Photoinhibition of Chloroplast Reactions. II. Multiple Effects[1]

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Summary. Ultraviolet light inhibits the photoreduction of 2,6-dichlorophenolindophenol or nicotinamide adenine dinucleotide phosphate with water as the electron donor (evolution of oxygen) but not the photoreduction of nicotinamide adenine dinucleotide phosphate with ascorbate as the electron donor. It inhibits photophosphorylation associated with either system. Experiments undertaken to test whether plastoquinone is the site of UV inhibition yielded inconclusive results.

Visible light (> 420 mμ) causes the loss of all chloroplast activities, photosystem I being more sensitive than system II. The data suggests 2 modes of action for visible light. The one sensitized by system II results in damage resembling that of UV light. The other, sensitized by system I, results in the destruction of the reaction center of this system.

Earlier studies of photoinhibition in whole algae or isolated chloroplasts concerned either UV (usually 253.7 mμ) or visible light (ref. see 7). No comparisons have been made, and it has been assumed that the effects of both were identical.

Our previously reported kinetic and spectral analysis of photoinhibition in chloroplast reactions (7) showed that the kinetics of the 2 inhibitions are similar throughout the spectrum. However, the findings that light of the 2 spectral regions differed greatly in effectiveness, and that the sensitizing pigments were different, strongly suggested more than one site of photoinhibition. This study reports experiments concerning the 2 effects of UV and visible upon various chloroplast reactions which confirm the above hypothesis.

Methods

The methods employed were in most respects identical to those described in our previous study (7). The light sources used for inhibition were either a 2000 w Xenon arc lamp or a 50 cm, 15-w germicidal lamp (GE-G30T8). In experiments using the Xenon arc, the light beam was collimated by quartz lenses, passed through 6.5 cm of water, color filters, and was focused upon the sample. For exposure by the germicidal lamp, the sample vessel was held in a fixed position about 1 cm from the lamp surface. We refer to the germicidal lamp as a 253.7 source since over 80 % of the light emitted by the low pressure Hg arc is in the 253.7 mμ mercury line.

The reaction mixtures used for the assay of dye and NADP reduction activity with water as the electron donor were described previously (7). For the measurement of NADP reduction with ascorbate as an electron donor, 0.5 nmole DCMU, 75 nmoles Na ascorbate and 7.5 nmoles DPIP were added to 40 μl of the mixture used in the O_2 evolving system.

Removal and readdition of hexane soluble chloroplast components was carried out following Krogman and Olivero's procedure (11). Plastoquinone A_{45} was obtained as a generous gift from Dr. O. Isler.

Comparison of the Effects of UV and Visible Light. Several chloroplast reaction systems were used to assay the effects of photoinhibition. 1) The full system of NADP reduction which involves both photoacts and includes the O_2 evolving step, water being the ultimate source of electrons. 2) The donor system of NADP reduction in which DCMU[3] inhibits O_2 evolution from water and ascorbate, mediated by DPIP, acts as an electron donor. Only photoact I is required for this reaction. 3) Photoreduction of DPIP with concomitant evolution of O_2. This reaction certainly requires photoact II and possibly both photosystems. 4) Photophosphorylation occurring concomitantly with assays 1 and 2

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[3] Abbreviations: DCMU, 3-(3,4-dichlorophenyl)-1,1-dimethylurea; PMS, phenazine methosulphate; P.Q., plastoquinone.

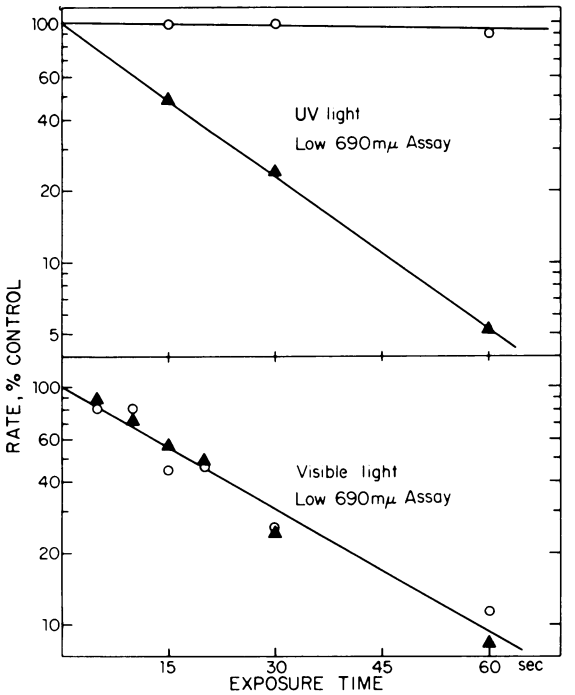

FIG. 1A (*top*) Comparison of photoinhibition by UV light (230–410 mμ) of the full (△) and donor (○) systems of NADP reduction assayed in low intensity 690 mμ actinic light. NADP reaction mixture as in Methods. Rate of controls: full system, 16; donor system, 15.5 μmoles NADP/mg Chl-hr. Chloroplasts from same irradiated sample were used for both assays. Fresh sample irradiated for each exposure time. Saturating rate of NADP reduction 86.5 μmoles NADP/mg Chl-hr. FIG. 1B (*bottom*). Same experiment as figure 1A but using visible (> 550 mμ) photoinhibitory light.

above. Figure 1 shows the effects of UV and visible light, respectively, on the full and donor system of NADP reduction. To allow a valid comparison, the activity of the chloroplasts before and after photoinhibition was assayed in weak light of 690 mμ. This wavelength was selected because, as found by Hoch and Martin (6), it is used with nearly identical quantum yields in both systems. The data show that in contrast to the full system the donor system of NADP reduction is completely insensitive to UV light. However, both systems are equally sensitive to inhibition by strong visible light.

