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Studies on the Mechanism of Photoinhibition in Higher Plants

I. EFFECTS OF HIGH LIGHT INTENSITY ON CHLOROPLAST ACTIVITIES IN CUCUMBER ADAPTED TO LOW LIGHT

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ABSTRACT

Cucumber plants (*Cucumis sativus* L.), grown at low quantum flux density (120–150 microeinsteins per square meter per second) were photoinhibited by a three-hour exposure in air to ten times the light intensity experienced during growth. Chloroplasts were isolated from photoinhibited and control leaves and the following activities determined: O_2 evolution in the presence of ferricyanide, photosystem I activity, noncyclic and cyclic photophosphorylation, and light-induced proton uptake. Chlorophyll and chloroplast absorbance spectra, and chloroplast fluorescence were also measured. It was found that photosystem II electron transport and noncyclic photophosphorylation were inhibited by about 50%, while cyclic photophosphorylation was less inhibited and photosystem I electron transport and light-induced proton uptake were unaffected. Electron transport to methylviologen could not be fully restored by electron donation to photosystem II. Chloroplast fluorescence induction at room temperature was strongly reduced following photoinhibition. There was no difference in the absorption spectra of the extracted chlorophylls from control and photoinhibited chloroplasts, but an increase of the absorption in the blue wavelength region was observed in the photoinhibited chloroplasts. It is suggested that high light stress does not result in alteration of the membrane properties, as is the case in low-temperature stress for example, but affects directly the photosynthetic reaction centers, primarily of photosystem II.

It was reported earlier (18) that photoinhibition in plants that were adapted to growth at low light intensities could not be completely prevented by the presence of CO_2 and/or O_2. It was also shown that the photoinhibition in low light plants was essentially similar to the photoinhibition occurring in high light plants when exposed to high irradiances in the absence of CO_2 (16–18). The inhibition of photosynthesis in both cases was found to be due to a substantial decrease in the capacity for electron transport associated with PSII. Jones and Kok (8, 9) and Björkman (3) had previously proposed that photoinhibition was due to inactivation by light of the photosynthetic reaction centers, but direct evidence has been lacking.

Electron transport from water to $NADP^+$ involves a number of intermediate electron carriers as well as the light-harvesting assemblies and the reaction centers of the two photosystems. To further characterize the biochemical events that are associated with photoinhibition, a simple method was employed to obtain uniformly photoinhibited plant material, and several photochemical activities measured in the isolated chloroplasts.

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MATERIALS AND METHODS

Plant Material. Cucumber plants (*Cucumis sativus* L.) were grown from seed in 10-cm pots of soil kept in a growth cabinet with a day/night temperature regime of 25/20 C and a RH of about 75%. The photoperiod was 13 h and quantum flux (400–700 nm) was 150 $\mu E\ m^{-2}\ s^{-1}$ at leaf level. The plants were 20 or 21 days old when used.

Photoinhibition Treatment. Intact plants were placed under a 400 w mercury vapor lamp and adjusted so that one leaf was fully exposed to an irradiance of about 1,500 $\mu E\ m^{-2}\ s^{-1}$. The light was filtered through 8 cm of water and a Schott KG 3 filter (250 × 250 × 2 mm) to ensure absorption of all ultraviolet and infrared irradiation. Unless otherwise stated, the treatment period was 3 h in air at room temperature (~25 C). Leaf temperature, as determined with a calibrated thermocouple, did not exceed 30 C during treatment. Twin plants that remained in the growth cabinet during the treatment period were used as controls.

Chloroplast Isolation. Chloroplast thylakoids were isolated from 1.5 to 2.5 g treated and control leaves by homogenization at 2 C for 10 s in a Sorvall Omnimix in 50 ml 0.05 M Sørensen's phosphate buffer (pH 7.5), 0.05 M NaCl, 0.001 M EDTA, and 0.5% (w/v) BSA. The homogenate was filtered through four layers of Miracloth and the filtrate centrifuged for 5 min at 1,000*g*. The chloroplasts were washed once (1,000*g* for 10 min) in 50 ml of blending medium minus EDTA and 0.05% (w/v) BSA and resuspended in the same buffer to a final concentration of 1,200 μg Chl/ml. Chlorophyll was determined according to Arnon (1).

