Proc. Nat. Acad. Sci. USA
Vol. 71, No. 3, pp. 807–810, March 1974

Carotenoid Transformations in the Chloroplast Envelope

(spinach/light-catalyzed changes)

S. W. JEFFREY*, ROLAND DOUCE†, AND A. A. BENSON

Scripps Institution of Oceanography, University of California, San Diego, La Jolla, Calif. 92037

Contributed by A. A. Benson, November 16, 1973

ABSTRACT **The envelope of the spinach chloroplast is a yellow membrane system with a unique carotenoid composition. Envelopes prepared from dark-treated leaves had a violaxanthin content up to 3.5 times the lutein plus zeaxanthin content, whereas in chloroplast envelopes from illuminated leaves this ratio was only 0.75. Light-catalyzed changes in violaxanthin content also occurred in the thylakoid fraction.**

The role of this reversible light-catalyzed de-epoxidation of violaxanthin in the function of the envelope of the chloroplast is discussed.

In a recent paper by Douce *et al.* (1) it was reported that violaxanthin constituted a greater proportion of the carotenoids of the chloroplast envelope than of the thylakoids of ordinarily prepared spinach chloroplasts. There is now considerable evidence (2) that the conversion of violaxanthin to zeaxanthin occurs when isolated spinach chloroplasts are illuminated. The biochemical mechanism of this conversion has been studied (3, 4), but its physiological significance remains obscure (2).

In this paper we report the recognition of a light-dependent conversion of violaxanthin to zeaxanthin in the envelope of spinach chloroplasts.

MATERIALS AND METHODS

Spinach obtained from local markets was stored briefly at 4°. Whole leaves were washed, placed in water at 20°, and exposed either to white light (1.2×10^6 ergs/cm^2 per sec) or to darkness for 2 hr. Chloroplasts were prepared according to the "aqueous" method of Walker (5). The purification of intact chloroplasts (class I) was carried out by a modification of the method of Leech (6). The chloroplast envelope, thylakoid, and stroma were prepared and characterized as described (1). The membrane fractions isolated by discontinuous sucrose gradient centrifugation were concentrated as pellets by centrifugation.

Protein content was determined by the method of Lowry *et al.* (7) with crystallized bovine-serum albumin (Miles Laboratory Inc.) as the standard.

Pigments were extracted from the thylakoid and the outer envelope preparations with 80% acetone. Chlorophylls *a* and *b* were measured spectrophotometrically, with revised equations of Jeffrey and Humphrey‡. Carotenoids were separated by the sucrose thin-layer chromatography method of Jeffrey (8) and measured after quantitative elution. A rapid and quantitative method was essential because of the lability of plant pigments, violaxanthin being particularly fragile. For chromatography, pigments were transferred from 80% acetone to diethyl ether (Mallinckrodt, peroxide-free), by addition of an equal volume of ether to the acetone extract, and washing with 20 volumes of cold 10% NaCl solution. The ether hyperphase containing the pigments was concentrated under nitrogen, and a little solid NaCl was added to remove traces of water from the ether phase. NaCl is preferable to Na_2SO_4, since no adsorption of pigments occurs.

Modifications adapting the chromatography method (8) to California & Hawaii (C & H) commercial powdered sugar (containing 3% cornstarch) involved carefully drying the sucrose at 85 ± 2° for 60 ± 5 min, and blending immediately for 1 min with a mixture of petroleum ether (ligroine, Eastman Kodak, 63–75° fraction) and chloroform (1:1, v/v). The slurry was immediately spread onto glass plates (12 × 16 cm or 8 × 10 cm), dried at room temperature for 15 min, and stored in a tightly sealed desiccator.

The solvent systems required to give identical resolution on the C & H brand sugar to those described in ref. 8 were 1.3% *n*-propanol in ligroine (63–75°) for the first dimension and 40% chloroform in ligroine for the second dimension. Total development time on 8 × 10-cm plates, which were routinely used, did not exceed 8 min. Chromatography and pigment extractions were performed in the dark to minimize harmful photo-oxidation, and precautions detailed previously (8) for successful chromatography of pigments on sucrose, particularly the necessity of keeping the sucrose plates, solvents, and extracts completely dry, were strictly observed. The use of the 63–75° fraction of ligroine in all the solvent systems was essential, and could not be duplicated by other boiling-point fractions of ligroine, or by hexane.

