Photoinhibition and Zeaxanthin Formation in Intact Leaves[1,2]

A POSSIBLE ROLE OF THE XANTHOPHYLL CYCLE IN THE DISSIPATION OF EXCESS LIGHT ENERGY

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ABSTRACT

Comparative studies of chlorophyll *a* fluorescence, measured with a pulse amplitude modulated fluorometer, and of the pigment composition of leaves, suggest a specific role of zeaxanthin, a carotenoid formed in the xanthophyll cycle, in protecting the photosynthetic apparatus against the adverse effects of excessive light. This conclusion is based on the following findings: (a) exposure of leaves of *Populus balsamifera, Hedera helix,* and *Monstera deliciosa* to excess excitation energy (high light, air; weak light, 2% O_2, 0% CO_2) led to massive formation of zeaxanthin and a decrease in violaxanthin. Over a wide range of conditions, there was a linear relationship between either variable, F_V, or maximum fluorescence, F_M, and the zeaxanthin content of leaves. (b) When exposed to photoinhibitory light levels in air, shade leaves of *H. helix* had a higher capacity for zeaxanthin formation, at the expense of β-carotene, than shade leaves of *M. deliciosa.* Changes in fluorescence characteristics suggested that, in *H. helix,* the predominant response to high light was an increase in the rate of nonradiative energy dissipation, whereas, in *M. deliciosa,* photoinhibitory damage to photosystem II reaction centers was the prevailing effect. (c) Exposure of a sun leaf of *P. balsamifera* to increasing photon flux densities in 2% O_2 and 0% CO_2 resulted initially in increasing levels of zeaxanthin (matched by decreases in violaxanthin) and was accompanied by fluorescence changes indicative of increased nonradiative energy dissipation. Above the light level at which no further increase in zeaxanthin content was observed, fluorescence characteristics indicated photoinhibitory damage. (d) A linear relationship was obtained between the ratio of variable to maximum fluorescence, F_V/F_M, determined with the modulated fluorescence technique at room temperature, and the photon yield of O_2 evolution, similar to previous findings (O Björkman, B Demmig 1987 Planta 170: 489–504) on chlorophyll fluorescence characteristics at 77 K and the photon yield of photosynthesis.

Carotenoids are present in the thylakoid membranes of all eukaryotes, and it has been suggested for many years that they protect the photosynthetic apparatus against the destructive effects of light and O_2 (9). One line of evidence supporting such a role can be found in mutants of algae and higher plants which are deficient in carotenoid biosynthesis and which are viable only when maintained in low light (9).

Exposure of photosynthetic tissues to light in excess of that which can be utilized in photosynthesis results in photoinhibition, a reduction in photosynthetic activity, due primarily to a sustained reduction in the photochemical efficiency of PSII. This inhibition of photosynthetic activity by light and, in particular, by the interaction between light and additional environmental stresses has received increasing attention (15). Photoinhibition may result from two different processes working singly or in combination (4): (a) a decrease in the rate constant for photochemistry of PSII caused by damage to the PSII reaction centers and (b) an increase in the rate constant for nonradiative dissipation of excitation energy. According to a model by Kitajima and Butler (7), photoinhibitory damage to PSII reaction centers, *i.e.* a decrease in the rate constant for PSII photochemistry, leads to a rise in initial fluorescence at open PSII traps, F_O,[3] whereas an increase in the rate constant of nonradiative energy dissipation leads to a decrease in both initial fluorescence, F_O, and maximum fluorescence at closed PSII traps, F_M. Such an increase in thermal de-excitation could function as an overflow valve to allow for nondestructive dissipation of excess excitation energy and may therefore be viewed as a potentially protective process rather than as an indication of damage, *e.g.* to proteins (10) of the thylakoid membrane. Increased nonradiative energy dissipation, as indicated by Chl 77 K fluorescence characteristics, was shown to occur under conditions of excess excitation in low light (in 2% O_2 and the absence of CO_2) with no evidence for simultaneous photoinhibitory damage to the PSII reaction centers, as well as at high light levels where there was evidence for simultaneous damage (4).

This paper presents a striking parallel between the occurrence of conditions, at both high and low light levels, under which increased nonradiative energy dissipation is observed, and the accumulation of the carotenoid zeaxanthin in leaves exposed to such conditions. Zeaxanthin is known to be formed by deepoxidation of violaxanthin in the so-called violaxanthin or xanthophyll cycle which is present in thylakoid membranes and which is fine-tuned to respond to the balance of electron transport reactions and CO_2 uptake (6, 23). The function of this cycle has been unknown thus far. We propose that the formation of zeaxanthin under photoinhibitory conditions is indicative of a specific function of this particular carotenoid compound in the prevention of photoinhibitory damage.

