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Light-Induced Spectral Absorbance Changes in Relation to Photosynthesis and the Epoxidation State of Xanthophyll Cycle Components in Cotton Leaves[1]

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ABSTRACT

When cotton (*Gossypium hirsutum* L., cv Acala SJC-1) leaves kept in weak light were suddenly exposed to strong red actinic light a spectral absorbance change took place having the following prominent characteristics. (a) It was irreversible within the first four minute period after darkening. (b) The difference in leaf absorbance between illuminated and predarkened leaves had a major peak at 505 nanometers, a minor peak at 465 nanometers, a shoulder around 515 nanometers, and minor troughs at 455 and 480 nanometers. (c) On the basis of its spectral and kinetic characteristics this absorbance change can be readily distinguished from the much faster electrochromic shift which has a peak at 515 nanometers, from the slow, so-called light-scattering change which has a broad peak centered around 535 nanometers and is reversed upon darkening, and from absorbance changes associated with light-induced chloroplast rearrangements. (d) The extent and time course of this absorbance change closely matched that of the deepoxidation of violaxanthin to zeaxanthin in the same leaves. (e) Both the absorbance change and the ability to form zeaxanthin were completely blocked in leaves to which dithiothreitol (DTT) had been provided through the cut petiole. DTT treatment also caused strong inhibition of that component of the 535-nanometer absorbance change which is reversed in less than 4 minutes upon darkening and considered to be caused by increased light scattering. Moreover, DTT inhibited a large part of nonphotochemical quenching of chlorophyll fluorescence in the presence of excessive light. However, DTT had no detectable effect on the photon yield of photosynthesis measured under strictly rate-limiting photon flux densities or on the light-saturated photosynthetic capacity, at least in the short term. We conclude that it is possible to monitor light-induced violaxanthin de-epoxidation in green intact leaves by measurement of the absorbance change at 505 nanometers. Determination of absorbance changes in conjunction with measurements of photosynthesis in the presence and absence of DTT provide a system well suited for future studies of mechanisms of dissipation of excessive excitation energy in intact leaves.

Under most natural conditions leaves are normally exposed to irradiance levels during the course of the day which exceed the capacity by which the resulting excitation energy can be used in photosynthesis. This excessive energy must be safely dissipated; otherwise the photosynthetic system will suffer photoinhibitory damage (17). Analysis of Chl fluorescence has provided strong evidence that leaves adapted to high irradiances have a greater capacity to dissipate excessive energy via a process of NRD[2] than leaves adapted to low irradiances (1). It has been proposed that such NRD, which is seen as q_{NP}, could protect against photoinhibitory damage (1, 4, 13). It has also been proposed that the molecular basis of 'safe' nonradiative dissipation in part involves the carotenoid zeaxanthin, hypothetically acting as a quencher of excited states of Chl in the antenna (4, 5). Good correlations between NRD (expressed as an increase in q_{NP} or as an increase in K_D, the rate constant for NRD) and zeaxanthin content of leaves were obtained under a wide range of conditions (5).

Zeaxanthin is formed by deepoxidation of violaxanthin (5,6,5′,6′-diepoxizeaxanthin) via the intermediate antheraxanthin (5,6-monoepoxizeaxanthin) (for reviews, see refs. 6, 25). Both deepoxidation steps are catalyzed by the same enzyme, violaxanthin deepoxidase (25). This enzyme is thought to be located on the lumen side of the thylakoid membrane and its activity has a sharp optimum around pH 5. Excessive light promotes the deepoxidation of violaxanthin to zeaxanthin, evidently because the resulting buildup of a pH gradient across the thylakoid membrane causes acidification of the lumen, thereby activating the deepoxidase (6, 25). The back reaction (epoxidation of zeaxanthin to violaxanthin) is catalyzed by an epoxidase thought to be located on the other side of the membrane. Its activity is promoted under the pH and redox conditions that exist under limiting light (25). Violaxanthin bound to thylakoid membranes appears to be mainly located in LHCI and LHCII (20). However, with the methods hereto used for isolating the pigment protein complexes, a considerable fraction of the violaxanthin appears in the free pigment band. The distribution of the xanthophyll cycle components in the thylakoid membrane is therefore uncertain; moreover, these components may also have a high degree of mobility.

In view of its proposed role in photoprotection, more information about the epoxidation state of the xanthophyll-

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[2] Abbreviations: NRD, nonradiative energy dissipation; A, antheraxanthin; DTT, dithiothreitol; ΔA_λ, absorbance change at wavelength λ; LHC, light harvesting complex; q_p, q_{NP}, photochemical and nonphotochemical Chl fluorescence quenching, respectively; $P[CO_2]$, photosynthetic CO_2 uptake; P[ET], photosynthetic electron transport; $P[O_2]$, photosynthetic O_2 evolution; PFD, photon flux density (400–700 nm); V, violaxanthin; z, zeaxanthin.

cycle *in vivo* under conditions of excessive light is needed. The techniques used so far to quantify the xanthophyll components of this cycle in green leaves, TLC and HPLC, have certain drawbacks. TLC is complicated and very time consuming. Although HPLC has many advantages over TLC, the usefulness of HPLC has been limited primarily because of the difficulties involved in separating zeaxanthin from lutein which is a major component of leaf xanthophylls. Recent improvements (22) have fully overcome this problem but the fact that these methods are destructive still limits their use in kinetic studies of the operation of the xanthophyll cycle in intact leaves.

Yamamoto *et al.* (26) and Sokolove and Marsho (21) described an absorbance change in chloroplast suspensions which was associated with the conversion of violaxanthin to zeaxanthin. This absorbance change had a peak at 505 nm. Recently, Pfündel and Strasser (16), working with photosynthetically incompetent etiolated bean leaves, were able to observe an increase in absorbance at 505 nm over several hours in darkness that took place after vacuum infiltration with ascorbate at pH 5. This slow increase in absorbance was accompanied by a large increase in zeaxanthin and decrease in violaxanthin. Measurements of absorbance changes at 505 nm in intact, photosynthesizing green leaves are complicated by their high optical density at this wavelength and by the presence of light-induced absorbance changes with considerable spectral overlaps. These are the electrochromic shift, peaking at 515 or 518 nm (24), the so-called, light-scattering change, centered around a broad optimum around 530 nm (7, 12), and changes caused by chloroplast rearrangements within the leaf (9).

Since light-induced absorbance changes associated with zeaxanthin formation in green leaves have not been previously reported, we investigated the potential of measurements of such changes for studies of the kinetics of the operation of the xanthophyll cycle. Using normal green cotton leaves having a high photosynthetic capacity, we were able to show that the deepoxidation that occurred when the leaves were illuminated with strong red light was clearly associated with a large and specific light-induced absorbance change with a peak at 505 nm. This absorbance change was blocked by a DTT application that had no effect on short-term photosynthetic performance.

