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Photooxidation by Photosystem II of Tris-washed Chloroplasts[1]

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Abstract. **Irradiation of tris-washed chloroplasts with moderate intensities of red light caused a partial bleaching of chloroplast pigments and an inhibition of the hydroquinone-supported photoreduction of NADP. The presence of an electron donor for photosystem 2 (PS2) during the irradiation prevented the bleaching and inhibition. It is concluded that the strong oxidant produced by PS2 accumulates in tris-washed chloroplasts during irradiation and an electron donor for PS2 protects against the photooxidation reactions.**

We have shown previously (4, 5, 6) that washing chloroplasts with tris buffer inhibits electron transport between water and PS2 and that a number of reduced compounds, *e.g.*, PD[2] and HQ can restore photosynthetic electron transport by donating electrons to PS2. It was noted in experiments in which ferricyanide was the electron acceptor for tris-washed chloroplasts (6) that irradiation in the absence of an electron donor resulted in an initial decrease in the absorbance ratio. A_{420}/A_{480} used to monitor ferricyanide photoreduction. Subsequent investigation revealed that this absorbance change was due to a bleaching of the chloroplast pigments. The work reported in the present paper continues from that observation and shows that irradiation of tris-washed chloroplasts by moderate intensities of red actinic light in the absence of an electron donor system caused bleaching of chlorophyll and carotinoid pigments, an inhibition of NADP photoreduction in the presence of PS2-specific electron donor and a loss of the fluorescence of variable yield. These destructive effects of actinic irradiation were all prevented if an electron donor system was present during the irradiation. It is concluded that the destructive effects are due to the strong oxidant produced by PS2 which tends to accumulate during irradiation in the absence of an electron donor.

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[2] Abbreviations: PD, *p*-phenylenediamine; HQ, *p*-hydroquinone; PS1 and PS2, photosystem I and photosystem II; DCMU, 3-(3,4-dichlorophenyl)-1,1-dimethylurea; DCPIP, 2,6-dichlorophenol-indophenol.

Experimental Procedures

Chloroplasts were prepared by grinding 50 g of spinach leaves in a Waring Blendor for 20 sec in 150 ml of solution containing 0.4 M sucrose, 0.05 M tris-HCl (pH 7.8), and 0.01 M NaCl (abbreviated as STN solution). The supernatant from a 1-min centrifugation at 300*g* was centrifuged again at 600*g* for 7 min in 4 centrifuge tubes. The pellet from 2 tubes was suspended at 0° in 20 ml of 0.8 M tris-HCl (pH 8.0) to about 0.1 mg chl/ml (tris-washed chloroplasts). The pellet from the other 2 tubes was suspended in 20 ml of STN solution (intact chloroplasts). After standing for 10 min, heavy particles of both batches of chloroplasts were removed by centrifugation at 300*g* for 1 min. The remaining supernatants were centrifuged at 1500*g* for 7 min and the precipitates were resuspended in 2 ml of STN solution. Measurements were made with chloroplasts suspended in a standard reaction medium consisting of 15 mM tris-HCl, 4 mM KPO_4, 1 mM ADP, 4 mM $MgCl_2$, and 20 mM NaCl, pH 7.8.

Chlorophyll concentration was determined by the method of MacKinney (2). Ferredoxin was purified from spinach leaves; DCMU donated by Dr. P. G. Heytler was used as a methanolic solution. ADP and NADP were obtained from Calbiochem Company. *p*-Hydroquinone was obtained from Aldrich Chemical Company; DCPIP, from the La Motte Chemical Products Company.

NADP photoreduction was measured under aerobic conditions with an Aminco-Chance dual wavelength spectrophotometer with monitoring wavelengths of 340 and 370 nm; ferricyanide photoreduction was measured with monitoring wavelengths of 420 and 480 nm; carotenoid photobleaching with wavelengths of 490 and 530 nm. The EMI 9524

phototube was blocked with Corning filters 5840 and 5970 for the NADP measurement and with 2 Corning 9788 filters and an Optics Technology 600 nm short pass cut-off filter for the ferricyanide and carotenoid measurements. The fluorescence-yield measurements were the same as those described previously (5).

