Phytotoxicity of Protoporphyrinogen Oxidase Inhibitors: Phenomenology, Mode of Action and Mechanisms of Resistance

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Introduction

Over the past twenty-five years, herbicides that inhibit the enzyme protoporphyrinogen oxidase (Protox) have grown from a few experimental compounds to one of the largest class of patented herbicides (1). These herbicides were termed "peroxidizing herbicides" by some because they caused rapid membrane lipid peroxidization (2). However, their molecular site of action remained unknown for almost twenty years after their initial commercial introduction.

This herbicide group consists of a large number of bicyclic compounds (Fig. 1) that can be categorized according to their structural characteristics. The first commercialized subgroup of Protox inhibitors was the para-nitro diphenyl ethers (e.g., nitrofen, acifluorfen, oxyfluorfen). Oxadiazon (an oxadiazole) was the first non-diphenyl ether Protox inhibitor to reach the market. It can be classified with another chemical subgroup of Protox inhibitors, the phenyl heterocycles, which includes a broad range of compounds characterized by a phenyl group attached directly to the nitrogen of a heterocyclic ring. The heterocycle is generally a five or six constituent ring but can be a phenylimide (e.g., flumiclorac). Anderson *et al.* (1) include in this group the *O*-phenylcarbamates that have a phenyl ring connected to heterocyclic rings with a carbamate linkage. The third broad category of Protox inhibitors is the heterocyclic carboxamides. These are generally pyridones linked to phenyl groups by a carboximide linkage.

In addition to these examples, the patent literature indicates that there are thousands of compounds that might have this same mode of action. Although it is assumed that most of these compounds with structures and activities similar to those of known Protox inhibitors also have this mode of action, some of these may have multiple sites of action (e.g., acetyl Co-A carboxylase and phytoene desaturase (2)).

Several reviews of the Protox-inhibiting herbicides are available (2-10). This review is meant only to provide a summary of what we consider the most important information about this topic.
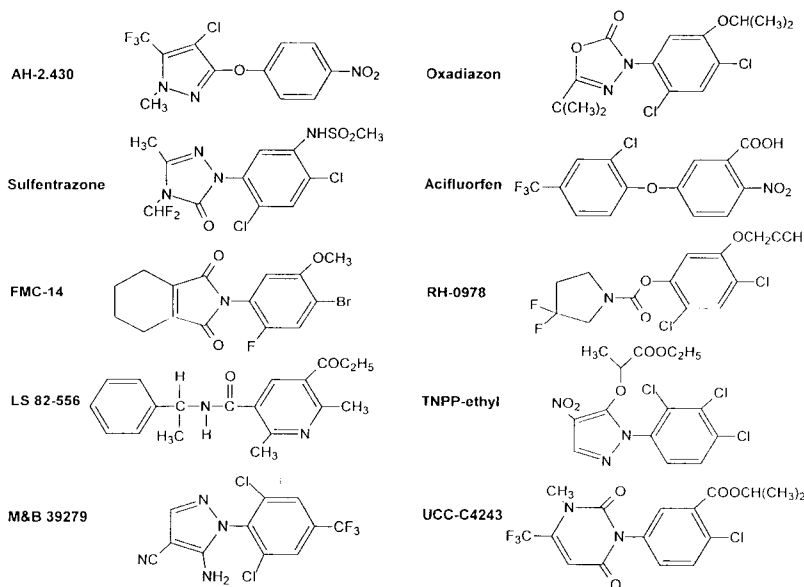
Figure 1. Protox-inhibiting compounds showing their typical phenyl heterocycle structural characteristics. Modified from (75).

Herbicidal Activity

Gross Effects

While Protox inhibitors are not intrinsically photodynamic, light is required for the development of injury to plants exposed to these herbicides. This structurally diverse group of herbicides induces fairly consistent gross effects on plants. Symptoms include leaf cupping, crinkling and bronzing (discoloration of the foliage with reddish brown appearance) and necrosis (11). As with most herbicides, the degree of damage is greater on younger tissues and proportional to the rate of application (12,13). These herbicides are fast acting, with evidence of injury occurring within a few hours of application. Symptoms generally progress from the appearance of water-soaked spots; color change from green to yellow, brown or black; wilting and desiccation (within 24 h); and chlorosis, usually after the appearance of necrotic lesions. Bronzing appears first in the veins and then as small spots over the foliage (14). The extent and persistence of symptoms depend upon the metabolic resistance of individual species. Resistant plants are temporarily injured and recover within weeks from the time of application (11,12,15-17). While most Protox inhibitors are commercialized as postemergence herbicides and have little soil activity, some of the more recently discovered compounds have excellent preemergence activity (18-20).

The selectivity of Protox inhibitors has been attributed to rapid metabolism of the toxophores into non-toxic derivatives (21,22). Conversely, sensitive weeds unable to convert the herbicide to inactive metabolites accumulate lethal concentrations of the parent compound, leading to extensive and irreversible cellular damage and ultimately to death (22). In addition to these well documented symptoms, Protox inhibitors may have other unusual effects on plants, the significance of which have not been determined. For example, root hair formation has been reported to be inhibited by a phenyl triazolinone Protox inhibitor in sensitive weeds (22).

Environmental Effects

Conflicting results have been reported about the effect of the environment on the phytotoxicity of Protox inhibitors. Relative humidity has a strong effect on their efficacy, with greater herbicidal activity at higher relative humidity (23,24). On the other hand, temperature does not directly influence the efficacy of these herbicides (23,25), although Lee and Oliver (12) reported higher herbicidal activity with higher temperature under low relative humidity. These environmental factors apparently modulate the absorption of the herbicides into plants (24) but do not influence the intrinsic herbicidal activity of the toxophores.

On the other hand, light is required for the development of injury in the treated tissues. The onset and degree of cellular damage to tissues exposed to light, following a period of incubation to acifluorfen in darkness, was temperature independent (26). This observation supports the view that the mechanism of membrane degradation leading to the injuries begins with a photochemical event. Elaborating on this concept, timing of herbicide application in the field is known to affect the efficacy of Protox inhibitors. Certain weeds are more affected by application of acifluorfen under conditions of darkness than applications in sunlight (12). This could be due to a fortuitous combination of the factors mentioned thus far. At night, herbicide absorption is greater because relative humidity is generally higher, and absorption can occur over a longer period, unhindered by light-induced cellular damage. This, in turn, causes greater accumulation of protoporphyrin IX (Proto IX), which leads to a more severe phytotoxic response upon exposure to light the next day. Overall, the undesirable effects of these herbicides on crops, such as foliar injury and stand reduction, are minimal relative to the increase in yields obtained by broad spectrum weed control (13,15,17,25,27,28).

A discussion of the mode of action of Protox inhibitors follows beginning at the molecular site of action and progressing to the physiological and biochemical consequences of inhibition. The literature on the mode of action of these herbicides was produced mostly in the reverse order, as it took a relatively long time to discover the mechanism of action of Protox inhibitors. However, we now have the knowledge to explain this complex mode of action in a logical, sequential manner.

