

# Photophosphorylation after Chilling in the Light<sup>1</sup>

## Effects on Membrane Energization and Coupling Factor Activity

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### ABSTRACT

The response of *in situ* photophosphorylation in attached cucumber (*Cucumis sativus* L. cv Ashley) leaves to chilling under strong illumination was investigated. A single-beam kinetic spectrophotometer fitted with a clamp-on, whole leaf cuvette was used to measure the flash-induced electrochromic absorbance change at 518 minus 540 nanometers ( $\Delta A_{518-540}$ ) in attached leaves. The relaxation kinetics of the electric field-indicating  $\Delta A_{518-540}$  measures the rate of depolarization of the thylakoid membrane. Since this depolarization process is normally dominated by proton efflux through the coupling factor during ATP synthesis, this technique can be used, in conjunction with careful controls, as a monitor of *in situ* ATP formation competence. Whole, attached leaves were chilled at 5°C and 1000 microeinsteins per square meter per second for up to 6 hours then rewarmed in the dark at room temperature for 30 minutes and 100% relative humidity. Leaf water potential, chlorophyll content, and the effective optical pathlength for the absorption measurements were not affected by the treatment. Light- and CO<sub>2</sub>-saturated leaf disc oxygen evolution and the quantum efficiency of photosynthesis were inhibited by approximately 50% after 3 hours of light chilling and by approximately 75% after 6 hours. Despite the large inhibition to net photosynthesis, the measurements of  $\Delta A_{518-540}$  relaxation kinetics showed photophosphorylation to be largely unaffected by the chilling and light exposure. The amplitude of the  $\Delta A_{518-540}$  measures the degree of energization of the photosynthetic membranes and was reduced significantly by chilling in the light. The cause of the decreased energization was traced to impaired turnover of photosystem II. Our measurements showed that the chilling of whole leaves in the light caused neither an uncoupling of photophosphorylation from photosynthetic electron transport nor any irreversible inhibition of the chloroplast coupling factor *in situ*. The sizeable inhibition in net photosynthesis observed after chilling in the light cannot, therefore, be attributed to any direct effect on photophosphorylation competence.

with illumination (10, 12, 22, 24). The inhibition is partially reversed by elevated CO<sub>2</sub> levels (2, 12), implicating a stomatal-mediated component. However, only a portion of the chilling-induced inhibition of photosynthesis can be overcome by saturating CO<sub>2</sub>, revealing that the largest component of the inhibition lies at the level of the chloroplast itself.

The chilling of whole plants in the light causes a reduction in the electron transfer activity of thylakoids isolated from the chilled leaves. However, Kee *et al.* (6) have shown that even though whole chain electron transport was substantially inhibited by chilling in the light, the residual activity was in substantial excess of that needed to support the measured rates of light- and CO<sub>2</sub>-saturated net photosynthesis. Thus, these reductions in the turnover capacity of electron transport carriers cannot be the direct cause of light- and chilling-induced inhibition of net CO<sub>2</sub> reduction.

The functioning of many chloroplast processes is vitally dependent upon the availability of ATP. Thus, before any potential direct effects of chilling can be assigned to these "ATP-dependent" processes, it is important to establish if the ATP formation competence of chill-sensitive plants is compromised by the stress. Photophosphorylation is a highly regulated and relatively labile process and thus seemingly a prime candidate for disruption by environmental stress. Indeed, adverse effects of chilling on ATP formation competence have been reported (3, 8, 17, 20), but the relevance of these *in vitro* studies to the *in situ* inhibition is difficult to evaluate. A special complication associated with studying stress-induced perturbations is distinguishing inhibitions that are actually part of the *in vivo* process from inhibitions induced through manipulation of tissues made labile by the stress.

In this study we investigated the response of membrane energization and coupling factor activity in attached leaves to chilling in the light by monitoring the flash-induced electrochromic absorption bandshift (26) measured as an absorption change at 518 minus 540 nm ( $\Delta A_{518-540}$ ). The basis for this measurement of *in situ* ATP formation is that the membrane depolarizing proton efflux through the coupling factor complex that drives the ADP phosphorylation reaction results in a more rapid relaxation of the electric potential produced by the light flashes. The degree of membrane energization can be judged from the amplitude of the flash-induced change. From an analysis of the electric field depolarization kinetics of cucumber leaves chilled in the light we conclude that,

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Temperatures lower than about 10°C induce in many species of higher plants persistent and even irreversible reductions in light-saturated photosynthesis after rewarming. Low temperature-induced inhibition of photosynthesis develops more rapidly and is more severe if the chilling occurs concurrent

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although photophosphorylation shows some marginal perturbation, the dysfunction is not severe enough to be the direct cause of the inhibition observed in light- and CO<sub>2</sub>-saturated photosynthesis.

## MATERIALS AND METHODS

### Plant Growth Conditions

Cucumber plants (*Cucumis sativus* L. cv Ashley) were raised from seed in a soil/peat/vermiculite mixture, watered daily and fertilized weekly. The plants were grown in a controlled environment chamber (600 to 800  $\mu\text{E}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$  PAR, 14 h photoperiod, day/night temperatures of 23°C/20°C) as detailed elsewhere (12). Attached leaves which had almost reached full expansion (approximately 3 weeks) were used for the various *in vitro* or *in situ* measurements.

