Typical Peroxidative Parameters Verified with Mung-Bean Seedlings, Soybean Cells and Duckweed

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Peroxidation is indicated by several physiological parameters: (1) leakage of cell membranes and production of short-chain hydrocarbons, (2) degradation of cell constituents, (3) inhibition of chlorophyll biosynthesis, (4) accumulation of tetrapyrroles (protoporphyrin IX), (5) inhibition of protoporphyrinogen oxidase by peroxidizers, (6) alleviation of peroxidation by inhibitors of chlorophyll biosynthesis, (7) alleviation of peroxidation by photosynthesis inhibitors ("diuron effect"), (8) counteraction of the diuron effect by glucose (heterotrophic conditions). Such parameters, described and elaborated with microalgae and higher plants, are verified in this study with soybean cells, duckweed and mung beans. Preliminary data indicate lack of quantitative relationship between inhibition of protoporphyrinogen oxidase, protoporphyrin (IX) formation and peroxidative consequences.

Introduction

Light-induced peroxidative degradations lead to phytotoxic consequences, and this mode of action is well acknowledged for possible future herbicides. *p*-Nitrodiphenyl ethers were the first herbicides of this type developed and introduced for weed control [1, 2], followed by the tetrahydrophthalimides as the next family of peroxidizing compounds [3, 4]. Although a breakthrough as widely-used herbicides is still missing for all of the "peroxidizers" interest both in the producing industry and in basic research has been focused on peroxidative mode of action, since phytotoxic activity is often remarkably high (the dosis is down to 1 g/ha a.i. and lower). Many active compounds have been synthesized in the last years (see ref. [5] for some examples).

Peroxidation exhibits typical physiological features, and most of them can be reliably measured when appropriate biological systems and conditions are used. Firstly, radicals are initiated by visible light, affecting cell constituents like acyl lipids thereby producing short-chain hydrocarbons, malondialdehyde and membrane deterioration with leakage of water and low-molecular weight vacuole contents [1, 6, 7]. Secondly, in contrast to biosynthesis inhibitors, which only stall the increase of the respective constituents in the cell, peroxidizers cause degradation leading to an absolute *decrease* of the radical-attacked cellular compounds (like lipids, chlorophylls, carotenoids). Thirdly, chlorophyll biosynthesis is immediately and strongly inhibited by peroxidizers [4, 8]. Forthly, tetrapyrroles accumulate [9–11], most probably protoporphyrin IX as has been analyzed for *Bumilleriopsis* [12]. However, it is unclear whether other porphyrin precursors may also accumulate under the influence of peroxidizing compounds, or whether chemical modifications take place at the tetrapyrrole(s). Peroxidizers inhibit protoporphyrinogen oxidase [13, 14] and recent measurements imply that this is a fifth reliable parameter. All compounds leading to accumulation of protoporphyrin inhibit this enzyme. Apparently, excess protoporphyrinogen is nonenzymatically oxidized to protoporphyrin. There are preliminary data suggesting that only part of it is effective in peroxidation, which possibly has to be bound to certain thylakoid peptides ([15], own observations) since soluble protoporphyrin IX has little peroxidative activity [12]. Protoporphyrin acts as photosensitizer to produce starter radicals whose nature,

Chemical names: chlorophthalim, N-(4-chlorophenyl)-3,4,5,6-tetrahydrophthalimide; diuron, 3-(3,4-dichlorophenyl)-1,1-dimethylurea; gabaculine, 3-amino-2,3-dihydrobenzoic acid; metribuzin, 4-amino-6-*tert*-butyl-3-methylthio-1,2,4-triazin-5(4H)one; oxyfluorfen, 2-chloro-4-trifluoromethylphenyl-3-ethoxy-4-nitrophenyl ether.

