Accumulation of Protoporphyrin IX in the Presence of Peroxidizing Herbicides

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Peroxidizing herbicides were assayed for their capacity to accumulate tetrapyrrole-like compounds in the liquid culture medium when growing the xanthophycean alga *Bumilleriopsis filiformis*. We have isolated and purified this compound by HPLC. For identification optical, fluorescence, NMR and mass spectroscopy were employed. All these techniques showed that the accumulation product is protoporphyrin IX. This tetrapyrrole was found, although to a different extent, using either chlorophthalim, oxadiazon, oxyfluorfen or LS 82-556. Accumulation of protoporphyrin IX caused by peroxidizing compounds was also found in light-grown *Bumilleriopsis* cells and heterotrophically (dark-)grown *Scenedesmus* cells, but neither in light-grown *Scenedesmus* cells nor in dark-grown *Neurospora*.

Introduction

Cyclic imide herbicides interfere with the chlorophyll content of algae and higher plants [1–3]. Furthermore, free-radical peroxidation is initiated by these compounds [4, 5]. It was shown recently that tetrapyrroles accumulate in etiolated soybean cells under the influence of acifluorfen-methyl and a lutidine derivative [6]. It is still unclear whether both herbicidal effects are independent or connected.

In our attempts to elucidate the mechanism of cyclic imide mode of action, we have looked for the accumulation of precursors in the chlorophyll-biosynthetic pathway in the presence of chlorophthalim. For this investigation we have chosen the autotrophic xanthophycean alga *Bumilleriopsis*, which shows strong tolerance against peroxidative herbicides [7]. We also extended our studies to chemically unrelated herbicidal compounds, such as oxadiazon which shows very similar effects on chlorophyll formation as cyclic imides [1], and the peroxidizing herbicides oxyfluorfen [8] and LS 82-556 [9]. With all these compounds the accumulation of a tetrapyrrole in the culture medium could be observed, which was isolated and identified by various analytical methods.

Chemical names, abbreviations: chlorophthalim, N-(4-chlorophenyl)-3,4,5,6-tetrahydrophthalimide); LS 82-556, (S)3-N-(methylbenzyl)carbamoyl-5-propionyl-2,6-lutidine; oxadiazon, 5-*tert*-butyl-3-(2,4-dichloro-5-isoproxyphenyl)-1,3,4-oxadiazol-2-(3H)-one; oxyfluorfen, 2-chloro-1-(3-ethoxy-4-nitrophenoxy)-4-(trifluoromethyl)benzene; Chl, chlorophyll *a*.

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Materials and Methods

The xanthophycean microalga *Bumilleriopsis filiformis* (own stock) was cultivated at a light intensity of 40 W/m^2 at 22 °C in mineral medium as described [10], using a thermostated water-bath and culture vessels containing 200 ml of algae suspension. *Scenedesmus acutus*, strain 276-3a, was grown in a medium according to Ref. [11] either in the light like *Bumilleriopsis* or in the dark with supplementation of 0.5% glucose and 0.25% yeast extract. *Neurospora crassa*, strain 2308, was cultivated in the dark in the medium given in Ref. [12]. All herbicides were added from 10^{-2} M stock solutions in methanol as indicated. After *Bumilleriopsis* had been cultivated for 2 days, chlorophyll and packed cell volume were determined and the medium collected by centrifugation at $4000 \times g$ for 15 min. The supernatant was evaporated and the residue dissolved in methanol/acetic acid (5:1, v/v). This solution was diluted with water, adjusted to pH 2 with HCl and partitioned twice against diethyl ether. The diethyl-ether phase was dried with anhydrous Na_2SO_4 and a subsequent esterification was carried out with diazomethane. The latter was obtained by mixing 0.3 g of nitrosomethylurea with 2 ml of a 20% NaOH solution and addition of 5 ml of diethyl ether. Excess diazometh-

ane in diethyl ether was added to the sample dissolved in diethyl ether. After 5 min the diethyl-ether phase was washed 3 times with 10% acetic acid, 2% NH_4OH and then with water. The ester fraction was separated by TLC on silica-gel G plates with 15% (v/v) ethyl acetate in toluene as solvent (R_f = 0.3) and purified by HPLC on a Spherisorb 5 ODS column with methanol (flow 1 ml/min) as eluent. The compound with a retention time of 14 min was used for identification by various spectroscopic procedures.

