

Radical Formation and Peroxidative Activity of Phytotoxic Diphenyl Ethers

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ESR Signals, Spin-Trapping, Nitro/Non-Nitro Diphenyl Ethers, Chloroplasts, Electron Transport

Phenyl-*t*-butylnitrone can be used to trap light-induced radicals with isolated chloroplasts, measurable by ESR technique. The radical signals are dramatically amplified and their lifetime prolonged in the presence of peroxidizing diphenyl ethers. A *para*-substitution with $-\text{NO}_2$, $-\text{Cl}$ or $-\text{I}$ is necessary, as well as an operating photosynthetic electron transport. The nature of radicals is not yet clear; they may be alkyl or linolenic-acid radicals, but their presence indicates peroxidation. Furthermore, the ESR measurements presented strengthen our hypothesis that peroxidative activity of paraquat and diphenyl ethers are not caused by the same mechanism.

Introduction

Depending on substituents diphenyl ethers exert a multifunctional mode of action (P. Böger, this issue). The *para*-nitrodiphenyl ethers, such as oxyfluorfen, exhibit their phytotoxic activity by peroxidative destruction of the photosynthetic membrane [1, 2]. Light is required for these compounds to induce herbicidal activity [3, 4].

From earlier experiments [1, 5] we can assume that the photosynthetic electron transport initiates radical formation leading to radicalic chain reactions. Evolution of volatile hydrocarbons is the consequence of degradation of polyunsaturated fatty acids. No experimental evidence for radicals showing up under the influence of peroxidizing DPEs has been presented so far. This communication is to support our previous hypothesis [5] and to demonstrate the existence of radical intermediates by activity of oxyfluorfen.

Only a few reactions in (bio)organic chemistry, which proceed via (free) radical mechanisms, can

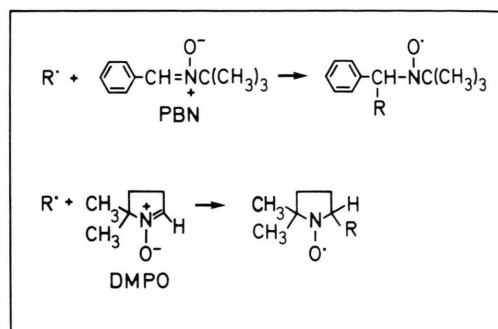


Fig. 1. Reactions leading to a stable spin adduct using the spin traps PBN and DMPO.

be studied directly by ESR. Generally, radical intermediates are below the detection limit, and short-living species have to be trapped [6, 7]. The addition of a free radical R^\cdot to a positively charged part of an acceptor molecule is a means of observing radical formation ("spin trapping"). This technique has been applied successfully to investigations of numerous biological systems avoiding higher concentrations of organic buffers or methanol, which themselves may lead to spin adducts [8, 9]. Particularly relevant are those spin traps leading to a kinetically stable new radical (Fig. 1). Thus the addition of R^\cdot to either a nitro [10], nitroso [11] or nitrone [12] produces a stable nitroxide radical suitable for appropriate ESR measurements.

Materials and Methods

Spinacia oleracea, var. Atlanta, was grown in the open during March to June and September to Octo-

Abbreviations: 1. Herbicides: acifluorfen-methyl (AFM), methyl 5-[2-chloro-4-(trifluoromethyl)phenoxy]-2-nitrobenzoate; DCMU (diuron), [3-(3,4-dichlorophenyl)-1,1-dimethylurea]; MC 15608, methyl 5-[2-chloro-4-(trifluoromethyl)phenoxy]-2-chlorobenzoate; oxyfluorfen, 2-chloro-4-trifluoromethylphenyl-3'-ethoxy-4'-nitrophenyl ether; paraquat (methylviologen), 1,1-dimethyl-4,4'-bipyridylium dichloride; RH 1939, 2-chloro-4-trifluoromethylphenyl-3'-ethoxyphenyl ether. 2. Others: DMPO, 5,5-dimethyl-1-pyrroline-1-oxide; DPE, diphenyl ether; ethoxyquin, 6-ethoxy-1,2-dihydro-2,2,4-trimethylquinoline; ESR, electron spin resonance; PBN, phenyl-*t*-butylnitrone.