MATERIALS AND METHODS

Plant Material

Cotton plants (*Gossypium hirsutum* L. cv Acala SJC-1) were grown in a controlled-growth room in flowing-nutrient culture as described in detail by Schäfer and Björkman (18). The incident PFD at leaf level was 700 to 800 μmol m^{-2} s^{-1}. Fully expanded leaves were used, usually from the fourth to sixth node, counting from the top of the plant. About 30 min before excision, the leaves were shaded by a panel of reflective aluminum foil, giving a PFD of about 100 μmol m^{-2} s^{-1} at the leaf surface. Some experiments were made on leaves from cotton plants grown in a field just outside the laboratory. These experiments were made during July at which the daily maximum PFD was approximately 2000 μmol m^{-2} s^{-1}. The whole plant had been kept shaded since the night before each experiment. The PFD at time of leaf excision was approximately 100 μmol m^{-2} s^{-1}. At this PFD the deepoxidation state of the xanthophyll cycle in cotton was found to be the same as in leaves which had been kept in darkness for several hours.

DTT Treatments

In all experiments the petiole was cut either under water (controls) or under a 1 mM solution of reduced or oxidized DTT with a piece of a sharp razor blade. When DTT or oxidized DTT was applied, the petiole was promptly transferred to a small cylinder containing the same solution. For the first 15 or 30 min, the leaves were kept at 100 to 200 μmol m^{-2} s^{-1} PFD to keep the stomata open so that uptake of the DTT solution via the transpiration stream was facilitated. The PFD was then decreased to approximately 15 μmol m^{-2} s^{-1} and maintained at this low PFD until the start of measurements. The volume of solution taken up by each leaf was recorded hourly. Depending on transpiration rate the time required for the leaves to take up an amount of solution equal to their fresh weight varied from 2 to 4 h. On the assumption that DTT freely entered the leaf with the transpiration stream, this would yield a concentration in the bulk leaf solution of at least 1 mM.

Induction of Violaxanthin Deepoxidation in Weak Light in the Presence of Ascorbate

For the experiment shown in Table II, leaf discs were infiltrated under vacuum with a Na-ascorbate buffer (150 mM), adjusted to pH 5.0 with citric acid (final concentration about 10 mM) with or without 1 mM DTT. After infiltration, the leaf discs were kept floating for 5 h on the same solution. To avoid anaerobiosis, the leaf discs were illuminated by weak light (approximately 10 μmol m^{-2} s^{-1}).

Chemicals

Na-ascorbate, citric acid, DTT, and oxidized DTT (trans-4,5-dihydroxy-1,2-dithiane) were purchased from Sigma, St. Louis, MO. Freshly prepared solutions of DTT or oxidized DTT were used for each experiment. The same batches of DTT and oxidized DTT were kindly tested by H. Yamamoto and A. Gilmore of the University of Hawaii for the potency of these compounds as inhibitors of activity of purified violaxanthin deepoxidase *in vitro*. They confirmed that this batch of reduced DTT was a highly effective inhibitor, whereas the batch of oxidized DTT had no effect on the activity of the isolated enzyme.

Measurements of Light-Induced Absorbance Changes in Leaves

Absorbance measurements were carried out in a Perkin Elmer model 356 Dual-Wavelength, Dual-Beam spectrophotometer. Leaf discs (20 or 28 mm in diameter) were placed in the primary sample compartment in front of the photomultiplier tube and fixed by means of a small holder. The distance between the leaf and the photomultiplier cathode was about 15 mm. All measurements of light-induced absorbance changes reported in this paper were made in the split beam

mode. The reference beam, which bypassed the leaf, was attenuated by neutral density filters to give approximately the same signal as the beam transmitted through the leaf. Actinic illumination was provided by a cold light fiber optic light source (model KL 1500, Schott, Mainz, FRG). The fiber optic entered the sample compartment from the rear side. The actinic light beam was filtered through a red-transmitting filter (Corning 2–60) and the photomultiplier tube was protected by insertion of a blue-green transmitting filter (Corning 4–96) immediately in front of it. At an actinic PFD of 1300 μmol m^{-2} s^{-1} this arrangement resulted in a small transient artifact upon light on or light off of an amplitude equal to 0.0003 absorbance units, as determined with a Whatman No. 1 filter paper in place of the leaf sample. To minimize water loss during the measurements, a small housing, lined with reflective foil on the outside and with moistened fabric on the inside, was placed around the leaf holder. Some measurements of light-induced absorbance changes were also made at 505, 515, and 540 nm with a custom-built instrument that permitted use of intact leaves attached to the plant. The results obtained in these measurements were very similar to those obtained with leaf discs using the system described above.

Photosynthesis Measurements

Photon yields of O_2 evolution were determined at 4 to 5% CO_2, 20 to 21% O_2, balance N_2, under strictly rate limiting photon flux densities on leaves that had not been preexposed to excessive light. These measurements were either made on 10 cm^2 leaf discs in a closed system using a Hansatech (King's Lynn, Norfolk, England) 12 leaf disc O_2 electrode as described in Björkman and Demmig (2) or using an open-flow system (18) on a 10 cm^2 area of a leaf whose cut petiole was kept in water or in a DTT solution. In the latter system, a humidified stream of 5 CO_2, 19.9% 0_2, balance N_2, was passed through the leaf chamber at a flow rate of 4 mL min^{-1} (using a mass flow controller model FC260, Tylan, Carson, CA) and the change in O_2 concentration caused by the leaf was continuously measured in the exit gas stream with a model DW1/CB1-D O_2 electrode system (Hansatech), modified for gas-phase measurements. No difference was found between the photon yield values obtained with the open and the closed systems.

Light-dependence curves and light-saturated rates of photosynthetic O_2 evolution were determined with the open-flow system only, which is much better suited to measurements of high photosynthetic rates than the closed leaf disc system. The open flow, whole leaf, system also avoids problems caused by heating of the O_2 electrode at high irradiance levels since the electrode chamber temperature is independent of that of the leaf chamber. Moreover, since the leaf is permitted to take up water, any effects caused by transpirational water loss from the leaf are minimized. Measurements of light-saturated rates of O_2 evolution were made at a gas flow rate of 14 mL min^{-1}, while in the determinations of light dependence curves the flow rate at each photon flux density was adjusted (4–14 mL min^{-1}) to give a suitable change in O_2 concentration. Rates of O_2 exchange are given as the sum of net O_2 evolution and dark respiration. The light dependence curves were measured as the response to increased PFDs. The rate at each PFD was taken after an apparent steady state had been reached which usually was obtained within 10 to 15 min after each increase in PFD. In the experiments shown in Figure 7, the leaf temperature rose from 27 to 33°C as the PFD was increased from 800 to 2000 μmol m^{-2} s^{-1}. Since the rate of light- and CO_2-saturated photosynthesis increases with increasing temperature in this range, the rates were adjusted for the temperature dependence determined at saturating PFD in separate experiments. Other experiments with comparable cotton leaves in the absence and presence of DTT where the light dependence of photosynthesis in the range 800 to 2000 μmol m^{-2} s^{-1} was measured at a constant leaf temperature confirmed that these adjustments for a rising leaf temperature were correct.

