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# DISTRIBUTION OF CAROTENOIDS IN SUB-CELLULAR AND SUB-PLASTIDIC FRACTIONS OF RADISH SEEDLINGS (*RAPHANUS SATIVUS*) GROWN IN THE PRESENCE OF BLEACHING HERBICIDES

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**Key Word Index**—*Raphanus sativus*; Cruciferae; carotenoids; plastids; herbicides; bleaching; phytoene; lycopene; biosynthesis.

**Abstract**—The intracellular and intraplastidic distribution of carotenoids has been investigated in radish seedlings grown in the presence of the herbicides amitrole and SAN 6706. Both herbicides caused bleaching and the plants became deficient in chlorophylls and the usual chloroplast cyclic carotenoids, but accumulated the acyclic carotenoid biosynthetic intermediates 15-*cis*-phytoene and all-*trans*-lycopene. In both the untreated and herbicide-treated plants all carotenoids, including phytoene and lycopene, were contained in the plastid. In all cases the normal cyclic carotenoids were located virtually exclusively in the thylakoid or prothylakoid fraction. In amitrole-treated plants, lycopene also was contained only in the thylakoid fraction, whereas phytoene, in these and in SAN 6706-treated plants, was detected in both the thylakoid fraction and an envelope preparation. Possible implications for the biosynthesis of the carotenoids are discussed.

## INTRODUCTION

Chloroplasts from green tissues of higher plants contain β-carotene, (frequently accompanied by traces of α-carotene), and the xanthophylls lutein, violaxanthin and neoxanthin, usually with some zeaxanthin and antheraxanthin [1]. All these carotenoids are considered to be present in pigment–protein complexes located in highly specific orientations in photosynthetic reaction centres and light-harvesting assemblies in the thylakoid membrane [2]. The presence of some carotenoid, particularly violaxanthin, in the chloroplast envelope has also been reported [3, 4]. The carotenoids appear to be biosynthesized within the chloroplast; several biosynthetic transformations have been achieved with isolated chloroplasts, including the incorporation of mevalonate into phytoene [5] and of phytoene into cyclic carotenes [6]. The roles of cytoplasm and chloroplast in the synthesis of mevalonate for chloroplast terpenoid biosynthesis have been subject to argument. Wherever in the cell the early stages may occur there is no real doubt that the late stages of carotenoid biosynthesis take place in the plastid, though the actual site or sites of synthesis within the chloroplast remain unknown.

It is well-known that, in the presence of certain herbicidal compounds, notably amitrole and pyridazinones, formation of the range of cyclic carotenoids normally found in the chloroplast is inhibited and biosynthetic intermediates, such as phytoene, phytofluene and lycopene, not detectable in untreated plants, may accumulate [7]. Such herbicide-treated plants, therefore, provide an opportunity to gain more insight into the location of carotenoid biosynthesis within the cell and within the plastid.

## RESULTS AND DISCUSSION

Radish plants treated with amitrole and especially SAN 6706 were bleached during growth in continuous white light and became deficient in chlorophyll and carotenoids. The carotenoid compositions are given in Table 1. In the SAN-treated plants, the total carotenoid content was very much reduced; the normal cyclic carotenoids were present only in very low amounts but were replaced to some extent by phytoene. The amitrole-treated plants also contained reduced amounts of the normal carotenoids, though the individual cyclic carotenoids were present in approximately the same ratio as in the controls; phytoene and lycopene were also present. These were identified initially by their light absorption and mass spectra [phytoene UV $\lambda_{max}^{hexane}$ nm: 275, 285, 296, $M^+$ at $m/z$ 544 ($C_{40}H_{64}$); major fragment ion at $m/z$ 337 $[M-205]^+$; lycopene UV $\lambda_{max}^{hexane}$ nm: 442, 470, 504, $M^+$ at $m/z$ 536 ($C_{40}H_{56}$), major fragment ion at $m/z$ 467 $[M-69]^+$]. Phytone and lycopene are important intermediates in the biosynthesis of cyclic carotenoids [1]. The lycopene intermediate is normally in the all-*trans* configuration, as in the cyclic products, but the phytoene formed in higher plants appears to be the 15,15′-*cis*-isomer. The phytoene and lycopene isolated from the herbicide-treated radish seedlings were examined by high resolution (300 MHz) $^1$H NMR spectroscopy. The lycopene spectrum was identical to that of all-*trans*-lycopene published by Vetter *et al.* [8] (in particular showing $H_{11,11'}$ and $H_{15,15'}$ at δ6.62, $H_{7,7'}$ at δ6.48, $H_{12,12'}$ at δ6.34, $H_{8,8'}$ and $H_{14,14'}$ at δ6.24, $H_{10,10'}$ at δ6.17 and $H_{6,6'}$ at δ5.94). Phytoene gave a spectrum characteristic of the 15,15′-*cis*-isomer [9], significant features being the signals for $H_{14,14'}$ and $H_{15,15'}$ at δ6.30 and 6.09, respectively. The phytoene and