Sucrose plates, used with the above solvents, do not resolve isomers such as α- and β-carotene or their dihydroxy derivatives, lutein and zeaxanthin. These pigments, which formed one spot, were treated as one fraction throughout this work. Although other thin-layer systems are available for the

* To whom correspondence should be addressed. Present address: CSIRO Marine Biochemistry Unit, Botany Building, Sydney University, N.S.W. 2006, Australia.

† Present address: Faculty of Science, University of Grenoble, Grenoble, France.

‡ Spectrophotometric equations for determination of chlorophylls *a* and *b* in 80% acetone (μg/ml) calculated from revised extinction coefficients of chlorophylls *a* and *b* (Jeffrey, unpublished) are as follows: chlorophyll $a = 11.73\ E_{664} - 1.97\ E_{647}$; chlorophyll $b = 20.56\ E_{647} - 5.42\ E_{664}$.

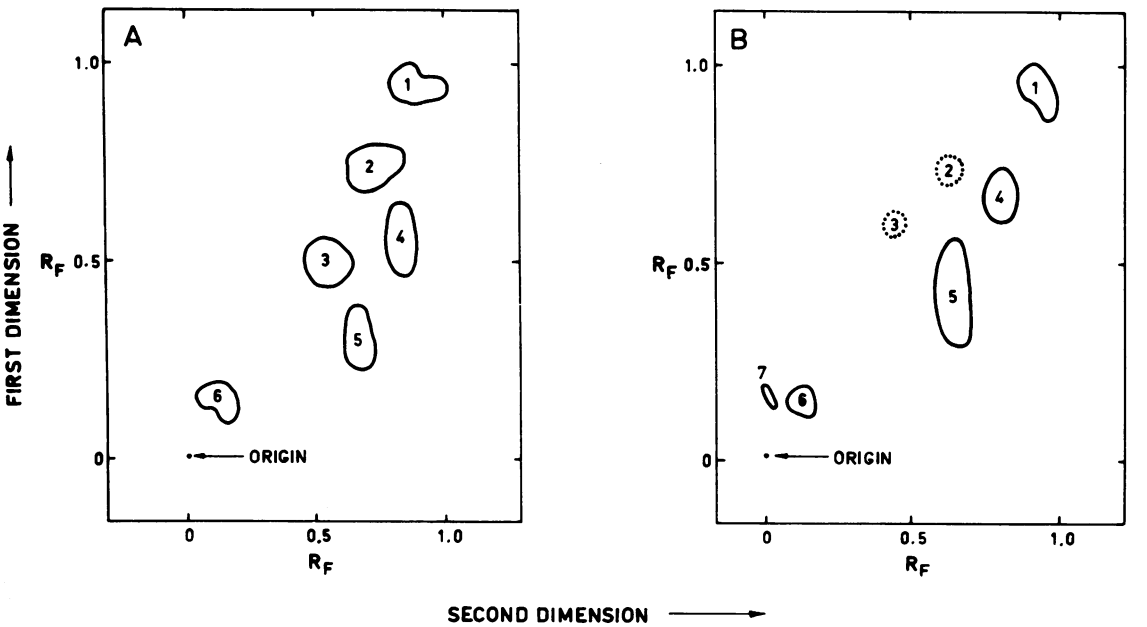

FIG. 1. Sucrose thin-layer chromatograms of pigments of (*A*) inner thylakoids and (*B*) outer envelope of the spinach chloroplast. First dimension, 1.3% *n*-propanol in ligroine (63–75°); second dimension, 40% chloroform in ligroine (63–75°). (*1*) β-carotene; (*2*) chlorophyll *a*; (*3*) chlorophyll *b*; (*4*) lutein + zeaxanthin; (*5*) violaxanthin; (*6*) neoxanthin; (*7*) chlorophyllide *a*.

separation of lutein and zeaxanthin (9–11), strong inorganic adsorbants are used, and evidence for quantitative recovery of unaltered pigments from these adsorbants has not been recorded. The sucrose plate, however, gives rapid quantitative recovery of unaltered pigments (8), particularly important in the present work because of the lability of violaxanthin. Furthermore, since the sucrose plate also separates major chlorophyll degradation products (pheophytins, chlorophyllides, and pheophorbides), accurate assessment of unaltered chlorophylls *a* and *b*, free from traces of breakdown products which could occur during thylakoid and envelope preparation, was also achieved.

