabetrinil significantly reduced germination of grain sorghum. Results from experiments on ^{14}C-acetate incorporation into lipids indicate that metolachlor and

the oxime ether safeners influence lipid metabolism causing a redistribution of carbon in the lipid fractions of germinating sorghum roots. Results from studies with acetyl-CoA carboxylase indicate that this enzyme is not a target site for either metolachlor or the oxime ether safeners. Metolachlor and the oxime ether safeners enhanced glutathione levels in grain sorghum seedlings at 12 to 48 hr after imbibition was initiated with oxabetrinil being more stimulatory than metolachlor or CGA-133205. Glutathione reductase activity was also stimulated in safener-treated grain sorghum seedlings. Both safeners slightly enhanced nonenzymatic and enzymatic conjugation of metolachlor with reduced glutathione. Oxabetrinil conjugated enzymatically or nonenzymatically with reduced glutathione at a slow rate, but CGA-133205 did not. These data suggest that during the early stages of seed germination and seedling development of grain sorghum, safeners can enhance the detoxication of metolachlor by enhancing glutathione levels and enzymatic and nonenzymatic conjugation of metolachlor with glutathione. It appears that oxabetrinil and CGA-133205 are conferring protection to grain sorghum by increasing the rate of metolachlor metabolism.

ACKNOWLEDGEMENTS

I would like to express my thanks to Dr. Kriton Hatzios for his guidance and support, and the remainder of my committee, Dr. Joyce Foster, Dr. John Hess, Dr. Jeff Derr, and Dr. Dave Orcutt for their assistance and review of this dissertation. Financial support of this research was provided by a research grant from the Herman Frasch Foundation of the American Chemical Society, Washington, DC. Partial support was also provided by grants from Grants-in-Aid of Research, Sigma Xi, The Scientific Research Society, New Haven, CT, and from Graduate Research Development Grant, Graduate School, Virginia Polytechnic Institute and State University, Blacksburg, VA. Gratitude is also expressed to Ciba-Geigy Corp., Greensboro, NC, for supplying untreated and safener-treated grain sorghum seeds as well as the technical and ^{14}C-labeled samples of metolachlor, oxabetrinil, and CGA-133205 used in these experiments.

I am deeply indebted to my fellow graduate students, particularly, for the scientific discussions, scientific assistance, and for assistance in maintaining the proper perspective on life. I am also indebted to for lending a helping hand when ever one was needed.

Finally, I am forever grateful to my wife, for standing by my side during this period of my life. With each others support we can go forth; conquering any adversities while achieving our goals and aspirations.

TABLE OF CONTENTS

LIST OF ABBREVIATIONS

acetyl-CoenzymeA carboxylase [EC 6.4.1.2]

alachlor, N-(2,6-diethylphenyl)-N-(methoxymethyl)acetanide

bentazon, 3-(1-methylethyl)-(1H)-2,1,3-
benzothiadiazin-4(3H)-one 2,2dioxide

CAMM, computer-aided molecular modeling

CDNB, 1-chloro-2,4-dinitrobenzene

CGA-133205, O-[1,3-dioxolan-2-yl-methyl]-2,2,2-trifluro-
4'-chloroacetophenone-oxime

cyometrinil, (Z)-a[(cyanomethoxy)imino]benzenacetonitrile

dichlormid, 2,2-dichloro-N,N-di-2-propenylacetamide

EPTC, S-ethyl dipropylcarbamothioate

flurazole, phenylmethyl-2-chloro-4-(trifluoromethyl)-5-
thiazolecarboxylate

GR, glutathione reductase, [EC 1.6.4.2]

GSH, reduced glutathione

GSSG, oxidized glutathione (glutathione disulfide)

GST, glutathione-S-transferase [EC 2.5.1.18]

haloxyfop,2,-[4-[[3-chloro-5-(trifluoromethyl)-2-
pyridinyl]oxy]phenoxy]propanoic acid

hGSH, homoglutathione

metolachlor, 2-chloro-N-(2-ethyl-6-methylphenyl)-N-
(2-methoxy-1-methylethyl)acetamide

oxabetrinil, N-(1,3-dioxolan-2-yl-methoxy)-imino-
benzacetonitrile

R-29148, 3-(dichloroacetyl)-2,2,5-trimethyl-1,3-oxazolidine

sethoxydim, 2-[1[(ethoxyiminio)butyl]-5-[2-(ethylthio)propyl]-3-hydroxy-2-cyclohexen-1-one

LIST OF TABLES

LIST OF FIGURES

I. INTRODUCTION

The use of safeners to protect crops from herbicide injury has become a significant agricultural practice in the past two decades. Otto Hoffman [1962] introduced the concept of chemical enhancement of crop tolerance to herbicides and later developed the first commercial safener, 1,8-naphthalic anhydride, which was patented in 1971 [Pallos & Casida, 1978]. In 1972, dichlormid was patented as the second commercial herbicide safener [Pallos & Casida, 1978]. Several other safeners, including the oxime ethers, have since been patented. The oxime ether safeners, cyometrinil, oxabetrinil and CGA-133205, have been developed by CIBA-GEIGY Corporation (Basel, Switzerland) to protect grain sorghum against injury caused by the chloroacetanilide herbicide, metolachlor [Ebert & Gerber, 1988; Hatzios, 1988; LeBaron et al., 1988].

The exact mechanism by which safeners protect grass crops against herbicide injury is not known. Hatzios [1983] summarized four general mechanisms of action for herbicide safeners. First, the safener may interfere with herbicide uptake and/or translocation; second, the safener may compete with the herbicide at a common site of action within the protected plant (competitive anagonism) third, the safener may

stimulate herbicide degradation within the plant degradation); and fourth, the safener may act through a combination of the above processes.

In some cases, the structural similarity between (enhanced herbicides and safener molecules is apparent, but in other cases, the similarities between these molecules are not so obvious. If safeners and herbicides are similar at the molecular level then this would lend support to the proposed hypotheses that safeners serve either as substrates for inducible herbicide degrading enzyme(s) or as as competitive antagonists of herbicides at a common target site.

Many of the plant metabolic processes influenced by the chloroacetanilide herbicides are synthesized via acetyl-Coenzyme A (Acetyl-CoA) intermediates [Fuerst, 1987]. Some of these metabolic processes include protein synthesis, terpenoid synthesis, gibberellic acid synthesis, and lipid synthesis [Fuerst, 1987]. Lipid and terpenoid metabolic pathways appear to be likely target sites for a competitive antagonism between safeners and herbicides [Wilkinson, 1988].

Safener-induced enhancement of herbicide detoxication also appears to be a likely mechanism of action of herbicide safeners. The majority of the chloroacetanilide herbicides are detoxified in plants

via conjugation with reduced glutathione (GSH). The GS-conjugate of these herbicides is catabolized further, but the importance of these metabolites is not known [Gronwald, 1988]. At the present time it is unclear as to what influence safeners have on the detoxication of the chloroacetanilide herbicides. Gronwald [1988] recently reported that the degree of protection provided by safeners to grass crops against injury from chloroacetanilide herbicides correlates rather strongly with the ability of safeners to enhance glutathione-S-transferase(s) activity in corn and grain sorghum. However, other questions regarding the influence of safeners on glutathione levels and the activity of other glutathione-related enzymes needs to be examined.

It is known that oxime ether safeners applied to grain sorghum will protect this crop from injury caused by a subsequent application of metolachlor. The fate of the safener after it has been applied to grain sorghum is not known. Uptake of cyometrinil through coleoptiles of sorghum seedlings has been observed [LeBaron et al., 1988], but involvement of cyometrinil in plant metabolism thereafter has not been evaluated. Breaux et al. [1988] demonstrated that flurazole, a thiazole carboxylate safener that is applied to grain

sorghum seed, was rapidly absorbed and metabolized in 3- to 5-day-old grain sorghum shoots. The major metabolite that was detected was the glutathione-flurazole conjugate [Breaux, 1988].

OBJECTIVES OF DISSERTATION RESEARCH

The overall objective of this dissertation research was to elucidate the mechanism(s) of action of the oxime ether safeners and to determine their fate within safened plants. Specifically, the objectives of these studies were:

a) to use computer-aided molecular modeling (CAMM) analysis to model the herbicides and their respective safeners and make molecular comparisons for determining the structural similarities of these molecules;

b) to evaluate the influence of the herbicide, metolachlor, on germination and root growth of grain sorghum grown from seed that was untreated or treated with the oxime ether safeners, oxabetrinil or CGA-133205;

c) to determine the influence of metolachlor alone and in combination with oxabetrinil or CGA-133205 on lipid metabolism in grain sorghum seedlings;

d) to determine the influence of seed-applied oxabetrinil and CGA-133205 on the levels of glutathione and the activity of glutathione reductase and glutathione-S-transferase of grain sorghum seeds during germination and early seedling establishment;

e) to determine the influence of pH and metolachlor on the potential nonenzymatic conjugation

of oxabetrinil and CGA-133205 with reduced glutathione;

f) to determine the uptake, translocation, and metabolism of the oxime ether safeners, oxabetrinil and CGA-133205, in grain sorghum; and

g) to determine the influence of oxabetrinil and CGA-133205 on the uptake, translocation, and metabolism of metolachlor in grain sorghum.

REFERENCES

Breaux, E. J., M. A. Hoobler, J. E. Patanella, and G. A. Leyes (1988). Mechanisms of action of thiazole safeners. in "Crop Safeners for Herbicides: Development, Uses, and Mechanisms of Action" (K. K. Hatzios and R. E. Hoagland, Eds.), Academic Press., New York, 163-175.

Ebert, E. and Gerber, H. R. (1988). Differential effects of oxabetrinil and fenclorim against metolachlor and pretilachlor injury on various grasses. in "Crop Safeners for Herbicides: Development, Uses, and Mechanisms of Action" (K. K. Hatzios and R. E. Hoagland, Eds.), Academic Press, Inc., New York, 177-194.

Fuerst, E. P. (1987). Understanding the mode of action of chloroacetanilide and thiocarbamate herbicides. Weed Technol. **1**, 270-277.

Gronwald, J. W. (1988). Influence of herbicide safeners on herbicide metabolism. in "Crop Safeners for Herbicides: Development, Uses, and Mechanisms of Action" (K. K. Hatzios and R. E. Hoagland, Eds.), Academic Press., New York, 163-175.

Hatzios, K. K. (1988). Development of Herbicide Safeners: Industrial and University Perspectives.

in "Crop Safeners for Herbicides; Development, Uses, and Mechanisms of Action" (K. K. Hatzios and R. E. Hoagland, Eds.), Academic Press, Inc., New York, 3-46.

Hatzios, K. K. (1983). Herbicide antidotes: Development, chemistry and mode of action. Adv. Agron. **36**, 265-316. Hoffman, O. L. (1962). Chemical seed treatments as herbicide antidotes. Weed Sci. **10**, 322-332.

LeBaron, H. M., McFarland, J. E., Simoneaux, B. J. and Ebert, E. (1988). Metolachlor. in "Herbicides: Chemistry, Degradation, and Mode of Action" (P. C. Kearney and D. D. Kaufman, Eds), Marcel Dekker, Inc., New York, Vol. 3, 335-382.

Pallos, R. M., and Casida, J. E. (1978). Chemistry and Action of Herbicide Antidotes. Academic Press, New York.

Wilkinson, R. E. (1988). Terpenoid Biosynthesis as a Site of Action for Herbicide Safeners. in "Crop Safeners for Herbicides: Development, Uses, and Mechanisms of Action" (K. K. Hatzios and R. E. Hoagland, Eds.), Academic Press., New York, 163-175.

II. MOLECULAR COMPARISONS OF SELECTED HERBICIDES AND SAFENERS BY COMPUTER-AIDED MOLECULAR MODELING

INTRODUCTION

Computer-aided molecular modeling (CAMM) is now recognized by chemists as a powerful tool for the design and development of new chemicals such as pharmaceuticals and pesticides [Davies, 1986; Seiter & Cohan, 1986]. CAMM allows chemists to compare three-dimensional (3-D) spatial and electronic properties of hypothetical compounds to those of compounds with known activity, providing a rational basis for selecting new molecules to be synthesized in the laboratory [Davies, 1986]. Besides the obvious applications of CAMM in chemical synthesis, CAMM is also useful to weed scientists and plant physiologists for comparing compounds with a known mechanism of action to compounds with an unknown mechanism of action. If the two compounds have similar chemical structures and properties, then it is likely that these chemicals will have similar mechanisms of action. Until recently, the use of CAMM was limited to scientists of major chemical corporations because of the powerful computer equipment needed to run the CAMM programs. With rapid advances in computer technology,

CAMM analysis can now be conducted with personal computers [Davies, 1986; Rodgers, 1987].

CAMM programs available for use with personal computers have some limitations. The major ones are related to the size of molecules that can be modeled, the graphic display of the modeled compound, and the lack of computational ability for some of the more complex quantum mechanical calculations [Davies, 1986]. However, even with these limitations, the information that can be generated about a series of molecules can be useful to chemists and plant physiologists. Programs available for use on the personal computer can provide scientists with enough information to compare the size, shape, and charge distribution of molecules containing up to 100 atoms.

Molecular comparisons of compounds possessing biological activity can be also evaluated by the use of quantitative structure-activity relationships (QSAR). QSAR provides information about the identity and location of functional groups on a general structure required for biological activity. Some of the more common parameters used in QSAR studies include the octanol/water partition coefficient; steric properties, usually determined by the Hansch equations; and inductive constants [Hansch, 1979]. QSAR methods are

useful in describing the importance of the functional groups when comparing molecules with the same general structure, but these methods do not allow the comparison of molecules that have different general structures. The major disadvantages of QSAR are: a) biological response data must be generated for each analog that is to be evaluated, and b) many analogs must be evaluated for statistical purposes. Generation of such biological data may be time consuming and very expensive.

Herbicide safeners, also known as antagonists, antidotes, and protectants, are used to protect crop plants from the applied herbicide; thus, allowing for the control of closely related weed species or hard-to-control weed species without crop injury. The safener may be applied in one of two ways, depending on the selectivity of the safener. If the safener is selective only for the crop then the safener can be applied as a tank mixture with the herbicide. This is the case with the carbamothioate herbicide, EPTC, and the dichloroacetamide safener, dichlormid (Figure 1), which are used extensively in corn as a formulated mixture. If the safener is nonselective it must be applied only to the crop. This specificity is usually achieved by treating the crop seed before planting.

The oxime ether safeners (Figure 2) and the thiazole carboxylate safener, flurazole, (Figure 3) are applied in this manner when used in combination with the chloroacetamide herbicides metolachlor (Figure 4) and alachlor (Figure 3), respectively on grain sorghum.

The exact mechanism by which these safeners protect the grass crops against herbicide injury is not known. Hatzios [1983] has summarized four general mechanisms of action for herbicide safeners. First, the safener may interfere with herbicide uptake and/or translocation; second, the safener may compete with the herbicide at a common site of action within the protected plant (competitive antagonism); third, the safener may stimulate herbicide degradation within the plant (enhanced degradation); and fourth, the safener may act through a combination of the above processes.

The "competitive antagonism theory" and the "enhanced degradation theory" proposed for explaining the mechanism of the protective action of herbicide safeners imply that the molecules of safeners and herbicides may have some common molecular characteristics. In some cases, the structural similarity of herbicide and safener molecules is apparent (Figure 1). However, in other cases, the similarities between herbicides and safeners are not so

obvious (Figures 2, 3, and 4).

Therefore, the objective of this study was to use CAMM analysis for modeling selected herbicides and their respective safeners and then to make molecular comparisons for determining the structural similarities of these molecules. Structural similarities between safeners and herbicides would support the proposed hypotheses that safeners serve either as inducible substrates for herbicide degrading enzymes or as competitive antagonists of herbicides at a common target site. The particular herbicide/safener combinations that were analyzed included EPTC/dichlormid, alachlor/flurazole, and metolachlor/oxime ether safeners (cyometrinil, oxabetrinil, and CGA-133205).

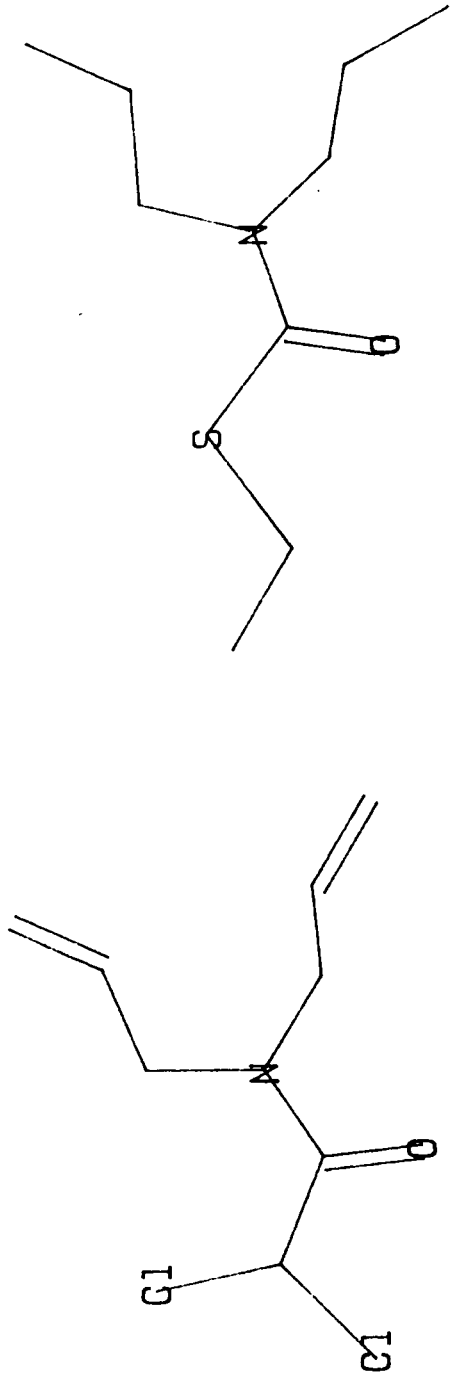


Figure 1. Molecular structures of dichlormid (left) and EPTC (right).

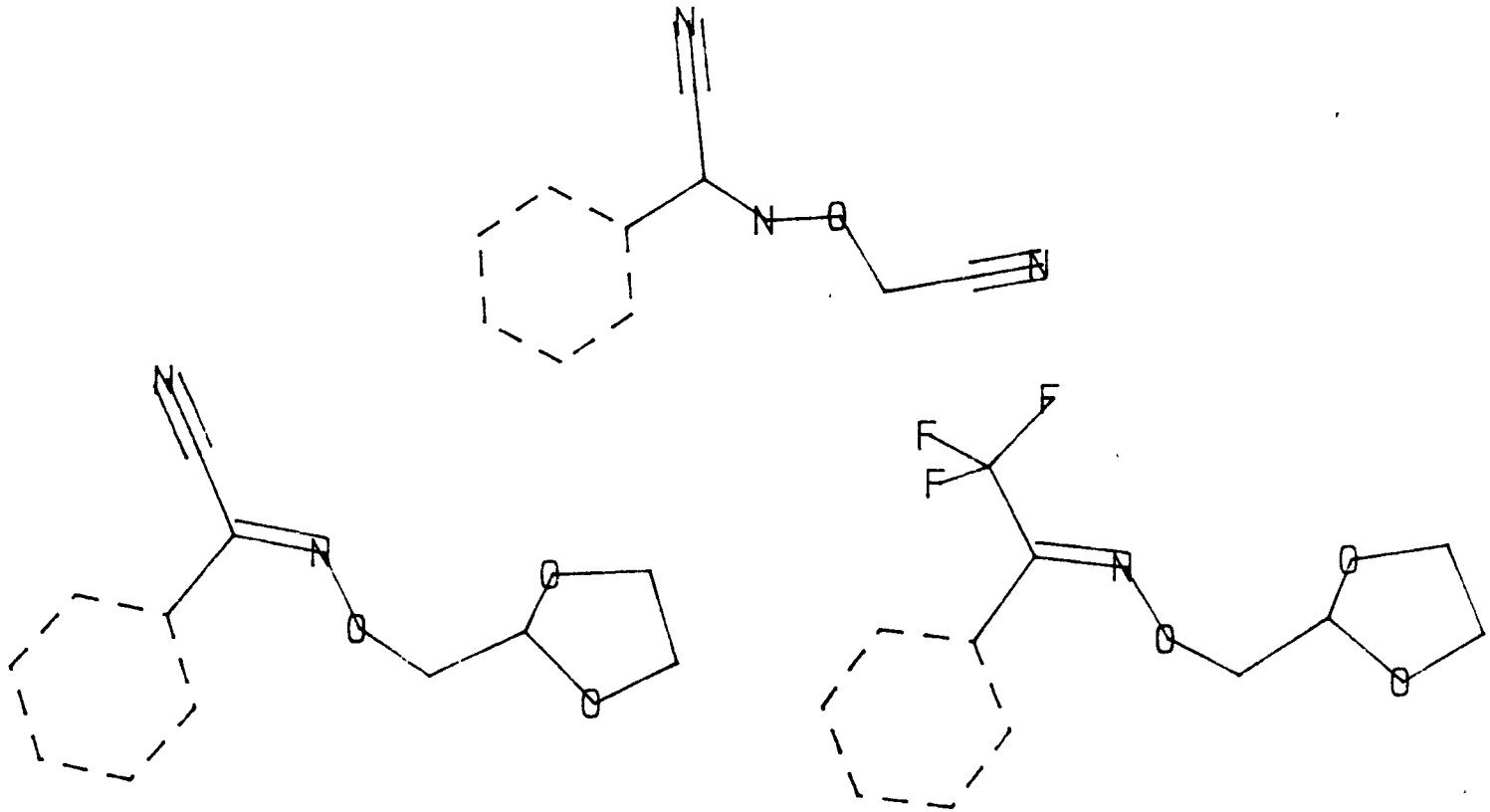


Figure 2. Molecular structures of the oxime ether safeners, cyometrinil (top), oxabetrinil (bottom left), and CGA-133205 (bottom right).

