SESSION 3B

MODE OF ACTION AND METABOLISM OF HERBICIDES: I

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HERBICIDES AFFECTING PLANT PIGMENTS

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ABSTRACT

Herbicides which initiate peroxidation or inhibit carotenoid biosynthesis cause bleaching of carotenoids and chlorophylls. The peroxidative reactions are light-dependent and proceed via a radical-chain mechanism resulting in the destruction of several cell components. Indicators to measure peroxidation are ethane evolution, malondialaldehyde formation, and degradation of the plastidic sulfolipid. Radical formation can be demonstrated by ESR spectroscopy after application of oxyfluorfen to isolated chloroplasts. Herbicides may interfere with different reactions of the carotenogenic pathway. In an *in vitro* system employing thylakoids from the blue-green alga *Aphanocapsa* or chloroplasts from *Chenopodium* it could be demonstrated that three enzymes, phytoene desaturase, ζ-carotene desaturase and lycopene cyclase are directly affected by difunon, J 852 and CPTA, respectively. The chlorophyll bleaching observed with these and similar compounds is a secondary photooxidation due to decreased levels of carotenoids.

Many herbicides may directly affect the leaf pigments. Although their modes of action may be different, they have in common a decrease in the levels of carotenoids and chlorophylls when applied in sub-lethal concentrations (Sandmann and Böger, 1982). The observed pigment deficiency is caused either by initiation of pigment degradation or by inhibition of carotenoid biosynthesis. Although chlorophyll biosynthesis can be influenced by potent inhibitors (comp. Böger, 1988) no herbicides exerting their direct mode of action on enzymes involved in chlorophyll formation are known. When carotenogenesis is inhibited, low endogenous concentrations of protective carotenoids result in a secondary photodestruction of chlorophylls (Bartels and Hyde, 1970). This

differential light effect on carotenoids and chlorophylls after addition of inhibitors of carotene biosynthesis can be reliably demonstrated with heterotrophic cultures of green algae. Scenedesmus can be grown with glucose as a carbon source in complete darkness with a fully developed photosynthetic apparatus. Then a compound interfering with an enzyme of the carotenogenic pathway will affect the endogenous concentration of carotenoids only, chlorophylls are decreased much less than carotenoids (Sandmann et al. 1984).

In higher plants, light is required for activation of p-nitro and p-chloro-diphenyl ethers. However, the nature of the light requirement and the active form of the herbicide which initiates peroxidative reactions has still to be elucidated. A controversy exists about the role of photosynthetic electron transport in diphenyl-ether activation. Some authors (Ensminger and Hess, 1985 a; Ensminger et al. 1985) did not find a protection by inhibitors of photosynthetic electron transport.

Working with the green alga Scenedesmus we found in our laboratory that diuron is a potent inhibitor of peroxidative ethane evolution (Kunert and Böger, 1984) and prevents damage to pigments when both oxyfluorfen and diuron are added together to algal cells at the beginning of the experiment (Figure 1, left). This result has also been confirmed with isolated spinach chloroplasts (Lambert and Böger, 1981). Moreover, using electron-spin resonance a trapped signal is measured with isolated spinach thylakoids which shows radical formation in the presence of oxyfluorfen (Figure 1, right). Again, diuron eliminated the oxyfluorfen-dependent ESR signal, possibly derived from a polyunsaturated fatty acid-free radical (Lambert et al. 1984). Action spectra for light requirement of diphenyl ethers show maxima around 450 and 650 nm (Ensminger and Hess, 1985 b), pointing at chlorophyll as the pigment involved.

Except for nitro-, chloro-diphenyl ethers and paraquat, the lutidine derivative LS 82-556 (Matringe et al., 1986), and the tetrahydrophthalimide S 23142 (Sato et al., 1986) are chemicals which also initiate radicalic peroxidation reactions in plants (unpublished results).

In our laboratory we have applied the noninvasive measurement of hydrocarbon gas products of lipid hydroperoxide decomposition (Kunert and Böger, 1981) as well as degradation of ^{35}S-prelabeled plastidic sulfolipid (Sandmann and Böger, 1983) and the formation of thiobarbituric

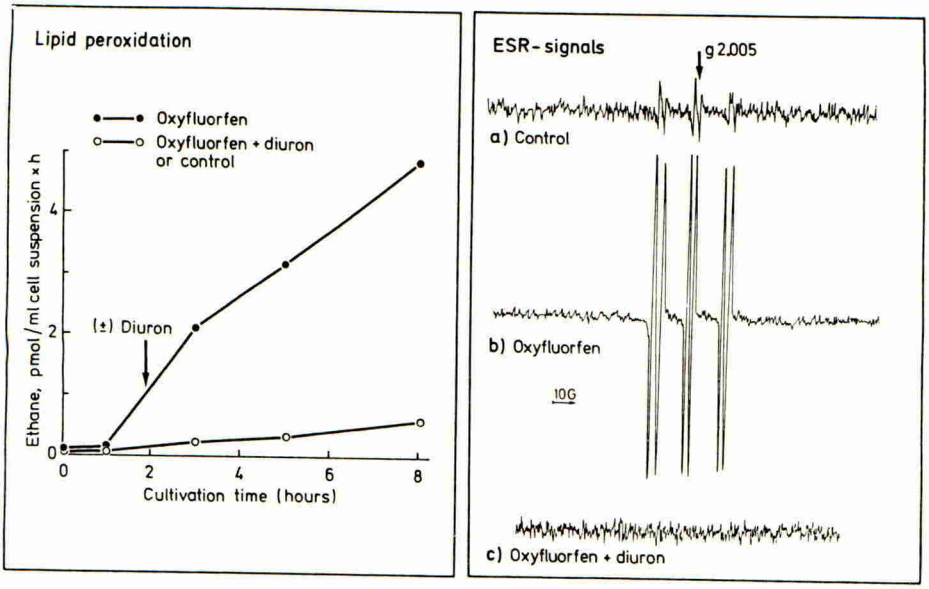

Figure 1: Peroxidative ethane evolution and radical formation in the presence of oxyfluorfen

Left part: Lipid peroxidation in the light measured as ethane production using Scenedesmus acutus. Diuron abolishes peroxidation when added together with oxyfluorfen. When added later (after 2 h, see arrow) no protection is observed anymore. This is believed as being due to the inefficiency of the endogenous antioxidative system (glutathione, ascorbate) having been impaired by the foregoing 2-h impact of oxyfluorfen. When the antioxidative system is unable to quench herbicide-generated radicals, a rather low radical concentration, possibly generated non-photosynthetically, should be effective to ensure peroxidation. Right part: Using isolated spinach chloroplasts, ESR signals are induced by oxyfluorfen in the light and suppressed by diuron.

acid reactive material which is predominantly malondialaldehyde (Kunert and Ederer, 1985). All diese compounds are peroxidation products of polyunsaturated fatty acids. As α-linolenic acid is the most abundant one in plant membranes, ethane is the major hydrocarbon formed (Sandmann and Böger, 1982).

Table 1 shows the behaviour of all three indicators of lipid peroxidation in the presence of oxyfluorfen. The application of 1 μM of this nitro-diphenyl ether over 48 h results in a significant accumulation of ethane, the degradation about 90% of the sulfolipid and a 3-fold increase of the malondialdehyde level over the control value.

The mechanism of peroxidation of fatty acids is initiated by formation of starter radicals. As already pointed out, the nature of this reaction sequence which comprises the so-called light-activation of diphenyl ethers is unknown. The subsequent mechanism is as follows (Sandmann and Böger, 1986): The starter radical can abstract a hydrogen from a polyunsaturated fatty acid (PUFA). After conjugation of two double bonds, this leads to the PUFA radical which reacts with molecular oxygen giving a peroxy radical. This in turn reacts with another PUFA molecule thereby starting a chain reaction. The resulting alkyl peroxide is reduced to an alkoxy radical through a Fenton-type reaction mediated by metal ions (e.g. Fe^{2+}). By β-scission this radical splits into the corresponding aldehyde and a short chain hydrocarbon radical which again reacts with Fe^{2+} to give a fully saturated hydrocarbon. The chain length of the evolving hydrocarbon is determined by the position of the double bonds of the degraded polyunsaturated fatty acid. The number of C-atoms is the same as that found beyond the double bond most distant from the carboxyl group (ω-1 rule). The most prominent PUFA in plants involved in this radicalic peroxidation reaction is α-linolenic acid and the resulting hydrocarbon is ethane. Malondialdehyde originates from the fatty acid aldehyde mentioned above when a trienoic or higher unsaturated fatty acid gets peroxidized.

Lipophilic antioxidants such as vitamin E and the non-biological compound ethoxyquin are strong protectors against peroxidative diphenyl ethers (Lambert et al., 1983; Kunert and Böger, 1984). Recently, this laboratory has reported on a highly significant correlation between in vivo lipid peroxidation, measured as ethane production, and the content of the hydrophilic vitamin C in mustard seedlings after treatment with p-nitro-diphenyl ethers (Kunert, 1984). Nevertheless, vitamin E (α-tocopherol) appears to be the most effective compound to interrupt the radical-chain reactions in biological membranes. Most likely, the antioxidative action of vitamin E is determined by a synergism with the water-soluble antioxidant vitamin C.

The colorless phytoene is the first carotene in the carotenoid pathway in plants. It is subject to a series of desaturation reactions followed by two cyclization steps. The first enzyme involved in phytoene conversion is phytoene desaturase. The final product of this enzyme is ζ-carotene which is also the substrate of ζ-carotene desaturase. The

TABLE 1. Indicators of peroxidation initiated by 1 μM of oxyfluorfen in Scenedesmus cells treated for 48 h in the light.

Parameters measured	Control	Oxyfluorfen
Ethane evolution (nmol/ml pcv)	0.3	6.1
MDA formation (nmol/ml pcv)	8.7	23.2
Degradation of sulfolipid, ^{35}S-labeled (10^3 dpm/l)	62.4	7.9

pcv, packed cell volume

carotene resulting from this reaction is lycopene which is cyclized to α - and β-carotene. Phytoene desaturase and ζ-carotene desaturase are the targets of many commercial experimental herbicides. Norflurazon(e), difunon, fluridone, flurochloridone and diflufenican are examples for phytoene desaturase inhibitors, J 852 and dihydropyrones inhibit ζ-carotene desaturase (Sandmann and Böger, 1988). Amitrole is a herbicide which accumulates ζ-carotene in intact cells. However, in vitro studies have shown that ζ-carotene desaturase is not directly inhibited by this compound (Kostal, 1986).

The differential action of amitrole on ζ-carotene desaturation *in vivo* and *in vitro* demonstrates the necessity of a cell-free system to pin-point the target enzyme. Furthermore, *in-vitro* studies are essential to investigate the molecular mechanism of these herbicides and to directly probe the inhibition site by correlation of the structure of chemically modified inhibitors with their inhibitory activity. It is difficult to establish an *in-vitro* system. There is the problem of how to provide the ^{14}C-labeled substrate (e.g. phytoene); the isolation of the thylakoid membranes has to be done extremely careful in order not to inactivate the membrane-bound enzymes. The most promising *in-vitro* system of an organism with oxygenic photosynthesis is the one from Aphanocapsa (Synechocystis) (Clarke *et al.*, 1982). Recently, cell-free preparations from Chenopodium

album were also used successfully in our laboratory.

