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CAROTENOID METABOLISM AND THE BIOSYNTHESIS OF ABSCISIC ACID

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Key Word Index—*Lycopersicon esculentum*; Solanaceae; *Phaseolus vulgaris*; Leguminosae; carotenoid metabolism; deuterium labelling; apo-carotenoids; neoxanthin; violaxanthin; abscisic acid.

Abstract—The conversion of all-*trans*-violaxanthin to 9′-*cis*-neoxanthin was shown to occur in fluridone-treated etiolated *Lycopersicon* and *Phaseolus* seedlings, following exposure to light. The results of deuterium oxide labelling experiments supported this precursor/product relationship, and provided further evidence for the origin of abscisic acid. Several apo-carotenoids, putative by-products of abscisic acid biosynthesis, were synthesised by chemical oxidation but were not detected in plant extracts. *In vitro*, lipoxygenase cleaved neoxanthin and violaxanthin down to small ($\leqslant C_{13}$) fragments. It may be that *in vivo* any apo-carotenoids formed by the specific cleavage of 9′-*cis*-neoxanthin, during abscisic acid biosynthesis, are rapidly metabolized by lipoxygenase or similar enzymes.

INTRODUCTION

There is now considerable evidence to support an indirect 'apo-carotenoid' pathway for abscisic acid (ABA; **7**) biosynthesis [1–3, and refs therein] with the most likely pre-cleavage precursors being 9′-*cis*-neoxanthin (**4**) and 9-*cis*-violaxanthin (**3**; [2, 3]). Cleavage of these xanthophylls across the 11,12 (11′,12′) double bond would produce xanthoxin (Xan; **5**), which is readily converted to ABA by plant tissues [4, 5]. The regulation of ABA biosynthesis occurs prior to the production of Xan, and could involve control of violaxanthin and neoxanthin interconversion and/or the cleavage reaction [5, 6].

At present little is known about xanthophyll biosynthesis in higher plants, especially that of the *cis* isomers and allenic xanthophylls such as neoxanthin [7, 8]. A pathway for the biosynthesis of neoxanthin in plants has been proposed from zeaxanthin, via an epoxide such as violaxanthin [9, 10] but this lacks experimental support. A cell-free system from the alga *Amphidinium carterae* converted ^{14}C-zeaxanthin to all-*trans*-neoxanthin (**2**) but no intermediates were isolated [11]. In part this paper describes attempts to ascertain the relationship between the all-*trans*- and 9-*cis*-isomers of violaxanthin and neoxanthin in leaves of higher plants.

A vast number of possible carotenoid breakdown products or apo-carotenoids, have been identified [12] but knowledge of the biochemistry of carotenoid catabolism is extremely limited. The majority of these apo-carotenoids are volatile (C_9–C_{13}) compounds, many of which are important aroma constituents, but others range in size up to C_{30}, for example apo-8′-violaxanthal which has been extracted from orange peel [13]. The existence of such a variety of apo-carotenoids, both in the type of end-group and size, may indicate that in certain tissues non-specific cleavage of carotenoids is occurring.

The photooxidation of carotenoids *in vitro* has produced a range of C_9–C_{15} apo-carotenoids, including Xan and *trans*-xanthoxin (*t*-Xan), most of which have been identified in plant extracts [12, 14]. The bleaching of carotenoid solutions by lipoxygenase (LOX) has been described frequently but the products of such degradations have not usually been identified [15–17], although Xan and *t*-Xan were isolated from incubations of LOX and violaxanthin [17]. Pigment bleaching by cytochrome *c* and peroxidases also takes place *in vitro* [15, 18]. The relevance of such observations to the non-specific or specific degradation of carotenoids *in vivo* is uncertain. Several potential by-products of neoxanthin and violaxanthin cleavage have been synthesized and their occurrence in plant tissues and LOX/xanthophyll incubations investigated, with the aim of revealing more about the cleavage of these xanthophylls *in vivo*.

RESULTS AND DISCUSSION

Origin of neoxanthin

Etiolated leaves contain the same major carotenoids as green leaves, namely lutein, violaxanthin, neoxanthin, antheraxanthin and β-carotene, but in different relative proportions [10]. Transfer of etiolated seedlings to the light results in a general stimulation of carotenoid synthesis, although the rates of accumulation for individual carotenoids vary widely [19, 20]. This accumulation of carotenoids is controlled both by phytochrome and the availability of chlorophyll and intact thylakoids [19]. After 72 hr in the light, levels of β-carotene and neoxanthin in leaves of five-day-old etiolated tomato seedlings increased 27- and 13-fold respectively (data not shown), whilst those of violaxanthin, antheraxanthin and lutein rose by only 1.7-, 6- and 4-fold respectively. Under

Abbreviations—DTT, dithiothreitol; LOX, lipoxygenase; *not*, *notabilis*; PA, phaseic acid; TEA, triethylamine; *t*-Xan, 2-*trans*-xanthoxin; Xan, xanthoxin.

these conditions it is difficult to determine the relationship between individual carotenoids.