MATERIALS AND METHODS

Plant Material. *Populus balsamifera* L., about 1.5 m tall, was grown in 25 L plastic pots filled with garden soil. Plants were

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[3] Abbreviations: F_O, instantaneous fluorescence emission; F_M, maximum fluorescence emission; F_V, variable fluorescence emission; PFD, photon flux density; Car, carotenoids; Z, zeaxanthin; A, antheraxanthin; V, violaxanthin; ϕ_i, photon yield (incident light).

kept outdoors in the Würzburg Botanic Garden from May to August. They were watered daily and received 2.5 L of Hewitt's type nutrient solution (22) containing 12 mM NO_3^- once per week. Leaves of *Hedera helix* L. were obtained from a natural population growing at a shaded site in the Würzburg Botanic Garden. Experiments on *P. balsamifera* and *H. helix* were performed in July and August. Shade plants of *Monstera deliciosa* Liebm. were obtained from a local nursery. Plants were kept in a glasshouse at a defined low level of PFD of 20 to 30 μmol photons m^{-2} s^{-1} for at least 4 weeks prior to onset of the experiments.

Photoinhibitory Treatments. Attached (*P. balsamifera, M. deliciosa*) or detached (*H. helix*) intact leaves were enclosed in a ventilated, temperature-controlled gas exchange chamber. Leaf temperature was at 25°C. Illumination was provided by a metal halide lamp (HRI-T 1000 W/D; Radium Elektrizitäts-Ges., Wipperfürth, F.R.G.). Infrared radiation was reduced using a 6 mm thick heat reflecting glass (113 Tempax; Schott, Mainz, F.R.G.). Leaves were supplied either with ambient air or with gas from a cylinder containing 2% O_2, 98% N_2 (no CO_2). The gases were brought to a dew point of 18°C before entering the leaf chamber. Discs punched from the leaves were used for measurements of fluorescence and O_2 exchange.

Fluorescence. Chl *a* fluorescence was measured at room temperature using a pulse amplitude modulation fluorometer (model PAM 101; H. Walz, Effeltrich, F.R.G.) (18). A leaf disc (diameter 1.9 cm) was enclosed in a small water-jacketed brass-cuvette. The disc was placed on foam rubber and the upper leaf surface pressed against a Perspex window which formed the bottom of the chamber lid, in which the fiber optic probe of the fluorometer was fixed. In this way, sample and fiber optics were maintained at a constant distance, allowing direct comparison in the absolute level of fluorescence emission from different samples. All fluorescence measurements were preceded by a 5-min period of complete darkness to allow for relaxation of any fluorescence quenching associated with thylakoid membrane energization (8). Fluorescence was excited with a measuring beam of weak light from a pulsed light-emitting diode to obtain F_O, which designates the fluorescence level when all reaction centers of PSII are open. Maximum fluorescence yield, F_M, was determined by application of a 1 s pulse of saturating light (typically 3000 μmol m^{-2} s^{-1}) to transiently close all reaction centers and completely reduce the acceptor Q of the PSII reaction center. The variable fluorescence, F_V, is given by the difference between F_M and F_O.

O_2 Exchange. Measurements of photon yields of photosynthetic O_2 evolution were made at 25°C and 5% CO_2 with a Hansatech LD-2 leaf disc O_2-electrode unit and a Hansatech LS-2 light source, as described previously (1).

Pigment Analysis. The carotenoids were analyzed quantitatively after separation by TLC (3, 21). Extraction and preparation procedures were performed in a darkened room and samples were kept under nitrogen between individual isolation steps. For the estimation of all carotenoids the same extinction coefficient was used ($E_{1cm}^{1\%}$: 2500; in ethanol for λ_{max}). Chl *a* and *b* were determined after Röbbelen (17), modified by Metzner *et al.* (14), in acetone (80% v/v) extracts of fresh leaves. The pigment content of leaves is given in μg cm^{-2}. Mol wt of the various carotenoids are very similar (β-carotene: 536.9; lutein (3,3'-dihydroxy-α-carotene): 568.9; violaxanthin (5,6,5',6'-diepoxyzeaxanthin): 600.9; antheraxanthin (5,6-epoxyzeaxanthin): 584.9; zeaxanthin (2,3'-dihydroxy-β-carotene): 568.9; neoxanthin: 600.9). The mol wt of Chl *a* and *b* are 893.5 and 907.5, respectively.

RESULTS

Relationship between Effect of Photoinhibitory Treatment on Photon Yield of O_2 Evolution and Room Temperature Chl Fluo-

rescence Characteristics. The photon yield (ϕ_i) of O_2 evolution and the corresponding F_V/F_M ratio in *Monstera deliciosa* leaves, exposed to a high PFD for different lengths of time, are shown in Figure 1. A linear relationship between these two variables was obtained. The ratio F_V/F_M obtained at room temperature with the modulated fluorescence technique can thus be used as a quantitative indicator of high-light-induced changes in photochemical efficiency, as has been demonstrated previously for the ratio F_V/F_M determined at 692 nm (emission of PSII) with low temperature (77 K) Chl fluorescence (1, 4).

