Origin of Hydroxyphytoene Derivatives Formed in the Presence of Bleaching Herbicides

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Algae and higher plants treated with the bleaching herbicide flurtamone accumulate not only phytoene but also several derivatives that have phytoene-like spectra. The formation of different hydroxyphytoenes was light-dependent and increased with light intensity. Hydroxylation of phytoene and phytoene epoxide could also be detected in solution with chlorophyll as photosensitizer. With hydroxy-1,2-epoxy-1,2-dihydrophytoene as substrate, the major reaction products could be identified by mass spectroscopy as hydroxy-1,2-epoxy-1,2-dihydrophytoenes. The mechanism responsible for the formation of hydroxy derivatives of phytoene or phytoene epoxide is a nonenzymatic photochemical reaction sensitized, e.g., by chlorophyll. This reaction is not specific for the substrate. The singlet oxygen involved preferentially attacks isolated double bonds of the substrate molecule.

Keywords: *Cress seedlings; 1,2-epoxy-1,2-dihydrophytoene; flurtamone; hydroxy-1,2-epoxy-1,2-dihydrophytoene; hydroxylation; photooxidation; phytoene; Scenedesmus*

INTRODUCTION

Bleaching herbicides prevent the formation of colored carotenoids. Most of these herbicides directly interact with phytoene desaturase, leading to the accumulation of colorless phytoene (Sandmann and Böger, 1989). It has been shown recently that functionally and structurally different phytoene desaturases exist (Sandmann et al., 1992). After their purification, it could be demonstrated that only the specific type of phytoene desaturase found in organisms with oxygenic photosynthesis including green algae and higher plants is inhibited by bleaching herbicides (Sandmann and Fraser, 1993).

In the presence of bleaching herbicides not only phytoene but also phytoene epoxide and hydroxy derivatives are accumulated (Britton et al., 1987; Sandmann and Albrecht, 1990). The occurrence of hydroxyphytoenes was taken as an indication for their participation in the catalytic process of introduction of a double bond between carbons 11 and 12, but detailed analysis of the position of the hydroxy groups revealed that neither 11- nor 12-hydroxy derivatives of phytoene existed (Albrecht et al., 1991). Therefore, a desaturation mechanism via hydroxyphytoenes as intermediates seems to be rather unlikely. Furthermore, enzymatic studies including cofactor requirements of purified herbicide-susceptible phytoene desaturases revealed that either NAD or NADP is essential for catalysis (Fraser et al., 1993). This result demonstrated a dehydrogenation mechanism and excludes hydroxyphytoenes as intermediates. Consequently, the origin of different hydroxyphytoenes remains an open question. However, we found some indications that light has an effect on the composition of the different phytoene derivatives (Sandmann and Albrecht, 1990).

The present paper is concerned with the origin of hydroxyphytoene derivatives and the way they are generated under the influence of light in the presence of the bleaching herbicide flurtamone (Sandmann et al., 1990). The experiments were carried out not only with cress seedlings but also with the green alga *Scenedesmus*, which offers additional experimental advantages.

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MATERIALS AND METHODS

Scenedesmus acutus (strain 276-3a, Algal Culture Collection, University of Göttingen) was cultivated either heterotrophically in a glucose-containing medium or in a mineral medium as previously described (Sandmann and Böger, 1981). Autotrophic cultures were grown for 3 days in the light. Seeds of cress *(Lepidium sativum)* were germinated in a Petri dish on filter paper in 5 mL of water containing 0.1 μM flurtamone for 3 days.

Carotenoids were extracted with hot methanol (15 min, 60 °C) containing 6% KOH and partitioned into 10% diethyl ether in petroleum ether (bp 35–80 °C). The organic phase was concentrated for HPLC analysis. HPLC separation was carried out on a Spherisorb 5μ ODS-1 column with acetonitrile/methanol/2-propanol (95:3.5:1.5 v/v) and a flow of 1 mL/min (Sandmann and Albrecht, 1990). Spectra were recorded in the elution peaks by a diode array detector. The standards of phytoene and derivatives used for cochromatography were the ones that were previously isolated and identified (Albrecht et al., 1991). Chlorophyll was extracted with hot methanol and purified by TLC on silica plates using toluene/ethyl acetate (70:30).