Fluridone, which inhibits carotenogenesis at the stage of phytoene dehydrogenase [21], was used to prevent the synthesis of new carotenoids following the transfer of etiolated seedlings to light. The increases in levels of phytoene (λ_{max}^{EtOH} 287 nm) and the constant low levels of β-carotene confirmed that the inhibitor was successfully preventing carotenoid synthesis (data not shown). Data on carotenoid content are presented as percentages of total xanthophylls because on a fresh weight or leaflet basis overall carotenoid levels remained constant or fell, especially in *Phaseolus*, due to light-induced leaf expansion (data not shown).

After a time lag (6–9 hr in *Lycopersicon*; 12–25 hr in *Phaseolus*), during which chlorophyll synthesis began and some phytoene accumulated, a decrease occurred in the percentage of all-*trans*-violaxanthin concommitant with an increase in the percentage of 9′-*cis*-neoxanthin (Figs 1–3). The combined percentage of these two xanthophylls remained virtually constant (42.5 ± 1.3, *Lycopersicon* wild-type; 37.7 ± 0.8, *L. notabilis*; 42.6 ± 1.4, *Phaseolus*) while the ratio of all-*trans*-violaxanthin: 9′-*cis*-neoxanthin changed from *ca* 5:1 to *ca* 1:1. There was a fall in the percentage of violaxanthin and neoxanthin in *Phaseolus* after 60 hr but this was probably due to preferential photodestruction of these compounds over lutein as leaf expansion occurred [see 22]. Longer term experiments have shown that the ratio of 9′-*cis*-neoxanthin: all-*trans*-violaxanthin does not change from *ca* 1:1 to 3:2, possibly as a result of structural constraints within the pigment-protein complexes.

These results suggest that during the transition of etioplasts into chloroplasts all-*trans*-violaxanthin acts as a precursor of 9′-*cis*-neoxanthin, and that this conversion is stimulated by light, either directly or as a consequence of chloroplast formation (Fig. 4). This is consistent with previous hypotheses regarding neoxanthin formation and the results of carotenoid interconversions in an algal cell-free system [9–11].

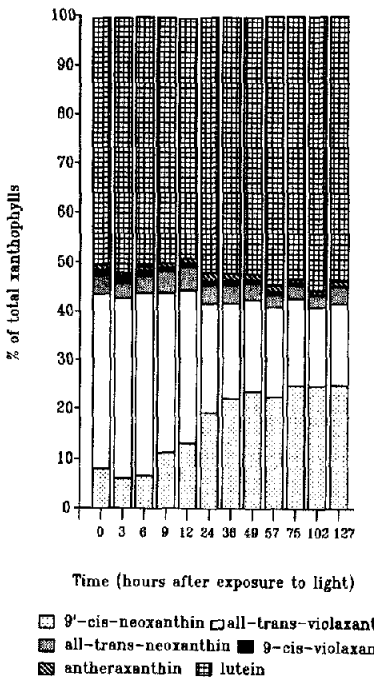

Fig. 1. Stacked bar graph showing changes in xanthophyll composition in leaves of fluridone-treated seven-day-old etiolated *L. esculentum* wild-type seedlings, following transfer from the dark to the light.

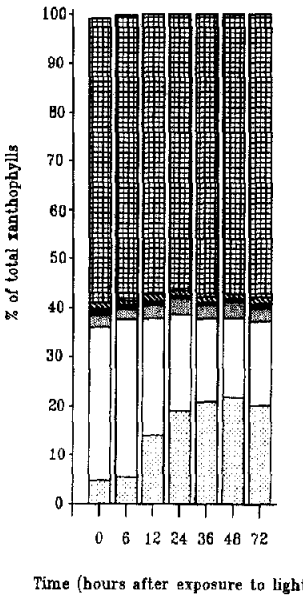

Fig. 2. Stacked bar graph showing changes in xanthophyll composition in leaves of fluridone-treated seven-day-old etiolated *L. esculentum notabilis* seedlings, following transfer from the dark to the light. Key as in Fig. 1.