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ber. Chloroplasts isolated according to [13] were used for the experiments described. To washed, freshly harvested leaves 50 ml homogenization medium was added: 0.4 M sucrose; 50 mM tricine, [N-tris(hydroxymethyl)methylglycine], adjusted with NaOH to pH 8.2; 10 mM NaCl; 5 mM MgCl₂. The homogenate was filtered (four layers of cheesecloth, one layer of nylon tissue, 40 µm mesh), centrifuged for 1 min at 3100 × *g*, and the pellet washed in 2 ml of the suspension medium: 0.4 M sucrose, 50 mM tricine; 20 mM NaCl; 4 mM MgCl₂, and 10 mM K₂HPO₄. The chlorophyll content was determined by extraction with 80% aqueous acetone [14].

The samples for the ESR experiments contained chloroplasts (equivalent to 200 µg of chlorophyll) suspended in 10 mM phosphate, pH 7.4; 50 µM Fe(NO₃)₃-EDTA; 5 mM NaCl; 2.5 mM NH₄Cl, in a final volume of 2 ml. The herbicides (98% pure), 0.10 M, stock solutions in dimethylformamide, were added to the reaction mixture keeping the final concentration of the solvent below 0.1%. Final DPE concentration had to be 0.1 mM. PBN, 0.15 M, and DMPO, 0.02 M [see ref. 8] were added just before starting the ESR experiments. These concentrations are generally used and have been found adequate for our purpose. Photolysis was performed according to [15] using the chloroplast-suspension medium, but without chloroplasts (see Results).

ESR spectra were recorded on a Varian E-109 spectrometer (Palo Alto, USA) at approx. 9.5 GHz and 100-KHz modulation frequency. Exact *g*-values and hyperfine coupling constants were determined on a Bruker B-ER 420 instrument (Karlsruhe, Germany) equipped with a frequency counter and a gaussmeter. Samples were transferred to a quartz aqueous solution cell; for anaerobic experiments, the cell was evacuated and flushed with an inert gas [16].

ESR measurements were performed at room temperature in a V-4534 optical transmission cavity which allowed direct illumination (light of about 90 W/m², passing through a 4-mm KG-1 heat filter from Schott) during signal tracing.

For the determination of C₂-hydrocarbons as markers of herbicide-induced peroxidation, 2-ml aliquots of *Scenedesmus* cultures with a packed cell volume (pcv) of 3 to 4 µl/ml were illuminated (white light, approx. 100 W/m²) for 15 h in gas-tight vessels, using a Warburg apparatus as described [5]. *Scenedesmus obliquus* (formerly *acutus*),

no. 276-3a, from the Culture Collection, University of Göttingen, Germany, had been cultivated autotrophically using light of about 30 W/m² according to [17]. Each experiment was performed at least four times. The amplitudes of the individual ESR signals had tolerances of ± 5% from the mean. In the graphs, a typical measurement is given.

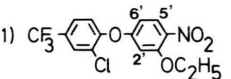
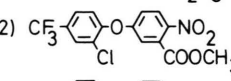
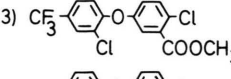
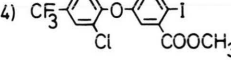
Chemicals, analytical grade, were purchased from Merck, Darmstadt; DMPO and PBN, Janssen Pharmaceutica, Beerse, Belgium; and methylviologen, Serva, Heidelberg.

Results and Discussion

Para-substituted DPEs, such as oxyfluorfen, induced peroxidative degradation of the photosynthetic membrane (Table I) in light. Ethane and some ethylene produced, used as markers of peroxidation, were related to the substitution pattern of the DPEs at 3'- and 4'-position. The highest peroxidative activity was shown by acifluorfen-methyl (AFM, no. 2), having a *para*-NO₂ group. Replacement by -Cl or -I (Nos. 3 and 4) decreased the amount of volatile hydrocarbons formed to 42% for the 4'-Cl, and 26% for the 4'-I compound, as compared to the values found with AFM. C₂-production with compounds 1 and 2 is also dependent on substitution at 3'-position, as documented previously [5].

Addition of the antioxidant ethoxyquin [18, 19] strongly decreased the evolution of C₂-hydrocarbons. Concurrent addition of 10 µM DCMU, known

Table I. Light-induced formation of C₂-hydrocarbons (nmol/ml pcv × 15 h) in *Scenedesmus* after incubation with different diphenyl ethers.

Additions (0.1 µM)	—	(+) Ethoxyquin (100 µM)	(+) DCMU (10 µM)
Control	0.6	—	—
1) 	9.3	1.7	} not detectable
2) 	14.2	3.7	
3) 	6.0	0	
4) 	3.7	0	

to block the photosynthetic electron flow, inhibited peroxidation with all compounds shown. Addition of oxyfluorfen (1 μM) to *Scenedesmus*, in the presence of PBN (0.1 mM), prevented peroxidation, *i.e.* the concurrent formation of C_2 -hydrocarbons (data not documented), a physiological evidence that radicals trapped in this study are related to phytotoxic effects. Also pigment destruction was alleviated.