Protoporphyrinogen Oxidase

General Considerations

The porphyrin pathway is ubiquitous to the biotic realm and leads to the synthesis of hemes (in all organisms) and chlorophylls (in photosynthetic organisms) (Fig. 2). Although initial precursors differ between plants and animals, the enzymes involved in these biosynthetic pathways are for the most part similar, particularly in the eukaryotes (29). The last enzyme in common between the two pathways is Protox. The subsequent reaction is catalyzed by either an Fe- or a Mg-chelatase that commits Proto IX toward either heme or chlorophylls biosynthesis, respectively.
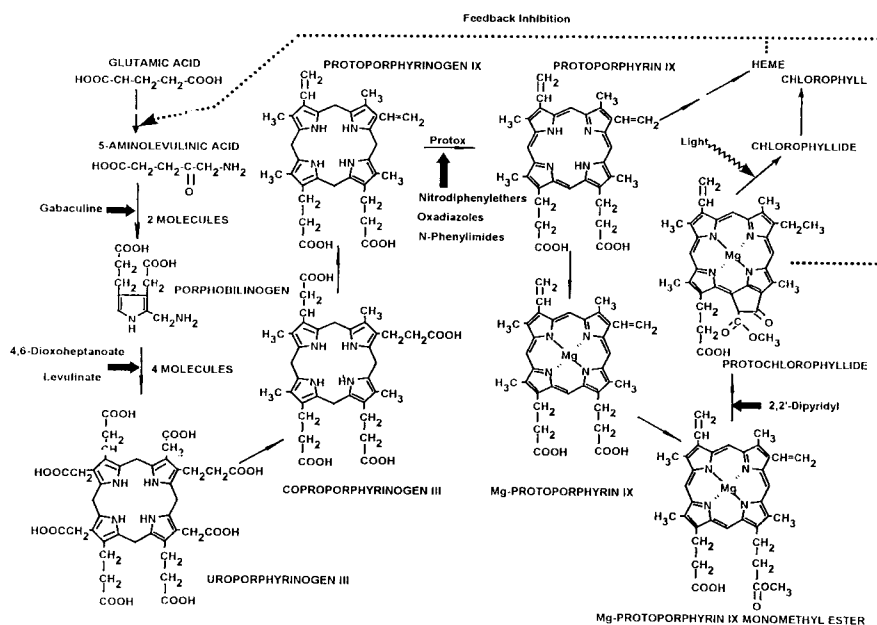

Figure 2. Chlorophyll biosynthetic pathway showing sites sensitive to chemical inhibition.

Mechanistically, Protox oxidizes the methylene groups linking the pyrrole rings to methenyl groups. Removing six hydrogens from the photodynamically inactive substrate, protoporphyrinogen IX (Protogen), converts it to a red, highly photosensitizing product, Proto IX (30,31). Almost nothing is known about sequence of events involved in this multiple oxidation process or the catalytic site on the enzyme. This lack of knowledge stems in part from the many difficulties encountered in studying this enzyme.

Because Proto IX is highly photodynamic and is self-sensitized in the presence of light (32,33), the biosynthetic pathway has been under strong selective pressure to minimize the transient cellular concentration of this chlorophyll precursor. Minimization of Proto IX concentration is evident in greening tissues, where Proto IX concentrations are barely

detectable, although carbon flow through the chlorophyll biosynthetic pathway is high. Tight control of free Proto IX appears to be accomplished in the mitochondria by having Protox and the subsequent enzymes organized in a loosely associated membrane complex (34), and one can reasonably envision a similar mechanism in the plastidic membranes.

Cellular Localization of Protox Activity

While Protox activity may be in the matrix or membrane-bound in prokaryotic organisms, it appears to be limited strictly to membranes in all eukaryotic systems. Activity is found in the mitochondrial inner membrane, chloroplast envelope and thylakoids, and the plasma membrane (35-37). Inhibition differs, with organellar forms being sensitive to herbicidal Protox inhibitors, and the plasma membrane Protox activity apparently not inhibited by the same compounds (Table 1) (38). Organellar Protox activity oxidizes various porphyrinogen substrates but has higher specificity for Protogen IX (35,36). Until recently, Protox activity found in the plasma membrane was not associated with the tetrapyrrole biosynthetic pathway and was believed to be a relatively unspecific peroxidase; it exhibited some substrate specificity, preferring Protogen IX over other porphyrinogen substrates (35). Jacobs and Jacobs (46) have now identified a plasma membrane ferro-chelatase, suggesting that heme synthesis may also occur in the plasmalemma. Most recently, a somewhat herbicide-resistant plant enzyme with Protox activity was found in the endoplasmic reticulum. Although the biological function of this enzyme has not yet been determined, it may also participate in the complex mode of action of Protox inhibitors (45).

Purification and Characterization

Although Protox has been partially purified in yeast and animal systems (44,47,48), purifying Protox in plants has proven difficult. Jacobs and Jacobs (36), first characterized Protox from barley organelles. This enzyme has a molecular weight of 36 kDa on a SDS-PAGE, and no differences between chloroplastic and mitochondrial forms were identified. The plant enzyme had a K_m of 5 μM for Protogen, which is similar to K_m values of Protox from other sources (Table 1). The pH optimum was between 5 and 6, a value much lower than that reported for mammalian enzyme (Table 1). Although the specific activity of the purified Protox was greatly enhanced relative to a crude etioplast preparation and the protein migrated as a single band on SDS-PAGE, antibodies elicited by this preparation were not able to inhibit Protox activity (unpublished data), leaving the exact nature of the purified band unresolved. It is possible, however, that the lack of inhibition by the antibodies could be due to conformational differences between the purified enzyme and the enzyme in its lipophilic native environment in membranes. The membrane may also protect the antigenic site.

Table 1.   Characteristics of protoporphyrinogen oxidase of different origins.

Origin	Mw (kDa)	Subunits	Cellular location	Herbicide sensitivity	$K_m{}^a$ (μM)	Optimum pH	Cofactor	Reference
Prokaryote								
D. Gigas	128	Three	Membrane	-	-	-	-	39
E. Coli	21	Single	Membrane	Tolerant	-	-	-	40
B. Subtilis	51	Single	Matrix	Tolerant	10.4	-	FAD/FMN?	49
M. Xanthus	49	Single	Membrane	Sensitive	1.6	-	FAD	42
Eukaryote								
Plastids	36	Single	OE	Sensitive	5.0	5-6	-	35,36,38
Mitochondrion								
Yeast	55	Single	IM	Sensitive	0.1	7.2	FAD	43
Animal	65	Single	IM	Sensitive	5.6	7-8	FAD/FMN?	41,44
Plant	36	Single	IM	Sensitive	5.0	5-6	-	35,36,38
PM	-	-	PM	Tolerant	-	-	-	35,38
ER	-	-	ER	Tolerant	0.4	-	-	45

[a]For protoporphyrinogen IX. Abbreviations: OE, outer envelope; IM, inner membrane; PM, plasma membrane; ER, endoplasmic reticulum.