Extraction of protoporphyrin IX from either *Bumilleriopsis* cells, cultivated for 3 days, *Scenedesmus* cells (3 days cultivated) or 5 days old *Neurospora* mycelium was carried out with a mixture of acetone/1 N NH_4OH/50 mM HEPES buffer, pH 8.0 (15:2:2, v/v) for 30 min at 55 °C. After washing the extract 3 times with hexane, the acetone phase was diluted with water, brought to pH 2 by HCl and extracted with diethyl ether. The diethyl-ether phase was further treated as described for the extracts from the *Bumilleriopsis* culture medium.

Chlorophyll *a* was quantified after hot methanol extraction (15 min, 65 °C) according to McKinney [13]. Packed cell volume (pcv) was determined by centrifugation in calibrated microcentrifuge tubes. Protoporphyrin IX and its dimethyl ester was purchased from Sigma Chemicals, Munich.

Results

Cultures treated with 20 μM of chlorophthalim excreted a brownish compound into the liquid medium which showed a tetrapyrrole-like red fluorescence. After isolation and methylation, a retention time of 14 min was found on a reversed-phase column with methanol as eluent (Fig. 1). The same retention was observed with protoporphyrin-IX dimethyl ester. A mixture of the methylated sample and protoporphyrin-IX dimethyl ester gave a single peak without a shoulder. Other tetrapyrroles were not excreted by *Bumilleriopsis* cells in the presence of chlorophthalim. The absorbance spectrum of the isolated compound in acetone is presented in Fig. 2A. The main maxima appeared at 408 nm, minor ones were found at 555 and 598 nm (Table I). All the maxima

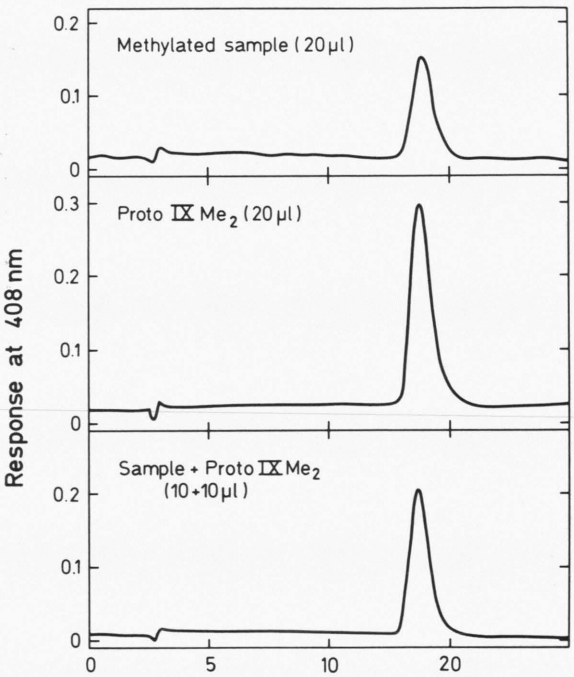


Fig. 1. HPLC separation of tetrapyrroles. (A) Methylated isolate from the supernatant of chlorophthalim-treated *Bumilleriopsis* cultures. (B) Protoporphyrin-IX dimethyl ester. (C) Co-chromatography of samples A and B.

Table I. Spectral characteristics of compounds excreted by *Bumilleriopsis* into the culture medium with chlorophthalim present during growth.