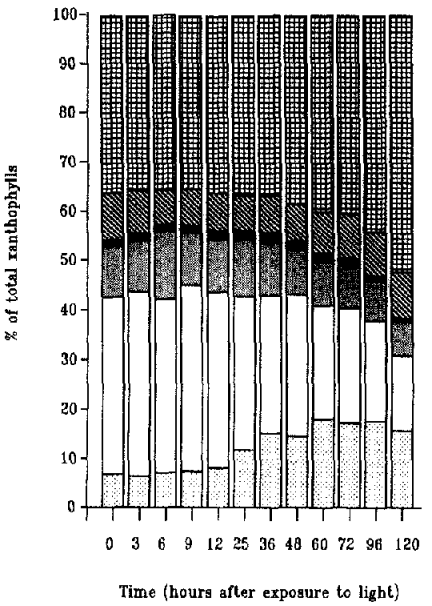

Fig. 3. Stacked bar graph showing changes in xanthophyll composition in leaves of fluridone-treated seven-day-old etiolated *P. vulgaris* seedlings, following transfer from the dark to the light. Key as in Fig. 1.

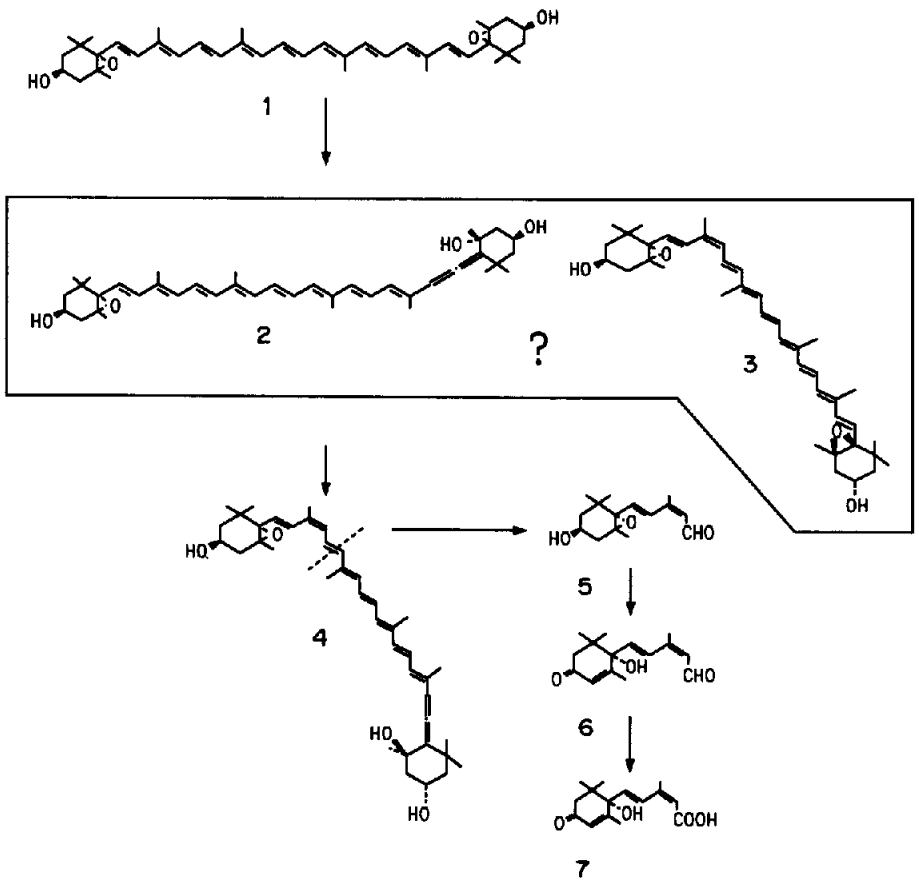

Fig. 4. Probable biosynthetic pathway for ABA from all-*trans*-violaxanthin.

There were no significant changes in the percentage of either all-*trans*-neoxanthin or 9-*cis*-violaxanthin during the conversion of all-*trans*-violaxanthin to 9′-*cis*-neoxanthin, and so it is not possible to predict which, if either, is an intermediate in this conversion.

Notabilis (*not*) is a wilty tomato mutant whose ABA deficiency may result from a lesion in the C_{40} part of the biosynthetic pathway [5, 6]. The combined percentage of all-*trans*-violaxanthin and 9′-*cis*-neoxanthin were consistently lower in leaves of *not* than those of wild-type *Lycopersicon* or *Phaseolus*, (Fig. 2) but the conversion of all-*trans*-violaxanthin to 9′-*cis*-neoxanthin proceeded as normal. The significance of this is unknown but examinations of the sub-plastidal localization of the xanthophylls in wild-type and *not* plastids would be interesting.