Assays. All electron transport reactions were measured polarographically in twin Clark-type O_2 electrodes. Temperature was maintained at 25 C and actinic light was supplied from 150 w quartz iodine lamps. The quantum flux density was 2,300 $\mu E\ m^{-2}\ s^{-1}$ white light. The final Chl concentration was 20 μg/ml.

O_2 evolution was measured with ferricyanide as electron acceptor in a reaction mixture (3 ml) containing 50 mM Hepes (pH 7.6), 0.5 mM $MgCl_2$, 1 mM EDTA, and 1.5 mM $K_3Fe(CN)_6$. PSI activity was assayed with ascorbate/DCIP[2] as electron donor and MV as electron acceptor in a medium containing 30 mM Na-pyrophosphate buffer (pH 8.0), 0.5 mM $MgCl_2$, 0.33 mM iso-ascorbate, 0.033 mM DCIP, 0.067 mM MV, and 3.3 μM DCMU. In control experiments it was established that the chloroplast preparations contained no catalase activity. NH_4Cl (2.5 mM) was used as an uncoupler for both reactions.

Cat-supported PSII activity was measured according to Ort and Izawa (14) in 50 mM Tricine-NaOH buffer (pH 7.8) containing

[2] Abbreviations: DCIP, 2,6-dichlorophenolindophenol; MV, methylviologen; ASC, iso-ascorbate; Cat, catechol; SOD, superoxide dismutase; DPC, 1,5-diphenylcarbazide; FeCN, K-ferricyanide; PMS, phenazine-methosulfate.

0.1 M sucrose, 2 mM $MgCl_2$, 0.5 mM MV, 0.5 mM ASC, 1 mM Cat, and 80 μg/ml SOD. PDC was added at 1 mM concentration to the same reaction mixture (a modification of the assay described by Vernon and Shaw [23]), and 2.5 mM NH_4Cl used as uncoupler for both reactions.

Photophosphorylation activities were measured by the pH method of Nishimura *et al.* (13) using a homemade pH meter and combined Radiometer GK 2321C electrode connected to a Perkin-Elmer 165 recorder. pH changes were measured in the O_2 electrode vessel and O_2 changes recorded simultaneously on a Rikadenki two-channel strip chart recorder. The continuous alkalization of the medium was strictly dependent on the presence of ADP. The photophosphorylation medium contained 0.42 mM Na-succinate, 5 mM $MgCl_2$, 4.2 mM KH_2PO_4, and 1 mM ADP. Noncyclic photophosphorylation was determined using 0.05 mM MV and cyclic photophosphorylation using 0.032 mM PMS, respectively. The pH was adjusted to between 8 and 8.1 immediately prior to measurement. The pH changes were calibrated by adding known amounts of 0.1 N HCl to each reaction medium.

Light-induced proton uptake was measured with the same apparatus as described for photophosphorylation, the assay mixture being either the phosphorylation buffer without ADP, or 50 mM KCl and 0.067 mM methylviologen as electron acceptor. pH was adjusted to 7.5 for these measurements and changes also calibrated by adding known amounts of 0.1 N HCl.

Absorption Spectra and Fluorescence Measurements. Absorption spectra of 80% acetone extracts and of chloroplasts in washing buffer were recorded in a Perkin-Elmer Model 356 spectrophotometer. Chl concentrations were exactly the same for both control and photoinhibited chloroplasts, *e.g.* 5 μg/ml 80% acetone and 17 μg/ml washing buffer, respectively. Chloroplast fluorescence was measured in the same instrument using only the photomultiplier, HTV R375 S-20, which was shielded from the actinic beam (blue light; Balzers K-45 broadband interference filter) by a Balzers B40-675 interference filter. The experimental arrangement is depicted in Figure 1. Chl concentration was 3.3 μg/ml washing buffer (pH 7.6), and the samples were kept in the dark. Additions were made as stated under "Results."

