

Peroxidizing Herbicides (I): Mechanism of Action

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General physiological properties of peroxidizing compounds

Such compounds, also called “peroxidizers” induce a rapid loss of water and ions, the pigments bleach out and eventually the leaf becomes necrotic and brown. The plant is “burning down” as greenhouse screeners say. Phytotoxicity develops most rapidly on a sunny day with plants having been sprayed the day before in late afternoon. Light is necessary for this mode of action why the term “photobleaching herbicides” is also used. A striking feature are the low use rates required often surpassing those of the sulfonylureas; a disadvantage is the limited crop selectivity. Up to now the latter has hampered a breakthrough of peroxidizing herbicides for weed control in major crops. Nevertheless, essentially all leading agrochemical companies are interested in these compounds and invest their efforts accordingly. Figure 1 demonstrates the structures of modern peroxidizers which have recently entered field testing. Crops, use rates and treatment are also indicated as far as disclosed. As can be seen, all compounds listed include a similar chemical basic structure, namely a 2,4,5-trisubstituted aryl linked to a heterocycle, preferably via an N-atom. There are, however, substituted pyrazoles or pyridine carboxamides available which show peroxidizing activity as well as the older p-nitrodiphenyl ethers. The development of the chemistry of peroxidizers together

with some structure-activity considerations will be outlined in part II of this review.

The mode of action can most conveniently be measured quantitatively with liquid suspension cultures of green microalgae. *Scenedesmus acutus*, as used in our laboratory, can be grown in light as well as in the dark offering many possibilities to determine adequate peroxidative parameters with the intact cell. Figure 2 shows several typical markers. The first indication for a peroxidizer in action is the immediate halt of chlorophyll biosynthesis as demonstrated by part A of Figure 2 (Kunert and Böger, 1981; Wakabayashi *et al.*, 1986). This is observed in heterotrophic dark cultures (Nicolaus *et al.*, 1989) as well as in illuminated autotrophic ones. In the light, in addition to inhibited chlorophyll biosynthesis, a strong degradation of chlorophylls and carotenoids is observed. Photosynthesis is affected after a couple of hours (part B) and subsequently ethane is produced in the light (part C). Polyunsaturated fatty acids, major constituents of the acylipids of thylakoids and cell membranes are rapidly degraded leading to evolution of saturated short-chain hydrocarbons. The chain length of the alkane formed depends on the ω -number that indicates the location of the double bond at the reduced end of the fatty acid. Linolenic acid, an ω -3 polyunsaturated fatty acid, will form ethane, an ω -4 acid propane, an ω -5 species butane etc. (= “ ω -1 rule”; Sandmann and Böger, 1982). Propane, for example, shows up besides ethane in treated soybean suspension cell cultures (Böger and Nicolaus, 1993). Ethane determination with *Scenedesmus* is a convenient and quick assay for peroxidizing com-

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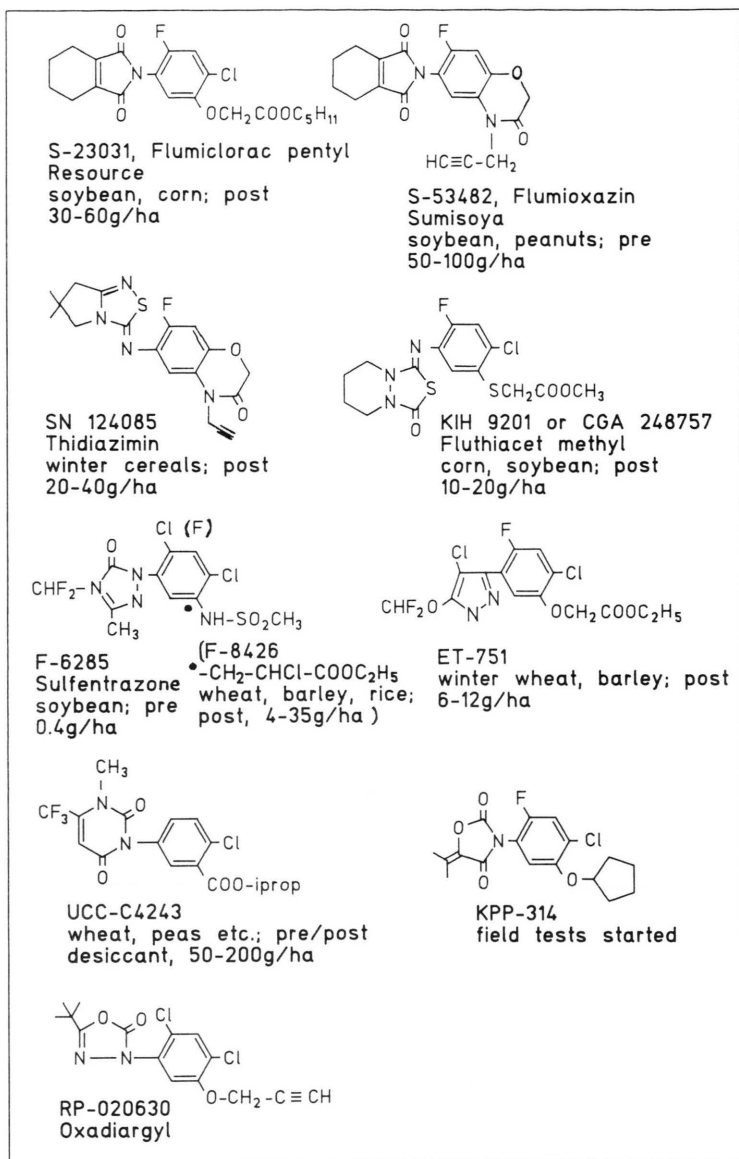