Dithiothreitol (DTT, 1 mM) has been reported to inhibit the de-epoxidation of violaxanthin to zeaxanthin, during the operation of the 'xanthophyll cycle', by an as yet unknown mechanism [23]. DTT was fed to etiolated *Phaseolus* seedlings via the transpiration stream 24 hr before the seedlings were transferred to light, but no effect on the conversion of violaxanthin to neoxanthin was apparent (data not shown).

We have previously shown that deuterium from D_2O was incorporated into carotenoids in etiolated *Phaseolus* seedlings [2]. Water stress-induced ABA was labelled with deuterium to a similar extent as neoxanthin and violaxanthin. We considered this as confirmatory evidence for the apo-carotenoid nature of ABA. To further investigate the relationship between violaxanthin, neoxanthin and ABA extracts were made of *Phaseolus* seedlings grown on 50% D_2O, in the dark for five days, and then either kept in the dark on 50% D_2O or water, or transferred to the light and fed with water or 50% D_2O for a further three days. Following transfer from D_2O to water, both in the dark and in the light, a reduction in the amount of labelling occurred (Table 1). In the dark this reduction was between 25 and 28% for violaxanthin and lutein and 16% for neoxanthin. In the light the extent of deuteration of leutein and violaxanthin fell by 44–50% compared to a fall of 32% for neoxanthin, even though the levels of neoxanthin had increased by over six-fold compared to increases of only 1.5–1.7-fold for the other xanthophylls.

This indicates that violaxanthin and lutein were being synthesized from newly formed precursors while neoxanthin was derived from an existing, labelled compound. These data further support the hypothesis that neoxanthin is synthesized from violaxanthin, at least in 'greening' leaves.

The extent of deuteration of ABA and phaseic acid (PA) produced when plants grown under the above conditions were stressed was also determined. As can be seen from Table 1 the extent of labelling of ABA and neoxanthin was comparable in all treatments. We therefore conclude that the xanthophyll pool available to the

Table 1. Extent of deuteration of selected xanthophylls, ABA and PA extracted from leaves of *P. vulgaris*, grown on 50% D_2O in the dark for 5 days and then either kept in the dark on 50% D_2O or water, or transferred to the light and fed with 50% D_2O or water

Treatment, after five days in the dark grown on 50% D_2O	Violaxanthin		Neoxanthin		Lutein		ABA	PA
	% ^2H	Conc.*	% ^2H	Conc.	% ^2H	Conc.	% ^2H	% ^2H
Dark, 3 days, 50% D_2O	18	147	19	30	16	199	15	16
Dark, 3 days, H_2O	13	111	16	25	12	134	12	12
Light, 3 days, 50% D_2O	14	197	16	118	14	284	17	n.d.
Light, 3 days, H_2O	9	256	13	188	9	299	11	n.d.

* = nmol g^{-1} fr. wt.
n.d. = not determined.

cleavage enzymes is in equilibrium with the bulk of these carotenoids produced by light-induced *de novo* synthesis. Thus while it seems very likely that the neoxanthin utilized for ABA biosynthesis is spatially separate from the bulk of the neoxanthin, which is presumed to exist in the thylakoid pigment-protein complexes, this neoxanthin cannot be detected as a separate pool by the above method.

Neoxanthin and violaxanthin cleavage

In order to investigate the nature of the *in vivo* cleavage of these xanthophylls, potential breakdown products were synthesized by zinc permanganate oxidation. All-*trans*-violaxanthin and 9′-*cis*-neoxanthin were purified in bulk from green *Phaseolus* leaves. Following oxidation fragments were purified by RP-HPLC (system 1) and identified on the basis of UV/VIS absorbance spectra, reactions with dilute acid and mass spectra (Table 2). All-*trans*-violaxanthin gave rise to *t*-Xan and the all-*trans* C_{25} and C_{27} epoxy apo-aldehydes (**11**, **12**), while 9′-*cis*-neoxanthin produced Xan, the C_{15} allenic apo-aldehyde (**8**), the 9′-*cis* C_{25} and C_{27} epoxy apo-aldehydes and the all-*trans* C_{25} and C_{27} allenic apo-aldehydes (**9**, **10**), consistent with previous studies (Fig. 5; [24–26]).