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however, is not yet known. It still has to be shown whether photosensitation produces singlet oxygen or a protoporphyrin anion, the latter leading to hydroxyl radicals (see ref. [16]). A sixth characteristic attribute is the alleviation of peroxidation by inhibitors in the biosynthetic pathway of chlorophyll, like gabaculine or 4,6-dioxoheptanoic acid. Only plastid-containing cells are able to respond to peroxidizing compounds. Herbicide-induced chlorophyll degradation is stalled with gabaculine. Decrease of peroxidation by applying both the peroxidizer in question and gabaculine (to *e.g.* duckweed) is appropriate evidence indicative of whether a phytotoxic compound has peroxidative activity or not.

In autotrophic cells photosynthetic electron transport is required for tetrapyrrole accumulation and subsequent peroxidation. Both processes are also observed under a semi-anaerobic (nitrogen) atmosphere [17]. Requirement of photosynthesis (in higher plants) is also evidenced by a recently published action spectrum [18]. Accordingly, as a seventh peroxidative feature, diuron, metribuzin and other photosynthesis inhibitors protect against peroxidation. Why electron transport is needed – either to supply NADPH or C-precursors for tetrapyrroles or both – is still an open question. It should be mentioned that photophosphorylation is stimulated with photosynthesis inhibitors present. As item number eight it should be mentioned that photosynthesis can be substituted by glucose *i.e.* by heterotrophic conditions. Then, a respiratory breakdown will allow for protoporphyrin formation in light or darkness. In the light, peroxidation will occur and diuron has no effect [17]. Seedlings or cotyledons which have often been used in herbicidal peroxidation studies apparently represent a mixed-type metabolism. This may explain why the alleviating effect of diuron – which has first been found with autotrophic microalgae [19] – could either been confirmed or not (see ref. [17] for literature con and pro). Improvement of tolerance (of field-grown soybeans) against a peroxidizer has recently been reported by spraying a mixture of bentazon and acifluorfen [20].

When peroxidizing herbicides are applied to the (autotrophic) plant in the field light is used in three ways: (a) for the build-up of protoporphyrin (IX), (b) for photosensitation of the tetrapyrrole and formation of the starter radical, (c) for photodestruction of the activated tetrapyrrole. It appears that these three processes need different light intensities [17]. Furthermore, process (a) is counteracted by process (c). Conceivably, these light effects on peroxidation are complex and have produced conflicting results. Careful discrimination by appropriate experimental conditions are needed.

The phytotoxic activity of a peroxidative compound can thus be measured by the typical parameters outlined. However, a quantitative correlation between inhibition of protoporphyrinogen oxidase, the level of protoporphyrin in the cell, and the peroxidative consequences is by no means certain at the moment. We do not know reaction constants or time courses for protoporphyrin formation and its photodestruction, the type of radical formed, or whether only a part of the protoporphyrin will effect peroxidation.

This paper presents data on typical peroxidation parameters as outlined using a member of the tetrahydrophthalimides (cyclic imides) as example and higher plant (cells) as biological specimen. It will be shown that typical parameters originally elaborated in part either with microalgae or higher plants can be measured with higher plant cells when certain precautions are taken.

Materials and Methods

Mung-bean seedlings (*Phaseolus radiatus* L., syn. *Ph. aureus* Roxb.) were grown for 1 week in pots on vermiculite under white light at 50 $\mu E/m^2 \times$ sec (for experiments described in Fig. 1) or as hydroponic cultures in darkness for all other experiments. The soaked seeds were placed on a net, and this was positioned adjacent to a water surface in a closed chamber. After a 6 day germination period in the dark the seedlings were illuminated with 30 $\mu E/m^2 \times$ sec white light for 3 h before harvest to start the greening process. Dry and fresh weights of leaves as well as their malondialdehyde content [21] were determined 24 h after spraying the plants with 5 ml per pot of the herbicide suspension including 0.1% Tween 40 (Fig. 1). Short-chain hydrocarbons (Fig. 4) were measured after incubation of 10 leaf pairs in 3 ml of 10 mM phosphate buffer, pH 7.5, for 16 h at about 200 $\mu E/m^2 \times$ sec and 22 °C. The hydrocarbons were automatically withdrawn from the sealed head-space vessels and

quantitated by gas chromatography according to [22]. Leakage of endogenous cellular phenolics was determined in the reaction medium by recording the absorbance at 320 nm and multiplication with the total volume (ml). Protoporphyrin in mung beans was determined from 1 week old etiolated seedlings by suspending 20–30 leaves in a chlorophthalim suspension as mentioned above for about 20 h in the dark (Fig. 4). The tetrapyrrole was extracted with a mixture of acetone/1 N NH_4OH/50 mM HEPES buffer, pH 8, for 30 min at 55 °C and quantitated by its fluorescence emission at 632 nm as previously described [12].