### Chilling Treatments

All chilling treatments were initiated at 10:00 am, 2 h into the photoperiod. Whole plants were wrapped in moistened Labmat<sup>2</sup> (a two-ply, plastic/absorbant paper, disposable bench covering) with only a 15 cm<sup>2</sup> area of the leaf left exposed to illumination in order to prevent excessive water loss which could otherwise confound our studies. This leaf was positioned between two horizontal frames and the entire plant/frame assembly placed in a refrigerated cooler set at 2°C. Light (1000  $\mu\text{E}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$  PAR) was provided by a 200 W spot light and passed through an IR reflecting mirror (Optical Coatings Laboratory, Inc., Santa Rosa, CA) and 5 cm of water before reaching the leaf. Abaxial leaf temperature was measured with a thermocouple thermometer and fluctuated between 4 to 6°C.

### Photosynthetic Measurements

Oxygen evolution from 10 cm<sup>2</sup> leaf discs was measured at 23°C and 5% (v/v) CO<sub>2</sub> using a Hansatech LD1 polarographic oxygen electrode (Hansatech Ltd., Norfolk, England) as described in Delieu and Walker (1). Irradiance was provided by a slide projector lamp and attenuated with neutral density filters. Light saturation curves were constructed by starting at the lowest irradiance and increasing, stepwise, to full intensity (1550  $\mu\text{E}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$  PAR). Quantum yields were calculated from the linear portion of the curve at irradiances below 200  $\mu\text{E}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$  PAR. Absorbed irradiance was determined by subtracting reflected and transmitted light from incident light (11). Reflected light was measured 1 cm above the leaf surface at an angle of 45° and transmitted light was measured directly beneath the leaf, with a LiCor LI-190S quantum sensor (LiCor Inc., Lincoln, NE). In 15 determinations made on attached cucumber leaves, the amount of reflected light was  $2.1 \pm 0.1\%$  and the amount of transmitted light was  $8.4 \pm 0.3\%$  of incident from 40 to 1400  $\mu\text{E}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$  PAR. On this basis, the

absorbed light was calculated to be 89.5% of incident irradiance.

### Leaf Water Potential Measurements

Leaf water potentials ( $\psi_L$ <sup>3</sup>) were determined using a laboratory-built isopiestic thermocouple psychrometer (11).

### Chl Determination

Chl concentrations in 80% acetone extracts were calculated according to equations derived elsewhere (4) using the specific absorption coefficients for Chl *a* and *b* of Ziegler and Egle (27).

### Spectrophotometric Measurements of *in Vivo* Flash-Induced Absorbance Changes

The flash-induced electrochromic absorption band shift was measured using a laboratory-built single beam spectrophotometer. An attached leaf was positioned in a foam-lined cuvette clamp. A shuttered, dim ( $6 \times 10^{-2}$  J·m<sup>-2</sup>·s<sup>-1</sup>) measuring beam (4 nm band width) was provided by a diffraction monochromator (Model AH10, Instrument SA Inc., Metuchen, NJ), and delivered to a 1 cm diameter spot on the adaxial leaf surface via a fiber optic light guide. The measuring beam passed through the leaf, was collected from the abaxial surface by one arm of a bifurcated light guide and routed to a photomultiplier tube (Hamamatsu R268). The saturating actinic flash was generated by a xenon lamp (6  $\mu\text{s}$  duration at half peak height; Model FX-193 Flash Tube, EG & G, Salem, MA), passed through a red blocking filter (Corning CS 2-59), and delivered to the abaxial surface through the second arm of the bifurcated light guide. Based on the predicted exponential saturation of the reaction centers as a function of increasing flash intensity, it was established from light attenuation experiments that the xenon flashes were more than 98% saturating. In certain experiments in which high flash frequencies were required, weaker flashes (~50% saturating) were used (3-8  $\mu\text{s}$  duration at half peak height; model FX-200 flash tube, E G & G, Salem, MA). The actinic flash was excluded from the photomultiplier tube by filters (a DT, green, wide band interference filter, 66.1055 Rolyon, Arcadia, CA and a Corning CS 4-96) and by the positioning of the light guides. The amplified signal was digitized and the digitized signals summed (Nicolet Model 1174, Nicolet Inst. Corp., Madison, WI) for signal averaging. The kinetic trace of  $\Delta A_{518-540}$  can be seen to have a biphasic decay. Since only the initial, faster component of decay is associated with photophosphorylation (18) the slow component was subtracted. The remaining fast decay was expressed as the relaxation time constant,  $\tau$ , according to the equation  $\Delta A_{518-540} = \Delta A_{518-540} \max e^{-t/\tau}$  where both *t* (time) and  $\tau$  are in ms.

<sup>3</sup> Abbreviations:  $\psi_L$ , leaf water potential; DCCD, *N,N'*-dicyclohexylcarbodiimide; Tween 20, polyoxyethylene sorbitan monolaurate;  $\beta$ , intensification factor (the extent to which leaf architecture increases the absorbance due to lengthening of the optical path);  $\lambda$ , wavelength;  $\Delta p$ , transmembrane protonmotive force;  $\Delta\text{pH}$ , transmembrane pH difference; CF<sub>1</sub>, coupling factor 1.

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### Treatment of Attached Leaves with DCCD

A 15 cm<sup>2</sup> area of both abaxial and adaxial sides of a leaf were lightly abraded with 400 grit carborundum and washed with water. A solution of 10 mM DCCD, 2% (v/v) methanol, and 1% (v/v) Tween 20 was applied to both surfaces for 30 min while the entire plant was kept in the dark at room temperature and 100% RH.

