Chilling-Enhanced Photooxidation[1]

EVIDENCE FOR THE ROLE OF SINGLET OXYGEN AND SUPEROXIDE IN THE BREAKDOWN OF PIGMENTS AND ENDOGENOUS ANTIOXIDANTS

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ABSTRACT

Chilling temperatures (5°C) and high irradiance (1000 microeinsteins per square meter per second) were used to induce photooxidation in detached leaves of cucumber (*Cucumis sativus* L.), a chilling-sensitive plant. Chlorophyll *a*, chlorophyll *b*, β carotene, and three xanthophylls were degraded in a light-dependent fashion at essentially the same rate. Lipid peroxidation (measured as ethane evolution) showed an O_2 dependency. The levels of three endogenous antioxidants, ascorbate, reduced glutathione, and α tocopherol, all showed an irradiance-dependent decline. α-Tocopherol was the first antioxidant affected and appeared to be the only antioxidant that could be implicated in long-term protection of the photosynthetic pigments. Results from the application of antioxidants having relative selectivity for $^1O_2$, O_2^-, or ·OH indicated that both $^1O_2$ and O_2^- were involved in the chilling- and light-induced lipid peroxidation which accompanied photooxidation. Application of D_2O (which enhances the lifetime of $^1O_2$) corroborated these results. Chilling under high light produced no evidence of photooxidative damage in detached leaves of chilling-resistant pea (*Pisum sativum* L.). Our results suggest a fundamental difference in the ability of pea to reduce the destructive effects of free-radical and $^1O_2$ production in chloroplasts during chilling in high light.

Photooxidation has been defined as a light- and O_2-dependent degradation of photosynthetic pigments (24). Several environmental factors can impair the photosynthetic membrane's ability to process excitation energy which may then cause the oxidative degradation of thylakoid constituents (see [24] for review). Low temperature stress is one such environmental factor. When chilling-sensitive plants are chilled (0–15°C) in the light, injury is either more severe or occurs sooner than if the same plant had been chilled in the dark. A few studies have demonstrated an oxygen requirement for, or an oxygen enhancement of, this light-induced chilling injury (25, 29, 32, 33). Chilling-sensitive plants can also be injured by dark chilling (*cf.* 15) but, by definition, this damage is of a different nature.

The light and oxygen dependencies of photooxidation suggest that the light reactions of photosynthesis may be "leaking" energy to molecular oxygen, forming one or more potentially toxic oxygen species (O_2^-, H_2O_2, ·OH, $^1O_2$). These activated oxygen molecules can have marked deleterious effects on biological systems (12). Not surprisingly, green plants possess quenching

mechanisms, concentrated in the chloroplast, that can reduce the potentially destructive effects of these toxic species. About 30 to 40% of the cellular ascorbate (6, 7, 9), 10 to 50% of the glutathione (31), all of the α tocopherol (1, 11), and all of the β carotene (11) are localized in the chloroplast even though chloroplasts occupy only 3 to 4% of the total cellular volume in a mature leaf mesophyll cell (21). Ascorbate and glutathione are both water-soluble antioxidants involved in the removal of such photosynthetically generated oxidants as O_2^- and H_2O_2 (12). α Tocopherol and β carotene are located within the membrane and, as such, probably directly quench lipid-peroxy radicals, the Chl triplet state, or $^1O_2$ in the thylakoid membrane (4, 16, 17).

In the present study, photooxidative conditions were generated by treating detached leaves of chilling-sensitive cucumber (*Cucumis sativus* L.) with cold (5°C) and light (1000 $\mu E \cdot m^{-2} \cdot s^{-1}$). Levels of Chl, four carotenoids, ascorbate, glutathione, and α tocopherol were monitored. In addition, antioxidants having limited specificities for different active oxygen species were used to ascertain the roles of these active oxygen species in photooxidation (measured as ethane production). Detached leaves of pea (*Pisum sativum* L.), a chilling-resistant plant, showed no symptoms of photooxidation when treated in parallel experiments. Evidence for lipid peroxidation and a concomitant injury to photosynthesis and chloroplast ultrastructure are reported in an accompanying article (35).