Protoporphyrinogen-oxidase activity of mung beans was determined according to [23]. Membranes used in this assay were isolated from etiolated leaves which were pre-illuminated for 6 h at 50 $\mu E/m^2 \times sec$ before harvest. The leaves were ground for 10 sec in a blender in a medium of 0.5 M saccharose; 1 mM $MgCl_2$; 30 mM HEPES buffer, pH 7.7, and 0.2% bovine serum albumin. Then the slurry was filtered through cheese cloth and centrifuged differentially first at $150 \times g$ for 1 min and the supernatant for another 2 min at $10{,}000 \times g$, the residue suspended in the homogenization medium and aliquots were taken for the assay. Experimental details for the corn oxidase were similar and will be published elsewhere.

Results and Discussion

Fig. 1 demonstrates the "drying" effect of a peroxidizing compound in the light. Chlorophthalim has been applied as example but oxyfluorfen gave the same results. The increase of dry weight (see ref. [23]) in the middle of the figure was counteracted by a simultaneous spray with gabaculine. Table I shows the influence of gabaculine and diuron on short-chain hydrocarbon formation induced by chlorophthalim. Both inhibitors decreased peroxidation. As expected from previous studies [17] the "diuron effect" is not that pronounced as with *e.g.* autotrophic algae cells. Seedlings greened for some hours as used here are not yet fully photosynthetically competent. However, heterotrophic and autotrophic conditions can be experimentally separated with *soybean cell* cultures. Fig. 2 demonstrates the influence of chlorophthalim (or oxyfluorfen which yielded the same results at 0.5 μM) on light-induced propane, ethane

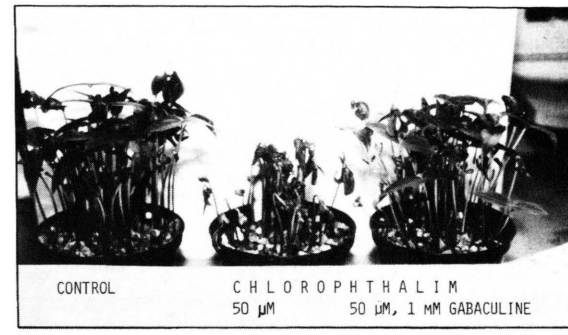

Fig. 1. One-day treatment of mung-bean seedlings with chlorophthalim and protection by gabaculine. The ratios of dry weight/fresh weight were 0.118, 0.158 and 0.119 for control, chlorophthalim treatment (middle), and treatment with a combination of chlorophthalim and gabaculine (right), respectively. The accumulated malondialdehyde in leaves was determined as 22.4, 44.8 and 26.8 nmol/g dry weight, respectively.

and ethylene formation. As demonstrated by the upper part of the figure in autotrophic cells diuron completely abolished light-induced hydrocarbon formation (No. 2), diuron alone had no effect (No. 1, upper part). The mixotrophic cell culture (grown with glucose in the light = mixotroph), did not show a response against diuron. No impairment of hydrocarbon evolution by diuron under the influence of the peroxidizing herbicide was seen (comp. lower part, Nos. 2, 3). It should be noted, that in contrast to autotrophic *Scenedesmus* ethylene always appeared in substantial amounts together with ethane and some propane. The heterotrophic cells evolve some ethylene with only

Table I. Influence of gabaculine (3 mM) and diuron (5 μM) on peroxidative formation of ethane and propane induced by chlorophthalim (5 μM) in the light.

