

# The Occurrence of $\beta$ -Carotene-5,6-epoxide in the Photosynthetic Apparatus of Higher Plants

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The occurrence of  $\beta$ -carotene-5,6-epoxide in higher plant photosynthetic tissue is described. The compound is found in isolated chloroplasts, thylakoids and other subchloroplast particles but can only be detected in intact leaves or cotyledons of higher plants when these are exposed to very high light intensities or to inhibitors such as monuron or paraquat. The distribution of the epoxide within the individual pigment-protein complexes is given. It is particularly associated with the PS I reaction centres (CP1 and CP1a) and less so with the PS II reaction centre (CPa).

Circular dichroism shows that the  $\beta$ -carotene-5,6-epoxide isolated from photosynthetic tissue is optically inactive. It is therefore not produced enzymically but is a product of photooxidative events in the photosynthetic apparatus. Its presence in photosynthetic tissue is a reliable indicator of photooxidative damage to the thylakoid membrane involving oxidation of  $\beta$ -carotene.

## Introduction

Carotenoids found in the photosynthetic tissues of higher plants are located within the separate pigment-protein complexes (PPCs) of the thylakoid membrane. The distribution of carotenoids within these PPCs is highly specific.  $\beta$ -Carotene is largely located in the PS I and PS II reaction centres whilst the xanthophylls, namely lutein, violaxanthin and neoxanthin, predominate in the light-harvesting chlorophyll *a/b* complexes (LHCPs). The distribution and the role of violaxanthin are somewhat complicated. Two separate pools of this carotenoid may exist [1] and only a certain proportion may be involved in the epoxidation/de-epoxidation reactions of the “violaxanthin cycle”. This cycle is thought by some workers to take place, at least in part, in the chloroplast envelope [2] as well as in the thylakoid pigment-protein complexes.

A major function of the carotenoids is to protect the chloroplast against photooxidative damage. Under photoinhibitory conditions or in the presence of compounds that interfere with photosynthetic

electron transport (for example, DCMU, paraquat) photodestruction of carotenoids and chlorophylls takes place. The rates of destruction for individual pigments are highly specific and are largely dependent on the precise location of these pigments in the photosynthetic apparatus.  $\beta$ -Carotene and neoxanthin are the most susceptible carotenoids to photooxidation.

Ashikawa and co-workers [3] have suggested that there is a correlation between the observed photobleaching of  $\beta$ -carotene in thylakoid membranes and the occurrence of all-*trans*  $\beta$ -carotene monoepoxide. They further suggested that the appearance of this latter compound was largely the result of an enzymic epoxidation/de-epoxidation cycle comparable with the violaxanthin cycle [4].

The presence of  $\beta$ -carotene monoepoxide in photosynthetic tissue has only recently been described. Britton *et al.* [5] reported its presence in leaves of *Hordeum vulgare* and Ashikawa *et al.* [3] detected it in the cyanobacterium *Synechococcus vulcanus* and in spinach thylakoids.  $\beta$ -Carotene-5,6-epoxide has been widely reported to be present in flowers and fruit [6], but an extensive reexamination by Eugster and co-workers (personal communication) has failed to detect natural optically active  $\beta$ -carotene-5,6-epoxide in these sources.

In this paper the isolation and spectroscopic characterization of  $\beta$ -carotene-5,6-epoxide (5,6-epoxy-5,6-dihydro- $\beta$ -carotene) are reported, and

**Abbreviations:** CD, circular dichroism; DCMU (3-(3,4-dichlorophenyl)-1,1-dimethylurea; monuron, 3-(4-chlorophenyl)-1,1-dimethylurea); paraquat, methyl viologen; PS I, photosystem I; PS II, photosystem II; SDS-PAGE, sodium dodecylsulphate polyacrylamide gel electrophoresis.

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its occurrence and distribution in the photosynthetic apparatus of higher plants examined.

Circular dichroism measurements have been used to determine whether the epoxide is an enzymic or non-enzymic product, and the possible physiological significance of its occurrence is discussed.

## Materials and Methods

### Plant material

Barley (*Hordeum vulgare* var. "Golden Promise") was grown for 7 days at 20 °C in normal greenhouse conditions with a light intensity of 150  $\mu\text{mol m}^{-2}\text{s}^{-1}$  (16/8). For treatment with inhibitors, first leaves were excised and floated on aqueous or aqueous/ethanolic solutions of paraquat (1 mM) or monuron (1 mM) respectively. For irradiation with high intensity light a Schott KL 1500-T lamp was used.