### Measurement of Flash-Induced H<sup>+</sup> Production from Water Oxidation

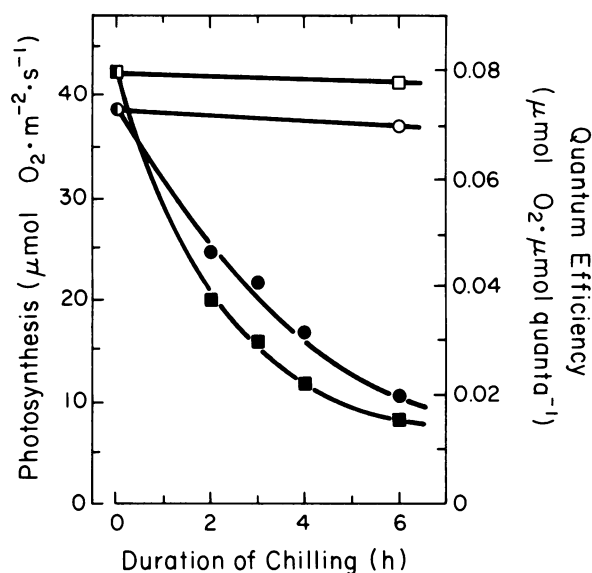
Cucumber leaves were chilled in the light as described above. Following a 30 min dark period of rewarming, one leaf disc (10 cm<sup>2</sup>) was removed from each of two illuminated leaves and both discs were ground in a mortar and pestle in 2.5 mL of medium containing 0.3 M NaCl, 30 mM Tricine-NaOH (pH 7.8), 3 mM MgCl<sub>2</sub>, and 0.5 mM Na<sub>2</sub>-EDTA. The homogenate was filtered through four layers of Miracloth, and centrifuged for 15 s in an Eppendorf benchtop microfuge. The pellet was resuspended in 1.25 mL of a medium containing 0.2 M sorbitol, 5 mM HEPES-NaOH (pH 7.5), 2 mM MgCl<sub>2</sub>, and 0.5 mg/mL BSA (essentially fatty acid free) and centrifuged as above. The pellet was washed in 1.25 mL of a medium containing 0.1 M sorbitol, 0.5 mM Tricine-NaOH (pH 7.8), 5 mM MgCl<sub>2</sub>, and 25 mM KCl and centrifuged as above. The final pellet was resuspended in 2.5 mL of this medium. This procedure resulted in the recovery of 80 to 120 nmol Chl; that is, approximately 10% of the total Chl originally present in the two leaf discs.

The number of PSII centers capable of liberating a proton upon activation by a sequence of single turnover saturating flashes was determined according to Whitmarsh and Ort (25) using an Orion 9103 pH electrode and Keithley 610c electrometer. Nigericin (2.0 nmol) and K<sub>3</sub>Fe(CN)<sub>6</sub> (1 nmol) were added to the 2.5 mL thylakoid suspension from above that contained 80 to 120 nmol Chl. The cuvette was thermostatted at 23°C. At 10 s intervals, the sample was given a series of 10 single-turnover saturating flashes (3 Hz). The pH change was calibrated by the addition of 5 or 10 nmol HCl to the reaction volume immediately after the 50 total flashes. The synchronous actinic flashes were delivered by a pair of xenon lamps (FX-200 flash-tube, E G & G, Salem, MA) positioned on both sides of the cuvette. The combined effect of these simultaneous flashes (3.8 μs at half-peak height) was greater than 98% saturating.

## RESULTS

### Decreases in the Quantum Efficiency and in the Light-Saturated Rate of O<sub>2</sub> Evolution Caused by Chilling in the Light

Chilling attached cucumber leaves at 5°C under strong illumination (1000 μE·m<sup>-2</sup>·s<sup>-1</sup> PAR) caused a progressively severe inhibition of photosynthesis measured after the leaf had been rewarmed in the dark at 23°C. This inhibition was seen both as a decline in the quantum efficiency (■) as well as a reduction in the light- and CO<sub>2</sub>-saturated rate (●) of leaf disc O<sub>2</sub> evolution (Fig. 1). After 6 h of illumination both had declined to scarcely 25% of control values whereas neither of



**Figure 1.** Decreased quantum efficiency and inhibited rates of light- and CO<sub>2</sub>-saturated photosynthesis induced by chilling in the light (5°C, 1000 μE·m<sup>-2</sup>·s<sup>-1</sup>). Open symbols (○, □) represent plants treated for 6 h at 23°C and 1000 μE·m<sup>-2</sup>·s<sup>-1</sup>. Chilled plants (●, ■) were rewarmed for 30 min in the dark at 100% RH and room temperature prior to measurements. Photosynthesis (○, ●) was measured as leaf disc oxygen evolution at 23°C and 5% (v/v) CO<sub>2</sub> at an irradiance of 1300 μE·m<sup>-2</sup>·s<sup>-1</sup> PAR. The quantum efficiency (□, ■) of photosynthesis was calculated based on an absorbance of 89.5% of the incident irradiance by the leaf disc. The data points presented are the mean of six independent measurements. Standard errors were 3 to 9% of the mean value.