Table 2 shows the formation of carotene intermediates in the in-vitro conversion chain of phytoene to β-carotene with a membrane preparation from the blue-green alga Aphanocapsa and Chenopodium. For experimental details see Sandmann (1987). Phytoene, ζ-carotene and lycopene are the substrates of the two desaturases and the cyclase, respectively. Radioactivity is accumulated exceeding the control values in the carotene pools when appropriate inhibitors are added. Difunon increases the residual radioactivity in phytoene because the reacting phytoene desaturase is inhibited. Corresponding observations are made for J 852, an inhibitor of ζ-carotene desaturase, and CPTA, an inhibitor of lycopene cyclase. The underlining indicates a significant increase. It can also be seen from Table 2 that the Aphanocapsa membranes exhibit a much higher carotenogenic activity than Chenopodium. In general, we use Chenopodium to demonstrate that the enzymes of the carotenogenic pathway expected to be inhibited by a herbicide is equally affected in Chenopodium as in Aphanocapsa. Then we continue our cell-free assays with the more efficient Aphanocapsa system.

TABLE 2. In vitro conversion of ^{14}C-phytoene into subsequent carotenes in the presence of different inhibitors of carotenogenic enzymes.

	Incorporation of radioactivity (dpm) into			
	Phytoene	ζ-Carotene	Lycopene	β-Carotene
A. Aphanocapsa				
Control	3924	1013	816	1070
Difunon, 1 µM	7354	397	227	377
J 852, 50 µM	4035	2180	399	561
CPTA, 50 µM	3649	941	1432	411
B. Chenopodium				
Control	4125	712	316	355
Difunon, 1 µM	5331	276	147	123
J 852, 50 µM	4291	1688	171	231
CPTA, 50 µM	3846	744	622	200

^{14}C-phytoene generated from 0.5 µCi D,L-^{14}C-mevalonic acid, memberanes equivalent to 120 µg of chlorophyll; incubation time was 2 h at room temperature.

Although this in-vitro system consists of a reaction chain which involves three subsequent enzymes, it is possible to perform enzyme kinetics for each of the enzymes involved. All herbicidal inhibitors of phytoene- and ζ-carotene desaturase are of a non-competitive type. For a dihydropyrone which inhibits ζ-carotene desaturase we have extended our kinetic studies to distinguish between a reversible non-competitive inhibitor or an irreversible inhibitor. The kinetics indicate that the compound mentioned is irreversibly bound to the enzyme (unpublished).

Several herbicides are multifunctional. Fomesafen is an example for a compound that initiates peroxidation and also inhibits carotenoid biosynthesis at the level of phytoene (Sandmann and Böger, 1986). With an in-vitro system for carotenogenesis it is possible to estimate the contributions of inhibition of carotene biosynthesis in addition to peroxidation by comparing the I_{50} values for both inhibitory activities. The I_{50}-value of 20 µM for peroxidation was determined in intact Scenedesmus cells by degradation of the chloroplast-specific sulfolipid (Sandmann et al., 1984). For in-vitro inhibition of the phytoene desaturase reaction an I_{50}-value of 80 µM was determined (Table 3). These results demonstrate that the major contribution to the herbicidal effect of fomesafen is induction of peroxidative processes and that inhibition of carotenogenesis is also substantial.

TABLE 3. Multifunctional modes of action of fomesafen. I_{50}-values for its different modes of action.

Fomesafen	Inhibition of carotenogenesis (in vitro)	Initiation of peroxidation (intact cells)
	80 µM	20 µM

Cell-free carotene biosynthesis was performed with Aphanocapsa thylakoids, peroxidation with intact cells of Scenedesmus acutus.

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HERBICIDES THAT INHIBIT AMINO ACID BIOSYNTHESIS: THE SULFONYLUREAS - A CASE STUDY

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ABSTRACT

Several structurally-unrelated herbicides act by blocking amino acid biosynthesis. Because amino acid metabolism is similar in plants and well-studied microbes, the microbial systems have been exploited for the determination of the molecular targets of the herbicides and the isolation and manipulation of genes that confer herbicide resistance. In the case of the sulfonylurea herbicides, the molecular target, acetolactate synthase (ALS), an enzyme which catalyzes the first common step in the biosynthesis of isoleucine, leucine and valine, was discovered using bacteria. Resistance to the herbicides can be brought about in bacteria, yeast or plants by overexpression of ALS or by mutations which result in herbicide-insensitive forms of the enzyme. Structural homology evident in microbial ALS genes lead to the use of the yeast ALS gene as a heterologous hybridization probe for isolation of plant ALS genes. Mutant plant genes, encoding herbicide insensitive ALS, have been used to transform plants to herbicide resistance.

INTRODUCTION

The use of crop protection chemicals to reduce loss due to weeds, insects and disease has become an integral part of modern agriculture. About one half of the annual $15 billion expenditure for pesticides is used to purchase herbicides. These products have traditionally been found by random screening of newly synthesized compounds to find a lead. Improvements are then sought by synthesizing analogs of the lead compound. To be developed as a commercial herbicide a compound must not only control weeds, but also show low toxicity to other organisms, in particular animals. As a result, herbicides are often compounds that interfere with metabolic processes unique to plants.

The most obvious metabolic distinction between the plant and animal kingdoms is that plants obtain energy via photosynthesis and, therefore, it is not surprising that many herbicides interfere with this process. Many other metabolic differences between plants and animals exist, including synthesis of plant-specific hormones, and the plant cell wall. Plants and animals also differ in their ability to synthesize amino acids and vitamins. While plants are able to synthesize all these compounds, animals must obtain vitamins and the so-called essential amino acids from their diet. Several herbicides, have been shown to act by inhibiting amino acid biosynthetic enzymes (Table 1). Because amino acid biosynthesis is similar in plants and such well studied microbes as the bacteria Escherichia coli and Salmonella typhimurium and the baker's yeast Saccharomyces cerevisiae, these organisms can be used to facilitate research and development work on such herbicides. We have taken advantage of this in our studies of the sulfonylurea herbicides.

TABLE 1

Herbicides which inhibit amino and biosynthesis

Herbicide	Active Ingredient	Inhibited Pathway	Target Enzyme	Apparent K_i (nM)	Enzyme Source
Roundup®	Glyphosate	Common aromatic	5-enolpyruvyl-shikimate-3-	10 000	bacteria
Oust®	Sulfometuron methyl	Branched-chain	acetolactate synthase	650 120 14	bacteria yeast pea
Glean®	Chlorsulfuron	Branched-chain	acetolactate synthase	7 21	tobacco pea
Arsenal®	AC 243,997	Branched-chain	acetolactate synthase	12 000	maize
Amitrole®	Aminotriazole	Histidine	imidazole glycerol phosphate dehydratase	30 000	bacteria
Basta®	Phosphinothricin	Glutamine	glutamine synthase	73 000	pea

These herbicides are notable for their high potency (application rates of a few grams per hectare) and low mammalian toxicity. The potency of the sulfonylureas is reflected in their lower inhibition constants compared to those reported for the other herbicides listed in Table 1. Here, we describe our past studies on the mechanism of action of the sulfonylurea herbicides, present status on selective herbicide toxicity through genetic modification of crops and future prospects. Similar strategies, achievements, and goals have characterized the research on other herbicides that act on amino acid biosynthetic enzymes.

MOLECULAR TARGET

The site of action of the sulfonylurea herbicides has recently been elucidated. Physiological studies in *S. typhimurium* suggested that the target of sulfometuron methyl (SM) was the enzyme acetolactate synthase (ALS) which is required for the synthesis of isoleucine, leucine and valine (LaRossa & Schloss 1984). Multiple ALS isozymes exist in enterobacteria and it has been shown that ALS II and ALS III, but not ALS I, are inhibited by sulfometuron methyl (LaRossa & Schloss 1984. LaRossa & Smulski 1984) *In vitro* analyses of ALS activity from yeast, pea and tobacco demonstrated that the eukaryotic enzymes are also very sensitive to SM (Falco & Dumas 1985. Ray 1984. Chaleff & Mauvais 1984). In addition, the presence of multiple copies of the wild type *ILV2* gene in yeast results in a six-fold increase in ALS activity and a

Fig. 1. Deduced amino acid sequences of wild type and SM-resistant yeast ALS and E. coli ALS isozyme II. The | indicates conserved amino acid residues. The arrows indicate amino acid substitutions that result in SM-resistant ALS.

five-fold increase in the SM minimal inhibitory concentration (Falco & Dumas 1985). SM resistant mutants of S. typhimurium, yeast and tobacco have been isolated. Most of the mutants from each organism produce ALS activity that is insensitive to the herbicide and the resistant enzyme activity cosegregates with cellular resistance in genetic crosses. The bacterial and yeast mutations map to the loci of the ALS structural genes ilvG and ILV2, respectively (LaRossa & Schloss 1984. Falco & Dumas 1985. Chaleff & Mauvais 1984. Chaleff & Ray 1984). Finally, the DNA sequence of a bacterial mutant gene and a yeast mutant gene revealed single nucleotide differences from their respective wild type genes. The mutations result in single amino acid substitutions in the structurally homologous amino-terminal regions of the two proteins, but at different positions (Figure 1). (Falco et al. 1985. Yadav et al. 1986).

Interestingly, ALS is also the target of a structurally unrelated class of herbicides, the imidazolinones (Shaner et al. 1984) (Table 1). Thus, ALS appears to be a particularly efficacious target for herbicides. Experiments of LaRossa et al. (1987) indicated that the activity of sulfometuron methyl in bacteria is enhanced by the accumulation of an ALS substrate, α-keto- butyrate, which is toxic. LaRossa et al. have suggested that it is both the deficiency of branched chain amino acids, and the increase in concentration of the toxic intermediate, that combine to make ALS a particularly good target for herbicides.

SELECTIVE HERBICIDE TOXICITY

For most agricultural applications a herbicide must act selectively, blocking growth of weeds without damaging the crop plant. Chlorsulfuron is an example of a selective sulfonylurea herbicide. Cereal crops show considerable tolerance to chlorsulfuron due to their ability to metabolize the herbicide (Sweetser et al. 1982). The discovery of such selective chemicals has often required the synthesis of thousands of compounds. An alternative approach, which has now been well documented, has relied upon genetic modification of crops to achieve selectivity.

In the case of the sulfonylureas, herbicide resistant tobacco was first obtained by selection of mutant cells in tissue culture and subsequent regeneration of mutant plants from the cell lines (Chaleff & Ray 1984). Resistance resulted from production of a herbicide-insensitive form of the target enzyme (ALS), rather than from detoxification (Chaleff & Mauvais 1984). It has been possible to isolate analogous resistant mutants of other plant species, but considerable effort and time is involved. The introduction of herbicide resistance by genetic transformation of crops using isolated genes is a methodology with tremendous potential, which is beginning to be realized.

In our work on the microbial ALS genes we observed DNA hybridization of the yeast and Salmonella genes. In addition, the predicted amino acid sequences of the ALS enzymes, derived from the nucleotide sequences of the yeast and bacterial genes, indicated an unanticipated level of conservation between the prokaryotic and eukaryotic enzymes, (Falco et al. 1985) and lead to an attempt to detect homology among ALS genes from other species. A segment of the yeast ALS gene that spanned most of the coding region was used as a probe, and hybridizations were carried out under low stringency conditions. Homology was detected between the yeast ALS gene and DNA from the prokaryotic cyanobacterium Anabaena 7120, and the higher plants Arabidopsis thaliana and Nicotiana tabacum. Phage carrying putative ALS genes were isolated from all three species (Mazur et al., 1985).

In order to confirm that these isolated clones carried ALS genes, the clones were mapped and partially sequenced (Mazur et al., 1985). Comparison of the partial deduced amino acid sequences of the putative cyanobacterial and plant ALS genes with those of the yeast and three Escherichia coli ALS genes confirmed the identity of the clones. All three putative ALS clones shared conserved sequences with the yeast and bacterial enzymes, which had been shown in previous studies to share three domains of homology interspersed with four domains of non-homology (Falco et al., 1985).