MATERIALS AND METHODS

Growth and Treatment Conditions. Cucumber (*Cucumis sativus* L., cv Ashley) and pea (*Pisum sativum* L., cv Early Alaska) plants were raised from seed in a 1:1:1 (v/v/v) mixture of gravel:vermiculite:Turface in an air-conditioned glasshouse (D/N temperature 26/20°C) of the Duke University Phytotron. Plants were watered twice daily, once with water and once with one-eighth strength Hoagland solution.

Recently expanded leaves (approximately 14 d) were excised and sealed in 22.5 ml, flat-sided, glass tissue culture flasks (T-15, Bellco Glass, Inc., Vineland, NJ). The flasks were submerged to a depth of 1 cm in a temperature bath, and irradiated by a 400 W incandescent lamp (Hi Tek Lighting, Crawfordsville, IN). Condensation was always present inside the flasks and indicated a 100% RH atmosphere.

HPLC. Leaf pigments were quantified using essentially the protocol of Eskins and Dutton (3). α Tocopherol was isolated by evaporating acetone extracts (3) to dryness onto Al_2O_3 powder *in vacuo*. The powder was transferred to a chromatographic column (80–100 mesh Al_2O_3) and eluted with hexane:ether (2:1, v/v) for the removal of α tocopherol; the Chl remained behind. The hexane/ether extract was dried *in vacuo* and the residue was redissolved in 100 μl acetone for injection into the HPLC. The column chromatography step was necessary to concentrate α tocopherol to detectable levels and to remove the Chl which

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absorb heavily in the UV. Separation was on an Altex (Berkley, CA) C18, 5 μm, R.P. column connected to a Waters Associates (Bedford, MA) model 441 detector. Detection was at 436 nm for the pigments and 280 nm for α tocopherol and quantified with a Hewlett Packard model 3900A reporting integrator. A double pump system was used to generate a gradient of methanol:water (9:1, v/v) mixed with increasing amounts of ethyl acetate (3). Peaks were identified and quantified based on their detection time as compared to authentic standards (Chl a and b from U.S. Biochemical Corp., β carotene from Sigma, α tocopherol from Eastman Kodak Co.), or by collecting them and determining their absorbance spectra in an Aminco DW2 spectrophotometer (for neoxanthin, violaxanthin, and lutein). Zeaxanthin was a kind gift of Dr. K. Eskins, Peoria, IL. Identification of the pigments was also confirmed by comparing chromatograms thus produced to Figure 2 from Eskins and Dutton (3).

Ascorbate Determination. Whole-tissue levels of ascorbate were measured using a modification of the technique of Foyer et al. (6). Leaf discs (1.8 cm^2) were given chilling treatments as described above in either light (1000 μE·m^{-2}·s^{-1}) or darkness. Single discs were ground in a glass tissue homogenizer at liquid N$_2$ temperature, homogenized in 3 ml of cold (0°C) 2.5 M HClO$_4$ (pH < 1.0), and centrifuged at 10,000g for 5 min. A 0.5 ml aliquot of the supernatant was added directly to 2.5 ml of a 200 mM succinate buffer (pH 12.7, adjusted with NaOH) in an Aminco DW2 spectrophotometer. The final pH was very near 5.6. The A at 265 nm was recorded immediately, and again 30 min after addition of 5 units of ascorbate oxidase (Sigma, from Curcubita pepo). Ascorbate was estimated from the decrease in A_{265} by comparison with a standard curve. The only modification of Foyer's et al. (6) technique was the direct use of the 2.5 M HClO$_4$ supernate instead of first raising the pH because considerable and rapid autooxidation of ascorbate was found to occur in solutions above pH 2.0 (34).