Fig. 1. Peroxidizing herbicides in development. The herbicides shown are from Hopkins (1994), the Brighton Crop Protection Conf. (several articles in Volume 1, Brit. Crop Prot. Council, Farnham, Surrey, UK, 1993), and from personal communications).

pounds applicable to greater series of chemicals using automatic sampling devices.

A further parameter easily detectable with either intact algae cells or with higher plant seedlings is the accumulation of protoporphyrin IX (Matringe and Scalla, 1988). Under moderate illumination a high level shows up within 2–3 hours after application and then gradually disappears apparently due to photooxidation (Fig. 2, part D). Gabaculine, blocking the tetrapyrrole biosynthesis

pathway (by inhibiting the formation of δ -amino-levalinate; Avissar and Beale, 1988) almost completely prevents proto-IX formation. In addition, degradation of pigments and light-induced alkane evolution is stopped. In the cell the accumulated protoporphyrin IX is sensitized leading to radicals which in turn attack the unsaturated fatty acids of the membranes, degrading them, causing ion leakage (Orr and Hess, 1981) and water loss of the cell. Accumulation of proto IX proceeds best in low

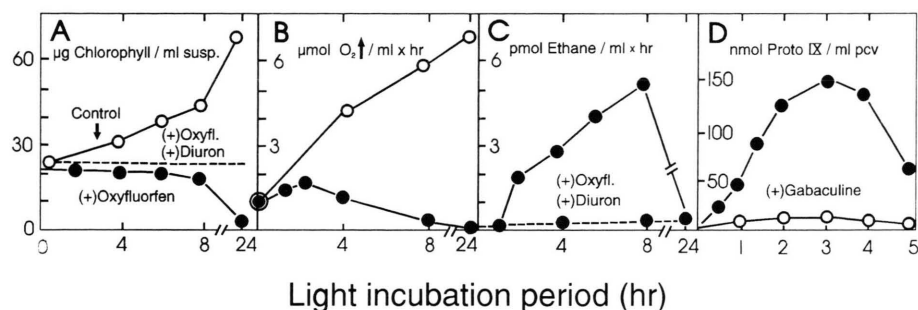


Fig. 2. Quantitative parameters measured with the intact autotrophic cell (*Scenedesmus acutus*) indicating peroxidative mode of action. In this example cells were treated with oxyfluorfen (see Table I for formula). Diuron is 3-(3,4-dichlorophenyl)-1,1-dimethylurea; gabaculine is 3-amino-2,3-dihydrobenzoic acid. Dashed lines: oxyfluorfen + diuron present.

light, which may explain the improved efficiency of peroxidizing herbicides applied before the plants are exposed to strong sunlight.

The diuron effect

Using photosynthetically competent autotrophic cultures, the "diuron effect" is observed with peroxidizers, namely the stop of chlorophyll degradation in the light with a photosynthesis inhibitor present. Also the light-induced production of ethane is almost completely stalled (Fig. 2, A, C, dashed lines; Kunert and Böger, 1981). The same holds for formation of protoporphyrin IX. With photosynthetic tissues or cells having stored carbohydrate reserves, this effect is absent. Apparently, not the photosynthetic electron transport itself is involved but pho-

tosynthates, ATP and reduced pyridine nucleotides are required to synthesize the components for the tetrapyrrole pathway (Nicolaus *et al.*, 1989). Nurit *et al.* (1987) showed the same effect with green cucumber cotyledon pieces as it could be demonstrated with autotrophic soybean suspension cultures and duckweed (Sandmann *et al.*, 1990; Böger and Nicolaus, 1993). This alleviation of peroxidation by photosynthesis inhibitors in autotrophic cells is essentially a safeguarding effect.