Table 1. Carotenoid compositions of leaves and plastids of radish seedlings grown for 6 days in continuous white light (8 W/m$^2$) in the presence of SAN 6706 ($1 \times 10^{-4}$ M) or amitrole ($1 \times 10^{-4}$ M)

| | Control (untreated plants) | | | | SAN 6706 | | | | Amitrole | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Leaves | | Plastids | | Leaves | | Plastids | | Leaves | | Plastids | |
| Carotenoid | μg* | %† | μg | % | μg | % | μg | % | μg | % | μg | % |
| β-Carotene | 1122 | 25 | 134 | 25.9 | 41 | 4.5 | 2.1 | 2.4 | 481 | 22.1 | 16 | 5.1 |
| Lutein‡ | 1862 | 41 | 238 | 46.0 | 49 | 5.3 | 5.3 | 5.9 | 560 | 25.8 | 128 | 40.8 |
| Antheraxanthin | 397 | 8 | 40 | 7.7 | 8 | 0.9 | 0.4 | 0.5 | 250 | 11.5 | 44 | 14.0 |
| Violaxanthin | 582 | 13 | 60 | 11.6 | 6 | 0.6 | 1.3 | 1.4 | 282 | 13.0 | 65 | 20.7 |
| Neoxanthin | 582 | 13 | 45 | 8.7 | 3 | 0.3 | 0.1 | 0.1 | 224 | 10.3 | 28 | 8.9 |
| Phytoene | n.d. | n.d. | n.d. | n.d. | 812 | 88.2 | 80.0 | 89.6 | 200 | 9.2 | 21 | 6.7 |
| Lycopene | n.d. | n.d. | n.d. | n.d. | 2 | 0.2 | 0.1 | 0.1 | 176 | 8.1 | 12 | 3.8 |

*Figures for leaves are μg/400 seedlings; for plastids are μg recovered from plastids isolated from 400 seedlings.
† % of total carotenoid.
‡The lutein normally contained *ca* 5% zeaxanthin.
n.d., Not detected.

lycopene that accumulate in herbicide-treated radish plants are, therefore, the normal 15-*cis*-and all-*trans* isomers, respectively.

As also shown in Table 1, plastids isolated from herbicide-treated or untreated radish plants contained all the carotenoids, including phytoene and lycopene, and the carotenoid compositions were broadly similar to those of the intact seedlings, though the β-carotene–xanthophyll ratio in the plastids from the amitrole-treated plants was low.