Glutathione Determination. Glutathione was measured as described by Smith (30).

Application of Antioxidants. Leaves were detached and floated abaxial side down for 30 min on a buffered (40 mM KH$_2$PO$_4$, pH 6.0) solution of Tiron[3] (Sigma), ascorbate, benzoate, formate or Dabco (Aldrich), or on unbuffered 99.9% D$_2$O (Sigma) then blotted dry. The leaves were then inserted into the glass flasks (above), submerged in the 5°C water bath, and illuminated for 8 h. One ml samples of the air above the leaves were collected for ethane measurement by GC.

Gas Chromatography. Ethane production was determined with a Varian model 3700 gas chromatograph by injecting (injector temperature = 80°C) samples of the gas inside the flasks onto a Poropak A$_2$O$_3$, 80 to 100 mesh column heated to 70°C and equipped with a flame ionization detector (detector temperature = 120°C). Ethane was identified on the basis of its retention time under the above conditions (0.79–0.81 min) as determined with authentic standards (Supelco, Bellefonte, PA) and quantified with a Hewlett Packard model 3900A reporting integrator calibrated with Supelco gas standards.

RESULTS AND DISCUSSION

Leaf discs from cucumber, a chilling-sensitive species, exhibited the classic symptoms of photooxidation when irradiated with high light (1000 μE·m^{-2}·s^{-1}) in the cold (5°C); namely, a loss of Chl (Fig. 1), and an O$_2$ dependency for injury (Table I). Concurrent damage to photosynthesis and ultrastructural alterations in the chloroplast are described in a subsequent report (35).

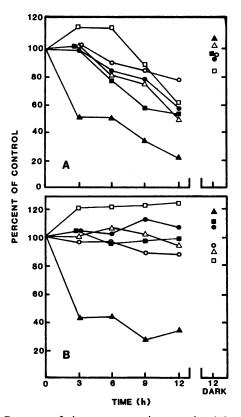In the presence of chilling stress and high light, Chl a, Chl b,

FIG. 1. Response of pigment content in cucumber (A) and pea (B) leaf discs to a 5°C chilling treatment in the presence (1000 μE·m^{-2}·s^{-1}) or the absence of light. Chl a (●); Chl b (○); β carotene (■); lutein (□); violaxanthin (▲); neoxanthin (△). Each point is the mean of 5 separate determinations. Standard errors were 5 to 15% of control values but error bars were left off of figure for clarity.

Table I. *Oxygen Dependency of Chilling- and Light-Induced Ethane Production in Cucumber Leaf Discs*

All experiments were conducted at 5°C and 1000 μE·m^{-2}·s^{-1} for 8 h under various partial pressures of O$_2$ and N$_2$. Mean ± SE, n = 8.

Atmosphere	Ethane Production (% of control)[a]
Air	100 ± 7
100% N$_2$	51 ± 4
50% O$_2$/50% N$_2$	39 ± 3
100% O$_2$	22 ± 3

[a] Control value was 10.0 ± 0.7 nmol·g^{-1} dry wt.

β carotene, and all xanthophylls in cucumber were degraded at comparable rates after a 3 h lag period (Fig. 1). Absolute, prechilling pigment levels are given in Table II. Ridley (27) demonstrated that during DCMU-induced chlorosis, β carotene is lost first, followed by Chl a then bulk xanthophylls before Chl b becomes oxidized. Our data do not statistically support that general trend. It is possible that the steps leading to bleaching in the presence of DCMU involve different processes then those found in chilling-enhanced photoxidation. Since the PSII electron transfer inhibitor atrazine was found to decrease a concurrent chilling-enhanced lipid peroxidation in cucumber (35), it is likely that DCMU-induced bleaching is not mechanistically identical to photooxidative ethane production.