Protoporphyrin IX, protoporphyrinogen-IX oxidase

The tetrapyrrole accumulating in the plant cells treated with peroxidizing compounds is mostly protoporphyrin IX. This was proven, however, by

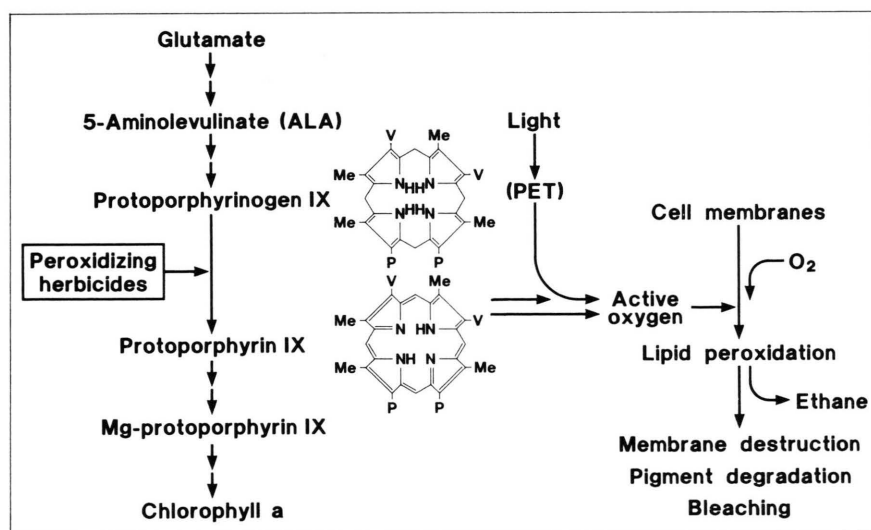


Fig. 3. Scheme of the three decisive steps leading to phytotoxicity of peroxidizing herbicides: (1) Inhibition of the chlorophyll biosynthesis pathway and accumulation of protoporphyrin IX in the cell. (2) Excitation of proto IX by light and activation of oxygen; radical formation. (3) Degradation of membranes, bleaching, ethane evolution. PET, photosynthetic electron transport, which is only indirectly involved (see text).

NMR and mass spectroscopy in *one* case only (Sandmann and Böger, 1988). There is evidence that tetrapyrroles other than proto IX may be formed (e.g. in liverwort cells; Iwata *et al.*, 1994). The target enzyme of peroxidizing compounds is protoporphyrinogen-IX oxidase (= protox, EC 1.3.3.4; Fig. 3) which oxidizes protoporphyrinogen (= protogen; Matringe and Scalla; 1988, Matringe *et al.*, 1989). The protogen precursors uro- and coproporphyrinogen are not oxidized (Duke *et al.*, 1994). The enzyme is located as a membrane-integrated protein in chloroplasts, mitochondria, and in the (outer) plasma membrane (Jacobs and Jacobs, 1987; Matringe *et al.*, 1992; Lee *et al.*, 1993). The plastidic and mitochondrial protox is instrumental in the biosynthetic pathway of chlorophylls and hemes. The peroxidizers are bound to protox competitively *vs.* the protogen (Camadro *et al.*, 1991; Nandihalli *et al.*, 1993) and reversibly (Nicolaus *et al.*, 1993a).

Protogen is overproduced since the feedback control by an endproduct of the chlorophyll biosynthesis chain is missing (protochlorophyllide?, Kotzabasis *et al.*, 1989). Protogen is readily oxi-

dized to proto IX which accumulates in the cell. Oxidation may occur non-enzymatically by oxygen and/or by other protox enzymes, which are not inhibited by peroxidizing herbicides (as claimed e.g. for the plasma membrane by Jacobs *et al.*, 1991; Nandihalli and Duke, 1993). Also a soluble protogen-oxidizing peroxidase has been reported (Yamato *et al.*, 1994). A soluble protox from chloroplasts of photomixotrophic tobacco cell cultures can also oxidize coproporphyrinogen (Yoshida *et al.*, 1993). For the assay of isolated membrane-bound protox see Nicolaus *et al.* (1993b).

