Accumulation of Colorless Carotenes and Derivatives during Interaction of Bleaching Herbicides with Phytoene Desaturation

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Z. Naturforsch. **45c,** 487–491 (1990); received November 1, 1989

Diflufenican, Difunon, Fluridone, Hydroxyphytoene, Norflurazon, Phytoene Epoxyde, Phytoene Isomers, Phytofluene Isomers

Colorless carotenoids were accumulated, analyzed and quantified in heterotrophically-grown *Scenedesmus* cultures treated with the bleaching herbicide norflurazon. By optical, IR and mass spectroscopy 15-*cis* as well as all-*trans* phytofluene, 15-*cis* phytoenes and traces of its all-*trans* isomer were identified. Furthermore, a phytoene epoxyde and several hydroxyphytoenes were assigned. Comparing the concentrations of these phytoene derivatives in heterotrophic cultures grown in darkness with those in light-grown autotrophic cells, an exchange of phytoene derivatives was observed. Levels of phytoene were much higher in the dark whereas concentrations of hydroxyphytoenes dominated in the light. Other herbicides like fluridone, diflufenican, and difunon accumulate the same colorless carotenoids including phytoene epoxyde and various hydroxyphytoenes. For all herbicides an almost constant hydroxyphytoene to phytoene epoxyde ratio was observed indicating an interrelated formation of both oxygenated phytoene derivatives which can be influenced by light conditions of the cultures.

Introduction

Phytoene desaturase (= phytoene dehydrogenase) is a key enzyme in the carotenogenic pathway. It utilizes both identical halves of the symmetrical phytoene molecule as substrate and introduces two double bonds at C-11 and C-11′. This reaction is dependent on NAD(P) and oxygen [1]. Phytoene desaturase is allosterically regulated by subsequent carotenes in the pathway and inhibited by various so-called bleaching herbicides [2].

Since the first report by Bartels and McCullough in 1972 on interference of the pyridazinone metflurazon with phytoene desaturation [3], many chemically different herbicides for this target have been developed [4]. After suitable *in vitro* systems for the carotenogenic pathway had been established [1, 5], it was possible to perform enzyme-kinetic studies on herbicide interaction with phytoene desaturase. In the case of another pyridazinone herbicide, norflurazon, it could be demonstrated recently that this compound is a reversible non-competitive inhibitor of phytoene desaturase [6].

In this paper we investigate the consequences of phytoene-desaturase inhibition by norflurazon on accumulation of colorless carotenes and oxygenated derivatives and compare it with the action of other phytoene desaturase-inhibiting herbicides. Without addition of an inhibitor the levels of phytoene, phytofluene and other carotene precursors of α- and β-carotene are below the detection limit. For our studies we employed heterotrophic cultures of the green alga *Scenedesmus*. This organism grows well on an organic substrate in complete darkness developing a chloroplast which is almost identical to higher plants with a similar carotenoid inventory. Using this alga in the dark, we can exclude interference of light with carotenoids *e.g.* photoisomerization and photooxidative degradation, and determine the amount of naturally occurring accumulation products and the proportion of *cis* and *trans* isomers.

Materials and Methods

Scenedesmus acutus (strain 276-3a, Algal Culture Collection, University of Göttingen) was cultivated heterotrophically in a glucose-containing medium or for the autotrophic experiment in Table I in a mineral medium as described in ref. [7] with 1 μM norflurazon present. Heterotrophic cultures were grown for one week in darkness and autotrophic ones for three days in the light. Carotenoids were extracted with hot methanol (15 min, 65 °C) containing 6% KOH and partitioned

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Verlag der Zeitschrift für Naturforschung, D-7400 Tübingen
0341–0382/90/0500–0487 $ 01.30/0

against 10% diethyl ether in petrol (b.p. 35–80 °C). The lipophilic phase was further used for separation and quantitation of colorless carotenoids or further purification necessary for subsequent spectroscopic analysis.