Absorption spectra were recorded on a Cary model 14 spectrophotometer. Pigments were eluted with the following solvents for which extinction coefficients are given: chlorophyll *a* (acetone), $E_{663\,nm}$ = 88.15 liters/g·cm (Jeffrey, unpublished); chlorophyll *b* (acetone), $E_{645\,nm}$ = 50.59 liters/g·cm (Jeffrey, unpublished); β-carotene (hexane), $E_{450\,nm}$ = 250.5 liters/g·cm (12); lutein (ethanol), $E_{447\,nm}$ = 255 liter/g·cm (13); violaxanthin (ethanol), $E_{441\,nm}$ = 225 liter/g·cm (13); neoxanthin (ethanol), $E_{438\,nm}$ = 227 liter/g·cm (13).

TABLE 1. *Pigment composition of thylakoids and envelopes with no light pretreatment*

Pigment	Thylakoids* (% composition)	Envelopes† (% composition)
β-Carotene	16.4	10.5
Lutein + zeaxanthin	35.6	18.8
Violaxanthin	32.5	66.2
Neoxanthin	15.5	4.5
Ratio: violaxanthin/(lutein + zeaxanthin)	0.9	3.5
Ratio: total chlorophyll/ total carotenoid	6.8	0.1

* Single preparation.
† Frozen pooled samples.

RESULTS

The pigment composition of the envelope and the thylakoids are shown in Table 1 and Fig. 1. Violaxanthin was the dominant xanthophyll in the envelope, exceeding the lutein plus zeaxanthin fraction by more than 3-fold. However, lutein plus zeaxanthin were in excess of violaxanthin in the thylakoids. Only negligible amounts of chlorophylls *a* and *b* were found in the envelope, with a ratio of total chlorophyll to total carotenoid of 0.1, compared to a ratio of 6.8 in the thylakoids. This very low and variable chlorophyll content of the envelope is considered to be a contamination from the light stroma lamellae containing plastoglobuli [fraction 3 on the sucrose gradient (1)]. This conclusion is supported by the appearance of the envelope pellet after centrifugation for 1 hr at 27,000 rpm (Beckman model L3-50, SW27:1 rotor) (1). A tiny green "pin head" spot occurred at the base of the centrifuge tube embedded at the center of the yellow pellet, strongly indicating that this chlorophyll was not associated with the yellow membrane. Fig. 2 shows the full absorption spectrum of 80% acetone extracts of the envelope and the thylakoids. The spectrum of the envelope shows the strong carotenoid absorption, with typical three-banded spectrum in the blue region, with very low chlorophyll absorption at 664 nm. On a protein basis, however, the envelope was less rich in carotenoid than the thylakoids, the envelope extract in Fig. 2 containing approximately 13 times more protein than the thylakoids. Envelopes contained about 8 μg of carotenoid per mg of protein, compared to 27 μg of carotenoid per mg of protein in the thylakoids.

TABLE 2. *Ratio of violaxanthin to lutein + zeaxanthin of thylakoids and envelopes prepared from leaves incubated in the light and dark*

	Thylakoids		Envelopes	
Exp.	Light	Dark	Light	Dark
1	0.35	0.85	0.75	2.21
2	—	—	0.52	2.48

Changes in the proportions of the xanthophylls in the outer envelope occurred if spinach leaves were incubated in full sunlight or darkness before preparation of the membranes. Table 2 shows that the outer envelope from leaves preincubated in the light (1.2×10^6 ergs/cm^2 per sec for 2 hr at 15°) contained slightly more lutein plus zeaxanthin than violaxanthin, whereas leaves kept in darkness under the same conditions yielded membranes in which violaxanthin was 2.5 times the lutein plus zeaxanthin content. Both these changes could be reversed, by reversing the light pretreatments.

The thylakoids showed less dramatic light-induced changes in the ratio of violaxanthin to lutein plus zeaxanthin, but because the total amount of carotenoid on a protein basis was 3 times that of the envelope, the violaxanthin transformation in the thylakoids was also significant.

Isolated envelopes incubated in the light with ATP, FMN, $NADPH_2$, and a water-soluble chloroplast extract containing violaxanthin de-epoxidase, showed small transformations of violaxanthin to zeaxanthin.

DISCUSSION

The envelope of the spinach chloroplast is a double-membrane system with a specific carotenoid composition and a unique Mg^{2+}-dependent ATPase activity (1). The present work describes an additional characteristic of the outer envelope, that of a functioning violaxanthin epoxide cycle.