Specific cleavage of 9-*cis*-violaxanthin or 9′-*cis*-neoxanthin across the 11,12 (11′,12′) double bond to produce Xan would also produce either the C_{25} epoxy apo-aldehyde (**11**) or the C_{25} allenic aldehyde (**9**). Random cleavage would lead to other fragments being formed. A RP-HPLC system was developed (system 2) in which the apo-aldehydes were well resolved, and in which no major components of the plant extracts examined (absorbing *ca* 300–500 nm) co-chromatographed with the C_{25} and C_{27} apo-aldehydes.

Extracts of a variety of tissues (etiolated and green leaves of *Lycopersicon* and *Phaseolus*, non-stressed and stressed, both for varying times and to varying degrees; green leaves of the wilty ABA-deficient mutants of *Lycopersicon*, *not*, *flacca* and *sitiens*; and roots of *Lycopersicon*) were analysed using this HPLC system but no traces of any of the C_{25} or C_{27} apo-aldehydes were detected. In one case the levels of all-*trans*-violaxanthin and 9′-*cis*-neoxanthin in etiolated *Phaseolus* leaves fell by almost 20 $\mu g\, g^{-1}$ fr. wt following stress, but no C_{25} or C_{27} apo-aldehydes were found, despite a limit of detection estimated to be $< 10\, ng\, g^{-1}$.

The C_{25} and C_{27} epoxy apo-aldehydes of interest here (**11**, **12**) have been isolated from various fruits [13, 27] but the corresponding apo-alcohols, which may be formed by

Table 2. Some properties of all-*trans*-violaxanthin, 9′-*cis*-neoxanthin and the apo-carotenoids formed from them by zinc permanganate oxidation

Compound	R_t (min; RP-HPLC system 2)	λ_{max} (nm; in eluate)*	Shift + acid (nm)	λ_{max} compared to published data		
				Present	Published	Ref./solvent
C_{15} Allenic apo-ald.	6.0	292	n.d.	291	290	24/EtOH
Xan/*t*-Xan	11.0	285	n.d.	282	283	37/MeOH
C_{25} Allenic apo-ald.	18.5	419	0	430, 405 (385)	431, 408, 389	24/C_6H_{12}
C_{27} Allenic apo-ald.	20.0	443	0	456, 430 (408)	—	—/C_6H_{12}
C_{25} *t*†-Epoxy apo-ald.	21.9	420	22–26	(439) 414	(440) 414	26/C_6H_6
C_{25} *c*-Epoxy apo-ald.	22.2	414	19–22	(435) 411	(434) 410	26/C_6H_6
C_{27} *t*-Epoxy apo-ald.	23.2	442	22	(469) 440	(470) 442	26/C_6H_6
C_{27} *c*-Epoxy apo-ald.	23.6	439	13	(462) 438	(468) 439	26/C_6H_6
9′-*cis*-Neoxanthin	27.0	465, 437, 413	14–16	466, 437, 414	467, 438, 415	35/EtOH
All-*t*-violaxanthin	29.5	472, 442, 418	39–43	470, 441, 420	470, 441, 422	36/EtOH

*Spectra recorded in eluate (HPLC system 2) similar (±1–2 nm) to those recorded in ethanol.
†*t* = all-*trans*, *c* = 9-*cis*.
n.d. = not determined.

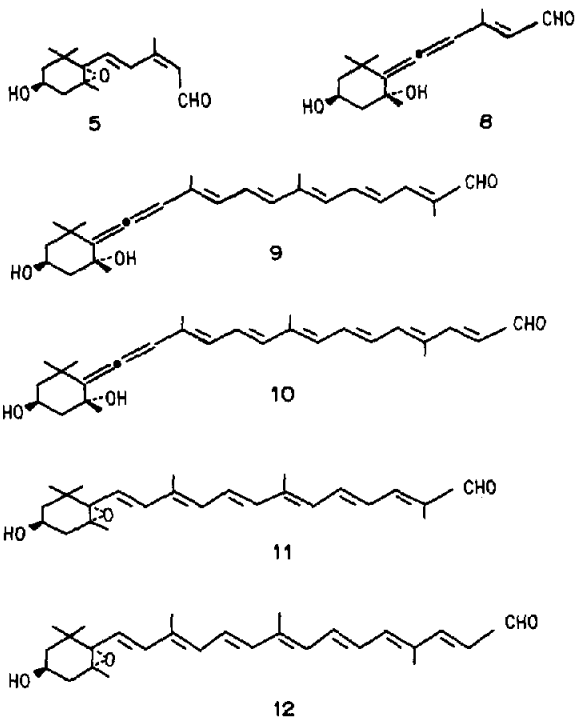

Fig. 5. Apo-aldehydes formed from zinc permanganate oxidation of violaxanthin and neoxanthin.

