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An Ascorbate-induced Absorbance Change in Chloroplasts from Violaxanthin De-epoxidation[1]

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ABSTRACT

A new ascorbate-induced chloroplast absorbance change which has the characteristics of a carotenoid shift is described. The absorbance change was light-dependent at pH 7 but not at pH 5. The difference spectra for the light and dark changes were similar, showing a large absorbance peak at 505 nanometers, smaller peaks near 468 and 437 nanometers, and a sharp valley around 483 nanometers. The absorbance change is assigned to violaxanthin de-epoxidation because various conditions affected the absorbance change and violaxanthin de-epoxidation similarly, and the difference spectrum resembled the spectrum of zeaxanthin *minus* violaxanthin in organic solvent.

Nigericin with KCl inhibited the light-dependent change at 505 nanometers. This effect, as well as the dark change at pH 5, indicated that de-epoxidation requires an acidic condition in chloroplasts. The effects of 3-(3,4-dichlorophenyl)-1,1-dimethyl urea, 2,6-dichlorophenolindophenol, and phenazine methosulfate indicated that the chloroplast acidification which mediates the 505 nanometers change is derived from hydrogen-ion transport linked to photosystem 1. Thus the 505 nanometers change could serve as an endogenous probe for chloroplast acidification and an indirect indicator of hydrogen-ion transport. At pH 5, the role of ascorbate appears to be to provide the reducing potential necessary for reductive de-epoxidation of violaxanthin. At pH 7, ascorbate could have an additional effect of stimulating electron transport and hence the hydrogen-ion transport necessary for de-epoxidation.

In contrast to leaves and algae, de-epoxidation in chloroplasts was irreversible under the conditions investigated. Under some conditions other absorbance changes which were apparently due to chlorophyll were superimposed on the de-epoxidation change. The relationship of these ascorbate-induced changes to other absorbance changes observed in chloroplasts and green algae remains to be determined.

In leaves, light induces reversible changes between violaxanthin (5,6,5′,6′-diepoxyzeaxanthin) and zeaxanthin through the monoepoxide antheraxanthin (17). This conversion, in which epoxide is alternately lost (de-epoxidation) and reincorporated from O_2 (16), appears to be a cyclic pathway for photosynthetic O_2 uptake (12) mediated indirectly by photosynthetic electron transport (15). Recently, Hager (5) concluded from the effects of pH and uncouplers that chloroplast acidification from hydrogen-ion transport mediated de-epoxidation and that de-epoxidation was therefore related in some way to photophosphorylation.

Since zeaxanthin absorbs light at longer wavelengths than violaxanthin, chloroplasts were examined for a difference spectrum which could be correlated with de-epoxidation. We report herein on a new light-induced absorbance change which is stimulated by ascorbate and which has the properties expected of a change from violaxanthin de-epoxidation. A preliminary report on part of this work has appeared (18).

MATERIALS AND METHODS

Chloroplasts were prepared at 0 C from market lettuce (*Lactuca sativa* var. Manoa). About 30 g of green leaves were chopped, homogenized in 115 ml of SNH solution (0.4 M sorbitol, 10 mM NaCl, 50 mM HEPES, pH 7) for 5 sec in a semi-micro Waring Blendor, filtered through 16 layers of gauze, and centrifuged at 500 or 1000*g* for 5 min. The resulting pellet was resuspended in SNH solution and, for spectrophotometric studies, filtered through glass wool to remove large particles.

Chloroplast absorbance changes were determined at 25 C with a Perkin-Elmer Model 356 two-wavelength double-beam spectrophotometer. Chloroplast suspensions in 1-cm cuvettes were illuminated with light from a tungsten lamp filtered through red Corning CS2-58 and two heat-absorbing filters. When required, the photomultiplier was shielded from actinic light with Corning filter CS4-96. Light intensity was measured with a YSI Model 65 radiometer.

Violaxanthin de-epoxidation activity in chloroplasts was determined by analysis of changes in pigment composition as described previously (8). The chloroplast suspensions were illuminated in test tubes at 20 C with light from a tungsten-iodine lamp filtered through red cellophane. Chlorophyll concentration was determined according to Vernon (13).