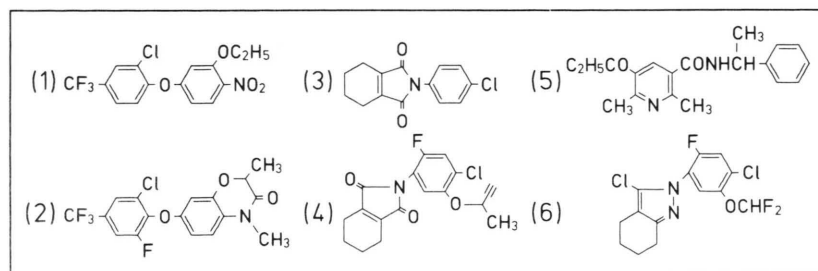
The herbicidal effect of peroxidizing herbicides could be quantitatively correlated with the inhibition of *Echinochloa* root growth (performed in the light in petri dishes; Wakabayashi *et al.*, 1979). Such studies were extended to other phytotoxic parameters as exemplified in Figure 2 (Wakabayashi *et al.*, 1988; Watanabe *et al.*, 1992). For some selected compounds Table I shows logarithmic half-inhibition and half-activation values (pI_{50} , pK_a) for the parameters mentioned in Figure 2 including data on short-term accumulation of proto IX. Active peroxidizers have low I_{50} values

Table I. Quantitative parameters of some selected peroxidizing compounds obtained with autotrophic *Scenedesmus acutus* cultures.

| Nos. | Peroxidizing compound | Growth inhibition pI_{50} | Decrease of chlorophyll pI_{50} | Ethane formation pK_a | Proto-IX accumulation $\frac{\text{nmol}}{\text{ml pcv}}$ | Protox* inhibition pI_{50} |
|------|-----------------------|--------------------------------|--------------------------------------|----------------------------|--|---------------------------------|
| (1) | Oxyfluorfen | 8.0 | 8.2 | 7.0 | 66 | 8.1 |
| (2) | Suam 16476 | 6.8 | 6.9 | 7.0 | 66 | 8.0 |
| (3) | Chlorophthelim | 7.0 | 7.1 | 6.0 | 68 | 7.6 |
| (4) | S-23121 | 7.4 | 7.5 | 7.0 | 75 | 9.0 |
| (5) | LS 82-556 | 5.7 | 5.6 | 5.0 | 20 | 5.3 |
| (6) | Shionogi 1 | 7.5 | 7.5 | 6.6 | 61 | 8.6 |

For the definition of the K_a value see Table II. Proto-IX accumulation refers to its short-term formation within 1 hr in the light using 1 μM of the peroxidizing compound indicated; pcv = packed cell volume (comp. Watanabe *et al.*, 1992).

* Inhibition of isolated maize protoporphyrinogen oxidase. The enzyme from *Scenedesmus acutus* cannot yet be assayed properly.



for protox. In Table II the regression equations are linearized by using the positive logarithms of the I_{50} values. The pI_{50} values for *Echinochloa* root growth and *Scenedesmus* growth inhibition, as well as the values for pigment decrease in autotrophic light and hetero-trophic dark *Scenedesmus* cultures, can be correlated quantitatively with the pI_{50} values for inhibition of isolated protox from maize (and other higher plants) and with light-induced ethane formation (pK_a values) by *Scenedesmus*. – Figure 4 demonstrates the regression lines for equations (4) and (6) of Table II. All data can be deduced from the primary target site, that is inhibition of protox leading to impaired chlorophyll biosynthesis as well as accumulation of proto IX. Accordingly, peroxidative data from *Scenedesmus* can be linked with those from higher plants; the investigator screening for peroxidative activity may choose that marker which fits best to his laboratory equipment.

The low I_{50} values (and even lower inhibition constants K_i ; see e.g. Nicolaus *et al.*, 1995 for details) for higher plant protox are responsible for the low use rates in the field. Furthermore, proto IX acts catalytically with respect to radical formation so that little proto IX present will give a substantial peroxidizing effect. At the moment, however, neither the stability of protogen or proto IX

in the cell is known, nor the turnover rate of oxygen activation by light-sensitized proto IX.