Rates of CO_2 uptake in the presence and absence of DTT under normal air concentrations of CO_2 were made at a leaf temperature of 30°C as previously described (18). The photon yield of CO_2 uptake was first determined at strictly limiting PFDs (35 and 90 μmol m^{-2} s^{-1}). The PFD was then raised to 1000 μmol m^{-2} s^{-1} and after 15 min it was raised to 2000 μmol m^{-2} s^{-1}. The rate determined when an apparent steady state had been reached (after approximately 15 min at this PFD) was taken as the light saturated rate. The sum of the rates of carboxylation and oxygenation (P[ET]) was calculated as described (18).

Xanthophyll Determinations

Contents of the components of the xanthophyll cycle were measured on leaf samples which had been frozen in liquid nitrogen. Extraction was in 80% acetone and separation by HPLC as described in detail (22).

Chl Fluorescence Measurements

Chl fluorescence was measured as previously described (18), using a system which incorporates a PAM Chl fluorometer system (Walz, Effeltrich, FRG). The petioles were immersed in water or a 1 mM DTT solution during these measurements. In experiments where we wanted to prevent gas exchange, both leaf surfaces were covered with a thin layer of silicon grease (Dow Corning high vacuum grease) just before the start of the fluorescence measurements. Nonphotochemical fluorescence quenching (q_{NP}) and photochemical quenching (q_P) were calculated as outlined in (19). However, the maximum fluorescence yield for each leaf used in the calculations of q_{NP} was not determined in leaves which had been kept in complete darkness but was instead determined for leaves kept at a low PFD (*cf.* 18). The saturating pulses used to cause temporary closure of all PSII traps had a PFD of 5000 μmol m^{-2} s^{-1} and a duration of 1 s.

RESULTS

Light-Induced Absorbance Changes and Violaxanthin Deepoxidation

Yamamoto *et al.* (26) described an absorbance change at 505 nm which was associated with zeaxanthin formation in isolated chloroplast preparations. Since strong light induces deepoxidation of violaxanthin, we measured light-induced absorbance changes in cotton (*Gossypium hirsutum* L. cv Acala SJC-1) leaves which were depleted of zeaxanthin by

preincubation in weak light for several hours. In Figure 1 are shown results of absorbance change measurements at 505 nm, compared with those at 535 nm, where the so-called light scattering change usually is measured. At 505 nm, absorbance rose monotonically upon illumination and showed no decline during the following dark period. After the light was turned off, a small further increase in absorbance took place. A second illumination produced little further rise, corroborating the short-term irreversibility of the first light-induced absorbance change. Like the absorbance at 505 nm, that at 535 nm rose during the first 2 min of the first exposure, but exhibited a longer lag time and the absorbance leveled off earlier. In contrast to the change at 505 nm, the change at 535 nm displayed a pronounced decay upon darkening, although the absorbance did not return to its initial level. The second illumination induced a larger change with faster kinetics than that observed during the first illumination and it was now fully reversed upon darkening.

Previous studies on light-induced absorbance changes around 535 nm have indicated that the maximum extent of this change is strongly dependent on the balance between the activity of ATP-consuming dark reactions and the intensity of the actinic light (7, 11). It also seems likely that factors such as stomatal opening, leaf temperature, and the activation state of Calvin cycle enzymes can all affect the amplitude of this absorbance change and, therefore, cause a large variation in the response of different leaf samples. However, a stable state of the photosynthetic apparatus which resulted in good reproducibility was obtained when the leaves were kept at low PFD (15 μmol m^{-2} s^{-1}) for 3 to 4 h prior to the absorbance change measurements.

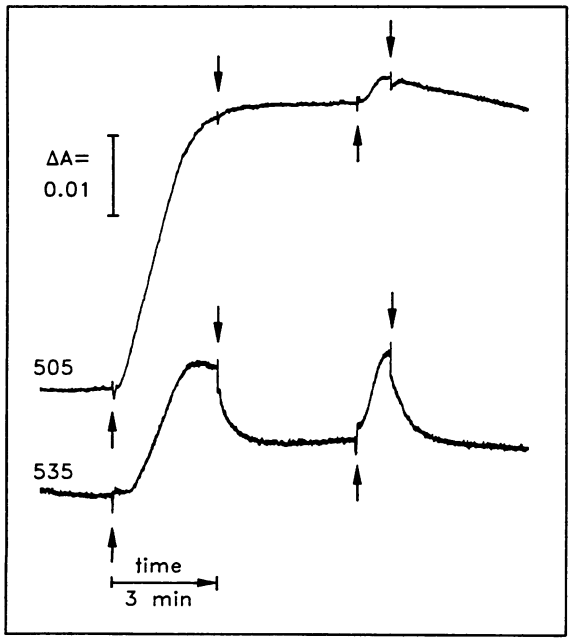

Figure 1. Original traces of light-induced absorbance changes at 505 (upper trace) and 535 nm (lower trace) in cotton leaf discs. Two different discs of the same leaf were used, after a preadaptation period at low PFD (15 μmol m^{-2} s^{-1}) of 5 h. The arrows denote the times at which red actinic light (500 μmol photons m^{-2} s^{-1}) was switched on (↑) or off (↓). The leaf discs were in air at a temperature of 26°C during measurement.

As shown in the traces in Figure 1 at both 505 and 535 nm, a small, rapid component is evident upon light on and light off. This component is presumably caused by spectral overlap from the electrochromic shift which is known to have a peak around 518 nm. The rise kinetics upon light on are generally similar at 505 and 535 nm but a pronounced difference is evident upon light off. The differences in the kinetics between the absorbance changes at these two wavelengths indicated that a good point to determine the extent of change in absorbance that is specific to 505 nm would be after at least 3 min following the actinic illumination period.

Figure 2 shows the kinetics of the absorbance change at 505 nm (ΔA_{505}) in comparison with those of the measured zeaxanthin or zeaxanthin plus antheraxanthin content in different discs of the same leaf. Violaxanthin deepoxidation followed a time course similar to that of ΔA_{505} not only in the light but also in the dark. On these grounds it seemed justified tentatively to distinguish absorbance changes, caused by violaxanthin deepoxidation from those caused by light scattering changes associated with membrane energization, on the basis of reversibility.

From measurements similar to those shown in Figure 1, the extent of the reversible and the extent of the irreversible part of ΔA, determined as indicated in the inset in Figure 3, were obtained at different wavelengths. The resulting difference spectra are shown in Figure 3. The irreversible part (curve B) shows a pronounced peak at 505 nm with an apparent shoulder at about 515 nm. Sometimes the absorbance change at 515 nm was even higher than that at 505 nm.

