Herbicidal Inhibition of Carotenogenesis Detected by HPLC

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Diflufenican, Dichlormate, Amitrole, Carotenoid Biosynthesis, Precursor Accumulation

The target sites of three herbicides which inhibit carotenoid biosynthesis have been characterized using HPLC analysis of pigment extracts from two higher plant systems, carrot cell suspension cultures and barley seedlings. Diflufenican causes an accumulation of phytoene and phytofluene. Dichlormate causes accumulation of phytoene, phytofluene, ζ-carotene, neurosporene and β-zeacarotene. Amitrole causes accumulation of phytoene, phytofluene, ζ- γ- and δ-carotenes and lycopene. Significant differences in the geometric and hydroxylated natures of the accumulated precursors occurred between the carrot cell and dark- and light-grown barley. These differences are discussed with respect to both the target sites of the three carotenogenic herbicides and the biosynthetic pathway leading to carotenoid biosynthesis in higher plants.

Introduction

The herbicidal inhibition of carotenoid biosynthesis has been demonstrated with several classes of unrelated molecules *e.g.* phenyl pyridazinones, phenoxybenzamides and phenoxynicotinamides [1]. Therefore, a procedure which could rapidly characterize such inhibitors in terms of their target site(s) *i.e.* desaturation and/or cyclization would be a valuable tool in assessing biochemical activity of potential bleaching herbicides.

A HPLC method has been developed and used in the characterization of a number of carotenogenic inhibitors *e.g.* diflufenican [1–3]. This method has been shown to separate, detect and quantify, in one single 30 min analysis, the full complement of chloroplastic pigments *i.e.* the xanthophylls, carotenes, chlorophylls *a* and *b* and also the unique precursors of carotenoid biosynthesis [1–3].

This system has been used here to examine the effects of three known inhibitors of carotenogenesis in two higher plant systems. Diflufenican was chosen as a known inhibitor of phytoene desaturation [2–5]; dichlormate was chosen as a known inhibitor of phytoene and ζ-carotene desaturation [6] and amitrole as an inhibitor of phytoene and ζ-carotene desaturation and lycopene cyclization [1]. Data is presented which shows the effects of these compounds on carotenogenesis in dark- and light-grown barley and in a carrot cell culture system. Special emphasis will be given to the fate of accumulated precursors in relation to their geometric conformation and their modification by the introduction of oxygen functions in the absence and presence of light.

Abbreviations: diflufenican, N-(2,4-difluorophenyl)-2-(3-trifluoromethylphenoxy)-3-pyridine carboxamide; dichlormate, (3,4-dichlorobenzyl methyl carbamate); amitrole, (1 H – 1,2,4-triazol-3-ylamine).

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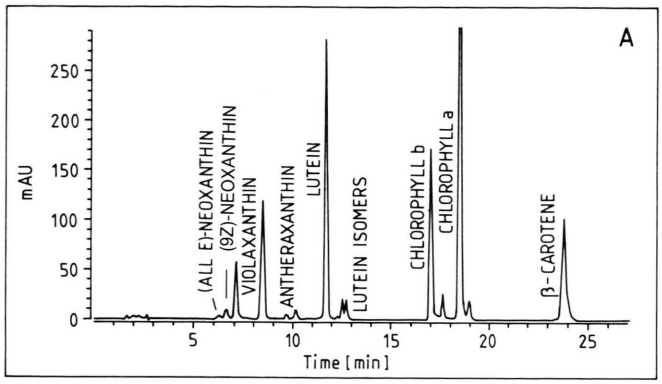
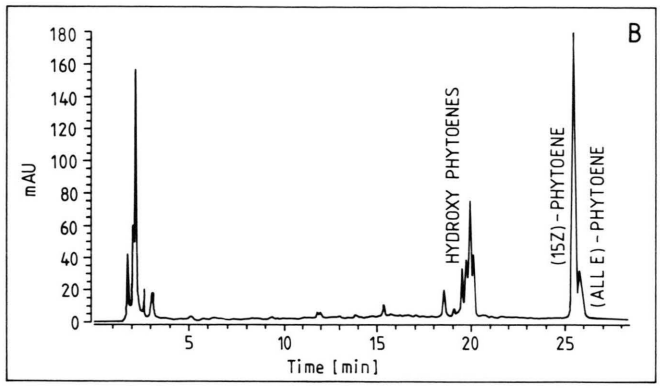
Materials and Methods