Gene Identification

Relatively little information is available on plant Protox at the molecular level. Dailey *et al.* (49) reported the expression of a cloned Protox from *B. subtilis* in *E. coli*, and they designated this gene *hem G*. This gene encodes for a monomeric protein soluble in the cytoplasmic fraction of cells with an approximate molecular weight of 52 kDa. Concurrently, this gene was cloned from *E. coli* by other researchers (40). Protox native to *E. coli* is apparently distinct from the *Bacillus* form in that it has an approximate molecular weight of 21 kDa and is resistant to DPEs. Most recently, a mouse Protox was successfully cloned in *E. coli*, and a novel herbicide-sensitive prokaryotic Protox from *M. xanthus* was cloned and characterized in *E. coli* (42,50). Cloning of these (and other) herbicide-tolerant Protoxes into plants in attempts to generate transgenic crops resistant to Protox inhibitors is under way (E. R. Ward and S. Volrath, Manipulation of Protoporphyrinogen Oxidase Enzyme Activity in Eukaryotic Organisms, International Patent Application No. WO 95/34659, 1995).

In Vitro *Effects*

Inhibition. The mechanism of action of Protox inhibitors eluded scientists for several years. One reason was its novel mode of action, with symptoms somewhat similar to those observed in plants treated with bipyridilium herbicides. Therefore, physiologists first investigated better known herbicide target sites. Because the requirement for light was reported early on, the participation of known photoreceptors was investigated. While carotenoids (51-54) and photosynthetic electron flow (55) were thought to be implicated, inconsistencies with other experimental results pointed to a different molecular target (56-59). Other theories were proposed, including the direct involvement of the herbicides in the photodynamic reaction (60), involvement of the mitochondria (61),

photophosphorylation (62,63) and activation of the phenylpropanoid pathway (64). None of these studies unequivocally explained the physiological responses observed.

Much progress was made when it was discovered that the chlorophyll precursor Proto IX accumulated in DPE-treated tissues (57). Oddly, enzymes located downstream from the synthetic step of Proto IX, namely the Mg-chelatase were not strongly inhibited by these herbicides. Finally, Matringe et al. (65,66) reported the unusual condition by which Proto IX accumulated as a result of the inhibition of Protox, the enzyme involved in its synthesis. Proto IX accumulation had been reported in humans affected by the genetic disorder, porphyria variegate, which is caused by a dysfunctional Protox (67,68). This condition had never been observed in plants.

Protox is now known to be the target of a host of herbicide classes (Fig. 1). The reader is directed to Duke et al. (6) and Nandihalli et al. (69) for the general properties of these inhibitors and why Protox may be susceptible to such diverse chemistry.

Binding Affinity. Using tritium-labeled acifluorfen with a high specific activity, Matringe and Scalla (70) determined that acifluorfen binds to high-affinity binding sites with an apparent dissociation constant of 6.2 ± 1.3 nM in a purified pea etioplasts suspension. The relatively rapid dissociation (30 s for half dissociation) indicates that binding of the herbicide is readily reversible. Radiolabeled acifluorfen can be displaced with excess concentration of unlabeled acifluorfen or Protogen (Fig. 3). Furthermore, other Protox inhibitors tested also competed for the same binding site as acifluorfen, and the binding affinity was proportional to the ability of individual molecules to inhibit Protox, i.e.
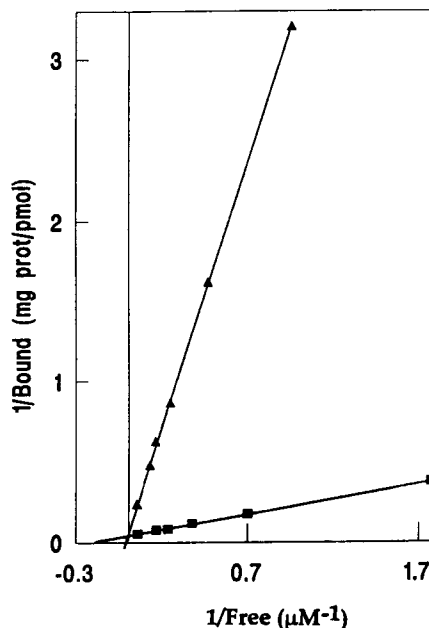

Figure 3. Double-reciprocal plot of competitive inhibition of [^{3}H]-acifluorfen binding to pea etioplasts by protoporphyrinogen IX. Binding in the presence of increasing concentration of [^{3}H]-acifluorfen alone (■) or with 5 μM protoporphyrinogen IX (▲). From (37).

Protox has greater affinity for compounds with a lower I_{50} than compounds with a higher I_{50} (71,72). We can infer from these sutudies that Protox inhibitors bind to an area associated with the catalytic site on the protein, since Protogen can also displace acifluorfen. The reaction catalyzed by Protox is not reversible because although Protogen could compete for the binding of acifluorfen, Proto IX could not displace acifluorfen from its binding to Protox, indicating a much lower affinity of the enzyme for Proto IX (70).

Similar results were obtained using solubilized Protox activity from etioplast membranes (73). *N*-phenylimide herbicides inhibited Protox in a competitive and reversible manner, much like the DPEs. The binding site affinity (Kd) was 8.9-9.8nM and this herbicide could be displaced by acifluorfen-ethyl. More recently, Nicolaus *et al.* (74) reported the binding affinity of [^{14}C]thidiazimin on membrane-bound Protox etioplast from corn. The mounting evidence confirms that the diverse classes of Protox inhibitors competitively bind to a similar region on Protox, and this region is associated with the catalytic site. Reddy *et al.* (75) found that unlabeled herbicides from ten different Protox inhibitor families displaced radiolabeled acifluorfen from etioplast membranes.

While mechanistic information may be deduced from these experiments, one need not go into such detail to evaluate the potency of Protox inhibitors. Inhibition of Protox in crude etioplast suspensions can be measured in a dose–response assay that permits calculation of the I_{50}s (Fig. 4).
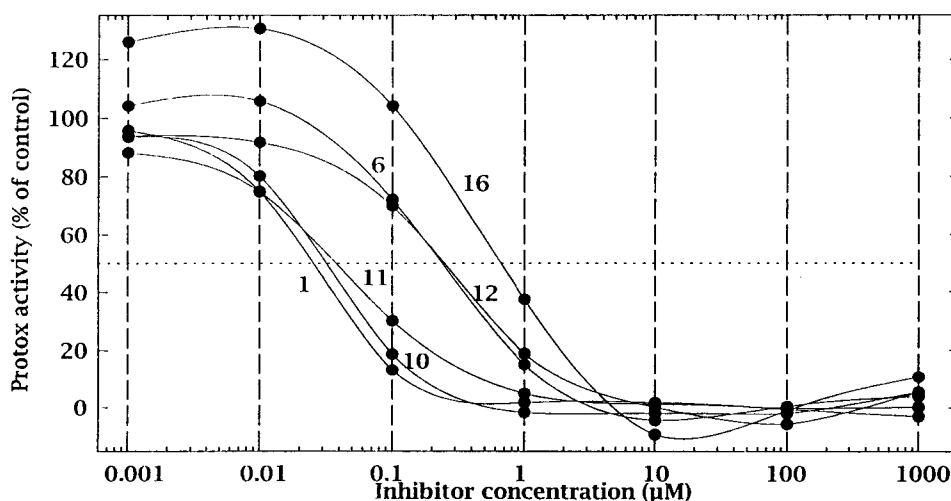

Figure 4. Typical dose-response curves for inhibition of Protox by herbicides. Dotted line indicates the I_{50}. From (78).