Chloroplasts were isolated following homogenization of 7-day-old leaves in the buffer medium described by Ridley [7] (330 mM sorbitol, 50 mM tricine, 10 mM  $\text{NaHCO}_3$ , 2 mM  $\text{KNO}_3$ , 2 mM EDTA, 1 mM  $\text{MgCl}_2$ , 1 mM  $\text{MnCl}_2$ , 0.5 mM  $\text{K}_2\text{HPO}_4$  pH 7.4). Chloroplasts at a chlorophyll concentration of approximately 250  $\mu\text{M}$ , were incubated for up to 24 h at a light intensity of 250  $\mu\text{mol m}^{-2}\text{s}^{-1}$  at 25 °C. DCMU (5  $\mu\text{M}$ ) was added from an ethanolic stock solution while maintaining the ethanol concentration at less than 0.01% in the final incubation. Aliquots (150  $\mu\text{l}$ ) of the chloroplast suspension were removed at selected intervals for pigment analysis by reversed-phase HPLC.

### Electrophoresis

Chloroplasts were ruptured in distilled water and a crude membrane fraction was obtained by centrifugation at  $15,000 \times g$  for 15 min. The pellet was twice washed in 50 mM Tris-HCl buffer (pH 7.8) containing 1 mM EDTA and recentrifuged. Thylakoid membranes were purified by centrifugation on a discontinuous sucrose gradient (50%, 40%, 30%, 20% w/v in 50 mM Tris-HCl (pH 7.8), 1 mM EDTA) which was centrifuged at  $60,000 \times g$  for 60 min. The membranes were collected at the 40/50% interface and pelleted by centrifugation at  $30,000 \times g$  for 10 min. The pellet was resuspended in 0.1 M Tris-glycine buffer (pH 9.0) and stored in liquid  $\text{N}_2$  prior to electrophoresis. Stick gels (resolving gel 7.5% acrylamide; stacking gel 4% acrylamide), each containing

approximately 10  $\mu\text{g}$  of chlorophyll, were run at 3 mA/stick for 30–40 min at 4 °C. Membranes were solubilized for 30 sec at an SDS:chlorophyll ratio of 5:1. Individual pigment-protein complexes were cut from the gel and extracted in ethanol for analysis by HPLC.

### HPLC

For HPLC analysis a reversed-phase procedure was used, with a 5  $\mu\text{m}$  Zorbax (DuPont Ltd.)  $\text{C}_{18}$  column (25.0  $\times$  0.46 cm) and a gradient of 0–100% ethyl acetate in acetonitrile/ $\text{H}_2\text{O}$  (9:1 v/v) and a flow rate of 1  $\text{ml min}^{-1}$ . Triethylamine (0.1% v/v) was added to the acetonitrile/ $\text{H}_2\text{O}$ . A Hewlett-Packard HP 1040 A Diode Array Detector was used to monitor the chromatogram at selected wavelengths for quantitative analysis and for determination of absorption spectra.

### Isolation of $\beta$ -carotene-5,6-epoxide

A total lipid extract was obtained by ethanolic extraction of leaves that had been homogenized in buffer (chloroplast isolation buffer, as described earlier). This extract was chromatographed on a column of neutral alumina (activity grade III), and a fraction containing the monoepoxide was eluted with 5% ether/hexane. This fraction was further purified by TLC on silica gel G with 10% ether/hexane as the developing solvent. Two bands (1.  $R_f$  0.9 and 2.  $R_f$  0.6) were removed and eluted. Band 2 was rechromatographed on MgO: Kieselgur G (1:1 w/w) with 5% acetone/hexane as the developing solvent, to give two components;  $\beta$ -carotene-5,6-epoxide ( $R_f$  0.6) and  $\beta$ -carotene-5,8-epoxide (mutatochrome) ( $R_f$  0.4). The  $\beta$ -carotene-5,6-epoxide was finally purified on a small column of grade III neutral alumina which was washed with 100% hexane prior to elution of the epoxide with 5% ether/hexane.

### Circular dichroism

Circular dichroism spectra of  $\beta$ -carotene-5,6-epoxide (in EPA: ether–isopentane–ethanol, 5:5:2) were measured in a Jasco J500 A spectropolarimeter at room temperature and at  $-180$  °C.