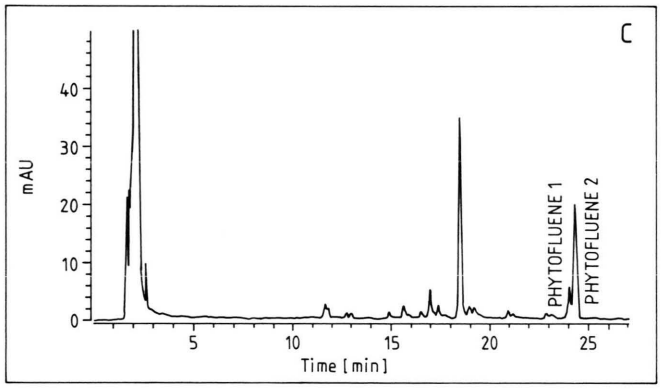
The two higher plant systems used in this study are dark-grown carotenogenic carrot cell suspension cultures and barley seedlings (etiolated and light-grown). All herbicide treatments, growth conditions, carotenoid extraction and analysis were as reported previously [5]. Details of herbicide concentrations employed and treatment times are given in the legends to Tables I, II and III.

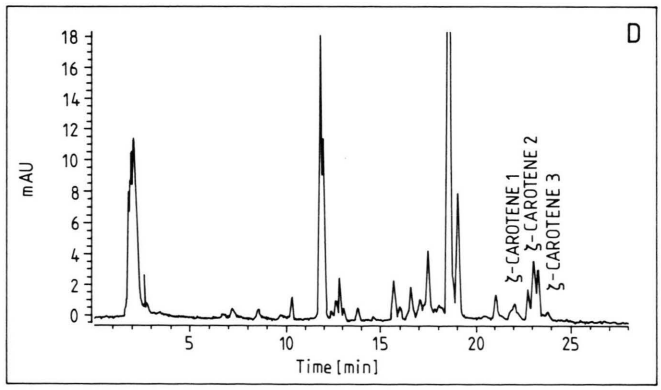
Results

Fig. 1A shows an HPLC chromatogram of chloroplastic pigments from untreated light-grown barley cotyledons. The composition and content of this tissue was as expected for normal light-grown tissue. This tissue does not contain detectable amounts of any of the unique carotenoid precursors *i.e.* phytoene, phytofluene, ζ-carotene, neurosporene and lycopene.


A
mAU
Time [min]
(ALL E)-NEOXANTHIN
(9Z)-NEOXANTHIN
VIOLAXANTHIN
ANTHERAXANTHIN
LUTEIN
LUTEIN ISOMERS
CHLOROPHYLL b
CHLOROPHYLL a
β-CAROTENE