The actinic light was obtained with a Unitron LKR microscope illuminator and Baird Atomic interference filters (approximately 10 nm half band width), with additional infrared blocking filters. Photobleaching and photoinhibition experiments were carried out by irradiating chloroplasts (10 μg chl/ml suspended in the standard reaction medium) in 1-cm cuvettes with 680 nm actinic light at 2×10^4 ergs cm^{-2} sec^{-1} except where other intensities are noted.

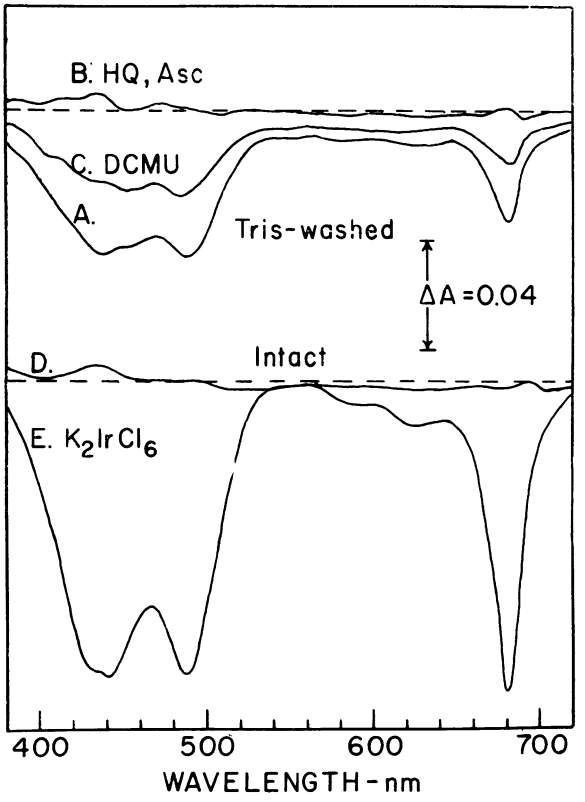

FIG. 1. Difference spectra (A through D) of irradiated *vs.* non-irradiated chloroplasts (10 μg chl/ml). 10 min irradiation with 680 nm light 2×10^4 ergs cm^{-2} sec^{-1} of A) tris-washed chloroplasts; B) tris-washed chloroplasts in the presence of 200 μM HQ and 330 μM ascorbate; C) tris-washed chloroplasts in the presence of 1 μM DCMU; D) intact chloroplasts; E) difference spectrum of intact chloroplasts treated with 10^{-4} M K_2IrCl_6 in the dark *vs.* nontreated.

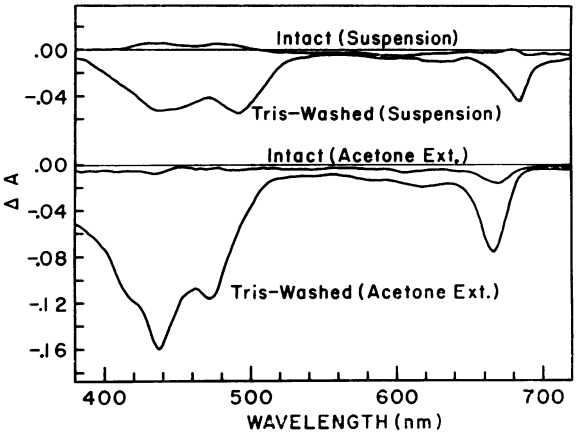

FIG. 2. Difference spectra of irradiated *vs.* non-irradiated intact and tris-washed chloroplasts and of acetone extracts of the chloroplasts (10 μg chl/ml). 20 min irradiation with 680 nm light 2×10^4 ergs cm^{-2} sec^{-1}.