Figure 2 demonstrates the loss of chlorophylls and colored carotenoids after addition of 0.1 μM DPEs as indicated. In contrast to compounds of the *m*-phenoxybenzamide type, which inhibited carotenogenesis [20], DPEs of the oxyfluorfen type, as used here, *degrade* carotenoids and chlorophylls. Again ethoxyquin alleviated this effect, at least partially, indicating that peroxidative radical mechanisms are responsible for pigment destruction.

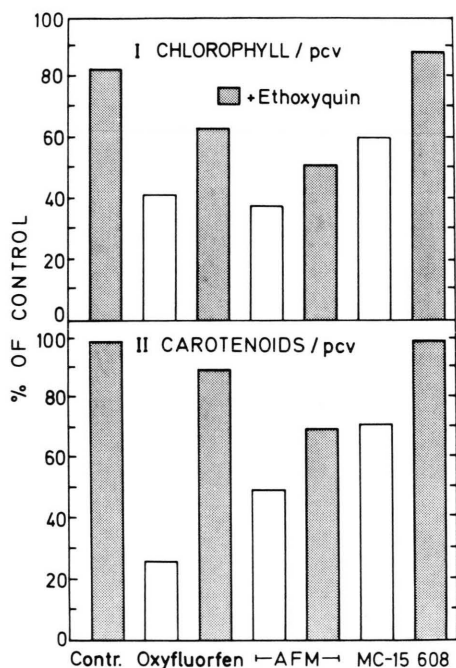


Fig. 2. Influence of different diphenyl ethers (0.1 μM) on chlorophylls and carotenoids of autotrophic *Scenedesmus* after a 24-h cultivation time. The hatched bars of both figure parts I, II show the protection by ethoxyquin (0.1 mM) against peroxidative destruction of the pigments. Control is with ethoxyquin, without herbicide. The cells without any additions (100%) had 11 mg chlorophyll/ml packed cell volume (pcv) and 1 mg/ml pcv total carotenoids. Acifluorfen-methyl, AFM = methyl 5-[2-chloro-4-(trifluoromethyl)phenoxy]-2-nitrobenzoate; MC 15608 = methyl 5-[2-chloro-4-(trifluoromethyl)phenoxy]-2-chlorobenzoate.

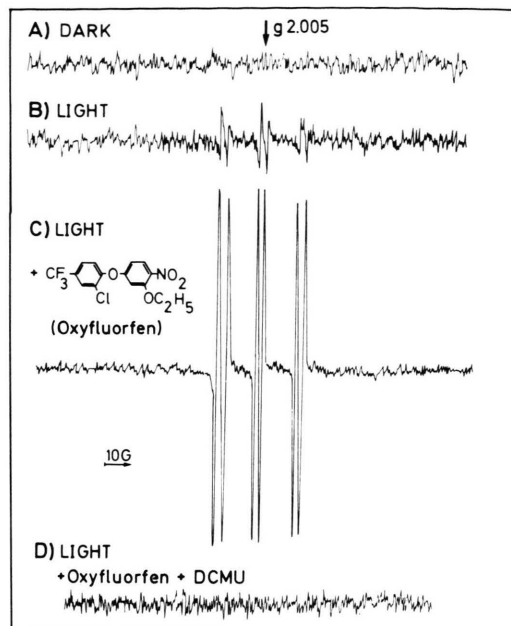


Fig. 3. ESR spectra of a spinach-chloroplast suspension, after illumination in the presence of the nitron trap PBN and oxyfluorfen. In (D) 10 μM DCMU was added concurrently with the diphenyl ether to inhibit photosynthetic electron transport. Controls: (A) with or without oxyfluorfen present, (B) without diphenyl ether. Frequency = 9.34046 GHz; modulation amplitude = 0.5 G (gauss); modulation frequency = 100 KHz; scan rate = 4 min/200 G.