Characteristics	Methylated isolate	Protoporphyrin-IX dimethyl ester
Absorbance maxima [nm]	408 555 598	408 554 598
Absorbance ratio A_{408}:A_{555}:A_{598}	45:1.8:1	45:2.3:1
Fluorescence maxima [nm]		
Excitation	408	408
Emission	602	602

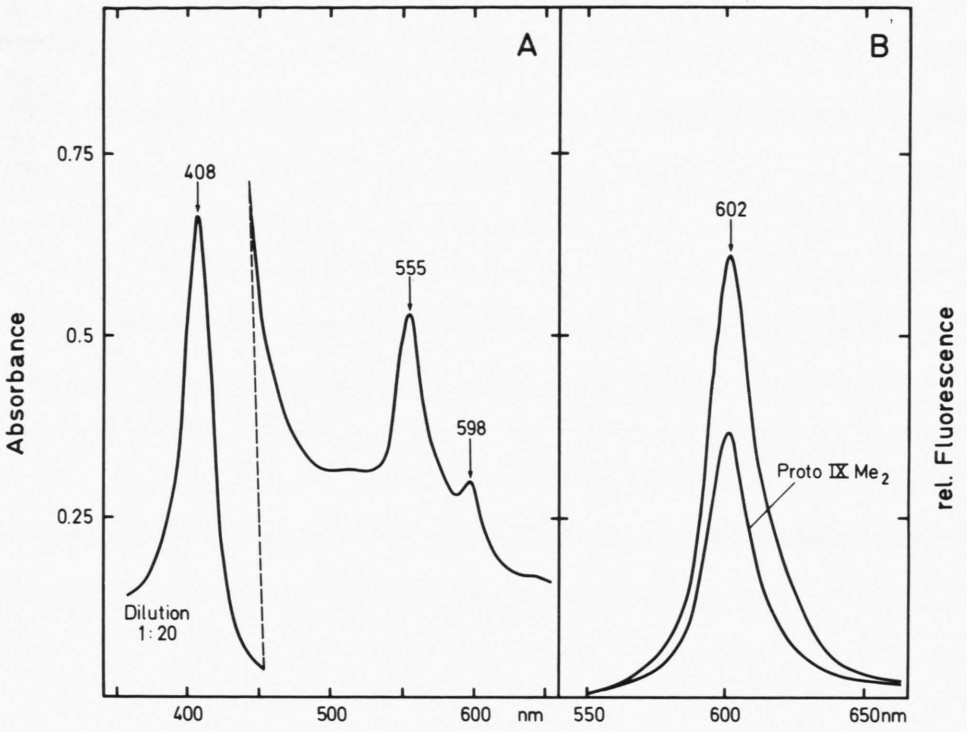


Fig. 2. Absorbance spectrum (A) and fluorescence emission spectrum (B) of the methylated isolate from the culture medium of herbicide-treated *Bumilleriopsis*.

corresponded with the absorbance of authentic protoporphyrin-IX dimethyl ester [14]. Also the absorbance ratios of the peaks were very similar. The peak at 408 nm was found 4–5-fold higher than the peak at 598 nm and the absorbance at 555 nm was about twice as strong as at 598 nm. Fluorescence-emission spectra gave the same shape for the methylated isolate and purchased protoporphyrin-IX dimethyl ester with identical maxima at 602 nm with acetone as solvent (Fig. 2B). The excitation maximum was determined at 408 nm (Table I).

Direct evidence that the excretion product of *Bumilleriopsis* is protoporphyrin IX was provided by mass spectrometry. Mass spectra were obtained from 10 µg of the dimethyl ester after ionization by fast-atom bombardment (FAB) from a cesium source under protonating conditions (Fig. 3). The resulting spectrum of the isolated tetrapyrrole in *m*-nitrobenzyl alcohol showed an intense protonated molecular ion $[M+H]^+$ of protoporphyrin-IX dimethyl ester (molecular weight 590.7) at m/z = 591.5 with isotope distribution at 591 and 593 as expected for this compound together with a typical fragmentation pattern [15]. Fragments from benzylic fission of one acetic acid methyl ester $[MH-74]^+$ from the methylated propionyl side chains were obtained at 518. Furthermore, cleavage of one molecule of formic