Both cucumber and pea showed a marked decrease in violaxanthin and an apparent increase in lutein during the first 3 h of treatment (Fig. 1). This is probably due to the violaxanthin/zeaxanthin epoxide cycle which is commonly induced under stress conditions and converts the former pigment to the latter

Table II. *Pigment and Antioxidant Content of Prechilled Cucumber and Pea Leaves*

Mean ± SE, $n \geq 5$.

	Cucumber	Pea
	$nmol \cdot g^{-1}$ fresh wt	
Chl a	1200 ± 60	1030 ± 50
Chl b	520 ± 30	510 ± 30
Lutein	110 ± 5	130 ± 20
β Carotene	94 ± 8	94 ± 2
Neoxanthin	37 ± 3	40 ± 5
Violaxanthin	48 ± 3	48 ± 5
α Tocopherol	51 ± 5	65 ± 5
Ascorbate	6250 ± 520	7440 ± 390
Glutathione	200 ± 20	110 ± 10

(27). Zeaxanthin could not be determined directly in our laboratory with reverse phase-HPLC in spite of numerous attempts to separate it from lutein. Therefore, the apparent lutein increase (which equaled the amount of violaxanthin lost) may well have actually been zeaxanthin synthesis. We do not believe that *de novo* lutein synthesis caused the apparent lutein increase. Following this initial change in violaxanthin and lutein, they were both degraded in cucumber at a rate similar to the other pigments. In pea, the only pigment changes noted were for lutein and violaxanthin; a steady state for these was reached in the first 3 h of treatment after which no further change occurred.

The reduction in ethane production under a 100% N_2 atmosphere (Table I) was consistent with the known oxygen dependency of chilling-induced photosynthetic inhibition (25, 29, 33) and pigment bleaching (24, 32). Interestingly, ethane levels also decreased in a 50% or 100% O_2 atmosphere (Table I) with a concomitant increase in an unidentified small mol wt compound. The latter ran between ethane and acetaldehyde on the GC but was shown not to be propane, butane, or pentane (34). We believe these data indicate that increased partial pressures of O_2 altered the degradation products of lipid peroxidation resulting in less ethane and more of the unidentified gas. The alternative explanation, that the high concentration of O_2 suppressed the peroxidation, seems much less plausible. Furthermore, ethylene production showed an expected oxygen stimulation in these plants (34), so O_2 was clearly entering the cells and exerting an influence on metabolism.

About 50% of the total cellular ascorbate in cucumber leaf discs was oxidized at a relatively constant rate during the chilling and high light treatment (Fig. 2A). In contrast, dark chilling of cucumber, and dark or light chilling of pea caused no such loss of ascorbate (Fig. 2). Ascorbate plays a major role in chloroplast metabolism; 30 to 40% of total ascorbate is chloroplastic (5, 7, 9) and stromal concentrations as high as 50 mM have been reported (6, 8). One recently elucidated role of ascorbate is in an enzymic NADPH/glutathione/ascorbate cycle that removes photosynthetically-generated O_2^- and H_2O_2 (12). Ascorbate may also directly reduce O_2^- (20), quench $^1O_2$ (28), and regenerate reduced α tocopherol (17, 23). Any of the routes for ascorbate oxidation listed above could be functioning in cold- and light-stressed cucumber leaf discs because the data presented in this and an accompanying report provide evidence for the presence of O_2^- (35), $^1O_2$ (Table I), GSSG (Fig. 3), and oxidized α tocopherol (Fig. 4).

Ascorbate levels in pre-chilled cucumber and pea leaves (Table II) were 4- to 6-fold higher than those reported for spinach leaf discs using the same assay (6). We found considerable ascorbate oxidation during extraction at pH above 2.0 (34) and feel that our modification of Foyer's *et al.* (6) technique (see "Materials and Methods") accounts for the high ascorbate levels we report.