Photochemical conversion of phytoene and phytoene epoxide was performed in 10 mL of methanol with a concentration of either 37 μg/mL phytoene or 26 μg/mL phytoene epoxide after addition of 1 mg of chlorophyll. The samples were illuminated in a test tube with an Osram L36W/30 fluorescence lamp with an intensity of 25 μE/m^2 s. Samples of 1 mL were taken, saponified, and extracted into 10% diethyl ether in petroleum ether and analyzed by HPLC. For mass spectral analysis of the reaction products after 20 h of illumination, the components were separated by HPLC and the newly appearing peak fractions collected. Spectra were recorded in a Finnigan mass spectrometer with an ion source temperature from 240 to 280 °C and an ionization potential of 70 eV.

Phytoene and phytoene epoxide (=1,2-epoxy-1,2-dihydrophytoene) were isolated from norflurazon-treated *Scenedesmus* as recently described (Albrecht et al., 1991). The bleaching herbicide flurtamone [=RE40885; 5-(methylamino)-2-phenyl-4-[3-(trifluoromethyl)phenyl]-3(2*H*)-furanone] was synthesized and provided by Chevron Chemical Co., Richmond, CA.

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Table 1. Carotenoids and Phytoene Derivatives in Flurtamone-Treated[a] Illuminated (110 μE/m^2 s) *Scenedesmus* and Cress Seedlings

	concn (μg/g of dry wt)	
	Scenedesmus	cress sedlings
colored carotenoids	1.64[b]	0.03[b]
dihydroxyphytoene[c]	0.51	
hydroxyphytoenes	1.67	0.03
phytoene epoxide	0.03	
phytoene	2.02	0.11

[a] The flurtamone concentrations used were 1 μM for *Scenedesmus* and 0.1 μM for cress seedlings. [b] In the cultures without flurtamone the concentrations of colored carotenoid were 1.05 μg/g of dry weight in cress leaves and 8.07 μg/g in *Scenedesmus*. Phytoene or derivatives were not detectable. [c] Tentative assignment.

RESULTS AND DISCUSSION

Detailed analysis of phytoene derivatives demonstrated that accumulation of phytoene as an effect of bleaching herbicides is accompanied by several hydroxyphytoene derivatives and by phytoene epoxide (Albrecht et al., 1991). Table 1 quantitates their occurrence and the decrease of colored carotenoids in cultures of the green alga *Scenedesmus* and in cress seedlings after application of flurtamone. In contrast to the untreated *Scenedesmus* culture, substantial amounts of phytoene were found. In addition, comparably high amounts of hydroxyphytoenes were accumulated. Another compound had a phytoene spectrum and showed a very high polarity. Because it exhibited an HPLC behavior similar to that described for dihydroxyphytoene (Britton et al., 1987), we tentatively identified it as this phytoene derivative. In cress seedlings phytoene and hydroxyphytoenes accumulated due to flurtamone treatment.

It was shown previously that the proportions of hydroxyphytoene and epoxyphytoene vary with illumination (Sandmann and Albrecht, 1990). For a detailed investigation of this light effect we used cultures of *Scenedesmus* which offer the advantage that they grow either autotrophically in the light or heterotrophically in total darkness with a complete pigment inventory (Sandmann et al., 1984).

In a time course experiment with *Scenedesmus* which was grown for 6 days in darkness and then transferred into light, from day 3 on (Figure 1) the formation of phytoene and derivatives (all with phytoene-like spectra) was measured. After 5 days in the dark, phytoene accumulation reached its maximum. In parallel, small amounts of hydroxyphytoenes and steadily increasing amounts of phytoene epoxide were formed. After illumination, a drop in the phytoene and epoxide contents was observed. In contrast, the concentration of hydroxyphytoenes strongly increased.

In Figure 2 light dependence of hydroxyphytoene formation was investigated with cress seedlings treated with 0.1 μM flurtamone. The relative hydroxyphytoene content as percent of total phytoene and derivatives was plotted against the light intensity. In seedlings grown in complete darkness hydroxyphytoenes were not detectable. Variation of the light intensity in the range from 13 to about 240 μE/m^2 s resulted in a steady increase of the hydroxyphytoene concentration. This result suggests that hydroxyphytoenes may originate from accumulating phytoene. Due to this light-dependent occurrence of hydroxyphytoenes in herbicide-treated plants, we carried out photooxidation experiments in solution using purified phytoene and phytoene epoxide as substrates with chlorophyll as photosensitizer (Figure 3). Over a period of 5 h of illumination, about 50% of the phytoene was converted (part A), and after 20 h, only a residual 20% was left. Simultaneously, different positional isomers of hydroxyphytoenes were formed (Table 1). These compounds were identified by their spectrum and by cochromatography with NMR-characterized standards (Albrecht et al., 1991). Typically, the