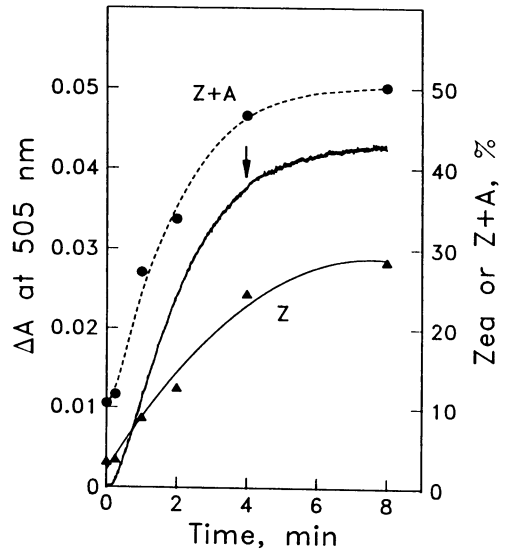

Figure 2. Comparison of the time courses of the absorbance change at 505 nm and xanthophyll pigment content in leaf discs upon illumination. Different discs of the same leaf were illuminated (PFD = 500 μmol m^{-2} s^{-1}) in the spectrophotometer for the indicated times and then frozen in liquid nitrogen within 2 s. Subsequently, pigment contents were determined by HPLC. The arrow denotes the time when actinic light was turned off. Pigment contents are given in percent of the total xanthophyll cycle pool size (V + A + Z). The discs had the same size (1.0 cm in diameter) as the actinic light beam. ΔA_{505} was determined under identical conditions in samples of the same leaf. A representative curve from three parallel measurements is shown.

The spectrum also has a small peak at 465 nm and two minor troughs at 480 and 455 nm. For comparison, the spectrum of the reversible part is also shown (curve A). This spectrum displayed a single peak around 535 nm and very small changes were evident below 500 nm. Although data are not shown here, the spectrum of the change obtained in the second illumination (see trace for 535 nm in Fig. 1) measured at maximal amplitude was almost identical to that shown for the reversible component in Figure 3. While the irreversible component contributed markedly to the total absorbance change at 535 nm, the reversible component contributed little to the change at 505 nm. That the contribution of the reversible change to the total change at 505 nm was small is also indicated by the finding that little additional change occurred upon a second illumination (Fig. 1, top trace). Part of the small change in ΔA_{505} in response to the second illumination may be caused by the electrochromic shift (see below).

The relationship between ΔA_{505} and zeaxanthin content is shown in Figure 4A and that between and zeaxanthin plus antheraxanthin is shown in Figure 4B. In both cases an approximately linear relationship was obtained. Different leaves showed somewhat different slopes, presumably because of slightly different optical characteristics.

Effects of DTT on Light-Induced Absorbance Changes

Application of DTT via the cut petiole invariably prevented light-induced zeaxanthin formation in cotton leaves (see below). When such DTT treated leaves were illuminated, the increase in absorbance at 505 nm was completely inhibited (Fig. 5, trace b). Only a small absorbance change with rapid induction and relaxation kinetics remained. DTT treatment also inhibited the irreversible component of the light-induced absorbance change at 535 nm (Fig. 5, trace d) and at other wavelengths as well (data not shown). Although a small and slow rise at 535 nm still occurred upon illumination, the slowly reversible transient, usually taken as indicative of light scattering changes due to membrane energization (11) had

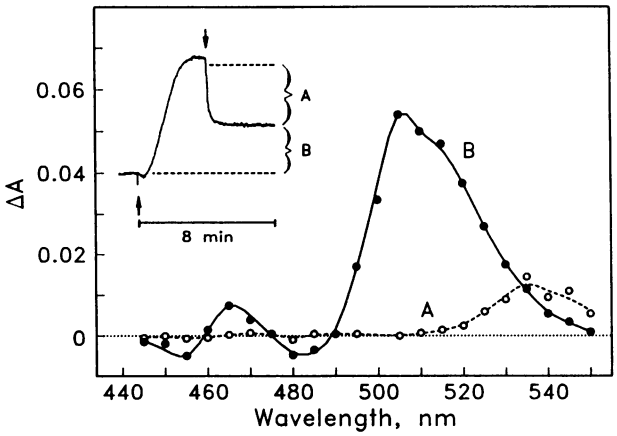

Figure 3. Difference spectra for light-induced absorbance changes in cotton leaf discs, determined after 4 min of a subsequent dark period. The inset, with a trace measured at 535 nm, shows how the reversible (A) and the irreversible (B) component were obtained. The ΔA scale also applies to the inset. For the spectrum, a new disc was used at each wavelength. Actinic PFD was 500 μmol m^{-2} s^{-1}. For other conditions, see Figure 1.

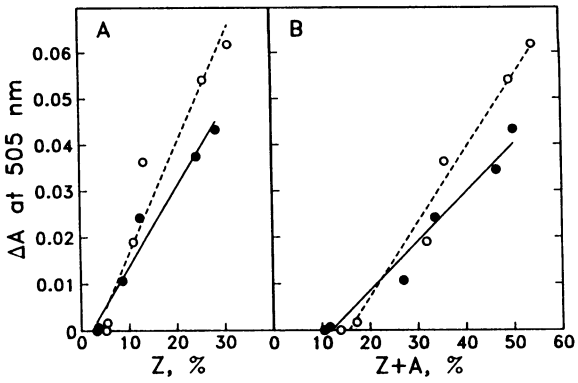

Figure 4. Relationship between and amount of zeaxanthin (A) and the sum of zeaxanthin and antheraxanthin (B) formed during illumination at 500 μmol photons m^{-2} s^{-1} and during the subsequent dark period. In addition to the data shown already in a different way in Figure 2 (closed symbols), data from another experiment with a different leaf are shown (open symbols). Pigment contents are given in percent of the total xanthophyll cycle pool size (V + A + Z). The measuring conditions were the same as in Figure 2.

almost completely disappeared (Fig. 5, trace d). The rapid reversal had approximately the same amplitude as the slow rise. This suggests that the slow rise was caused by a slow buildup of an electric membrane potential. A similar slow rise can be seen at 505 nm (Fig. 5, trace b), although the amplitude is smaller [*cf.* (24)]. However, the kinetics at 505 nm are more complex and obviously composed of the electrochromic changes and an additional fourth component. The latter displays a decline in the light during the first minute and may reflect the same process as the relatively rapid rise after light off, which accounts for most of the kinetics after light off in the control as well.

Effect of DTT on Photosynthesis

In spite of the inhibitory effect of DTT treatment on zeaxanthin formation and on the associated ΔA_{505} as well as on ΔA_{535} (Fig. 6, lower panels), such treatments had no effect on the rate of CO_2-saturated O_2 evolution determined on the same leaves (Fig. 6, upper panel). Moreover, the response of CO_2-saturated O_2 evolution to increased PFD in the presence of DTT was indistinguishable from that in the absence of this compound (Fig. 7). The DTT treated leaf used in this experiment had taken up a relatively high amount of DTT and ΔA_{505} was completely eliminated (data not shown). Other measurements confirmed that the photon yield of O_2 evolution (ϕ_a), determined at limiting PFDs was unaffected by DTT. ϕ_a values of 0.104 ± 0.004 O_2 photon^{-1} (n = 3) were obtained for DTT treated leaves compared with 0.105 ± 0.004 O_2 photon^{-1} for untreated leaves [*cf.* (18)]. No zeaxanthin was found in control leaves kept in the range where the rate of photosynthesis was linear with PFD.