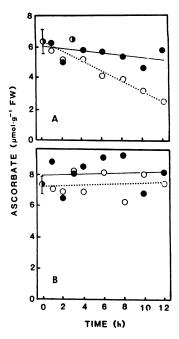Glutathione is a sulhydryl-containing tripeptide thought to be

FIG. 2. Response of ascorbate in cucumber (A) and pea (B) leaf discs treated at 5°C and either dark (●, ——) or light (1000 $\mu E \cdot m^{-2} \cdot s^{-1}$) (O, - - -). Results are the mean of 5 replicates. Representative SE given for control values; all others were similar.

involved in stabilizing enzymes possessing exposed thiol groups. It may also, through its ability to reduce dehydroascorbate (5), function in the chloroplast O_2^- and H_2O_2 scavenging system (12). Ten to 50% of the cellular glutathione is chloroplastic while the remainder is cytosolic (9, 31). Over 95% of each pool has been reported to be reduced (5) although Smith *et al.*(31) report that up to 35% of the chloroplast glutathione may be oxidized.

Levels of reduced glutathione are maintained in two ways. First, glutathione reductase (EC 1.6.4.2), which uses NADPH to reduce GSSG, has been localized in the chloroplast, and acts to maintain GSH:GSSG ratios above 0.9 (13). Second, biosynthesis and degradation processes influence levels of total glutathione; glutathione synthetase (EC 6.3.2.3) may be under feedback inhibition control by GSH (26). The dramatic increase in GSSG and decrease in total GSH in cold- and light-treated cucumber leaves (Fig. 3B) suggest that both oxidation and degradation were removing glutathione. This correlates well with the other indicators of photooxidation shown to be occurring in these tissues. Cucumber chilled in the dark, as well as pea chilled in either the light or dark, demonstrated a small but steady decrease in total glutathione (Fig. 3).

Levels of the lipid-soluble antioxidant, α tocopherol, decreased by a factor of five during the first 3 h of treatment in cucumber leaf discs exposed to 5°C and high light then continued to decline at a much slower rate for the remainder of the 12 h treatment (Fig. 4A). In contrast, α tocopherol levels in pea remained unchanged (Fig. 4B). The biphasic nature of α tocopherol degradation in chilled and irradiated cucumber suggests two available pools, one near the site of oxidative injury and one further removed. All of the cellular α tocopherol in higher plants has been localized in the chloroplast (1, 11), with approximately two-thirds in the thylakoid membrane and the remaining one-third in the envelope (calculated from the data of [2] and [18]).

Chl and carotenoid degradation began 3 h after degradation of α tocopherol was already underway in chilled and irradiated cucumber leaves (Fig. 4A). If the first phase of α tocopherol breakdown represents a larger, thylakoid pool, *i.e.* the one nearest the site of triplet state Chl and $^1O_2$ generation, then this pool
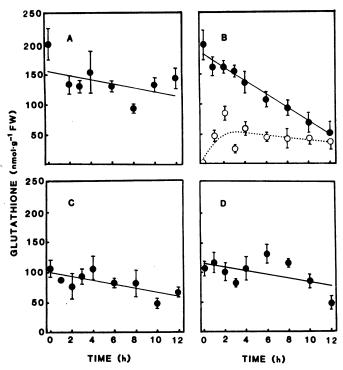
FIG. 3. Response of glutathione in cucumber (A, B) and pea (C, D) leaf discs treated at 5°C and either dark (A, C) or light (1000 μE·m^{-2}·s^{-1}) (B, D). Total glutathione (●, ——); GSSG (O, - - -). Results are the mean ± SE for six replicates. Solid lines are linear regressions through all 54 points. Dashed line for GSSG fitted by eye.
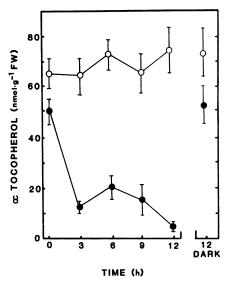

FIG. 4. Response of α tocopherol in cucumber (●) and pea (O) leaf discs treated at 5°C and 1000 μE·m^{-2}·s^{-1} or 12 h and darkness. Results are the mean ± SE for five separate determinations.

may be the first line of defense for protecting photosynthetic pigments. Pigment destruction presumably then began only after the thylakoidal α tocopherol was depleted.