Additions	Ethane, propane (pmol/mg dry weight)
Control, + diuron	1.04
(+) Chlorophthalim	30.1
(+) Chlorophthalim, gabaculine	0.7
(+) Chlorophthalim, diuron	12.5

Etiolated mung-bean leaves were illuminated for 6 h at 50 $\mu E/m^2 \times sec$ to induce greening. Then 10 leave pairs were suspended in 3 ml phosphate buffer, pH 7.5, the compounds added as indicated and illuminated for 16 h at 200 $\mu E/m^2 \times sec$ white light, 23 °C, for peroxidative short-chain hydrocarbon production.

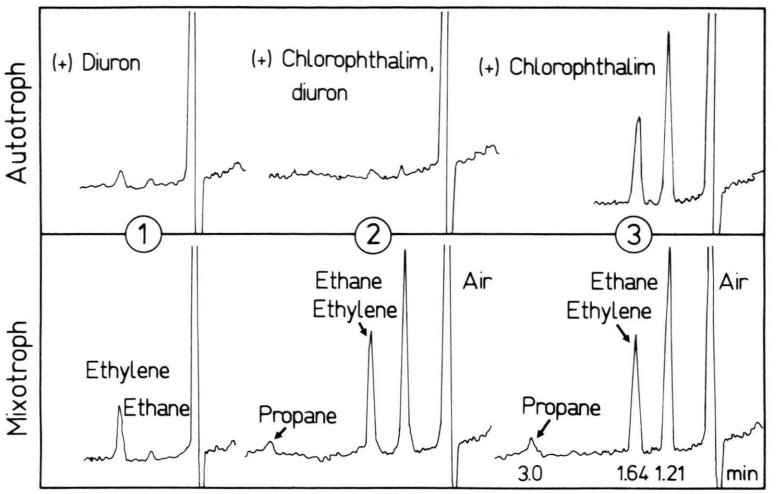

Fig. 2. Light-induced formation of short-chain hydrocarbons by soybean cell cultures (*Glycine max*, Merr.) Cells were grown in Murashige-Skoog medium in Erlenmeyer flasks for two weeks (24 °C, approx. 70 $\mu E/m^2 \times$ sec white light) either with 1%, w/v, glucose (mixotroph) or under a CO_2-enriched air atmosphere without added glucose (autotroph). Then the compounds were added as indicated (see Nos. 1, 2, 3) and the cultivation continued for about 20 h. Subsequently, aliquots were incubated in vials as described (4), and illuminated with about 350 $\mu E/m^2 \times$ sec white light for 24 h at 23 °C for short-chain hydrocarbon determination. 1 mM bicarbonate was added to autotrophic cells during this period. Chlorophthalim, 0.5 µM; diuron 10 µM. No propane was seen with the autotrophic cells. Retention times for the three gases are given in No. 3, bottom.

diuron present. Part of the ethylene undoubtedly is due to a higher-plant stress response and degradation of membrane fatty acids is not the source of this hydrocarbon.

The different response of both types of soybean cell cultures is reflected in their ability to produce protoporphyrin (Fig. 3). The fluorescence emission peak was found at 632 nm and coincided with