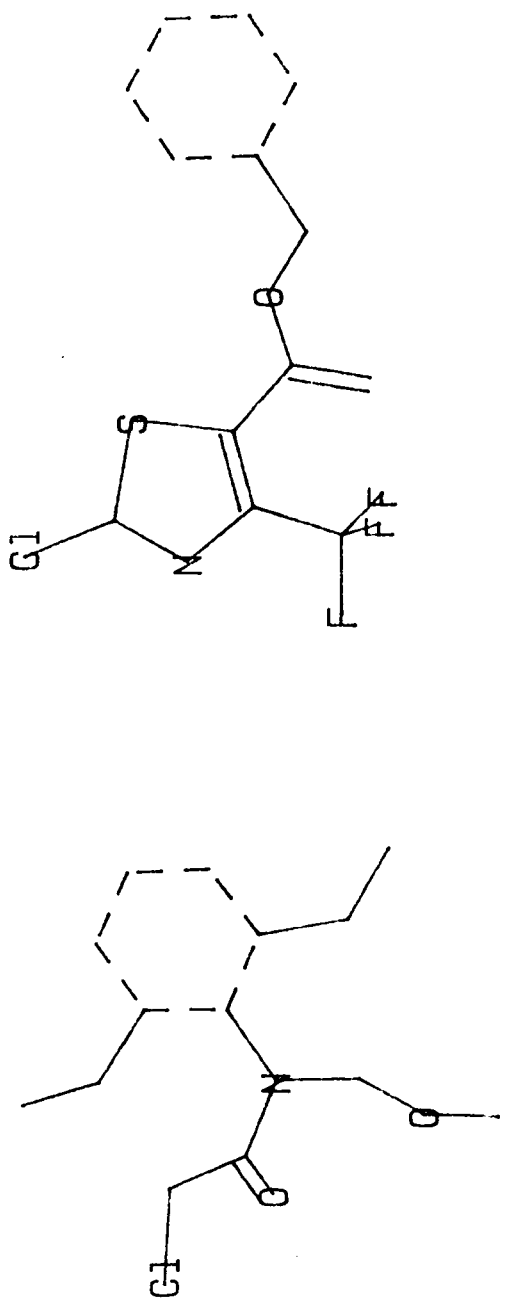


Figure 3. Molecular structures of alachlor (left) and flurazole (right).

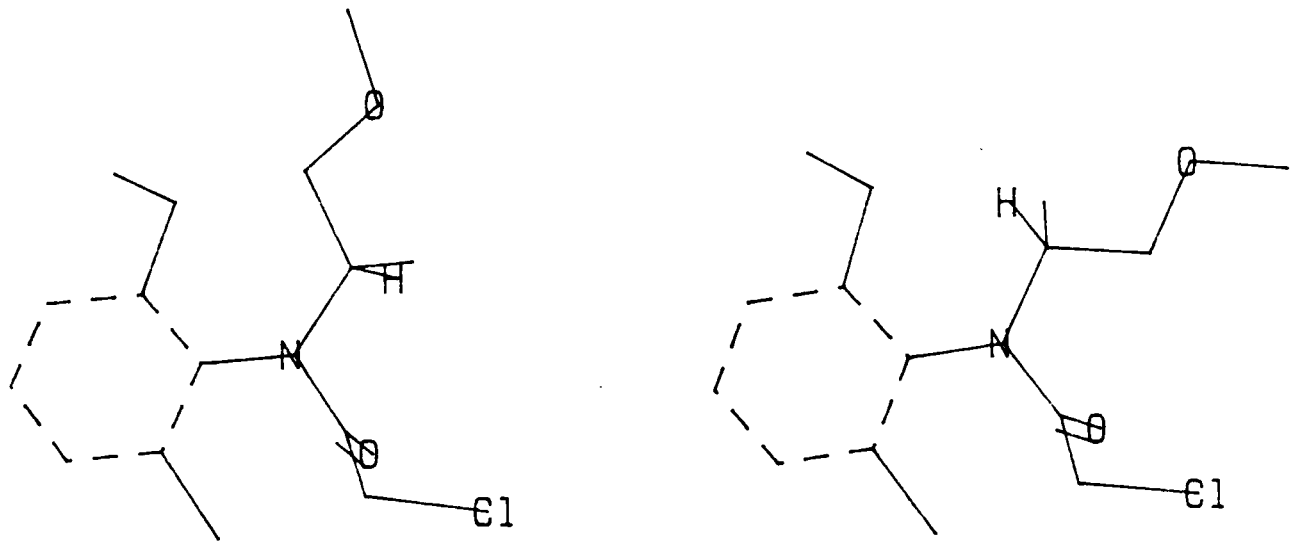


Figure 4. Molecular structures of the less active isomer of metolachlor (left) and active isomer of metolachlor (right).

MATERIALS AND METHODS

Overview of software program. XICAMM® (XIRIS Corp., P. O. Box 787 New Monmouth, NJ 07748), a molecular modeling program that is compatible with personal computers was used in this experiment. The first step in using this program is to enter the molecule of interest. Entering the molecule can be accomplished by drawing the molecule on the screen using the cursor keys to position the cursor, the letter keys to designate the atom after the cursor is positioned, and the number keys to designate the bond type. An easier and faster method is to enter fragments such as rings using the function keys and then to bond these with side chains or other rings that were entered using either the function keys or letter, number, and cursor keys. When working with a series of related molecules that have the same general structure, an easier and faster method is to enter the general structure, model the general structure, and then edit this general structure using the methods mentioned above. Using this method of entering and modeling molecules reduces the amount of time to model the molecules and also insures that the core of the molecules is in the same configuration.

After the molecule is entered, the next step is to model it. Molecular modeling consists of a series of algorithms to minimize the internal molecular energy associated with bonding and nonbonding interactions. The algorithms used may vary with the molecular modeling program that is being used; therefore, one should use caution comparing results obtained from different programs. Even though different algorithms are used to obtain the final results, the same basic parameters are used in each alogrithm. In some cases the program may locate a local energy minimum that is not the true minimum for the molecule. To correct for this one must examine the bond and atom strains along with the 3-D structure and if the strains appear high or if an atom appears to be trapped then the atom must be moved and the molecule remodeled.

Information obtained. The first piece of information obtained from XICAMM is data concerning each atom in the molecule. These data consist of the atomic number, atomic weight, valence, hybrid (Sp1, Sp2, or Sp3), number of implied hydrogens associated with the atom, cyclic or acyclic, and bond data such as the atoms involved in each bond, the bond length, the bond angle, the dihedral angle, and the amount of energy associated with the angle strain, chiral strain,

and non-bonding strain. After this information is obtained and evaluated by the researcher to determine if the molecule appears to be modeled correctly other parameters about the molecule are calculated. The first of these calculated parameters is the connectivity indices described later. Other calculated parameters are the principal moments and the molecular volume.

The final molecular comparison is a visual comparison that includes superimposing one molecule over the other for a geometric evaluation of one molecule relative to the other. The superimposing of these two molecules is accomplished by designating at least three strategic atom pairs between both molecules; after which, the distance between the atom pairs is minimized without altering the molecular configuration of either molecule. After the distance between the two molecules is minimized, the structures are redrawn on the screen; one in red, one in green, and the area of exact overlap is yellow. Also, these two molecules can be rotated in space as one unit so that they can be observed from any angle.

Calculation of parameters. Our molecular comparisons included size, shape, principal moments, molecular volume, and connectivity indices. In order

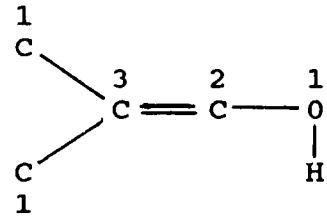
to make these comparisons the molecules must first be modeled i.e. their most stable configuration is calculated. This is accomplished by using CAMM. When doing this type of research one must remember that the most stable configuration may not be the most active configuration. But, even if the most stable configuration is not the most active configuration, it is a good starting point for CAMM and for the molecular comparisons. After the most stable configuration is generated, the molecular parameters can be obtained. The commercial formulation of metolachlor, DUAL®, contains four stereoisomers [Moser, 1985]. Two of the four isomers exist because the two different ortho substituents hinder rotation around the N-aromatic bond giving rise to a chiral axis (Figure 4). The other two isomers arise from the chiral-C atom (C-1′) giving rise to an S and an R-enantiomer. These four stereoisomers exhibited differential herbicidal activity when applied preemergence to seven grass and three broadleaf plants with the two S-enantiomers being the most herbicidally active [LeBaron et al., 1988; Moser et al., 1985]

From the modeled structure, principal moments and the molecular volume are calculated. Principal moments describe the charge distribution along the x, y, and z-axes. They are calculated from the center of mass of

the molecule using the symmetrical tensor of gyration matrix from which eigenvalues and eigenvectors are obtained [Anonymous, 1986]. Principal moments are sorted from largest to smallest. The molecule is then rotated to align the largest with the x-axis and the smallest with the z-axis. Finally, the molecular volume is calculated using the van der Waals radius for each atom minus the amount of overlap from adjacent atoms [Anonymous, 1986]. The amount of overlap is determined from bond lengths obtained from the modeled bond distances. The calculated volume for each atom is summed and the total volume is reported as cm^3/mole.

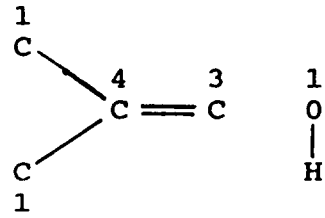
Connectivity indices are a numerical descriptor of the number of atoms and the amount of branching in a molecule [Randic, 1975]. Connectivity indices have been used to predict the adsorption of pesticides by soils with limited success [Gerstl & Helling, 1987] and to predict the protein binding and cellular uptake of pesticides in cultured human cells with good success [Murakami & Fukami, 1985]. For a complete review of connectivity indices and how they are calculated see Kier and Hall [1986]. Three different connectivity indices are calculated: the 'normal connectivity index', the 'bonds connectivity index', and the 'valence connectivity index'. These different

connectivity indices differ in their consideration of multiple bonds and charged atoms within the molecule. To calculate a normal connectivity index each non-hydrogen atom is assigned a number corresponding to the number of non-hydrogen atoms to which it is bound (Figure 5). Next, the values for adjacent atoms are multiplied, the reciprocal square root is calculated, and these are summed (Figure 5) to give the normal connectivity index. The bonds connectivity index makes an adjustment for multiple bonds and then the bonds connectivity index is calculated as above. Valence connectivity indices take into account valence electrons associated with heteroatoms (non-carbon atoms) and is calculated as described above (Figure 5) [Kier and Hall, 1986].



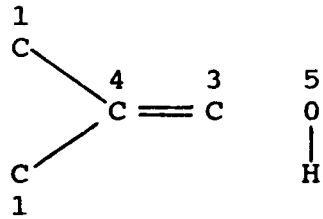
$$(1.0*3.0)^{-0.5}+(1.0*3.0)^{-0.5}+(3.0*2.0)^{-0.5}+(2.0*1.0)^{-0.5}=2.270$$

(normal connectivity index)



$$(1.0*4.0)^{-0.5}+(1.0*4.0)^{-0.5}+(4.0*3.0)^{-0.5}+(3.0*1.0)^{-0.5}=1.866$$

(bond connectivity index)



$$(1.0*4.0)^{-0.5}+(1.0*4.0)^{-0.5}+(4.0*3.0)^{-0.5}+(3.0*5.0)^{-0.5}=2.270$$

(valence connectivity index)

Figure 5. Sample calculations of connectivity indices. Numbers next to atoms represent the number of bonded non-hydrogen atoms (normal connectivity index) and adjusted for multiple bonds (bonds connectivty index) and for valence electrons (valence connectivity index). Indices are then calculated by taking the reciprocal square root of all possible combinations and summing them.

RESULTS AND DISCUSSION

Molecular comparison of EPTC and dichlormid by CAMM analysis. Comparison of the calculated parameters of EPTC and dichlormid indicate that these two molecules have almost the same number of atoms (normal connectivity index, Table 1.) However, when evaluating the bonds connectivity index, it is revealed that dichlormid contains more multiple bonds than does EPTC (Table 1) and this is also illustrated in Figure 1. Evaluation of the principal moments revealed that the charge associated with EPTC is spread over a slightly longer distance along the x-axis, but the charge distribution along the y- and z-axes is almost identical between EPTC and dichlormid. The molecular volume of these two chemicals is almost identical (Table 1). The superimposing of these two structures with the carbamate portion of the two molecules being aligned revealed that these two molecules are in fact similar. The only major difference is that the two chlorine atoms of dichlormid do not superimpose over any functional group of EPTC. However, Gronwald [1988], has reported that the sulfur atom of EPTC undergoes an oxidation reaction generating the herbicidal EPTC-sulfoxide. Now if EPTC-sulfoxide and

Table 1. Comparison of the calculated molecular parameters of the herbicide, EPTC, and the safener, dichlormid.

Calculated parameters	EPTC	EPTC sulfoxide	dichlormid
Connectivity indices			
normal	5.757	6.167	5.629
bonds	5.434	5.537	4.535
valence	5.740	5.998	4.362
Principal moments			
x-axis	6.055	5.591	5.501
y-axis	1.809	1.924	1.865
z-axis	0.055	0.071	0.056
Modeled strain	2.727	1.352	3.510
Molecular volume (cm^3/mole)	144.768	123.152	138.793

dichlormid structures are superimposed, the two compounds are similar with active functional groups in the same location on both molecules (Figure 6).

Molecular comparison of alachlor and flurazole by CAMM analysis. When comparing alachlor with the safener, flurazole, most of the calculated parameters are similar except for the principal moment along the x-axis and the molecular volume (Table 2). Figure 3 and 6 reveal that flurazole is a larger molecule than alachlor; therefore, these differences are not surprising. However, all three connectivity indices are similar indicating that the number of atoms, bonds, types of bonds, and valence electrons contained in alachlor and flurazole are similar (Table 2). When the alachlor structure is superimposed over the flurazole structure (Figure 7), the phenyl rings superimpose almost directly while the acyl side chain of alachlor superimposes over the side chain of flurazole with the chlorine atom being near the trifluromethyl group, and the alkyl side chain does not superimpose directly over the flurazole molecule. However, the end of the alkyl side chain of alachlor is located near the heterocyclic ring of flurazole (Figure 7).

Molecular comparison of metolachlor stereoisomers by CAMM analysis. As mentioned earlier the commercial formulation of metolachlor contains four stereoisomers. If the four stereoisomers are separated only by the chiral-C, ignoring the isomers originating from the chiral axis, and then evaluated for herbicidal activity, the R-enantiomer is inactive while the S-enantiomer is very active against three broadleaf and seven grass weeds [LeBaron et al., 1988; Moser et al., 1985]. It is interesting to note that while the S-enantiomer at the chiral-C atom has high herbicidal activity, it has virtually no fungicidal activity, but the R-enantiomer has high fungicidal activity and very little herbicidal activity [Moser et al., 1985]. Comparison of the calculated parameters and the visual features of the inactive versus active enantiomers of metolachlor did not reveal any major molecular differences (Table 3). Connectivity indices will not change because they are based on the number of atoms, bonds, and types of atoms and bonds which are the same in all isomers. When comparing the principal moments for the two enantiomers of metolachlor there exists a difference in the numerical value of the charge distribution along each axis (Table 3). This difference is explained by the placement of the methyl

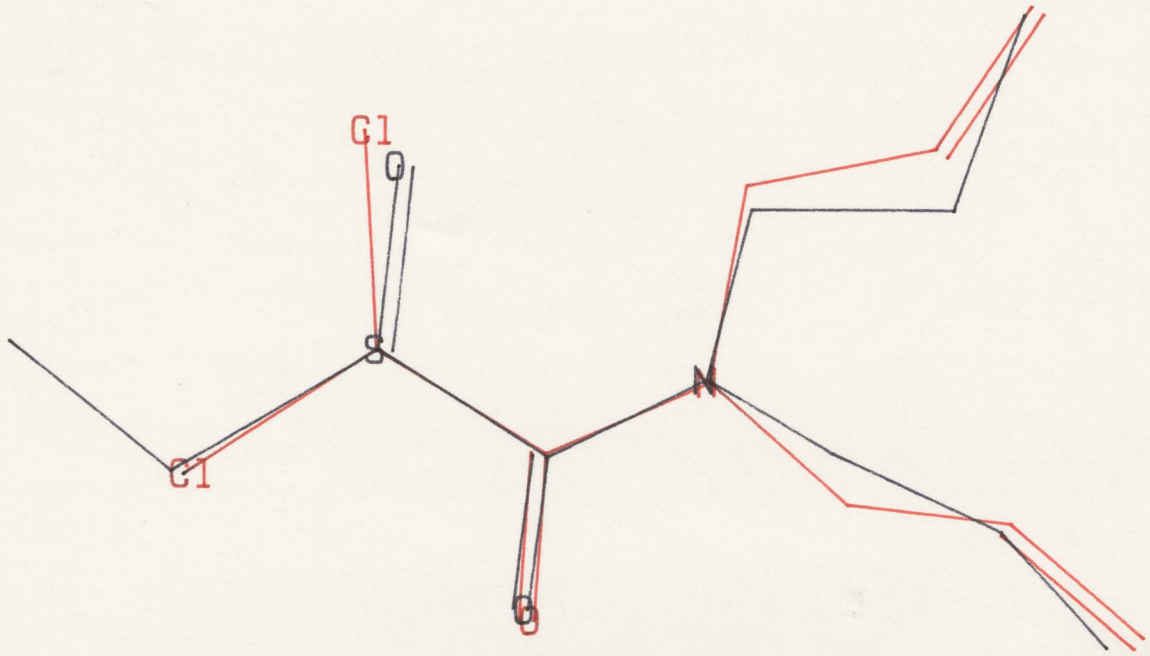


Figure 6. Structure of EPTC-sulfoxide (black) superimposed over the structure of the safener, dichlormid (red).

Table 2. Comparison of the calculated molecular parameters of the herbicide, alachlor, and the safener, flurazole.

Calculated parameters	Alachlor	Flurazole
Connectivity indices		
normal	8.275	8.941
bonds	7.182	7.487
valence	6.438	6.675
Principal moments		
x-axis	5.474	10.158
y-axis	3.158	3.422
z-axis	0.171	0.200
Modeled strain	2.547	10.130
Molecular volume (cm^3/mole)	168.297	225.940

Figure 7. Structure of the herbicide, alachlor, (black) and the safener, flurazole, (red) superimposed.