The main part of the work was that in which plastids from plants which had been grown in the dark or in continuous far-red or white light were disrupted and thylakoid and envelope membrane fractions prepared according to Douce *et al.* [3]. The carotenoid compositions of these fractions from SAN 6706- and amitrole-treated plants are given in Table 2. Although the samples were necessarily much smaller, the carotenoid compositions of the thylakoid fractions from plastids of radish seedlings grown in white light in the presence of SAN 6706 or amitrole were essentially similar to those of the isolated chloroplasts. Much lower carotenoid levels were obtained in the fractions from plastids from plants grown in the dark or in far-red light. In all cases, the cyclic carotenoids were found virtually exclusively in the thylakoid or prothylakoid fractions, and were essentially absent from the envelope preparations. Since this result appears to contradict the findings of Douce *et al.* [3, 4] who reported the presence of violaxanthin and other carotenoids in their envelope preparations from spinach leaf chloroplasts, untreated white-light-grown radish seedlings and 4-week-old spinach plants were investigated; again, the carotenoids were found to be restricted to the thylakoid fraction, less than 1% of the total or of any individual carotenoid, including violaxanthin, being detected in envelopes prepared by the Douce procedure [3]. The lycopene which appeared in the radish plants in response to amitrole treatment was also restricted to the thylakoid fraction. The situation with phytoene was different, however, with both SAN- and amitrole-treated plants, grown in the dark or in far-red or white light, although most of the phytoene was recovered in the thylakoid or prothylakoid fraction, a substantial proportion (17–32% in amitrole, 20–45% in SAN-treated plants) was found in the membrane preparation containing plastid envelopes. This may be taken as evidence that the earlier stages of carotenoid biosynthesis in the plastid, i.e. up to the phytoene stage, take place in the envelope, with the later stages—desaturation, cyclization, hydroxylation—then occurring in the thylakoids. This would necessitate a mechanism for transporting the hydrocarbon intermediate, phytoene, from envelope to thylakoid within the plastid. Alternative possibilities must also be considered. The herbicides amitrole and SAN 6706 are known to disturb photosynthetic membrane development, so that plastids of the herbicide-treated plants are not identical to the normal chloroplasts of untreated plants. It is, thus, possible that the thylakoid and envelope membrane fractions prepared by methods used for normal plants may contain incompletely developed or modified membranes so that, for example substances that are found in an envelope preparation may in fact be located in some abnormal thylakoid or prothylakoid membrane whose properties are so modified that it is obtained in the envelope fraction. It must also be noted that although the carotenoids are eventually localized in the mature functioning membrane, they are synthesized along with other components as part of the overall process of construction of these membranes, and their synthesis may occur at a time when the properties of the 'immature' membranes differ greatly from those of the final mature thylakoid.

Thus, although this work has revealed an interesting feature about the distribution of the biosynthetic intermediate phytoene in sub-plastid fractions, it is necessary to know more about the structures of the plastids and membranes of the herbicide-treated plants in order to determine whether carotenoid biosynthesis requires the co-operation of different sites within the plastid, e.g. whether some steps are performed by the chloroplast envelope.

## EXPERIMENTAL

Seedlings of radish (*Raphanus sativus* L. var. Saxa) were grown for 6 days either in the dark or in continuous far-red or white light (intensity normally 8 W/m$^2$). Herbicide treatment was applied by watering the plants with amitrole (aminotriazole, $1 \times 10^{-4}$ M) or SAN 6706 (Metflurazon, 4-chloro-5-(dimethylamino)2-(α,α,α-

Table 2. Distribution of carotenoids in prothylakoid, thylakoid and envelope fractions of plastids of radish seedlings grown for 6 days in the presence of SAN 6706 ($1 \times 10^{-4}$ M) or amitrole ($1 \times 10^{-4}$ M) in the dark, or continuous far-red or white light

| | Dark | | | | Far-red light | | | | White light (8 W/m²) | | | | White light (0.3 W/m²) | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Carotenoid | μg | % total | % PT† | % E† | μg | % total | % PT | % E | μg | % total | % T† | % E | μg | % total | % T | % E |
| SAN 6706 | | | | | | | | | | | | | | | | |
| β-Carotene | 2.0 | 2.0 | 100 | — | 9.0 | 3.4 | 100 | — | 2.6 | 2.2 | 100 | — | 18.6 | 7.2 | 100 | — |
| Lutein | 3.0 | 3.0 | 100 | — | 11.0 | 4.2 | 100 | — | 4.4 | 3.7 | 100 | — | 26.0 | 10.0 | 100 | — |
| Antheraxanthin | 0.4 | 0.4 | 100 | — | 2.0 | 0.8 | 100 | — | 0.4 | 0.3 | 100 | — | 4.6 | 1.8 | 100 | — |
| Violaxanthin | 0.7 | 0.7 | 100 | — | 4.0 | 1.5 | 100 | — | 1.6 | 1.4 | 100 | — | 4.6 | 1.8 | 100 | — |
| Neoxanthin | 0.1 | 0.1 | 100 | — | 1.0 | 0.4 | 100 | — | 0.4 | 0.3 | 100 | — | 4.0 | 1.5 | 100 | — |
| Phytoene | 94.0 | 94.0 | 78 | 22 | 238.0 | 89.8 | 82 | 18 | 108.9 | 92.1 | 68 | 32 | 201.0 | 77.6 | 83 | 17 |
| Lycopene | — | — | — | — | — | — | — | — | — | — | — | — | — | — | — | — |
| Amitrole | | | | | | | | | | | | | | | | |
| β-Carotene | 1.5 | 6.7 | 100 | — | 7.3 | 8.4 | 98 | 2 | 20.0 | 5.1 | 99 | 1 | 44.8 | 28.4 | 99 | 1 |
| Lutein | 4.2 | 18.7 | 98 | 2 | 16.6 | 19.2 | 97 | 3 | 163.0 | 41.5 | 99.5 | 0.5 | 46.0 | 29.2 | 99 | 1 |
| Antheraxanthin | 1.8 | 8.0 | 97 | 3 | 4.7 | 5.4 | 98 | 2 | 54.0 | 13.7 | 99 | 1 | 14.0 | 8.9 | 99 | 1 |
| Violaxanthin | 2.6 | 11.6 | 97 | 3 | 10.9 | 12.6 | 98 | 2 | 80.0 | 20.4 | 100 | — | 22.3 | 14.1 | 100 | — |
| Neoxanthin | 0.4 | 1.8 | 98 | 2 | 3.1 | 3.6 | 97 | 3 | 32.0 | 8.1 | 99.5 | 0.5 | 11.5 | 7.3 | 99 | 1 |
| Phytoene | 12.0 | 53.3 | 80 | 20 | 44.0 | 50.8 | 75 | 5 | 30.0 | 7.6 | 54 | 46 | 18.2 | 11.5 | 70 | 30 |
| Lycopene | — | — | — | — | — | — | — | — | 14.0 | 3.6 | 100 | — | 1.0 | 0.6 | 100 | — |