Effect of Excessive Excitation Energy at Low Light Levels on Fluorescence Characteristics and Pigment Composition of Populus Leaves. Figure 2 shows the effect of conditions preventing both photosynthesis and photorespiration in low light, on fluorescence characteristics and pigment composition in a sun leaf of *P. balsamifera*. Exposure of the leaf to 2% O_2, 0% CO_2 resulted in a pronounced and reversible quenching of initial fluorescence at open PSII traps, F_O, maximum fluorescence at closed traps, F_M, and variable fluorescence, $F_V = F_M - F_O$ (Fig. 2A). The pronounced quenching of F_M and F_O was accompanied by only a slight and rapidly reversible decrease in the ratio F_V/F_M suggesting that no photoinhibitory damage had occurred but solely an increase in nonradiative energy dissipation.

Figure 2B illustrates the quantitative changes in the three components of the xanthophyll cycle: (a) the diepoxide violaxanthin, (b) a monoepoxide fraction consisting of antheraxanthin and lutein-5,6-epoxide, and (c) zeaxanthin. Under control conditions, in weak light and air, violaxanthin was predominant and there was very little antheraxanthin/lutein-5,6-epoxide and zeaxanthin. We take this as evidence that no significant formation of zeaxanthin occurred during the isolation and separation procedure of the pigments. When the leaves were exposed to 2% O_2 and 0% CO_2, an increasing amount of zeaxanthin was found in the tissue, concomitant with the quenching of fluorescence. The increase in zeaxanthin was quantitatively matched by a decrease in violaxanthin such that the sum of the three xanthophylls remained constant throughout the treatment. The conversion of violaxanthin to zeaxanthin was almost complete after approximately 35 min and was stoichiometric, *i.e.* zeaxanthin increased by 3.75 nmol cm^{-2} (from 0.67 to 4.42 nmol cm^{-2}) and violaxan-
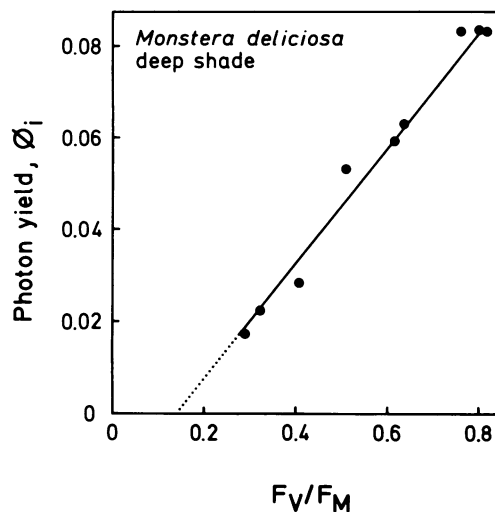

FIG. 1. Relationship between the photon yield of O_2 evolution (ϕ_i) and the F_V/F_M ratio in a shade leaf of *M. deliciosa* exposed to a PFD of 1650 μmol m^{-2} s^{-1} for various periods of time (30–200 min). The exposed leaf samples were kept at a PFD of 30 μmol m^{-2} s^{-1} for 3 h before measurements of photon yields and fluorescence were made. Leaf samples were kept in complete darkness for 5 min prior to fluorescence measurements.
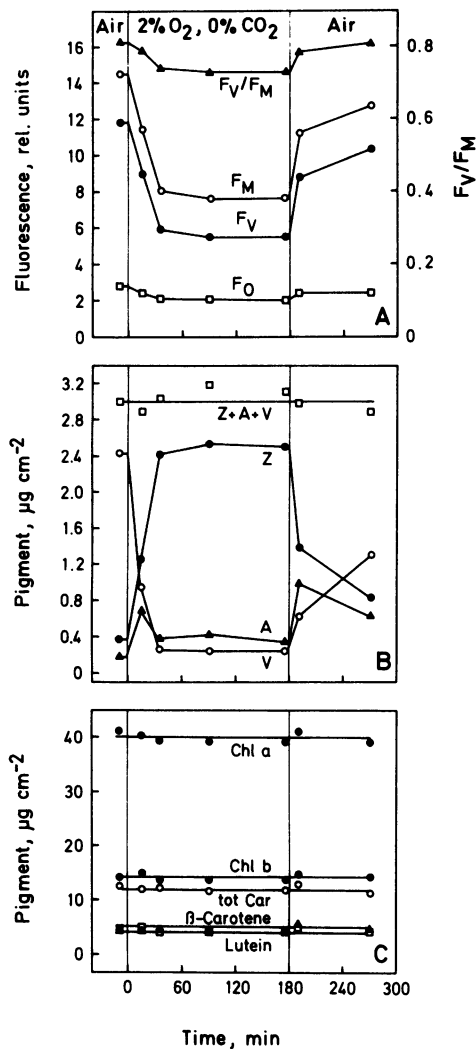
FIG. 2. Time course of changes in Chl fluorescence (A) and pigment content (B and C) induced by an exposure of a *P. balsamifera* leaf to 2% O_2, 0% CO_2 at a PFD of 100 μmol m^{-2} s^{-1}. All leaf samples were kept in complete darkness for 5 min prior to fluorescence measurements, and prior to freezing of samples for pigment analysis, respectively.
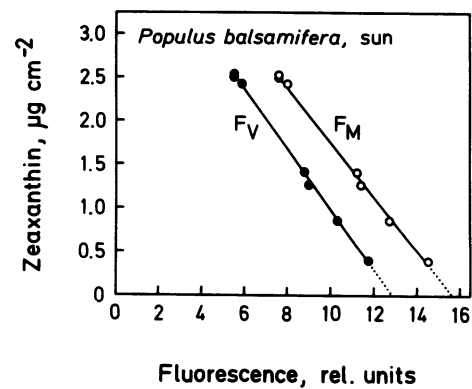