RESULTS

Photoinhibitory treatment of cucumber leaves by exposing them for 3 h to about 10 times the quantum flux density experienced during growth did not result in bleaching or other macroscopic damage. Some plants showed transient wilting upon being transferred to the high light intensity. Results from these experiments are not included among the data presented here. The Chl *a/b* ratios of the isolated chloroplasts were not significantly affected by the treatment as is shown in Table IA. O_2 evolution with FeCN as electron acceptor, electron transport from H_2O to MV, cyclic and noncyclic photophosphorylation were all inhibited substantially (Table I, B and C), but there was no decrease in PSI activity at all (Table IB), confirming earlier results obtained with bean plants (18). Electron transport and noncyclic photophosphorylation, when measured simultaneously, were inhibited to the same extent (data not shown). From these experiments ATP:2e ratios could be calculated and were found to be unaffected by photoinhibition treatment, ranging from 1.2 and 1.5 for chloroplasts from both control and photoinhibited leaves.

The time-course for inhibition of O_2 evolution in the presence of FeCN (Fig. 2) showed a decline over 3 h of high light treatment, while PSI electron transport remained largely unaffected. It was not possible to restore PSII mediated electron transport to MV by Cat or DPC in the chloroplasts from photoinhibited leaves to any significant extent (Table IB). In the presence of DPC, inhibition appeared to be about 10% less than that observed for O_2 evolution with ferricyanide. This was probably due to an increase of the PSII activity affected during the chloroplast isolation procedure, since a 10 to 15% increase in activity of control chloroplasts was also observed when DPC or Cat were present. Tris- (23) or NH_4OH-treated (14) chloroplasts from photoinhibited tissue showed a 30% reduction in their DPC- and Cat-dependent electron transport activities compared to those from control leaves, suggesting different sites for photoinhibition and Tris- or NH_4OH-inactivation. Chl fluorescence of the isolated chloroplasts from photoinhibited leaves was also significantly reduced after 3 h of photoinhibitory treatment. The results of some typical experiments are shown in Figures 3 and 4. Under conditions where light intensity is not limiting electron transport rates, relative fluorescence yield is inversely related to electron transport (11). This was also found for the control chloroplasts in this study, where fluorescence yield was very high when no electron acceptor was present (Fig. 3A), appreciably reduced upon addition of MV (Fig.

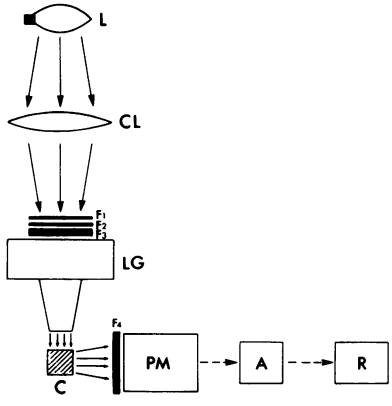

FIG. 1. Experimental arrangement for measuring fluorescence induction in isolated chloroplasts. L, actinic light source; CL, bi-convex lens; F_1, F_2, Corning 1-75 heat filters; F_3, Balzers K-45 broadband interference filter; LG, light guide; C, cuvette-containing chloroplast suspension; F_4, Balzers B40-674 interference filter; PM, photomultiplier; A, amplifier; R, recorder.

Table I. *Photochemical Functions of Chloroplasts Isolated from Cucumber Leaves Before and After Photoinhibition*

The values represent means of 2 to 10 experiments and the standard deviation is given.

	Control	Photoinhibited	Inhibition
			%
A. Chl *a/b* ratio	2.53 ± 0.12	2.50 ± 0.61	
B. Electron transport activities, uncoupled (μmol O_2 evolved or consumed/mg Chl·h)			
H_2O → FeCN	200 ± 30	114 ± 20	43
H_2O → MV	139 ± 29	68 ± 18	51
Cat → MV (+ SOD)	161 ± 29	87 ± 27	46
DPC → MV	152 ± 10	99 ± 1	35
ASC. DCIP → MV (+ DCMU)	145 ± 36	143 ± 42	0
C. Photophosphorylation activities (μmol H^+/mg Chl·h)			
Non-cyclic (MV)	87 ± 40	38 ± 25	56
Cyclic (PMS)	549 ± 66	327 ± 76	40
Cyclic (PMS, DCMU)	462 ± 122	276 ± 43	40
D. Light-induced H^+ uptake (nmol H^+/mg Chl)			
Steady-state (MV)	352 ± 125	353 ± 67	0