Phytoene and phytofluene, respectively, were obtained after TLC on activated Al_2O_3 60 F_{254} plates (Merck, Darmstadt, F.R.G.) with 5% toluene in hexane by scraping-off the fluorescence-quenching band at R_f = 0.7 and the fluorescent band at R_f = 0.4 and elution with diethyl ether. Subsequently, the 15-*cis* and *trans* isomers were separated by column chromatography on Al_2O_3 (Woelm, Eschwege, F.R.G.), grade I [8]. The details on purification of epoxy- and hydroxyphytoene are given in a previous publication [9].

Prior to HPLC analysis the extracts were run on silica-gel TLC strips in toluene for 5 cm and a high and a low polar fraction was obtained by scraping-off and elution with acetone of the zones from 1 to 3 cm and 4 to 5 cm, respectively. HPLC separation was carried out on a Spherisorb 5 ODS-1 column with acetonitrile/methanol/2-propanol 95:3.5:1.5 and a flow of 2 ml/min [10]. Elution was monitored with a Philips Pye Unicam PU 4021 multichannel diode-array detector with simultaneous recording of the spectra in the elution peaks.

Infra-red (IR) spectra were recorded in KBr pellets in the range from 600 to 1500 cm^{-1} with a Polaris ™FM IR spectrometer. UV spectra were obtained in spectroscopic pure hexane in a Shimadzu UV 360 spectrophotometer. Mass spectra were determined in a MAT 112 S mass spectrometer with an ion-source temperature from 240 to 280 °C and a ionization potential of 70 eV.

Bleaching herbicides used in this study were norflurazon (4-chloro-5-methylamino-2-(trifluoromethylphenyl)-pyridazin-3(2H)one), difunon (5-(dimethylaminomethylene)-2-oxo-4-phenyl-2,5-dihydrofurane-carbonitrile-(3)), fluridone (1-methyl-3-phenyl-5-(3-trifluoromethylphenyl)-4(1H)-pyridone), and diflufenican (N-(2,4-difluorophenyl)-2-(3-trifluoromethylphenoxy)nicotinamide).

Results and Discussion

After separation of a carotenoid extract from a dark-grown *Scenedesmus* culture treated with norflurazon into a non-polar and a polar fraction by TLC, both fractions were analyzed by HPLC. As phytoene desaturase, the target of norflurazon, converts phytoene *via* phytofluene into ζ-carotene, occurrence of both carotenes and possible derivatives were monitored by on-line recording of corresponding absorbance spectra. This procedure revealed four major UV-absorbing carotenoids in the non-polar fraction (Fig. 1A), two with phytofluene-like spectra, **1** and **2,** with absorbance maxima at 331, 348, 368 nm, and two phytoene-like compounds, **3** and **4,** with absorbance maxima at 276, 286, 297 nm [8]. In the polar fraction four additional carotenoids, all with a phytoene-like spectrum, were detected.

Subsequent mass spectroscopy was employed to identify the accumulation products. In the mass spectra compounds **1** and **2** showed the molecular ion of phytofluene at *m/e* 542 [11] together with a prominent fragment at 337 resulting from the scission of three isoprene units (data not shown). Compounds **3** and **4** exhibited molecular ions of *m/e* 544 and the fragment of 339 which is typical for phytoene. These results indicate that carotenoids **1** and **2** are two different phytofluene and carotenoids **3** and **4** two different phytoene stereo isomers.

One way of analyzing stereo isomers is by IR spectroscopy [11, 12] or in case of phytofluene by comparing the peak heights in the optical absorbance spectrum [13, 14]. In Fig. 2A the IR spectra

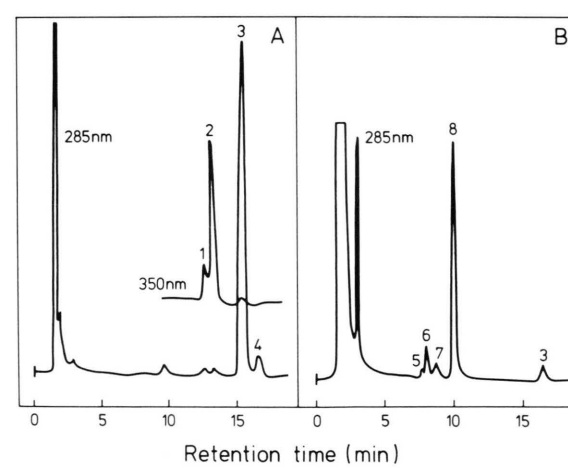

Fig. 1. HPLC separation of the non-polar (A) and polar (B) UV-absorbing carotenoids which accumulate in the presence of norflurazon.