RESULTS

The light-induced difference spectra of lettuce chloroplasts in the presence of ascorbate both during and after continuous illumination are shown in Figure 1. The spectrum during continuous illumination showed characteristics of a carotenoid shift with peaks at 505, 468, and 437 nm and valleys at 483 and 447 nm. The peak at 437 nm was small and not always evident. After the actinic light was turned off, the spectrum persisted but with a general increase in absorbance below 483 nm. Thus the absorbance change in the presence of ascorbate at 505 nm was not reversible. The partly reversible changes at shorter wavelength appeared to be maximal near 430 nm, which suggested that changes in this re-

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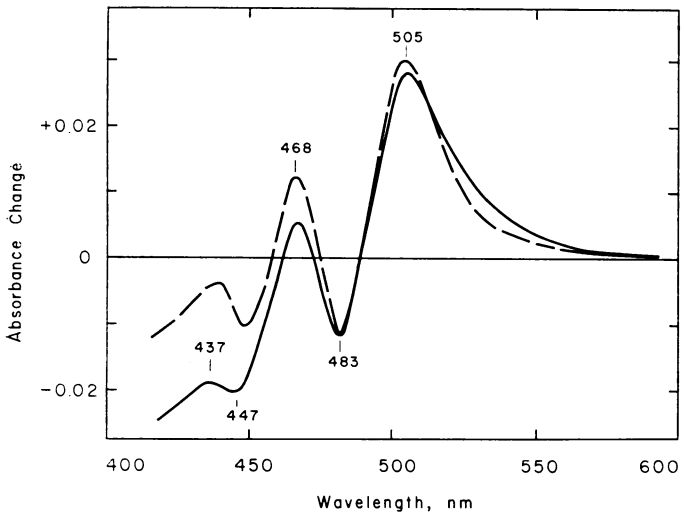

FIG. 1. Light minus dark chloroplast difference spectra during (——) and after (– – –) continuous illumination. Chloroplasts (41 μg chlorophyll per ml) suspended in SNH solution containing 30 mM sodium ascorbate were illuminated with actinic light at 2 × 10^5 ergs cm^{-2} sec^{-1}. Scanning was at 60 nm per min and was begun 2 min after the actinic light was turned on or off.

gion were due to at least two components, one part of the 505 nm change and the other a reversible component, probably chlorophyll.

Hager (5) reported that at pH 5, the presence of ascorbate-induced de-epoxidation of violaxanthin in the dark. Figure 2 shows the difference spectrum of chloroplasts induced by ascorbate at pH 5 following dark incubation. The similarity in shape and peak positions between this spectrum and those in Figure 1 is clearly evident.

Figure 3 shows the kinetics of the light-induced 505 nm change. In the presence of ascorbate there was a very small but rapid initial rise of about 0.002 *A*, followed by a slower but much larger change. DCMU inhibited this absorbance increase but not when DPIP[2] or PMS was also added. In the presence of PMS or DPIP the change at 505 nm was partly reversible. Nigericin, a potent hydrogen-ion transport inhibitor (11), inhibited the change at 505 nm. The results of parallel experiments on light-dependent de-epoxidation and on pH 5 dark de-epoxidation are summarized in Table I. A close correlation between the 505 nm change and violaxanthin de-epoxidation is evident.

To investigate the possible contribution of chlorophyll to the irreversible absorbance change, difference spectra from 750 to 400 nm following illumination were determined. Figure 4 shows that changes in chlorophyll absorbance did occur, as indicated by changes in the red region, but such changes were variable depending on conditions. No chlorophyll absorbance change was observed in the presence of DPIP, although the change associated with violaxanthin de-epoxidation was present. When absorbance decreases in the red region from chlorophyll were present, there were corresponding changes in the 400 to 460 nm region and maximally near 437 nm. The 500 nm region was not affected greatly except when the chlorophyll change was large relative to the de-epoxidation change as in the presence of PMS. In such cases the peak of the positive absorbance change appeared to be shifted to varying degrees from 505 to 515 nm in different preparations.

The difference spectra of purified carotenoids, zeaxanthin *minus* violaxanthin, in acetone and in carbon disulfide are shown in Figure 5. The characteristics of these spectra resembled closely the light-induced and pH 5 changes in chloroplasts. The major peaks in acetone and in carbon disulfide were 488 and 518 nm respectively as contrasted to 505 nm for the light-induced chloroplast change. These peak positions probably reflect the differences in the absorbance maxima for these pigments in organic solvents and in chloroplasts.

DISCUSSION

Recently, Maslova and Meister (9) observed broad light-induced changes in the second derivative absorbance spectra of leaves with low chlorophyll *b* content. Such changes appeared to be related to violaxanthin interconversion but were not detectable in leaves with normal pigment content. Butler and Hopkins (1) have shown that although second derivative spectra can enhance the resolution of superimposed peaks, under certain conditions artifacts can result.