Earliest studies of the violaxanthin cycle used whole leaves and green algae (14–19), and more recently, isolated chloroplasts (3, 20). The changes involve a light-stimulated conversion of violaxanthin to zeaxanthin, by way of the monoepoxide intermediate antheraxanthin, and a dark reversal. The function of this cycle in photosynthetic tissues is unclear. Evidence has been cited that the epoxide cycle plays a role in protecting the chloroplast against lethal photosensitized oxidations (21, 22), that it is involved in photosynthetic oxygen evolution (23), photosynthetic oxygen uptake (24), is inhibited by inhibitors of photosynthetic phosphorylation (3), and operates only at high light intensities under conditions where CO_2 incorporation becomes limiting (25). The cycle has also been implicated in the 515-nm change that occurs when photosynthetic systems are illuminated (2, 26).

The present work clearly localizes an active light-dependent violaxanthin de-epoxidation cycle and its dark back reaction in the outer envelope of the chloroplast, a membrane system that contains only negligible amounts of chlorophyll and no recognized enzymatic activities of the inner thylakoids. In the same experiments a violaxanthin cycle was also operating in the thylakoids. It is possible that the cycle may perform different functions depending on the particular membrane system in which it is operating. Relevant to these changes in the thylakoids are the observations of Vernon *et al.* (27) that

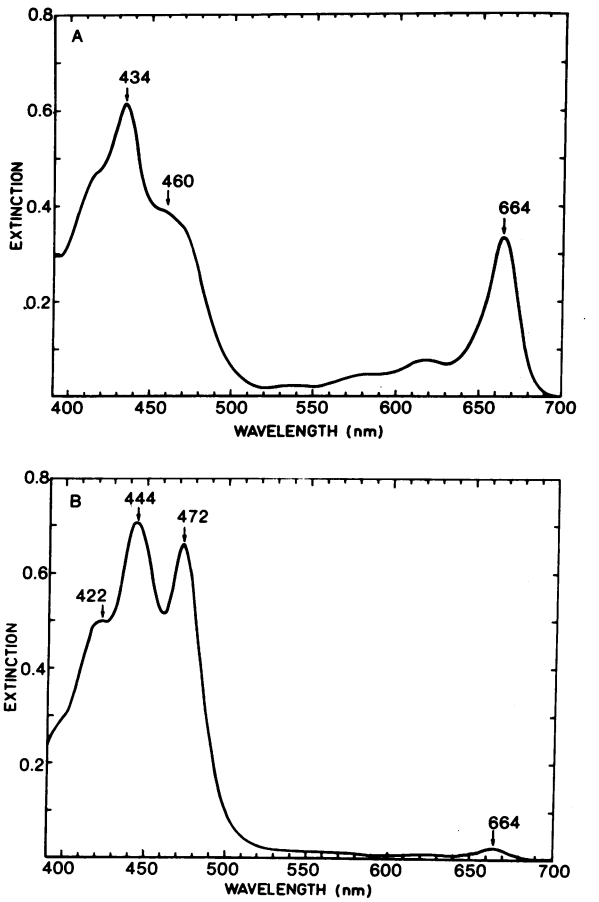

FIG. 2. Absorption spectra of 80% acetone extracts of (*A*) thylakoids and (*B*) envelopes of the spinach chloroplast. The protein contents of (*A*) and (*B*) were 25 and 350 μg/ml, respectively.

thylakoids fractionated with Triton X-100 yield photosystem-II particles enriched with violaxanthin. The present work raises the question whether the violaxanthin content of such particles would be lowered or negligible if leaves were treated with light before fractionation. The necessity to take into account the light history of leaves in future carotenoid distribution and function studies cannot be over emphasized.

Whatever the function of the carotenoids in the thylakoids [discussed by Krinsky, (2)] the outer envelope is clearly a yellow membrane system with absorption properties suggestive of a blue light filter (Fig. 2). One role of the envelope might be to protect the chlorophyll of the thylakoids from harmful photooxidations at high light intensities. The possible release of elemental oxygen from the de-epoxidation of violaxanthin in the light also suggests that the envelope could be the site of formation of molecular oxygen and involved in its transfer out of the chloroplast. Finally there is evidence from nonphotosynthetic systems that carotenoids in membranes may act by stabilizing protein conformation (2). Such a role might be particularly important in a membrane system that is continually synthesizing all the galactolipid of the thylakoids (28).