Fluorescence, rel. units

FIG. 3. Relationship between zeaxanthin content and Chl fluorescence (variable and maximum fluorescence) in a sun leaf of *P. balsamifera* exposed to 2% O_2, 0% CO_2 at a PFD of 100 μmol m^{-2} s^{-1} (data from Fig. 1).

thin decreased by 3.63 nmol cm^{-2} (from 4.03 to 0.40 nmol cm^{-2}). Upon return to normal air the amount of zeaxanthin rapidly decreased to about 50% of the steady state level in 2% O_2, 0% CO_2, and then continued to decline slowly.

Although the components of the xanthophyll cycle underwent major changes in their relative proportions, virtually no changes occurred in the amounts of the other 'photosynthetic' pigments such as Chl *a* and *b*, β-carotene, lutein, and the *sum* of the total carotenoids (β-carotene, lutein, zeaxanthin, violaxanthin, antheraxanthin, and neoxanthin) (Fig. 2C). Zeaxanthin was the only (measured) pigment which responded to excessive light with an instantaneous increase. The time course of the changes in zeaxanthin content of the tissue mirrored the time course of the quenching of F_V and F_M. Both F_V and F_M were linearly correlated with the zeaxanthin content of the leaf tissue (Fig. 3).

Effect of High Light Treatment on Fluorescence and Pigment Content in Leaves Differing in Photoinhibitory Response. To investigate the involvement of carotenoids in the response of leaves to high light conditions, two species were chosen which displayed different types of photoinhibition, as indicated by fluorescence characteristics. In young shade leaves of *H. helix*, a strong increase in the rate constant of nonradiative energy dissi-

pation appeared to be the predominant effect, since there was a net decrease in F_O (Table I). Mature shade leaves of the highly shade-tolerant rain forest species *M. deliciosa*, on the other hand, showed evidence of pronounced photoinhibitory damage, as indicated by a strong net increase in F_O (Table I). The complete set of fluorescence and pigment data for *H. helix* shade leaves, before and after the high light treatment, is shown in Table I. The data give a typical example of the remarkably small variation between duplicate samples. The 2-h treatment resulted in a pronounced decrease of variable fluorescence (from 11.6 to 3.7) which was accompanied by a moderate decrease in F_V/F_M from 0.79 to 0.57. The zeaxanthin content of the tissue increased strongly, as did the sum of the three xanthophylls (zeaxanthin, antheraxanthin, and violaxanthin). The net increase in xanthophylls was matched by a decrease in β-carotene (Table I). These changes are given in μg cm^{-2} and correspond to a decrease in β-carotene content by 2.1 nmol cm^{-2} (from 11.3 to 9.2 nmol cm^{-2}) and a concomitant increase in the three xanthophylls of the cycle, by 2.01 nmol cm^{-2} (zeaxanthin: +3.96; antheraxanthin: −0.65; violaxanthin: −1.30 nmol cm^{-2}). The level of the other major xanthophyll, lutein, did not decrease. Consequently, the sum of the total carotenoids remained constant. This photoinhibitory treatment did not result in any decrease in Chl *a* and *b* content. Instead, there was a small increase, particularly in Chl *a*. For *H. helix*, a similarly strong correlation between the quenching of variable fluorescence and the amount of zeaxanthin present in the tissue was observed (Fig. 4) as was seen in *P. balsamifera* (Fig. 3). Figure 4 includes data obtained with *H. helix* leaves exposed to either low light in 2% O_2, 0% CO_2, or to high light (500 or 1500 μmol photons m^{-2} s^{-1}) in air for 2 h. The two sets of data points fit the same regression line. Clearly, high light treatments (air) have a similar effect on fluorescence as treatments in an atmosphere of 2% O_2, 0% CO_2 in low light. This result corroborates the interpretation that, in *H. helix*, the effect of the high light treatment is mainly to increase the rate of nonradiative energy dissipation.