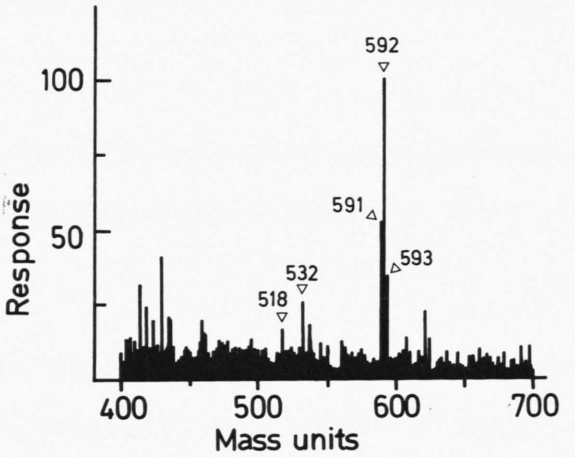
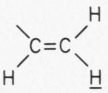
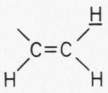
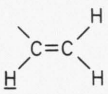


Fig. 3. FAB-mass spectrum of the methylated tetrapyrrole from 400 to 700 mass units after fast-atom bombardment ionization (see Results).

Table II. Chemical shifts for isolated protoporphyrin IX after methylation.

Assignment	Chemical shift [ppm]
	−3.62
 1-4	10.1 10.3
$-CH_3$ 1-4	3.7 3.8
	6.2
	6.4
	8.3
$-CH_2-\underline{CH_2}-COOCH_3$	3.3
$-CH_2-CH_2-COO\underline{CH_3}$	3.7
$-\underline{CH_2}-CH_2-COOCH_3$	4.4

Spectra measured at 250 MHz with tetramethylsilane as standard; concentration of protoporphyrin-IX dimethyl ester was 1 mM.

acid methyl ester (m/z = 532) was also observed. A reference spectrum of purchased protoporphyrin-IX dimethyl ester gave the same mass peaks.

From our *Bumilleriopsis* culture we could isolate enough material even to perform proton magnetic resonance spectrometry in deuterated chloroform. In comparison with the data in Ref. [16], the protons of the porphyrin skeleton and the various side chains could be identified. Table II shows the chemical shifts for protoporphyrin-IX dimethyl ester. We could assign the methine bridge protons, the amine protons, the various olefinic protons of the vinyl-side chain and the protons of the methyl propinate groups and the methyl groups.

In Table III cell growth as packed cell volume (pcv) per ml culture volume was determined as well as chlorophyll *a* and protoporphyrin-IX formation with various herbicides present during cultivation. All these herbicides induced the accumulation of protoporphyrin IX. The control culture did not excrete detectable protoporphyrin IX. Oxadiazon showed the smallest effect even when applied in a concentration of 100 μM. The same concentration was used for LS 82-556 and a 3-fold higher protoporphyrin-IX level than for oxadiazon was measured. 20 μM of oxyfluorfen accumulated almost the same amount as 100 μM of LS 82-556. The most effective compound is chlorophthalim which was employed in a concentration of 20 μM. This herbicide was 2 times more potent than oxyfluorfen. Except for oxadiazon which showed a very moderate effect, all herbicides caused a slight decrease of growth only and a strong decrease of the chlorophyll content (comp. Ref. [1]).

Protoporphyrin IX was also detectable in control cells of *Bumilleriopsis* grown in the light (Table IV). Treatment with chlorophthalim increased the endogenous level fivefold. We have also determined the effect of chlorophthalim on protoporphyrin-IX accumulation in cells of the green alga *Scenedesmus*, which is very sensitive to peroxidizing herbicides [7]. In addition, this alga offers the advantage to grow either autotrophically in the light or heterotrophically in the dark forming chlorophyll. In cells treated with 0.08 μM chlorophthalim protoporphyrin IX was found after separation by HPLC 13 times more than

Table III. Formation of chlorophyll *a* and excretion of protoporphyrin IX by *Bumilleriopsis* treated with various herbicides for 48 h.

Additions	Packed cell volume [μl/ml]	Chl [μmol/ml pcv)	Proto-IX conc. of the medium [nmol/ml pcv]
Control	15.6	3.05	0
Chlorophthalim 20 μM	13.1	1.81	504
Oxadiazon 100 μM	16.9	3.04	89
Oxyfluorfen 20 μM	14.5	1.74	255
LS 82-556 100 μM	13.3	1.62	361

ml refers to algae culture medium; density at start was 4 μl pcv/ml.