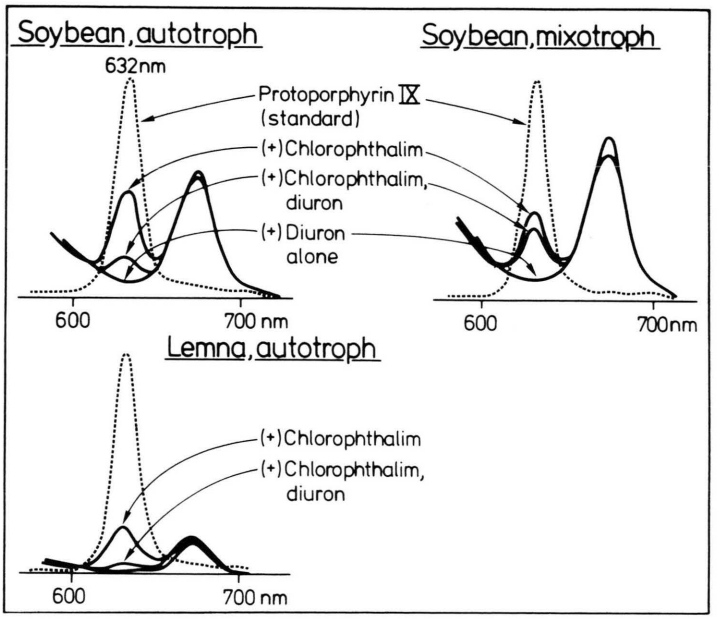

Fig. 3. Formation of protoporphyrin IX in autotrophic and mixotrophic soybean cells as well as in duckweed (*Lemna minor*): Influence of diuron. The original fluorescence emission spectra between 600 and 700 nm are shown (excitation at 405 nm) using 0.9 µM protoporphyrin IX as standard; the peak at 690 nm belongs to chlorophyll. Soybean cells were grown as indicated in the legend of Fig. 2, duckweed in normal hydroponic culture medium. With the compounds added as indicated (see Fig. 2 for concentrations) *Lemna* was incubated for about 20 h in vials (used to determine hydrocarbons, see ref. 4) while the soybean cells were cultured as previously. In both assays about 70 $\mu E/m^2 \times$ sec white light and 24 °C were used.

that of standard protoporphyrin IX. Most probably, the tetrapyrrole formed in both soybean and duckweed is protoporphyrin IX, as has been proven for chlorophthalim with the alga *Bumilleriopsis* [12]. The autotrophic cells produced protoporphyrin in the light (but not in the dark, not documented), and diuron inhibited to about 80% under the conditions used. Diuron alone had no effect. The opposite is true for the mixotrophic, glucose-supplemented culture. Diuron exhibited only some inhibition on tetrapyrrole formation. These soybean cultures showed the same response as was documented and explained in detail with *Scenedesmus* [17].

Photosynthesis is necessary for build-up of the tetrapyrrole and consequently the diuron effect is evident. When photosynthesis is replaced by glucose the inhibition of electron transport does not severely affect protoporphyrin formation. It should be noted that the difference of the fluorescence peak height between the (+) diuron samples is real. Light contributed to protoporphyrin formation in glucose-supplemented soybean cultures. Based on cell material protoporphyrin formation was found about 30% less in mixotrophic cultures than in the autotrophic ones. Nevertheless, the hydrocarbon evolution of the mixotrophic cells was somewhat higher. – The diuron effect was also found with *Lemna*. Gabaculine effectively abolished tetrapyrrole formation as well as phytotoxic consequences like chlorophyll bleaching (degradation, data not shown).

Fig. 4 demonstrates the short-chain hydrocarbon evolution as well as membrane leakage with mung beans. Both parameters show the same concentration dependence. Interestingly, protoporphyrin formation markedly increased with concentrations higher than 1 μM chlorophthalim at which the plateau of peroxidative degradations was reached. This is reminiscent to recent findings with *Scenedesmus* where also no quantitative relationship between the tetrapyrrole level and peroxidation was observed [17]. Small amounts of the sensitizer were found sufficient to achieve maximum peroxidation. Excess tetrapyrrole did not contribute to phytotoxicity.

This line of thought is continued by the data in Fig. 5. As was reported for other peroxidizers [13] very low concentrations of chlorophthalim as well as oxyfluorfen are needed for half-inhibition of