In the present study a well-defined difference spectrum (Figs. 1 and 2) that was stimulated by ascorbate was observed in isolated lettuce chloroplasts with normal pigment content. The absorbance change required light at pH 7 but not at pH 5. The difference spectra for the pH 7 and pH 5 changes were similar, having the characteristics of a carotenoid shift. A direct relationship was found between the effects of various reagents (Fig. 3) and pH (Fig. 2) on the absorbance change and the effects of similar conditions on de-epoxidation as assayed by pigment analysis (Table I). Furthermore, the chloroplast difference spectrum resembled the difference spectrum of purified zeaxanthin *minus* violaxanthin in organic solvents (Fig. 5). These results support the conclusion that the ascorbate-dependent absorbance change is from violaxanthin de-epoxidation.

The extent of the absorbance change was large; at 505 nm it was as much as 3.5% of the initial absorbance. De-epoxidation also had other characteristic absorbance changes at 483 and 468 nm, but the change at 505 nm appeared to be a better indicator of its extent than the changes at shorter wavelength which, under some conditions, had superimposed chlorophyll changes.

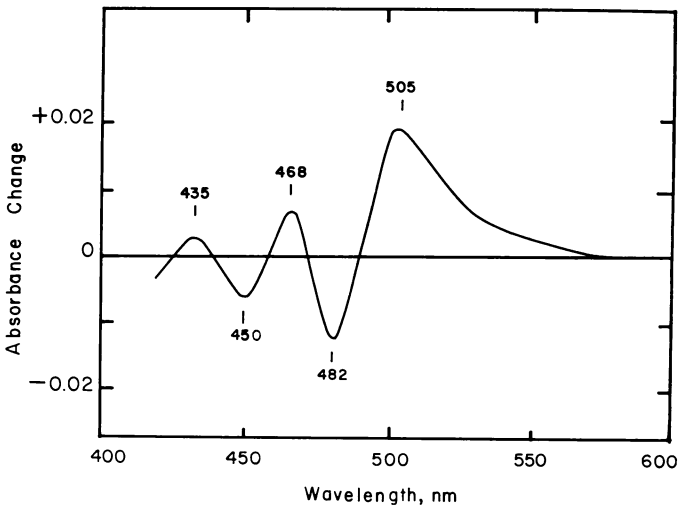

FIG. 2. The dark difference spectrum induced by ascorbate at pH 5. Chloroplasts isolated in SNH were suspended (18 μg chlorophyll per ml) in 50 mM sodium citrate buffer at pH 5. The spectrum was determined following addition of ascorbate (30 mM) and 5-min dark incubation. The reference was a sample without added ascorbate.

[2] Abbreviations: DPIP: 2,6-dichlorophenolindophenol; PMS: phenazine methosulfate.