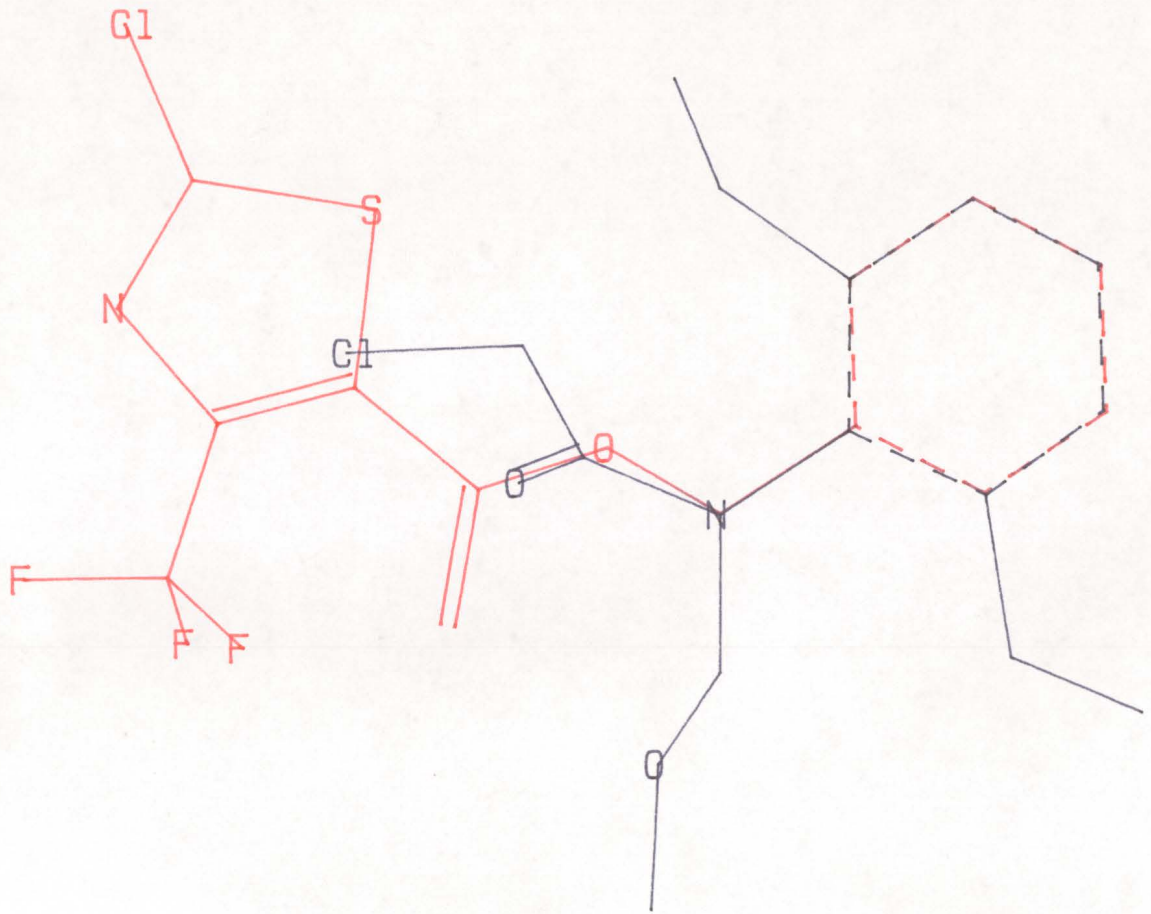


Table 3. Comparison of the calculated molecular parameters for the least and most active isomers of the herbicide, metolachlor, and the oxime ether safeners, cyometrinil, oxabetrinil, and CGA-133205.

Calculated parameters	Cyometrinil	Oxabetrinil	CGA-133205	Metolachlor (active)	Metolachlor (inactive)
Connectivity indices					
normal	6.411	7.932	8.605	8.647	8.647
bonds	4.220	6.237	7.448	7.532	7.532
valence	3.588	4.972	5.315	6.805	6.805
Principal moments					
x-axis	8.586	11.969	10.090	5.712	5.802
y-axis	1.390	1.331	1.856	2.911	3.147
z-axis	0.002	0.024	0.199	0.690	0.666
Modeled strain	0.422	4.090	4.017	6.135	5.747
Molecular volume (cm^3/mole)	134.031	159.316	188.331	180.885	181.215

group and the hydrogen atom on the chiral-C and their influence on the molecular configuration and charge distribution of the metolachlor isomers. Analysis of the principal moments reveals that the charge distribution is slightly more compact along the x-and y-axes in the active isomer than in the inactive isomer, but the visual evaluation of the two isomers revealed no major differences in the location of the functional groups.

Molecular comparison of metolachlor and oxime ether safeners by CAMM analysis. The first commercial oxime ether safener, cyometrinil, contains two nitrile groups (Figure 2). Oxabetrinil, the second oxime ether safener in this series, has only one nitrile group and a trifluromethyl functional group (Figure 2). CGA-133205, the third analog in this series does not contain any nitrile groups (Figure 2). It has a trifluromethyl group and a 1,3-dioxolan ring (Figure 2). Molecular comparisons of the oxime ether safener analogs revealed that chemical modifications of cyometrinil and oxabetrinil to yield CGA-133205, resulted in a molecule which is a better safener of grain sorghum and whose calculated parameters are close to those calculated for metolachlor (Table 3). Therefore, the comparison of the most effective

safener, CGA-133205, with metolachlor will be emphasized.

Almost identical comparisons can be made with metolachlor and CGA-133205 as made for alachlor and flurazole. The connectivity indices, the principal moments along the y- and z-axes, and the molecular volume are similar for metolachlor and CGA-133205. Normal connectivity indices indicate that both molecules contain about the same number of atoms (Table 3). Bond connectivity indices indicate that both compounds contain about the same number of multiple bonds while the valence connectivity index indicates that CGA-133205 contains more valence electrons than does metolachlor (Table 3).

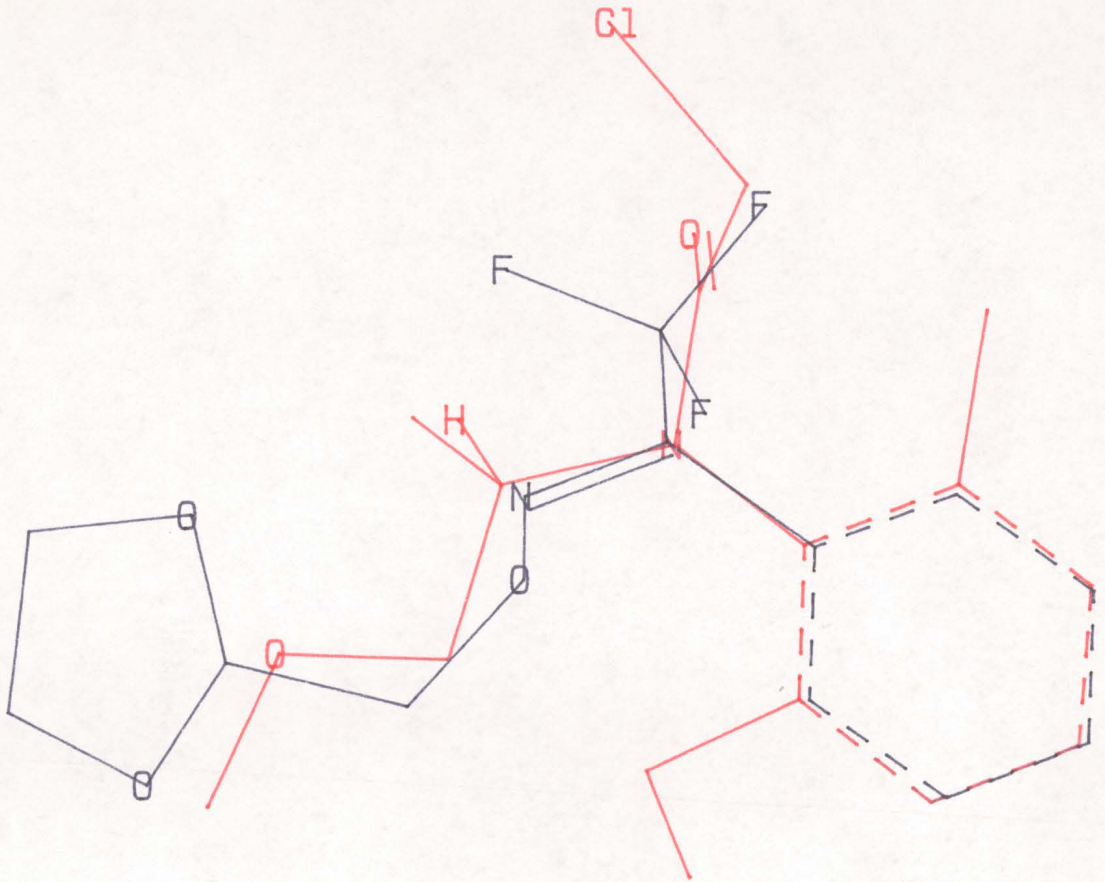
The only parameter that is not very similar is the principal moment along the x-axis indicating that the charge associated with CGA-133205 is spread over a longer distance along the x-axis (Table 3). Further analysis of the principal moments and a visual analysis of the modeled structures indicate that both of these molecules are long and flat with very little penetration into the z-plane (Figures 2 and 3).

When the structures of the active isomer of metolachlor and CGA-133205 are superimposed with their phenyl rings being alinged, the trifluromethyl group of

CGA-133205 is aligned over the alkyl side chain of the metolachlor (Figure 8). The 1,3-dioxolan ring of the CGA-133205 molecule and the acyl side chain of metolachlor do not align well, but they do orient themselves in the same general direction (Figure 8). The comparison of metolachlor with CGA-133205 (Figure 8) is similar to the comparison of alachlor and flurazole (Figure 6). If the metolachlor structure is altered by changing the bond angle at the chiral-C and the location of the chlorine atom (Figure 9) and then superimposed over CGA-133205, the structures are similar (Figure 10). However, this configuration of metolachlor is not the most stable configuration. In both Figures 8 and 10 it is easy to see the similarities between metolachlor and CGA-133205 and that these two molecules could very easily fit into the same binding site on a protein.

During the development of the chloroacetamide herbicides and the acylaniline fungicides many QSAR experiments were conducted to determine the most active substituents [Lebaron et al., 1988]. McFarland and Hess [1985] reported that nonhalogenated acetamides were less active in an oat (Avena sativa L.) root growth bioassay than were the halogenated derivatives and that an analog with fluorine was not inhibitory to

Figure 8. Herbicidally active S-enatiomer of metolachlor (red) superimposed over CGA-133205 (black).



oat root growth. However, if the α-halogen is replaced by a methoxyacetyl moiety then this acetamide becomes fungicidal and not herbicidal [McFarland & Hess, 1985]. Given the similarities of CGA-133205 with metolachlor one would suspect that if the triflurомethyl group of CGA-133205 was substituted with a halogen such as chlorine or bromine, then possibly this compound would have herbicidal activity. Alternatively, if the triflurомethyl group was substituted with a methoxyacetyl moiety then this compound might have fungicidal activity.

In summary, the results of these molecular comparisons indicate that in most of the successful and commercial herbicide/safener combinations, the chemical structures of safeners and herbicides are similar at the molecular level. These similarities include the size (volume and physical dimensions), shape, and charge distribution. It is apparent that a herbicide and its associated safeners may bind to the same active site of the target protein(s) (competitive antagonists) and/or they may serve as substrates of inducible metabolic enzymes which detoxify herbicides in protected plants.

Figure 9. Altered structure (changed bond angle at chiral C and location of Cl atom) of metolachlor with the chlorine atom oriented in the x-plane.

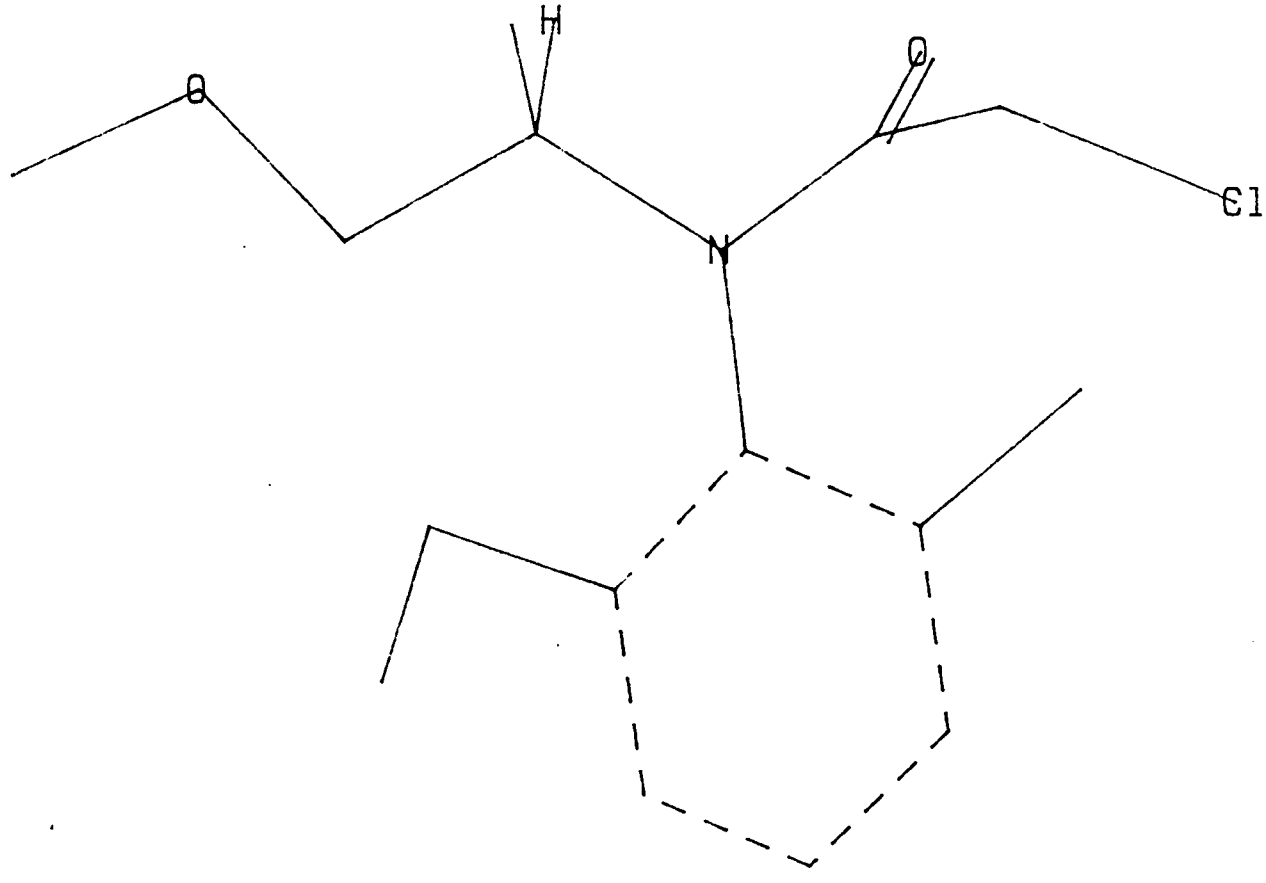
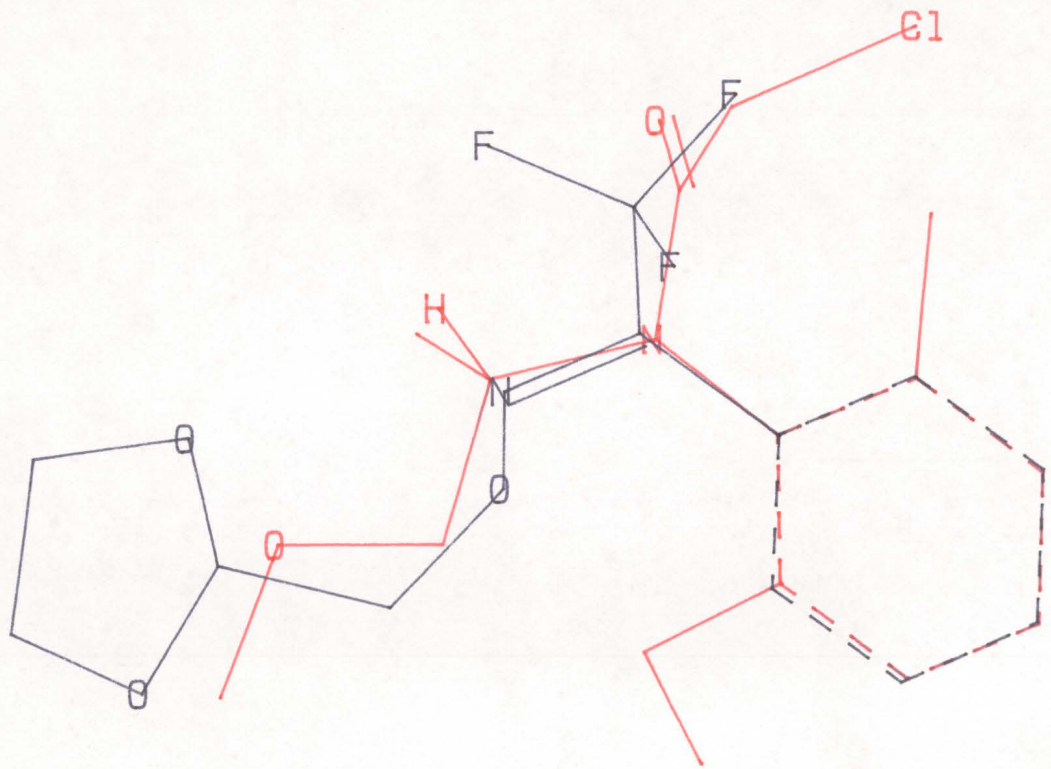


Figure 10. Altered structure of metolachlor (see Figure 9; red) superimposed over CGA-133205 (black).



REFERENCES

Davies, K. (1986). Choosing hardware for molecular modeling: Pitfalls and possibilities. Scientific Computing and Automation. Dec., 10-13.

Gerstl, Z. and Helling, C. S. (1987). Evaluation of molecular connectivity as a predictive method for the adsorption of pesticides by soils. J. Environ. Sci. Health, **B22(1)** 55-69.

Gronwald, J. W. (1988) Influence of herbicide safeners on herbicide metabolism. in "Crop Safeners for Herbicides: Development, Uses, and Mechanism of Action" (eds. K. K. Hatzios and R. E. Hoagland). Academic Press, Inc., New York. 103-128.

Hansch, C. and Leo, A. (1979). Substituent constants for correlation analysis in chemistry and biology. Wiley, New York.

Hatzios, K. K. (1983). Herbicide Antidotes: development, chemistry, and mode of action. Adv. Agron., **36**, 265-316.

Kier, L. B. and Hall, L. H. (1986). Molecular Connectivity in Structure-Activity Analysis. John Wiley & Sons, Inc., New York.

LeBaron, H. M., McFarland, J. E., and Simoneaux, B. J. (1988). Metolachlor. in "Herbicides: Chemistry, Degradation, and Mode of Action" (eds. P. C. Kearney & D. D. Kaufman). Marcel Dekker, Inc. New York. 335-382.

McFarland, J. E. and Hess, F. D. (1985). Herbicidal activity of acetanilides parallels alkylation potential. Abstr. Weed Sci. Soc. Am. **25**, 199.

Moser, H., Ribs, B., Sauter, H. P., and Bohnor B. (1985). Atropisomerism, chiral centre and activity of metolachlor. in "Pesticide Chemistry: Human Welfare and the Environment." (eds. J. Miyamoto and P. C. Kearney) **1**, 315-320.

Murakami, M. and Fukami, J. (1985). Specific molecular connectivity analysis of pesticides and related compounds: A preliminary study. Bull. Environ. Contam. Toxical. **34**, 775-779.

Rodgers, D. W., (1987). Molecular mechanics using a personal computer. Americ. Lab. **19(4)**, 28-39.

Seiter, C. and Cohan, P. (1986). Designing new compounds with a PC database. Americ. Lab. **18(9)**, 40-47.

Anonymous. (1986). XICAMM®, Users Manual. XIRIS Corporation, P.O. Box 787, New Monmouth, New Jersey, 07748.

III. INFLUENCE OF OXIME ETHER SAFENERS AND METOLACHLOR ON ACETATE INCORPORATION INTO LIPIDS AND ON ACETYL-CoA CARBOXYLASE OF GRAIN SORGHUM

INTRODUCTION

The chloroacetanilide herbicide, metolachlor, has been used as a preemergence herbicide for the control of annual grass weeds in corn (Zea mays L.), soybeans [Glycine max (L.) Merr.] and other broadleaf crops since the early 1970's (1). The recent development of seed-applied safeners for chloroacetanilide herbicides has increased the margin of crop selectivity of metolachlor and has allowed its use in sensitive crops such as grain sorghum (1, 2). Commercially developed safeners for the protection of grain sorghum against chloroacetanilide herbicides include the oxime ether safeners, cyometrinil, oxabetrinil, and CGA-133205 and the substituted thiazole carboxylate, flurazole (2).

The potential interactions between safeners and herbicides in protected plants have been studied extensively by numerous investigators, and several theories have been proposed (for review see ref. 3 and 4). It is presently accepted that safeners may act either by influencing the amount of a given herbicide that reaches its target site in an active form or as

antagonists, influencing the herbicidal effects at a common site of action (5).

Computer-assisted molecular modeling studies with oxime ether safeners and metolachlor revealed that the chemical structures of this herbicide and its safeners are very similar at the molecular level (6). Thus, it is possible that oxime ether safeners may compete with metolachlor at a common site of action inside the protected seedlings of grain sorghum.

Metolachlor and other chloroacetanilide herbicides have been reported to inhibit the early development of susceptible plants, but their exact mechanism of action is unknown (1). Selected physiological processes known to be influenced by chloroacetanilide herbicides include protein synthesis, terpenoid synthesis, gibberellic acid synthesis, and lipid synthesis (1, 7). Specific steps of lipid and terpenoid metabolism of grass plants appear to be likely target sites for a competitive antagonism between safeners and chloroacetanilide herbicides and the topic has been reviewed by Wilkinson (8).