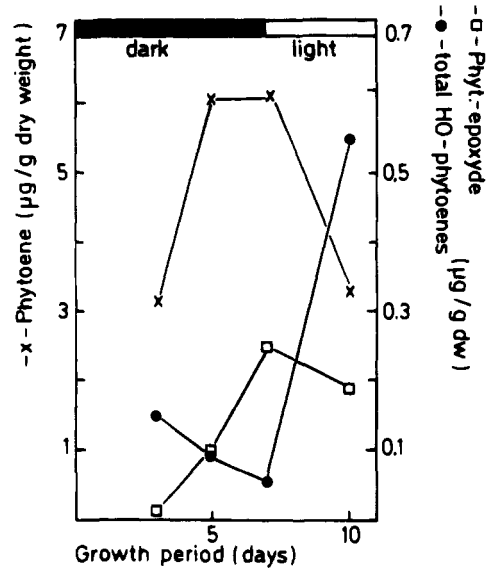

Figure 1. Synthesis of phytoene derivative in flurtamone-treated *Scenedemus* which was cultivated in darkness followed by transfer into light.

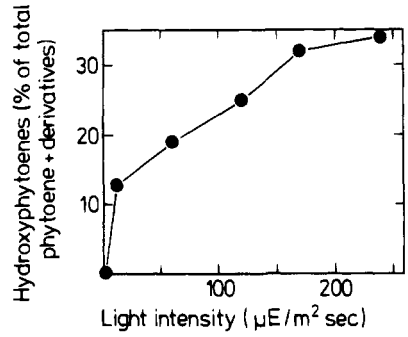

Figure 2. Relative amounts of hydroxyphytoenes as percent of total phytoene and derivatives in cress seedlings grown at different light intensities in the presence of 0.1 μM flurtamone.

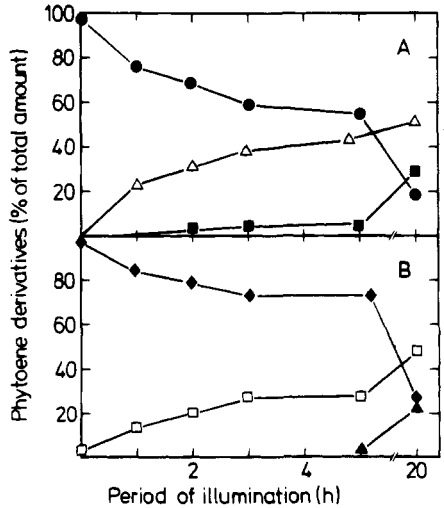

Figure 3. Accumulation of products during photooxidation of phytoene (A) and phytoene epoxide (B): ●, phytoene; ▽, hydroxyphytoene; ■, dihydroxyphytoene; ♦, phytoene epoxide; □, hydroxyphytoene epoxide; ▼, dihydroxyphytoene epoxide.

Table 2. Characterization of Phytoene Derivatives Originating from Phytoene or Phytoene Epoxide in Photosensitized Oxidation Reactions

retention time (min)	identified as
phytoene	
7.9	dihydroxyphytoene[a]
14.8	hydroxyphytoene[b]
15.5	hydroxyphytoene[c]
16.5	hydroxyphytoene[d]
18.3	phytoene epoxide[e]
31.7	15-*cis*-phytoene
33.6	*all-trans*-phytoene
phytoene epoxide	
6.8	dihydroxyphytoene epoxide[a]
10.3	hydroxyphytoene epoxide
18.3	phytoene epoxide[e]

[a] Tentative assignment. [b] 1-Hydroxy-2,3- together with 5-hydroxy-6,7-didehydrophytoene. [c] 6-Hydroxy-5,18-didehydrophytoene. [d] 9-Hydroxy-10,11- together with 10-hydroxy-9,19-didehydrophytoene. [e] 1,2-Epoxy-1,2-dihydrophytoene.

hydroxy group is located at carbon atoms next to an isolated double bond which had been shifted upon hydroxylation. The major hydroxyphytoene derivatives detected either in *Scenedesmus* or in the cell-free experiment were 5-hydroxy-6,7- and 6-hydroxy-5,18-didehydrophytoene. After 5 h, trace amounts of a very polar phytoene derivative with a retention time of 7.9 min in our HPLC system (Table 2) were detectable, which finally accumulated to about 30% after 20 h of illumination. Most likely, this product of phytoene is the dihydroxyphytoene previously reported to be formed in radish seedlings treated with a bleaching herbicide (Britton et al., 1987). This compound was also the most polar phytoene derivative in their reversed-phase HPLC system.