Subsequent complete sequence analysis of the two plant ALS genes has indicated that they code for proteins of 667 and 670 amino acids. Neither gene has introns. The two plant ALS sequences are conserved relative to each other throughout most of the length of the genes, including those domains which are not conserved even among the three E. coli isozymes. Approximately 75 percent of the nucleotides and 85 percent of the encoded amino acids are conserved between the two genes (Mazur et al., 1987). A comparison of the deduced amino acid sequences of the tobacco and Arabidopsis ALS genes is shown in Figure 2.

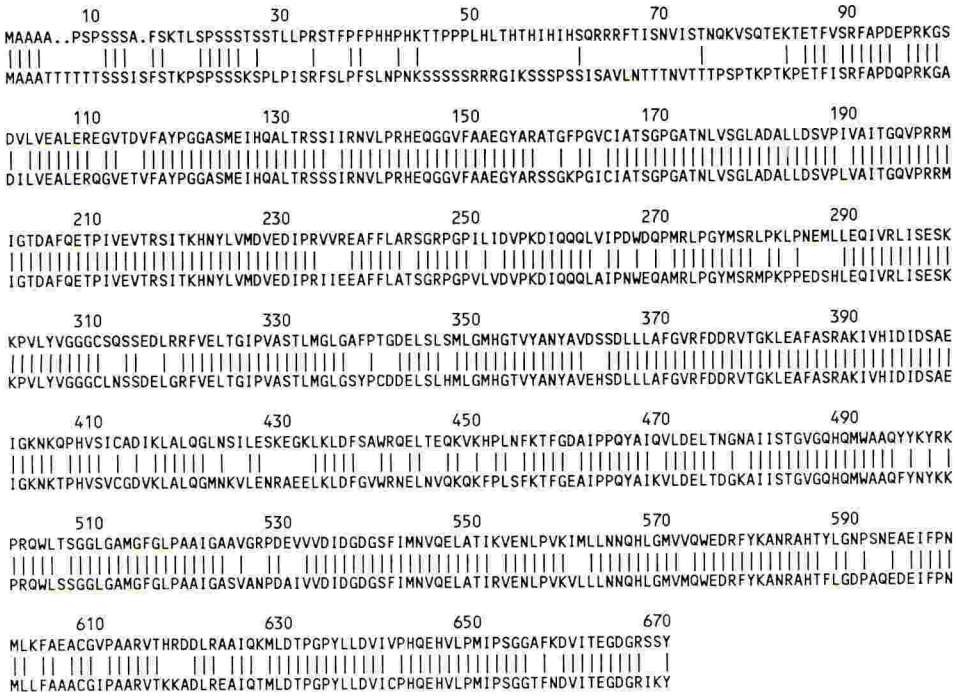

Fig. 2. Deduced amino acid sequences of plant ALS. The deduced amino acid sequence of a tobacco ALS gene is shown on the top line, and that of an Arabidopsis ALS gene is shown on the bottom line. Vertical lines highlight identical amino acids.

There is, however, one region which is not highly conserved between the two plant ALS sequences, at the 5' end of the coding sequences. This regionis assumed to encode chloroplast transit sequences, in that ALS is a nuclear encoded but chloroplast localized enzyme in plants (Miflin, 1974; Jones et al., 1984; Chaleff and Ray, 1984). The overall homology is in fact greater at the nucleotide level than at the amino acid level, suggesting that there are few constraints upon the amino acid sequence in this region.

The physical organization of the ALS genes in tobacco and Arabidopsis has been determined by Southern blot analyses. Homologous cloned ALS genes were used as probes. A single ALS gene hybridizes to the probe in Arabidopsis, while two hybridize in tobacco, which is an allotetraploid (Mazur et al., 1987). The presence of two ALS genes in tobacco is consistent with genetic data, which had indicated that tobacco mutations which confer herbicide resistance define two loci (Chaleff and Ray, 1984). Southern blot analyses of DNA from several other crop species have indicated that many carry multiple ALS genes.

TABLE 2

ALS Activity in Transformed Tomato

	Percent Uninhibited ALS Activity[1]			
	0ppb	10ppb	100ppb	1000ppb
Wild type	100	15	5	4
Transformant #3	100	42	25	12
Transformant #4a	100	60	42	26
Transformant #4b	100	29	15	5
Transformant #4c	100	58	43	25
Transformant #4d	100	29	15	10

[1]The ALS activities in each line are relative to the activity in the absence of sulfonylurea herbicide which is taken as 100 percent.

The cloned plant ALS genes have been used as probes to isolate genes carrying ALS mutations from herbicide resistant plants. In tobacco, the HRA line, which is mutated at the SURB locus and which is 1000 fold more resistant to sulfonylureas than are wild type lines (Chaleff et al., 1987), has been used as one source of mutant ALS genes. A second tobacco line, C3, which carries a mutation at the SURA locus (Chaleff and Ray, 1984), has also been used as a source of mutant ALS genes. Four genes, representing all the ALS loci from the two mutant lines have been isolated. The molecular characterization of mutant and wild type genes from each plant line has permitted the assignment of the genes to the appropriate genetic locus. Reintroduction of isolated genes into tobacco has indicated that a mutant gene from either locus can confer herbicide resistance in transformed cells. Plants regenerated from cells transformed with the SURB-Hra gene are resistant to field application rates of sulfonylurea herbicides (Lee et al., manuscript in preparation).

The tobacco SURB-Hra gene has been used to transform a number of heterologous species, and has conferred selectable levels of herbicide resistance in them. As an example, the sensitivity of ALS activity in transgenic tomato plants to a sulfonylurea herbicide is shown in Table 2. ALS activity was measured in leaf extracts of regenerated plants. Three different herbicide inhibition profiles are apparent among the five transformants shown; two pairs of transformants probably originate from single transformation events. At a concentration of 10 parts per billion of herbicide, approximately 60 percent of the activity in plants 4a and 4c, 40 percent of the activity in plant 3, and 30 percent of the activity in

TABLE 3

ALS Activity and Inheritance of Herbicide Resistance in Transformed Tobacco

	% Uninhibited ALS Activity[1]	Progeny Resistant/Sensitive[2] 100ppb	1000bbp	Segregation Ratio Resistant/Sensitive
NK326(WT)	7	---	---	---
NK326 #1	36	98/37	90/35	3/1
NK326 #9c	47	163/49	99/63	3/1
NK326 #9d	37	288/67	150/58	3/1
NK326 #10	26	93/31	96/24	3/1
NK326 #10c	56	333/45	290/76	3/1
K14 (WT)	7	---	---	---
K14 #7	71	990/4	109/1	255/1
K14 #11	52	208/85	127/76	3/1
K14 #27	45	129/45	108/42	3/1
K14 #29	30	192/46	163/67	3/1
K24 #31	44	106/35	99/34	3/1
K14 #32c	32	140/65	63/86	3/1
K14 #40	41	218/18	212/26	15/1
K14 #41	40	255/35	296/74	3/1
K14 #42	29	162/74	77/72	3/1
K14 #53	37	130/59	149/139	3/1
K14 #54	34	99/38	92/43	3/1
K14 #54A	28	137/55	100/72	3/1

[1]The ALS activity in each line is related to the activity in the absence of a sulfonylurea herbicide which is taken as 100 percent.

[2]Resistant progeny are able to grow at the indicated concentrations of herbicide.

plants 4b and 4d remains uninhibited by the sulfonylurea. These results demonstrate that the tobacco SURB-Hra gene can be highly effective in conferring herbicide resistance in heterologous species.

The SURB-Hra gene has been introduced into a number of commercial lines of tobacco, and regenerated plants have been assayed for levels of sulfonylurea resistance. Resistance was measured by assaying leaf ALS activity in the presence of herbicide, by measuring secondary callus growth in the presence of increasing concentrations of herbicide, by monitoring the ability of progeny seeds to germinate and grow in the presence of increasing concentrations of herbicide, and by monitoring plant phytotoxicity after foliar spray applications of herbicide. The results of each of these tests were consistent, yet indicated the need for monitoring resistance by several methods in order to identify those lines most suitable for crop breeding.

Table 3 shows the percentage of leaf ALS activity in transgenic tobacco plants which is uninhibited by 10 parts per billion of a sulfonylurea herbicide. Herbicide resistant ALS activity varies between 30 and 70 percent of the total ALS activity. The ability of the secondary callus to grow on particular levels of herbicide is generally, but not strictly, correlated with the amount of herbicide resistant ALS. Segregation analyses of progeny plants produced from self-fertilization is also shown in Table 3. The highest level of resistant ALS activity is found in a plant in which ALS genes are integrated at four loci, indicated by a segregation ratio of 255 resistant to one sensitive progeny. Most plants have ALS genes integrated at only one site (segregation ratio 3:1). The number of loci at which resistant ALS genes are integrated is not the sole factor which affects the degree of resistance of the transformants; position effects on gene expression and/or the presence of tandem gene copies at a locus also appear to influence expression. For example, secondary calli from plants 40 and 41 grows equally well in the presence of herbicide, and the plants have equivalent levels of resistant enzyme. Yet, segregation analyses indicate that plant 40 has two resistant ALS loci, while plant 41 has one. It can therefore be inferred that either the ALS gene in plant 41 is integrated in a particularly favorable position for expression, or that tandem copies of the gene are present at the single locus. For plant breeding purposes, a high level of herbicide resistance originating from a single genetic locus is preferred.

As a measure of agronomically useful herbicide resistance, the tobacco transformants were sprayed with sulfonylurea herbicides and evaluated for phytotoxic symptoms. Foliar sprays were applied at rates corresponding to 0, 4, 8, and 16 grams of herbicide/hectare; a typical field application rate is equivalent to 8 grams/hectare of herbicide. Transformed plants show no damage at application rates as high as 16 grams/hectare, a rate twice that of the field application rate. Wild type plants show damage at an application rate of 4 grams/hectare. Thus, transformation with the SURB-Hra gene provides a highly effective means of achieving selective herbicide toxicity.

We have made use of a microbial system to further study mutations that result in sulfonylurea herbicide resistance. Spontaneous mutations in the yeast ILV2 gene which encodes ALS have been isolated and characterized by DNA sequencing to determine the amino acid substitutions responsible for herbicide resistance. The substitutions occur at sites where the amino acid residues present in the wild type herbicide sensitive yeast enzyme are also found in all plant ALS enzymes known. The herbicide-resistant plant enzymes for which sequence information is known also have amino acid substitutions at these sites. We have used site-directed mutagenesis to make and characterize additional mutations at these sites in the yeast ILV2 gene. In this way the microbial system has continued to contribute to our understanding of sulfonylurea herbicide inhibition of ALS.

FUTURE PROSPECTS

The discovery that several herbicides act by inhibiting amino acid biosynthetic enzymes suggests possibilities for rational design of new herbicides. Amino acid biosynthesis is a particularly attractive target area for such a directed approach because so much is known about the genetics, biochemistry and regulation of these pathways. The first step in this approach is the choice of target enzyme. Amino acid pathways provide

material that is used in the production of NAD, folate, pantothenate, S-adenosylmethionine and purines. Judicious choice of a target enzyme can thus not only interfere with amino acid synthesis but also disrupt transfer of one-carbon units, acyl groups, protons or methyl groups.

The cellular response to such a metabolic blockade should also be considered in choosing an enzyme target. End-product limitation usually results in more metabolites entering the pathway as a consequence of enzyme derepression and relief of feedback inhibition. Upon sufficient substrate accumulation, catalysis may occur even in the presence of an inhibitor. However, accumulation of certain intermediates is toxic, precluding this cellular response. For example, the ALS inhibitors lead to high levels of α-ketobutyrate, which has deleterious physiological consequences.