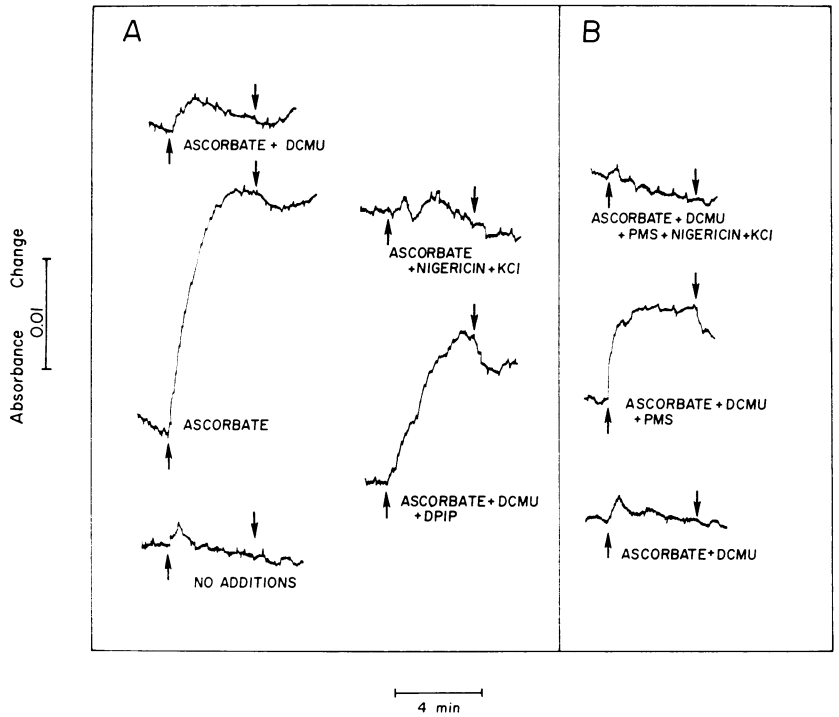

FIG. 3. Kinetics of absorbance change at 505 nm. The reference and sample wavelengths were at 580 and 505 nm respectively. When present, the concentrations were 30 mM sodium ascorbate, 10 μM DCMU, 50 μM DPIP, 50 μM PMS, and 182 nM nigericin with 50 mM KCl. Chloroplasts in A and B were equivalent to 23 and 13 μg chlorophyll per ml respectively. Actinic light intensity was 2×10^5 and 5×10^4 ergs cm^{-2} sec^{-1} for A and B respectively. The arrows indicate when the light was turned on ($\uparrow$) and off ($\downarrow$).

Table I. *Properties of Violaxanthin De-epoxidation as Determined by Analysis of Pigment Composition*

In experiments 1 through 4, chloroplasts in 10 ml of SNH solution (30 μg chlorophyll per ml) were illuminated with red light at 2×10^5 ergs cm^{-2} sec^{-1} for 15 min in air and analyzed for changes in pigment composition. In experiment 5, chloroplasts were suspended in 50 mM pH 5 citrate buffer and analyzed after 15-min incubation in the dark. The concentrations of various additions were as indicated in Figure 2.

Additions	De-epoxidation
	mmole violaxanthin per mole chl
Experiment 1	
Ascorbate	17
Ascorbate + DCMU	0
Experiment 2	
Ascorbate + DCMU	3
Ascorbate + DCMU + DPIP	11
Experiment 3	
Ascorbate	18
Ascorbate + DCMU	0
Ascorbate + nigericin + KCl	1
Experiment 4	
Ascorbate + DCMU	0
Ascorbate + DCMU + PMS	16
Experiment 5 (Dark)	
pH 5	0
pH 5 + ascorbate	22

The fact that the absorbance change was light-dependent at pH 7 (Fig. 1) but not at pH 5 (Fig. 2) suggests that the light-induced change stimulated by ascorbate reflects acidification of chloroplasts that is linked with photosynthetic electron transport. Accordingly, nigericin with KCl inhibited the light-

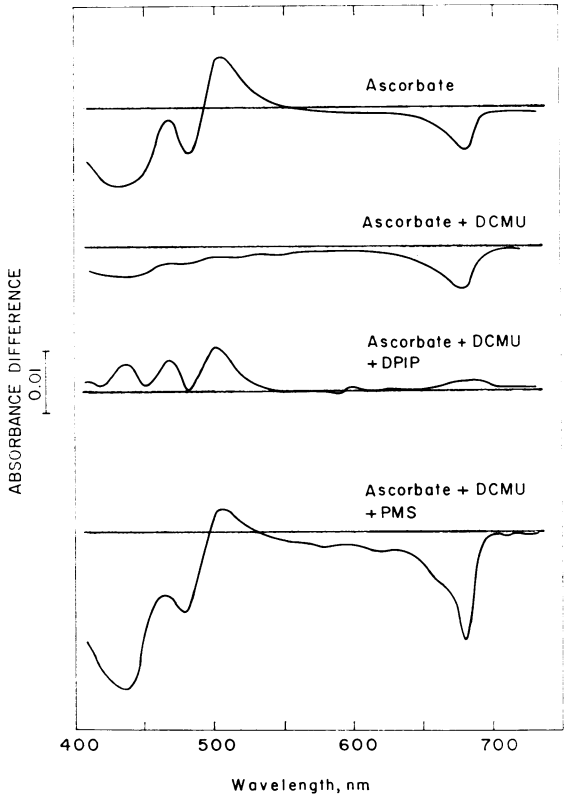

FIG. 4. Difference spectra of chloroplasts from 750 to 400 nm following continuous illumination. Chloroplasts (12 μg chlorophyll per ml) were illuminated at 5×10^4 ergs cm^{-2} sec^{-1} for 3 min, then scanned at 60 nm per min immediately after the light was turned off. The concentrations of various additions were as indicated in Figure 3.