reduction of the apo-aldehydes, are more commonly found [27–30]. Oxidation of apo-aldehydes to the carboxylic acids could also occur, as several apo-acids have been isolated from plants [8, 31, 32]. Comparisons were made between extracts of non-stressed and stressed etiolated bean leaves, by HPLC (systems 2–4), but no compounds were found to accumulate which could have been C_{25} or C_{27} apo-alcohols or apo-acids. Retention times of the corresponding apo-carotenals and apo-carotenols on RP-HPLC are similar [8]. Methylated extracts were also examined, in the hope that any methyl-apo-acids present would have polarities similar to the apo-aldehydes, but no evidence was obtained to suggest that any apo-acids were present.

A further possibility was that any apo-carotenoid acids formed were being esterified. HPLC (systems 1–4) analysis of normal or hydrolysed extracts (pectinase for 18 hr or pH 11/60° for 30 min) did not suggest that this was occurring.

In tomato leaves a C_{10} 'by-product' of ABA biosynthesis has been identified, which may be derived from the central portion of a C_{40} xanthophyll [33]. If this is the case then the 'other' C_{15} end of 9'-*cis*-neoxanthin or 9-*cis*-violaxanthin might also accumulate. Previous work has shown that following stress *t*-Xan, the 'other' end of 9-*cis*-violaxanthin, does accumulate to a limited extent in mature tomato leaves but not at all in etiolated bean leaves [2, 5]. The presence of the C_{15} allenic apo-aldehyde (**8**) that would be produced from neoxanthin was investigated by HPLC (systems 2, 3 and 5) and GC-MS but none was found in any extract examined in this present study. Of all the possible C_{13} apo-carotenoids derivable by the simple oxidation of tobacco carotenoids only two have not been isolated, one of these is the C_{13} allenic ketone related to neoxanthin [12, 34]. It has been suggested that this may result from its rapid metabolism to more stable compounds [34].

Lipoxygenase (LOX), in the presence of certain fatty acids such as linoleic, acid, can cause carotenoid cleavage *in vitro* [15–17], and was used here to bleach solutions of neoxanthin and violaxanthin. Conditions were optimized using zeaxanthin. The bleaching reaction could be stopped completely by the addition of the anti-oxidant *tert*-butylated hydroxyquinoline (TBHQ; [16]). Incubations interrupted with TBHQ were found to contain low levels of C_{25} and C_{27} apo-aldehydes ($< 5\%$ of the peak area of remaining C_{40}), both epoxy and allenic apo-aldehydes from neoxanthin and epoxy apo-aldehydes from violaxanthin. However the presence of these compounds was transient and after 15–20 min no traces were found. Xan/*t*-Xan and the C_{15} allenic apo-aldehyde were produced as well but in low yields (Xan/*t*-Xan, 1.9–4.1% of initial violaxanthin, 1.7–3.0% of neoxanthin; C_{15} allene, only *ca* 25% of the levels of Xan/*t*-Xan based on A_{280} nm), even though over 95% of the C_{40} xanthophylls were cleaved during the incubations. LOX/fatty acid free radical cleavage of xanthophylls appeared to show no substrate specificity and resulted in complete fragmentation, producing molecules of $\leqslant C_{13}$.

The stoichiometry observed between losses of neoxanthin and violaxanthin and increases in ABA and its metabolites in stressed etiolated bean leaves suggests that on a molar basis a 1:1 relationship exists [2, 3]. This implies that a specific cleavage, across the 11,12 (11',12') double bond, of the 9-*cis* xanthophylls is occurring to liberate Xan. It may be that a specific enzyme, possibly a dioxygenase, performs this first cleavage after which LOX and/or related enzymes rapidly degrade any C_{25} apo-aldehydes released.

EXPERIMENTAL

Plant material. Seeds of *Lycopersicon esculentum* Mill. cv Ailsa Craig wild-type and *notabilis* were obtained from Dr I. B. Taylor (University of Nottingham School of Agriculture, Sutton Bonington, Leics., U.K.). Etiolated *L. esculentum* and *Phaseolus vulgaris* seedlings were grown on vermiculite at 22° in the dark for up to 7 days. Plants to be treated with fluridone were grown on H_2O for 5–6 days and then sprayed with fluridone (13 mg l^{-1}) for 2 days before being transferred to weak light (17 μmol m^{-2} sec^{-1}), after which they were sprayed twice daily with 1 mg l^{-1} fluridone. Leaves were harvested at intervals, frozen and stored in liquid N_2. Water stressed and light-grown material was obtained as previously [2].