As shown in Table I, measurements of photosynthetic CO_2 uptake in air of normal atmospheric CO_2 and O_2 concentration also did not show any detectable effect on photosynthesis of DTT treatments that fully eliminated ΔA_{505} and strongly suppressed light-induced absorbance changes at 535 nm. Neither the photon yield at limiting PFDs nor the light-saturated rate of CO_2 uptake was affected by DTT. The electron flow

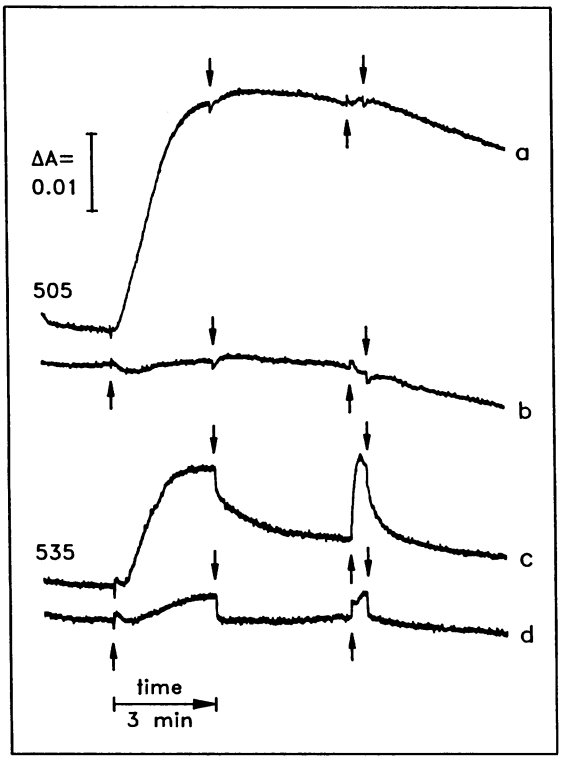

Figure 5. Original traces of absorbance changes at 505 and 535 nm in absence (curves a and c) or presence of DTT (curves b and d) in cotton leaf discs. The samples for the curves with DTT were taken from the same leaf, which had taken up a 1 mM DTT solution. The average concentration in the leaf was 1.27 (535 nm) and 1.47 mM DTT (505 nm), respectively. The actinic PFD was 1300 μmol m^{-2} s^{-1}. The arrows indicate when actinic light was switched on (↑) or off (↓). For other conditions, see Figure 1.

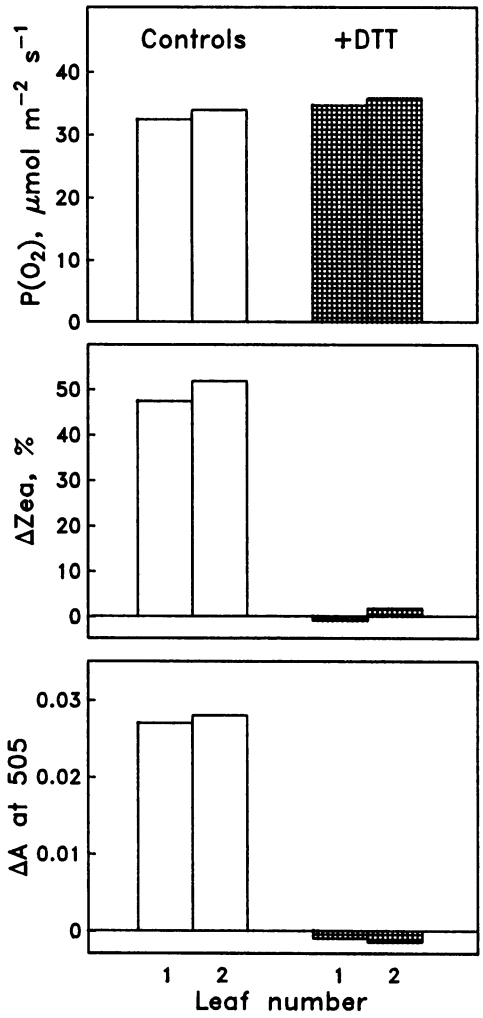

Figure 6. Effect of DTT on photosynthetic O_2 evolution, zeaxanthin formation, and ΔA_{505}. Data are shown for two different leaves. Before cutting the leaves under DTT solution, control discs (10 cm^2) were punched from the leaves and kept in a humid chamber under conditions otherwise identical to those for the remainder of the leaves until measurement. The final average DTT concentration was 1.40 and 1.33 mM in leaf 1 and 2, respectively. Upper panel: steady state rate of O_2 evolution in 5% CO_2 in air at a PFD of 1220 μmol m^{-2} s^{-1}. Leaf temperature was 28°C. Center panel: zeaxanthin concentration relative to the total pool size of xanthophyll cycle intermediates was determined in the samples used for O_2 evolution measurement. The samples were frozen in liquid nitrogen 6 min after termination of illumination. The data are given as the difference between the zeaxanthin content of an illuminated and a nonilluminated sample (7.8 and 5.1%, respectively). Lower panel: irreversible component of the absorbance change at 505 nm induced by a PFD of 500 μmol m^{-2} s^{-1}. $_{\Delta}A$ was determined in the samples before the measurements of O_2 evolution were made.

P[ET], calculated as the sum of carboxylation and oxygenation as described (18), also did not show any effect of DTT. It is noteworthy that the photon yield of electron transport calculated in this way (Table I) was identical to the measured value for the photon yield of CO_2-saturated O_2 evolution and that the light saturated rate of electron transport (Table I) was very similar to the measured CO_2- and light-saturated rate of O_2 evolution (Fig. 7), both in treated and untreated leaves.

It should be emphasized that all photon yield measurements reported here were made at strictly rate-limiting PFDs on leaves which had not been preexposed to high PFDs and that all measurements of light-saturated rates were taken after a relatively short exposure to saturating light (see "Materials and Methods"). Therefore, these results do not permit any conclusions regarding possible effects of DTT on photosynthetic performance in the longer term.

Effect of DTT on Fluorescence Quenching

Since it has been proposed (5) that zeaxanthin formation is a major cause of q_{NP} that takes place when leaves are exposed to excessive light, we investigated the effect of DTT on fluorescence quenching. In one kind of experiment we induced zeaxanthin formation in dim light by infiltrating the cotton leaves with ascorbate at pH 5 in the absence of DTT. These conditions are known to promote the deepoxidation of violaxanthin to zeaxanthin in isolated chloroplasts (6). The low pH activates the deepoxidase and the ascorbate provides the necessary reductant for the deepoxidation reaction even in the absence of a ΔpH and photosynthetically generated reductants. As shown in Table II the cotton leaves accumulated large amounts of zeaxanthin during an incubation of 5 h in the absence of DTT but very little zeaxanthin was formed when DTT (1 mM) was present in the infiltration solution.