Identifying a potent plant growth inhibitor, either by the random screening or rational design method, will not in general yield a herbicide which shows selectivity. However, the ability to introduce selective herbicide resistance into crops via transformation using isolated genes provides many exciting possibilities. A major constraint on any potential chemical herbicide, the requirement for low toxicity to the crop, is eliminated. The herbicide resistance genes can also be used as a dominant selectable genetic markers for both basic research and plant breeding. For basic research, these genes will serve as analogs of the antibiotic resistance genes which are so useful to microbial genetics. For plant breeding the resistance genes can be physically linked to other genes conferring agronomically useful traits, and introduced into plants via transformation using selection for resistance to the herbicide. Subsequently, in a plant breeding program the agronomically useful trait can be transferred to various cultivars through standard genetic crosses by following the easily assayed herbicide resistance phenotype associated with the linked genetic marker.

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HERBICIDES AFFECTING LIPID METABOLISM

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ABSTRACT

Three classes of herbicides have been found to have rather specific effects on plant lipid metabolism. Substituted pyridazinones, exemplified by San 9785, inhibit linoleate desaturation while thiocarbamates, such as EPTC or diallate, prevent very long chain fatty acid synthesis and, hence, alter wax or suberin formation. The monocotyledon-specific oxyphenoxy-propionic acids appear to act by inhibiting the fatty acid synthetase of sensitive plants. The details of these sites of action are discussed as well as the underlying reactions of lipid metabolism which are affected.

THE NATURE OF PLANT LIPIDS

The lipid composition of plant tissues has been reviewed (Hitchcock & Nichols, 1971; Harwood, 1980) and a few general features will be summarised here. Leaves from a wide variety of plants show a broadly similar lipid composition with the internal membranes containing mainly acyl lipids. Since the major organelles in leaves are chloroplasts, the overall lipid composition is dominated by that of the thylakoid membranes. The chloroplast thylakoids from higher plants and algae (as well as the photosynthetic membranes of cyanobacteria) have a very consistent lipid composition and one which is quite different from other cellular membranes (Gounaris et al., 1986). Glycosylglycerides (monogalactosyldiacylglycerol, digalactosyldiacylglycerol and sulphoquinovosyldiacylglycerol) predominate and the only important phospholipid is phosphatidylglycerol. In higher plants, the glycosylglycerides do not occur in significant amounts outside the plastids. In contrast, extra-chloroplastic membranes have phosphatidylcholine and phosphatidylethanolamine as major constituents. Typical leaf compositions are shown in Table 1 where it will be seen that the major plastid lipid, monogalactosyldiacylglycerol, and the main extra-chloroplast lipid, phosphatidylcholine, make up 26-46% and 7-24% of the total, respectively.

TABLE 1

The acyl lipid composition of plant leaves and roots

Tissue	% total acyl lipid (wt/wt)						
	MGDG	DGDG	SQDG	PG	PC	PE	PI
Barley leaves	43	26	5	6	11	4	1
Broad bean leaves	38	30	6	6	7	4	2
Rye grass leaves	39	29	4	7	10	5	2
Spinach leaves	37	28	5	7	13	6	2
Pea roots	11	10	2	2	45	18	6

Abbreviations:
MGDG = monogalactosyldiacylglycerol; DGDG =digalactosyldiacylglycerol:
SQDG = Sulphoquinovosyldiacylglycerol; PG = phosphatidylglycerol;
PC = phosphatidylcholine; PE = phosphatidylethanolamine;
PI = phosphatidylinositol

The low content of plastids in roots (proplastids are present) ensures that such tissues contain mainly phospholipids (Table 1) with shoots having an intermediate lipid composition. Other lipid classes (sterols, sterol esters, etc) are also present but are much less important quantitatively than the acyl lipids (Harwood 1980).

The surfaces of plant tissues are covered with a complex mixture of lipids and polymers thereof. Leaves are coated with wax and cutin whereas stems and roots are covered by suberin. For a comprehensive discussion of the composition of cutin and suberin see Kolattukudy (1980). Many of the constituents of these substances have very long hydrocarbon chains thus ensuring the water-barrier necessary for normal plant function. The formation of such hydrocarbon chains has implications for the thiocarbamate herbicides which will be referred to later.

All acyl lipids contain a mixture of esterified fatty acids. The position and nature of such acids have important implications for membrane function and can be altered by environmental parameters. Typically, a given lipid class will have a characteristic fatty acyl composition and examples for the major leaf lipids are shown in Table 2. Palmitic, oleic and especially, linoleic and α-linolenic acids are major components. In certain plants, called '16:3' species, hexadecatrienoic acid occurs but only in monogalactosyldiacylglycerol. A unique unsaturated acid, trans-3-hexadecenoate, is found only at the sn-2-position of chloroplast phosphatidylglycerol (Gounaris et al., 1986).

TABLE 2

The fatty composition of different acyl lipids

Tissue	Fatty acid composition (% total acids)						
Spinach leaves	16:0	16:1	16:3	18:0	18:1	18:2	18:3
MGDG	tr	-	25	-	1	2	72
DGDG	3	-	5	-	2	2	87
SQDG	29	1	n.d.	1	7	26	36
PG	11	*32	n.d.	-	2	4	47
PC	16	1	n.d.	2	9	31	40
Barley leaves							
MGDG	3	1	n.d.	1	1	3	91
DGDG	9	2	n.d.	1	3	7	78
SQDG	32	3	n.d.	1	2	5	55
PG	18	*27	n.d.	3	2	11	38
PC	25	3	n.d.	2	5	28	35

*16:1 = trans - 3-hexadecenoate in PG and cis- 9-hexadecenoate in other lipids. Abbreviations as in Table 1. n.d. = none detected, tr = trace (<0.5%).

Thus, in considerations of herbicide action or potentially-useful new compounds, a limited number of plant lipids and fatty acids need to be considered. Interestingly, some of these lipids and fatty acids do not occur in significant amounts outside the plant kingdom. This, of course, raises the possibility of selecting such compounds as herbicide targets with less chance of environmental and human toxicological problems.

LIPID METABOLISM

In order to provide background for further discussion on the site of action of individual herbicides, a few brief comments will be made concerning lipid metabolism in plants.

Fatty acids are synthesised de novo by a plastid-localised multifunctional acetyl-CoA carboxylase and a dissociable Type II fatty acid synthetase. The end-product of the synthetase is normally palmitoyl-acyl carrier protein (although certain plants, such as coconut, may accumulate medium-chain fatty acid products) which is elongated to stearate using a specific condensing enzyme. Desaturation to oleate then occurs also on an acyl carrier protein-bound substrate. Further desaturations to the polyunsaturated linoleic and α-linolenic acids then occur by a complex series of reactions involving acyl lipid substrates and cooperation of organelles. In contrast to the de novo production of C_{16} and C_{18} fatty acids, the very long chain fatty acids are produced by specific elongases located in the endoplasmic reticulum. Full details of the above reactions and the enzymes involved are reviewed in Harwood (1980).

For the production of acyl lipids, fatty acids are acylated to the 1- and 2-positions of glycerol 3-phosphate and the resultant phosphatidate is dephosphorylated to 1,2-diacylglycerol (Harwood and Price-Jones, 1987). The latter represents the key precursor for both galactolipid and phospholipid formation (c.f. Stumpf and Conn, 1980; Gunstone et al., 1986). These pathways are broadly similar in different plant types with the glycosylglycerides being made in plastids and the phosphoglycerides mainly being synthesised on the endoplasmic reticulum. However, there are minor differences, most noticably between the '16:3' and '18:3' plants (Moore, 1986; Harwood and Price-Jones, 1987).

SUBSTITUTED PYRIDAZINONES

Depending on the structure of the pyridazinone and the plant test species, these compounds can produce a variety of different effects including changes in fatty acid composition (Duke, 1985). In particular, interest has centred on two materials San 9785 (BASF 13-338; 4-chloro-5(dimethylamino)-2-phenyl-3(2H)pyridazinone) and San 6706 (metflurazone; 4-chloro-5-(dimethylamino)-2-(α,α,α,trifluoro-m-tolyl)-3-(2H)-pyridazinone) which both seem to affect fatty acid composition preferentially.

The initial observation that San 9785 altered the proportions of linoleic and α-linolenic acids in monogalactosyldiacylglycerol (St. John, 1976) suggested that the 15-desaturase was inhibited directly. This was tested using radiolabelled substrates. Such experiments have confirmed that there considerable species variation in the susceptibility of plants to San 9785 (Murphy et al., 1980). The generally higher susceptibility of monocotyledons in such experiments agreed with the in vivo studies of Khan et al (1979) who found that barley was more affected than broad bean at all herbicide concentrations. The desaturation of labelled linoleate to linolenate was severely inhibited in susceptible species and, interestingly, the accumulation of [^{14}C]linoleate was almost exclusively confined to monogalactosyldiacylglycerol. In contrast, extra-chloroplastic phosphatidylcholine which was highly labelled from both [^{14}C]acetate and [^{14}C]linoleate was hardly affected (Table 3; Murphy et al., 1985). Similar effects have also been noted for San 6706 with regard to its species selectivity (Khan et al., 1979) and its action on the desaturation of linoleate associated with monogalactosyldiacylglycerol. In addition, San 6706 also inhibits the desaturation of the palmitate at the sn-2-positions of phosphatidylglycerol to trans- 3-hexadecenoate (Davies & Harwood, 1983).

TABLE 3

Inhibition of the desaturation of linoleate associated with monogalactosyldiacylglycerol by San 9785

Plant	Lipid	San 9785	Fatty acid labelling (% total) 16:0	18:1	18:2	18:3
Barley	MGDG	0	-	24	49	27
[^{14}C]oleate	MGDG	10^{-4}M	-	31	64	5
	PC	0	-	41	59	tr
	PC	10^{-4}M	-	45	55	n.d.
Pea*	MGDG	0	7	13	33	47
[^{14}C]acetate	MGDG	$2x10^{-4}$M	6	11	69	14
	PC	0	16	26	55	3
	PC	$2x10^{-4}$M	14	25	59	2

Data for barley (Murphy et al., 1985), pea(Willemot et al., 1982)
Abbreviations as Table 1.
* Analysis of pea used $AgNO_3$-t.l.c. but the saturated and unsaturated acids have been shown to correspond to those listed (c.f. Harwood et al., 1987).

San 9785 appears to be more selective in its effects on lipid metabolism than other pyridazinones, such as norfluorazone (San 9789) which have been tested (c.f. Harwood et al., 1987). San 9785 has a low phytotoxicity in most systems and has little action on pigment accumulation (St. John, 1982). At 10^{-4}M levels, the herbicide only appeared to reduce the desaturation of linoleate in monogalactosyldiacylglycerol and was without effect on the synthesis of other fatty acids or on lipid metabolism. Higher concentrations, however, have been reported to cause a net loss in chloroplast galactosylglycerides and chlorophyll and to interfere with photosynthesis (c.f. Harwood et al., 1987).

The selective inhibition of linoleate desaturation in monogalactosyl-diacylglycerol by San 9785 is related to the preferential use of this lipid as a substrate for the 15-desaturase (c.f. Harwood, 1988). However, because the detailed metabolism of fatty acids in '16:3' and '18:3' plants is different, then it is not surprising that such plant types show differences in their reaction to San 9785 treatment (Norman & St. John, 1987). In general, however, substituted pyridazinones are inhibitors of fatty acid desaturation, particularly the conversion of linoleate to α-linolenate with San 9785 being the most specific.