In his recent review on the mechanisms of action of chloroacetanilide herbicides, Fuerst (7) proposed that the site of action of these herbicides may be related to some aspect of acetyl-Coenzyme A (acetyl-CoA) metabolism since many of the plant metabolic products influenced by

these herbicides are synthesized via acetyl-CoA intermediates. Acetyl-CoA is a direct precursor, serving as the main source of carbon for the synthesis, of fatty acids and of other lipids in higher plants (9). Subsequently, acetyl-CoA reacts with bicarbonate in an ATP-dependent reaction catalyzed by acetyl-CoA carboxylase (EC 6.4.1.2) to form malonyl-CoA. This reaction is the first committed step for de novo fatty acid synthesis in higher plants (9).

Acetyl-CoA carboxylase is a soluble chloroplastic enzyme that does not limit fatty acid biosynthesis under normal conditions (10-13). Recently, acetyl-CoA carboxylase has been identified as the target site involved in the phytotoxic action of two new classes of herbicides, the aryloxyphenoxypropionates and the cyclohexanediones (14-17). Similar to chloroacetanilides, these herbicides have been reported to strongly inhibit lipid biosynthesis in susceptible grass species, but not in tolerant broadleaf species (14, 15). The influence of chloroacetanilide herbicides and of oxime ether safeners on the activity of acetyl-CoA carboxylase from grain sorghum has not been examined. Inhibition of this enzyme by chemical treatments may result in the redistribution of acetyl-CoA into the lipid fractions of treated plants during the early stages of

the inhibition of plant growth following herbicide application.

The objectives of the experiments reported in this study were: a) to evaluate the influence of the herbicide, metolachlor, on germination and root growth of grain sorghum grown from seed that was untreated or treated with the oxime ether safeners, oxabetrinil or CGA-133205, b) to determine the influence of metolachlor alone and in combination with oxabetrinil or CGA-133205 on the incorporation of ^{14}C-acetate into total and specific classes of lipids in roots of sorghum seedlings, and c) to determine the influence of metolachlor, oxabetrinil, and CGA-133205 on the activity of acetyl-CoA carboxylase isolated from safened or unsafened sorghum seedlings.

MATERIALS AND METHODS

Plant material and chemical treatments for lipid extraction. Grain sorghum seed var 'Funk G-522 DR' was obtained from Ciba-Geigy Corporation, Greensboro, NC, USA. The seed was either untreated or treated with oxabetrinil (1.25 g a.i./kg seed) or with CGA-133205 (0.4 g a.i./kg seed). Grain sorghum seeds were exposed to ^{14}C-acetate and metolachlor, then prepared for lipid extraction according to the methodology of Warmund et. al. (18). Thirty seeds of each safener treatment were placed in individual scintillation vials. One-half ml of ^{14}C-acetate solution containing 1 μCi (sp. act. 94 mCi/mmol) was added to all vials. To half of the vials 0.5 ml of 10 μM metolachlor was added and to the other half 0.5 ml of distilled water was added. These vials were capped, placed into a 30°C incubator, and allowed to imbibe the ^{14}C-acetate solution for 12 hr. The seeds and any remaining solution were transferred to a petri dish with filter paper and 5 ml of distilled water and were returned to the incubator for 48 hr. The germinated seedlings were rinsed and 5 mm of root tissue taken from behind the root cap of each seedling was used for lipid extraction.

Extraction of lipids. The root tissue was frozen at -70°C for 30 min and then crushed with a glass stirring rod.

Two ml of 70°C isopropanol containing 50 μg/ml of the antioxidant, butylated hydroxy toluene, was added to the crushed tissue. This mixture was incubated in a 70°C water bath for 30 min and then cooled to room temperature. Next, 3 ml of chloroform/methanol (2:1 v/v) was added to extract the lipids from the isopropanol solution. One-tenth ml of the chloroform/methanol extract was radioassayed for total ^{14}C-acetate incorporation into lipids using standard liquid scintillation counting methods. The remaining extract was taken to dryness under nitrogen at 55°C. The residue was taken up in 200 μl of chloroform and 10 μl were spotted onto 200 μm high-performance silica gel thin-layer chromatography (TLC) plates (Whatman LHP-KD). Plates were developed using hexanes:diethyl ether:acetic acid (40:10:0.5 v/v). Then, the plates were sprayed with 0.1% 2,7-dichloro-fluorscein/methanol stain. Silica gel was scraped from areas on the plate corresponding to co-chromatographed standards of polar lipids, free sterols, free fatty acids, triglycerides, and sterol esters standards and placed in scintillation vials. The ^{14}C on the silica gel was quantified using standard scintillation counting methods. This experiment contained three replications and was repeated in time. Means were separated using Fishers' protected LSD.

Acetyl-CoA carboxylase extraction. Sorghum seeds that were either untreated or treated with oxabetrinil (1.25 g a.i./kg seed) or CGA-133205 (0.4 g a.i./kg seed) were planted approximately 2.5 cm deep in water-saturated vermiculite. Pots were placed into a growth chamber at 21°C with a 12 hr light period (60 $\mu Em^{-2}sec^{-1}$). After 7 days, the above ground portions were harvested and pulverized in liquid nitrogen using a mortar and pestle.

The extraction and purification procedures were those of Secor and Cseke (17) with slight modifications. All steps were performed at 4°C unless otherwise noted. Two grams of the pulverized plant material were ground in 6 ml of cold extraction buffer containing 100 mM tricine-KOH (pH 8.3), 10% (v/v) glycerol, 10 mM β-mercaptoethanol, 1 mM phenylmethyl sulfonyl fluoride, and 1 mM Na-EDTA. The plant slurry was filtered through two layers of Miracloth and the filtrate was centrifuged at 30,000 x g for 20 min. The pellet was discarded. Polyethyleneglycol (Sigma P-2139) was added to the supernatant while stirring for 20 min to make a 6% (w/w) solution. This solution was centrifuged at 30,000 x g for 20 min and the resulting pellet was discarded. Additional polyethyleneglycol was added while stirring for 20 min to make a 14% (w/w) solution and then centrifuged at 30,000 x g for 20 min. The resulting

pellet was resuspended in suspension buffer containing tricine-KOH (pH 7.8) and 10% (v/v) glycerol and centrifuged at 10,000 x g for 10 min. The pellet was discarded and protein was assayed in the supernatant using the Coomassie Blue G-250 dye binding assay method (19). This supernatant was used in the acetyl-CoA carboxylase assay described below.

Acetyl-CoA carboxylase assay. Acetyl-CoA carboxylase activity was assayed in microcentrifuge tubes under a fume hood following the method of Secor and Cseke (17). The final reaction volume was 250 μl. The reaction mixture contained 50 mM tricine-KOH (pH 8.3), 5 mM $MgCl_2$, 2 mM dithiothreitol, 2 mM ATP, 15 mM $NaH^{14}CO_3$ (0.33 μCi/μmol), approximately 1.5 mg protein/ml, 50 μM of inhibitor, and the reaction was initiated with a final concentration of 0.3 mM acetyl-CoA. Inhibitors included metolachlor, oxabetrinil, CGA-133205, and metolachlor/safener combinations at 50 μM each. The reaction mixture, excluding acetyl-CoA, was centrifuged in a microcentrifuge for 30 sec and then preincubated for 15 min at 35°C in a water bath. After the preincubation, the acetyl-CoA was added to initiate the reaction. After 15 min, 50 μl of 6 N HCl was added to terminate the reaction. A 150-μl subsample was taken and placed into a 4-ml scintillation vial. The scintillation vials were

put into a heating block at 90°C for approximately 1 hr until the samples are dry. Any unreacted $^{14}CO_2$ was volatilized by the heat and absorbed in an ascarite trap. 250 μl of distilled water was used to dissolve the dried residue. Scintillation cocktail (3 ml) was added to each vial and the samples were counted using standard liquid scintillation procedures. This experiment contained three replicates and was repeated in time. Means were separated uing Fisher's protected LSD.

RESULTS AND DISCUSSION

Influence of metolachlor and safeners on sorghum growth. Metolachlor at 10 μM had no effect on germination of grain sorghum nor did it significantly reduce root growth (Table 1). This is consistent with other reports of plant responses to chloroacetanilides when used at comparable rates (1, 7). At 60 hr after initiating the experiment, oxabetrinil significantly reduced germination and root growth. The addition of metolachlor to oxabetrinil-treated sorghum did not reduce germination or root growth further, nor did it reverse the oxabetrinil-induced inhibition (Table 1). The reduction in germination caused by oxabetrinil became less apparent at 108 hr, but root growth was still severely inhibited (data not presented). Sorghum root growth has been shown to be inhibited by flurazole (2.5 g a.i./kg seed) and to a greater extent by flurazole and alachlor (8.2 μM) combinations (18). Flurazole has been shown to reduce respiration (18) indicating a reduced catabolic rate and oxabetrinil has been shown to reduce the metabolic activity of sorghum protoplasts isolated from oxabetrinil pretreated plants (1.25 g a.i./kg seed) (25); thus, one would expect slower growth of oxabetrinil-treated seeds. Ketchersid and Merkle (20) reported that cyometrinil and oxabetrinil slowed shoot

TABLE 1

The Influence of Seed-applied Oxabetrinil and CGA-133205, Alone and in Combination with 10 μM Metolachlor, on Germination and Root Length of Grain Sorghum[a].

Treatment	Germination (%)[c]	Root length[b] (% of control)
untreated	92.3[A]	100.0[A]
metolachlor	94.3[A]	113.2[A]
oxabetrinil	74.3[B]	63.9[B]
oxabetrinil/metolachlor	73.3[B]	65.3[B]
CGA-133205	90.0[A]	102.3[A]
CGA-133205/metolachlor	97.6[A]	111.4[A]

[a]The safeners, oxabetrinil and CGA-133205 were applied as seed dressings at 1.25 and 0.4 g a.i./kg seed,respectively.

[b]Root length and germination determined at 60 hr after initiating the germination process.

[c]Means within the same column followed by the same letter are not significantly different as determined by Fishers' protected $LSD_{(0.05)}$.

growth of two sorghum varieties with cyometrinil being more inhibitory than oxabetrinil. They also demonstrated that at three times the use rate, oxabetrinil did not reduce germination (20). Ketchersid and Merkle (20) also reported that cyometrinil, oxabetrinil, and flurazole reduced respiration, again with cyometrinil being more active than oxabetrinil. CGA-133205, an analog of cyometrinil and oxabetrinil that is more effective as a sorghum safener (21), did not reduce germination or root growth (Table 1). The influence of the combined application of metolachlor with CGA-133205 was not different than CGA-133205 or metolachlor alone (Table 1).

Interactive effects of metolachlor and safeners on ^{14}C-acetate incorporation into lipids. Metolachlor at 10 μM did not inhibit or stimulate ^{14}C-acetate incorporation into total lipids or the individual lipid classes of sorghum seedlings (Table 2). These results are in agreement with those of Warmund et. al. (18) who reported that alachlor, at 8.2 μM, had no effect on ^{14}C-acetate incorporation into total lipids or the individual lipid classes of sorghum seedlings. Ebert and Ramsteiner (22) also reported that metolachlor did not influence the synthesis of any major sterol in grain sorghum. However, in the same study (22), metolachlor significantly inhibited the synthesis of long-chain alcohols and fatty

TABLE 2

The Influence of Seed-applied Oxabetrinil and CGA-133205, Alone and in Combination with 10 M Metolachlor, on ^{14}C-Acetate Incorporation into Lipids of Germinating Grain Sorghum Roots[a].

Treatment	Total lipids	Polar lipids	Free sterols	Free fatty acids	Triglycerides	Sterol esters
	nmol ^{14}C-acetate incorporated ($\times 10^{-4}$)[b]					
control	61.6^{B}	9.6^{BC}	9.0^{BC}	3.1^{B}	2.2^{B}	2.9^{C}
metolachlor	55.3^{B}	11.2^{B}	9.7^{ABC}	4.9^{B}	3.6^{B}	3.8^{BC}
oxabetrinil	119.4^{A}	19.5^{AB}	11.3^{AB}	6.0^{B}	4.9^{B}	6.5^{B}
oxabetrinil/metolachlor	135.7^{A}	25.4^{A}	15.2^{A}	15.2^{A}	13.7^{A}	10.9^{A}
CGA-133205	58.6^{B}	7.7^{B}	5.3^{C}	2.9^{B}	2.1^{B}	2.7^{C}
CGA-133205/metolachlor	68.3^{B}	13.5^{BC}	13.6^{AB}	10.0^{AB}	6.8^{B}	5.0^{BC}

[a]The safeners, oxabetrinil and CGA-133205, were applied as seed dressings at 1.25 and 0.4 g a.i./kg of seed, respectively.

[b]Means within the same column followed by the same letter are not significantly different as determined by Fishers' protected $LSD_{(0.05)}$.

acids in epicuticular waxes of sorghum. Other researchers have also found that at higher concentrations(50 to 100 μM), chloroacetanilide herbicides can inhibit lipid synthesis in treated plants (23-26). Weisshaar and Boger (26) reported that metazachlor, a chloroacetanilide herbicide, at 100 μM significantly inbited the incorporation of ^{14}C-acetate into several lipid classes of Chlamydomonas.

Oxabetrinil, applied as a seed dressing at 1.25 g a.i./kg seed, stimulated ^{14}C-acetate incorporation into total lipids and some lipid classes (Table 2). This safener-induced stimulation of ^{14}C-acetate incorporation was even more evident when oxabetrinil was applied with metolachlor (Table 2). Seed applied CGA-133205 at 0.4 g a.i./kg seed did not stimulate ^{14}C-acetate incorporation into any lipid fraction while the combined CGA-133205/metolachlor treatment tended to stimulate incorporation into all lipid classes (Table 2). Flurazole (2.5 g a.i./kg seed), a safener used to protect sorghum from the chloroacetanilide herbicide, alachlor, has been reported to slightly reduce the incorporation of ^{14}C-acetate into total lipids and the lipid classes of sorghum (18). Zama and Hatzios (25) reported that oxabetrinil at 100 μM inhibited ^{14}C-acetate incorporation into lipids of sorghum leaf protoplasts. Flurazole

inhibited ^{14}C-acetate incorporation into triglycerides and reduced the ratio of triglycerides to phospholipids (18). The addition of alachlor (8.2 μM) with flurazole restored the ^{14}C-acetate incorporation into triglycerides above the level of the control, but did not completely restore the triglyceride to phospholipid ratio (18).

The stimulation of the incorporation of ^{14}C-acetate into sorghum lipids by all oxabetrinil treatments needs to be viewed with some caution. As mentioned earlier, oxabetrinil reduced root growth of germinating sorghum seedlings (Table 1). Thus, the apparent stimulation of ^{14}C-acetate incorporation might be an indirect effect resulting from the distribution of ^{14}C into a smaller amount of tissue when compared to the control. However, the increased stimulation resulting from the safener/metolachlor combinations is probably not an indirect effect, but the result of a redistribution of carbon in the lipid fractions. When the results of the ^{14}C-acetate experiment with oxabetrinil or oxabetrinil/metolachlor in Table 2 are adjusted for the reduced root growth caused by oxabetrinil or oxabetrinil/metolachlor treatments (Table 1), the observed stimulation into the individual lipid fractions is similar to the stimulation observed with the CGA-133205/metolachlor treatments (Tables 2 and 3). Each

TABLE 3

The Influence of Oxabetrinil, Applied Alone and in Combination with 10 μM Metolachlor, on ^{14}C-acetate Incorporation into Lipids of Grain Sorghum Adjusted for Reduced Root Growth[a].

Lipid fraction	Oxabetrinil	Oxabetrinil/metolachlor
	nmole ^{14}C-acetate incorporated (x 10^{-4})[a]	
Total	76.3	88.6
Polar	12.5	16.6
Free sterols	7.2	9.9
Free fatty acids	3.8	9.9
Triglycerides	3.1	8.9
Sterol esters	4.2	7.1

[a]Values presented here are those given in Table 2 following adjustment for the oxabetrinil-induced reduction in root growth of grain sorghum given in Table 1.

value presented in Table 3 was calculated by multiplying the results of oxabetrinil or oxabetrinil/metolachlor treatments on ^{14}C-acetate incorporation given in Table 2 by the percent of control root length values caused by oxabetrinil (63.3%) or oxabetrinil/metolachlor (65.3%) treatments given in Table 1 and divided by 100. These results of the ^{14}C-acetate incorporation experiments indicate that metolachlor and the oxime ether safeners influence lipid synthesis, but they do not help explain the exact mechanism of action of either the safeners or the herbicide.

Interactive effects of metolachlor and safeners on acetyl-CoA carboxylase. Given the fact that the chloroacetanilides influence lipids (1, 7) which are synthesized from acetyl-CoA, acetyl-CoA carboxylase was investigated as a possible site for the antagonism of metolachlor and oxime ether safeners. Acetyl-CoA carboxylase catalyzes the first committed step in fatty acid biosynthesis; therefore, if metolachlor and/or oxime ether safeners are influencing acetyl-CoA carboxylase, this could explain the influence that these compounds have on lipid synthesis and aid our understanding of their mechanism of action.

As mentioned earlier, acetyl-CoA carboxylase is a light-dependent chloroplastic enzyme; therefore,

acetyl-CoA carboxylase was extracted from young green leaf tissue. Acetyl-CoA carboxylase extracted from safener-treated tissue contained as much activity as acetyl-CoA carboxylase extracted from untreated tissue (Table 4, means pooled over plant treatments). In fact, the amount of total protein was slightly higher in extracts from safener-treated tissue than from untreated tissue (data not presented). Metolachlor or the safeners added to the assay medium alone at 50 μM did not inhibit the activity of acetyl-CoA carboxylase of sorghum, irregardless of the source of the enzyme preparation (Table 4, means pooled over assay inhibitor). However, the addition of metolachlor/oxabetrinil combiniations to the reaction mixture did inhibit the activity of this enzyme obtained from untreated or safener-treated tissue (Table 4).

The amount of inhibition observed in these experiments is much less than that observed by Secor and Cseke using similar concentrations of haloxyfop and tralkoxydim (17) and also by Burton et. al. (14) using sethoxydim and haloxyfop. Rendina et al. (15) found that acetyl-CoA carboxylase activity was severely inhibited by the substituted 1,3-cyclohexanedione herbicides, alloxydim, sethoxydim, and clethodim, (0.12 μM to 4.86 μM) but not with the unrelated herbicides, acifluorfen or

TABLE 4

The Influence of Oxabetrinil, CGA-133205, and Safener/metolachlor Combinations on Acetyl-CoA Carboxylase Activity Extracted from Untreated and Safener Treated Sorghum Seedlings.

	Activity of Acetyl-CoA Carboxylase Extracted from Grain Sorghum Shoots[a]			
Assay inhibitor[b]	Untreated	Oxabetrinil[c]	CGA-133205[d]	Mean[e]
	----nmol CO_2/min/mg protein[f]---			
control	96.5^{AB}	99.7^{A}	106.4^{A}	100.8
metolachlor	89.5^{ABC}	90.5^{AB}	95.1^{AB}	91.7
oxabetrinil	87.4^{BC}	80.6^{BC}	80.4^{BC}	82.8
oxabetrinil/metolachlor	78.9^{C}	67.8^{C}	71.9^{C}	72.9
CGA-133205	102.9^{A}	85.8^{AB}	96.8^{AB}	95.2
CGA-133205/metolachlor	86.0^{BC}	79.3^{BC}	93.7^{AB}	86.3
Mean[g]	90.2	84.0	90.7	

[a]Plant treatment by assay inhibitor $LSD_{(0.05)}$=21.6.

[b]Metolachlor or oxime ether safeners were used at 50 uM.

[c]Seeds were treated with 1.25 g ai/kg seed of oxabetrinil.

[d]Seeds were treated with 0.4 g ai/kg seed of CGA-133205.

[e]Assay inhibitor $LSD_{(0.05)}$=12.5 (pooled over plant treatment).

[f]Means within the same column are not significantly different as determined by Fishers' protected $LSD_{(0.05)}$.

[g]Plant treatment $LSD_{(0.05)}$=8.8 (pooled over assay inhibitor).

bentazon. Rendina et. al. (15) also found that acetyl-CoA carboxylase from tolerant broadleaf crops was 400 to 60,000 times more tolerant to sethoxydim and clethodim compared to acetyl-CoA carboxylase from susceptible grass crops.

However, results of the ^{14}C-acetate incorporation into lipids experiment and the results of previous reports (7) indicate that the mechanism of action of metolachlor is related to acetyl-CoA metabolism. Because of the metolachlor-induced reallocation of carbon in the acetate incorporation experiment, it seems likely that metolachlor influences the synthesis of acetyl-CoA and not the incorporation of acetyl-CoA into fatty acids or other lipids via the function of acetyl-CoA carboxylase. In a recent report, Wilkinson (27) suggested that metolachlor may be inhibiting the activity of α-amylase [EC 3.2.1.1] and isocitric lyase [EC 4.2.1.3], two key enzymes that are involved in the breakdown of seed storage products to form acetate and acetyl-CoA. Application of oxabetrinil in that study reversed the metolachlor-induced inhibition of α-amylase extracted from grain sorghum (27). Furthermore, the results of the present study indicate that the oxime ether safeners, oxabetrinil and CGA-133205, can also influence the metabolism of acetyl-CoA in treated sorghum plants.