High light treatment of a mature leaf of *M. deliciosa* which had developed in deep shade resulted in a decrease of F_M comparable to that observed in *H. helix* (Table I). In *M. deliciosa* there was, however, a much more pronounced decrease in the ratio F_V/F_M, indicating severe photoinhibition. In contrast to the net decrease of F_O observed in *H. helix*, a pronounced (40%) net increase of F_O occurred in *M. deliciosa* (Table I). In *M. deliciosa*, the high light treatment also caused an increase in zeaxanthin which was, however, much less pronounced than the one observed in *H. helix*. Under control conditions (100 μmol photons m^{-2} s^{-1}, air), zeaxanthin could be detected in *M. deliciosa* but not in *H. helix*. The sum of the three components of

Table I. Changes in Pigment Content and Chl Fluorescence Characteristics in Two Species Induced by a 3-h Exposure to a PFD of 1500 μmol m⁻² s⁻¹ in Air. The changes are given in percent of the untreated controls.

Species	Chl a	Chl b	Car	β-Carotene	Lutein	Zeaxanthin	Antheraxanthin/ Luteinepoxide	Violaxanthin	Neoxanthin	Z + A + V	Fo	FM	FV	FV/FM
				μg cm⁻²							*relative units*			
H. helix														
Before a	51.6	24.1	14.8	6.32	6.11	0	0.94	1.08	0.32	2.01	3.08	14.2	11.1	0.783
b	52.3	23.9	14.4	5.85	6.53	0	0.74	1.00	0.32	1.74	3.19	15.2	12.0	0.790
X̄	52.0	24.0	14.6	6.09	6.32	0	0.84	1.04	0.32	1.88	3.14	14.7	11.6	0.787
After a	57.1	24.7	15.0	4.86	6.68	2.52	0.42	0.29	0.22	3.22	2.80	6.5	3.7	0.571
b	54.0	23.8	14.5	5.04	6.59	1.97	0.49	0.22	0.20	2.68	2.87	6.6	3.7	0.566
X̄	55.5	24.3	14.8	4.95	6.64	2.25	0.46	0.26	0.21	2.96	2.84	6.6	3.7	0.569
Percent change	+6.9	+1.0	+1.4	−19	+5.0		−46	−76	−36	+58	−9.6	−55	−68	−28
M. deliciosa														
Before	57.0	17.4	12.8	4.50	6.10	0.71	0	0.95	0.53	1.66	3.40	15.8	12.4	0.785
After	51.0	22.1	12.8	4.24	6.51	1.24	0	0.42	0.40	1.66	4.77	7.2	2.4	0.338
Percent change	−11	+27	±0	−5.8	+6.7	+74		−56	−25	0	+40	−54	−81	−57

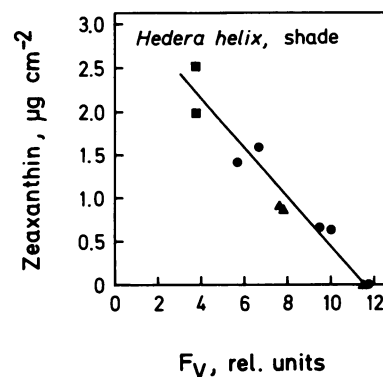FIG. 4. Relationship between zeaxanthin content and Chl fluorescence (FV) in shade leaves of H. helix exposed to different combinations of PFDs and exposure times. The different symbols depict treatments in 2% O_2, 0% CO_2 at a PFD of 100 μmol m⁻² s⁻¹ (●) and treatments for a 3-h period in air at PFDs of 500 (▲) and 1500 (■) μmol m⁻² s⁻¹, respectively.

the xanthophyll cycle on a leaf area basis was approximately the same in the two species under control conditions. The fresh weight per unit area of the two types of leaves was also similar (27.9 mg cm⁻² for M. deliciosa and 23.7 mg cm⁻² for H. helix) resulting in similar pigment concentrations on a fresh weight basis. Because of the higher Chl content of M. deliciosa leaves, the ratio xanthophyll/Chl was smaller in M. deliciosa. The high light treatment did not result in any increase in the sum of the three components of the xanthophyll cycle in M. deliciosa. In contrast to H. helix, M. deliciosa also exhibited only a very small decrease in β-carotene.

Effect in Low Light of Excessive Excitation Energy on Relative Proportions of Xanthophylls and β-Carotene. The net increase in the sum of the three xanthophylls, zeaxanthin, antheraxanthin, and violaxanthin, which was observed in H. helix in high light and air (Table I), could also be induced in low light by removal of the terminal electron acceptors (2% O_2, 0% CO_2) (Table II). The increase in the sum of the three components of the xanthophyll cycle again was quantitatively matched by a reversible decrease in β-carotene while the lutein content was unaltered and the sum of the total carotenoids remained constant throughout the treatment. In H. helix, part of the zeaxanthin was apparently formed at the expense of β-carotene, similar to the above response of this species during the high light treatment. The conversion of β-carotene to zeaxanthin occurred relatively rapidly (within 90 min) and was reversible.