To trace the radicals induced by 4'-substituted DPEs the spin traps PBN and DMPO were used. Only in the presence of molecular oxygen and in the light radical concentration was substantial to be measured. In Fig. 3, a set of spin-trapping experiments with chloroplasts and the nitron PBN is illustrated. In the dark, the chloroplast suspension (see Methods) with oxyfluorfen present did not show ESR signals (A). Upon illumination, but with oxyfluorfen absent, a small signal was observed with a hyperfine splitting typical for a PBN-spin adduct [21, 22] (B), which disappeared after about 30 min illumination time. An amplified, but identical signal, centered at g 2.005 ($A_N = 15.83$, $A_H^{\beta} = 3.31$ G) was formed immediately after illumination in the presence of oxyfluorfen (C). In contrast to (B), the signal amplitude was enlarged by a factor of 5 to 6 and observable for about 3 h. In the light DMPO was not effective with DPEs.

The same experiments carried out with a DPE having a -Cl (Fig. 4, E) instead of a 4'-NO₂ group showed the same signal of marked longevity as detected with oxyfluorfen (C). The lower phytotoxic

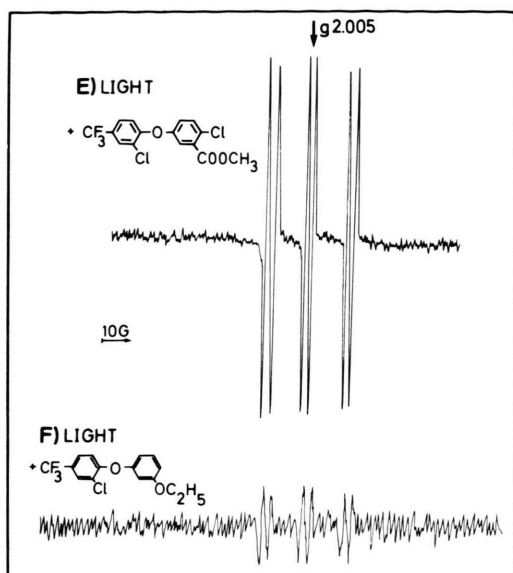


Fig. 4. Light-induced ESR signals with PBN present and two non-nitro diphenyl ethers.

activity of the Cl-DPE *vs.* the NO₂-DPE (Table I, Fig. 2) and the identical height of their signals are due to the high herbicidal concentration used throughout for both DPEs to obtain the ESR spectra (see Methods). This concentration, by far exceeding the dosis needed for phytotoxic effects, was necessary to produce radicals enough to be measurable in the ESR instrument. Photosynthetic electron transport is involved in radical formation as seen by signal D of Fig. 3, since inhibition of electron flow by diuron (10 μ M) abolished the weak signal of the control as well as the amplified signal of the *p*-NO₂ and *p*-Cl-DPEs (Figs. 3, 4; C, E). With the diphenyl ether RH 1939, having a hydrogen in 4'-position, no peroxidative activity was evident [23], and no amplified ESR signal was seen (Fig. 4, F). Both amplitude and shape of the small basic signal obtained were the same as observed in the light without herbicide present (see Fig. 3, B).

These experiments show a nitro [23] or halogen [24] substituent in 4'-position as necessary for the generation of radicals, as was concluded previously by assaying peroxidative activity. Our hypothesis for the activation of para-substituted DPEs is that radical intermediates (a DPE-anion radical?), generated by photosynthetic electron transport, abstracts a hydrogen atom from a polyunsaturated α -fatty acid (here: α -linolenic acid) leading to a radical center at a β -carbon. After conjugation of

two double bonds, giving a linolenic-acid radical followed by a reaction with molecular oxygen, a free peroxy radical is formed. This again reacts with another linolenic-acid molecule, starting a peroxidative chain reaction [25].

The ESR parameters of the spin adducts of PBN, obtained in this study, indicate that the signal may be due to either an alkyl radical, or a radical originating from linolenic acid. This interpretation is mainly based on the large value of 3.31 G found for the proton-coupling constant A_H^{β} . Thus, A_H^{β} for the methyl adduct is 3.45 G [6], for the adduct of linolenic acid 3.35 G [22], whereas 2.75 G were determined for the hydroxyl-radical adduct of PBN [15]. In this context it should be mentioned that the magnitudes of both A_N and A_H^{β} are solvent dependent to some extent: A_H^{β} of PBN-OH in benzene was reported as 2.88 *vs.* 2.75 G in water [15]. Apparently, a transient radical of the peroxidative diphenyl ether itself is not observed here. Only a light-induced (trapped) signal of illuminated control chloroplasts is amplified (Fig. 3, B, C; Fig. 4, E, F).