**Table I.** Leaf Water Potential ( $\Psi_L$ ) and Chl Content of Leaves after Chilling (5°C) in the Light (1000 μE·m<sup>-2</sup>·s<sup>-1</sup>)

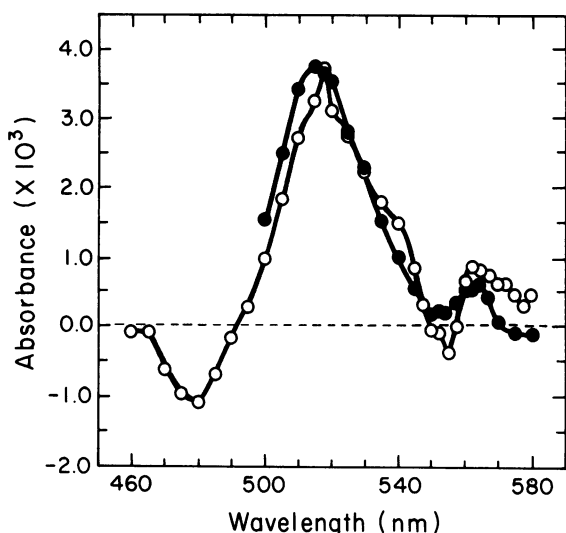
Mean values and standard errors are reported with the sample size given in parentheses.

Duration of Chilling	$\Psi_L$	Chl
<i>h</i>	<i>MPa</i>	<i>nmol·cm<sup>-2</sup></i>
0	-0.30 ± 0.01 (5)	43.3 ± 1.6 (17)
3	-0.35 ± 0.01 (5)	42.0 ± 0.7 (5)
6	-0.39 ± 0.02 (5)	40.8 ± 1.8 (8)

these photosynthetic parameters was much affected by 6 h of illumination at 23°C (○, □). The observed inhibition to photosynthesis was not accompanied by significant leaf water deficit or Chl loss (Table I).

### Measurement of the Flash-Induced Electrochromic Absorption Change in Attached Leaves

Shown in Figure 2 is the wavelength dependence of the absorption change measured 8 ms after an actinic flash in an attached cucumber leaf (○). The flash-induced spectrum is indistinguishable from spectra we have observed for other plants (15, 16) and very similar to the spectra observed by us (Fig. 2, ●) and others (*e.g.* ref. 26) in isolated thylakoids. The spectrum is dominated by the electrochromic absorption bandshift but a contribution from the flash-induced turnover



**Figure 2.** Wavelength dependence of the flash-induced absorbance change in attached cucumber leaves (O) and isolated spinach thylakoids (●). Absorbance change amplitudes were taken 8 ms after the actinic flash. Depending upon wavelength, between 8 and 64 flashes (delivered at 2 Hz) were summed to improve the resolution of measurement.

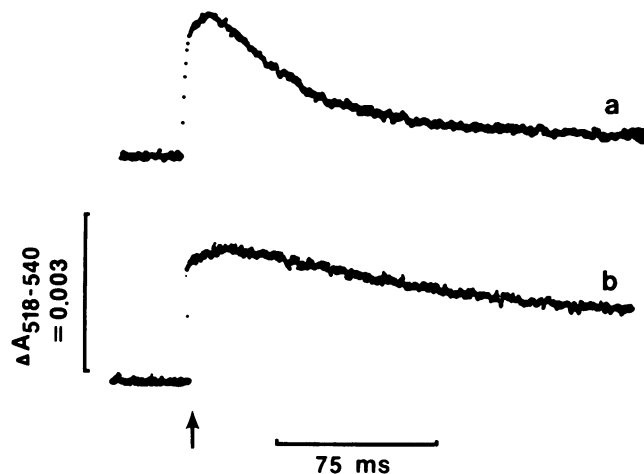
of Cyt *f* and *b*<sub>6</sub> is evident in the 550 to 570 nm region. As is common practice with measurements on isolated thylakoid membranes, for the data presented on attached leaves in Figures 3, 4, and 5, we used a reference wavelength of 540 nm to isolate the electrochromic absorption change from any other contributory factors such as the cytochrome redox changes mentioned above and scattering changes that exhibit a relatively broad and featureless spectrum.

A typical signal-averaged trace of the flash-induced electrochromic change ( $\Delta A_{518-540}$ ) in an attached dark-adapted cucumber leaf is shown in Figure 3a. The 40 ms relaxation time constant of the electrochromic change is characteristic of the rate of membrane depolarization associated with active ATP synthesis. Evidence that the relaxation of the electrochromic change is dominated by proton egress through the ATP synthetase is provided by the effect that the ATP synthesis inhibitor DCCD has on the kinetics. A ten-fold increase in the relaxation time constant to 400 ms (Fig. 3b) is consistent with the known action of DCCD preventing proton efflux through the integral membrane protein portion of the coupling factor complex.

#### Effect of Chilling in the Light on the Size and Relaxation Kinetics of the Flash-Induced Electrochromic Absorption Change

Chilling attached cucumber leaves in the light caused a progressive loss in the flash-induced amplitude of the  $\Delta A_{518-540}$  (Figs. 4 and 5A). After 6 h of exposure to low temperature and high light the flash-induced absorption change had all but disappeared.