Carotenoid Levels and Onset of Photoinhibitory Damage. The following data show that photoinhibitory damage by high light, as indicated by an increase in Fo accompanied by a strong decrease in FV/FM, occurred precisely at the point at which the conversion of violaxanthin to zeaxanthin had been completed and no further increase in zeaxanthin content took place. We chose a species, P. balsamifera, in which the sum of the three xanthopylls of the cycle did not increase within the time frame examined. Figure 5 shows fluorescence characteristics and pigment composition of a sun leaf of P. balsamifera upon stepwise transfer to increasing PFD levels in an atmosphere of 2% O_2 and 0% CO_2. All parameters are plotted against FV in order to show the quantitative relationship between the increase in zeaxanthin and the decline in FV. Between 0 and 320 μmol photons m⁻² s⁻¹, Fo decreased, accompanied by only a small decrease in FV/FM and, hence, in photochemical efficiency (Fig. 1). Between 320 and 2600 μmol photons m⁻² s⁻¹, however, Fo increased again and at the same time a precipitous decline in FV/FM was observed. The changes in fluorescence characteristics between 0 and 320 μmol m⁻² s⁻¹ (phase I) are indicative of an increase in

Table II. *Changes in Pigment Content and Composition Induced by Exposing H. helix Leaves to 2% O_2, 0% CO_2 in Weak Light*

Leaves were kept in air for 60 min, thereafter in 2% O_2, 0% CO_2 for 90 min, and then returned to air for 90 min. PFD was 100 μmol m^{-2} s^{-1} throughout the treatment.

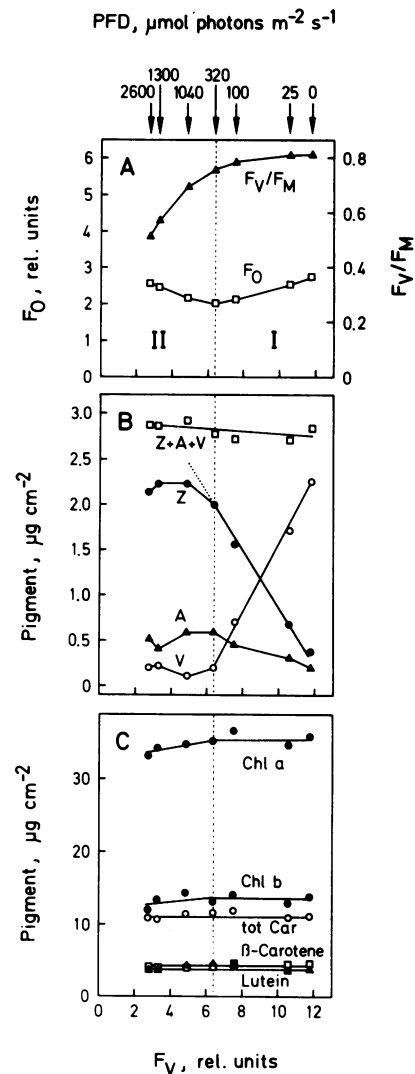| Composition of Air	Chl a	Chl b	Car	β-Carotene	Lutein	Zeaxanthin	Antheraxanthin/ Luteinepoxide	Violaxanthin	Neoxanthin	Z + A + V	Z + A + V + β-Car
						μg cm^{-2}					
Air	41.5	17.9	11.60	4.15	5.51	0.65	0	1.02	0.236	1.67	5.82
2% O_2, 0% CO_2	39.6	17.2	11.65	3.65	5.63	1.50	0.398	0.23	0.226	2.13	5.78
Air	40.7	17.0	11.47	4.25	5.24	0	0.604	1.02	0.347	1.62	5.87

FIG. 5. Relationship between variable fluorescence, F_V, and other Chl fluorescence characteristics (F_O, F_V/F_M), and pigment composition in a sun leaf of *P. balsamifera*. The leaf was stepwise exposed to increasing photon flux densities, for 30-min periods each, from 0 to 2600 μmol m^{-2} s^{-1}. Samples were taken at the end of the 30-min period at each light level and were kept in complete darkness for 5 min prior to fluorescence measurements or prior to freezing of samples for pigment analysis.

the rate of nonradiative energy dissipation, *i.e.* of the occurrence of a potentially protective process, whereas between 320 and 2600 μmol m^{-2} s^{-1} (phase II) fluorescence characteristics indicate damage to PSII.