Similar to metolachlor, this influence is probably due to a safener-induced effect on acetyl-CoA formation rather than a direct effect on the incorporation of acetyl-CoA into fatty acids or other lipids.

REFERENCES

1. H. M. LeBaron, J. E. McFarland, B. J. Simoneaux, and E.Ebert, Metolachlor. in "Herbicides: Chemistry, Degradation, and Mode of Action" (P. C. Kearney and D. D. Kaufman, Eds), Volume 3, pp. 335-381, Marcel Dekker, Inc, 1988.
2. K. K. Hatzios, Development of Herbicide Safeners: Industrial and University Perspectives. in "Crop Safeners for Herbicides: Development, Uses, and Mechanisms of Action" (K. K. Hatzios and R. E. Hoagland, Eds.), pp. 3-45, Academic Press, Inc., New York, 1988.
3. K. K. Hatzios and R. E. Hoagland, "Crop Safeners for Herbicides: Development, Uses, and Mechanisms of Action," p. 400, Academic Press, Inc., New York, 1988.
4. F. M. Pallos and J. E. Casida, "Chemistry and Action of Herbicide Antidotes," p. 171, Academic Press, Inc., New York, 1978.
5. K. K. Hatzios, Mechanisms of Action of Herbicide Safeners An Overview. in "Crop Safeners for Herbicides: Development, Uses, and Mechanisms of Action" (K. K. Hatzios and R. E. Hoagland, Eds.), pp. 65-101, Academic Press, Inc., 1988.

6. S. P. Yenne and K. K. Hatzios, Molecular comparison of oxime ether safeners and metolachlor. Abstr. Weed Sci. Soc. Am. 28, 95 (1988).

7. E. P. Fuerst, Understanding the mode of action of chloroacetanilide and thiocarbamate herbicides. Weed Technol. 1, 270 (1987).

8. R. E. Wilkinson, Terpenoid Biosynthesis as a Site of Action for Herbicide Safeners. in "Crop Safeners for Herbicides: Development, Uses, and Mechanisms of Action" (K. K. Hatzios and R. E. Hoagland, Eds.), pp. 221-240, Academic Press, Inc., 1988.

9. J. L. Harwood, Fatty acid metabolism. Annu. Rev. Plant Physiol. Plant Mol. Biol. 39, 101-138 (1988).

10. B. J. Nikolau, J. C. Hawks, and C. R. Slack, Acetyl-Coenzyme A carboxylase in maize leaves. Arch. Biochem. Biophys. 211, 605 (1981).

11. B. J. Nikolau and J. C. Hawke, Purification and characterization of maize leaf acetyl-CoA carboxylase. Acrh. Biochem. Biophys. 228, 86 (1984).

12. J. C. Hawke and R. M. Leech Acetyl-CoA carboxylase activity in normally developing wheat leaves. Planta 171, 489 (1987).

13. B. J. Nikolau, E. S. Wurtele, and P. K. Stumpf, Tissue distribution of acetyl-CoA carboxylase in leaves. Plant Physiol. 75, 895 (1984).

14. J. D. Burton, F. W. Gronwald, D. A. Somers, J. A. Connelly, B. G. Gengenback, and D. L. Wyse, Inhibition of plant acetyl-CoA carboxylase by the herbicides sethoxydim and haloxyfop. Biochem. Biophys. Res. Comm. 184, 1039 (1987).

15. A. R. Rendina and J. M. Felts, Cyclohexanedione herbicides are selective and potent inhibitors of acetyl-CoA carboxylase from grasses. Plant Physiol. 86, 983 (1988).

16. K. Kobek, M. Focke, and H. K. Lichtenthaler, Fatty-acid biosynthesis and acetyl-CoA carboxylase as a target of diclofop, fenoxaprop and other arloxy-phenoxy-propionic acid herbicides. Z. Naturforsch. 43c, 47 (1988).

17. J. Secor and C. Cseke, Inhibition of acetyl-CoA carboxylase activity by haloxyfop and tralkoxydim. Plant Physiol. 86, 10 (1988).

18. M. R. Warmund, H. D. Kerr, and E. J. Peters, Lipid metabolism in grain sorghum (Sorghum bicolor) treated with alachlor plus flurazole. Weed Sci. 33, 25 (1985).

19. M. M. Bradford, A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein dye binding. Anal. Biochem. 72, 248 (1976).

20. M. L. Ketchersid and M. G. Merkle, Effect of protectants on sorghum seedling respiration. Proc. South. Weed Sci. Soc. 36, 384 (1983).

21. N. T. Helseth and T. R. Dill. Greenhouse results with CGA-133205 on sorghum. Proc. South. Weed Sci. Soc. 39, 35 (1986).

22. E. Ebert and K. Ramsteiner, Influence of metolachlor and the metolachlor protectant CGA-43089 on the biosynthesis of epicuticular waxes on the primary leaves of Sorghum bicolor Moench. Weed Res. 24, 383 (1984).

23. W. B. Duke, F. W. Slife, J. B. Hanson, and H. S. Butler, An investigation on the mechanism of action of propachlor. Weed Sci. 23, 142 (1975).

24. J. M. Mellis, P. Pillai, D. E. Davis, and B. Truelove, Metolachlor and alachlor effects on membrane permeability and lipid synthesis. Weed Sci. 30, 399 (1982).

25. P. Zama and K. K. Hatzios, Interactions between the herbicide metolachlor and the safener CGA-92194 at the levels of uptake and macromolecular synthesis in

sorghum leaf protoplasts. Pestic. Biochem. Physiol. 27, 86 (1987).

26. H. Weisshaar and P. Boger, Primary effects of chloroacetanilides. Pestic. Biochem. Physiol. 28, 286 (1987).

27. R. E. Wilkinson, Consequences of metolachlor induced inhibition of gibberellin biosynthesis in sorghum seedlings. Pestic. Biochem. Physiol. 32, 25 (1988).

IV. INFLUENCE OF OXIME ETHER SAFENERS ON GLUTATHIONE CONTENT AND GLUTATHIONE-RELATED ENZYME ACTIVITY IN SEEDS AND GERMINATING SEEDS OF GRAIN SORGHUM

INTRODUCTION

The oxime ether safeners, cyometrinil, oxabetrinil and CGA-133205, have been developed by CIBA-GEIGY Corporation (Basel, Switzerland) to protect grain sorghum against injury caused by the chloroacetanilide herbicide, metolachlor [1-3]. With a broadcast application, these oxime ether safeners will also protect some grass weed species of the Sorghum genus against metolachlor; therefore, to achieve crop selectivity, these safeners are applied directly to grain sorghum as seed dressings [3, 4]. This mode of application allows for the uptake of these safeners into the germinating seed and the young seedling of grain sorghum and confers protection to this crop against a subsequent application of the herbicide, metolachlor [3].

Our current understanding of how treatment with a seed-applied safener protects grass crops against chloroacetanilide herbicide injury is equivocal [5, 6]. Two possible theories have been proposed [5, 6]: (1) a safener-induced enhancement of herbicide detoxication in the safened plant, and (2) a competitive antagonism

between the safener and the herbicide at a common site of action [5, 6]. At present, most of the accumulated evidence supports the enhanced degradation theory for the mode of action of herbicide safeners [5-7].

The metabolic detoxication of metolachlor in grasses such as corn and grain sorghum proceeds primarily via the formation of a conjugate of metolachlor with reduced glutathione (GSH) [3]. This reaction can be either nonenzymatic or enzymatic with the enzymatic reaction being catalyzed by glutathione-S-transferase enzymes (GSTs) [5, 8]. Recent reports have indicated that at least three isozymes of GSTs exist in corn and these isozymes exhibit differential substrate specificity towards chloroacetanilide herbicides [9-11]. These isozymes have been designated GST I, GST II, and GST III and are distinguished by their specificity towards the chloroacetanilide herbicides and by their separation characteristics [11]. GST I is a constitutive isozyme that catalyzes the conjugation of 1-chloro-2,4-dinitrobenzene (CDNB) and of the herbicide, alachlor, with GSH. GST II appears to be induced by safener treatments, and also catalyzes the conjugation of CDNB and alachlor, but has different separation characteristics from GST I. GST III appears to be a constitutive enzyme with a higher specific activity for

alachlor and metolachlor than GST I [11]. O'Connell [10] demonstrated that at least 80% of the enzymatic activity for the conjugation of metolachlor and alachlor with GSH resides in GST III with the remainder of the activity in GST I. The existence of GST isozymes in grain sorghum has been speculated [12], but detailed characterization of such isozymes is currently unavailable.

Gronwald [7] recently reported that the degree of protection provided by safeners to grass crops against injury from chloroacetanilide herbicides correlates rather strongly with the ability of safeners to enhance GST(s) activity in corn and grain sorghum. However, other questions regarding the influence of safeners on glutathione levels and the activity of other glutathione-related enzymes such as glutathione reductase (GR) [EC 1.6.4.2] need to be examined. Komives et al. [13] reported that treatment of 2.5-day old corn shoots with the dichloroacetamide safener, dichlormid, for 24 and 48 hr resulted in a 1.78 and 2.5-fold increase in GR activity, respectively. Questions regarding the potential enzymatic and nonenzymatic conjugation of the oxime ether safeners with glutathione should also be addressed. Flurazole, a thiazole carboxylate safener, has been shown to conjugate with reduced glutathione in corn and sorghum shoots within 2 hr after treatment [14].

Breaux et al. [14] postulated that the formation of the GS-flurazole conjugate may override the normal feedback inhibition of glutathione biosynthesis, resulting in the observed increases in glutathione levels. In addition, he noted that the molecules of most of the currently available safeners are reactive enough to conjugate with GSH and that the formation of GS-safener conjugates may be an important aspect involved in their protective nature.

All of the currently available information on the safener-induced enhancement of metolachlor conjugation with GSH has been generated using shoot tissues of corn or grain sorghum. The influence of seed-applied safeners on glutathione levels and the activity of glutathione-related enzymes during seed imbibition and early seedling establishment of grain sorghum have not been examined.

Therefore, the objectives of this research were to determine: a) the influence of seed-applied oxabetrinil and CGA-133205 on the levels of glutathione and the activity of GR and GST of grain sorghum seeds during the imbibition phase of germination and early seedling establishment, b) the influence of oxabetrinil and CGA-133205 on the nonenzymatic conjugation of metolachlor with reduced glutathione, and c) the potential

nonenzymatic conjugation of oxabetrinil and CGA-133205 with reduced glutathione as influenced by pH and metolachlor.

MATERIALS AND METHODS

Plant material. Since oxabetrinil and CGA-133205 are applied as seed-coat treatments and would be taken into the seed early in the germination process, seeds imbibed for 0 to 24 hr and germinating seedlings (36 to 72 hr) were used as plant material for these studies. For the 0 to 8 hr period of imbibition phase of germination, 16 g of seed was placed into a scintillation vial with 8 ml of distilled water. At 0, 1, 2, 4, and 8 hr after germination was initiated (biginning of imbibition), 2 g of seed was removed, frozen in liquid nitrogen, and ground in a mortar and pestle. For the 12 to 72 hr time points, seeds were put into petri dishes containing filter paper and 5 ml of distilled water. At 12 and 24 hr, seeds were removed, frozen, and ground as above. At 36, 48, and 72 hr, the shoots and roots were removed from the seed tissue and both parts were frozen and ground separately. All seeds were incubated at 30°C. Final concentrations of all reagents are given in parenthesis.

Glutathione extraction. A modified method of Tietze [15] was used for this study. Plant tissue (0.25 g) was ground in a mortar and pestle with 1.5 ml of trichloroacetic acid (5% w/v). The slurry was

centrifuged for 15 min in a microcentrifuge (approximately 13,000 x g). A portion of the extract (0.4 ml) was diluted with 2.8 ml of Na_2HPO_4-KOH buffer (0.36 M, pH 7.5) for a 1:8 dilution. The shoot and root tissue was diluted 1:80. Diluted extracts were used in the glutathione assays as follows:

Glutathione assay. This assay [15] and the GR assay [16] described below are based on the conjugation of 5,5'-dithiobis(2-nitrobenzoic acid) (DTNB) with reduced glutathione to form a GS-TNB conjugate and 2-nitro-5-thiobenzoic acid (TNB). The formation of TNB was monitored spectrophotometrically at 412 nm. Reduced glutathione was formed by the reduction of oxidized glutathione (GSSG) catalyzed by NADPH-dependent GR. Commercial yeast GR (Sigma Chemical Co., St. Louis, MO) was used for the glutathione assays. For total glutathione determination, the reaction was conducted in a 1 ml cuvette which contained; 400 μl of sample or standard; 400 μl of reagent I (110 mM Na_2HPO_4, 40 mM NaH_2PO_4, 15 mM EDTA, 0.04% BSA, and 0.3 mM DTNB; approximate pH is 6.9), 320 μl of reagent II (1 mM EDTA, 0.02% BSA, 50 mM imidazole, and 0.48 units of glutathione reductase; approximate pH is 7.1). The reaction was initiated by addition of 80 μl of NADPH (0.9 mM). The

reaction is monitored at 412 nm for 2 to 4 min at 24 to 26°C.

To analyze for oxidized glutathione, 40 μl of 2-vinylpyridine was added to 1 ml of the diluted extract and vigorously shaken every 15 min for 1 hr. Reduced glutathione reacts with the 2-vinylpyridine and is effectively removed. This solution is then assayed as above to obtain the oxidized glutathione content of the sample. Reduced glutathione in the crude extract is obtained by subtracting the oxidized glutathione from the total glutathione. Glutathione concentration in the samples was calculated from standard curves using 0 to 2.0 μM of GSH and GSSG. These curves are linear over this concentration range and the GR is active for at least 15 min.

Glutathione reductase and GST extraction. Plant tissue (0.25 g) prepared as described above plus 0.13 g of poly-vinylpolypyrrolidone were briefly ground with a mortar and pestle. Then 2.5 ml of extraction buffer (0.1 M K-phosphate buffer plus 0.5 M EDTA, pH 7.5) was added and the slurry was ground again. This slurry was filtered through two layers of Miracloth and then centrifuged for 20 min at 20,000 x g. The pellet was discarded and the supernatant was used as the crude extract for the following GR and GST assays. Protein was

determined using the Coomassie Blue G-250 dye-binding assay procedure [17].

Glutathione reductase assay. GR was assayed according to the procedure of Smith et. al. [16]. All reaction components except the crude extract were at room temperature and the reaction chamber temperature was maintained at 24 to 26°C. The reaction mixture contained 1 ml of 0.2 M K-phosphate buffer (0.1 M) plus 1 mM EDTA (0.5 mM), pH 7.5; 0.5 ml of 3 mM DTNB (0.75 mM) in 0.01 M K-phosphate buffer; 0.25 ml distilled water; 0.1 ml of 2 mM NADPH (0.1 mM); 0.05 ml of crude extract; and the reaction was initiated by the addition of 0.1 ml of 5 mM GSSG (0.25 mM) to make a final volume of 2 ml. Formation of TNB was monitored at 412 nm for 2 to 4 min, but the reaction was linear for at least 15 min. The rate of TNB formation is proportional to the amount of GR activity. The extinction coefficient for the TNB is 11500 moles cm^{-1} min^{-1} [18].

Glutathione-S-transferase assay. The procedures of Mozer et. al. [19] and Ezra et. al. [20] were used for assaying the activity of GST with slight modifications. The reaction mixture contained; 30 μl of 0.1 M K-phosphate buffer (pH 6.5); 10 μl of 60 mM reduced glutathione (6 mM); 10 μl of crude extract. The reaction was initiated by the addition of 10 μl of 6 mM of either

carbonyl labeled-^{14}C-metolachlor (1 mM; sp. act. 59.5 mCi/mmole), phenyl labeled-^{14}C-oxabetrinil (1 mM; sp. act. 12.2 mCi/mmole), or phenyl labeled-^{14}C-CGA-133205 (1 mM; sp. act. 9.8 mCi/mmole) in a final volume of 60 μl. The reaction vessel was centrifuged in a microcentrifuge for 30 sec for thorough mixing and then incubated at 35°C for 60 min. After incubation, the reaction was terminated by adding 60 μl of distilled water and 1 ml of dichloromethane. The reaction vessel was shaken vigorously and then micro-centrifuged for 3 min. Sixty μl of the aqueous phase was counted using liquid scintillation counting to determine the amount of conjugate formed.

All extractions for glutathione determinations, GR, and glutathione-S-transferase, were twice repeated in time and all assays were run in duplicate for each extraction.

Nonenzymatic conjugation of metolachlor with GSH. To determine the influence of the safeners on nonenzymatic conjugation of metolachlor with glutathione, the methods of Mozer et al. [19] and Ezra et al. [20] for enzymatic conjugation were modified. Reaction mixtures contained 30 μl of 0.1 M K-phosphate buffer (pH 7.0), 10 μl of 6 mM reduced glutathione (1 mM), 10 μl of 0.3 mM ^{14}C-metolachlor (50 μM), and either 10 μl of oxabetrinil

or CGA-133205 (0, 1, 10, 20, 40, 80, or 160 μM) in a final volume of 60 μl. The reaction vessel was microcentrifuged for 30 sec for thorough mixing and then incubated at 30°C for 1 hr. Addition of 60 μl of distilled water and 1 ml of dichloromethane terminated the reaction by partitioning any unreacted metolachlor and safener into the organic phase and any conjugate into the aqueous phase. The aqueous phase was subsampled (60 μl) for liquid scintillation spectrometery. This experiment contained three replicates and was repeated in time.

Influence of pH on nonenzymatic conjugation. To determine the influence of pH on the conjugation of metolachlor, oxabetrinil, and CGA-133205 with glutathione, and to determine the influence of metolachlor and pH on the conjugation of safeners with glutathione, the above procedure was used with the following modifications. The reaction mixture contained 30 μl of 0.1 M K-phosphate buffer (pH 6.0, 7.0, or 8.0), 10 μl of 6 mM reduced glutathione (1 mM), and 10 μl of 0.3 mM ^{14}C-metolachlor (50 μM), ^{14}C-oxabetrinil (1 mM), or ^{14}C-CGA-133205 (1 mM) depending on the conjugate that was being determined in a final volume of 60 μl. Reaction times and separation procedures were the same as

above. This experiment contained three replicates and was repeated in time.

RESULTS AND DISCUSSION

Influence of oxabetrinil and CGA-133205 on glutathione content of grain sorghum seeds and germinating seedlings. The levels of total and reduced glutathione in untreated seeds of grain sorghum decreased during the first 12 hr after the initiation of seed imbibition, reaching a minimum at 4 hr (Table 1). From 8 hr up to 72 hr, the levels of total and reduced glutathione increased gradually in seeds as well as shoots and roots of germinating seedlings of grain sorghum reaching a maximum at 48 hr (Table 1). At 36 hr after the initiation of seed germination, the majority of total and reduced glutathione was present in the shoots and roots of the emerging seedlings of grain sorghum rather than the seed tissue (Table 1).

Before the initiation of germination (0 hr), seeds of grain sorghum treated with oxabetrinil (1.25 g ai/kg seed) and CGA-133205 (0.4 g ai/kg seed) contained total and reduced glutathione levels that were 20 to 30% higher than those of untreated seeds (Table 1). Storage of safener-treated seeds of grain sorghum for periods greater than a year does not adversely influence seed germination or the protective activity of the oxime ether safeners [1]. Measurable effects of oxime ether safeners

Table 1. Influence of Seed-applied Oxabetrinil and CGA-133205 on Total and Reduced Glutathione Levels of Grain Sorghum During Seed Germination and Early Seedling Establishment.