THIOCARBAMATE HERBICIDES

In the period around 1970, several thiocarbamate herbicides were shown to alter epicuticular waxes in a number of plant species (c.f. Rivera& Penner, 1979; Duke, 1985). Moreover, several of the compounds wereshown to inhibit lipid or fatty acid radiolabelling (c.f. Harwood et al., 1987). A connection between lipid metabolism and wax production comes from the fact that many wax components are produced from very long chain ($>C_{20}$)fatty acids (Kolattukudy, 1980). Therefore, the production of such acids was examined directly for any thiocarbamate effects. As

expected, such compounds as EPTC (S-ethyl dipropylthiocarbamate) diallate (S-2,3-dichloroallyl N,N-diisopropylthiocarbamate) and CDEC (2-chloroallyl diethyldithiocarbamate) were found to prevent very long chain fatty acid formation in vitro. Although the labelling of such compounds was severely reduced, that of other fatty acids, such as palmitate and stearate, was unaffected (Harwood & Stumpf, 1971; Table 4). Similar effects on fatty acid elongation have also been reported for trichloroacetic acid which also affects wax production and increases leaf wettability.

TABLE 4

Changes in radiolabelling of fatty acids from [^{14}C]acetate caused by thiocarbamates

Tissue		Fatty acid labelling (% total) (% total ^{14}C-fatty acids)				
		16:0	18:0	20:0	22:0	24:0
Pea Seed	Control 0	24	65	5	5	1
	EPTC(10^{-5}M)	20	79	1	0	0
	Diallate(10^{-5}M)	26	70	4	tr	0
Potato discs	Control	31	43*	9	7	3
	EPTC(10^{-5}M)	33	47*	11	1	tr
Potato discs	Control	27	56*	10	4	-
	Diallate(10^{-5}M)	43	49*	2	1	-

Data taken from Harwood and Stumpf (1971; pea) and Bolton and Harwood (1976; potato)* Includes 18:0 and 18:1 labelling.

Kolattukudy and Brown (1974) extended these observations to study the labelling of individual wax components which could be produced from very long chain fatty acids. Not only did they confirm the results of Harwood and Stumpf (1971) in showing a selective effect of diallate, CDEC and EPTC on fatty acid elongation but they also found that these compounds particularly reduced labelling of alkane and secondary alcohol fractions. Thus, major effects of thiocarbamates in whole leaves seem to be confined to wax components or their immediate precursors.

Very long chain fatty acids are also precursors and components of a different surface covering layer, suberin. An experimental system which is very active at synthesising both very long chain fatty acids and suberin is the ageing potato disc. This system was also tested for the effects of thiocarbamate herbicides. EPTC, diallate and triallate (S-(2,3,3-trichloroallyl) diisopropylthiocarbamate) were used and all three compounds were found to reduce severely the proportion of very long chain fatty acids labelled (Table 4; Bolton & Harwood, 1976). In contrast, palmitate and stearate labelling was virtually unchanged.

The effects of thiocarbamates have been examined in more detail recently using subcellular fractions. If pea seeds were treated with either diallate or triallate then the microsomal fractions subsequently isolated were unable to synthesise very long chain fatty acids (Table 5).

Such a result could be explained either by postulating that the herbicide (or an active metabolite) binds to the endoplasmic reticulum and remains there during isolation or, alternatively, that the herbicides prevent the synthesis of elongase proteins which has to occur during germination (c.f. Harwood & Stumpf, 1970). When diallate and triallate were tested directly on the microsomal fraction, rather high concentrations were needed to prevent very long chain fatty acid formation. Moreover, palmitate and stearate synthesis was also affected at these concentrations (Table 5). The difference between *in vivo* and *in vitro* observations may have been due to the metabolism of thiocarbamates to a more active (and selective) metabolite *in vivo* or, alternatively, because very long chain fatty acid formation is concentrated in the outer leaf cells (Lessire & Stumpf, 1982) and is extra-chloroplastic. Thus, because of poor translocation or rapid inactivation by pea tissues, the thiocarbamates may only be able to easily inhibit very long chain fatty acid synthesis *in vivo* (c.f. Harwood *et al.*, 1987).

TABLE 5

Effect on thiocarbamates on fatty acid synthesis by microsomal fractions from germinating peas

	Total Synthesis (d.p.m)	Distribution of radioactivity (% ^{14}C-fatty acids) 16:0	18:0	VLCFA's
Control	11040	30	34	22
Seeds treated 10^{-4}M diallate	7760	40	60	0
Control	14250	56	26	9
Seeds treated 10^{-4}M triallate	12570	64	19	0
Control	14170	49	15	2
+ 10^{-4}M diallate) in assay	5850	48	28	0
+ 10^{-4}M triallate) in assay	5800	54	20	0

Incubations were with ^{14}C-malonyl-CoA. VLCFA's = very long chain ($>C_{20}$) fatty acids. Data from K. Abulnaja and J. L. Harwood (unpublished results).

OXYPHENOXYPROPIONIC ACIDS AND RELATED COMPOUNDS

A number of phenoxy-phenoxypropionic acids have been described which are selective against graminaceous species (c.f. Nestler, 1982). These compounds are post-emergence herbicides which are rather poorly active against dicotyledons. Necrosis of meristematic tissues is a common symptom of their action in sensitive species.

Diclofop-methyl (methyl 2-[2'4'-(dichlorophenoxy)phenoxy]propanoate) has been shown to severely affect lipid synthesis in sensitive plants but was without action on the formation of proteins, carbohydrates or nucleic acids (Hoppe, 1981). Similarly, we have shown that fluazifop-butyl (butyl2-[4-(5-trifluoromethyl-2-pyridyloxy)phenoxy]propionate) had no

effect on amino acid uptake, protein synthesis, carbon dioxide fixation or the formation of water-soluble organic compounds at concentrations where a virtually complete inhibition of acyl lipid production was found (K.A. Walker & J.L. Harwood, unpublished observations).

The effects of diclofop-methyl on lipid labelling in maize were explained by an inhibition of fatty acid synthesis at the level of fatty acid synthetase (Hoppe & Zacher, 1985). Moreover, measurement of de novo fatty acid synthesis in chloroplasts isolated from sensitive and tolerant species also showed a selective effect of diclofop-methyl. The D-isomer, which is the active herbicide is also the active stereoisomer for fatty acid synthesis in vitro (Hoppe & Zacher, 1985). Similarly, we have shown that fluazifop-butyl only affects fatty acid synthesis in chloroplasts from sensitive species and that the physiologically-important R-stereoisomer is the active form (Table 6).

TABLE 6

Stereospecific effects of diclofop-methyl and fluazifop on fatty acid synthesis by chloroplasts from sensitive and tolerant species

Plant	Herbicide	Total fatty acid synthesis (% control)
Maize	D-diclofop (1μM)	10
	L-diclofop (1μM)	70
Barley	R-fluazifop (17.5μM)	13
	S-fluazifop (17.5μM)	43
	RS-fluazifop (35μM)	14
Pea	RS-fluazifop (100μM)	96
	RS-fluazifop (5μM)	104

Data for maize chloroplasts (Hoppe & Zacher, 1985), for barley and pea chloroplasts (K.A. Walker, S.M. Ridley and J.L. Harwood, unpublished observations). *the S-stereoisomer contained 7% of the R-isomer as contaminent.

Differential radioisotope experiments have shown that labelling of very long chain fatty acids is unaffected by fluazifop-butyl (Table 7) again emphasising the action of such compounds on the fatty acid synthetase. This synthetase is a dissociable Type II enzyme in plants containing seven individual enzymes and acyl carrier protein (c.f. Harwood, 1988). Because chain length-specific condensing enzymes have been isolated, and because fluazifop-butyl affects the ratio of C_{16} and C_{18} products (Table 7), it is possible that such reactions are the actual target site for the oxyphenoxypropionic acids. We are now testing this directly by measuring individual partial reactions.

TABLE 7

Pattern of fatty acids labelled from ^{14}C-acetate or ^{14}C-malonate in the presence of low inhibitory concentrations of fluazifop

	Fatty acids (% total ^{14}C-fatty acids)						
^{14}C-acetate	16:0	18:0	18:1	18:2	20:0	22:0	24:0
Control	11	2	21	57	10	0	0
5µM Fluazifop(R)	26	6	11	31	10	13	0
^{14}C-malonate							
Control	4	8	5	8	8	31	36
100µM Fluazifop (RS)	0	16	0	0	12	40	32

Data from K.A. Walker, S.M. Ridley and J.L. Harwood (unpublished observations) using barley tissue.

Apart from the two compounds mentioned above, other oxyphenoxypropionate herbicides which have been shown to inhibit fatty acid or lipid synthesis include clofop-isobutyl, fenthiaprop-ethyl, fenoxaprop-ethyl and their free acids and haloxyfop(c.f. Harwood et al., 1987). Although they are not oxyphenoxy propionic acids, two cyclohexene-one derivatives alloxydim and sethoxydim show herbicidal selectivity and symptoms which are very similar to the oxyphenoxy compounds (Duke & Kenyon, 1987). Furthermore, sethoxydim has been shown to inhibit fatty acid synthesis in sensitive systems such as maize (Burgstahler & Lichtenthaler, 1984) and barley leaves (K.A. Walker, S.M. Ridley & J.L. Harwood, unpublished results). However, the selectivity of sethoxydim may be due partly to its differential metabolism or transport which contrasts with the oxyphenoxy propionic acids where all available evidence indicates that this mechanism of selectivity is based on the target site.

CONCLUSIONS

Three classes of herbicides have now been shown to inhibit lipid synthesis in plants directly. The oxyphenoxy propionic acids are particularly exciting because they are highly effective at low concentrations and, moreover, have a selectivity which may be based on difference between the fatty acid synthetases of different plants. Unfortunately, we know little about the details of the proteins involved in plant lipid metabolism so that it is currently not possible to use such information to design effective new herbicides. However, the production of plant specific compounds such as the glycosylglycerides, α-linolenate and trans- 3-hexadecenoate are obvious areas which should be explored further. What seems to be needed at present is an investment in research into basic plant lipid biochemistry. Only when sufficient background information is available is it likely that fundamental advances in herbicide design can take place.

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C-14 DEMETHYLATION IN PHYTOSTEROL BIOSYNTHESIS - A NEW TARGET SITE FOR HERBICIDAL ACTIVITY

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ABSTRACT

Application of a γ-keto triazole herbicide as a root drench at low dosages to seedlings reduced normal shoot phytosterols and led to the accumulation of C-14α-methyl-sterols. These were 14α-methyl-Δ^8-cholestenol in oat and 14α-methyl-Δ^8-ergostenol, obtusifoliol and dihydroobtusi-foliol in barley and pea. The appearance of these sterols was correlated with growth inhibition and symptoms of phytotoxicity. It is suggested that, as with fungi, 14α-methyl sterols do not adequately support efficient membrane function and development and that this could be the basis of the mode of action of the herbicide.

INTRODUCTION

The cell membrane is an important target site for many biocides. The SBIs (Sterol Biosynthesis Inhibitors) are a large class of systemic fungicides which operate by inhibiting the biosynthesis of ergosterol or an equivalent membrane sterol (Kato 1986). A most important sub-group, which includes such well-known members as triadimenol, propiconazole, imazalil and prochloraz, inhibit the cytochrome P-450 dependent monooxygenase responsible for the removal of the C-14α-methyl group. The resulting depletion of ergosterol and the accumulation of C-14α-methyl sterols leads to the inhibition of growth in vitro and the effective control of many fungal diseases in vivo. The precise cause of growth inhibition is still unclear although adverse changes in cell membrane fluidity, permeability and the performance of membrane-bound enzymes have all been invoked (Weete 1987).