Between 0 and 320 μmol photons m^{-2} s^{-1}, a strong increase in zeaxanthin content and a concomitant decrease in violaxanthin content took place (Fig. 5B). The increase in zeaxanthin content was linearly related to the decrease in variable fluorescence as had been observed in young *H. helix* shade leaves under both high and low light conditions (Fig. 4, compare also Fig. 3). At 320 μmol photons m^{-2} s^{-1}, violaxanthin was almost totally converted to zeaxanthin and no further increase in zeaxanthin content or decrease in violaxanthin content was observed up to 2600 μmol photons m^{-2} s^{-1}. Yet, variable fluorescence continued to decrease between 320 and 2600 μmol photons m^{-2} s^{-1}. Thus, there is a range of extremely high light levels which cause a quenching of both F_V and F_M (not shown) that is not accompanied by any further increase in the content of zeaxanthin. Although there were no marked changes in β-carotene, lutein, or

in the total carotenoids, there appeared to be a small decrease in Chl a and b between 320 and 2600 μmol photons m^{-2} s^{-1} (Fig. 5C).

DISCUSSION

This study shows that the carotenoid composition of leaves specifically responds to photoinhibitory treatments and that there is a strong quantitative correlation between the formation of zeaxanthin in the xanthophyll cycle and a type of fluorescence quenching which is indicative of increased nonradiative energy dissipation. Furthermore, a high capacity for the formation of zeaxanthin is related to a high tolerance to photoinhibition.

The quenching of Chl fluorescence was used as an indicator of an increased rate of nonradiative energy dissipation under conditions where the rate of absorption of excitation energy exceeded the rate of its utilization via photosynthetic electron transport. One kind of nonradiative energy dissipation has been related to thylakoid membrane energization ('high energy quenching') and relaxes rapidly (8). The study presented here is concerned with a type of quenching with longer induction and relaxation times, which persists in the dark (4). This type of fluorescence quenching could be induced at low light levels (e.g. 100 μmol photons m^{-2} s^{-1}) by preventing photosynthesis and photorespiration (2% O_2, 0% CO_2) and at high light levels (e.g. 1500 μmol photons m^{-2} s^{-1}) in air. At the high but not at the low light level, this type of fluorescence quenching, indicative of an increased rate of nonradiative energy dissipation, was accompanied by an additional effect, photoinhibitory damage to the PSII reaction centers. According to a model of Kitajima and Butler (7) for Chl 77 K fluorescence, a decrease in the rate constant for photochemistry of PSII (K_P), i.e. photoinhibitory damage to the PSII reaction centers, leads to a rise in F_O. In contrast, an increase in the rate constant of nonradiative energy dissipation (K_D) leads to a decrease in F_M and a concomitant though smaller decrease in F_O. In a previous study it has been suggested that the changes in 77 K fluorescence caused by high light may be accounted for by a combination of these two processes (4). Since they have opposite effects on F_O, the net changes in F_O in relation to the changes in F_M may be taken as an indicator of the relative contribution of the two processes.

A quantitative relationship between the photon yield of photosynthetic O_2 evolution and the ratio F_V/F_M obtained with room temperature modulated fluorescence, existed during photoinhibitory treatments as had been shown for the ratio F_V/F_M, measured at 692 nm in low temperature (77 K) Chl fluorescence (1, 4). Therefore, F_V/F_M in room temperature fluorescence, as well as in low temperature fluorescence, can serve as a quantitative indicator of high light-induced changes in photochemical efficiency.

Carotenoid Levels under Photoinhibitory Conditions. Exposure of leaves to conditions of excess excitation energy, which resulted in an increased rate of nonradiative energy dissipation, invariably led to a specific increase in zeaxanthin and a decrease in violaxanthin (16). This was the case both at low light levels in 2% O_2, 0% CO_2 (Fig. 2, Table II), and at high light levels in air (Fig. 4, Table I). None of the other carotenoids which were present in larger amounts than the components of the xanthophyll cycle (at least under control conditions) responded with an increase to such photoinhibitory conditions. Rather, β-carotene in *Hedera* decreased in both low and high light. It is conceivable, that the zeaxanthin formed in excess of that which could be accounted for by the decrease in the original level of violaxanthin results from conversion of β-carotene. The fact that a reversible decrease in β-carotene content was induced by 2% O_2, 0% CO_2 in low light (Table II), i.e. under conditions excluding photoinhibitory damage, strongly suggests a regulatory function of these conversions and shows that the reversible conversion of β-carotene to

zeaxanthin does not require high light *per se* but is triggered by the balance between the absorption and the utilization of excitation energy.

Relationship between Fluorescence Quenching Indicative of Increased Nonradiative Energy Dissipation, and Increase in Zeaxanthin. There was a striking quantitative correlation between either F_V or F_M and the zeaxanthin content of the leaves, for the low light response (100 μmol photons m^{-2} s^{-1}, 2% O_2, 0% CO_2) in *P. balsamifera* (Figs. 2 and 3) and in *H. helix* (Fig. 4). This type of fluorescence quenching of both F_M and F_O can be interpreted as an increase in the rate constant for nonradiative energy dissipation caused by 'the creation of alternative quenchers that compete with the reaction centers for excitation energy' (7). It is an attractive possibility that zeaxanthin may act as a quencher of fluorescence. It is unknown whether zeaxanthin is a better quencher than violaxanthin. Alternatively, differences in the microcompartmentation of the two carotenoids in the thylakoid membrane could lead to the increased fluorescence quenching reported here. That 'a carotenoid triplet is a quencher of Chl fluorescence' was concluded by Mathis *et al.* (13) from flash excitation studies using thylakoid suspensions, in view of a similar kinetic behavior of rapid fluorescence quenching and the formation of carotenoid triplet states as indicated by the absorbance change at 505 nm.