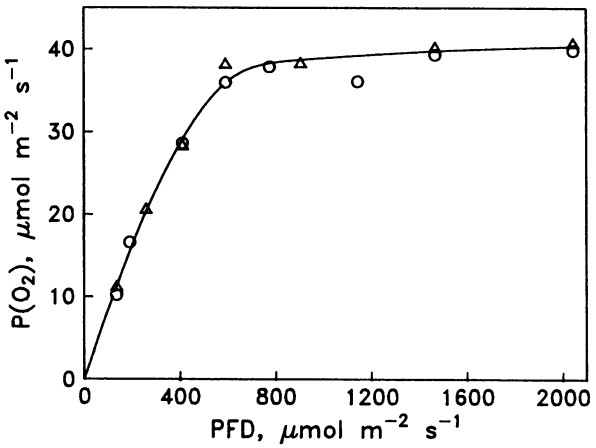

Figure 7. Light dependence of O_2 evolution in two cotton leaves at 27°C in absence (circles) or presence (triangles) of DTT (1.8 mM average concentration in the leaf).

Table I. *Photon Yield of CO_2 Uptake [$\phi(CO_2)$] at Limiting PFDs, Light-Saturated Rate of CO_2 Uptake [$P(CO_2)$] in Normal Air (0.035% CO_2, 20.9% O_2) and Corresponding Calculated Values for the Photon Yield [$\phi(ET)$] and Light-Saturated Rate [$P(ET)$] of Electron Flow via Carboxylation + Oxygenation of Ribulose-1,5-bisphosphate in Cotton Leaves in the Presence and Absence of DTT*

Electron flows are in equivalent values of μmol O_2 m^{-2} s^{-1}, and photon yields in mol CO_2 or O_2 per absorbed mol photon.

	no DTT	+ DTT
$\phi[CO_2]$	0.0720	0.0728
ϕ[ET]	0.105	0.104
P[CO_2]	23.6	23.2
P[ET]	41.2	40.6

Table II. *Effect of DTT on the Relative Zeaxanthin Content (Z/Z+A+V) and on Maximum Fluorescence Yield (F_M) in Cotton Leaf Disks Infiltrated with 150 mM Ascorbate Buffer (pH 5)*

The leaf disks were kept at a PFD of 10 μmol m^{-2} s^{-1} for 5 h. If present, DTT concentration in the buffer was 1 mM. F_M was determined in a saturating light pulse.

	$\frac{Z}{Z+A+V}$		F_M	
	no DTT	+ DTT	no DTT	+ DTT
			%	
Initial value	0.01	0.01	100.0	100.0
After 5 h	0.60	0.04	77.5	78.7

Nevertheless, DTT had no effect on maximum fluorescence yield (F_M) in the same leaf discs, showing that the presence of large amounts of zeaxanthin as such does not necessarily cause any increase in q_{NP}.

In another kind of experiment, we determined the effect of DTT on the light-induced fluorescence quenching that occurs when the leaves are exposed to a high PFD. These experiments were made on whole leaves whose cut petioles had been placed in either a DTT solution or in water. Figure 8A shows the results obtained with a leaf that was free to exchange CO_2 and O_2 with normal air. In Figure 8B gas exchange was prevented by covering the leaf surfaces with a thin layer of silicon grease. The determinations of q_{NP} and the reduction state of Q ($1 - q_p$) were derived from the time course of fluorescence yield during exposure to an actinic light of high PFD. Zero time refers to the time at which the actinic light was increased from 0 to 1150 μmol photons m^{-2} s^{-1}. Figure 8 (upper panels) clearly shows that DTT caused considerable inhibition of q_{NP} both in the presence and in the absence of gas exchange. However, considerable nonphotochemical quenching took place in the presence of DTT even though ΔA_{505} was almost completely absent. The difference in q_{NP} between DTT-treated and untreated control leaves reached a maximum after 2 to 3 min and tended to decline at longer times. As shown in the lower panels of Figure 8, the reduction state of Q was markedly higher in the presence than in the absence of DTT, *i.e.* DTT caused a larger fraction of the PSII reaction centers to be closed. Similar degrees of inhibition of q_{NP} by DTT as those shown in Figure 8 were obtained over a range of excessive PFDs (data not shown). No inhibition of q_{NP} and q_p was obtained at rate-limiting PFDs (data not shown). It is noteworthy that application of oxidized DTT had quantitatively the same effect as that of reduced DTT both on q_{NP} and the reduction state of Q (Fig. 8). This was also true for the effects of these two compounds on ΔA_{505} and ΔA_{535} (data not shown).

DISCUSSION

In this work we have characterized a light-induced absorbance change in leaves, whose properties strongly indicate that it is caused by deepoxidation of violaxanthin. It is of crucial importance, therefore, that the absorbance change related to the operation of the xanthophyll cycle can be distinguished from other absorbance changes occurring in the leaf and overlapping spectrally.

First, it was important to rule out any influence of leaf water status and chloroplast movements on the observed absorbance changes. Changes in the water status can considerably affect the absorbance of a leaf (8). Since we minimized water loss in our procedure and no continuous rise component was present in the absence of other major absorbance changes, we conclude that changes in leaf water status played no role in our system. Absorbance changes caused by chloroplast movements (9) are induced by blue light only and we used red actinic light. Moreover, such absorbance changes are quite different from those described here both with respect to their difference spectrum and their time course. Any contribution of chloroplast movements to the absorbance changes reported here can therefore be ruled out.

Other light-induced absorbance changes are the so-called light scattering change, peaking at 535 nm (7), and the electrochromic shift around 518 nm (24). Because of its very rapid kinetics, the latter change can be readily distinguished from the relatively slow conversion of violaxanthin to zeaxanthin. A differentiation between the absorbance change caused by violaxanthin deepoxidation and the change at 535 nm is more difficult, for both are probably driven by the light-induced acidification of the thylakoid lumen (6, 15, 25). Whereas the ΔpH and the light scattering signal can be considered to be reversible within a few minutes after light off (7), in our experiments zeaxanthin formation was irreversible (on the time scale used here) after a short light exposure (Fig. 2; *cf.* also [21] and [27]).