On a given species, there is often a good correlation between the I_{50} of various chemicals and their toxicity. For example, the level of Proto IX accumulation and phytotoxicity of phenopylate and 13 derivatives correlated well with their respective I_{50}s (72). The reliability of this technique enables approximation of activity by QSAR analysis (69,75). However, the situation becomes more complicated when the activity of a compound is compared among several species. The I_{50} obtained for a particular herbicide on a species may not reflect the species susceptibility. For example, the I_{50} of acifluorfen on mustard, cucumber and morningglory was similar (1-4 μM), although these species varied greatly in sensitivity to this herbicide (76).

Structure–Activity Relations. The influence of molecular structure on the effects of a herbicide on an intact plant is dependent on many processes. In the case of Protox inhibitors, the molecule must move across waxy or suberized barriers of leaves or roots, cross the plasma membrane and plastid envelope and bind to Protox. During the transport to the molecular site, the molecule might be metabolically transformed to a more-or-less active Protox inhibitor. The molecular structure can influence any of these processes. Thus, most studies relating molecular structure to activity of herbicides have dealt with how structure affects whole-plant herbicidal activity or physiological functions unrelated to Protox inhibition (e.g., 77). Very often there is little correlation between the activity of a herbicide at the molecular and whole-plant levels. However, we have found that in several cases that there does seem to be general correlation between whole-plant and molecular activities of Protox inhibitors (72,75,78,79).

Several studies have been published relating molecular characteristics of different groups of Protox inhibitors to their inhibitory potential (69,72,75,78,79). Reddy *et al.* (75) related the molecular characteristics of ten different chemical families of Protox inhibitors with Protox inhibition, Proto IX accumulation and herbicidal activity. However, considering the number of parameters needed to obtain multilinear regression equations indicating significant relationships, one or more important parameters may have been omitted. Indeed, these equations have not been useful in predicting molecular activity of stereoisomeric pairs (75), indicating that more steric parameters are needed to be successful in accurately predicting activity.

Perhaps the most useful information about Protox inhibitors obtained by computational chemistry has been to show that the more active compounds more closely mimic portions of the Protogen IX molecule than do the less effective inhibitors (Table 2, Fig. 5). Geometric parameters seem to best indicate this similarity. There is a less obvious similarity in electrostatic charge distribution with Protogen IX (75). Rather than empirically modeling the activity of Protox inhibitors at the molecular level with multilinear regression analysis and a large number of quantitative molecular parameters, it would be preferable to design new compounds based on knowledge of the Protox binding site(s) and on substrate/transition state(s).

Whether there is much demand for QSAR-assisted design of Protox inhibitors is debatable because finding compounds with excellent activity at the molecular level has been relatively easy without this approach. The most difficult aspect is understanding the factors affecting their selectivity, a problem perhaps best approached by other strategies (see section on Mechanisms of Resistance).

Table 2. Mean three-dimensional geometries and van der Waals volumes of one half of Protogen and five most active and five least active analogues from the diphenyl ether and phenopylate classes of Protox-inhibiting herbicides.

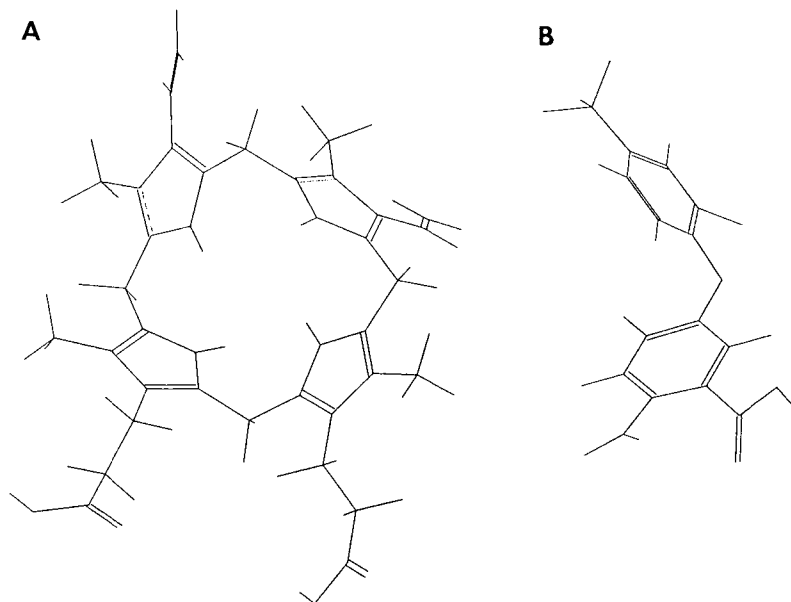| Substrate/ inhibitor	Å			Å³
	Length (y-axis)	Width (x-axis)	Height (z-axis)	van der Waals vol.
Protogen (half fraction)	11.6	10.6	11.9	220
Diphenyl ether (most active)	13.4	8.3	6.2	263
Phenopylate (most active)	10.8	9.3	4.9	241
Diphenyl ether (least active)	10.4	5.3	5.3	201
Phenopylate (least active)	8.6	6.8	4.5	181

Figure 5. 3-Dimensional optimized structures of (**A**) Protogen and (**B**) acifluorfen.

In Vivo Effects

Accumulation of Proto IX

Phenomenon. Proto IX rapidly accumulates to abnormally high levels in plant tissues or cells that contain Protox inhibitors (Fig. 6). Accumulation is often detectable less than an hour after treatment. This phenomenon is consistent in a large number of different plant tissues with numerous Protox inhibitors (6). Proto IX can accumulate to levels as high as 20 nmoles per gram of fresh weight; however, levels of only a few nmoles per gram of fresh weight are generally lethal. The kinetics of Proto IX accumulation is affected by the presence or absence of light. In the dark, Proto IX accumulates rapidly and reaches a concentration that remains constant for several hours (Fig. 6) (80,81). Proto IX does not accumulate indefinitely because of the feedback inhibition of the porphyrin pathway by protochlorophyllide (PChlide), which also accumulates (82). Levels of Proto IX were 350 to 450% greater in tissues exposed to red light than in those maintained in darkness. The light-driven conversion of PChlide to chlorophyllide (Chlide) alleviates the feedback inhibition of the porphyrin pathway. Far-red light did not affect the concentration of Proto IX (82), probably because it does not affect the level of PChlide.

In the presence of white light, a transient accumulation is observed within 2 to 4 h after treatment, but Proto IX concentrations rapidly decrease and return to pretreatment concentrations (Fig. 6) (80,83). Proto IX, which is known to be photooxygenated in the presence of light and oxygen in non-biological systems (33), may be converted to the green pigment photoprotoporphyrin. To our knowledge the existence of this molecule has not been reported in biological systems. Rapid herbicidal damage to the porphyrin pathway may also cause the concentration of Proto IX to decrease. Studies with tissues exposed to long periods of light prevented some researchers from detecting significant effects on Proto IX in their studies (e.g., 84). Maximal accumulation should occur under dim light. Under these conditions PChlide levels should be low with no significant photooxidative damage.