Results

Irradiation of tris-washed chloroplasts with moderately strong red light in the absence of an electron donor system caused a partial bleaching of the chloroplast pigments. The difference spectrum of the irradiated *vs.* dark chloroplasts (Fig. 1, Curve A) shows that both chlorophyll and carotenoid pigments were bleached. The bleaching was completely prevented by adding an electron donor system (Fig. 1, Curve B) and partially prevented by 1 μM DCMU (Fig. 1, Curve C). Intact chloroplasts were not bleached by the irradiation treatment (Fig. 1, Curve D). A similar bleaching could be achieved chemically in the dark by adding the oxidizing agent K_2IrCl_6 at 10^{-4} M (Fig. 1, Curve E). The bleaching with tris-washed but not with intact chloroplasts was also confirmed in difference spectra of 80 % acetone extracts of the irradiated and dark samples (Fig. 2) showing that light caused an actual destruction of the pigments. Measurements with the acetone extracts indicated that about 10 % of the chlorophyll *a* was bleached by irradiating the chloroplasts for 20 min with 2×10^4 ergs cm^{-2} sec^{-1} of 680 nm light although the extent of bleaching varied between chloroplast preparations. The intensity dependence and time course of the photobleaching are shown in Fig. 3. Bleaching of the carotenoid and chlorophyll bands followed the same kinetics.

Heat-treated chloroplasts (50° for 3 min) which are also inhibited between water and PS2 (5, 6) are also subject to the photobleaching reaction provided ferricyanide is present (Fig. 4). The lack of bleaching in the absence of ferricyanide may be due to the protective action of endogenous electron donors which are scavanged by the ferricyanide.

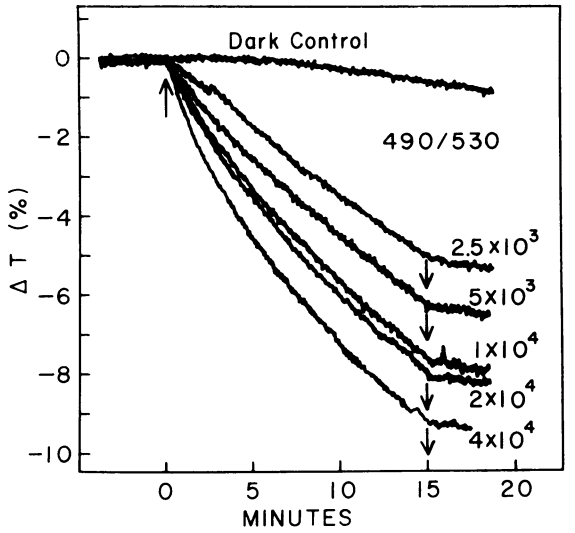

FIG. 3. Time course of photobleaching of carotenoid absorption band in tris-washed chloroplasts (10 μg chl/ml) at various intensities of 680 nm actinic light.

The irradiation treatment also inhibited the capacity of the tris-washed chloroplasts to photoreduce NADP in the presence of the hydroquinone-ascorbate electron donor system (Fig. 5). If the irradiation was carried out in the presence of the electron donor system, little or no inhibition occurred. The effectiveness spectra for the bleaching and the inhibition of the HQ-supported NADP photoreduction (Figs. 6 and 7, respectively) are essentially the same, both indicating the effectiveness of light absorbed by chlorophyll *a*. The inhibition appeared to be primarily PS2. The DCPIP-supported photoreduction of NADP driven by PS1 in the presence of DCMU was not inhibited by the irradiation treatment whereas the HQ-supported reaction driven by PS2 plus PS1 was inhibited 50% (Fig. 8).

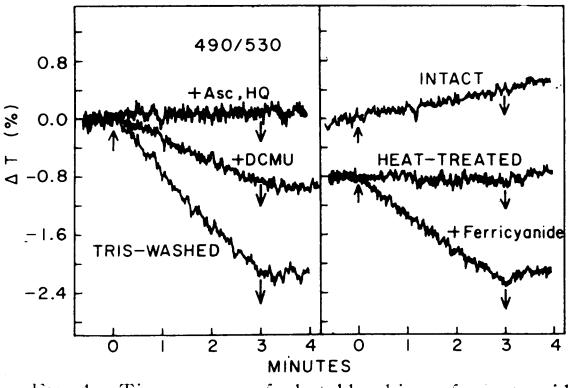

FIG. 4. Time course of photobleaching of carotenoid absorption band in tris-washed, heat treated (50° for 3 min) and intact chloroplasts (10 μg chl/ml) in the presence of 220 μ HQ and 330 μM ascorbate, 1 μM DCMU or 1 mM ferricyanide where indicated.