Synthetic (5*R*,6*S*)  $\beta$ -carotene-5,6-epoxide exhibited strong C.D. when measured under identical conditions. The C.D. measurements were obtained by Prof. C. H. Eugster, Dr. S. Mohanty, and Mr. P. Uebelhart, University of Zurich.

### Mass spectrometry

Electron impact mass spectra were obtained by Mr. M. Prescott on a VG Micromass 7070F mass spectrometer (positive ion mode) coupled with a Finnigan Incos Data System.

## Results

### Characterization of $\beta$ -carotene-5,6-epoxide

The isolated compound had  $\lambda_{\max}$  at 423, 449 and 478 nm in the HPLC elution solvent and a retention time consistent with a carotene epoxide structure. The absorption spectrum of this compound was also checked in conventional solvents [8]. The chromatographic characteristics on TLC and HPLC and the absorption spectra in several solvents were identical to those of a synthetic standard compound, kindly provided by Professor Eugster and Dr. M. Acemoglu (University of Zurich), and were consistent with the identification of the isolated compound as  $\beta$ -carotene-5,6-epoxide. Treatment with dilute HCl gave an immediate hypsochromic shift of 20 nm, confirming isomerization of the 5,6-epoxide to the 5,8-epoxide ( $\lambda_{\max}$  405, 429, 454 nm). Confirmation of the structure was obtained by mass spectrometry (MS  $m/z$ : 552 [ $M^+$ ], 472 [ $M-80$ ] $^+$ , 205, 165.

### Occurrence of $\beta$ -carotene-5,6-epoxide in photosynthetic tissue

Analysis of pigment extracts obtained directly from intact leaves of a number of higher plants in-

cluding *H. vulgare*, radish, pea and spinach, and from some green algae (*Scenedesmus* spp. and *Dunaliella*) by reversed-phase HPLC did not reveal the presence of  $\beta$ -carotene-5,6-epoxide in any of these healthy photosynthetic tissues.  $\beta$ -Carotene-5,6-epoxide could only be detected when the tissue was subjected to conditions which would result in photo-oxidation in the chloroplast, *i.e.*, photoinhibitory conditions (very high light intensities,  $> 2000 \mu\text{mol m}^{-2}\text{s}^{-1}$ ), or addition of substances which affect photosynthetic electron transport (for example, monuron and paraquat [unpublished results], or diflufenican [9]). In such cases the amount of epoxide detected amounted to 2–4% of the total carotenoid (Table I). The appearance of  $\beta$ -carotene-5,6-epoxide coincided with a decrease in the  $\beta$ -carotene level, but could not account for more than a small part of the observed loss. No evidence for the de-epoxidation of  $\beta$ -carotene-5,6-epoxide to  $\beta$ -carotene following such treatments, either *in vivo* or *in vitro*, could be found.

Buffer homogenization of plant material, an essential step in the preparation of chloroplasts, thylakoids etc., led to the appearance of the epoxide in these preparations even when it could not be found in the intact plant (Fig. 1). Generally the production of the epoxide was greater after the use of longer and more complicated isolation procedures and was particularly enhanced when plant material was frozen in liquid  $N_2$  and then thawed.

Incubation of isolated chloroplasts or thylakoids in light under aerobic conditions caused photobleaching of the individual carotenoids and chlorophylls in a well-defined order.  $\beta$ -Carotene was the most sus-

Table I. Carotenoid composition of individual barley leaves incubated in various bleaching treatments.

	% Pigment in barley leaves			
	Control	30 min high light	48 h 1 mM monuron	48 h 1 mM paraquat
Neoxanthin	11.8	12.9	12.4	13.7
Violaxanthin	16.7	8.3	17.6	8.7
Antheraxanthin	0.5	1.0	0.7	1.6
Lutein	42.6	43.0	55.0	52.1
Zeaxanthin	N.D.	8.8	N.D.	2.3
$\beta$ -Carotene-5,6-epoxide	N.D.	1.5	1.7	1.2
$\beta$ -Carotene	27.9	24.3	12.6	19.6
Chlorophyll <i>a/b</i> ratio	1.9:1	2.0:1	1.4:1	1.8:1
Lutein/ $\beta$ -carotene ratio	1.5:1	1.8:1	4.4:1	2.6:1
$\beta$ -Carotene/ $\beta$ -carotene-5,6-epoxide ratio	—	16.2:1	7.4:1	16.3:1

N.D., not detected.

ceptible pigment to photooxidative damage. An increase in both the total amount of  $\beta$ -carotene-5,6-epoxide and its amount relative to the other carotenoids were measured (Fig. 2). The increase was most marked in chloroplasts incubated in the presence of  $5 \mu\text{M}$  DCMU, the level doubling after 2 h incubation (Fig. 3). Longer incubations resulted in a decrease in the epoxide level suggesting that it was also being destroyed during the incubation.