Determination and identification of carotenoids and ABA. As described in ref. [2].

Deuterium oxide labelling experiments. These were performed as described previously [2], using *Phaseolus vulgaris* cv Canadian Wonder seedlings. After 5 days on 50% D_2O in the dark some seedlings were placed in weak light (17 μmol m^{-2} sec^{-1}), and some transferred to H_2O. The latter had their 'growth pouches'/root systems thoroughly flushed with H_2O. The extent of deuteration of the carotenoids, ABA and PA was calculated using a BASIC computer programme bassed on the method of Wendt and McClosky [38], as in ref. [2].

Large-scale extraction of carotenoids. Two-week-old light-grown *P. vulgaris* leaves (106 g) were homogenized in 500 ml of redistilled Me_2CO with 20 g of Celite-535, filtered, reduced to aq. (*ca* 50 ml) and partitioned against C_6H_{12} (30 ml). The sample, in

C_6H_{12}, was subjected to flash chromatography (17 × 5 cm i.d.; silica gel; eluted with C_6H_{12}–*iso*-PrOH–TEA, 800:200:1), 100 ml frs collected and UV/VIS spectra recorded. Violaxanthin (frs 5–6) and neoxanthin (frs 7–11) were collected separately and reduced to dryness. Further purification was by RP/HPLC (system 1), after which the xanthophylls (all-*trans*-violaxanthin, 0.7 mg; 9′-*cis*-neoxanthin, 1.6 mg) were stored in EtOH at −20°.

Zinc permanganate oxidation. This was based on methods used by [24–26] but performed on a smaller scale. The technique was optimized using commercially prepared zeaxanthin. Between 250 and 500 μg of xanthophyll was dissolved in Me_2CO (2:1; v/w) in a mini-vial, covered with foil, and aq. $Zn(MnO_4)_2$ (2 mg ml^{-1}) added dropwise [0.25 mg $Zn(MnO_4)_2$/mg xanthophyll] over 1 hr with vigorous stirring. After 1 hr the mixt. was reduced to aq., made up to 5 ml with H_2O and partitioned twice against EtOAc (10 ml). The EtOAc was washed with H_2O prior to being reduced to dryness. The samples were stored in EtOAc at −20°. Analysis was by RP-HPLC (systems 1 and 2, samples injected in 20–30 μl EtOAc), linked to a photodiode-array detector, and GC-MS.

C_{15} allenic apo-aldehyde (**8**) (3,5-dihydroxy-6,7-didehydro-5,6-dihydro-apo-β-caroten-11-al): EIMS (GC-MS), *m/z* (rel. int.): 250 $[M]^+$ (7), 232 (100), 214 (33), 199 (61), 149 (57), 133 (64), 105 (42), 91 (39), 77 (36), 69 (25), 43 (57).

cis-C_{25} epoxy apo-aldehyde (9′-*cis*-5′,6′-epoxy-3′-hydroxy-5′,6′-dihydro-12-apo-β-caroten-12-al): probe MS, *m/z* (rel. int.): 382 $[M]^+$ (100), 364 (9), 302 (32), 287 (19), 234 (10), 221 (26), 173 (26), 159 (26), 145 (32), 119 (35), 105 (37), 91 (49).

trans-C_{25} epoxy apo-aldehyde (**11**) (5,6-epoxy-3-hydroxy-5,6-dihydro-12′-apo-β-caroten-12′-al): probe MS, *m/z* (rel. int.): 382 $[M]^+$ (100), 364 (30), 302 (35), 287 (21), 234 (14), 221 (32), 173 (37), 159 (39), 145 (46), 119 (49), 105 (54), 91 (88).

cis-C_{27} epoxy apo-aldehyde (9′-*cis*-5′,6′-epoxy-3′-hydroxy-5′6′-dihydro-10-apo-β-caroten-10-al): probe MS, *m/z* (rel. int.): 408 $[M]^+$ (88), 390 (5), 328 (22), 299 (10), 287 (10), 234 (21), 221 (41), 181 (38), 145 (43), 119 (55), 105 (60), 91 (100).

trans-C_{27} epoxy apo-aldehyde (**12**) (5,6-epoxy-3-hydroxy-5,6-dihydro-10′-apo-β-caroten-10′-al): probe MS, *m/z* (rel. int.): 408 $[M]^+$ (31), 390 (9), 328 (7), 299 (4), 287 (5), 234 (12), 221 (22), 181 (22), 145 (26), 119 (36), 105 (38), 91 (100).