* % of total carotenoid.

† % PT, % E, % T indicate the percentage of each individual carotenoid recovered in the prothylakoid, envelope and thylakoid fractions, respectively.

trifluoro-*m*-tolyl)-3(2*H*)-pyridazinone, $1 \times 10^{-4}$ M). Plastids were isolated from the cotyledons by the methods of refs. [10] or [11]. Chloroplasts were also isolated by the same methods from 4-week-old spinach plants grown in the Botanical Gardens of the University of Karlsruhe. Isolated plastids were disrupted and envelope and thylakoid (or prothylakoid) fractions obtained by the method of ref. [3]. Carotenoids were extracted from seedlings, plastids and sub-plastid fractions, these extracts were saponified to remove chlorophyll and the carotenoids were separated and purified all according to the procedures of ref. [12]. Quantitative carotenoid analyses were performed by standard spectrophotometric procedures [13].

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## REFERENCES

1. Britton, G. (1976) in *Chemistry and Biochemistry of Plant Pigments* (Goodwin, T. W., ed.) 2nd edn, Vol. 1, p. 262. Academic Press, London.
2. Thornber, J. P. (1975) *Annu. Rev. Plant Physiol.* **26**, 127.
3. Douce, R., Holtz, R. B. and Benson, A. A. (1973) *J. Biol. Chem.* **248**, 7215.
4. Siefermann-Harms, D., Joyard, J. and Douce, R. (1978) *Plant Physiol.* **61**, 530.
5. Buggy, M. J., Britton, G. and Goodwin, T. W. (1974) *Phytochemistry* **13**, 125.
6. Subbarayan, C., Kushwaha, S. C., Suzue, G. and Porter, J. W. (1970) *Arch. Biochem. Biophys.* **137**, 547.
7. Ridley, S. M. (1982) in *Carotenoid Chemistry and Biochemistry* (Britton, G. and Goodwin, T. W., eds.) p. 353. Pergamon Press, Oxford.
8. Vetter, W., Englert, G., Rigassi, N. and Schwieter, U. (1971) in *Carotenoids* (Isler, O., ed.) p. 189. Birkhäuser, Basel.
9. Khan, N., Loeber, D. E., Toube, T. P. and Weedon, B. C. L. (1975) *J. Chem. Soc. Perkin Trans.* **1**. 1457.
10. Heber, W. and Santarius, K. A. (1970) *Z. Naturforsch. Teil B* **25**, 718.
11. Jensen, R. G. and Bassham, J. A. (1966) *Proc. Natl. Acad. Sci. U.S.A.* **56**, 1095.
12. Britton, G. and Goodwin, T. W. (1971) *Methods Enzymol.* **18c**, 654.
13. Davies, B. H. (1976) in *Chemistry and Biochemistry of Plant Pigments* (Goodwin, T. W., ed.) 2nd edn, Vol. 2, p. 38. Academic Press, London.