Although Proto IX is a reddish-brown pigment, it cannot be seen accumulating in treated green cells because there is much more chlorophyll than Protox IX. However, in tentoxin-treated tissues void of chlorophyll, Proto IX can be detected *in situ* with fluorescence microscopy (85). These studies indicate that it accumulates primarily outside the plastid, concentrating in the plasma membrane.

The only other pigments reported to increase upon Protox inhibition are Proto IX monomethyl ester (86) and a pigment fluorescing at 590 nm (87). The first of these, Proto IX ME, is a compound not thought to participate in the normal porphyrin pathway. We have speculated it is formed in the cytoplasm, where Proto IX levels are normally very low. The pigment fluorescing at 590 nm has not been identified; however, it has only been found in liverwort cells, and its absorption spectrum is similar to that of Zn-Proto IX.

PChlide content increases in light-grown tissues placed in the dark. However, levels are generally reduced by Protox inhibitors, but the inhibition is not complete. Data from several studies suggest that some cells are not affected by the herbicide (85,88). In a few cases, PChlide levels have been reported to be increased by Protox inhibitors (e.g., 85). The kinetics of accumulation suggest that the increase may be the result of eventual reentry of excess Proto IX generated by the herbicide into the chlorophyll pathway (85). In the light, chlorophyll levels in Protox inhibitor-treated tissues decrease in time (76,83), probably due to the loss of membrane integrity of the chloroplast apparatus, resulting in photooxidation of chlorophyll.
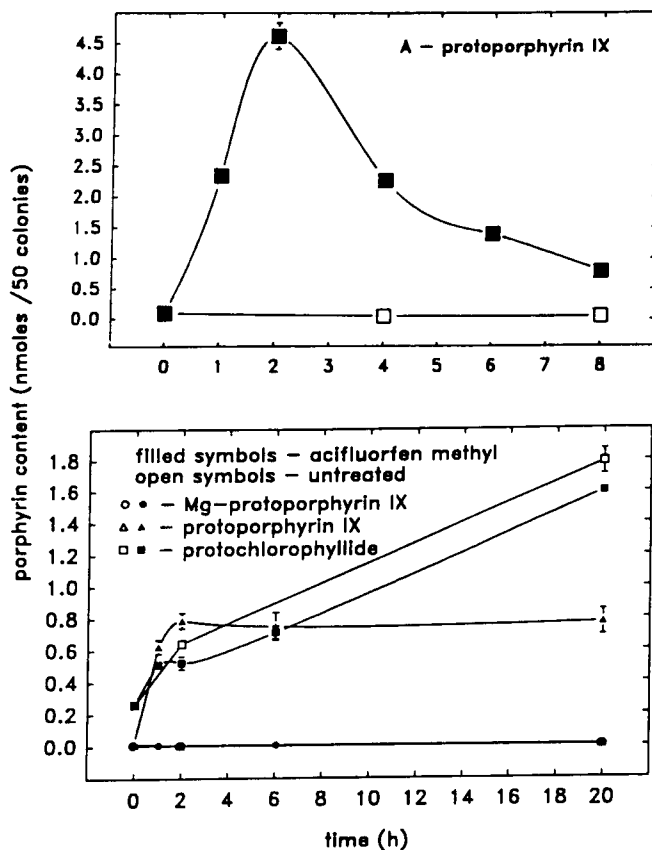
Figure 6. Kinetics of Proto IX accumulation in acifluorfen–methyl-treated duckweed in the presence of light (top) or incubated for 20 h in darkness (bottom). From (80).

Other intermediates in the chlorophyll pathway have been measured and do not accumulate in plants treated with herbicidal doses of the Protox inhibitors with which these analyses have been made (e.g., 80, Fig. 7). However, an analysis for all porphyrin and porphyrinogen intermediates has been carried out for only a few Protox inhibitors and in only a few plant species.

Mechanism. How does inhibition of an enzyme result in abnormally high accumulation of its catalytic product? It was broadly assumed that in animal cells with dysfunctional Protox, Protogen IX autooxidized to Proto IX (67). However, the rate of accumulation had never been measured. The very rapid accumulation of Proto IX in herbicide-treated plant tissues suggested that its production might be enzymatic (38). A great deal of circumstantial evidence indicates that this is the case.
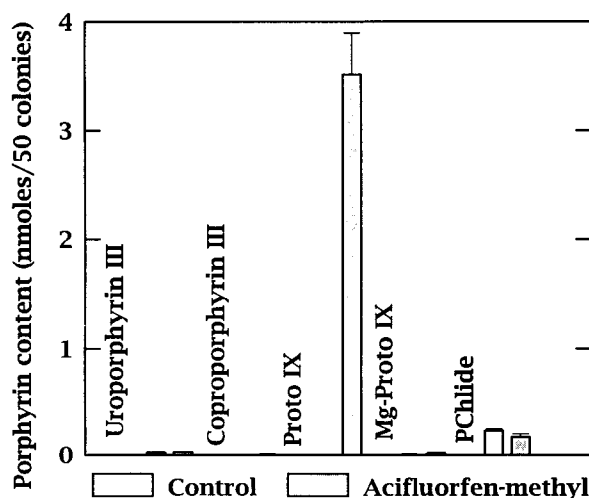
Figure 7. Effects of 1 μM acifluorfen-methyl on porphyrin content of *Lemna pausicostata* colonies after incubation in darkness for 20 h with or without herbicide in the media. From (6).

After inhibition of plastid Protox, the substrate, Protogen IX , is lost from the plastid to the cytoplasm (81,89), where it can be rapidly converted to Proto IX by a plasma membrane-bound, herbicide-insensitive, peroxidase-like enzyme (Fig. 8) (35,90). The actual biological role of this enzyme in plants has not been ascertained, although it has recently been shown that heme is produced by the plant plasma membrane (91), suggesting that this enzyme may be involved in an alternative, extraorganellar, heme biosynthetic pathway. Proto IX accumulates to levels in the plasma membrane and cytosolic components high enough to cause photodynamic damage in the light. Protox inhibitors are very effective, in part because partial inhibition of Protox is sufficient for Proto IX to accumulate. Furthermore, these competitive inhibitors are not likely to be displaced from their binding sites on the enzyme by an increasing pool of substrate because Protogen leaks from the plastid and is converted to Proto IX in the plasma membrane. The extraplastidic regions of the cell are less protected from photooxidative damage than is the plastid.

Secondary Effects

Membrane Damage. Proto IX is a strong photodynamic pigment that induces formation of singlet oxygen in the light (30,31). These highly reactive species induce oxidative peroxidation of polyunsaturated fatty acids. The mechanism of lipid peroxidation by oxygen radicals is beyond the scope of this review. Therefore, readers are referred to Devine *et al.* (92) for more information.