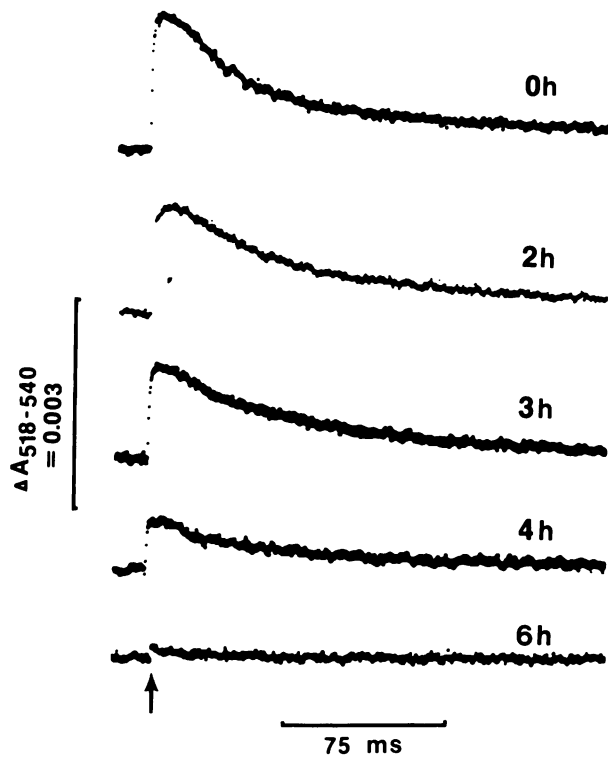
Despite the severe effect that chilling in the light had on the flash-induced amplitude, there was only a modest effect on the rate of decay; that is, on the rate of ATP formation. Analysis of the exponential decay of the traces in Figure 4



**Figure 3.** Effect of the coupling factor inhibitor DCCD on the relaxation kinetics of the flash-induced electrochromic absorption change. A control leaf was dark-adapted for 30 min prior to recording trace a. The following day the same leaf was treated for 30 min in the dark with a surface application of 10 mM DCCD in 2% methanol and 1% Tween 20 prior to recording trace b. Similar treatments of abraded leaves with solutions lacking DCCD were without effect. The arrow indicates the positioning of the excitation flash. The absorbance changes induced by eight flashes delivered at 3 Hz were summed for each trace.

reveals an increase in the time constant from 45 ms in control plants to about 70 ms for plants chilled in the light (Fig. 5B, ■). Increasing the frequency (Fig. 5B, ▲) at which the flashes are delivered to the leaf both lowers the value of relaxation time constant as well as reduces the effect that chilling in the light has on it. The higher flashing rate results in a larger driving force for ATP formation because, as the sequential flashes become more closely spaced, their contribution to membrane energization becomes increasingly additive (*e.g.* ref. 16).

It was of interest to discover whether the decrease in amplitude of the  $\Delta A_{518-540}$  caused by chilling in the light (Figs. 4 and 5A) reflected a correspondingly smaller membrane potential or, alternatively, was caused by a decrease in the optical pathlength through the leaf. The influence of chilling in the light on the optical path taken by the measuring beam passing through the leaf was investigated following the procedure of Rühle and Wild (19). Figure 6 shows the absorbance spectrum of an attached cucumber leaf, before (trace a) and after (trace b) chilling in the light. Trace c is the absorbance spectrum of a suspension of cucumber thylakoids in which the Chl content was adjusted to be equivalent to that of the leaf on a surface area basis. Trace d is the spectrum of a methanol-extracted, pigment-free leaf in which the apparent absorbance from 500 to 750 nm is due only to reflection and light scattering intrinsic to the leaf's architecture. By subtracting trace d from the leaf spectra (traces a and b) the contribution of light dispersion can be minimized and the absorbance of the pigments themselves estimated. The intensification of absorbance due to increased pathlength caused by leaf architecture is then the ratio of the leaf absorbance ( $A_L$ ) to that of the thylakoid suspension ( $A_T$ ) at any discrete wavelength. Values

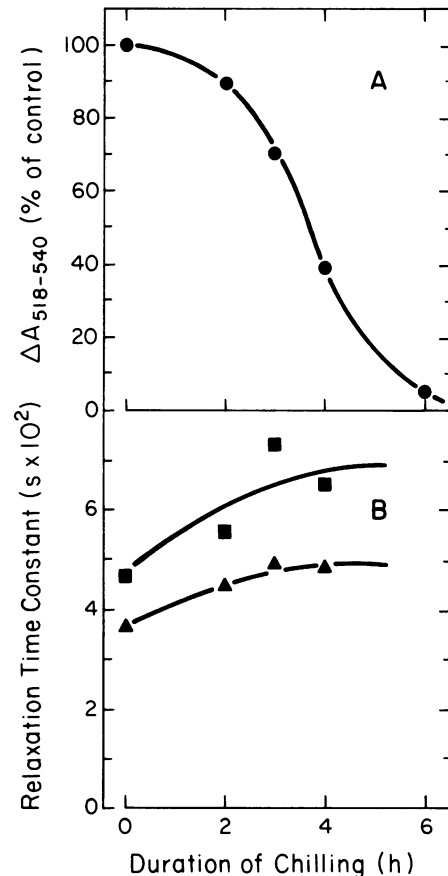


**Figure 4.** Effect of chilling in the light on the  $\Delta A_{518-540}$  relaxation kinetics in attached cucumber leaves. Five individual plants were chilled ( $5^{\circ}\text{C}$ ) under an irradiance of  $1000 \mu\text{E}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$  PAR for up to 6 h as indicated. The signal averaged traces presented are the result of the normalized average of 16 actinic events at 540 nm subtracted from the normalized average of 8 actinic events at 518 nm. The vertical arrow indicates the positioning of the single turnover flash. The flashes were given at 0.1 Hz.

for the intensification factor ( $\beta$ ) are given for four wavelengths in Table II for control and leaves chilled in the light. The values of  $\beta$  at 700 nm (2.55) and 677 nm (1.41) are in good agreement with those reported by Rühle and Wild (19) at 700 nm ( $2.44 \pm 0.10$ ) and 680 nm ( $1.70 \pm 0.10$ ) for *Sinapsis alba*. Chilling the attached cucumber leaf from trace a for 3 h in the light (trace b) did not significantly affect the leaf absorbance at 518 or 540 nm.