Questions, problems

A review as presented here, cannot discuss all questions and controversies which still exist relating to mode of action of peroxidizing herbicides. Some of them shall be briefly mentioned:

1) Why is chlorophyll biosynthesis blocked? In heme biosynthesis of mammalian mitochondria “substrate channeling” has been shown (Ferreira *et al.*, 1988) which may be effective in plastids, too. Overproduced protogen is oxidized outside the membrane-bound chlorophyll biosynthesis channel. Proto IX apparently cannot reenter the channel, unless present in high concentration. High levels of proto IX, indeed, stimulate some proto-chlorophyllide formation (Kouji *et al.*, 1989).

2) How is protoporphyrinogen oxidized, non-enzymatically or enzymatically? Both may happen, depending on the presence of reducing agents in the cell (glutathione, cysteine). When these are absent oxygen chemically reacts with protogen, and secondly membrane-bound protox enzymes are poorly inhibited without reducing compounds present. Conceivably, a plasmalemma-bound protox may oxidize protogen (exported) from the

Table II. Quantitative correlations of phytotoxic parameters obtained from various peroxidizing compounds.

| | | | | | | |
|-----|-----------|--------------------------------------|---|---------------------------------|--------|---------------------------------|
| (1) | pI_{50} | (<i>Echinochloa</i> root growth) | $= 0.775 pI_{50}$ (± 0.042) | (<i>Scenedesmus</i> growth) | -0.191 | [n = 167, r = 0.944, s = 0.354] |
| (2) | pI_{50} | (<i>Scenedesmus</i> growth) | $= 0.966 pI_{50}$ (± 0.019) | (Chlorophyll, L) | +0.093 | [n = 188, r = 0.991, s = 0.168] |
| (3) | pI_{50} | (Chlorophyll, D) | $= 0.910 pI_{50}$ (± 0.154) | (Protox) | +1.350 | [n = 23, r = 0.903, s = 0.538] |
| (4) | pI_{50} | (Chlorophyll, L) | $= 0.626 pI_{50}$ (± 0.134) | (Protox) | +2.331 | [n = 30, r = 0.875, s = 0.475] |
| (5) | pK_a | (Ethane) | $= 0.038$ [Proto-IX (1.0 μ M)] (± 0.005) | | +4.231 | [n = 55, r = 0.916, s = 0.390] |
| (6) | pK_a | (Ethane) | $= 0.729 pI_{50}$ (± 0.187) | (Protox) | +1.239 | [n = 18, r = 0.900, s = 0.493] |
| (7) | pI_{50} | (Chlorophyll, L) | $= 1.230 pK_a$ (± 0.232) | (Ethane) | -0.589 | [n = 16, r = 0.950, s = 0.480] |
| (8) | pI_{50} | (Carotenoid, L) | $= 1.205 pK_a$ (± 0.250) | (Ethane) | -0.424 | [n = 11, r = 0.964, s = 0.428] |

Data refer to *Scenedesmus acutus*, *Echinochloa*, and maize protox (isolated from etiolated seedlings). The algae were grown in heterotrophic dark liquid cultures (D) or cultivated autotrophically in the light (L). Therefore (D) refers to inhibition of chlorophyll biosynthesis only, while (L) includes this inhibition plus degradation of chlorophyll. For proto-IX formation of eq. (5) see Table I. *Echinochloa utilis* (sawa millet), line (1), refers to inhibition of its root growth (petri-dish assay in the light). The K_a value (“activity value”) is the molar concentration giving half of the maximum of light-induced ethane formation (Lambert *et al.*, 1983). In some papers this figure has been called I_{50} or pI_{50} , respectively.

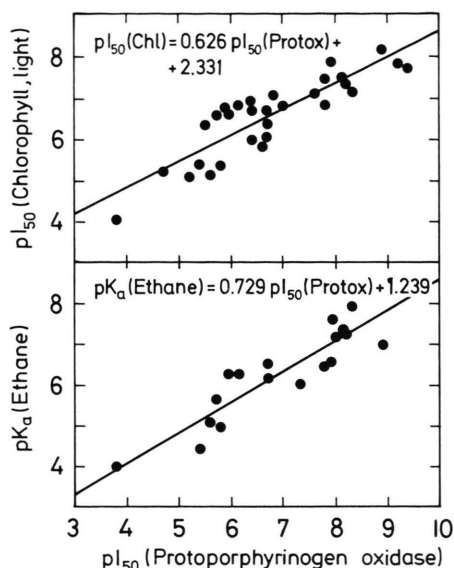


Fig. 4. Regression lines showing equations (4) and (6) of Table II, namely the quantitative correlation of inhibition of isolated maize protoporphyrinogen oxidase (pI_{50} Protox) to light-induced degradation of either chlorophyll (pI_{50} Chl) or ethane formation (pK_a Ethane) in *Scenedesmus*. Each data point refers to a different peroxidizing compound with different inhibitory activity, mostly of the cyclic-imide type.

plastid even in the presence of peroxidizers providing the level of reductants is low in its vicinity. Furthermore, there are peroxidases in the cell which may oxidize protogen unspecifically and which are insensitive to peroxidizing herbicides (Yamato *et al.*, 1995).