Membrane peroxidation can be estimated in several ways. Ethane and malondialdehyde are byproducts of fatty acid degradation, and their levels increase in herbicide-treated tissues exposed to light (Fig. 9). Synthesis of ethane in the presence of DPE and cyclic
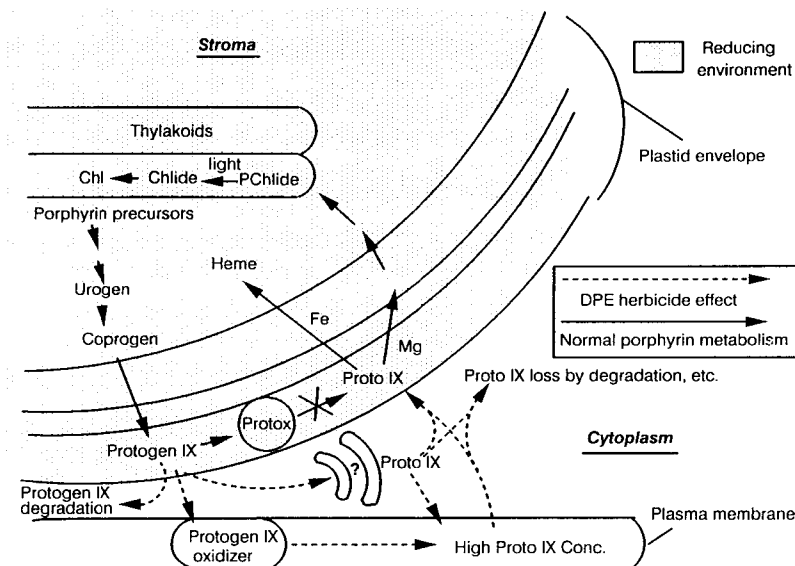
Figure 8. Scheme for porphyrin metabolism in diphenyl ether herbicide-affected plant cells. From (35).

imides appears to be correlated to Proto IX levels and has be used as a marker of membrane peroxidation from light-induced oxygen radical peroxidation (83,93,94). The same research team also found that herbicidal injury could be partially prevented by exogenous addition of antioxidant (α-tocopherol) and oxygen radical scavengers.

Loss of membrane integrity is also readily observed at the ultrastructural level. Kenyon *et al*. (93) reported the sequence of membrane degradation in acifluorfen-treated cucumber cotyledons. The first indication of herbicide damage is retraction of the plasma membrane from the cell wall and chloroplast swelling. Vesiculation is prominent in the cytoplasm after 1.5 h of exposure to light. The first signs of membrane degradation occur on the plasma membrane, and the density of mitochondria is dramatically reduced. Because plastids are designed to cope with oxidative stress during normal photosynthetic activity, their membranes were more tolerant to peroxidation than others. However, after 5 h of light exposure, thylakoids were dilated and the chloroplast envelope was destroyed. Similar results were obtained with oxadiazon.

This loss of membrane integrity can be conveniently, although indirectly, quantified by measuring changes in electrical conductivity of a bathing medium containing leaf tissues incubated with Protox inhibitors (Fig. 10) (93-95). There is a strong correlation between accumulation of Proto IX and the level of electrolyte leakage (69,72,78,81,96). Furthermore, conductivity increases with the intensity and/or duration of light received by treated tissues (94).
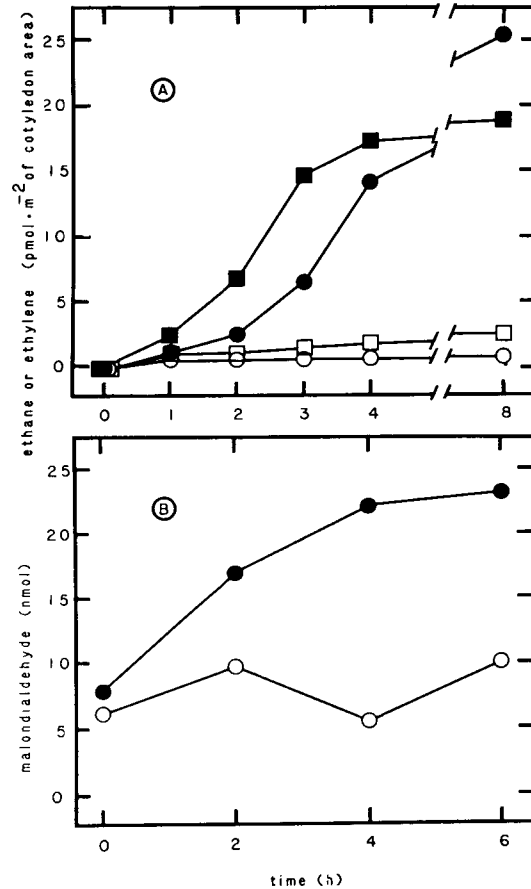
Figure 9. Changes in ethane (circles) and ethylene (squares) (A) and malondialdehyde (circles) (B) generated during illumination of cucumber cotyledons in the presence (filled symbols) or absence (open symbols) of acifluorfen-methyl. From (93).

Respiration. Acifluorfen decreases the CO_2-dependent O_2 evolution in cucumber cotyledons while not affecting cellular respiration (93). However, degradation of the mitochondrial apparatus in the presence of Protox inhibitors is evident, suggesting that the steady O_2 consumption might not be associated with respiration but with other physiological oxygen-consuming processes such as membrane peroxidation (92).

Induction of Stress Metabolism. Biotic and abiotic stresses long have been known to induce ethylene synthesis. As expected, ethylene synthesis increases in plants treated with Protox inhibitors (93). Although this might be an indiscriminate stress response rather than being herbicide specific, there is evidence that in some way ethylene synthesis enhances phytotoxic responses to diphenyl ether Protox inhibitors. Abdallah *et al.* (97) reported that plant lesions from acifluorfen treatments may be alleviated by pretreatment with ethylene
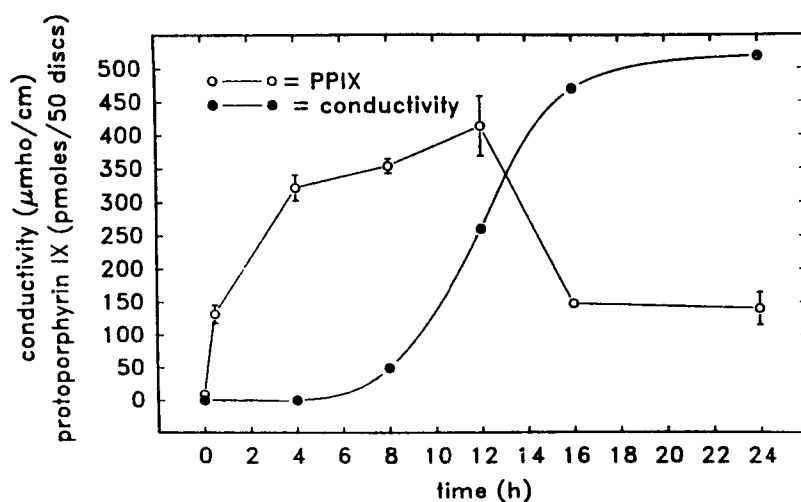
Figure 10. Simultaneous Proto IX accumulation and electrolyte leakage in cucumber cotyledons exposed to 10 μM acifluorfen in the presence of light. Error bars are 1 SE of the mean. From (88).

inhibitors. While more research is needed to elucidate the exact physiological participation of ethylene in the complex mode of action of Protox inhibitors, there are some data available on the activation of stress metabolism by acifluorfen.