#### Inhibition of PSII Turnover by Chilling in the Light

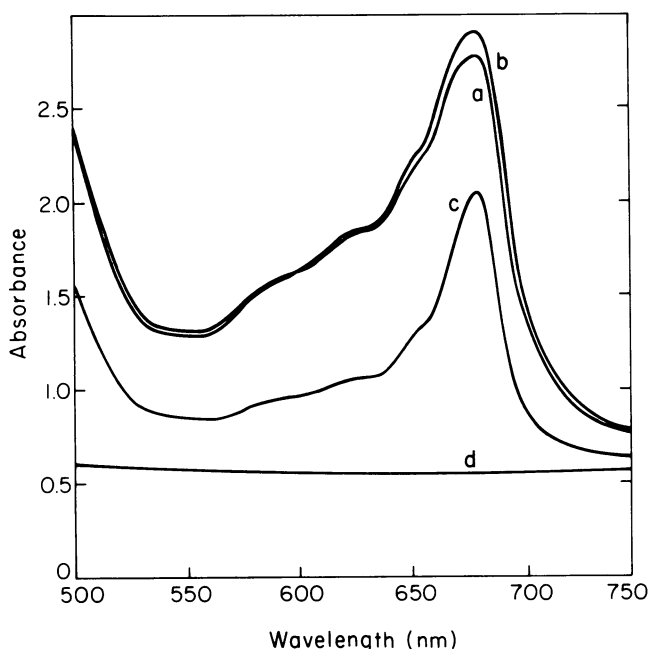
Since the light chilling-induced decrease in the  $\Delta A_{518-540}$  cannot be accounted for by a change in optical path length it probably reflects a true decrease in the flash-induced electric field across the thylakoid membrane. To determine if the decrease in energization arose from an inhibition of electron transfer, we used 10 closely spaced (40 Hz) half-saturating flashes to energize the leaves. The rationale of the experiment is that nearly the same membrane potential should be attainable from the cumulative energization of fewer active components turning over a greater number of times providing that the flashes are spaced closely enough to be additive. Indeed, although requiring more flashes, leaves that had been chilled for 3 h in the light (Fig. 7, trace b) generated a



**Figure 5.** A,  $\Delta A_{518-540}$  in attached cucumber leaves as a function of chilling exposure. The amplitude of the rapidly relaxing component of the absorption change at the time of the flash was determined by regression analysis of the exponential decay (see "Materials and Methods"). The data presented are the mean values from 24 control and 24 (*i.e.* six plants for each time of chilling) chilled plants. The standard errors ranged from 6 to 12% of the mean values. The mean  $\Delta A_{518-540}$  value for the 24 control plants was  $2.1 \times 10^{-3} \pm 0.0001$ . B, Relaxation time constants of the flash-induced  $\Delta A_{518-540}$  in attached cucumber leaves as a function of chilling exposure. The rate of relaxation of the electrochromic change was measured at flash frequencies of 0.1 Hz (■) and 2.0 Hz (▲). The mean values of six independent determinations are plotted for which the standard errors ranged from 4 to 10% of the mean values. The differences in the  $\tau$  values before and after chilling obtained for different plants were tested using an analysis of variance on a repeated measures design, testing  $\tau$  against the within-plant residual error. Differences among the three chilling treatments were tested using a one-way analysis of variance at a 5% level of significance.

comparable "steady state" membrane potential as the unchilled controls (Fig. 7, trace a).

To assess the competency of PSII, we measured flash-induced water oxidation in thylakoid membranes isolated from control leaves and leaves that had been chilled in the light. The concentration of active PSII centers was determined from measurements of proton production due to water oxidation (25). Because each flash was intense enough to excite every reaction center (>98% saturating) yet short enough to ensure that each reaction center turned over only a single time (*i.e.* less than 10% double hits), the number of protons



**Figure 6.** Measurement of absorption intensification in control and chilled cucumber leaves. Absorbance spectra for an attached cucumber leaf before (trace a) and after (trace b) a 3 h treatment of 5°C and 1000  $\mu\text{E}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$  are shown. Trace c is a spectrum of a suspension of cucumber thylakoids at a Chl content per  $\text{cm}^2$  equal to that of the control leaf (trace a). Trace d is of a pigment-free, methanol-extracted cucumber leaf that was used as the baseline for the determination of the true absorbance of the leaf and the thylakoid suspension. See "Materials and Methods" and ref. 19 for specifics of the calculations.

**Table II.** Effect of 3 h of Chilling (5°C) in the Light (1000  $\mu\text{E}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$ ) on the Intensification Factor of Cucumber Leaf Absorbance at Various Wavelengths

The intensification factor,  $\beta$ , is the ratio of the leaf absorbance ( $A_L$ ) to the thylakoid suspension absorbance ( $A_T$ ) and represents the degree to which the leaf architecture increases the effective optical pathlength for absorbance measurements.

$\lambda$	Control			Chilled			$\beta_L/\beta_T$
	$A_L$	$A_T$	$\beta_1$	$A_L$	$A_T$	$\beta_2$	
nm							
700	0.54	0.21	2.55	0.56	0.21	2.64	0.96
677	2.00	1.42	1.41	2.04	1.42	1.44	0.98
540	0.53	0.21	2.48	0.51	0.21	2.38	1.04
518	0.81	0.41	1.97	0.80	0.41	1.95	1.01

released from water was equal to the number of PSII centers present in the sample that were able to turn over at a rate of at least three times per s. The data in Figure 8 show that 3 h of chilling in the light (●) caused about a 25% decrease in flash-induced water oxidation, comparable to the decrease in the flash-induced  $\Delta A_{518-540}$  (Fig. 5A).