The fluorescence-yield changes induced by actinic light in tris-washed chloroplasts were shown previously to be quite small compared to those of normal chloroplasts (4, 5), presumably because the source of electrons for the photoreduction of Q, the hypothetical fluorescence-quenching reaction centers of PS2, was blocked by the tris-wash treatment. Much of the fluorescence of variable yield could be restored to the tris-washed chloroplasts, however, by adding an electron donor system for PS2. Preirradiation of the tris-washed chloroplasts for 5 min with 2×10^4 ergs cm^{-2} sec^{-1} of 680 nm light in the absence of an electron donor system prevented the restoration of the variable-yield fluorescence on subsequent addition of the electron donor (Fig. 9A, Curve R). If the preirradiation was carried out in the presence of the electron donor system, the light-induced fluorescence-yield changes were the same as those of the dark control (Fig. 9A, Curve R with HQ and Asc *vs.* dark). Irradiation of intact chloroplasts had no effect on the fluorescence of variable

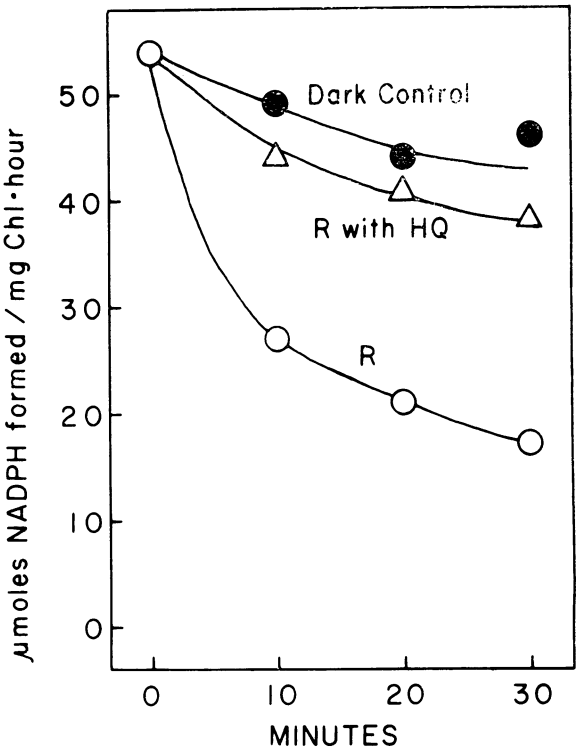

FIG. 5. Inhibition of HQ-supported photoreduction of NADP with tris-washed chloroplasts (10 μg chl/ml) by irradiation with 2×10^4 ergs cm^{-2} sec^{-1}, 680 nm light for the time shown. R, irradiated with no additions; R with HQ, irradiated in the presence of 200 μM HQ and 330 μM ascorbate; dark control, no irradiation. 1 mM ADP, 330 μM NADP, 3 μM ferredoxin, 200 μM HQ and 330 μM ascorbate were added to the reaction medium after the irradiation treatment for the measurement of rates of NADP photoreduction.

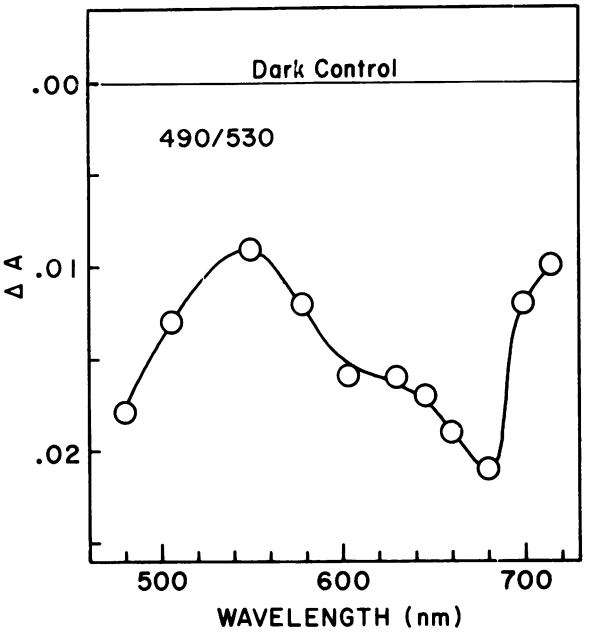

FIG. 6. Effectiveness spectrum for photobleaching of tris-washed chloroplasts (10 μg chl/ml). Bleaching of carotenoid absorption band measured after 10 min irradiation with 5×10^3 ergs cm^{-2} sec^{-1} of monochromatic light (10 nm pass band) at the wavelengths shown.