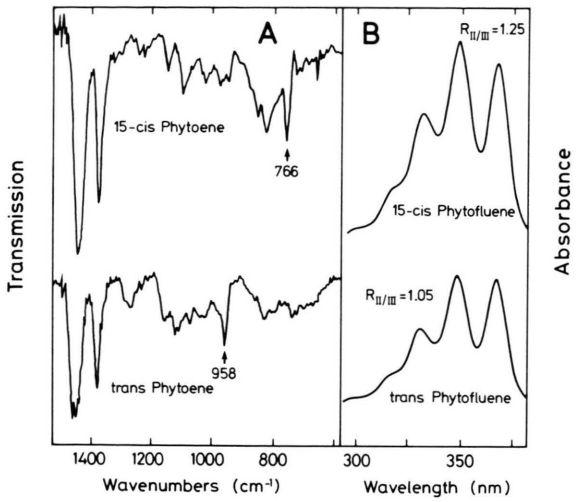

Fig. 2. Assignment of carotenoids **3** as the 15-*cis* and **4** as the all-*trans* isomers of phytoene (A) by IR spectroscopy and **1** as the 15-*cis* and **2** as the all-*trans* phytofluene (B) by UV spectroscopy.

of the phytoene in peak No. 3 (top spectrum) and in peak No. 4 (bottom spectrum) are compared. The absorbance strongest band of carotenoid **3** at 766 cm^{-1} corresponds to a C–H out of plane deformation in a disubstituted *cis*-double bond. In the case of phytoene this absorbance determines exclusively the double bond at position 15 to occur in a *cis* configuration. In carotenoid **4** the 766 cm^{-1} band is absent and is replaced by a band at 958 cm^{-1} which is characteristic of a corresponding disubstituted 15-*trans*-double bond [11].

The 15-*cis* and all-*trans* isomers of phytofluene show the same UV absorbance maxima but are different in their fine structure [8]. A good indicator to discriminate between these two isomers can be calculated from the ratio of heights of the middle absorption band *versus* the longest wavelength band with a base line through the minimum between the two maxima (= $R_{II/III}$). This value is 1.25 for carotenoid **1** (top spectrum in Fig. 2B) and 1.05 for carotenoid **2** (bottom spectrum in Fig. 2B). Comparing these values with those obtained for authentic phytofluene isomers [13, 14], the phytofluenes from peaks No. 1 can be assigned as 15-*cis* and No. 2 as all-*trans*.

The mass spectra of the polar phytoene derivatives are presented in Fig. 3. Spectrum A shows the major peaks of carotenoid **8.** They are at *m/e* 560, 355 and 339. The mass peak of 560 corresponds to a phytoene molecule carrying one additional oxygen and the fragment ions correspond to the typical loss of 3 isoprenic units [10], one without ($M^+ - 205$) and one with an additional oxygen ($M^+ - (205 + 16)$). These mass-spectroscopy data are the same as obtained for a phytoene epoxyde from tomato [15]. The possibility that compound **8** is a hydroxyphytoene can be excluded from the absence of a fragment *m/e* 542 ($M^+ - 18$) due to