#### Effect of Chilling in the Light on Light-Adaptation of *In Situ* ATP Synthesis

Preillumination of a leaf with continuous light results in more rapidly relaxing flash-induced electrochromic changes

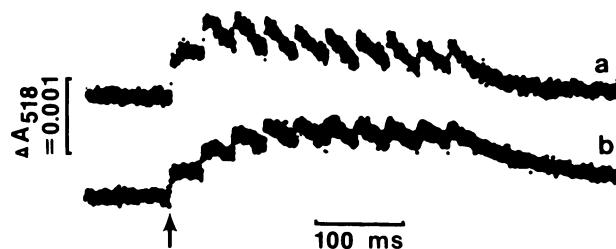
(Fig. 9, trace b). This effect of preillumination almost certainly arises from thioredoxin-dependent reduction of the coupling factor (9), a process that is being extensively studied on the isolated enzyme (e.g. refs. 7, 23) and has been implicated in the regulation of coupling factor *in vivo* (e.g. refs. 13, 14, 21, 23). Reduction of the coupling factor lowers the energetic threshold for the activation of the enzyme (5), which would account for the more rapid relaxation of the transmembrane electric potential seen after preillumination (Fig. 9, cf. traces a and b).

In this study we examined the effect of chilling in the light on the capacity for this light adaptation by preilluminating for 90 s at room temperature with 500  $\mu\text{E}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$  PAR, followed by a 2 min dark interval. The 2 min dark interval was long enough to allow for the dissipation of any energization caused by the preillumination but too short to allow for significant reoxidation of the coupling factor. Thereafter, the flash-induced electrochromic change was measured. Comparison of traces b and d in Figure 9 shows that the light adaptation, that we interpret to be reduction of coupling factor by thioredoxin during the 90 s preillumination, is not disrupted by the chilling treatment. We do not presently know the significance of the decrease in amplitude observed in both control and chilled leaves following the preillumination.

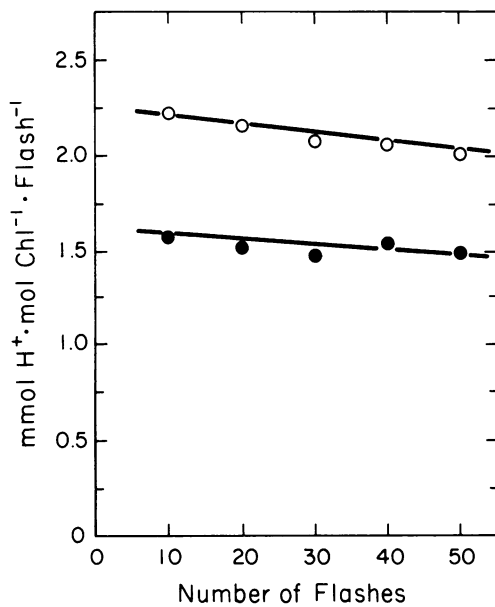
## DISCUSSION

The capacity of thylakoid membranes to store charge (*i.e.* their electrical capacitance) is considerably smaller than their capacity to bind protons (*i.e.* their  $\text{H}^+$ -buffering capacity). Consequently, the electrical and chemical components of the overall electrochemical potential that drive ATP formation develop out of phase. The first protons that are accumulated by the thylakoid vesicle after the onset of illumination result in a sizeable electric potential due to the charge imbalance across the membrane while the transmembrane pH difference remains small. As proton accumulation continues the  $\Delta\text{pH}$  becomes larger as membrane buffering groups are successively titrated and the electric potential declines to very low values as charge compensating ions distribute in the electric field and nullify the charge imbalance.

In this study we took advantage of the contribution by the electric potential to single turnover flash-driven ATP synthesis



**Figure 7.** Effect of chilling in the light (3 h) on the development of a membrane potential from a series of 10 subsaturating flashes. A control leaf was dark-adapted for 30 min prior to recording trace a. Trace b was taken after the leaf was chilled at 5°C in the light (1000  $\mu\text{E}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$  PAR) for 3 h and then dark-adapted for 30 min at room temperature. The flashes used in this experiment were approximately half saturating and delivered at 40 Hz.



**Figure 8.** Effect of chilling in the light on flash-induced water oxidation measured in isolated cucumber thylakoids. Attached leaves were chilled and illuminated for 3 h as described in "Materials and Methods," allowed to rewarm in the dark at room temperature for 30 min, then thylakoids were isolated and the average proton yield per flash of 10 cumulative flashes was determined. Mean values of nine replicate control (○) and six chilled (●) samples were reported. The calculated standard errors ranged from 3 to 9% of the mean values.