#### *Location of $\beta$ -carotene-5,6-epoxide in the photosynthetic apparatus*

Small amounts of  $\beta$ -carotene-5,6-epoxide are produced during the isolation procedure for the pigment-protein complexes. The amounts (approx. 3% total carotenoid) in the thylakoids and within the complexes are similar, indicating that the technique of SDS-PAGE itself did not result in epoxide formation

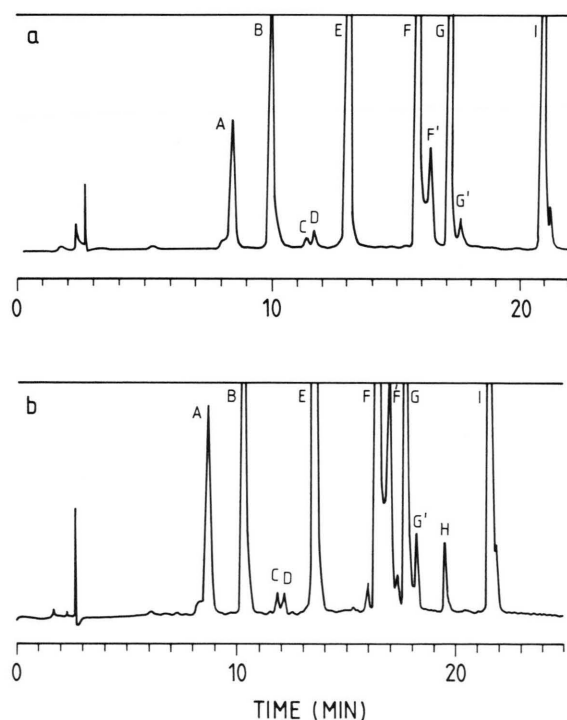


Fig. 1. Reversed-phase HPLC of carotenoids and chlorophylls from (a) "control" barley seedlings and, (b) barley chloroplasts incubated with  $5 \mu\text{M}$  DCMU in the light. Peak identifications: A, neoxanthin; B, violaxanthin; C, lutein-5,6-epoxide; D, antheraxanthin; E, lutein; F, F', chlorophyll *b*; G, G', chlorophyll *a*; H,  $\beta$ -carotene-5,6-epoxide; I,  $\beta$ -carotene.

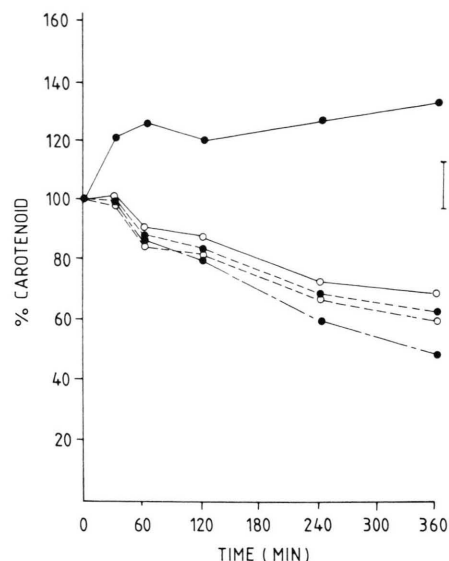


Fig. 2. Photodestruction of individual carotenoids in isolated barley chloroplasts in the absence of DCMU (light control).  $\beta$ -Carotene-5,6-epoxide (●—●), lutein (○—○), violaxanthin (●---●), neoxanthin (○---○),  $\beta$ -carotene (●---●).