Kömives and Casida (14) reported that application of the diphenyl ethers, acifluorfen and oxyfluorfen, increased the leaf content of several phytoalexins and stress metabolites in several crops (Fig. 11). This may be a significant factor under certain circumstances, where weeds outside the spectrum of the herbicide may also become more resistant to biocontrol agents or where crops may accumulate undesirable secondary metabolites. A positive aspect of this unexplained phenomenon is that Protox inhibitor herbicides could make naturally resistant crops more resistant to pathogens by this mechanism. Protox inhibitors participate in such a phenomenon because they induce relatively high oxidative stress at the cellular level, and oxidative stresses have been linked to phytoalexin accumulation and hypersensitive reactions (98).

More specifically, phenylalanine ammonia-lyase activity has been shown to increase 1,250% in acifluorfen-treated tissues exposed to light, while the activity was not changed by acifluorfen in the dark (64). In addition, acifluorfen stimulates the phenyl propanoid pathway, leading to accumulation of a particular phenolic amide (64). This phenomenon was not attributed to a generic herbicide stress response because 13 other herbicides tested did not induce similar levels of this particular phenolic compound. We have observed a similar phenomenon (unpublished) where acifluorfen-treated sicklepod accumulate high levels of a phytoalexin normally synthesized in response to *Alternaria cassia* (99). It should be noted that activation of phenolic metabolism is not unique to Protox inhibitors. Other herbicides with remarkably different modes of action may also stimulate this pathway in plants. PAL and chalcone isomerase activities are enhanced by chloroacetamide and sulfonylureas and lead to elevated levels of hydroxyphenolic compounds in maize and soybean (100). Levels of the phytoalexin gossypol levels were increased with triazines,
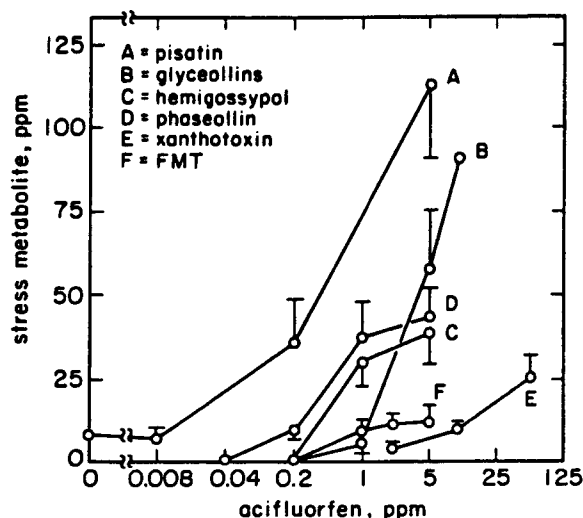
Figure 11. Effect of acifluorfen on accumulation of secondary metabolites. Leaf content of six secondary metabolites at various levels of acifluorfen with a 48 h exposure time. From (14).

prometryn and dalapon (101). However, Hoagland and Duke (102) found few herbicides to produce such an effect.

Mechanisms of Resistance

Plants resist most herbicides through three general mechanisms: 1) resistance at the molecular site of action, 2) rapid metabolic degradation of the herbicide, and 3) preventing the intact herbicide from moving from the site of application to the site of action (103). However, the complicated mechanism of action of Protox inhibitors provides several more sites at which resistance could evolve. Several of these sites are taken advantage of in cases of natural resistance. The mechanisms of natural resistance to Protox inhibitor herbicides have been thoroughly reviewed previously (104,105); we present only an overview of the current understanding on this matter.

Metabolic degradation of certain Protox-inhibiting herbicides such as acifluorfen and sulfentrazone has been associated in the resistance of both crops and weeds (21,22,104). We have found evidence in a more detailed study that the initial oxidative product of sulfentrazone metabolism in soybean and sicklepod had less *in vivo* activity than the parent material and that most also were less active on Protox (Dayan *et al.*, unpublished data). However, in most cases, natural resistance cannot be correlated with metabolic degradation (104-106).

Rice appears to be largely resistant to the photodynamic effects of Proto IX through protective mechanisms against the singlet oxygen-induced oxidative stress (105). We have found evidence that a soybean cultivar resistant to sulfentrazone was also more tolerant to peroxidative stress induced by rose bengal than were other cultivars (Dayan *et al.*, unpublished). Paraquat-resistant weeds with elevated levels of enzymes that protect against

peroxidative damage may be cross resistant to acifluorfen, suggesting that this mechanism could evolve in response to selection pressure with Protox inhibitor herbicides (107). However, claims that resistance of several crops was associated with ratios of specific endogenous antioxidants (108) have been unsubstantiated (76,104).

While resistance to Protox inhibitors at the molecular site of action has been speculated to occur in some naturally resistant species (104), most studies suggest that this mechanism may not exist in higher plants (10). The strongest evidence is that resistance at the site of action typically is associated with more than 100-fold resistance compared to that at non-altered target sites. This degree of difference in the I_{50} values of Protox inhibitors for Protox preparations from different sources has not been reported (10,76,104). Some of the more naturally resistant plant species such as rice and mustard have quite low I_{50} values (76,104). The relatively small variation in I_{50}s observed between Protox preparations from different species is likely due to factors other than intrinsic differences in susceptibility to the herbicide. For example, mustard Protox is sensitive to chemical inhibition, but Proto IX does not accumulate because Protogen is degraded to less phytotoxic forms (109).

Differences in activity of the herbicide-insensitive plasma membrane peroxidase that converts Protogen to Proto IX could also be a resistance mechanism. We have shown that *in situ* manipulation of the activity of this enzyme can lead to less Proto IX accumulation and thereby protect the plant against Protox inhibitors (Lee and Duke, unpublished). This mechanism deserves further study. The only clear differences in Protox response to inhibitors are between totally resistant bacterial Protox and the always-susceptible mitochondrial and plastidic forms of eukaryotic organisms.

Reduced uptake of oxyfluorfen was determined to be one of the factors contributing to natural resistance of rice to oxyfluorfen (104,110), and there is some evidence that it may contribute to natural resistance to other Protox inhibitors in other species (105). However, sulfentrazone was equally taken up and translocated to the foliage of resistant and less-resistant soybeans (unpublished data). There are no cases of sequestration of Protox inhibitors accounting for any case of natural resistance. Fig. 12 summarizes the known and potential mechanisms of resistance to Protox inhibitors.

Although several crop and weed species have natural resistance to Protox-inhibiting herbicides, there are no verified cases of evolution of resistance to these herbicides due to selection pressure with Protox inhibitor herbicides. Some weeds that have evolved resistance to paraquat in the field have some cross resistance to Protox inhibitors (107). In selection studies with mutagenized *Arabidopsis thaliana,* no non-lethal, acifluorfen–methyl-resistant mutants were found in several hundred thousand viable seeds (M. Yamamoto and S.O. Duke, unpublished). Sato *et al.* (111) reported a mutant of the unicellular alga, *Chlamydomonas reinhardtii,* selected with a Protox inhibitor to be cross resistant to a range of Protox inhibitors but not to DCMU. Direct proof that the mutant had a resistant Protox was not produced. However, oxyfluorfen-resistant, nonchlorophyllous soybean cells were selected that contained Protox that was 12-fold less sensitive to the herbicide than the wild-type cells (112). The resistant cells accumulated only about 25% less Proto IX in the light than the unselected cells. It is not known whether this would translate into a significant increase in resistance in a plant regenerated from these cells.