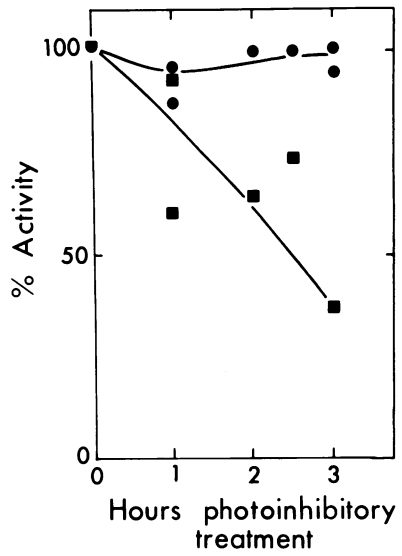

FIG. 2. Effect of length of photoinhibitory treatment of cucumber leaves on electron transport activities of the isolated chloroplasts. ■, H_2O → FeCN; ●, ASC.DCIP → MV(DCMU).

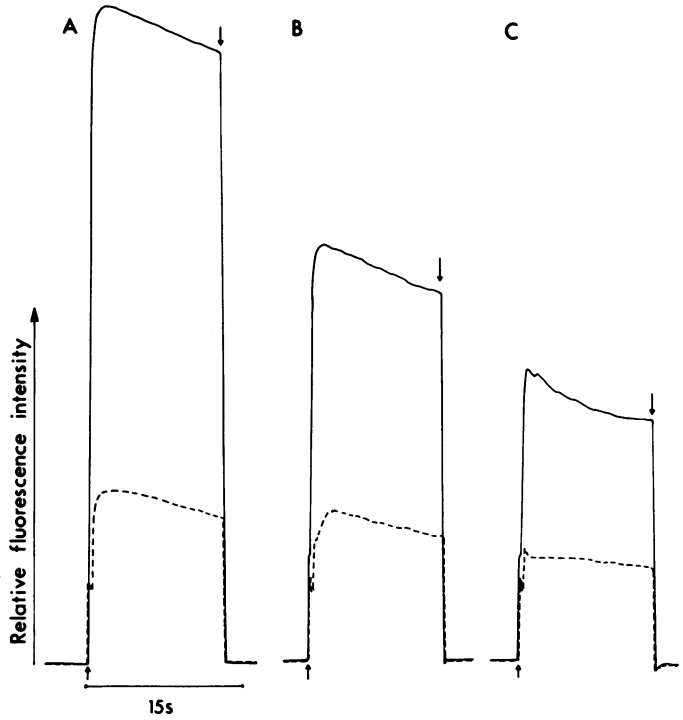

FIG. 3. Fluorescence induction in chloroplasts isolated from leaves of cucumber before (——) and after (- - -) photoinhibition. High excitation light, 1,000 μE m^{-2} s^{-1}. Total volume, 3 ml; assay medium, 0.05 M phosphate buffer (washing buffer); 3.3 μg Chl/ml; MV, 67 μM; NH_4Cl, 2.5 mM; DCMU, 3.3 μM.

3B) and even further decreased by uncoupling (Fig. 3C). These effects were not as pronounced in the chloroplasts from photoinhibited leaves but the total fluorescence yield was significantly decreased (Fig. 3, A–C).

In Table II the results of several experiments are summarized. They indicate also a modified response of the chloroplasts from the photoinhibited leaves toward addition of electron acceptors and uncouplers, since the reduction in F_{max} was not the same under all conditions. From these experiments it also appeared that the rate of the fluorescence rise was somewhat slower after photoinhibition. This was confirmed by results obtained from measurements of fluorescence yield at only 1% of the above light intensity, which are shown in Figure 4. The rise to F_{max} in the chloroplasts from photoinhibited leaves was considerably slower than in the chloroplasts from the control (Fig. 4A). In the presence of DCMU (Fig. 4B), however, the rates appeared to be much the same.