3) Location: Protox enzymes, membrane-bound and sensitive to peroxidizing compounds, are located in thylakoids, the plastid envelope, and in mitochondria (Camadro *et al.*, 1991; Matringe *et al.*, 1992). The Duke group, however, claims protox absent from thylakoids (Lee *et al.*, 1993).

4) What type of activated oxygen is formed? Generally, light-activated proto IX leads to singlet oxygen. We could show that proto IX can reduce e.g. nitro compounds in the light using glutathione as electron donor (unpubl. results). This implies that an "electron transport" through sensitized proto IX is possible which may produce superoxide anions (O_2^-) under certain conditions. Also illuminated chlorophyll can generate superoxide anions (You and Fong, 1986). The radical(s) pro-

duced in the illuminated GSH/proto IX-system are stable for an hour (checked by epr-data; Kastrau, 1993). This finding is corroborated by our observation that a substantial amount of ethane is evolved in the dark after *Scenedesmus* has been pre-illuminated with oxyfluorfen present. Ethane production in the dark depended on the length of the previous light incubation (B. Nicolaus, unpubl. results). Both dark and light ethane formation could be alleviated by radical quenchers like ethoxyquin (1,2-dihydro-6-ethoxy-2,2,4-trimethylquinoline).

5) Little work has been done to systematically check for differences in inhibitor sensitivity of plastidic and mitochondrial protox. With four different diphenyl ethers somewhat divergent I_{50} values and inhibition constants have been reported (Camadro *et al.*, 1991). Comparative inhibition studies including mammalian mitochondrial protox with modern peroxidizers in development should be intensified with reference to possible toxicology.

6) Characterization of purified protox: The biosynthetic enzyme from (etiolated) lettuce has been purified as well as that from yeast mitochondria (Camadro *et al.*, 1994). A problem is loss of activity of the membrane-integrated protein during purification with concurrent alteration of enzyme kinetics (own observations, maize). Plant protox appears to use oxygen or quinones as electron acceptors (Tietjen, 1991) but no decisive data are available at the moment (see also the discussion on protogen-IX oxidizers by Duke *et al.*, 1994).

7) Resistance due to impaired protox inhibition is known for mutants of eukaryotic microalgae like *Scenedesmus* (own results, unpubl.) or *Chlamydomonas* (Shibata *et al.*, 1992). As shown for the latter species the gene for herbicide-sensitive protox is nuclear encoded. No data on amino-acid exchanges are available as yet to indicate the binding region of protox for protogen and the inhibiting herbicides. Regrettably, only the prokaryotic gene is known and cloned (from *Bacillus subtilis*; Dailey *et al.*, 1994). This enzyme is not sensitive to inhibitors.

Resistance (tolerance) against peroxidizers has been reported for some plant species (Sherman *et al.*, 1991). It appears that generally tolerance is not based on an (altered) herbicide-insensitive protox but results from small proto-IX accumula-

tion. This may be caused by an (enzymic) degradation of proto-gen to non-porphyrin products (Jacobs *et al.*, 1994), impaired oxidation of proto-gen, or possibly by an altered translocation of proto-gen within the cell (see discussion of this topic in Jacobs and Jacobs, 1994). Possible enzymic degradations of these tetrapyrroles should be studied. Furthermore, antioxidative systems of the cell are effective against herbicide-induced radicals (Finckh and Kunert, 1985; Gullner *et al.*, 1991). The antioxidative components (e.g. ascorbate, glutathione) and enzymes can be manipulated (Sandmann and Böger, 1990) and induced by peroxidizing herbicides (Schmidt and Kunert, 1986) leading to differential phytotoxicity.

8) Safening: This has not yet been studied in detail. Matsunaka and Wakabayashi (1989) and recently Devlin and Zbiec (1993) have shown that injury of maize by peroxidizing cyclic imides could be prevented by safeners. Data on very effective impairment of proto-IX accumulation in maize and cress seedlings have been published by treatment with safeners like naphthalic anhydride or BAS 145 138 (Böger and Miller, 1994). The mechanism is studied at the moment.

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