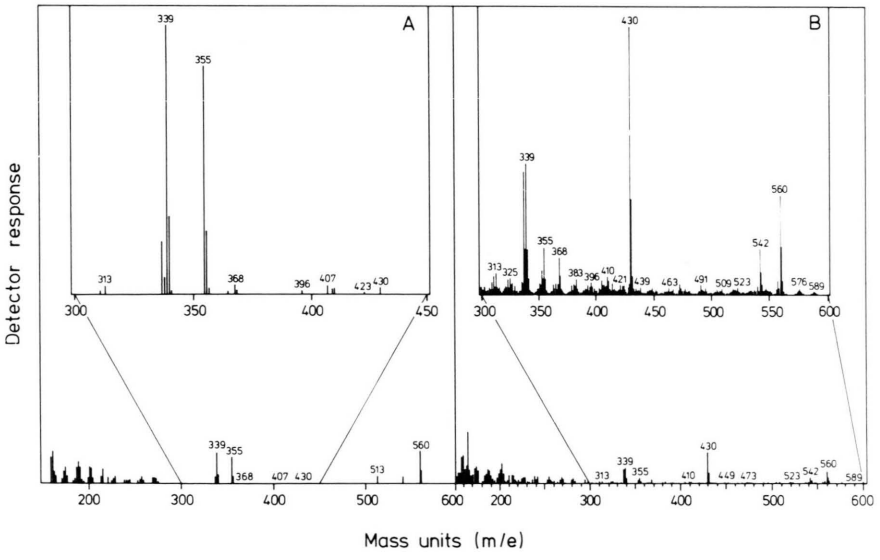

Fig. 3. Mass spectra of carotenoids **8** (A) and **7** (B) identifying them as epoxy- and hydroxyphytoene, respectively.

water loss which would be detectable otherwise and by a negative sililation reaction [11]. In a previous publication [9] it was demonstrated by NMR-spectroscopic analysis that this phytoene epoxyde is 1,2-epoxy-1,2-dihydrophytoene.

The mass spectrum of carotenoid **7** (Fig. 3B) shows the same mass peak at 560. However, other fragment ions in addition to *m/e* 339 and 355 at 337 and 542 are present which can be denoted to an oxygenated phytoene derivative [11]. The most conclusive fragment *m/e* 337 ($M^+ - 18 - 205$) results from scission of 3 isoprenic units after loss of water. Carotenoids from HPLC peaks No. 6 and 7 showed essentially the same fragmentation pattern. All three polar phytoene derivatives (**5** to **7**) can therefore be assigned as monohydroxyphytoenes. The difference in polarity of these 3 hydroxyphytoenes which enables their separation on reversed-phase HPLC results from the different positions of the hydroxy groups. Due to their decreasing polarity, the hydroxyphytoenes were numbered **I** (carotenoid **5** in Fig. 1), **II** (carotenoid **6**), and **III** (carotenoid **7**). The mechanism of hydroxyphytoene formation in norflurazon-treated cells has been discussed to occur either by secondary oxidation of phytoene especially in the light or by rearrangement of 1,2-epoxy-1,2-dihydrophytoene and other possible phytoene epoxydes *via* carbonium anion intermediates [9].

In Table I the amounts of phytofluene, phytoene and its derivatives which accumulate in heterotrophic *Scenedesmus* were determined and compared with the autotrophic cultures grown in the light. With 1 μM of norflurazon the colored carotenoids are strongly decreased regardless of the cultivation conditions. The main accumulation product is 15-*cis* phytoene accompanied by 1 to 2% of its all-*trans* isomer. Only in heterotrophic *Scenedesmus* substantial amounts of phytofluene were determined. Obviously, phytofluene is very sensitive to photodestruction in the light. The main isomer in heterotrophic cells was *trans* phytofluene together with about 11% of the 15-*cis* stereo isomer present.

1,2-Epoxy-1,2-dihydrophytoene was the dominating phytoene derivative but only in dark-grown cultures. In the autotrophic-illuminated cells very low amounts of this compound was found. The hydroxyphytoenes **I, II,** and **III** (numbered in order of decreasing polarity on reversed-phase HPLC) were found in small concentrations compared to accumulated epoxyde in heterotrophic cultures. However, in autotrophic cultures the distribution was reversed. Except for hydroxyphytoene **I** much more hydroxy derivatives were accumulated than epoxyde. This result on replacement of phytoene epoxyde by hydroxylated derivatives in the light supports the assumption that in illuminated cultures the hydroxy phytoenes are formed at the expense of phytoene epoxydes. Therefore, a polar phytoene derivative which has been detected in light-grown wheat seedlings treated with metflurazon [16] is most likely a hydroxyphytoene rather than an epoxyde as assumed by the authors.