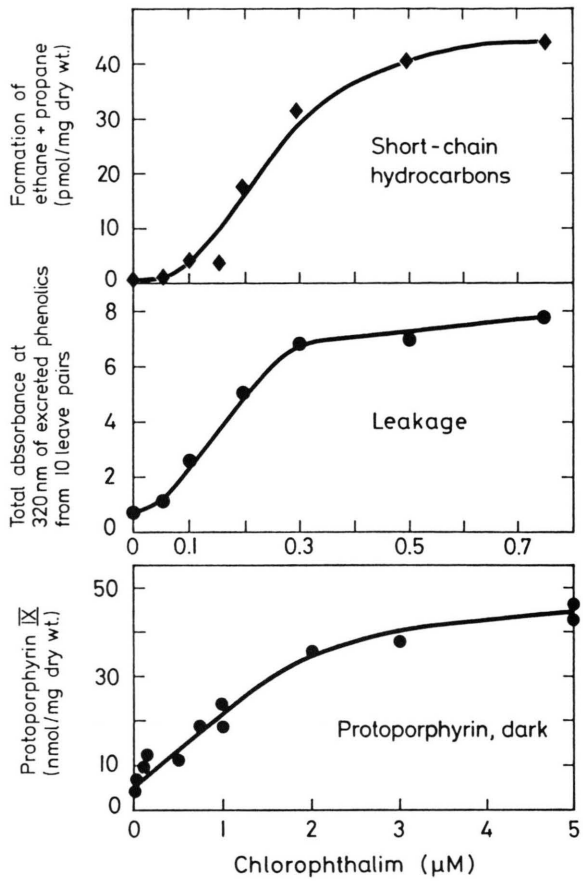

Fig. 4. Light-induced peroxidative activity of chlorophthalim in mung-bean seedlings measured as evolution of ethane plus propane, or leakage of endogenous phenolics compounds of the cell vacuole, and accumulation of protoporphyrin IX in the dark.

protoporphyrinogen oxidase. Although the inhibition curve with the homogenate from mung beans exhibited some irregularity (an insensitive plateau was attained, possibly due to impurities in the homogenate) for both chlorophthalim and oxyfluorfen an inhibition constant of about 6×10^{-9} M can be calculated (Fig. 5A). A comparative value was obtained at about 10^{-9} M for oxyfluorfen using a homogenate from corn seedlings (Fig. 5B). From Fig. 4 the "activity value" (concentration for half maximum peroxidation) can be estimated as considerably higher than the inhibition constant, namely 2.2×10^{-7} M and 1.6×10^{-7} M for formation of hydrocarbon gases and leakage, respectively, while the value for half-maximum forma-

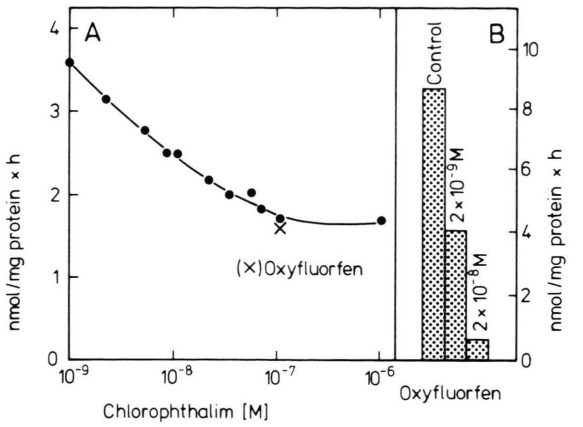

Fig. 5. Inhibition of protoporphyrinogen oxidase by two peroxidizing compounds. Formation of protoporphyrin IX (nmol/mg protein × h) was measured using homogenates of mung bean (A) and corn seedlings (B). In contrast to the oxidase activity in the corn-seedlings homogenate, with the mung-bean preparation inhibition could not be decreased to zero by 10^{-7} M chlorophthalim or oxyfluorfen. Apparently, there is a stronger non-enzymatic oxidation of protoporphyrinogen in the crude mung-bean homogenate which may be overcome by purification.

tion of protoporphyrin (in the dark) is up to 10^{-6} M.

These data give evidence that a clear-cut relationship between target-enzyme inhibition, the protoporphyrin level and peroxidative consequences does not exist. As indicated in the Introduction a quantitative relation can hardly be expected between these data. Cell-free data (enzyme inhibition) are difficult to compare with subsequent multistep processes in the cell. Particularly, data on protoporphyrin reactivity are necessary and on a possible functional location of protoporphyrin in the cell.

Acknowledgement

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