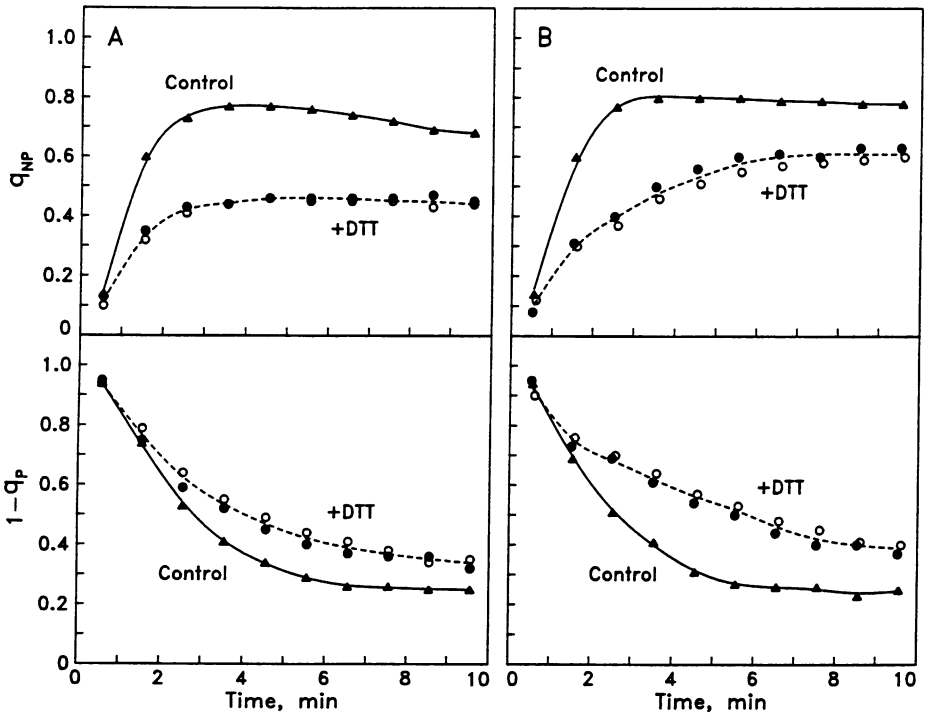

Figure 8. Nonphotochemical (q_{NP}) and photochemical (expressed as $1 - q_p$) fluorescence quenching in cotton leaves after DTT treatment. Three different leaves of the same plant were allowed to take up pure water (control) or a 1 mM solution of either reduced (closed circles) or oxidized DTT (open circles) for 4 h at a low PFD (10 μmol m^{-2} s^{-1}). Actinic PFD was 1150 μmol m^{-2} s^{-1}. Panel B shows the result obtained when different spots of the same leaves were illuminated while gas exchange was prevented by application of silicon grease. When ΔA_{505} was determined under the same conditions as in Figure 1 on the same leaves, the irreversible component was suppressed by at least 97% in the leaves treated with reduced or oxidized DTT, compared with the control.

The change in absorbance still present after a dark period of 4 min has two other properties which indicate that it is caused by violaxanthin deepoxidation. First, its difference spectrum (Fig. 3) is completely different from spectra for other known absorbance changes, but very similar to that reported in Yamamoto *et al.* (26) for violaxanthin deepoxidation in isolated chloroplasts. Its peaks are located at 505 and 465 nm, its troughs at 480 and 455 nm, while Yamamoto *et al.* (26) observed peaks at 505 and 468 nm and troughs at 482 and 450 nm in the respective wavelength band. There is only one apparent difference between the spectrum determined by Yamamoto *et al.* and that determined by us: we invarably found an additional shoulder, or sometimes a peak, around 515 nm. However, Yamamoto *et al.* did report a shift of the maximum from 505 nm to longer wavelengths and tentatively ascribed it to overlapping changes in the absorbance by Chl. Alternatively, the shoulder at 515 nm could be explained by the occurrence of selective scattering (14, 23) caused by the appearance of a new absorption band.

The second major evidence for ascribing the changes at 505 nm to violaxanthin deepoxidation is the complete suppression of the irreversible component by DTT. DTT is a powerful inhibitor of violaxanthin deepoxidation in chloroplasts (27), etiolated leaves (16), and green leaves. Although it is likely that DTT does not affect only the xanthophyll cycle (see Fig. 5 and comments below), its action still seems quite specific, since photosynthesis was totally unaffected by DTT.

Although the spectral change caused by violaxanthin deepoxidation is large (Figs. 1 and 3) and also makes an important contribution to the total absorbance change observed at 535 nm (Fig. 3), it has not been previously reported for intact leaves. To obtain a maximal response it was important that the leaf had been kept under conditions that favor a high epoxidation state (weak light or darkness). It is noteworthy that the cotton leaves used in our experiments also had a large pool of xanthophyll cycle components (violaxanthin + antheraxanthin + zeaxanthin) (22).

At 505 nm a relatively large absorbance change was obtained, even for small variations in the deepoxidation state (Fig. 4). At this wavelength the relative contribution from other processes was small (Fig. 5), so that ΔA_{505} could be used directly to estimate the deepoxidation state. However, to obtain quantitative data, calibration for each leaf may be needed since the apparent extinction coefficient may vary from leaf to leaf. It still remains to be determined if ΔA_{505} is caused by the difference in absorbance between violaxanthin and zeaxanthin alone or between violaxanthin and zeaxanthin plus antheraxanthin.

We found that the inhibitory effects of supplying oxidized DTT were indistinguishable from the effects of reduced DTT on q_{NP}, q_p, ΔA_{505} (Fig. 8) and ΔA_{535}. This finding is in contrast to the effects of the two forms of DTT on the isolated violaxanthin deepoxidase, where the reduced form acts as very potent inhibitor while the oxidized form has no effect (see "Materials and Methods"). A simple explanation for the effectiveness of exogenously applied oxidized DTT as an inhibitor of violaxanthin deepoxidation *in vivo* would be that it is reduced to DTT in the leaf. However, we do not have any direct evidence that such a reduction takes place.

In addition to inhibition of violaxanthin deepoxidation and associated light-induced absorbance changes, DTT inhibited the reversible component of the 535 nm absorbance change which is unlikely to be caused by violaxanthin deepoxidation (Fig. 5). The 535 nm absorbance change has been thought to be caused by altered light scattering properties of the chloroplasts due to conformational changes (7, 15) induced by the acidification of the thylakoid lumen. Our observations would support the view that the reversible component of ΔA_{535} is

rather an indirect than a direct consequence of thylakoid acidification (3), since it is difficult to see how the proton gradient could be dissipated by the presence of DTT without any concomitant effect on photosynthesis. It seems more likely that rather than affecting the ΔpH, DTT affects the process by which acidification is transformed into structural changes in the thylakoid membrane or the process by which such structural changes are expressed as light scattering changes.

A third effect of DTT discovered in the present study was inhibition of a major portion of nonphotochemical fluorescence quenching induced by excessive light (Fig. 8). This effect is consistent with the proposed fluorescence quenching action of zeaxanthin. However, zeaxanthin formation induced by low pH and ascorbate in dim light was not associated with an increased nonphotochemical fluorescence quenching (Table II). It is clear, therefore, that if zeaxanthin does indeed mediate quenching in the presence of excessive light there are additional requirements for zeaxanthin to be effective. For example, proper location of zeaxanthin within the thylakoid membrane and a close association with the excited Chl are likely to be critical factors affecting the effectiveness of zeaxanthin in mediating fluorescence quenching. On the assumption that the DTT-sensitive portion of the total fluorescence quenching was caused by the presence of zeaxanthin, then the remainder might tentatively be attributed to a direct ΔpH effect. The relative contribution of the DTT-sensitive quenching to NRD depends on the model used to calculate NRD. If we assume that a given degree of nonphotochemical quenching contributes equally to NRD, irrespective of the mechanism involved, and calculate NRD according to Kitajima and Butler (10), then the component inhibited by DTT in these cotton leaves could be as high as 50%. Further studies of the DTT sensitive component of fluorescence quenching are needed to identify the underlying process.