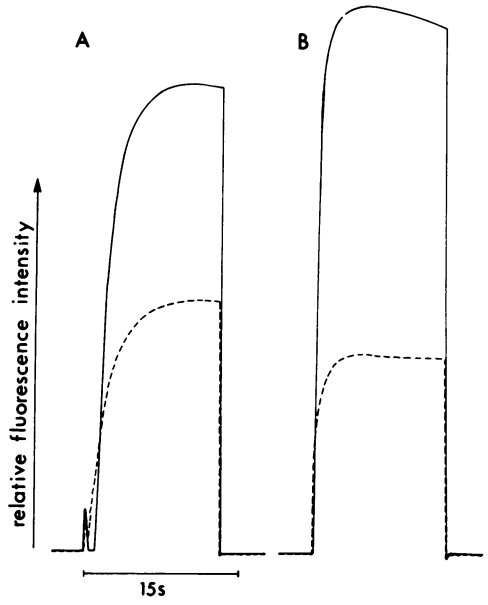

FIG. 4. Fluorescence induction in chloroplasts isolated from leaves of cucumber before (——) and after (- - -) photoinhibition. Low excitation light, 10 μE m^{-2} s^{-1}. Conditions as in Figure 3.

Table II. *Effect of Photoinhibition on Fluorescence Yield of the Isolated Chloroplasts*

The data are the mean of four experiments ± SD.

	Reduction in F_{max}
	%
No e^- acceptor	67 ± 7
+ MV	55 ± 8
+ MV, NH_4Cl	51 ± 6
+ MV, NH_4Cl, DCMU	57 ± 5

Whereas photophosphorylation, fluorescence yield, and electron transport from water to ferricyanide or methylviologen were considerably decreased after photoinhibition, the extent of the light-induced proton uptake was not affected at all (Table ID). The absorption spectra of the Chl extracted from isolated chloroplasts that showed about 50% inhibition of O_2 evolution were not different from those of the controls (Fig. 5A). The chloroplasts themselves from photoinhibited leaves were found to have an increased absorption in the blue region of the spectrum (Fig. 5B).

DISCUSSION

From the results presented in this paper it appears that photoinhibition indeed affects directly the photochemical reaction centers of PSII, as has been postulated before (3, 8, 9). The results confirm the previous observation (18) that, following photoinhibition treatment of higher plant leaves, electron transport associated with PSII is significantly decreased, while PSI-related electron transport remains unaffected. It was reported for *Rhodospirillum rubrum* chromatophores, that photoinhibition *in vitro* did not affect the coupling factor (CF_1) (19). The concomitant inhibition of the photophosphorylations observed here was probably

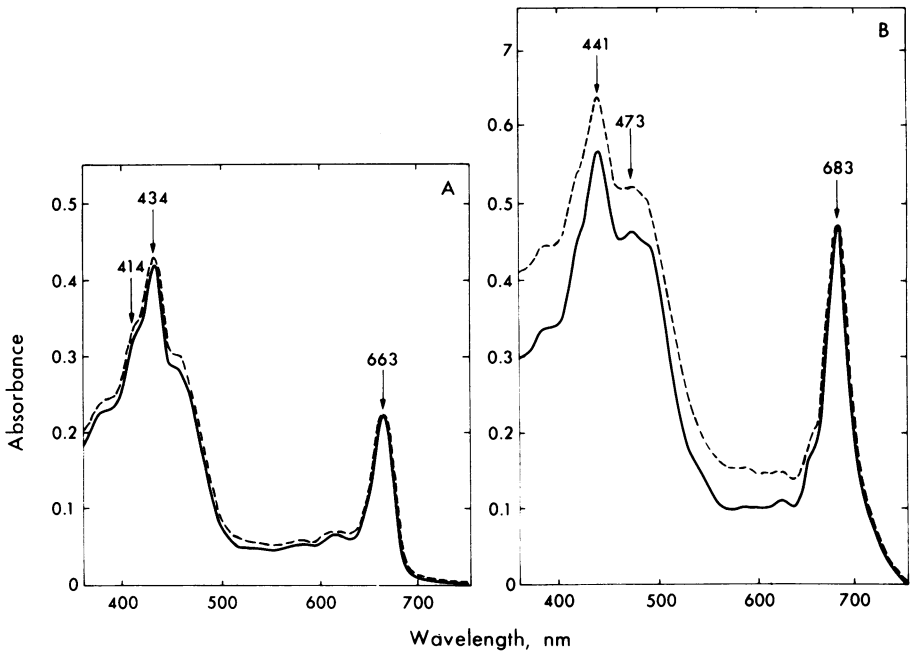

FIG. 5. Absorption spectra of (A), 80% acetone extracts of chloroplasts isolated from cucumber leaves before (——) and after (- - -) photoinhibition, and (B) of chloroplasts isolated from cucumber leaves before (——) and after (- - -) photoinhibition.