A fundamental question, which has received comparatively little attention, is why SBI fungicides are inhibitory or toxic to the fungus and not to the host plant to which they are applied. The reason for this selective toxicity is by no means clear as plants contain sterols which are considered necessary for efficient membrane function as well as for possible hormonal roles (Grunwald 1978). Moreover, the enzyme involved in the removal of the C-14α-methyl group in phytosterol biosynthesis has also been shown recently to be cytochrome P-450 dependent (Rahier and Taton 1986).

A priori it may be argued that the plant C-14α-demethylase could be inherently less sensitive to nitrogen heterocyclic xenobiotics. Alternatively, it could be equally as sensitive to the fungicides as the fungal enzyme but the resulting C-14α-methyl sterols may not be detrimental to plant growth. A possible compromise explanation, supported by recent evidence (Buchenauer et al. 1984, Burden et al. 1987a) is that the plant demethylase can be inhibited by SBI fungicides, with resulting inhibition of growth, but only at dosages in excess of correct field rates.

If indeed the C-14α-methyl sterols do not adequately support plant growth and development then the C-14α-demethylase must be considered a

target enzyme for herbicidal activity. The relative ineffectiveness of SBI fungicides in inhibiting this enzyme may then be seen to be due to the lack of optimum structural features for efficient binding. It was considerations of this nature which led us to examine the effects on phytosterol biosynthesis of a representative of the γ-keto triazoles, a group of triazoles synthesised by ICI scientists, which are related to triazole fungicides and plant growth regulators (Fig. 1) but which are active as non-selective pre-emergence herbicides (Balasubramanyan and Lewis 1976, Anderson et al. 1983). The results provide evidence that inhibition of C-14α-demethylation may be the principal mode of action of these compounds.

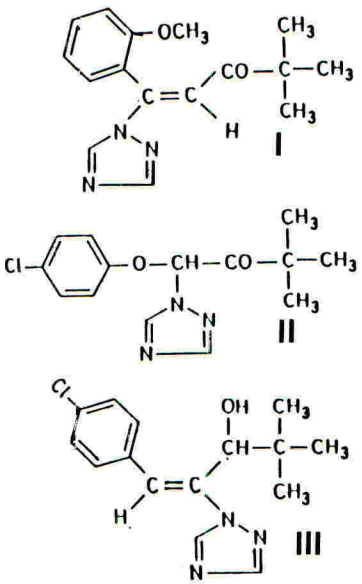

Fig. 1. Structures of triazole herbicide (I), fungicide (II), and plant growth regulator (III).

MATERIALS AND METHODS

Chemicals

The γ-keto triazole herbicide (I) was kindly provided by ICI Jealotts Hill Research Station.

Plant material

Barley, oat and pea seeds were germinated and grown in pots containing moist sand/vermiculite with conditions as described previously (Burden et al. 1987a). The seedlings were treated at emergence with a single dose of the γ-keto triazole (10.0, 1.0 and 0.1 μM), applied as a root drench in 1% ethanolic solution (200 ml per pot), after which they were watered with a nutrient solution. Shoot growth was measured and 3 weeks after emergence shoot samples were harvested and the tissue frozen prior to extraction and analysis.

Sterol analysis and identification

Total sterol analysis of shoot tissue was carried out using a previously described method (Burden et al. 1987). However, the tlc purification stage was done after acetylation, to provide a fraction containing the combined 4,4-dimethyl, 4-monomethyl and 4-demethyl sterols.

Analysis was by glc of the acetate derivatives using a bonded SE-52 column (25 m x 0.25 mm), 120° → 255° at 10°/min using hydrogen carrier gas (10 psi). β-cholestanol was used as internal standard for quantification and the assumption was made that all sterol acetates gave equal detector responses.

Sterol analysis of membrane tissue was as described (Burden et al. 1987b). Mass spectra were obtained using a Hewlett-Packard gc-mass selective detector coupled to the SE-52 column.

Preparation of membrane fraction

Procedure B of Dupont and Hirkman (1985) was used, with some minor modifications (Burden et al. 1987b) to produce a 100,000g microsomal fraction.

RESULTS

Plant growth

All dosages of the γ-keto triazole (I) affected the shoot growth of the three species examined. Root growth (data not reported here) was less affected. In general, shoot inhibition was first observed about 5 days after emergence. Later, at the higher dosages, symptoms of necrosis and chlorosis were observed on the foliage, and oat and barley plants, treated at 10 μM, did not survive much longer than 3 weeks. Pea plants, although considerably reduced in height and vigour, were not killed even at the greatest dose rate. Shoots of barley plants, treated at the lowest dose rate (0.1 μM), were still retarded in growth, compared to controls, when the plants were grown to the stage of ear and spike production (12 weeks). However, the plant morphology was unlike that of typical azole-PGR treated plants in that, for example, the leaves were narrower than those of control plants and not broad with a dark green colouration. Some growth data for barley and pea plants are shown in Table 1.

TABLE 1

Growth of shoots following γ-keto herbicide (I) treatment as a root drench

Dosage (μM)	Shoot height (cm) 1 week	2 weeks	3 weeks
Barley[a]			
0	17.6	26.6	31.3
0.1	14.4	19.9	20.8
1.0	11.8	16.3	16.6
10.0	9.1	10.7	12.4
Pea[b]			
0		16.4	21.7
0.1		13.7	17.0
1.0		8.5	10.1
10.0		6.5	7.6

[a] average of 6 plants per treatment

[b] average of 5 plants per treatment

Sterol analysis and identification

Sterols, as their acetate derivatives, were identified by their retention times relative to β-cholestanol acetate and their mass spectra. The mass spectra of C-14α-methyl sterol acetates were as follows:- 14α-methyl-Δ^8-cholestenol 442 (18%), 427 (100), 367 (32), 273 (10); 14α-methyl-Δ^8-ergostenol 456 (18%), 441 (100), 381 (36), 273 (12); obtusifoliol 468 (44%), 453 (100), 393 (45), 287 (36); dihydroobtusifoliol 470 (24%), 455 (100), 395 (49), 287 (13). These mass spectra, which are characterised by very strong M-15 ions, are comparable with values recorded in the literature (Schmitt and Benveniste 1979, Pascal and Schroepfer 1980).

The sterol content of 3 week old barley, oat and pea shoots is shown in Table 2. In barley, at all three dosages of the herbicide, there was a dramatic decrease in the normal phytosterols campesterol, stigmasterol and sitosterol. The C-14α methyl sterols 14α-methyl-Δ^8-ergostenol, obtusifoliol and dihydroobtusifoliol accumulated in large quanitites, particularly at the greatest dose rate. A qualitatively similar effect was observed in pea although the C-14α-methyl sterol levels were much smaller. A different situation was observed in oat. This species possess more cholesterol than is usual for a plant species (Burden et al. 1987b) and treatment with the γ-keto triazole produced large quanitites of 14α-methyl-Δ^8-cholestenol.

TABLE 2

Dosage (μM)	Sterol μg/g dry wt) "Normal" sterols a	b	c	d	C-14α-methyl sterols e	f	g	h
Barley								
0	0.01	0.22	0.23	0.57	---	---	---	---
0.1	0.01	0.12	0.13	0.14	---	0.06	0.13	0.06
1.0	trace	0.09	0.12	0.16	---	0.09	0.19	0.12
10.0	trace	0.05	0.11	0.09	---	0.10	0.24	0.17
Oat								
0	0.11	0.05	0.15	0.32	---	---	---	---
0.1	0.10	0.04	0.10	0.23	0.05	---	---	---
1.0	0.04	0.01	0.03	0.04	0.23	---	---	---
10.0	0.02	---	0.05	0.11	0.55	---	---	---
Pea								
0	---	0.17	0.48	0.78	---	---	---	---
0.1	---	0.16	0.44	0.65	---	0.01	0.01	---
1.0	---	0.11	0.32	0.42	---	0.02	0.04	0.02
10.0	---	0.08	0.19	0.11	---	0.07	0.09	0.08

Identity of sterols: a = cholesterol, b = campesterol, c = stigmasterol, d = sitosterol, e = 14α-methyl-Δ^8-cholestenol, f = 14α-methyl-Δ^8-ergostenol, g = obtusifoliol, h = dihydroobtusifoliol.

Analysis of a microsomal fraction prepared from 2 week old barley shoots showed changes in sterols comparable to those observed in whole tissue (Table 3).

TABLE 3

Sterol composition of microsomal fraction of 2 week old barley shoots following treatment with γ-keto herbicide (I) as a root drench

Dosage (μM)	Sterol (% of total) "Normal" sterols			C-14α-methyl sterols		
	b	c	d	f	g	h
0	21.8	25.2	53.0	---	---	---
0.1	22.0	19.9	42.6	3.4	8.7	3.4
1.0	15.0	15.2	22.5	10.6	21.5	15.3
10.0	14.9	13.2	21.4	13.7	23.2	13.5

Identity of sterols as in Table 2.

DISCUSSION

The γ-keto triazole herbicide (I) used in this study is a typical member of its class (Anderson et al. 1983). While there are many structural similarities with SBI fungicides such as triadimefon and ent-kaurene oxidase inhibitor PGRs such as uniconazol (III), (Fig. 1), a γ-disposition of the triazole ring in relation to the carbonyl, an E configuration of the double bond and an ortho substitution of the aryl ring are all necessary for high herbicidal activity (Anderson et al. 1983).

The results presented here demonstrate that administration of the herbicide (I) at low dosages inhibited normal phytosterol formation and induced the accumulation of C-14α-methyl sterols (Fig. 2). The sterol 14α-methyl-Δ^8-cholestenol (2) accumulating in oats differed from those (4,5 and 6) found in barley and pea. This is likely to result from an inhibition of the cholesterol branch pathway which is believed to be particularly active in oat (Burden et al. 1987b, Cooke and Burden 1987). However, the absence of other C-14α-methyl sterols in this species is surprising.

There was generally a good correlation between growth inhibition and interference with sterol biosynthesis. However, the important question is whether these effects are causally related.

Sterols - usually cholesterol in mammals, ergosterol in fungi, campesterol, stigmasterol and sitosterol in plants - are believed to be important constituents of biomembranes where they align with the phospholipids and modulate the physical state (fluidity or viscosity) of the membrane (Bloch 1983). Studies with animal, fungal and artificial membranes have demonstrated that C-14α-methyl sterols cannot efficiently substitute for the normal sterols because of the protruding axial 14α-methyl group (Bloch 1983), Van den Bossche et al. 1980). There is abundant evidence for adverse effects of 14α-methyl sterols on fungal

Fig. 2. Biosynthetic pathways for phytosterols
⟶ Normal pathway
⇢ Pathways after inhibition of C-14α demethylase
Identity of sterols: 1 = cycloartenol, 2 = 14α-methyl-Δ^8-cholestenol,
3 = norlanosterol, 4 = obtusifoliol, 5 = dihydroobtusifoliol,
6 = 14α-methyl-Δ^3-ergostenol, 7 = cholesterol, 8 - campesterol,
9 = sitosterol, 10 = stigmasterol

growth (Weete 1987) but much less for plant growth. However, it has been noted that fenarimol treated bramble cells which accumulated obtusifoliol and 14α-methyl-$\Delta^{8,24}$-ergostadienol eventually became necrotic and died (Schmitt et al. 1982).

Thus a working hypothesis is that herbicide (I) interferes with sterol biosynthesis at the C-14α-demethylation stage and consequently damages cell membranes leading to symptoms of necrosis and chlorosis in existing foliage and retarded development from the apical meristem.