The finding that the reduction state of Q, measured under excessive light, was higher in the presence than in the absence of DTT (Fig. 8) is consistent with the hypothesis that the reduction of nonphotochemical fluorescence quenching by DTT reflects an increase in the rate constant for NRD in the antenna Chl. At first sight the absence of an effect of DTT on any measured characteristics of photosynthetic gas exchange might seem inconsistent with its marked effect on nonphotochemical quenching. It should be noted, however, that the photon yield measurements were made at low PFDs before exposure of the leaves to excessive light. Under these conditions there is little or no nonphotochemical quenching that can be attributed to NRD (18) and the reduction state of Q approaches zero. Moreover, little or no zeaxanthin is found. The effect of DTT on q_{NP} and $(1 - q_p)$ was only observed when the rate of absorption of excitation energy exceeded the rate with which the energy was deexcited via photosynthesis and photorespiration. Under the latter conditions a decrease in NRD is unlikely to affect the rate of photosynthesis but it would cause an increase in the reduction state of Q and in the probability that photoinhibitory damage would occur.

The present results do not permit us to conclude whether or not prevention of zeaxanthin formation and associated decrease in nonphotochemical quenching by DTT results in an increased susceptibility to photoinhibitory damage. To determine if zeaxanthin formation serves as a photoprotective mechanism, we are currently studying the effects of DTT treatments under longer term exposures of leaves to different levels of excessive light on subsequent photon yield of photosynthesis and PSII photochemistry. The results will be presented in a forthcoming paper.

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LITERATURE CITED

1. **Björkman O** (1987) High-irradiance stress in higher plants and interaction with other stress factors. *In* J Biggins, ed, Progress in Photosynthesis Research, Vol IV. Martinus Nijhoff Publishers, Dordrecht, pp 11–18
2. **Björkman O, Demmig B** (1987) Photon yield of O_2 evolution and chlorophyll fluorescence characteristics at 77 K among vascular plants of diverse origins. Planta **170:** 489–504
3. **Coughlan S, Schreiber U** (1984) The differential effects of short-time glutaraldehyde treatments on light-induced thylakoid membrane conformational changes, proton pumping and electron transport properties. Biochim Biophys Acta **767:** 606–617
4. **Demmig B, Winter K, Krüger A, Czygan F-C** (1987) Photoinhibition and zeaxanthin formation in intact leaves. A possible role of the xanthophyll cycle in the dissipation of excess light energy. Plant Physiol **84:** 218–224
5. **Demmig B, Winter K, Krüger A, Czygan F-C** (1988) Zeaxanthin and the heat dissipation of excess light energy in *Nerium oleander* exposed to a combination of high light and water stress. Plant Physiol **87:** 17–24
6. **Hager A** (1980) The reversible, light-induced conversions of xanthophylls in the chloroplast. *In* F-C Czygan, ed, Pigments in Plants, Ed 2. Fischer, Stuttgart, pp 57–79
7. **Heber U** (1969) Conformational changes of chloroplasts induced by illumination of leaves in vivo. Biochim Biophys Acta **180:** 302–319
8. **Heber U, Neimanis S, Lange OL** (1986) Stomatal aperture, photosynthesis, and water fluxes in mesophyll cells as affected by the abscission of leaves. Simultaneous measurements of gas exchange, light scattering, and chlorophyll fluorescence. Planta **167:** 554–562
9. **Inoue Y, Shibata K** (1973) Light-induced chloroplast rearrangements and their action spectra as measured by absorption spectophotometry. Planta **114:** 341–358
10. **Kitajima M, Butler W** (1975) Quenching of chlorophyll fluorescence and primary photochemistry in chloroplasts by dibromothymoquinone. Biochim Biophys Acta **376:** 105–115
11. **Kobayashi Y, Köster S, Heber U** (1982) Light scattering, chlorophyll fluorescence and state of the adenylate system in illuminated spinach leaves. Biochim Biophys Acta **682:** 44–54
12. **Krause GH** (1973) The high-energy state of the thylakoid system as indicated by chlorophyll fluorescence and chloroplast shrinkage. Biochim Biophys Acta **292:** 715–728
13. **Krause GH, Behrend U** (1986) pH-dependent chlorophyll fluorescence quenching indicating a mechanism of protection against photoinhibition of chloroplasts. FEBS Lett **200:** 298–302
14. **Latimer P, Rabinowitch E** (1959) Selective scattering of light by pigments *in vivo*. Arch Biochem Biophys **84:** 428–441
15. **Packer L, Crofts AR** (1967) The energized movement of ions and water by chloroplasts. *In* DR Sanadi, ed, Current Topics in Bioenergetics, Vol 2. Academic Press, New York, pp 23–64
16. **Pfündel E, Strasser RJ** (1988) Violaxanthin de-epoxidase in etiolated leaves. Photosynth Res **15:** 67–73
17. **Powles SB** (1984) Photoinhibition of photosynthesis by visible light. Annu Rev Plant Physiol **35:** 15–44
18. **Schäfer C, Björkman O** (1989) Relationship between photosyn-

thetic energy conversion efficiency and chlorophyll fluorescence quenching in upland cotton *(Gossypium hirsutum* L.). Planta **178:** 367–376

19. **Schreiber U, Schliwa U, Bilger W** (1986) Continuous recording of photochemical and non-photochemical chlorophyll fluorescence quenching with a new type of modulation fluorometer. Photosynth Res **10:** 51–62
20. **Siefermann-Harms D** (1985) Carotenoids in photosynthesis. I. Location in photosynthetic membranes and light-harvesting function. Biochim Biophys Acta **811:** 325–355
21. **Sokolove PM, Marsho TV** (1976) Ascorbate-independent carotenoid de-epoxidation in intact spinach chloroplasts. Biochim Biophys Acta **430:** 321–326
22. **Thayer SS, Björkman O** (1989) Leaf xanthophyll content and composition in sun and shade as determined by HPLC. Photosynth Res (in press)
23. **Thorne SW, Horvath G, Kahn A, Boardman NK** (1975) Light-dependent absorption and selective scattering changes at 518 nm in chloroplast thylakoid membranes. Proc Natl Acad Sci USA **72:** 3858–3862
24. **Witt HT** (1979) Energy conversion in the functional membrane of photosynthesis. Analysis by light pulse and electric pulse methods. The central role of the electric field. Biochim Biophys Acta **505:** 355–427
25. **Yamamoto HY** (1979) Biochemistry of the violaxanthin cycle in higher plants. Pure Appl Chem **51:** 639–648
26. **Yamamoto HY, Kamite L, Wang Y-Y** (1972) An ascorbate-induced absorbance change in chloroplasts from violaxanthin de-epoxidation. Plant Physiol **49:** 224–228
27. **Yamamoto HY, Kamite L** (1972) The effects of dithiothreitol on violaxanthin de-epoxidation and absorbance changes in the 500-nm region. Biochim Biophys Acta **267:** 538-543