Similar photooxidation experiments were performed with phytoene epoxide. A period of 5 h of illumination resulted in a conversion of only 25%, which increased to about 50% after 20 h (Figure 3B). The major reaction product was analyzed by mass spectroscopy and identified as a hydroxyphytoene epoxide. Figure 4 shows its

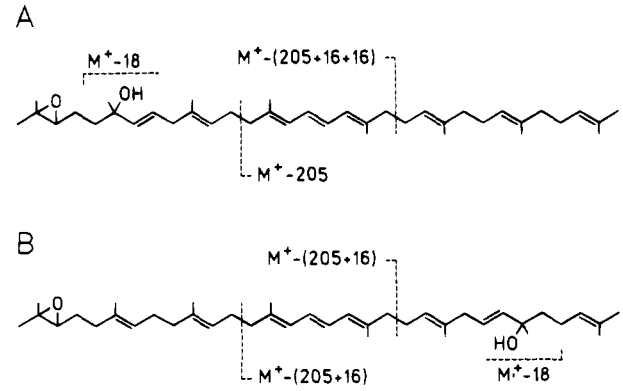

Figure 5. Thermal fragmentation pattern during mass spectroscopy of hydroxy-1,2-epoxyphytoenes exemplified with either the 5- (A) or the 5′-hydroxy derivative (B).

mass spectrum. The mass peaks in this spectrum are the molecular ion *m/e* 576 together with prominent fragment ions at *m/e* 558, 371, 355, 353, 339, and 337. Ion *m/e* 558 ($M^+ - 18$), originating from water loss of a newly introduced hydroxy group, is present in large amounts. The high intensity of this peak is typical for an allylic hydroxy derivative (Kayser, 1976). The other specific fragment ions allow us to discern whether the hydroxy group is introduced near the side where the 1,2-epoxy moiety is located or into the other half of the molecule. The typical fragmentation pattern of both alternatives is examplified for either 5- or 5′-hydroxy-1,2-epoxyphytoene in Figure 5. In the case of the 5-hydroxy derivative (part A), the fragment *m/e* 371 ($M^+ - 205$) results from the scission of three isoprenic units (Moss and Weedon, 1976). This fragmentation is typical for the unsubstituted end of a carotene. In addition, the residual substituted part can lose the hydroxy group as water, giving a fragment ion of *m/e* 353 ($M^+ - 205 - 18$). Alternatively, the side carrying the epoxy together with the hydroxy group can be split, yielding the fragment ion *m/e* 339 [$M^+ - (205 + 16 +$