The carotenoid triplet state is probably populated by triplet-triplet energy transfer from the triplet state of Chl a (11). The probability of the formation of Chl triplet states *in vivo* remains unclear; in Chl solutions this probability was shown to be high (2). Carotenoid triplets could then also quench Chl fluorescence by reaction with the singlet state of Chl a, as suggested by Mathis and Schenk (12), thereby competing with the photosynthetic reaction centers for excitation energy.

In an intact, photosynthesizing leaf, two contrasting requirements have to be met by an 'alternative quencher.' Besides operating as an overflow valve under conditions of excess excitation, such a quencher should be absent or ineffective under control conditions to allow for a highly efficient excitation energy transfer, which is indicated by the finding that photon yields of photosynthesis are close to their maximum theoretical value in nonphotoinhibited leaves (1). Zeaxanthin is the only carotenoid compound to meet *both* requirements, and the xanthophyll cycle is perfectly regulated to sense the point at which absorbed light becomes excessive. This is so because the cycle is known to respond to the balance between electron transport reactions and CO_2 fixation, via its sensitivity (a) to the acidification of the intrathylakoid lumen (5), (b) to the redox state of 'some' electron carrier (19), and (c) to the levels of NADPH and O_2 (20).

Alternatively to a function of zeaxanthin as a fluorescence quencher via direct interaction with excited states of Chl, zeaxanthin could react with the products of further reactions of triplet Chl, such as radicals of hydrocarbon compounds or singlet oxygen, and thereby prevent damaging photooxidations. All of these mechanisms are consistent with protection by carotenoid pigments against the destructive effects of excessive excitation energy but more work is needed to distinguish between them. There is clearly a range of high light intensities in which no further increase in zeaxanthin occurred, whereas there still was further quenching of F_V (Fig. 5) and, to a smaller extent, of F_M (not shown). This was the case under conditions where accumulation of excitation energy went far beyond the level that is normally experienced. This additional quenching of F_V and F_M (phase II in Fig. 5) could be explained by the assumption that photoinhibitory damage *per se* results not only in a rise in F_O but also in a decrease in F_M.

Relationship between Capacity for Zeaxanthin Formation and Avoidance of Photoinhibitory Damage. The point at which photoinhibitory damage became evident, as indicated by an increase

in F_O and a precipitous decline in F_V/F_M (Fig. 5), coincided with the point at which no further increase in zeaxanthin was observed. One may speculate that photoinhibitory damage occurs only when the capacity of the system to increase the concentration of the potentially protective compound, zeaxanthin, is exceeded. To further elucidate this relationship, the changes in pigment composition were studied in two species differing in their susceptibility to photoinhibition and in the type of photoinhibitory response. Young shade leaves of *H. helix* responded to high light treatments with changes in fluorescence characteristics (net *decrease* in F_O, moderate decrease in F_V/F_M) which indicated a strong increase in the rate of nonradiative energy dissipation (Table I). These leaves recovered completely from photoinhibition within 4 h. In mature shade leaves of a second species, *M. deliciosa*, the high light treatment resulted in a pronounced photoinhibitory damage, as indicated by the net *increase* in F_O and a strong decrease in F_V/F_M (Table I). In *M. deliciosa*, the recovery from photoinhibition required days and remained incomplete. These two types of leaves could also clearly be distinguished with respect to the magnitude of the changes in pigment composition. In young shade leaves of *H. helix*, a strong increase in zeaxanthin content beyond that which can be accounted for by a decrease in violaxanthin was induced within a few hours both in the low light (2% O_2, 0% CO_2) and in the high light treatment. In contrast, *M. deliciosa* leaves did not exhibit the capacity for xanthophyll synthesis, apparently from β-carotene, within the same time frame. The different responses may be related to a generally higher level of biosynthetic activity in the young *Hedera* leaf. These findings further suggest a protective function of carotenoid pigments: the *H. helix* leaf which exhibited the higher capacity for rapid zeaxanthin formation also showed the lower susceptibility to photoinhibitory damage, and in the leaf of *P. balsamifera* (Fig. 5) in which the sum of the xanthophylls did not increase, pronounced photoinhibitory damage became manifest at the point at which no further increase in zeaxanthin occurred.

Considering (i) the previous notion that carotenoid pigments engage in protection of the photosynthetic apparatus and (ii) our finding that zeaxanthin was the only carotenoid pigment which did respond to conditions of excessive excitation energy with an increase, we conclude that the operation of the xanthophyll cycle plays a specific role in the protection of the photochemical apparatus against high light.

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