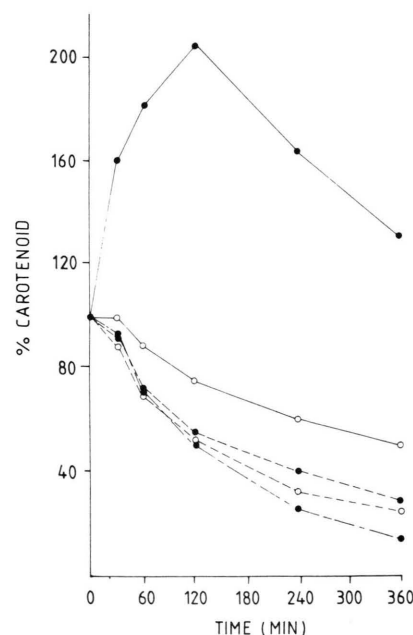


Fig. 3. Photodestruction of individual carotenoids in isolated barley chloroplasts in the presence of  $5 \mu\text{M}$  DCMU.  $\beta$ -Carotene-5,6-epoxide (●—●), lutein (○—○), violaxanthin (●---●), neoxanthin (○---○),  $\beta$ -carotene (●---●).

and that the epoxide was a product of the buffer homogenization. Analysis of the individual pigment-protein complexes from a variety of plant species, grown under normal greenhouse conditions, showed that  $\beta$ -carotene-5,6-epoxide is confined to the CP1 and CP1a (PS I reaction centre) complexes (Table II).

Following incubation of barley chloroplasts under aerobic conditions and a light intensity of  $150 \mu\text{mol m}^{-2}\text{s}^{-1}$ ,  $\beta$ -carotene-5,6-epoxide was detected in the LHCP<sub>2</sub>/CPa, PS II complexes and in the free pigment fraction (Table III). Incubation of chloroplasts in the presence of  $5 \mu\text{M}$  DCMU for 2 h enhanced this effect. Longer exposure to DCMU increased the total amount of epoxide in the CP1/CP1a complexes to between 11 and 15% of total carotenoid. However, the epoxide could not be detected in the PS II complex (LHCP<sub>2</sub>/CPa), principally due to the high level of photooxidation taking place there. Levels of  $\beta$ -carotene-5,6-epoxide in the free pigment fraction

were higher than those after only 2 h exposure. At no time could the epoxide be detected in the major light-harvesting complexes LHCP<sub>1</sub> and LHCP<sub>3</sub>.

## Discussion

Ashikawa and his co-workers [3] detected all-*trans*  $\beta$ -carotene monoepoxide in the photosynthetic membranes of both spinach (thylakoids) and of a cyanobacterium. They suggested that epoxidation/de-epoxidation reactions, involving  $\beta$ -carotene monoepoxide levels were diurnal, *i.e.*, levels of the epoxide were low following a dark period and rose following exposure to natural day-light. This is, in fact, the opposite of the "violaxanthin cycle" which involves a light-driven de-epoxidation [4].

In the present study, however, neither  $\beta$ -carotene-5,6-epoxide, nor its furanoid oxide derivative  $\beta$ -carotene-5,8-epoxide (mutatochrome) could be detected

Table II. Percentage distribution of pigments in each pigment-protein complex of barley chloroplasts: control.

	% Pigment in the pigment-protein complexes				
	CP1/CP1a	LHCP <sub>1</sub>	LHCP <sub>2</sub> /CPa	LHCP <sub>3</sub>	FP
Neoxanthin	2.5	18.1	9.3	22.1	4.7
Violaxanthin	8.5	11.5	9.7	11.9	29.6
Lutein	21.6	62.3	37.7	61.6	49.8
$\beta$ -Carotene-5,6-epoxide	3.2	N.D.	N.D.	N.D.	N.D.
$\beta$ -Carotene	67.4	8.2	43.3	4.5	15.9
Chlorophyll <i>a/b</i> ratio	10.8:1	1.4:1	3.6:1	1.4:1	4.0:1
$\beta$ -Carotene/ $\beta$ -carotene-5,6-epoxide ratio	21.0:1				

N.D., not detected.

Table III. Percentage distribution of pigments in each pigment-protein complex of barley chloroplasts: +5  $\mu\text{M}$  DCMU (2 h illumination).

	% Pigment in the pigment-protein complexes				FP
	CP1/CP1a	LHCP <sub>1</sub>	LHCP <sub>2</sub> /CPa	LHCP <sub>3</sub>	
Neoxanthin	2.6	13.2	5.0	14.4	1.5
Violaxanthin	6.7	10.5	12.8	13.2	20.2
Lutein	24.0	73.7	42.9	68.8	60.6
$\beta$ -Carotene-5,6-epoxide	7.7	N.D.	5.2	N.D.	2.6
$\beta$ -Carotene	58.9	2.6	34.2	3.7	15.2
Chlorophyll <i>a/b</i> ratio	9.3:1	1.4:1	5.5:1	1.5:1	5.5:1
$\beta$ -Carotene/ $\beta$ -carotene-5,6-epoxide ratio	7.6:1		6.8:1		5.8:1