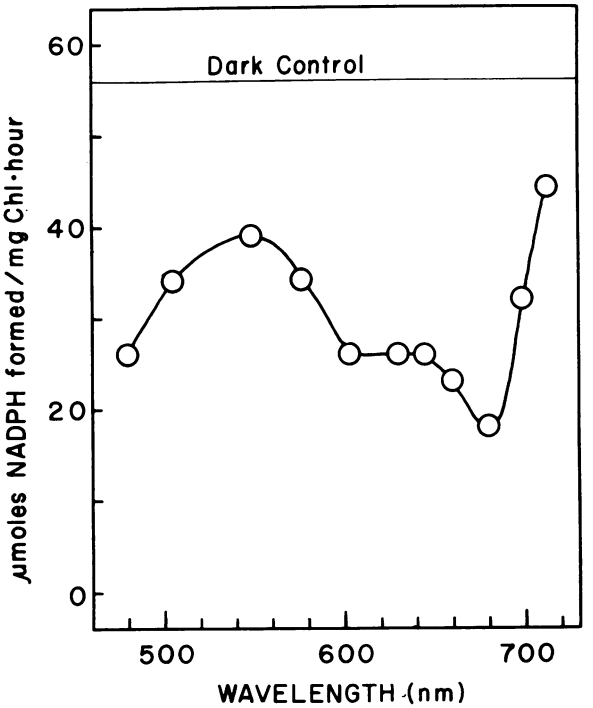

FIG. 7. Effectiveness spectrum for inhibition of HQ-supported NADP photoreduction with tris-washed chloroplasts (10 μg chl/ml). NADP photoreduction measured after 10 min irradiation with 10^4 ergs cm^{-2} sec^{-1} of monochromatic light (10 nm pass band) at the wavelengths shown. 1 mM ADP, 330 μM NADP, 3 μM ferredoxin, 200 μM HQ and 330 μM ascorbate added to the reaction medium after the irradiation treatment.

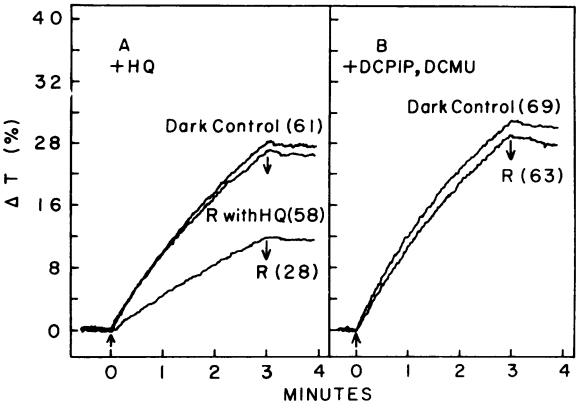

FIG. 8. NADP photoreduction with tris-washed chloroplasts. A, supported by 200 μM HQ and 330 μM ascorbate; B, supported by 200 μM DCPIP and 330 μM ascorbate in the presence of 1 μM DCMU. R, irradiated with no additions; R with HQ, irradiated in the presence of 200 μM HQ and 330 μM ascorbate. Numbers in parenthesis indicate NADPH formed/mg chl/hr.

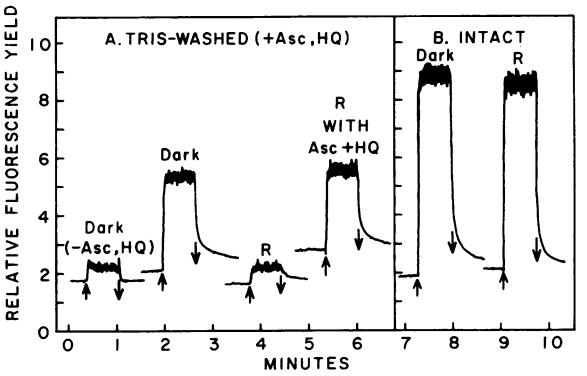

FIG. 9. Relative fluorescence yield of tris-washed (A) and intact (B) chloroplasts in the presence and absence of 650 nm actinic light (on at upward arrows, off at downward arrows). Tris-washed chloroplasts were measured in the presence of 200 μM HQ and 330 μM ascorbate except where noted as (- Asc, HQ). Dark indicates no preirradiation treatment, R indicates preirradiation treatment of 5 min of 680 nm light of 2×10^4 ergs cm^{-2} sec^{-1}, R with Asc + HQ indicates same preirradiation in presence of 200 μM HQ and 330 μM ascorbate.