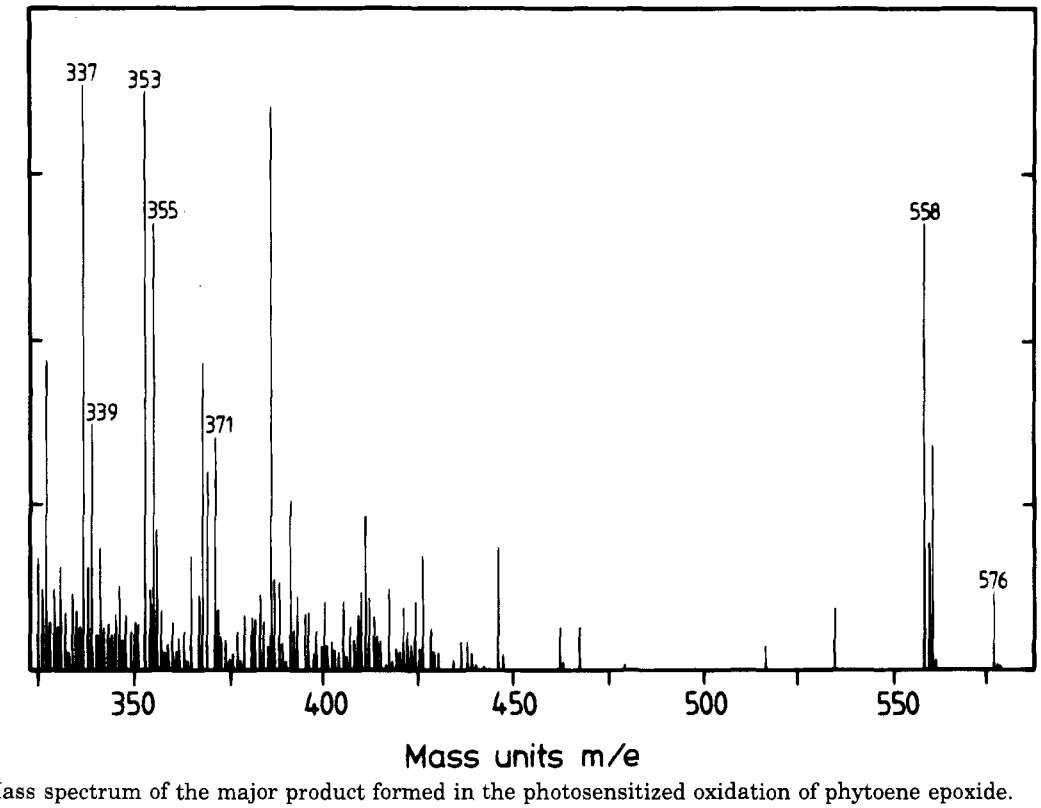

Figure 4. Mass spectrum of the major product formed in the photosensitized oxidation of phytoene epoxide.

16)]. When both oxygen groups are separated as in Figure 5B, scission of three isoprenic units results in fragment m/e 355 [$M^+ - (205 + 16)$] regardless of which side is split off. When the side with the epoxy group is lost together with water from the residual fragment, a fragment ion of m/e 337 [$M^+ - 18 - (205 + 16)$] is retained. The occurrence of all possible fragment ions (Figure 4) demonstrates that during photooxidation of 1,2-epoxyphytoene a series of hydroxy isomers are formed including those with a hydroxy group at either positions 3−11 or 1′−11′. This diversity resembles the formation of hydroxyphytoene isomers in *Scenedesmus* (Albrecht et al., 1991).

A second product derived from photooxidation of phytoene epoxide could be detected after only 5 h of illumination (Figure 3B). After 20 h, it resembled about 20% of the initially applied phytoene epoxide. This compound showed a higher polarity than the hydroxyphytoene epoxide with a retention time of 6.8 min in our HPLC system (Table 2). In analogy to the products formed in the experiment with phytoene as substrate, this compound is most likely a dihydroxyphytoene epoxide.

In the experiments of Figure 3 it was possible to simulate the formation of hydroxyphytoenes in intact plant cells which occurs under conditions of high phytoene accumulation in a photochemical reaction. A nonenzymatic photooxidative mechanism could be demonstrated. The reaction is photosensitized by either chlorophyll, a porphyrin precursor, or a breakdown product which may be abundant in chloroplasts where the desaturation reaction is located (Linden et al., 1993) and the phytoene is accumulated. The different positional hydroxylated isomers (Albrecht et al., 1991) indicate that the same type II mechanism involving singlet oxygen as described for chlorophyll-sensitized photooxidation of the olefin 2-methyl-2-pentene (Gollnik, 1968) is responsible for the introduction of hydroxy groups into phytoene. Singlet oxygen attacks the double bond of the olefin between C-2 and C-3. Upon reduction of the resulting hydroperoxides, 2- or 3-hydroxylated derivatives are yielded in a ratio of 1:1 in which the double bond is shifted (Foote and Denny, 1971). In the results presented here the nature of the reducing agent is unknown. As phytoene possesses three isolated double bonds at each side of the symmetrical molecule, six different positional isomeric monohydroxy derivatives are possible. Consequently, photooxidation of the 1,2-phytoene epoxide can result in the generation of 10 different hydroxylation products. Our interpretation of the mass spectrum (Figure 4) showed that there is no preference for a specific carbon atom of the phytoene epoxide molecule to be hydroxylated. Furthermore, the occurrence of different hydroxyphytoenes in herbicide-treated *Scenedesmus* has previously been shown (Albrecht et al., 1991).

At least for the large amounts of hydoxyphytoenes formed in illuminated plants we can exclude the possibility that they are formed from phytoene epoxide by opening of the epoxy ring as recently discussed (Albrecht et al., 1991). However, the small amounts of hydroxyphytoenes found in dark-grown flurtamone-treated *Scenedesmus* (Figure 1) may originate from the large quantities of phytoene epoxide formed. Another possibility is the formation directly from phytoene by radicalic reactions. The photooxygenation reaction is not specific to phytoene. With phytoene epoxide the same analogous product was obtained in solvent but with a slower rate. The resulting reaction products can also serve as additional substrates for a further hydroxylation to dihydroxy derivatives (Table 2).

In conclusion, it was possible to identify the mechanism leading to the formation of hydroxyphytoene derivatives as a nonenzymatic photochemical reaction sensitized, e.g., by chlorophyll. In contrast, phytoene epoxide originates from an independent reaction. It is not generated by a photosensitized oxidation and may be enzymatically formed by an epoxidase which, due to a broad substrate specificity, exhibits some affinity for phytoene as previously suggested (Albrecht et al., 1991).

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