C_{25} allenic apo-aldehyde (**9**) (3,5-dihydroxy-6,7-didehydro-5,6-dihydro-12′-apo-β-caroten-12′-al): probe MS, *m/z* (rel. int.): 382 $[M]^+$ (21), 364 (40), 346 (76), 331 (19), 275 (12), 263 (15), 247 (24), 233 (24), 221 (27), 207 (29), 197 (45), 167 (100), 119 (43), 105 (45), 91 (59).

C_{27} allenic apo-aldehyde (**10**) (3,5-dihydroxy-6,7-didehydro-5,6-dihydro-10′-apo-β-caroten-10′-al): probe MS, *m/z* (rel. int.): 408 $[M]^+$ (12), 390 (24), 372 (31), 357 (8), 311 (12), 267 (20), 247 (16), 230 (35), 215 (43), 197 (67), 167 (55), 119 (53), 105 (61), 91 (100).

Lipoxygenase incubations. The procedure used was that of ref. [15] but on a small scale. Aq. linoleic acid soln: linoleic acid in EtOH (7.5% w/v; 1 ml) mixed with Tween 80 in EtOH (10% v/v; 0.3 ml), aq. EDTA (0.5%; 5 ml) added, the pH adjusted to 9 (0.1 M NaOH) and the vol. made up to 10 ml with H_2O. Aq. carotenoid solns: 250 μg of carotenoid plus 9 μl Tween 80 dissolved in 250 μl $CHCl_3$, reduced to dryness under vacuum, dissolved in EDTA (0.25%; 1 ml). Incubation: 1 ml of linoleic acid soln and 1 ml of carotenoid soln made up to 12.5 ml with H_2O, aliquots removed (2–10 ml) to plastic cuvettes and LOX (lipoxidase; EC 1.13.11.12; Sigma type V; 737 000 units mg^{-1}) added (300 units ml^{-1} of incubation). Bleaching monitored continuously at 460 nm (zeaxanthin), 445 nm (neoxanthin) or 450 nm (violaxanthin). TBHQ added in EtOH (5 μl ml^{-1} of incubation) to final conc. of 480 μm. After 5–20 min the incubation mixt. was loaded on to a C_{18} Sep-Pak cartridge (pre-washed with 5 ml MeOH and 10 ml H_2O), washed with 10 ml of H_2O and the reaction products eluted with EtOAc (2–3 ml). Analysis of the products was by RP-HPLC (systems 2–4) and HPLC (system 5).

Chromatography. HPLC systems: 1. ODS-Spherisorb (250 × 10 mm i.d.) eluted with a linear gradient of 10–50% B in A over 25 min and maintained at 100% B for a further 15 min, at 2 ml min^{-1}, where A is 85% MeOH (+0.1% TEA; v/v) and B is CH_2Cl_2–MeOH (3:2). Eluate monitored between 300 and 500 nm. 2. ODS-Spherisorb (250 × 4.5 mm i.d.) eluted with 30–100% A_1 in B_1 over 15 min followed by 0–100% C_1 in A_1 over 30 min at 1 ml min^{-1}, where A_1 is MeCN–H_2O (9:1; +0.1% TEA; v/v), B_1 is H_2O (+0.1% TEA; v/v) and C_1 is EtOAc. Eluate monitored between 220 and 500 nm. 3. As 2 except eluted with 0–100% A_1 in B_1 over 45 min. 4. ODS-Spherisorb (250 × 4.5 mm i.d.) eluted with a linear gradient of 20–100% MeOH in 0.1 M HOAc over 40 min at 1 ml min^{-1}, eluate monitored between 220–500 nm. 5. Spherisorb (250 × 4.5 mm i.d.) eluted isocratically with C_6H_{12}–*iso*-PrOH (22:3) at 2 ml min^{-1}, eluate monitored between 220 and 500 nm.

GC-MS. GC: Phase Sep. OV-1 bonded-phase column (25 m × 0.32 mm i.d.; 0.4 μm film thickness) with a temp. prog. of 35–180° (ballistic)–250° (at 8° min^{-1}), He at 20 ml min^{-1}, linked to MS via a single stage jet separator at 230°. MS: resolution >600, ionising voltage 70 eV, source 190°, probe temp. 190–220°.

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