B
mAU
Time [min]
HYDROXY PHYTOENES
(15Z)-PHYTOENE
(ALL E)-PHYTOENE


C
mAU
Time [min]
PHYTOFLUENE 1
PHYTOFLUENE 2


D
mAU
Time [min]
ζ-CAROTENE 1
ζ-CAROTENE 2
ζ-CAROTENE 3

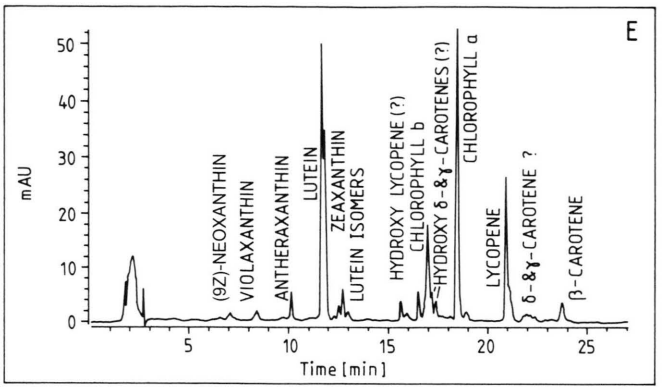


Fig. 1. Typical chromatograms of pigment extracts using the HPLC system employed in this study. An untreated light-grown barley with detection at 441 nm. B, C, D and E light-grown barley treated with 100 μM amitrole and detection at 287, 350, 401 and 441 nm respectively. NB. in light-grown barley no carotenoid precursors were detected at 287, 350 and 401 nm.

Tables I, II and III represent summaries of typical data obtained using the two carotenogenic systems treated with diflufenican, dichlormate and amitrole respectively. Fig. 1 B–E shows the pigment profiles from light-grown barley treated with 100 μM amitrole. They exemplify the separations obtained for the carotenoid precursors including a number of geometric and hydroxylated conformations.

All diflufenican treatments resulted in an accumulation of phytoene and to a lesser extent phytofluene (Table I). In dark treatments (carrot and dark-grown barley) phytoene accumulates solely in the 15*Z* (*cis*)-form. Whereas in the light-grown barley treatment both the 15*Z* and all *E* (*trans*)-conformations were detected. The ratio of (15*Z*)- to (all *E*)-isomers decreased with increasing time of exposure to light (data not shown). Hydroxylat-

Table I. Summary of carotenoid precursor profiles following diflufenican treatment.

Precursor	Carrot cells	Barley dark-grown	Barley light-grown
(15*Z*)-Phytoene	95.3	81.9	39.5
(all *E*)-Phytoene	n.d.	n.d.	17.7
Phytofluene 1	2.3	15.2	0.4
Phytofluene 2	2.3	2.9	1.0
Hydroxyphytoenes	n.d.	n.d.	39.9
Dihydroxyphytoenes	n.d.	n.d.	1.8

Data represents a summary of typical data, expressed as a percentage of total carotenoid precursors from carrot cells and barley treated with 1.0 and 100 μM diflufenican respectively for 7 days. n.d. = not detected.

Table II. Summary of carotenoid precursor profiles following dichlormate treatment.

Precursor	Carrot cells	Barley dark-grown	Barley light-grown
(15*Z*)-Phytoene	52.8	33.1	43.5
(all *E*)-Phytoene	n.d.	tr.	tr.
Phytofluene 1	11.7	7.7	4.0
Phytofluene 2	1.0	1.9	9.7
ζ-Carotene 1	6.0	18.0	5.5
ζ-Carotene 2	24.2	29.5	13.4
ζ-Carotene 3	n.d.	n.d.	14.8
β-Zeacarotene	2.1	3.2	0.5*
Neurosporenes*	1.0	5.5	tr.
β-Zeacarotene-related	n.d.	n.d.	1.7**
Hydroxyphytoenes	n.d.	n.d.	tr.
Hydroxy-ζ-carotenes	n.d.	n.d.	tr.
Hydroxy-β-carotenes	1.2	1.1	3.0***

Data represents a summary of typical data, expressed as a percentage of total carotenoid precursors from carrot cells and barley treated with 50 and 100 μM respectively for 7 days. n.d. = not detected; tr. = trace amounts; * = more than one peak resolved with similar polarity and identical absorption spectra characteristics; ** = identical absorption spectra to β-zeacarotene but less polar, also less polar than β-carotene; *** = more than one peak resolved with similar polarity and identical absorption spectra characteristics (revealed after saponification).

Table III. Summary of carotenoid precursor profiles following amitrole treatment.