Imbibition Time	Seed Treatment: Untreated Total	Untreated Reduced(A)	Oxabetrinil[a] Total	Oxabetrinil[a] Reduced(B)	CGA-133205[b] Total	CGA-133205[b] Reduced(C)	B/A Ratio	C/A Ratio
(hr)	--------Glutathione content (umol/g tissue)[c]----------							
0	69.8^{D}	41.1^{C}	92.3^{D}	50.7^{D}	88.1^{D}	48.4^{D}	1.23	1.18
1	60.2^{D}	37.4^{C}	93.6^{D}	51.3^{D}	77.7^{D}	43.2^{D}	1.37	1.15
2	54.6^{D}	28.5^{C}	84.1^{D}	48.9^{D}	63.4^{D}	34.1^{D}	1.72	1.20
4	50.6^{D}	28.4^{C}	75.3^{D}	46.0^{D}	43.5^{D}	22.6^{D}	1.62	0.80
8	56.2^{D}	32.1^{C}	56.2^{D}	30.8^{D}	42.7^{D}	22.4^{D}	0.96	0.70
12	61.8^{D}	35.3^{C}	36.6^{D}	18.5^{D}	47.3^{D}	26.8^{D}	0.52	0.76
24	95.5^{D}	53.0^{C}	79.3^{D}	44.0^{D}	105.1^{D}	55.8^{D}	0.83	1.05
36[d]	89.9^{D}	51.2^{C}	90.9^{D}	56.8^{D}	119.2^{D}	70.3^{D}	1.11	1.37
36[e]	408.4^{C}	255.4^{B}	779.0^{C}	380.8^{C}	603.4^{C}	441.8^{C}	1.49	1.73
48	1558.5^{A}	761.3^{A}	3185.1^{A}	2012.8^{A}	1224.9^{A}	819.5^{A}	2.64	1.08
72	1044.5^{B}	690.6^{A}	1746.4^{B}	1006.2^{B}	950.9^{B}	561.9^{B}	1.46	0.81

[a]Sorghum seed was treated with oxabetrinil at 1.25 g ai/kg of seed.
[b]Sorghum seed was treated with CGA-133205 at 0.4 g ai/kg of seed.
[c]Columns followed by the same letter are not significantly different as determinied by Fishers' protected $LSD_{(0.05)}$.
[d]Seed tissue only.
[e]Shoot and root tissue removed from seed.

on the growth and respiration of grain sorghum during early stages of seed germination have been reported by Ketchersid and Merkle [21]. However, information on the potential influence of seed safeners on the metabolic activity of grain sorghum seeds during storage is currently unavailable. Therefore, the differences in glutathione content of untreated and safener-treated seeds of sorghum is difficult to explain at this time.

The levels of total and reduced glutathione in safener-treated seeds of grain sorghum during germination and seedling establishment followed a trend similar to that observed with untreated seeds (Table 1). Glutathione levels decreased gradually following the initiation of germination, reaching a low at 8 to 12 hr, and then increased continuously, reaching a maximum at 48 hr (Table 1). At most time periods following the initiation of seed germination, safener-treated seeds of grain sorghum contained higher levels of total and reduced glutathione than unsafened seeds (ratios of B/A and C/A in Table 1). This was particularly true for oxabetrinil-treated seedlings which at 36 to 72 hr contained 46 to 164% more reduced glutathione than untreated seeds (Table 1). However, treatment with oxabetrinil for 8 to 24 hr or with CGA-133205 at 4 to 12 hr reduced the glutathione content of sorghum seeds when

compared to level in untreated grain sorghum (Table 1).

Reduced glutathione is an obligatory reactant for the enzymatic or nonenzymatic formation of the glutathione conjugate of metolachlor or other chloroacetanilide herbicides [8, 12]. The oxabetrinil-induced increases in the GSH content of grain sorghum seedlings at 36 to 72 hr are significant and they may contribute to the mechanism of protective action of this safener. Data reported by Gronwald et al. [12] showed that oxabetrinil caused a slight (11%), but not significant increase in the levels of total and reduced glutathione of excised shoots of etiolated 2-day old grain sorghum seedlings. The use of a different sorghum cultivar (G-623 GBR) as well as time-course differences may have accounted for the differential results obtained by Gronwald et al. [12] and the present study.

Relatively little is known about the content, synthesis, and metabolic fate of glutathione in mature or germinating seeds of higher plants. In a recent study on the thiol content of legume seeds, Klapheck [22] reported that the major thiol in Vicieae seeds was glutathione while in Phaseoleae seeds the major thiol was homoglutathione. Seeds from the Trifoleae tribe contained both glutathione and homoglutathione [22]. The results of the present study show that the levels of

glutathione found in grain sorghum seeds are greater than the levels of glutathione or homoglutathione found in the seeds of legume species [22]. The glutathione reserves of grain sorghum seeds are depleted during early germination indicating that the biosynthesis of glutathione in grain sorghum seeds may be initiated between 12 and 24 hr following the initiation of seed imbibition.

Influence of safeners on glutathione reductase activity. The activity of GR extracted from seeds or germinating seedlings of grain sorghum remained rather stable during the 72 hr time-course of this study (Table 2). Before the initiation of imbibition (0 hr), the activity of GR extracted from safener-treated seeds of grain sorghum was 15 to 24% higher than that of untreated seeds (Table 2). At the early stages of seed germination (1 to 12 hr), the activity of GR from safener-treated seeds was lower than that of untreated seeds (B/A and C/A ratios in Table 2). At 24 hr or later time points following the initiation of seed imbibition, the activity of GR in safener-treated seeds or shoots and roots of sorghum seedlings was at or above the activity level of GR extracted from corresponding tissues of untreated grain sorghum (Table 2). However, most of these safener influences did not appear to be significant.

Table 2. Activity of Glutathione Reductase Extracted from Untreated, Oxabetrinil-treated and CGA-133205-treated Grain Sorghum During Seed Germination and Early Seedling Establishment.

Imbibition Time	Seed Treatment: Untreated (A)	Oxabetrinil[a] (B)	CGA-133205[b] (C)	B/A Ratio	C/A Ratio
(hr)	---------(umol/min/mg protein---------				
0	337.0^{BC}	387.3^{BAC}	417.2^{DC}	1.15	1.24
1	353.0^{BC}	330.3^{BC}	328.2^{DE}	0.94	0.93
2	483.3^{A}	371.4^{BAC}	333.2^{DE}	0.77	0.69
8	314.4^{BC}	247.4^{C}	343.0^{DE}	0.68	1.09
12	361.2^{BAC}	318.3^{BC}	250.8^{E}	0.88	1.69
24	264.3^{C}	470.9^{BA}	310.4^{DE}	1.78	1.17
36[d]	478.1^{A}	528.2^{A}	635.6^{A}	1.10	1.33
36[e]	476.1^{A}	488.7^{BA}	598.0^{BA}	1.03	1.26
48	401.1^{BA}	478.9^{BA}	493.2^{BC}	1.19	1.23
72	342.9^{BC}	435.5^{BA}	363.4^{DE}	1.27	1.06

[a]Sorghum seed was treated with oxabetrinil at 1.25 g ai/kg of seed.
[b]Sorghum seed was treated with CGA-133205 at 0.4 g ai/kg of seed.
[c]Columns followed by the same letter are not significantly different as determinied by Fishers' $LSD_{(0.05)}$.
[d]Seed tissue only.
[e]Shoots and roots removed from seed.

Based on these data, it is safe to conclude that a safener-induced enhancement of GR activity does not appear to play a major role in the safening action of oxabetrinil. This statement is further supported by data on the GSH/GSSG ratios calculated for untreated and oxabetrinil-treated sorghum tissues and presented in Table 3. Averaged over time the GSH/GSSG ratios of control and oxabetrinil-treated sorghum tissues were 1.39 and 1.32, respectively (Table 3).

Thus, the significant increase in reduced glutathione levels of grain sorghum seedlings caused by the safener oxabetrinil (Table 1) can not be explained as a result of safener-induced influence of GR activity. Instead it may be the result of a direct influence of oxabetrinil on the biosynthesis of glutathione in grain sorghum. Dichloro-acetamide safeners such as dichlormid and R-29148 have been shown to directly influence the de novo synthesis of GSH from sulfate by enhancing the activity of the enzyme ATP sulfurylase in corn [23].

CGA-133205 caused an increase in the GSH/GSSG ratio in shoots and roots of germinating sorghum seedlings at 36 and 48 hr after the initiation of seed imbibition (Table 3). At the same time periods, CGA-133205 increased the activity of GR extracted from these tissues by 26 and 23%, respectively (Table 2). Thus, it is

Table 3. Ratio of Reduced (GSH) to Oxidized (GSSG) Glutathione Extracted from Untreated, Oxabetrinil-treated and CGA-133205-treated Grain Sorghum during Seed Germination and Early Seedling Establishment.

Imbibition	Seed Treatment		
Time	Untreated	Oxabetrinil[a]	CGA-133205[b]
(hr)	----------(GSH/GSSG ratio)----------		
0	1.43	1.22	1.22
1	1.64	1.21	1.25
2	1.09	1.39	1.16
4	1.28	1.57	1.08
8	1.33	1.21	1.11
12	1.34	1.02	1.30
24	1.25	1.25	1.13
36[c]	1.32	1.66	1.43
36[d]	1.67	0.96	2.73
48	0.96	1.72	2.02
72	1.95	1.36	1.44
Average	1.39	1.32	1.44

[a]Sorghum seed was treated with oxabetrinil at 1.25 g ai/kg seed.

[b]Sorghum seed was treated with CGA-133205 at 0.4 g ai/kg seed.

[c]Seed tissue only.

[d]Shoot and root tissue removed from seed.

likely that CGA-133205 might act by enhancing the activity of GR to maintain a high GSH/GSSG ratio in the cells of protected grain sorghum. A 2.5-fold increase in the activity of GR extracted from 2.5-day old corn treated with the safener dichlormid for 48 hr, has been reported recently by Komives et al. [13]. Neverheless, further research is needed to define more clearly the potential influences of CGA-133205 on GR activity.

The ratios of reduced to oxidized glutathione determined in grain sorghum seeds and seedlings were low compared to ratios observed in photosynthetic tissue of other plant species [18] (Table 3). Given the activity of GR determined in these experiments (Table 2) it is difficult to explain these low GSH/GSSG ratios. Nevertheless, information on the levels of GSH and GSSG in germinating sorghum seeds is not available in the literature.

Influence of safeners on GST activity. The activity of crude extracts of glutathione S-transferase (GST) obtained from untreated and safener-treated seeds or germinating seedlings of grain sorghum was assayed by monitoring formation of GS-metolachlor conjugate with ^{14}C-metolachlor as substrate (Table 4). GST activity from untreated grain sorghum tissues remained somewhat stable during the first 8 hr following the initiation of

seed imbibition. However, from 12 to 72 hr, GST activity increased constantly, reaching a maximum at 72 hr. At time points equal to or greater than 36 hr, most of the GST activity was associated with the shoots and roots of germinated sorghum seedlings rather than the seed (Table 4). GST activity obtained from untreated tissues of the Funk G522-DR cultivar of grain sorghum used in the present study was significantly higher than that reported by Gronwald et al [12] for the Funk G-623 GBR cultivar of grain sorghum.

Similar to the aforementioned studies on glutathione content and GR activity, at time 0 hr, the activity of GST obtained from oxabetrinil- and CGA-133205-treated seeds of grain sorghum was 67 and 89% greater than that of untreated seeds (ratios of B/A and C/A in Table 4). Again this safener effect is difficult to explain based on current information available in the literature. However, it is evident that oxime ether safeners have an apparent influence on the metabolism of grain sorghum seeds during storage which needs to be examined more thoroughly in the future.

Following the initiation of seed imbibition, GST activity in oxabetrinil-and CGA-133205-treated seeds remained enhanced compared to that of untreated seeds up to the time point of 8 hr (B/A and C/A ratios, Table 4).

Table 4. Metolachlor-conjugating Activity of Glutathione-S-transferase Extracted from Untreated, Oxabetrinil-treated and CGA-133205-treated Grain Sorghum during Seed Germination and Early Seedling Establishment.

	Seed Treatment				
Time	Untreated (A)	Oxabetrinil[a] (B)	CGA-133205[b] (C)	B/A Ratio	C/A Ratio
(hr)	--GST activity (umol/mg protein/min)[c]--				
0	154.9^{C}	258.9^{A}	292.8^{BA}	1.67	1.89
1	161.7^{C}	190.6^{A}	272.4^{BA}	1.17	1.68
2	197.4^{C}	387.3^{A}	135.8^{B}	1.96	0.69
4	239.8^{C}	298.9^{A}	288.8^{BA}	1.25	1.20
8	198.0^{C}	205.8^{A}	215.6^{A}	1.39	1.46
12	288.8^{CB}	302.8^{A}	282.3^{BA}	1.04	0.93
24	318.5^{CB}	209.6^{A}	271.9^{BA}	0.66	0.85
36[d]	114.0^{C}	111.3^{A}	275.2^{BA}	0.98	0.66
36[e]	596.8^{CB}	325.3^{A}	228.0^{BA}	0.54	0.38
48	799.3^{B}	481.2^{A}	624.4^{A}	0.60	0.78
72	1825.0^{A}	541.2^{A}	355.2^{A}	0.29	0.19

[a]Sorghum seed was treated with oxabetrinil at 1.25 g ai/kg of seed.
[b]Sorghum seed was treated with CGA-133205 at 0.4 g ai/kg of seed.
[c]Columns followed by the same letter are not significantly different as determinied by Fishers' protected $LSD_{(0.05)}$.
[d]Seed tissue only.
[e]Shoots and roots removed from seed.

From 12 to 72 hr, GST activity in safener-treated seeds or germinating seedlings of grain sorghum was consistently lower than that of untreated seeds or seedlings (Table 4).

Gronwald et al. [12] recently reported that oxabetrinil significantly enhanced GST activity extracted from excised apical sections of 48-hr-old etiolated sorghum shoots. The oxabetrinil-induced enhancement of GST activity was relative and it decreased as metolachlor concentration in the assay medium increased [12]. Thus, while oxabetrinil enhanced GST activity of grain sorghum shoots by 21.8-fold when metolachlor was used at 0.5 μM, it caused only a 4.6-fold enhancement of GST activity when metolachlor was used at 0.5 mM. In the present study, the concentration of metolachlor used to assay GST activity was 1 mM and crude extracts were obtained from a sorghum cultivar different than that used by Gronwald et al. [12]. These two facts may explain the differences in the degree of safener-induced enhancement of GST activity between the present study and that conducted by Gronwald et al. [12].

Influences of safeners on nonenzymatic conjugation of metolachlor with GSH. Oxabetrinil used at concentrations ranging from 1 to 40 μM increased significantly (41 to 126%) the rate of nonenzymatic

conjugation of metolachlor with GSH at pH 7.0 (Table 5). These data are in agreement with those reported by Zama and Hatzios [24] who also reported an oxabetrinil-induced enhancement of the nonenzymatic conjugation of metolachlor with GSH. Gronwald et al. [12] have reported that some nonenzymatic conjugation of metolachlor with GSH does occur in vitro at pH 7.4, and that the rate of this reaction increased with increasing concentrations of metolachlor.

At higher concentrations (80 and 160 μM), oxabetrinil significantly inhibited the nonenzymatic conjugation of metolachlor with GSH (Table 5). CGA-133205 enhanced the nonenzymatic conjugation of metolachlor with GSH when used at concentrations of 1 to 160 uM (Table 5). A 106% increase in the rate of the nonenzymatic conjugation of metolachlor with GSH was observed when CGA-133205 was used at 160 uM (Table 5).

Comparison of the data presented in Tables 4 and 5 shows that the rate of nonenzymatic conjugation of metolachlor with GSH is much slower than that of the enzymatic conjugation catalyzed by GST. The rate of nonenzymatic conjugation of metolachlor with GSH was found to be strongly dependent on the pH of the reaction solution with conjugation increasing with increasing pH

Table 5. Influence of Safener Concentration on Nonenzymatic Conjugation of Metolachlor with Reduced Glutathione.[a]

Safener Concentration	Oxabetrinil	Percent of Control	CGA-133205	Percent of Control
(uM)	(nmol/hr)[b]	(%)	(nmol/hr)[b]	(%)
0	37.2 ± 12.5	100	37.2 ± 12.5	100
1	75.3 ± 22.6	201	63.7 ± 13.1	170
10	84.8 ± 27.3	226	50.3 ± 12.7	134
20	52.9 ± 9.3	141	54.7 ± 8.6	146
40	68.9 ± 14.7	184	51.4 ± 18.8	137
80	13.4 ± 17.1	36	57.4 ± 21.9	153
160	0	0	77.4 ± 36.1	206

[a]Data represent the mean ± SE of 2 experiments with 3 replicates per experiment.

[b]nmoles of metolachlor conjugated with GSH in one hr at pH 7.0.

(Table 6). Similar results have been reported by Leavitt and Penner [8] who showed that the nonenzymatic conjugation of the herbicide alachlor with GSH was dependent on the pH of the reaction solution and on the initial concentration of glutathione. The results of the present study show that the potential contribution of the nonenzymatic conjugation of metolachlor with GSH in the metabolic detoxication of this herbicide in grain sorghum and the protective action of the oxime ether safeners can not be ruled out.

Conjugation of oxime ether safeners with GSH. Data presented in Table 6 show that the safener oxabetrinil can conjugate with GSH nonenzymaticaly. The rate of this reaction is slow and is dependent on the pH of the reaction solution. CGA-133205 did not conjugate substantially with GSH at any pH (Table 6).

Comparison of the data presented in Table 6 shows that the rate of the nonenzymatic conjugation of oxabetrinil with GSH is much slower than that of the nonenzymatic conjugation of metolachlor with GSH. In addition, the initial ratio of glutathione to metolachlor (33:1) is lower than the ratio of glutathione to oxabetrinil (20:1). These two facts indicate that metolachlor is much more reactive than oxabetrinil or CGA-133205 in terms of conjugating with GSH under

Table 6. Influence of pH of the Reaction Solution on Nonenzymatic Conjugation of Metolachlor, Oxabetrinil and CGA-133205 with Reduced Glutathione.[a]

pH	Metolachlor	Oxabetrinil	CGA-133205
	(nmol/hr)[b]		
6.0	0.0	0.0	0.0
7.0	46.1 ± 25.5	0.4 ± 0.4	0.0
8.0	124.3 ± 9.9	2.6 ± 1.3	0.2 ± 0.1

[a]Data represent the mean ± SE of 2 experiments with 3 replicates per experiment.

[b]nmoles of metolachlor, oxabetrinil or CGA-133205 conjugated to GSH in one hour.

nonenzymatic conditions *in vitro*. This is most likely due to the high reactivity of the electrophilic chlorine ion of metolachlor which is not present on the molecules of the oxime ether safeners.

At pH 8.0, 2.6 nmoles of oxabetrinil conjugated with GSH in one hour (Table 6). However, when metolachlor at 50 μM was added to the reaction solution, only 0.2 nmoles of oxabetrinil conjugated with GSH in one hour at the same pH (8.0). Thus, the presence of metolachlor reduced the rate of nonenzymatic conjugation of oxabetrinil with GSH by more than 90% indicating again that metolachlor is more reactive than oxabetrinil in terms of nonenzymatic conjugation with GSH.

Influences of oxime ether safeners on GST activity using the safeners as substrate. The possibility that the conjugation of the safener oxabetrinil with GSH may proceed at a greater rate under enzymatic conditions was also examined. Crude extracts of GST obtained from untreated and oxabetrinil-treated tissues of grain sorghum catalyzed the conjugation of oxabetrinil with GSH (Table 7). However, GST activity from grain sorghum tissues utilizing oxabetrinil as a substrate was significantly lower than that utilizing metolachlor as a substrate (Tables 4 and 7). Pretreatment of sorghum seeds with the safener, oxabetrinil, did not appear to

Table 7. Oxabetrinil-conjugating Activity of Glutathione-S-transferase Extracted from Untreated and Oxabetrinil-treated Grain Sorghum during Seed Germination and Early Seedling Establishment.

Imbibition Time	Tissue Examined	Seed Treatment: Untreated (A)	Seed Treatment: Oxabetrinil[a] (B)	B/A Ratio
(hr)		GST activity (umol/mg protein/min)[b]		
24	Seed	6.0[C]	5.3[B]	0.88
36[c]	Seed	5.3[C]	6.2[B]	1.17
36[d]	Shoot and Root	28.1[C]	14.6[B]	0.52
48	Shoot and Root	107.9[B]	107.5[A]	1.00
72	Shoot and Root	160.4[BA]	45.6[B]	0.28

[a]Sorghum seed was coated with oxabetrinil at 1.25 g ai/kg of seed.

[b]Columns followed by the same letter are not significantly different as determinied by Fishers' protected $LSD_{(0.05)}$.

[c]Seed tissue with shoot and root removed.

[d]Shoot and root tissue with seed tissue removed.

enhance GST activity when this safener was used as a substrate (Table 7). Crude extracts of GST obtained from untreated or CGA-133205-treated tissues of grain sorghum did not catalyze the conjugation of the safener CGA-133205 with GSH (data not shown). These results illustrate, again, the poor reactivity of this safener with GSH.

Similar to studies conducted with the safener flurazole [14], the aforementioned evidence for the enzymatic and nonenzymatic conjugation of the safener oxabetrinil with GSH needs to be complimented by further analytical work (i.e. mass spectrometry or NMR spectroscopy) to conclusively demonstrate the formation of a GS-oxabetrinil conjugate in plant tissues. Unfortunately, the levels of the GS-oxbetrinil conjugate detected in this study were too low to be useful for such analysis. The biological significance of the conjugates of oxabetrinil with GSH also awaits further experimentation. Recent reports [14] have postulated that GS-conjugates of herbicide safeners such as flurazole could enhance GSH levels by deregulating the feedback control of GSH synthesis in tissues of the protected plants.