due to the decreased capacity for PSII electron transport and not to an effect on CF_1 itself. Cyclic photophosphorylation was inhibited whether measured with or without DCMU present. No artificial reductant, *e.g.* ASC or DTT was included in the assays for cyclic photophosphorylation, so that PMS was largely oxidized. Cyclic photophosphorylation, although mediated by PSI, requires proper redox poising of the intermediate electron carriers which in turn is dependent on the activity of PSII (12). This may be more difficult to achieve in chloroplasts from plants grown at low quantum flux densities, because, in those, light energy is preferredly distributed to PSII and PSI activity comparatively low. It was reported recently that in chloroplasts from *Pinus* grown in the dark or in yellow light, which had very little or no PSII activity, cyclic photophosphorylation was greatly enhanced in the presence of reduced PMS as compared to oxidized PMS (22). It is quite possible that in the chloroplasts from low light-adapted, photoinhibited leaves a similar restriction with regard to cyclic photophosphorylation was observed. This does not explain the results obtained when cyclic photophosphorylation was measured in the presence of DCMU and presently this question is under further investigation.

That these effects could not be due to changes in the permeability properties of the thylakoid membranes is strongly suggested by the fact that the light-induced proton uptake was not changed. It is assumed here that net proton uptake measured in the absence of ATP-synthesis (*i.e.* amount of leakiness to protons) provides an indication of the degree of physical damage to the membrane due to stress. Indirect evidence supporting this view is provided by the finding that the isolated chloroplasts were always very tightly coupled; *e.g.* 10- to 15-fold stimulation of Hill reaction rates by addition of NH_4Cl was routinely observed for all chloroplast preparations from both control and photoinhibited tissue. The unchanged ATP:2e ratios corroborated this notion. Similar results were obtained by Gross *et al.* (7), who reported that low salt-treated chloroplasts had drastically reduced electron transport rates but showed unchanged light-induced proton uptake. It has been found that isolated chloroplasts, which at 6 C had only 50% of the electron transport activity as compared to 23 C, showed no change in the extent of the light-induced proton uptake (C. Critchley, R. M. Smillie, in preparation). The decrease in chloroplast fluorescence also indicates that PSII is impaired, since at room temperature all the fluorescence is derived from Chl associated with PSII only (4). Lesions on either side of PSII lead to a reduced output of variable fluorescence (15). Furthermore, photoinhibition has been shown to decrease considerably the 683 nm fluorescence emission at 77 K, characteristic of PSII, in thylakoids from bean leaflets exposed to high irradiances in the absence of CO_2 and O_2 (17).

Chloroplast aging is also accompanied by a concomitant decrease in fluorescence and PSII mediated electron transport (6). Chilling injury in photosynthetic tissue too has been found to manifest itself in severe damage of electron transport capacity associated with PSII and a concomitant reduction in the induction rates and total yield of Chl fluorescence (20, 21). While in both systems the damage could be compensated for by addition of electron donors to PSII (2, 21), photoinhibition effects could not be alleviated by bypassing the water splitting apparatus. The results presented here are therefore consistent with the view that electron donation to PSII is impaired after photoinhibition. The sensitive site does not appear to be the water splitting complex itself, but must be located much closer to the reaction center and behind the site for entrance of electrons from PSII donors.

The chloroplasts themselves appear not to have changed significantly in their spectral characteristics, although this possibility cannot be completely ruled out on the basis of simple absorption spectra obtained from the acetone extracts. What seems possible, however, is that the imposed light stress had an effect on the molecular arrangement or orientation of the Chl within the photosynthetic units, and thus caused the observed increased absorption in the blue region of the spectrum. A similar increase in chloroplast absorption in the blue region was observed upon cooling chloroplasts *in vitro* and was attributed to changes in Chl aggregation (5). An effect on the associated carotenoids, which absorb in this region and have been found to play a protective role in photo-oxidation of Chl (10), can not be excluded. Further investigations into these possibilities are in progress.

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