Hydroxyphytoene has also been demonstrated when carotenoid biosynthesis was inhibited by diflufenican, another bleaching herbicides [17]. Therefore, we have investigated the influence of other chemically unrelated bleaching herbicides besides of norflurazon on the formation of phytoene and other oxygenated derivatives in heterotrophic *Scenedesmus* cultures (Table II). In gener-

Table I. Accumulation of carotenoids (μg/mg d.w.) in heterotrophic and autotrophic *Scenedesmus* treated with norflurazon (1 μM).

Carotenoids	Heterotrophic cultures		Autotrophic cultures	
	Control	Norflurazon	Control	Norflurazon
Colored carotenoids	4.85	0.33	6.12	1.51
15-*cis* Phytoene	0	3.31	0	5.88
trans Phytoene	0	0.07	0	0.09
15-*cis* Phytofluene	0	0.14	0	traces
trans Phytofluene	0	1.09	0	
Hydroxyphytoene **I**	0	0.13	0	0.01
Hydroxyphytoene **II**	0	0.30	0	1.52
Hydroxyphytoene **III**	0	0.18	0	1.25
1,2-Epoxy-1,2-dihydrophytoene	0	1.23	0	0.04

Table II. Influence of chemically different bleaching herbicides on the accumulation of phytoene, phytoene epoxyde (P-epoxyde), and total hydroxyphytoenes (HO-P).

Herbicides (1 μM)	Concentrations (μg/mg d.w.) of		Ratios (%) of	
	total phytoene derivatives	phytoene	P-epoxyde phytoene	HO-P P-epoxyde
Norflurazon	4.09	3.81	6.9	39.3
Fluridone	3.36	3.17	5.7	47.4
Difunon	4.07	3.60	11.6	44.4
Diflufenican	5.57	5.31	4.7	34.0

al, their effect is very similar. In addition to phytoene, they all accumulate phytoene epoxyde and hydroxyphytoenes. When applied in concentrations of 1 μM, diflufenican accumulated the highest amount of phytoene followed by norflurazon, difunon, and fluridone. The ratios of phytoene epoxyde to phytoene varied for the different herbicides and were highest for difunon and lowest for diflufenican. In contrast, the ratios of hydroxy- to epoxyphytoene were comparably constant which is another indication that formation of both oxygenated derivatives is interrelated.

From all the results we can conclude that the direct effect of the investigated bleaching herbicides is inhibition of phytoene desaturation. They accumulate phytoene almost exclusively as *cis* isomer which indicates that phytoene synthase specifically forms the *cis* isomer as substrate for phytoene desaturase in *Scenedesmus*. The occurrence of about 90% of *trans* phytofluene and only 10% of the 15-*cis* isomer can be explained by the mechanism suggested by Goodwin [18] on desaturation of carotenes. He proposed that a strained molecule present in an enzyme complex is the active intermediate. Consequently, when the phytoene conversion is inhibited, phytofluene should be released mainly in the thermodynamically favoured all-*trans* configuration.

So far, no indications were obtained to link the hydroxyphytoenes and phytoene epoxyde which have been identified with the catalytic process of phytoene desaturation. From the data available we assume that hydroxyphytoenes and phytoene epoxydes are formed by secondary effects of the herbicides *e.g.* by metabolization of phytoene which is accumulated in excess. These oxidation reactions could occur either enzymatically by enzymes which have a broad substrate specificity or by free radicalic reactions.

Acknowledgements

This work was supported by the Deutsche Forschungsgemeinschaft. Due thanks are expressed to Dr. G. Britton, Department of Biochemistry, University of Liverpool, for helpful discussion.

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