At the biochemical level it seems likely that the meta nitrogen of the triazole ring binds to the protohaem ion of the cytochrome P-450 component of the sterol C-14α-demethylase (Weete 1987). (I) was not fungicidal (Anderson et al. 1983, G.A. Carter, personal communication) and had no anti-gibberellin activity when assayed at up to 10 μM against ent-kaurene oxidase in a pumpkin endosperm cell free system (P. Hedden, personal communication). Thus it seems likely that there are different cytochrome P-450 dependent monooxygenases involved in fungal sterol demethylation, plant sterol demethylation and plant kaurene oxidation and that they may differ considerably in substrate specificity. Previous work has shown that some SBI fungicides such as triadimenol and plant growth regulators such as paclobutrazol, particularly as its 2R,3R and 2R,3S isomers, can also inhibit plant sterol C-14α-demethylation (Buchenauer 1984, Burden et al. 1987a, 1987c). However, the dosages required to achieve comparable effects in barley were considerably in excess of those required for herbicide (I).

A logical extension of the work described here would be the development of a cell-free system with which to quantify the effects of triazoles and related chemicals on phytosterol biosynthesis. If, as seems likely, an interference with phytosterols is predominantly a phytotoxic effect then this could be important in the discovery and development of new herbicides. It would also be useful in monitoring possible side effects of PGRs whose mode of action is intended to be based primarily on an inhibition of gibberellin biosynthesis.

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AUXIN-INDUCED H^+-EFFLUX: HERBICIDE ACTIVITY AND ANTAGONISM

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ABSTRACT

A method is described for the accurate measurement of proton-efflux from Avena coleoptile segments. Auxin induces rapid, linear rates of H^+-efflux after a short lag-period and the ability of various herbicides to mimic this process is reviewed. The 'dose-response' characteristics of 2,4-D have been determined in the presence and absence of fluazifop-butyl, a phenoxypropanoate graminicide. The antagonism observed between these two herbicides in the field is reflected in H^+-efflux assays in a manner indicating fluazifop-butyl has anti-auxin activity. The general importance of the anti-auxin behaviour of phenoxypropanoate graminicides is discussed.

INTRODUCTION

Despite forty years of intensive study devoted to synthetic and natural auxins there are still many features of their herbicidal activity which are poorly understood and merit further investigation (Pillmoor and Gaunt, 1981). Perhaps one reason for this has been the choice of experimental systems used to study 'auxin-type' responses. A common method used to detect auxin activity is the Avena coleoptile straight-growth bio-assay. However,auxins do not induce rapid growth directly but act via the induction of H^+-efflux leading to the acidification of the cell-wall matrix (Rayle, 1973). Proton efflux, therefore, is the first detectable effect of an auxin on sensitive (i.e. receptive) plant cells and as such precedes all other auxin-induced phenomena (altered genome expression etc.). Indeed, the ability of a compound to induce H^+-efflux can be viewed as a measure of its potential toxicity as an auxin-type herbicide (assuming it is not metabolised significantly by the target species), even though H^+-efflux itself may not be the cause of plant death.

An important advantage of studying H^+-efflux (instead of growth) is that the resulting data may be interpreted in terms of a simple molecular model. H^+-efflux is brought about through the activation of a plasmalemma-bound H^+-pumping ATPase (Evans, 1985). Auxin (IAA) does not appear to directly affect this enzyme in vitro but in vivo stimulates its activity via a putative secondary-messenger system, itself initiated by auxin binding to receptors in the plasmalemma and possibly elsewhere (Libbenga et al., 1986). It should be possible to determine the relative affinity of various auxins for the receptor protein binding site. In order to do this we require detailed information about rates of H^+-efflux over a wide range of 'auxin' concentrations (Weyers et al., 1987).

In this paper we describe a method for accurately measuring rapid, linear H^+-efflux from Avena coleoptiles and present comparative data for a number of well known synthetic auxins and auxin-type herbicides. We have analysed the activity of 2,4-D in detail and present data comparing the effect of this compound in the presence and absence of fluazifop-butyl, a phenoxypropanoate (ester) graminicide. These herbicides were chosen as an example of a mixture in which a broad-leaf weed-killer antagonises graminicide action in the field (Cobb, 1987). It is suggested that the

anti-auxin activity of the phenoxypropanoates may be a more important facet of their herbicidal activity than previously considered.

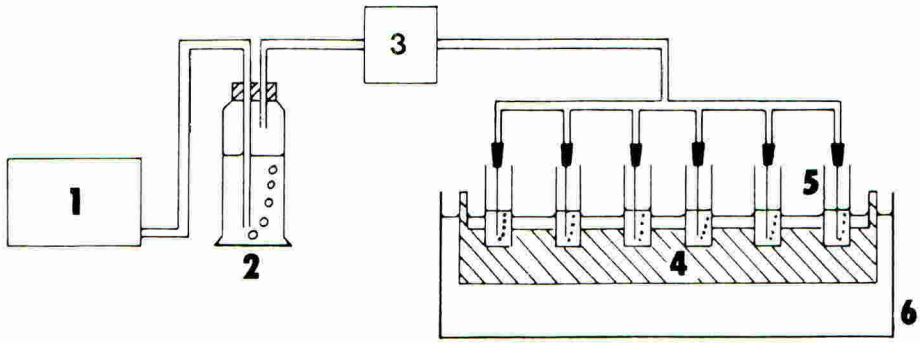

Fig. 1. The apparatus. 1) Air pump 2) Humidifier 3) Flow meter 4) Cuvette holder 5) Aeration chambers 6) Water bath.

MATERIALS AND METHODS

Avena sativa var. Maris Tabard seeds were sown in moist vermiculite (tap-water) and seedlings harvested after 88-94 h growth at 24 ^{o}C (darkness, high humidity). For each experiment c. 100 coleoptiles (35-60 mm) were gently abraded with a carborundum paste (800-grit silicon carbide powder in water) and stored/washed in distilled water. Segments were cut 3-18 mm behind the apex and stored (in darkness) in 50 cm^{3} of 0.15 mM MES-Tris buffer, pH 6.5. The segments were washed and kept stirred by vigorous aeration (600 cm^{3}/min) for 15 min, rinsed and transferred (10 x 10) into plastic vials (7 cm^{3} total volume) containing 1.8 cm^{3} of MES-Tris buffer plus 1.0 mM KCl (dim lab-light conditions, c. 10 µmol photons/m^{2}/s, PPFD). The vials were maintained at a constant temperature of 25 ^{o}C and individually aerated (50 cm^{3}/min) with humidified lab-air (Fig. 1). After 40-50 min continuous aeration various chemicals were added as required and pH readings commenced (110-120 min from starting tissue preparation). Approximately 1.5 cm^{3} of each bathing medium was transferred in sequence to a constant-temperature stirring vessel (Clark-type O_2-electrode cuvette) and pH measured to the nearest 0.01 unit (Russell semi-micro pH electrode, Corning 140 pH meter). 'Auxins' were dissolved in acetone (10-100 mM), diluted with Tris buffer to neutralize, then further diluted with the K^{+}-MES-Tris buffer to give stock solutions 100-times the final concentration required in the assay. Fluazifop-butyl (PP009, 90%) was dissolved in acetone (10 mM) before use. Over the pH range 6.3 - 5.5, the bathing medium buffering capacity was linear thereby allowing the rates of change of pH to be used as a direct measure of increase in $[H^{+}]$. For each treatment the rate of H^{+}-efflux was determined from at least two-to-four consecutive estimates of pH. Dose-response curves were analysed using a computer program developed by Weyers et al. (1987), based on a model of hormone-receptor complex formation at specific binding sites in plant cells.

RESULTS AND DISCUSSION

The effect of IAA on the pH of the coleoptile segment incubation medium is shown in Fig. 2. In the experiment shown 10 µM auxin induced rapid H^{+}-efflux after a lag period of 12 min and the rate of efflux was linear with respect to time for at least 30 min thereafter. Eventually, the rate of change of pH slowed down so that after 60 min no further substantial change in the pH difference relative to control was seen. At the lower

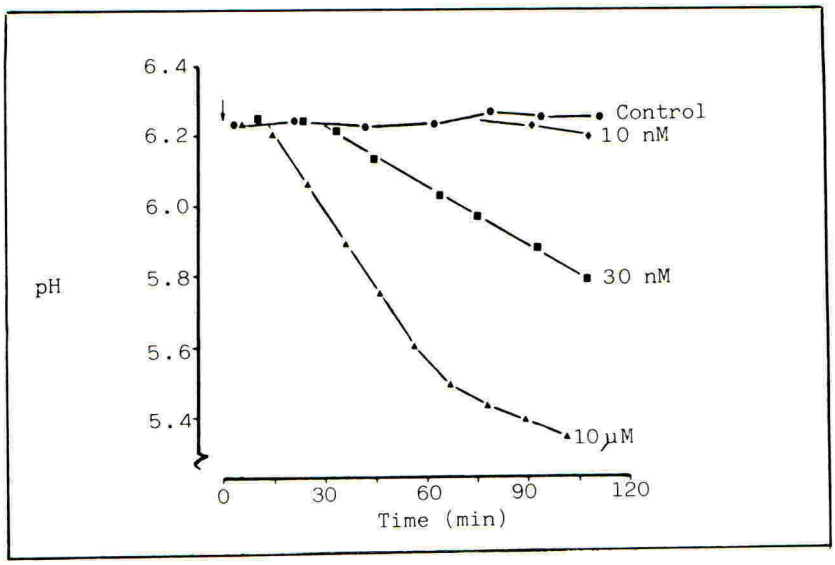

Fig. 2. The effect of various concentrations of IAA on bathing medium pH.

concentration of 30 nM, auxin was still able to promote appreciable rates of H^+-efflux in a linear fashion, with only a slightly extended lag period. At concentrations of 10 nM or less virtually no change in bathing medium pH was observed at all. Each curve of pH change against time has two important features: a) Lag period (L) ; b) Initial rate of response (R)

The lag-period for auxin-induced H^+-efflux varied as a function of external concentration (12-40 min, 10^{-4} - 10^{-8} M), however, irrespective of the length of the lag-period the rate of H^+-efflux was always linear with respect to time at least until the external pH had fallen to c. 5.5.

The relationship between the initial rate of H^+-efflux and external auxin concentration shows features common to a 'classical' hormone-receptor interaction (Fig. 3, O'Brien, 1979). The transition from 10 to 90% effect takes place over a narrow (physiologically relevant) concentration range and there is an even level of near-maximum response over a wide range of higher concentrations (this contrasts with low specificity and distinct optima often seen in growth curves; Nissen, 1985). These observations show that the rapid measurement of H^+-efflux is the most appropriate means of describing the response of Avena coleoptiles to various 'doses' of auxin. Data of this type (Fig. 3) can be analysed with respect to a simple model (based on Michaelis-Menten principles) which compares the rate of reaction (R) with the rate of 'hormone'(H)-receptor (Rec) complex formation.

$$\text{i.e.} \qquad H + Rec \underset{k_2}{\overset{k_1}{\rightleftharpoons}} HRec \xrightarrow{k_r} \text{Response } (H^+\text{-efflux})$$

where k_1, k_2 are rate-constants for HRec association/ dissociation and k_r is the rate constant for the series of events leading from the formation of HRec to the induction of H^+-efflux. It has been shown that such responses can be described in the following terms:

$$R = \frac{R_{max}\,[H]^{p}}{[H]^{p} + K_D}$$

where R_{max} = maximum initial rate of response
K_D = dissociation constant for HRec
$[H]_{50}$ = K_D^{1}/p, the 'hormone' concentration giving 50% response
p = interaction coefficient, allows deviations from Michaelis-Menten kinetics to be accommodated into the model.