N.D., not detected.

in "unstressed" leaves. The 5,6-epoxide was however, found in isolated chloroplasts from the same plants. The sensitivity of the HPLC method used, typically detecting 2–3 ng of carotenoids, would have detected  $\beta$ -carotene-5,6-epoxide if this were present as less than 0.05% of the total carotenoid in the intact leaf. This is far below the levels measured in isolated chloroplasts and thylakoids where the epoxide accounted for as much as 3% of total carotenoid. Unfortunately Ashikawa *et al.* [3] give no data on the relative levels of  $\beta$ -carotene monoepoxide in spinach leaves.

The location of  $\beta$ -carotene-5,6-epoxide in the photosynthetic apparatus of higher plants is given in Tables II and III. The epoxide is mainly located in the PS I reaction centre complexes (CP1 and CP1a) from oats, radish and barley thylakoid membrane. Isolated chloroplasts and thylakoids illuminated in the absence and presence of DCMU, show increased levels of the epoxide. It is only under such extreme conditions, where pigment destruction has taken place, that  $\beta$ -carotene-5,6-epoxide is found in the PS II reaction centre (CPa). This is in contrast to the report of Ashikawa *et al.* [3] that  $\beta$ -carotene monoepoxide is preferentially located in PS II particles. It is evident, however, that oxidation of  $\beta$ -carotene to  $\beta$ -carotene-5,6-epoxide takes place in the individual pigment-protein complexes of the thylakoid membrane. It is reasonable to assume that differences between the results of our study and that of Ashikawa and co-workers [3] may be due, in part, to differences in the preparation of these complexes. Indeed Ashikawa *et al.* [3] suggest that the amount of the epoxide found in isolated thylakoid membranes may be dependent on conditions of harvesting and preparation.

This is also illustrated by the fact that preparations of individual PS I and PS II (BBY) particles, as opposed to electrophoretic separation of complexes, were found to contain approximately equivalent amounts of  $\beta$ -carotene-5,6-epoxide (3% of the total carotenoid). Levels of the epoxide in isolated LHC II, obtained from the preparation of PS I particles [10], were very low by comparison and often

undetectable (Young and Britton, unpublished results).

Exposure of leaves or intact chloroplasts to high light or certain inhibitors of electron transport results not only in the bleaching of carotenoids and chlorophylls but also in a transient increase in both the total amount of  $\beta$ -carotene-5,6-epoxide and its amount relative to other carotenoids, particularly  $\beta$ -carotene. The transformation of  $\beta$ -carotene to its monoepoxide is, however, only a relatively minor route for the destruction of  $\beta$ -carotene (S. W. M. Lee, unpublished results). It has been demonstrated that the oxidation of unsaturated lipids by isolated sugar-beet chloroplasts correlated with the observed oxidation of  $\beta$ -carotene [11]. In contrast, it has been suggested that "enzymic" destruction of carotenoids, especially  $\beta$ -carotene, occurred during chloroplast isolation both in the light and the dark [12] and that  $\beta$ -carotene-5,6-epoxide is a product of the enzymic epoxidation of  $\beta$ -carotene [3].

Evidence which shows that the production of  $\beta$ -carotene-5,6-epoxide is not enzymic comes from C.D. studies. Some of the major carotenoids found in the chloroplast, namely the xanthophylls violaxanthin and neoxanthin, have 5,6-epoxide structures. These xanthophylls are biosynthetic products and their epoxide groups are introduced enzymically and stereospecifically to generate chiral centres at C-5 and C-6 [13]. When the isolated  $\beta$ -carotene-5,6-epoxide was analyzed by C.D. it was found to be optically inactive. It therefore cannot have been produced enzymically but must be derived from  $\beta$ -carotene by a purely photochemical or chemical reaction which gives a racemic mixture of the (5*R*,6*S*) and (5*S*,6*R*) isomers.

$\beta$ -Carotene-5,6-epoxide could only be detected in intact leaves or algal cells where destruction of  $\beta$ -carotene has taken place. This may be mediated by herbicide action or by the use of high light intensities to cause photooxidative destruction of the chloroplast carotenoids, especially  $\beta$ -carotene. The presence of  $\beta$ -carotene-5,6-epoxide in photosynthetic tissue is a reliable indicator of oxidative damage to the thylakoid membranes.

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