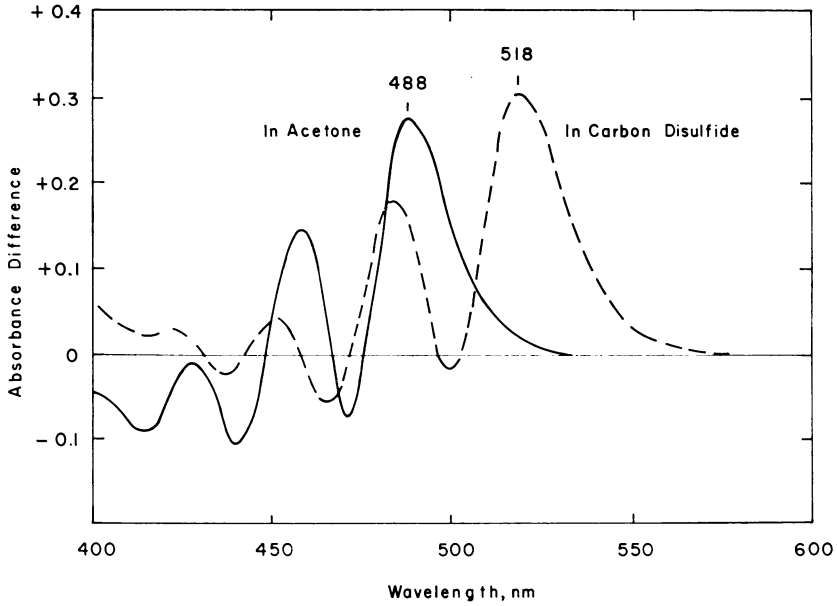

FIG. 5. Difference spectra of zeaxanthin *minus* violaxanthin in acetone (——) and in carbon disulfide (– – –). In acetone, the absorbances of violaxanthin and zeaxanthin were 0.58 at 443 nm and 0.56 at 452 nm, respectively. In carbon disulfide the absorbances were 0.61 for both violaxanthin at 468 nm and zeaxanthin at 477 nm. The purified pigments were isolated from lettuce by procedures described previously (17).

induced change. The acidification which drives de-epoxidation appears to be derived from hydrogen-ion transport associated with photosystem 1, since DCMU did not inhibit the light-induced change when DPIP or PMS were also present (Fig. 3). However, the possible involvement of other hydrogen-ion transport sites in de-epoxidation cannot be excluded at this time. Hager (5) also concluded that violaxanthin de-epoxidation was related to chloroplast acidification based on the effects of pH and uncouplers on pigment composition. It is interesting that, although photosystem 1 appears to mediate violaxanthin de-epoxidation, structurally violaxanthin may be more closely associated with photosystem 2. In studies on the fractionation of chloroplast with detergents such as Triton X-100, violaxanthin concentrates with fragments enriched in photosystem 2 (14).

Ascorbate was required for both the light-induced change at pH 7 and the dark change at pH 5. This requirement suggests that de-epoxidation could occur through reduction of epoxide to hydroxyl followed by elimination of water. In the case of the light-induced change, ascorbate could have an additional effect. Preliminary studies indicate that ascorbate concentrations which stimulated de-epoxidation also stimulated NADP reduction. This increased electron transport could result in concomitant hydrogen-ion transport and consequently stimulation of de-epoxidation.

Carotenoid shifts have been observed in some brown and red algae (3, 7), and in a chlorophyll *b* deficient barley mutant (2). The well known 515 nm change in green algae and higher plants has been assigned to carotenoids (4, 6) as well as to chlorophyll *b* (10). The specific carotenoids involved and the nature of the changes themselves have not been determined. The carotenoid shift reported herein appears to be a new change which, in the presence of relatively high ascorbate concentrations, manifests itself as a large, slow and irreversible change. It appears to be an endogenous probe for acidity in chloroplasts and hence an indirect indicator of hydrogen-ion transport.

The relationship, if any, between carotenoid shifts from de-epoxidation and other carotenoid shifts observed in various photosynthetic cells is uncertain (2, 3, 6, 7). Proof of relationships is difficult to obtain since the extent of carotenoid shifts are usually so small that even if a shift is from a chemical change, it may escape detection by present chemical methods. In the present case, assignment of the carotenoid shift to violaxanthin de-epoxidation was possible in part because the change induced with ascorbate was not only large but also irreversible. The reason for this irreversibility is unknown. Various procedures such as removing ascorbate and adding chloroplast extract have been tried but without success. Lack of reversibility is in contrast with the situation in leaves where de-epoxidation induced under intense light and anaerobiosis was reversed under low level illumination in air (15). Reversibility is also implicit in the light-induced steady-state turnover of violaxanthin in leaves which has been demonstrated with $^{18}O_2$ (12). Under reversible conditions the characteristics of absorbance changes from violaxanthin could be appreciably different from those reported herein. Nevertheless, the present results indicate that absorbance changes from de-epoxidation do occur and raise the possibility that such changes could be a factor in reversible carotenoid shifts observed in photosynthetic organisms that contain epoxy carotenoids.

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