α Tocopherol is freely dissolved in the thylakoid membrane (36) and serves as a powerful antioxidant with several possible modes of action. First, it can function as a "chain-breaking" antioxidant by trapping fatty acyl peroxy radicals formed during lipid peroxidation, breaking the chain reaction at that point. The α tocopherol can be subsequently regenerated by ascorbate (17, 23). Second, α tocopherol can reduce O_2^- to produce H_2O_2 and α tocopherolquinone (20). Third, α tocopherol can react with

Table III. *Effect of Preincubation with Various Antioxidants or D_2O on Ethane Production during a Chilling and Light Treatment*

All experiments were conducted for 8 h at 5°C and an irradiance of 1000 μE·m^{-2}·s^{-1}. Antioxidants were applied during a 30 min preincubation as a solution in 40 mM KH$_2$PO$_4$ buffer (pH 6.0). Mean ± SE, n = 8.

Treatment	Ethane Production (% of control)[a]
40 mM KH$_2$PO$_4$	100 ± 9
50 mM Tiron	29 ± 2
10 mM Tiron	50 ± 1
5 mM Tiron	67 ± 4
1 mM Tiron	69 ± 4
100 mM Ascorbate	52 ± 2
10 mM Ascorbate	94 ± 4
10 mM Benzoate	94 ± 5
10 mM Formate	105 ± 5
100 mM Dabco	58 ± 2
10 mM Dabco	73 ± 4
D$_2$O	146 ± 10

[a] Control rate was 10.5 ± 0.9 nmol·g^{-1} dry wt.

$^1O_2$ and either directly quench the latter or react with $^1O_2$ and be degraded (4). A single molecule of α tocopherol can undergo approximately 100 $^1O_2$ quenching events before it is oxidized to α tocopherolquinone (4). In our studies, considerable lipid peroxidation occurred in cucumber (35) concurrent with the loss of α tocopherol (Fig. 4). This peroxidation would provide an ample supply of lipo-peroxy radicals for reaction with α tocopherol. In addition, evidence for the presence of both O_2^- (Table III; Ref. 35) and $^1O_2$ (Table III) has been presented. Therefore, multiple routes for α tocopherol degradation probably existed in the chilled and irradiated cucumber leaf discs. Of special significance is the absence of α tocopherol degradation in similarly treated pea leaves.

We have previously reported that cucumber leaf discs suffer extensive lipid peroxidation in the cold and light (35). Therefore, the identity of the oxygen species involved in this photooxidative injury was investigated in cucumber leaves via the use of known, and fairly specific, antioxidants (Table III). Two superoxide anion scavengers, Tiron (10) and ascorbate (20), decreased ethane production. We therefore suggest a role for O_2^- during photooxidation (35). Singlet oxygen, however, was also implicated because Dabco, a $^1O_2$ quencher (22), decreased ethane production while D$_2$O, an enhancer of $^1O_2$ (19), increased it. The lack of an effect with benzoate and formate, scavengers of the hydroxyl radical (14), cannot be used to disprove conclusively a role for ·OH due to the extreme complexity of lipid peroxidation in a heterogenous system (12). These data are strictly qualitative because the antioxidant or D$_2$O concentration inside the chloroplast is not known. The data do, however, provide strong supporting evidence that both $^1O_2$ and O_2^- are involved in chilling-enhanced photooxidation in cucumber as has been suggested by Powles *et al.* (25). It is quite possible that similar types of mechanisms are responsible for lipid and pigment breakdown in other systems exhibiting photooxidation (24, 27, 32).

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