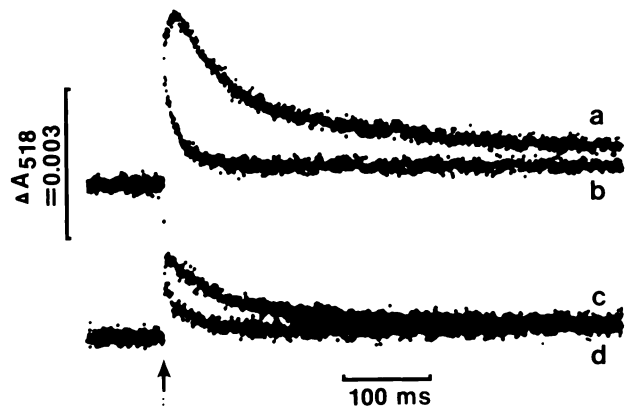
to investigate photophosphorylation in leaves. In photosynthetic membranes, the fate of the electric potential can be conveniently monitored through the effect that the electric field has on the absorption spectrum of a specialized group of pigments within the membrane. Membrane depolarizing proton efflux through the coupling factor complex associated with ATP synthesis results in an accelerated relaxation of the electric field-indicating absorption change. Factors that disrupt the naturally low conductance of the thylakoid bilayer to protons and other ions would also cause an accelerated decay but, in that case, the rate of decay would not respond to factors that control coupling factor activity (*e.g.* light-adaptation and treatment with DCCD) and can be distinguished on this basis. Factors that interfere with the phosphorylation of ADP by the coupling factor complex prevent the expected acceleration of the relaxation that is associated with ATP formation. We maintain a fairly conservative interpretation of the electrochromic bandshift and are, for instance, reluctant to estimate actual rates of ATP formation or magnitude of the flash-induced transmembrane electric field from these measurements. Nevertheless, measurement of the flash-induced electrochromic bandshift is proving to be a powerful approach for investigating the effect of environmental stress on photosynthetic energy transduction competence in leaves.

Chilling attached cucumber leaves in the light had only minor effects on the decay kinetics of the flash-induced electrochromic shift (Fig. 5 B). Only at a low flash repetition rate of 0.1 Hz was the relaxation time constant larger after chilling in the light than before. Flashing at 2.0 Hz restored the decay kinetics to close to those of the unchilled plant showing that

the slowed decay seen at the low flash frequency after chilling can be overcome by the larger  $\Delta p$  values produced when the individual contributions of closely spaced flashes to the  $\Delta p$  (mostly to the  $\Delta p\text{H}$ ) became additive.

Peeler and Naylor (17) provided evidence for an uncoupling of photosynthetic electron transfer from photophosphorylation in cucumber thylakoids from plants chilled in the light. Uncoupling causes an unmistakable acceleration of the decay of the  $\Delta A_{518-540}$  and was not seen in the present study in over 100 experiments with plants chilled in the light for 1 to 6 h. It seems likely that the thylakoid uncoupling reported by Peeler and Naylor was a differential effect of the isolation procedures. Indeed, they showed in the same study that cucumber thylakoids are particularly sensitive to uncoupling during isolation.

It is well established in chloroplast bioenergetic literature that high rates of ATP hydrolysis by thylakoid membranes require preillumination in the presence of sulfhydryl reducing compounds such as dithiothreitol. It is only recently, however, that the relevance of thiol reduction of  $\text{CF}_1$  to ATP synthesis has become evident. It is now known that the involvement of such thiol reagents in the coupling factor activation process is through the reduction of a single disulfide linkage in the  $\gamma$  subunit of  $\text{CF}_1$  (7, 23). Furthermore, it seems likely that thioredoxin, located in the stroma and photoreduced by PSI, mediates the reduction of the  $\gamma$  subunit of  $\text{CF}_1$  *in situ* (13, 21). While reduction of the  $\gamma$  subunit is not an obligatory requirement for activation it has been shown that reduction significantly lowers the energetic threshold required for catalytic activity (5). Thus, in the experiments reported in this study we have shown that cucumber plants chilled in the light are not only capable of activating coupling factors in their oxidized state (*i.e.* after 30 min dark adaptation) but are also able to carry out normal light-dependent reduction of the enzyme (Fig. 9).



**Figure 9.** Effect of chilling in the light on light adaptation of *in situ* ATP synthesis. An attached leaf was dark-adapted for 30 min prior to recording trace a. The leaf was then illuminated for 90 s at  $500 \mu\text{E} \cdot \text{m}^{-2} \cdot \text{s}^{-1}$  PAR, held for 2 min in the dark and trace b was taken. Traces c and d are comparable to a and b but taken the next day after chilling at  $5^\circ\text{C}$  for 3 h in the light ( $1000 \mu\text{E} \cdot \text{m}^{-2} \cdot \text{s}^{-1}$ ). Regardless of whether the plant had been chilled or not, the 90 s preillumination caused a nearly twofold decrease in the relaxation time constant. Each trace is the sum of four actinic flashes.

As a secondary issue to the behavior of the coupling factor we were interested to define the cause for the decrease in the  $\Delta A_{518-540}$  caused by the chilling treatment. Since the effective optical pathlength through the leaf was unchanged by chilling in the light (Fig. 6), the smaller flash-induced amplitude reflects either a smaller flash-generated membrane potential or a change in the sensitivity of the absorption bandshift to the size of the transthylakoid electric field. For instance, if the pigments within the thylakoid that respond to electrochromism were bleached, then no amount of electric potential would generate the same  $\Delta A_{518}$  in a chilled leaf as in a control leaf. Our experiments showed that the smaller  $\Delta A_{518}$  was actually indicating a smaller flash-induced membrane potential in light-chilled plants (Fig. 7).

In summary, this study shows that there is no direct effect of chilling in the light on coupling factor activity. The modest effect that chilling in the light has on photophosphorylation is the result of the thylakoid's reduced capacity to generate the driving force for ATP formation. This work forms the necessary basis for studies probing the effects of chilling on ATP-dependent reactions and processes.

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