Precursor	Dark-grown barley	Light-grown barley
(15*Z*)-Phytoene	86.5	74.8
(all *E*)-Phytoene	n.d.	9.2
Phytofluene 1	7.3	0.6
Phytofluene 2	3.1	2.1
ζ-Carotene 1	0.2	0.4
ζ-Carotene 2	0.6	0.6
ζ-Carotene 3	n.d.	0.4
Lycopene	0.6	3.7
δ- and γ-Carotene	0.2	0.5
Hydroxy-δ- and -γ-carotenes	1.7	0.3
Hydroxylycopenes	n.d.	0.5
Hydroxyphytoenes	n.d.	6.9

Data represents a summary of typical data, expressed as a percentage of total carotenoid precursors from barley treated with 100 μM amitrole for 7 days. n.d. = not detected.

ed and dihydroxylated derivatives of phytoene were also only detected in light-grown treatments. Monohydroxy, dihydroxy and didehydrodihydroxy phytoenes have previously been detected in light-grown treatments of barley with diflufenican [2, 3].

Sizeable accumulations of phytoene and ζ-carotene were detected in all dichlormate treatments. Less substantial accumulations of phytofluene, neurosporene and β-zeacarotene were also observed (Table II). At least two geometric isomers of phytofluene and ζ-carotene were detected and the ratio of the phytofluene isomers changed significantly in the presence of light (Table II). Phytoene accumulated in the (15*Z*)-form only, in dark treatments as observed with diflufenican treatments. Hydroxylated and dihydroxylated derivatives of phytoene and ζ-carotene were detected only in light-grown treatment. Whereas hydroxylated derivatives of β-zeacarotene (two peaks were detected in light-grown treatments) were detected in both light- and dark-grown treatments (Table II).

Amitrole treatment resulted in accumulations of phytoene, phytofluene, ζ-carotene and lycopene (Table III and Fig. 1). Therefore it appears that amitrole causes an inhibition of desaturation and cyclization reactions. The geometric conformations of phytofluene and ζ-carotene appeared to follow the same trends as those observed in light and dark treatments with dichlormate. The same trends were also observed with the phytoene accumulations. Lycopene accumulated as the all *E* (*trans*)-isomer with a small amount of the central mono-*cis*-isomer detected. As with diflufenican and dichlormate treatments hydroxylation of the acyclic precursors occurred only in the presence of light. This is in contrast to the hydroxylations of the monocyclic precursors δ- and γ-carotene which occurred in both light and dark treatments (Table III).

Discussion

A scheme labelling proposed target site of the molecules used in this study is presented (Fig. 2). This scheme also indicates further modifications which occur to accumulated precursors in the absence and presence of light.

The identification of all precursors is based on their absorption spectra, chromatographic behaviour and, where possible, on co-chromatography with standards. Only limited time-course and dose-response studies have been performed to date. It is likely that precursor profiles alter with time and herbicide concentration, however, this is more likely to be a quantitative rather than a qualitative difference.

The HPLC system provided a rapid and accurate method for determining diagnostic data concerning the target sites of the three inhibitors used. The system can clearly classify the inhibitors as follows: diflufenican, dichlormate and amitrole (phytoene/phytofluene desaturation); dichlormate and amitrole (ζ-carotene/neurosporene desaturation); amitrole (lycopene cyclization). The HPLC system also separated numerous geometric isomers and hydroxylated derivatives of the above precursors. This information may add to our understanding of the biosynthetic sequence and its control and organization in higher plant tissues.

The three sequences mentioned above *i.e.* phytoene/phytofluene desaturation, ζ-carotene desaturation and lycopene cyclization have been shown to be three discrete and separable events in a *Narcissus* cell-free system [7, 8]. Using this cell-free system dichlormate has been shown to inhibit phytoene/phytofluene desaturation and ζ-carotene desaturation independently. Whereas norflurazon