yield (Fig. 9B). The fluorescence of variable yield was more sensitive to the irradiation than either the bleaching or electron transport (Fig. 10). An irradiation period of 2 and one-half min with 2×10^4 ergs cm^{-2} sec^{-1} of 680 nm light was sufficient to eliminate most of the variable-yield fluorescence.

Discussion

The bleaching results suggest that the strong oxidant made photochemically by PS2 tends to accumulate during irradiation in the absence of an

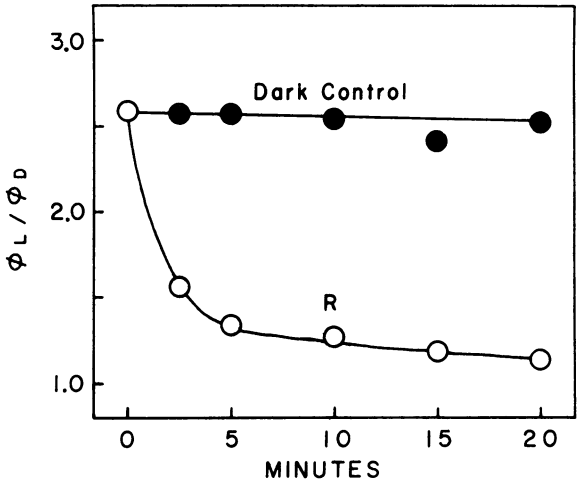

FIG. 10. Ratio of relative fluorescence yields of tris-washed chloroplasts with 200 μM HQ and 330 μM ascorbate in the presence, Φ_L, and absence, Φ_D, of 650 nm actinic light (*i.e.*, a measure of the fluorescence of variable yield). R, with chloroplasts preirradiated with 2×10^4 ergs cm^{-2} sec^{-1} of 680 nm light for the time shown; dark control, no preirradiation.

electron donor and destructively oxidizes chloroplast pigments. Protection against photooxidation is afforded by an electron donor for PS2 which is water in the case of normal chloroplasts or an artificial donor in the case of tris-washed chloroplasts. The results with heat-treated chloroplasts suggest that sufficient endogenous electron donors are present to protect against photobleaching. Once the endogenous electron donors are drained off by using ferricyanide as an electron acceptor, the bleaching occurs. The partial protection against photobleaching afforded to tris-washed chloroplasts by DCMU may have a similar basis. Fluorescence measurements previously indicated that there were sufficient endogenous electron donors in tris-washed chloroplasts to reduce Q if electron transport out of Q was blocked by DCMU (2). DCMU would prevent these electron donors from being drained off so that they might protect against the accumulation of the strong oxidant. Alternatively, a back reaction between reduced Q, which accumulates behind the DCMU block, and the oxidant would also protect against the photooxidation reactions.

A radiation dose which bleached about 10 % of the chlorophyll resulted in at least a 50 % inhibition of HQ-supported photoreduction of NADP. This inhibition appeared to be primarily at PS2 since the DCPIP-supported photoreduction of NADP in the presence of DCMU, a PS1 reaction, was not inhibited by the irradiation treatment. The presence of an electron donor for PS2 during the irradiation, however, protected against the photoinhibition as well as the photobleaching.

The fluorescence of variable yield was particularly sensitive to irradiation in the absence of an electron donor for PS2. An irradiation sufficient to eliminate most of the variable-yield fluorescence caused relatively little bleaching or photoinhibition. A similar uncoupling of electron transport from fluorescence yield changes was noted previously with UV-irradiated chloroplasts (6). In that case, addition of the HQ-ascorbate electron donor system restored the photoreduction of NADP *via* PS2 but did not restore the light-induced fluorescence yield changes.

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