The absence of evolved resistance to Protox inhibitors in the field is surprising for several reasons. Protox inhibitors appear to have a single site of action at which they are highly potent as inhibitors at the molecular level. These inhibitors mimic Protogen, competitively inhibiting the enzyme. Thousands of structurally diverse, very herbicidally effective Protox inhibitors have been discovered, implying that the Protogen binding site is promiscuous, like the binding sites for other herbicides that have large numbers of
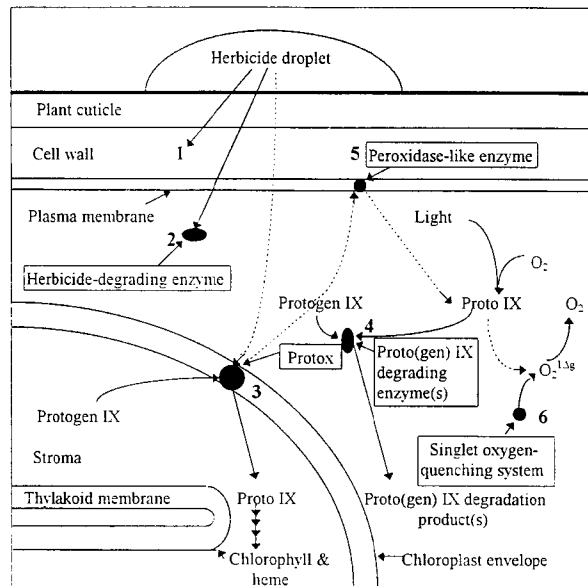
Figure 12. Schematic of possible mechanisms of resistance to Protox-inhibiting herbicides. The normal herbicide mechanism is denoted by dotted lines. Enzymes are black dots or ovals with names in boxes. Potential sites of resistance are in bold numbers: **1** - inhibition of uptake or sequestration of the herbicide; **2** - rapid metabolic degradation of the herbicide; **3** - herbicide-resistant Protox; **4** - degradation of extraplastidic Protogen IX and/or Proto IX; **5** - inactivated herbicide-resistant, extraplastidic Protox; **6** - quenching of singlet oxygen and other toxic oxygen species.

effective herbicides (e.g., D-1, acetyl-CoA carboxylase and acetolactate synthase-binding herbicides). While resistance to these other herbicides has evolved relatively rapidly, there have been no reported cases of a higher plant Protox being highly resistant to Protox inhibitors.

There is, however, natural variation in the susceptibility of weed populations to Protox inhibitors (e.g., 113). Weed resistance may be limited because of the usually short-lived selection pressure of most of these herbicides. However, if the present methods and rates of use of these herbicides are continued, resistance should evolve, as it has for paraquat, another fast-acting herbicide. In addition, the commercial availability of soil-active Protox inhibitors will lead to longer selection pressure, thus favoring the evolution of naturally occurring resistant mutants.

Crop resistance to Protox inhibitors could be manipulated by alteration of: the enzymes that degrade the herbicide, Protox, the enzyme(s) that degrade Protogen and/or Proto IX, and the herbicide-resistant, peroxidase-like enzyme that generates Proto IX in herbicide-treated plants. The herbicide-resistant Protox of prokaryotic organisms such as *E. coli* (40; E.R. Ward and S. Volrath, Manipulation of Protoporphyrinogen Oxidase Enzyme Activity in Eukaryotic Organisms, International Patent Application No. WO 95/34659, 1995) could

be moved to higher plants, but it is unlikely that this would produce a resistant plant unless the introduced enzyme could substitute for the plant's plastid, or mitochondrial Protox. This would entail adding the molecular processing information for targeting the proper organellar location as well as inactivation of the plastid and mitochondrial versions of Protox, perhaps by antisense genetic engineering. Insertion of a gene into the crop that codes for a herbicide-degrading enzyme (e.g., 114) might seem simple. However, the action of this enzyme would have to be rapid because Protox inhibitors work faster than any herbicides other than the bipyridiliums. The development of a Protox inhibitor-resistant crop is more problematic than for most other herbicides.

Toxicology

The toxicological effects of some Protox inhibitors have been reported (e.g., 115,116). Of course, commercially available Protox-inhibiting herbicides have passed toxicological evaluations to be approved for sale. However, most of these evaluations were made before it was generally known that these herbicides were inhibitors of mammalian Protox. Certainly, impaired Protox causes metabolic disorders in humans (67), and mammalian mitochondrial Protox is quite susceptible to Protox-inhibiting herbicides (10).

The effects of commercial Protox inhibitors on mammalian porphyrin metabolism has been reviewed (116). Protox-inhibiting herbicides generally elevate liver, bile and fecal porphyrins in mice; however, within days after withdrawal of herbicide doses that cause approximately ten-fold increases, levels returned to normal (116). The authors concluded that considering the doses of herbicides required to elicit an effect and the reversible nature of the effect, these compounds are not likely to be of significant toxicological risk because of their effects on porphyrin metabolism. Effects of Protox inhibitors on porphyrin metabolism vary considerably between species (115). However, others (117) have suggested that Protox-inhibiting herbicides might pose a toxicological risk for individuals with genetic defects in the porphyrin pathway, especially those with variegate porphyria, a serious disease caused by genetically impaired Protox. To date, there are no published reports of altered porphyrin metabolism in humans attributable to exposure to these compounds.

Protox inhibitors have been proposed as an alternative approach to photodynamic tumor therapy (118). Cultured human *HeLa* cells can accumulate toxic Proto IX concentrations when treated with very high concentrations of certain Protox inhibitors (118). Such treatments could lead to novel cancer treatment if these compounds could be selectively targeted to oncogenic tissues.

Summary

Protox-inhibiting herbicides have been commercially available for more than two decades. These chemicals are now widely used in agriculture. The development and recent commercialization of soil-active Protox inhibitors will further establish these herbicides in this competitive market. This is all the more likely since cases of cross-resistance to several other classes of herbicides targeting similar weeds are becoming more common. No cases of evolved resistance to Protox inhibitors have so far been reported.

The molecular site of Protox inhibitors has been established. Yet, many aspects of the interaction between these herbicides and their molecular target as well as the exact

mechanism of the reaction catalyzed by the enzyme remain unresolved. Furthermore, the physiological and biochemical events leading to the accumulation of catalytic product of the inhibited enzyme have not been completely characterized.

Although Protox inhibitors appear to have similar broad structural characteristics that apparently enable them to mimic the conformation of half the biological substrate, the nature of the binding site remains a mystery. Fortunately, answers may soon be available, as the recent cloning of a herbicide-sensitive Protox into *E. coli* now allows production of purified enzyme in large quantities. This undoubtedly will launch a series of new investigations into conformation of the binding site and the nature of the interaction between the substrate and these inhibitors at this site.

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