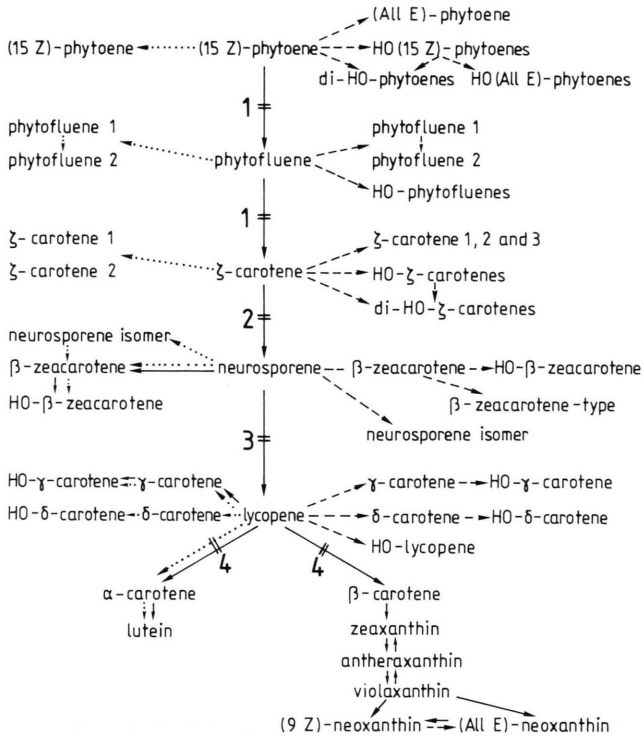


Fig. 2. Scheme of the proposed events following inhibition of carotenogenesis at different sites. Key: → known biosynthetic reactions; ····▶ precursors which accumulate with treatment in the dark; ---▶ precursors which accumulate with treatment in the light; HO represents hydroxylated precursors; (1) inhibition site for diflufenican, amitrole and dichlormate; (2) inhibition site for amitrole and dichlormate; (3) inhibition site for dichlormate; (4) inhibition site for amitrole.

was shown only to inhibit phytoene/phytofluene desaturation (Mayer *et al.* [8]). These results tie in with the precursor accumulations witnessed in *in vivo* studies here with dichlormate and previously with norflurazon [1].

The absence and presence of light resulted in an alteration of the composition and content of accumulated precursors in all treatments. Acyclic precursors *i.e.* lycopene, neurosporene, ζ-carotene, phytofluene and phytoene were only hydroxylated (at detectable levels) in light-grown treatments whereas the monocyclic precursors, β-zeacarotene, γ- and δ-carotenes, were hydroxylated in both light and dark treatments.

Phytoene and ζ-carotene accumulated in at least two different geometric forms. The relative amounts of the phytofluene isomers were significantly different in dark and light treatments. The significance of these isomeric conformations are unknown. Phytoene accumulated in the (15*Z*)-form only in dark treatments but in light-exposed treatments both the (15*Z*)- and (all *E*)-isomers were detected. The ratio of the (15*Z*)- to (all *E*)-isomers decreased with increasing time exposure to light.

The above information may add to our understanding of the organization, controls and constraints of carotenoid biosynthesis in green higher plant tissues:

i) In all treatments, with the exception of phytoene in dark-grown treatments, the precursors accumulated in more than one geometric form. This was most obvious with the phytofluene isomers.

ii) β-zeacarotene, a monocyclic carotenoid, was detected in all dichlormate treatments, thus demonstrating that the cyclization process in green higher plant tissues does not specifically require lycopene as its substrate. The cyclization process can occur with any carotenoid in which one half of the molecule has reached the lycopene level of desaturation *i.e.* in this instance, neurosporene.

iii) Hydroxylated β-zeacarotenes and hydroxylated δ- and γ-carotenes were detected in dichlormate and amitrole treatments respectively. This

type of hydroxylation is observed in dark- and light-exposed treatments. This is in contrast to the hydroxylations of the acyclic precursors which occur only in light-grown treatments. It is assumed therefore that the monocyclic carotenoids are most likely hydroxylated by the normal carotenogenic hydroxylases. This suggests that ring hydroxylation of monocyclic carotenes can occur in green tissue.

In conclusion, the data presented in this paper clearly identifies the target sites of three carotenogenic herbicides using a rapid and sensitive HPLC procedure applied to higher plant systems.

Acknowledgement

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