Table IV. Accumulation of protoporphyrin IX in the cells of different organisms.

Species, culture type	Protoporphyrin IX [nmol/ml pcv]	
	Control	+ Chlorophthalim
Bumilleriopsis, autotrophic (20 μM)	15.0	76.8
Scenedesmus, autotrophic (0.08 μM)	0	0
Scenedesmus, heterotrophic (0.08 μM)	3.68	53.7
Neurospora, heterotrophic (0.5 μM)	0	0

in the untreated control. In (autotrophic) cells grown in the light, protoporphyrin IX could neither be detected in the control nor in cultures treated with chlorophthalim. We also assayed for accumulation of protoporphyrin IX in dark-grown cells of *Neurospora crassa* after addition of chlorophthalim. Neither in the medium nor in the mycelium traces of any tetrapyrrole could be found.

Discussion

The herbicidal compounds used in this study induce peroxidation [1, 17]. However, the initial reactions of all these herbicides eventually leading to peroxidative destruction of cell components are unknown so far. By comparing the absorbance and fluorescence spectra with those of protoporphyrin IX and by HPLC analysis, we could demonstrate that a common feature of these compounds is the accumulation of protoporphyrin IX in the culture medium of *Bumilleriopsis*. The NMR spectral data and the mass spectra showing the protonated molecular ion together with a typical fragmentation pattern unequivocally identify protoporphyrin IX. Matringe and Scalla [4] have recently demonstrated the enrichment of a tetrapyrrole in non-chlorophyllous soybean cells during cultivation in darkness in the presence of the diphenyl ether acifluorfen-methyl and of LS 82-556. Our results suggest that it is protoporphyrin IX what they found.

Protoporphyrin IX was not detectable in *Neurospora* and in autotrophic *Scenedesmus* cells (Table IV). We conclude that protoporphyrin-IX accumulation in the presence of peroxidative herbicides is dependent on the existence of the chlorophyll biosynthetic pathway. However, in the light when peroxidation occurs, apparently protoporphyrin IX is destroyed. Therefore, protoporphyrin IX is found only in heterotrophically-grown *Scenedesmus*. In the case of *Bumilleriopsis* which is highly resistent against peroxidative herbicides, the protection mechanism (antioxidative systems) may also prevent protoporphyrin-IX degradation and even more protoporphyrin IX is present than in heterotrophic *Scenedesmus* cells. Nevertheless, the majority (*i.e.* 85%) of the protoporphyrin IX formed is excreted into the medium by *Bumilleriopsis* (Table III).

The accumulation of protoporphyrin IX after treatment with various peroxidative herbicides (Table III) raises the question on the possible connection between the effect on tetrapyrrole metabolism and peroxidative action. There could be an interdependent sequence of both events: Either peroxidation is the primary process leading to a destruction of enzyme(s) converting protoporphyrin IX, or the accumulated protoporphyrin IX acts as photosensitizer initiating peroxidation as has been proposed [6].

Both possibilities cannot be discriminated with *Bumilleriopsis* since this organism is extremely insensitive against peroxidizing herbicides and excretes protoporphyrin IX. In contrast, autotrophic *Scenedesmus* is very sensitive to peroxidizing herbicides. Concentrations 50 times less than used here are phytotoxic. However, protoporphyrin IX can neither be found inside nor outside the autotrophic cells when treated with peroxidizing herbicides. Only heterotrophic cells cultured in the dark accumulate protoporphyrin IX.

The possibility that four chemically different compounds are multifunctional by showing two different

and independent modes of action – accumulation of protoporphyrin IX and peroxidation – is unlikely. The demonstration of protoporphyrin-IX accumulation as a common feature of all peroxidizing herbicides known so far provides a new aspect which has to be taken into account when considering their mode of action. Experiments are under way to correlate protoporphyrin formation with peroxidative activity in photosynthetic cells.

Acknowledgements

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