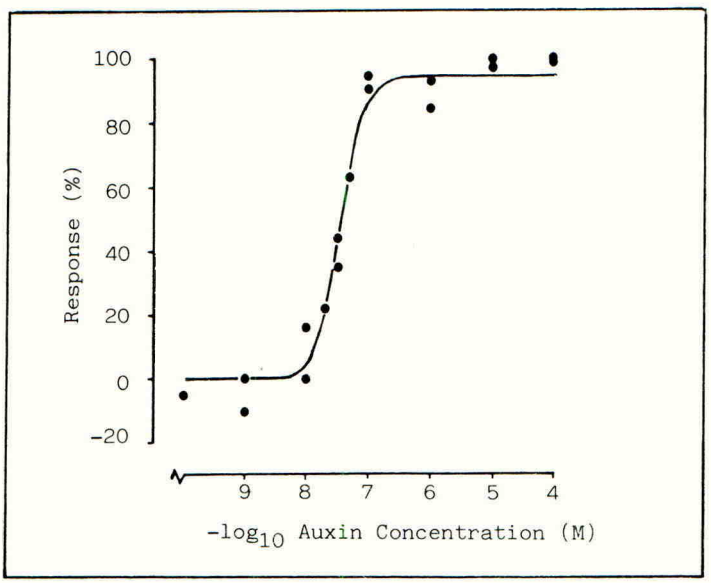

Fig. 3. IAA dose-response curve : initial rate of H^+-efflux. The data plotted were obtained in two separate experiments. The rate of response has been standardised so that the maximum observed rate (at 10^{-4}M) = 100. Maximum rates for each experiment were 1) 0.62 pH units/h 2) 0.87 pH units/h.

The above three parameters may be used to describe any dose-response curve and are termed 'sensitivity' parameters (Weyers et al., 1987). Using a computer program we have been able to make objective estimates of these parameters for auxin-induced H^+-efflux.

R_{max}	K_D	$[H]_{50}$	p
94.4	4.05×10^{-10}M	3.41×10^{-8}M	2.31

The relative activity of a number of different synthetic auxins (plus fusicoccin) is shown in Table 1. Fusicoccin is a phytotoxin known to promote high rates of H^+-efflux in a wide range of plant tissues, irrespective of their sensitivity to auxin (Marrè, 1979). The chemicals are grouped into activity classes as a crude means of defining their ability to mimic IAA in H^+-efflux assays. Of the herbicides tested, only benazolin was

able to promote H^+-efflux at appreciably faster rates than auxin (100-500 µM, 109-125%) although 2,4-D was able to induce final effects greater than auxin (data not shown). Clearly, fusicoccin and the auxin-herbicides may be less potent than auxin in terms of concentration but are characterised by causing more sustained levels of H^+-efflux. The resulting apoplast pH values of less than 5.0 are likely to cause toxic effects in target tissues.

Table 1. The H^+-efflux activity of Fusicoccin, synthetic auxins and auxin herbicides.

Chemical	Concentration (µM)	R (%)	L (min)	ACTIVITY
Fusicoccin	0.1	77	5	
"	1.0	179	3	HIGH
NAA	1.0	96	14	
Fluroxypyr	1.0	75	25	
"	10	101	17	
IPA	10	98	21	MEDIUM
2,4,5-T	10	85	16	
BAS 518	10	91	19	
2,4-D	10	83	20	
Benazolin	100	109	13	
BAS 514	100	75	20	
Fenoprop	100	70	16	LOW
Clopyralid	100	50	35	
"	1000	80	20	
2,3-D	10			
Ioxynil	100			NONE
Acifluorfen	100			
Fluazifop-H^+	100			

The data shown are those for the minimum concentration which gave rates of H^+-efflux close to those induced by an auxin standard (1-10µM; R = % relative effect).

Table 2. 2,4-D Sensitivity parameters.

Sensitivity parameter	2,4-D (A)	2,4-D + 100 µM Fluazifop-butyl (B)	$\frac{B}{A}$
R_{max}	117	119	1.0
K_D (µM)	2.1	14.8	7.2
$[H]_{50}$ (µM)	2.3	20.5	8.9
p	0.86	0.89	1.0

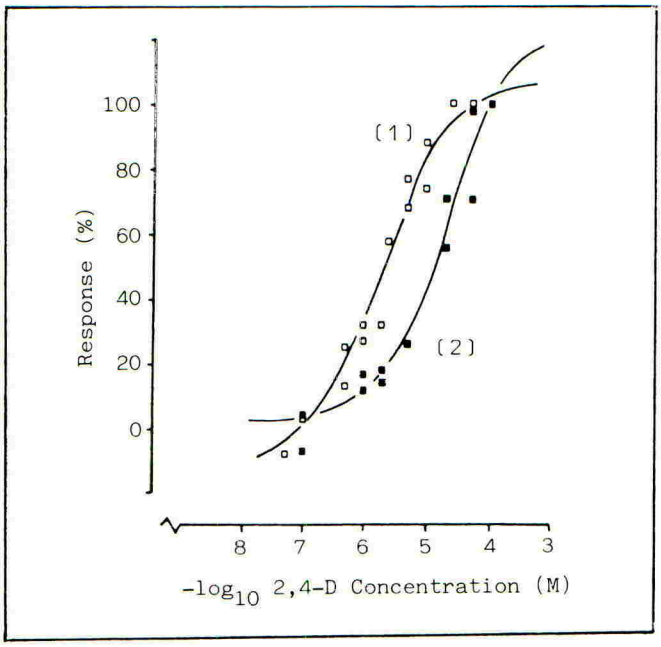

Fig. 4. 2,4-D dose-response curve ± 100 µM Fluazifop-butyl. Curve 1; 2,4-D only; Curve 2; plus fluazifop-butyl. Each curve represents the pooled data from two separate experiments. 2,4-D only : 100 = 0.65, 0.69 pH units/h. Plus fluazifop-butyl : 100 = 0.70, 0.82 units/h.

Fig. 4 (curve 1) shows the dose-response characteristics for initial rates of 2,4-D-induced H^+-efflux. 2,4-D (20-100 µM) can induce H^+-efflux at the same rate as auxin but is almost completely ineffective below 0.5 µM ($[H]_{50}$ = 2.3 µM, c. 70 x auxin $[H]_{50}$). Fluazifop-butyl alone was inactive in promoting H^+-efflux but was able to modify the activity of 2,4-D when the two herbicides were added simultaneously. Fig. 4 (curve 2) shows the dose-response curve for 2,4-D-induced H^+-efflux in the presence of 100 µM fluazifop-butyl. At high 2,4-D concentrations (100 µM) the graminicide did not affect rates of H^+-efflux but as the 2,4-D concentration was lowered an increasing level of inhibition was observed. Analysis of the two dose-response curves (Table 2) reveals that the affinity of the 'auxin' receptor for 2,4-D is reduced (K_D increased) by fluazifop-butyl although R_{max} remains unaffected. Such a pattern of effect is indicative of a simple competitive inhibition between these two herbicides for binding at an auxin-receptor site.

CONCLUSIONS

Whilst the elongation of Avena coleoptiles is a convenient bioassay for auxin activity, the rapid promotion of growth is not a direct response to auxin but an indirect consequence of apoplast acidification (Evans, 1985). The method described here for studying H^+-efflux allows the accurate determination of a rapidly induced auxin-mediated effect. Using this method we have obtained data which is suitable for analysis with respect to a molecular model of plant 'hormone' responses (Weyers et al., 1987).

As expected, several synthetic auxins and auxin-herbicides were active in a qualitatively similar fashion to IAA. The relative activity of each compound was determined by comparison with 1 to 10 μM auxin-induced rates of H^+-efflux (Table 1). The ability of each compound to induce H^+-efflux can be seen as a reflection of its ability to fit the auxin-binding receptor protein. Interesting information about structure-activity relationships can be obtained. For instance, the closely related herbicides fluroxypyr and clopyralid or BAS 514 and BAS 518 occupy different activity groups in Table 1. Even more acute dependency on specific molecular structure can be seen when comparing the activity of 2,4-D with that of 2,3-D.

We have applied the principles of hormone-receptor binding kinetics (cf. Michaelis-Menten analysis) to describe the auxin activity of 2,4-D in detail. Fluazifop-butyl, a graminicide susceptible to antagonism by 2,4-D in field applications, alters the dose-response characteristics for 2,4-D-induced H^+-efflux in a manner consistent with a simple competitive inhibition of 2,4-D/auxin-receptor complex formation. Fluazifop-butyl has no auxin activity alone but in combination with 2,4-D behaves as an anti-auxin. It cannot be determined from these studies whether fluazifop-butyl binds at the same or a different site as 2,4-D on the auxin receptor. The observed kinetics could be explained by fluazifop-butyl actually occupying the auxin binding site itself (scheme 1, Fig. 5) or by binding to a separate site on the receptor (scheme 2, Fig. 5) giving the same overall effect.

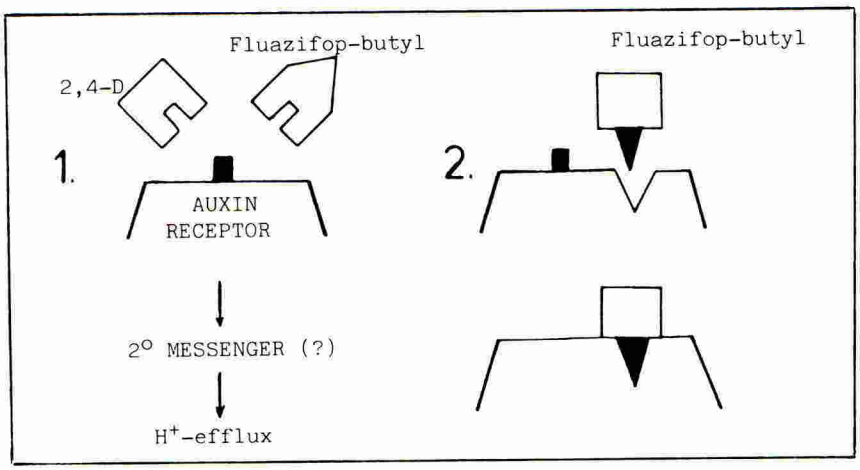

Fig. 5. Schematic representation of two models of fluazifop-butyl/2,4-D antagonism. Scheme 1: competition for common binding site. Scheme 2: separate binding sites; occupation of fluazifop-butyl site results in loss of 'auxin'-binding ability.

Can our experimental observations explain graminicide/broad-leaf weedkiller antagonisms observed in the field? The anti-auxin behaviour of fluazifop-butyl can be seen in target grass weeds which exhibit reduced internode elongation (eg. in Setaria viridis; Carr, 1986). In the presence of 2,4-D these sub-lethal symptoms may not develop, consequently resulting in increased vigour in the target weeds. The ultimate phytotoxicity of the phenoxypropanoates seems to be dependent on the accumulation of the acidic moiety (fluazifop, diclofop) in apical tissues where it inhibits lipid synthesis leading to necrosis and plant death (e.g. Carr et al., 1985, 1986; Shimabukuro et al., 1979). More information about the uptake,

hydrolysis and movement of graminicides in treated plants may reveal whether the short-term or long-term effects of 2,4-D on grass weeds can best explain the observed antagonism. We are currently extending our studies of H^+-efflux to include other graminicide/broad-leaf weedkiller mixtures. It should be possible in future to demonstrate whether anti-auxin properties are a general feature of the phenoxypropanoates and the extent to which such properties are the basis of herbicide antagonisms observed in the field.

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