In summary, the results of the present study demonstrated that glutathione and glutathione-related

enzymes in seeds and seedlings of grain sorghum play a key role in the mechanism of action of the oxime ether safeners. However, despite their chemical similarity, the oxime ether derivatives, oxabetrinil and CGA-133205, appear to behave differently when used as safeners of grain sorghum against injury from the chloroacetanilide herbicide, metolachlor. Oxabetrinil caused a significant enhancement of the levels of total and reduced glutathione in tissues of grain sorghum during seed germination and early seedling establishment with the levels increasing dramatically after 12 hr following initiation of germination. This influence of oxabetrinil appeared to be a direct effect on GSH synthesis rather than an indirect one resulting from an oxabetrinil-induced stimulation of the activity of GR. Deregulation of the feedback control of GSH synthesis by oxabetrinil is a possible mechanism of action since this safener was reactive enough to form a conjugate with GSH. In contrast, CGA-133205 appeared to enhance slightly the activity of GR causing high GSH/GSSG ratios in tissues of grain sorghum during the early phases of seed germination. CGA-133205 did not conjugate with GSH either enzymatically or nonenzymatically. Both safeners enhanced grain sorghum GST activity for conjugating metolachlor with GSH very early in the germination

process (0-8 hr following seed imbibition), but reduced activity at 24 to 72 hr. In addition, both safeners increased the rate of the nonenzymatic conjugation of metolachlor with GSH in a concentration-and pH-dependent fashion.

REFERENCES

[1] K. K. Hatzios, in "Crop Safeners for Herbicides: Development, Uses, and Mechanisms of Action" (K. K. Hatzios and R. E. Hoagland, Eds.), Academic Press, Inc., New York, 3 (1988).

[2] E. Ebert and H. R. Gerber, in "Crop Safeners for Herbicides: Development, Uses, and Mechanisms of Action" (K. K. Hatzios and R. E. Hoagland, Eds.), Academic Press, Inc., New York, 177 (1988).

[3] H. M. LeBaron, J. E. McFarland, B. J. Simoneaux, and E. Ebert, in "Herbicides: Chemistry, Degradation, and Mode of Action" (P. C. Kearney and D. D. Kaufman, Eds), Marcel Dekker, Inc., New York, Vol. 3, 335 (1988).

[4] F. J. Ellis, J. W. Peek, J. Boehle, Jr., and G. Muller, Weed Sci. 28, 1 (1980).

[5] K. K. Hatzios, in "Crop Safeners for Herbicides: Development, Uses, and Mechanisms of Action" (K. K. Hatzios and R. E. Hoagland, Eds.), Academic Press, Inc., New York, 65 (1988).

[6] K. K. Hatzios and R. E. Hoagland, in "Crop Safeners for Herbicides: Development, Uses, and Mechanisms of Action" (K. K. Hatzios and R. E. Hoagland, Eds.), Academic Press, Inc., New York, 355 (1988).

[7] J. W. Gronwald, in "Crop Safeners for Herbicides: Development, Uses, and Mechanisms of Action" (K. K. Hatzios and R. E. Hoagland, Eds.), Academic Press, Inc., New York, 103 (1988).

[8] J. R. C. Leavitt and D. Penner, J. Agric. Food Chem. 27(3), 533 (1979).

[9] R. Edwards and W. J. Owens, Planta. 175, 99 (1988).

[10] K. M. O'Connell, E. J. Breaux, and R. T. Fraley, Plant Physiol. 86, 359 (1988).

[11] K. P. Timmerman, Submitted to Physiol. Plant. (1989).

[12] J. W. Gronwald, E. P. Fuerst, C. V. Eberlein, and M. A. Egli, Pestic. Biochem. Physiol. 29, 66 (1987).

[13] T. Komives, V. A. Komives, M. Balazas, and F. Dutka, British Crop. Prot. Conf.-Weeds. 3, 1155 (1985).

[14] E. J. Breaux, M. A. Hoobler, J. E. Patanella, and G. A. Leyes, in "Crop Safeners for Herbicides: Development, Uses, and Mechanisms of Action" (K. K. Hatzios and R. E. Hoagland, Eds.), Academic Press, Inc., New York, 163 (1989).

[15] F. Tietze, Anal. Biochem. 27, 502 (1969).

[16] I. K. Smith, T. L. Vierheller, and C. A. Thorne, Anal. Biochem. 175, 408 (1988).

[17] M. M. Bradford, Anal. Biochem. 72, 248 (1976).

[18] J. Hess, Virginia Polytechnic Institute and State University, Blacksburg, Virginia, Personal Communication (1989).

[19] T. J. Mozer, D. C. Tiemeier, and E. G. Jaworski, Biochem. 22, 1068 (1983).

[20] G. Ezra, D. G. Rusness, G. L. Lamoureaux, and G. R. Stephenson, Pestic. Biochem. Physiol. 23, 108 (1985).

[21] M. L. Ketchersid and M. G. Merkle, Proc. South. Weed Sci. Soc. 36, 386 (1983).

[22] S. Klapheck. Physiol. Plant. 74, 727 (1988).

[23] C. A. Adams and J. E. Casida, Pestic. Biochem. Physiol. 19, 350 (1983).

[24] P. Zama and K. K. Hatzios, Weed Sci. 34, 834 (1986)

V. UPTAKE, TRANSLOCATION, AND METABOLISM OF THE SAFENERS, OXABETRINIL AND CGA-133205, IN GRAIN SORGHUM (*Sorghum bicolor*) AND THEIR INFLUENCE ON METOLACHLOR METABOLISM

INTRODUCTION

The chloroacetanilide herbicide, metolachlor, exhibits good selectivity in corn (Zea mays L.), but exhibits only marginal selectivity in grain sorghum. This differential plant susceptibility to metolachlor does not appear to result from differential uptake or translocation of this herbicide [LeBaron et al., 1988]. Root exposure of 2-day-old seedlings of corn, a tolerant species, and yellow nutsedge (Cyperus esculentus L.), a susceptible species, to ^{14}C-metolachlor for 48 hr resulted in greater root uptake of the herbicide by corn than by the yellow nutsedge. Following shoot exposure to ^{14}C-metolachlor, uptake of the herbicide was greater in the yellow nutsedge than in corn [LeBaron et al., 1988]. Uptake of the soil-applied chloroacetanilide herbicides appears to occur primarily through the shoots or cotyledons of grasses and through the roots of dicotyledonous plants [LeBaron et al., 1988]. Once inside the plant, chloroacetanilide herbicides are translocated acropetally [LeBaron et al., 1988].

Since differential plant sensitivity to metolachlor can not be explained by differential uptake, then differential metabolism within the plant may be the basis for the observed crop selectivity of metolachlor. In fact, tolerant species such as corn have been shown to metabolize metolachlor at a much faster rate than susceptible species such as yellow nutsedge [LeBaron et al., 1988]. Even though both corn and yellow nutsedge are capable of metabolizing metolachlor, corn detoxifies metolachlor faster and more efficiently than yellow nutsedge [LeBaron et al., 1988].

The first step in the metabolism of metolachlor in most plants is conjugation with reduced glutathione (GSH) [LeBaron et al., 1988; Gronwald, 1988; Breaux, 1988]. In some legumes such as soybeans, conjugation of metolachlor with homoglutathione (hGSH) is known to occur [Gronwald, 1988]. The conjugation of metolachlor with reduced glutathione is mainly an enzymatic reaction catalyzed by specific glutathione-S-transferase(s) [GSTs; EC 2.5.1.18]. After formation of the GS-metolachlor conjugate, peptidases remove the glycine and glutamic acid residues from the glutathione molecule leaving the cysteine conjugate of metolachlor. The cysteine conjugates is further catabolized by deamination to thiolactic acid conjugates or by N-acylation with malonic

acid [Gronwald, 1988]. In tolerant plants such as corn, thiolactic acid conjugates are considered terminal metabolites of metolachlor [Gronwald, 1988]. The conjugation of metolachlor with GSH can also proceed nonenzymatically, but at a much lower rate than the enzymatic reaction [Gronwald and Fuerst, 1987].

Cyometrinil, an oxime ether safener, was introduced to protect the marginally tolerant grain sorghum from injury caused by metolachlor. Cyometrinil was later replaced by oxabetrinil, a more effective safener of grain sorghum against metolachlor. Another oxime ether analog that is being evaluated as a safener for grain sorghum is CGA-133205. All of the currently available oxime ether safeners for use on grain sorghum are applied as seed treatments; thus, they are readily available for uptake by the germinating grain sorghum seed. Early research with cyometrinil and oxabetrinil has shown that these safeners do not reduce the amount of metolachlor taken up by grain sorghum seedlings [LeBaron et al., 1988]; therefore, the protection conferred by the application of these safeners to grain sorghum must be due to a safener-induced increase in the rate or pattern of metolachlor metabolism or due to effects at the site of action. Gronwald et al. [1987] recently reported that the degree of protection provided by safeners to grass crops against chloroacetanilide

herbicides correlates rather strongly with the ability of safeners to enhance glutathione-S-transferase activity.

Currently, information on the uptake and metabolism of the safeners, oxabetrinil and CGA-133205, in grain sorghum is not available. Similar to metolachlor, cyometrinil is taken up through the coleoptile of sorghum seedlings although some uptake has also been shown to occur through the seed coat of grain sorghum [LeBaron et al., 1988]. Breaux et al. [1986] demonstrated that flurazole, a thiazole carboxylate safener that is seed-applied to grain sorghum, was rapidly absorbed by 3- to 5-day-old etiolated shoots of corn and grain sorghum. In both crops, flurazole was metabolized rapidly and the major metabolite detected was the GSH conjugate of flurazole [Breaux, 1988].

The objectives of this research were to determine: a) the uptake, translocation, and metabolism of the oxime ether safeners, oxabetrinil and CGA-133205, in grain sorghum and b) the influence of oxabetrinil and CGA-133205 on the uptake, translocation, and metabolism of the herbicide, metolachlor, in grain sorghum.

MATERIALS AND METHODS

Plant material. Grain sorghum seed var 'Funk G-522 DR' was obtained from CIBA-GEIGY Corporation, Greensboro, North Carolina. The seed was either untreated or treated with oxabetrinil (1.25 g a.i./kg seed) or CGA-133205 (0.4 g a.i./kg seed). Approximately 40 seeds of each treatment were placed on moistened filter paper in petri dishes and incubated at 30°C for varying lengths of time depending on the experiment.

Uptake and translocation of oxabetrinil and CGA-133205. Untreated seed, prepared as described above, was incubated for 12 hr at 30°C. After the initial 12 hr incubation, 2 μl of 1 mM ^{14}C-oxabetrinil (phenyl labeled; sp. act. 12.2 mCi/mmole) or 1 mM ^{14}C-CGA-133205 (phenyl labeled; sp. act. 9.8 mCi/mmole) were placed on untreated seed. At 0, 0.5, 1, 2, 4, 24, 48, and 72 hrs after ^{14}C-safener application, the seed or seedling was rinsed in 80% methanol to determine the amount of ^{14}C-safener that remained on the exterior of the seed. At 0, 0.5, 1, 2, and 4 hrs the seed was oxidized (Packard Sample Oxidizer Model B306), trapping $^{14}CO_2$ originating from the ^{14}C-safener that is a product of the combustion of the plant material, after the methanol rinse to determine radiolabel, originating from the ^{14}C-safener, uptake into the seed. At 24 hr after the methanol rinse, the root was

removed and oxidized to evaluate the amount of translocation from the seed to the root. At 48 and 72 hrs, the coleoptile and the root were removed from the seed and all three parts were oxidized to determine the amount of translocation or radiolabel into the respective tissues.

Uptake and translocation of metolachlor. Seeds prepared as described above were incubated for 5 days; after which, 2 μl of 1 mM ^{14}C-metolachlor (carbonyl labeled; sp. act. 59.5 mCi/mmole) was placed on the coleoptile of each untreated and safener-treated grain sorghum seedling. At 0, 0.5, 1, 2, and 4 hr after ^{14}C-metolachlor application, the coleoptiles were removed from the seed, rinsed in 80% methanol, and oxidized for total radiolabel uptake. The seed and root were also oxidized to analyze for basipetal translocation of the radiolabel from ^{14}C-metolachlor.

Safener and metolachlor metabolism. Seeds were prepared as above and incubated for 3 days. After incubation, 2 μl of 1 mM ^{14}C-metolachlor (carbonyl labeled; sp. act. 59.5 mCi/mmole), ^{14}C-oxabetrinil (phenyl labeled; sp. act. 12.2 mCi/mmole), or ^{14}C-CGA-133205 (phenyl labeled; sp. act. 9.8 mCi/mmole) were applied to coleoptiles of untreated and safener-treated grain sorghum seedlings. At 0, 1, 2, 4, and 8 hr after application of

radiolabeled safener or herbicide, the coleoptiles were removed and rinsed in 80% methanol. After the rinse, coleoptiles were ground in 80% methanol with a mortar and pestle. The methanol extract was centrifuged for 5 min at 13000 x g. The supernatant was decanted and saved for further analysis. The pellet was oxidized to determine the amount of unextracted radiolabel from ^{14}C-metolachlor or ^{14}C-safener.

A subsample of the methanol extract was radioassayed for total radiolabel uptake from ^{14}C-metolachlor or ^{14}C-safener. To determine the amount of water soluble metabolites, a 60 μl subsample of the total extract was diluted with 60 μl of distilled water and mixed vigorously with 1 ml of dichloromethane. This solution was centrifuged for 3 min at 13,000 x g for thorough mixing. The aqueous phase was then subsampled and radioassayed.

To further characterize the metabolites, the extract from the 4 hr time point was dried under nitrogen gas. The residue was dissolved in 50 μl of methanol and 20 μl was spotted onto a 150 Å silica gel thin-layer chromatography plates. Plates were developed with a 1-butanol:acetic acid:water (12:3:5 v/v) solvent mixture. After development, silica gel was removed from the plates in 1 cm increments to quantify the amount of ^{14}C in each increment. ^{14}C-metolachlor, ^{14}C-oxabetrinil,

^{14}C-CGA-133205, and synthetic conjugates of GS-metolachlor or GS-safener were co-chromatographed with the plant extracts to aid in the identification of the detected metabolites.

RESULTS AND DISCUSSION

Uptake and translocation of oxabetrinil and CGA-133205. Safener uptake by germinating seeds of grain sorghum increased for the 72 hr duration of this experiment (Table 1). Uptake of the radiolabel from ^{14}C-oxabetrinil by grain sorghum seed was greater and more rapid than that of CGA-133205 (Table 1). Within the first 0.5 hr over 50% of the applied radiolabel from ^{14}C-oxabetrinil had been taken up by sorghum seeds compared to only 20% of the ^{14}C-CGA-133205 (Table 1). Following seed application of either ^{14}C-safener, most of the radiolabel was concentrated in the coleoptile of the germinating seedlings of grain sorghum at 48 and 72 hr after application (Table 2). With both safeners a small amount of the applied radioactivity was distributed in to the roots and seeds of the germinated seedlings of grain sorghum at 48 and 72 hr (Table 2). With this application method, it appears that the radiolabel from either the intact or metabolized ^{14}C-safener is either taken up by the coleoptile as it emerges from the seed or that the safener is translocated to the coleoptile after uptake of the safener through the seed.

Metabolism of oxabetrinil and CGA-133205 in grain sorghum. Total extractable radiolabel from ^{14}C-oxabetrinil

Table 1. Uptake of ^{14}C following application of ^{14}C-Oxabetrinil and ^{14}C-CGA-133205 by Germinating Seeds and Seedlings of Grain Sorghum.

time	^{14}C-oxabetrinil	^{14}C-CGA-133205
(hr)	----------% of applied[a]---------	
0.5	54.2^{D}	11.1^{E}
1	55.4^{DB}	20.0^{D}
2	66.7^{CB}	20.2^{D}
4	72.5^{BA}	27.6^{C}
24	78.0^{A}	35.9^{B}
48	81.5^{A}	41.9^{A}
72	84.1^{A}	46.2^{A}

[a]Means within a column followed by the same letter are not significantly different as determined by Fishers' protected $LSD_{(0.05)}$.

Table 2. Distribution of ^{14}C from ^{14}C-oxabetrinil or ^{14}C-CGA-133205 in grain sorghum seedlings derived from seed-treated with the respective safener.

time	oxabetrinil			CGA-133205		
(hr)	coleoptile	root	seed	coleoptile	root	seed
	----------% of applied[a]----------					
48	64.3^{A}	10.9^{A}	6.3^{A}	31.3^{A}	5.6^{A}	5.0^{A}
72	68.3^{A}	12.2^{A}	3.6^{B}	34.6^{A}	5.5^{A}	6.1^{A}

[a]Means within a column followed by the same letter are not significantly different as determined by Fishers' protected $LSD_{(0.05)}$.

Table 3. Oxabetrinil metabolism in coleoptiles of 3-day old untreated and oxabetrinil-treated grain sorghum seedlings.

time (hr)	untreated		oxabetrinil[a]		ratio	ratio
	total extract(A)[b]	water soluble(C)[c]	total extract(B)	water soluble(D)[d]	B/A	D/C
	% of applied[d]	% of extracted	% of applied	% of extracted		
1	22.8^{B}	8.8^{B}	14.4^{BC}	3.5^{B}	0.63	0.40
6	23.9^{B}	14.6^{B}	13.8^{BC}	15.2^{B}	0.58	1.04
4	33.0^{BA}	24.5^{B}	25.3^{B}	26.9^{B}	0.76	1.10
8	51.4^{A}	55.1^{A}	52.0^{A}	58.7^{A}	1.01	1.07

[a]Sorghum seed was treated with oxabetrinil at 1.25 g ai/kg seed.

[b]Total extractable ^{14}C-oxabetrinil.

[c]Water soluble portion of the ^{14}C-oxabetrinil contained in the total extract.

[d]Means within a column followed by the same letter are not significantly different as determined by Fishers' protected $LSD_{(0.05)}$.

increased with time for the 8 hr duration of this experiment in coleoptiles from both untreated and oxabetrinil-treated seedlings of grain sorghum (Table 3). The amount of radiolabel from ^{14}C-oxabetrinil extracted was greater in the untreated grain sroghum at 1, 2, and 4 hr, but not significantly different from that extracted from the oxabetrinil-treated grain sorghum at 8 hr (Table 3). The water soluble portion of the radiolabel from ^{14}C-oxabetrinil contained in the total extract also increased with time in the untreated and oxabetrinil-treated grain sorghum; thus, indicating that oxabetrinil is metabolized into a water soluble compound (Table 3).

TLC analysis of the total extract for oxabetrinil metabolites revealed that the majority of the extracted radiolabel from ^{14}C-oxabetrinil applied was still in the parent form 4 hr after application in the untreated grain sorghum and the oxabetrinil-treated grain sorghum (Table 4). In the methanol extract of coleoptiles derived from untreated grain sorghum, 11.8% of the extracted radiolabel from ^{14}C-oxabetrinil chromatographed as the GS-oxabetrinil conjugate, but no GS-oxabetrinil conjugate was detected in the extract from coleoptiles derived from oxabetrinil-treated grain sorghum (Table 4) even though 58.7% of the total extract was water soluble (Table 3).

Table 4. Separation of 80% methanol extract on TLC plates from 3-day old sorghum coleoptiles 4 hr after application of ^{14}C-oxabetrinil.

		untreated[a]	oxabetrinil[b]
Metabolite	Rf	------% of ^{14}C recovered[c]------	
Unknown #1	0.33	2.4 ± 0.8	25.6 ± 4.5
GS-oxabetrinil	0.53	11.8 ± 2.9	ND[d]
Unknown #2	0.69	ND	24.2 ± 2.9
Oxabetrinil	0.88	81.0 ± 3.3	52.6 ± 4.7

[a]Coleoptiles were derived from untreated grain sorghum seed.

[b]Sorghum seed was treated with oxabetrinil at 1.25 g ai/kg seed.

[c]Mean ± S.E.

[d]ND = Not detected.

An unidentified metabolite (Rf = 0.33) was detected in extracts from both untreated and oxabetrinil-treated grain sorghum, and the amount of this metabolite was 10 times higher in the extract from the oxabetrinil-treated than from the untreated grain sorghum (Table 4).

Similar to the studies with oxabetrinil, total extractable radiolabel following application of ^{14}C-CGA-133205 increased with time for the duration of the experiment with no differences between untreated and CGA-133205-treated grain sorghum at 1, 2, and 4 hr after application (Table 5). At 8 hr after ^{14}C-CGA-133205 application, the amount of total extractable radiolabel from ^{14}C-CGA-133205 was greater from the CGA-133205-treated grain sorghum than from the untreated grain sorghum (Table 5). The amount of the radiolabel from ^{14}C-CGA-133205 in the water soluble portion of the total extract does not appear to be different between treatments (Table 5).