We acknowledge the helpful suggestions of Prof. Richard McCarty and support by National Science Foundation, Grant GB 15500.

1. Douce, R., Holtz, B. & Benson, A. A. (1973) *J. Biol. Chem.*, **248**, 7215–7222.
2. Krinsky, N. I. (1971) in *Carotenoids*, ed. Isler, O. (Birkhäuser Verlag, Basel), pp. 669–716.
3. Hager, A. (1969) *Planta* **89**, 224–243.
4. Hager, A. & Perz, H. (1970) *Planta* **93**, 314–322.
5. Walker, D. A. (1971) in *Methods in Enzymology*, ed. Anthony San Pietro (Academic Press, New York), Vol. 23, pp. 211–220.
6. Leech, R. M. (1964) *Biochim. Biophys. Acta* **79**, 637–639.
7. Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, R. J. (1951) *J. Biol. Chem.* **193**, 265–275.
8. Jeffrey, S. W. (1968) *Biochim. Biophys. Acta* **162**, 271–285.
9. Hager, A. & Stransky, H. (1970) *Arch. Mikrobiol.* **71**, 132–163.
10. Hager, A. & Stransky, H. (1970) *Arch. Mikrobiol.* **72**, 68–83.
11. Chapman, D. J. (1966) Ph.D. Dissertation, University of California, San Diego, Calif.
12. Goodwin, T. W. (1955) in *Modern Methods of Plant Analysis*, eds. Paech, K. & Tracy, M. (Springer Verlag, Heidelberg), Vol. 3, p. 272.
13. Strain, H. H. (1938) *Leaf Xanthophylls* (Carnegie Institution, Washington, D.C.).
14. Sapozhnikov, D. I. Krasovskaya, T. A. & Maerskaya, A. N. (1957) *Dokl. Akad. Nauk SSSR* **113**, 74–76.
15. Anderson, J. M., Blass, U. & Calvin, M. (1960) in *Comparative Biochemistry of Photoreactive Systems*, ed. Allen, M. B. (Academic Press, New York), pp. 215–225.
16. Yamamoto, H. Y., Nakayama, T. M. & Chichester, C. O. (1962) *Arch. Biochem. Biophys.* **97**, 168–173.
17. Yamamoto, H. Y., Chang, J. L. & Aihara, M. S. (1967) *Biochim. Biophys. Acta* **141**, 342–347.
18. Hager, A. (1967) *Planta* **76**, 138–148.
19. Krinsky, N. I. (1954) *Biochim. Biophys. Acta* **88**, 487–491.
20. Yamamoto, H. Y., Kamite, L. & Wang, Y. (1972) *Plant Physiol.* **49**, 224–228.
21. Krinsky, N. I. (1966) in *Biochemistry of Chloroplasts*, ed. Goodwin, T. W. (Academic Press, New York), Vol. 1, pp. 423–430.
22. Krinsky, N. I. (1968) in Photophysiology, ed. Giese, A. C. (Academic Press, New York), Vol. 3, 123–195.
23. Sapozhnikov, D. I. (1969) in *Progress in Photosynthesis Research*, ed. Metzner, H. (H. Laupp Jr., Tübingen Germany), Vol. 2, pp. 694–700.
24. Yamamoto, H. Y. & Takeguchi, C. H. (1972) in *Proceedings of International Congress on Photosynthesis 2nd*, eds. Forti, Georgi, Avron, Mordhay & Melandri, Andrea (Junk, W. NV Publishers, the Hague), Vol. 1, pp. 621–627.
25. Sieferman, D. (1972) in *Proceedings of International Congress on Photosynthesis 2nd*, eds. Forti, Georgi, Avron, Mordhay & Melandri, Andrea (Junk, W. NV Publishers, the Hague), Vol. 1, pp. 629–635.
26. Yamamoto, H. Y., Wang, Y. & Kamite, L. (1971) *Biochem. Biophys. Res. Commun.* **42**, 37–39.
27. Vernon, L. P., Ke, B., Mollenhauer, H. H. & Shaw, E. R. (1969) in *Progress in Photosynthesis Research*, ed. Metzner, H. (H. Laupp Jr., Tübingen, Germany), Vol. 1, pp. 137–148.
28. Douce, R. (1974) *Science*, in press.