Bipyridylum salts, like paraquat, reduced by photosystem I to radical cations, are reoxidized by O₂ [26], which can be easily measured as oxygen

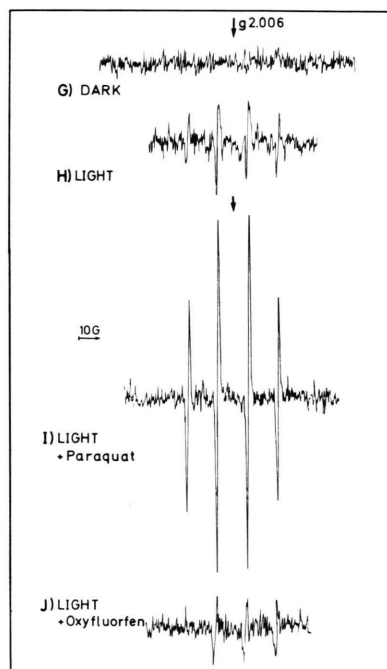


Fig. 5. Light-induced formation of DMPO adducts in the presence of 10 μ M paraquat and 0.1 mM oxyfluorfen. Signal H is the herbicide-free control.

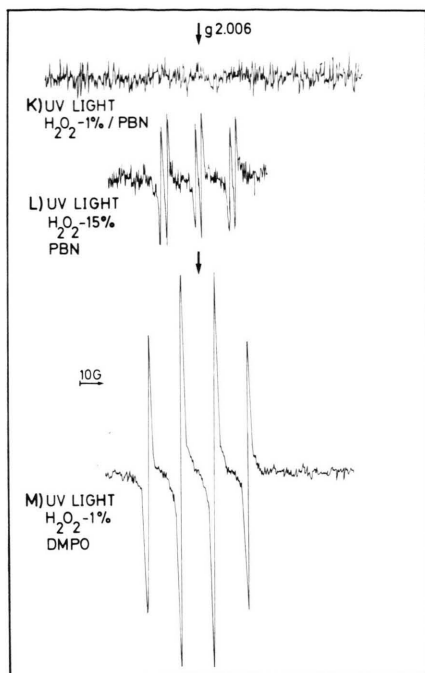


Fig. 6. ESR spectra obtained by photolysis of hydrogen peroxide in the presence of DMPO and PBN traps. With 15% instead of 1% H_2O_2 in the presence of DMPO, the signal was not amplified further (see text).

uptake. The originating reactive oxygen species cause peroxidative destruction of the photosynthetic membrane [27, 28]. Oxygen uptake is also observed with paraquat in the dark, when electrons are denoted by NADPH (via ferredoxin [29]). These two oxygen-uptake systems do not work with peroxidizing DPEs, which – in addition to the ESR measurements reported here – gives clear evidence that DPEs and paraquat have a different mode of driving radical (chain) reactions.

Figure 5 illustrates the results of spin-trap experiments with *DMPO* and isolated spinach chloroplasts plus herbicides. Similar to PBN, no significant amounts of ESR-active species were measured in the dark (signal G). Upon illumination a small signal, centered at $g = 2.006$, was observed (H). In contrast to signal (C) of Fig. 3 using PBN, with

oxyfluorfen plus *DMPO*, no increase concentration was detected as compared to the herbicide-free control (see signals H and J).

Illumination of chloroplasts in the presence of paraquat ($10\ \mu\text{M}$) gave a strong signal at $g = 2.006$, which was split into four equidistant lines (signal I). This was only observed for about 5 to 6 min. Thereafter, a new signal appeared. The first signal with a splitting of 15.3 G originated most likely from the hydroxyl-radical adduct of *DMPO* [15]. The second signal, exhibiting a more complex hyperfine pattern than the first one, was identical with the one described [30] and arises from the paraquat-radical cation produced by electron transport after consumption of oxygen in the reaction mixture. The same signal was found with paraquat, when reducing equivalents were delivered by NADPH through a system of hepatic microsomes from rats. Addition of paraquat and diuron ($10\ \mu\text{M}$) abolished the signals due to inhibition of the photosynthetic electron transport (data not shown).

For the standardization of the radicals trapped in our experiments with *DMPO* and PBN, we used the photolysis of 1 and 15% (v/v) H_2O_2 [15]. The signals generated thereby with *DMPO* present (Fig. 6M) were similar to those found with illuminated chloroplast material plus paraquat (Fig. 5, I). Again PBN did not show a spin adduct in 1% H_2O_2 (Fig. 6, K), but a small signal showed up using 15% H_2O_2 (signal L). Nevertheless, the amplitude of this signal was 2 to 3 times less than that generated by PBN plus oxyfluorfen (Fig. 3, C).

Acknowledgments

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