TLC analysis of the total methanol extracts for radiolabel from ^{14}C-CGA-133205 revealed that the majority of the ^{14}C-CGA-133205 in the total extract from coleoptiles derived from untreated and CGA-133205-treated grain sorghum chromatographed as the parent compound (Rf = 0.90; Table 6). One other major metabolite (Rf = 0.68) was detected in the extract from untreated and

Table 5. CGA-133205 metabolism in coleoptiles of 3-day old untreated and CGA-133205-treated grain sorghum seedlings.

	untreated		CGA-133205[a]			
time (hr)	total extract(A)[b] % of applied[d]	water soluble(C)[c] % of extracted	total extract(B) % of applied	water soluble(D) % of extracted	ratio B/A	ratio D/C
1	14.4^{BA}	20.8^{B}	10.6^{B}	25.5^{B}	0.74	1.23
2	11.7^{A}	17.9^{B}	13.3^{B}	23.0^{B}	1.14	1.28
4	19.1^{BA}	16.2^{B}	18.5^{B}	23.8^{B}	0.97	1.47
8	22.3^{A}	41.7^{A}	39.5^{A}	50.1^{A}	1.77	1.20

[a]Sorghum seed was treated with CGA-133205 at 0.4 g ai/kg seed.

[b]Total extractable ^{14}C-CGA-133205.

[c]Water soluble portion of the ^{14}C-CGA-133205 contained in the total extract.

[d]Means within a column followed by the same letter are not significantly different as determined by Fishers' protected $LSD_{(0.05)}$.

Table 6. Separation of 80% methanol extract on TLC plates from 3-day old sorghum coleoptiles derived from untreated or CGA-133205-treated grain sorghum seeds 4 hr after application of ^{14}C-CGA-133205.

		untreated	CGA-133205[a]
Metabolite	Rf	--------% of ^{14}C recovered[b]-------	
Unknown #1	0.68	27.0 ± 2.8	54.5 ± 9.2
CGA-133205	0.93	63.9 ± 2.3	44.3 ± 11.2

[a]Sorghum seed was treated with CGA-133205 at 0.4 g ai/kg seed.

[b]Mean ± S.E.

CGA-133205-treated grain sorghum (Table 6). These results indicate that pretreatment of grain sorghum with CGA-133205 increased the rate of CGA-133205 metabolism. The resulting metabolite is currently unidentified.

Uptake and translocation of metolachlor. Uptake of radiolabel following ^{14}C-metolachlor application by grain sorghum coleoptiles significantly increased over time in both untreated and safener-treated seedlings (Table 7). At 4 hr after ^{14}C-metolachlor application, the amount of radiolabel uptake was greater in coleoptiles of oxabetrinil-treated grain sorghum seedlings compared to that taken up by coleoptiles of untreated or CGA-133205-treated grain sorghum (Table 7). An increase in metolachlor uptake resulting from pretreatment with oxabetrinil has been demonstrated by Zama and Hatzios [1986 & 1987] using either isolated leaf protoplasts or hydroponically grown seedlings of grain sorghum. Fuerst and Gronwald [1986] have also reported an enhancement of metolachlor uptake by excised leaves of grain sorghum following treatment with oxabetrinil.

At 2 and 4 hr after metolachlor application, the amount of radioactivity translocated to the roots and seeds of grain sorghum seedlings was enhanced by the safener CGA-133205. Oxabetrinil caused a slight increase in the amount of radioactivity translocated to roots and

Table 7. Metolachlor uptake by grain sorghum coleoptiles and translocation to roots and seeds as influenced by a seed-applied treatment of oxabetrinil and CGA-133205.

	untreated		oxabetrinil[a]		CGA-133205[b]					
time (hr)	coleoptile(A)	root and seed(B)	coleoptile(C)	root and seed(D)	coleoptile(E)	root and seed(F)	ratio (C/A)	ratio (D/B)	ratio (E/A)	ratio (F/B)
	% of applied[c]									
0.5	14.8^{BC}	1.9^{A}	14.4^{C}	3.0^{A}	13.6^{CB}	3.1^{BC}	0.97	1.58	0.92	1.63
1	29.2^{BA}	2.1^{A}	21.2^{BC}	2.2^{A}	24.2^{BC}	3.2^{BC}	0.72	1.05	0.83	1.52
2	27.8^{BA}	3.0^{A}	21.2^{B}	3.1^{A}	25.5^{BC}	6.2^{BA}	0.76	1.03	0.92	2.07
4	34.0^{A}	3.0^{A}	58.2^{A}	4.1^{A}	38.5^{AB}	8.5^{A}	1.71	1.37	1.12	2.83

[a]Sorghum seed was treated with oxabetrinil at 1.25 g ai/kg seed.

[b]Sorghum seed was treated with CGA-133205 at 0.4 g ai/kg seed.

[c]Means within a column followed by the same letter are not significantly different as determined by Fishers' protected $LSD_{(0.05)}$.

seeds of grain sorghum seedlings at 4 hr after metolachlor application.

Metolachlor metabolism in safened or unsafened sorghum. Total extractable ^{14}C-metolachlor from grain sorghum coleoptiles was decreased by treatment with the safener, CGA-133205, at 1 hr, and with either safener at 2 and 4 hr after metolachlor application (Table 8). At 8 hr after ^{14}C-metolachlor application, greater than 94% of the applied radioactivity was recovered from the coleoptiles of untreated and safener-treated grain sorghum (Table 8). The water soluble portion of radiolabel originating from ^{14}C-metolachlor contained in the total extract from oxabetrinil-and CGA-133205-treated plant material was higher than the water soluble portion of radiolabel originating from ^{14}C-metolachlor contained in the total extract from the untreated at 1, 2, and 4 hr after ^{14}C-metolachlor application. At 8 hr after ^{14}C-metolachlor application there were no significant differences in the amount of the water soluble portion of the total radiolabel from ^{14}C-metolachlor extracted from untreated or safener-treated coleoptiles of grain sorghum (Table 8). The water soluble portion of the total extract of ^{14}C-metolachlor contains the metolachlor-glutathione conjugate and probably other polar metabolites that may have been formed; thus, it appears that the safeners are

Table 8. Metolachlor metabolism in coleoptiles of 3-day old untreated, oxabetrinil-treated, and CGA-133205-treated grain sorghum seedlings.

	untreated		oxabetrinil[a]		CGA-133205[b]					
time (hr)	total extract(A)[c]	water soluble(B)[d]	total extract(C)	water soluble(D)	total extract(E)	water soluble(F)	ratio (C/A)	ratio (D/B)	ratio (E/A)	ratio (F/B)
	% of applied[e]	% of extracted	% of applied	% of extracted	% of applied	% of extracted				
1	18.0^{B}	7.2^{B}	19.3^{B}	12.4^{C}	11.8^{B}	29.4^{C}	1.07	1.72	0.66	4.08
2	30.8^{B}	14.9^{B}	20.5^{B}	41.5^{BA}	14.8^{B}	66.4^{BA}	0.67	2.79	0.48	4.46
4	41.8^{B}	15.1^{B}	24.4^{B}	36.5^{BA}	20.9^{B}	41.6^{B}	0.58	2.41	0.50	2.75
8	107.3^{A}	71.2^{A}	94.1^{A}	64.6^{A}	94.7^{A}	77.4^{A}	0.88	0.90	0.88	1.09

[a]Sorghum seed was treated with oxabetrinil at 1.25 g ai/kg seed.

[b]Sorghum seed was treated with CGA-133205 at 0.4 g ai/kg seed.

[c]Total extractable ^{14}C-metolachlor.

[d]Water soluble portion of the ^{14}C-metolachlor contained in the total extract.

[e]Means within a column followed by the same letter are not significantly different as determined by Fishers' protected $LSD_{(0.05)}$.

increasing the rate of metolachlor metabolism for the first 4 hours after metolachlor application. The metabolism of metolachlor increased significantly with time following the application of ^{14}C-metolachlor to coleoptiles of both untreated or safener-treated seedlings of grain sorghum (Table 8).

TLC analysis of the total methanol extract revealed that 46% to 49% of the radiolabel from ^{14}C-metolachlor in the total extract from the coleoptiles of oxabetrinil-and CGA-133205-treated material chromatographed as the glutathione conjugate (Rf = 0.5) while only 22.5% of the ^{14}C-metolachlor in the total extract from the untreated grain sorghum chromatographed as the glutathione conjugate (Table 9). The amount of ^{14}C-metolachlor in the total extract that chromatographed as the parent metolachlor (Rf = 0.89) decreased in the extracts from oxabetrinil- and CGA-133205-treated grain sorghum when compared to the amount in the extract from the untreated grain sorghum (Table 9). Two additional unidentified metabolites of ^{14}C-metolachlor were present in the extracts from the untreated and oxabetrinil-treated grain sorghum, but not in the CGA-133205-treated grain sorghum (Table 5).

These results indicate that pretreatment of grain sorghum with either safener increases the amount of metolachlor that is metabolized by conjugation to

Table 9. Separation of 80% methanol extract of 3-day old sorghum coleoptiles 4 hr after application of ^{14}C-metolachlor.

Metabolite	Rf	untreated[a]	oxabetrinil[b]	CGA-133205[c]
		% of ^{14}C extracted[d]		
GS-metolachlor	0.5	22.5 ± 9.9	45.9 ± 7.4	49.0 ± 11.9
Unknown #1	0.56	ND[d]	14.5 ± 7.5	ND
Unknown #2	0.75	21.6 ± 8.1	ND	ND
Metolachlor	0.89	54.1 ± 9.3	40.4 ± 10.1	42.5 ± 10.1

[a]Coleoptiles were derived from untreated seed.

[b]Coleoptiles were derived from sorghum seed was treated with oxabetrinil at 1.25 g ai/kg seed.

[c]Coleoptiles were derived from sorghum seed was treated with CGA-133205 at 0.4 g ai/kg seed.

[d]Mean ± S.E.

[e]ND = Not detected.

glutathione. Gronwald and Fuerst [1987] recently reported that a seed-treatment of grain sorghum with oxabetrinil or flurazole increased the rate of formation of the metolachlor-glutathione conjugate and decreased the amount of unmetabolized parent metolachlor during the first 100 min after application of ^{14}C-metolachlor to etiolated grain sorghum shoots. Breaux [1986] reported that treatment with the safener, flurazole, increased the rate of acetochlor detoxication in corn and sorghum. Further analysis of the acetochlor detoxication revealed that flurazole increased the rate of acetochlor conjugation with glutathione and cysteine [Breaux, 1988]. At the present time it is unclear as to whether the cysteine conjugate of acetochlor results from the catabolism of the glutathione-acetochlor conjugate or whether acetochlor conjugates directly with cysteine [Breaux, 1988].

In summary, uptake of both safeners and metolachlor by grain sorghum increases in a time-dependent manner. Uptake of oxabetrinil is almost 2 times greater than CGA-133205 uptake and little redistribution of either safener occurs. Treatment of grain sorghum with oxabetrinil increased metolachlor uptake, but treatment with CGA-133205 did not. Treatment of grain sorghum with oxabetrinil and CGA-133205 increased the metablolism of metolachlor to a water soluble metabolite by 26.6 and

51.5%, respectively, compared to untreated grain sorghum at 2 hr after metolachlor application, but by 8 hr after application, no difference in metolachlor conjugation to GSH between safener-treated and untreated grain sorghum was evident (Table 8). Metabolism of the safeners was also time-dependent.

The rate of oxabetrinil metabolism was greater than the rate of CGA-133205 metabolism in coleoptiles of grain sorghum seedlings. Identification of the metabolites by TLC indicate that oxabetrinil and CGA-133205 increase the rate of metolachlor conjugation with glutathione. TLC analysis of the oxabetrinil and CGA-133205 metabolites in the total extract indicate that some metabolism of these safeners does occur, but currently these metabolites are unidentified. It has been speculated that oxabetrinil and CGA-133205 are reactive enough to conjugate with glutathione [Breaux, 1988]. The results of the present study show that this is particularly true for oxabetrinil which appeared to conjugate with GSH in coleoptiles of grain sorghum seedlings. Nevertheless, further research with additional analytical techniques (eg. mass spectrometry) is needed to conclusively identify the GS-conjugates of oxime ether safeners or of any metabolites of these conjugates.

Oxabetrinil and CGA-133205 confer protection to grain sorghum by increasing the rate of metolachlor metabolism by conjugation to glutathione. It is possible that the safeners and metolachlor are metabolized by the same mechanism and that pretreatment of grain sorghum with these safeners increases the activity of the associated metabolic pathway.

REFERENCES

Breaux, E. J. (1986). Identification of the initial metabolites of acetochlor in corn and soybean seedlings. J. Agric. Food Chem. **34**, 884-888.

Breaux, E. J., M. A. Hoobler, J. E. Patanella, and G. A. Leyes. (1988). Mechanisms of action of thiazole safeners. in "Crop Safeners for Herbicides: Development, Uses, and Mechanisms of Action" (K. K. Hatzios and R. E. Hoagland, Eds.), Academic Press., New York, 163-175.

Fuerst, E. P. and J. W. Gronwald. (1986). Induction of rapid metabolism of metolachlor in sorghum (Sorghum bicolor) shoots by CGA-92194 and other antidotes. Weed Sci. **34**, 354-361.

Gronwald,, J. W., E. P. Fuerst, C. V. Eberlein, and M. A. Egli. (1987). Effect of herbicide antidotes on glutathione content and glutathione S-transferase activity of sorghum shoots. Pestic. Biochem. Physiol. **29**, 66-76.

Gronwald, J. W. (1988). Influence of herbicide safeners on herbicide metabolism. in "Crop Safeners for Herbicides: Development, Uses, and Mechanisms of Action" (K. K. Hatzios and R. E. Hoagland, Eds.), Academic Press., New York, 163-175.

LeBaron, H. M., J. E. McFarland, and G. J Simoneaux. (1988). Metolachlor. in 'Herbicides: Chemistry,

Degradation, and Mode of Action. (P. C. Kearney and D. D. Kaufman, Eds.), Marcel Dekker, Inc., New York, 335-381.

K. K. Hatzios, (1986). Effects of CGA-92194 on the chemical reactivity of metolachlor with glutathione and metabolism of metolachlor in grain sorghum (Sorghum bicolor). Weed Sci. **34**, 834-841.

Zama, P. and K. K. Hatzios. (1987). Interactions between the herbicide metolahclor and the safener CGA-92194 at the levels of uptake and macromolecular synthesis in sorghum leaf protoplasts. Pestic. Biochem. Physiol. **27**, 86-96.

VI. SUMMARY AND CONCLUSIONS

The overall objectives of the experiments reported in this dissertation were to elucidate the mechanism of action of the oxime ether safeners for metolachlor and to determine the fate of these safeners in grain sorghum. In order to gain an insight to the mechanism of action of the oxime ether safeners, molecular comparisons of the safener and the respective herbicide were conducted using computer-assisted molecular modeling (CAMM). If the safener and herbicide molecules share similar structural features, then it is likely that the safener and the herbicide would be similar enough to influence the same metabolic pathway(s) in plants. Molecular comparison of selected herbicide/safener combinations using CAMM revealed that even when the safener and the herbicide did not have similar chemical structures, they were similar on the basis of three-dimensional molecular arrangement and charge distribution features. The results of these molecular comparisons of safeners and herbicides support the previously proposed hypotheses that the safener and the herbicide may compete for the same target site or serve as substrates for the same inducible xenobiotic degrading enzymes [Hatzios, 1988].

Several reports in the literature [Fuerst, 1987; LeBaron et al., 1988] suggest that the chloroacetanilide herbicides such as metolachlor influence acetyl-CoA metabolism in plants. If, in fact, this pathway is influenced by metolachlor, and if in fact, the oxime ether safeners and metolachlor are competing for the same target site, then treatment of grain sorghum with the oxime ether safeners should have some influence on this metabolic pathway. Incorporation of ^{14}C-acetate into lipids of grain sorghum seedlings, as influenced by safeners applied alone and in combination with metolachlor, was studied to determine if the oxime ether safeners and metolachlor are influencing acetyl-CoA metabolism. Results from these studies revealed that oxabetrinil stimulated incorporation of ^{14}C-acetate, and the amount of incorporation increased when oxabetrinil was applied with metolachlor. CGA-133205 did not have as large of an influence when applied alone or in combination with metolachlor; however, some stimulation of ^{14}C-acetate incorporation was observed. These results indicate that metolachlor and the oxime ether safeners can influence acetyl-CoA metabolism causing a reallocation of carbon, but this influence was not strong enough to explain the exact mechanism of action of either

the safeners or metolachlor and their interactions in safened plants.

Another possible mechanism of action of the oxime ether safeners is that they influence the detoxication of metolachlor [LeBaron et al., 1988; Hatzios, 1988; Gronwald, 1988]. Given the similarities of the safener and herbicide molecules it is likely that these compounds could serve as substrates for the same constitutive and/or inducible herbicide degrading enzymes. Metolachlor is detoxified via conjugation to reduced glutathione and this reaction is catalyzed by glutathione-S-transferase(s) [Gronwald, 1988; Breaux, 1988]. Another likely possibility is that safeners may induce the synthesis of other substrates involved in the detoxication of metolachlor via conjugation to GSH. Levels of glutathione and the activity of glutathione-S-transferase and glutathione reductase were evaluated in germinating seeds and seedlings of grain sorghum to determine if the oxime ether safeners are stimulate glutathione production and/or activity of glutathione-related enzymes. Results from these studies indicate that oxabetrinil and CGA-133205 enhanced the production of glutathione with oxabetrinil being more stimulatory than CGA-133205. Treatment with oxime ether safeners resulted in only slight increases in glutathione

reductase activity and glutathione-S-transferase activity. In vitro nonenzymatic conjugation of metolachlor with GSH was enhanced by both safeners. Overall, these results indicate that the safeners enhance the rate of metolachlor detoxication during late germination or early seedling development. In older seedlings the enhancement was not as apparent. Oxabetrinil was also shown to conjugate with reduced glutathione, but at a much lower rate than the conjugation of metolachlor with glutathione.

Information concerning the uptake, translocation, and metabolism of the oxime ether safeners is also needed in order to better understand how the oxime ether safeners influence metolachlor detoxication. Results from experiments conducted to evaluate the uptake of the oxime ether safeners and their influence on the uptake of metolachlor revealed that the safeners are taken up in a time-dependent manner. Uptake of oxabetrinil is more rapid than uptake of CGA-133205. Oxabetrinil stimulated the uptake of metolachlor while CGA-133205 did not. Translocation of the safeners and metolachlor was mostly acropetal, but in the CGA-133205 treated grain sorghum, significantly more metolachlor was translocated to the roots than in the untreated or oxabetrinil treated grain sorghum. Metolachlor was metabolized rapidly in

coleoptiles of grain sorghum. The major detected metabolite of metolachlor was identified as the glutathione-metolachlor conjugate. Application of safeners enhanced the amount of metolachlor that was metabolized into the glutathione-conjugate and into some unidentified water soluble metabolites. Metabolism of oxabetrinil was also evident in the coleoptiles of grain sorghum and the major metabolite of oxabetrinil was identified as the glutathione-oxabetrinil conjugate. However, an unidentified metabolite was also detected. Some metabolism of CGA-133205 did occur in grain sorghum coleoptiles, but the resulting metabolite was unidentified.

Overall, the results of these experiments indicate that the safeners and metolachlor indeed share enough molecular similarities to influence the same metabolic pathways in grain sorghum. These results also support both hypotheses: a) that the safeners and metolachlor are competitive antagonists at some common target site, and b) that both the safeners and metolachlor are serving as inducible substrates for herbicide degrading enzymes. A combination of these two mechanisms may be involved in the actual protection of grain sorghum by oxime ether safeners against the injury caused by metolachlor under field conditions. Currently, safeners are used only with

large seeded monocots. Results from these experiments indicate that the reason for this is the ability of monocots to detoxify herbicides via enzymatic or nonenzymatic conjugation with metolachlor. Application of the safeners does not induce a novel detoxication pathway, but enhances the rate of a constitutive detoxication pathway.

REFERENCES

Breaux, E. J., M. A. Hoobler, J. E. Patanella, and G. A. Leyes. (1988). Mechanisms of action of thiazole safeners. in "Crop Safeners for Herbicides: Development, Uses, and Mechanisms of Action" (K. K. Hatzios and R. E. Hoagland, Eds.), Academic Press., New York, 163-175.

Fuerst, E. P. (1987). Understanding the mode of action of chloroacetanilide and thiocarbamate herbicides. Weed Technol. 1, 270-277.

Gronwald, J. W. (1988). Influence of herbicide safeners on herbicide metabolism. in "Crop Safeners for Herbicides: Development, Uses, and Mechanisms of Action" (K. K. Hatzios and R. E. Hoagland, Eds.), Academic Press., New York, 103-128.

Hatzios, K. K. (1988). Mechanisms of Action of Herbicide Safeners: An Overview. in "Crop Safeners for Herbicides: Development, Uses, and Mechanisms of Action" (K. K. Hatzios and R. E. Hoagland, Eds.), Academic Press., New York, 65-102.

LeBaron, H. M., J. E. McFarland, and G. J Simoneaux. (1988). Metolachlor. in "Herbicides: Chemistry, Degradation, and Mode of Action" (P. C. Kearney and D. D. Kaufman, Eds.), Marcel Dekker, Inc., New York, 335-381.

The vita has been removed from the scanned document