The same sensitivity pattern is exhibited if the light used to assay chloroplasts activity is increased to above saturation. As shown in figure 2A, UV light destroys the full system but the activity of the donor system is unaffected. In high intensity visible light (fig 2B), the activity of the donor system (whose saturation rate is normally lower than that of the full system) does not start to decline until the activity of the full system has dropped to a comparable value, after which both activities decline in identical manner. One can therefore conclude that the dark reaction which limits the rate of the donor system at light saturation is not the reaction which is affected by strong visible light. Figure 2C illustrates that a light source which emits both UV and visible radiation such as a Xenon or Mercury arc lamp, filtered through only glass and water, produces results intermediate to the 2 above cases: both NADP reduction systems are inhibited, but the donor system to a lesser degree than the full

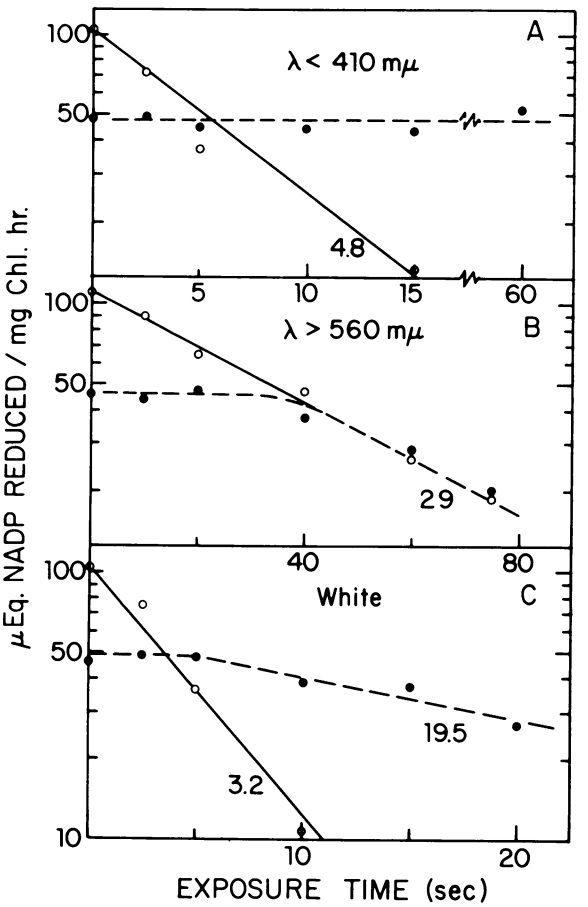

FIG. 2. Comparison of photoinhibition by 3 spectral regions of the full system (○) and the donor system (●) of NADP reduction in rate saturating red actinic light. Chloroplasts suspensions containing 4 μg chlorophyll in 40 μl were exposed; 1 μg aliquots taken and assayed for the 2 systems of NADP reduction. Reaction mixture as in Methods. Photoinhibitory light: Xenon arc plus filters to give desired spectral region. Curve C filtered only through 4 layers of glass and 15 cm of water. Half times (seconds) are labeled on each curve.

system. The UV type photoinhibition is predominant in these sources. Insufficient removal of UV radiation therefore explains the results of Kok et al. (9) who used a Hg arc and found photoact II to be more sensitive to photoinhibition than photoact I.

The simplest explanation of our results is that UV and visible light affect the photosynthetic mech-

anism at different sites. UV light inactivates a site which is part of photoact II and lies outside system I (is bypassed in the donor system). Visible light on the other hand, inactivates a site which is common to the full and the donor systems of NADP reduction, possibly a component of photoact I.

If UV and visible light truly have different sites of action, preirradiation with UV light should not change the rate or the kinetics of the decay of the (UV resistant) donor system in a subsequent exposure to strong visible irradiation. Results of an experiment using visible light with and without previous UV treatment are shown in figure 3. One of 2 aliquots of chloroplasts was irradiated with UV light during 9 minutes after which the activity of the full system was inhibited to about 18 % of the original value (left hand portion of fig 3). Both samples were then irradiated with visible light and the decline of their activity compared (right hand portion of fig 3). The rate and time course of the inhibition by visible light are identical in both samples. The effect of visible light upon the donor system is independent of previous UV treatment and of the activity of the full system, thus the 2 effects must be entirely different.

Although the donor system of NADP reduction is not sensitive to UV light, phosphorylation associated with this mode of electron transport is (fig 4). In both systems of NADP reduction, phosphorylation decays at a rate closely identical to the rate of decay of electron transport in the full system (not shown). DPIP and ascorbate thus restore the activity lost by UV photoinhibition in respect to NADP

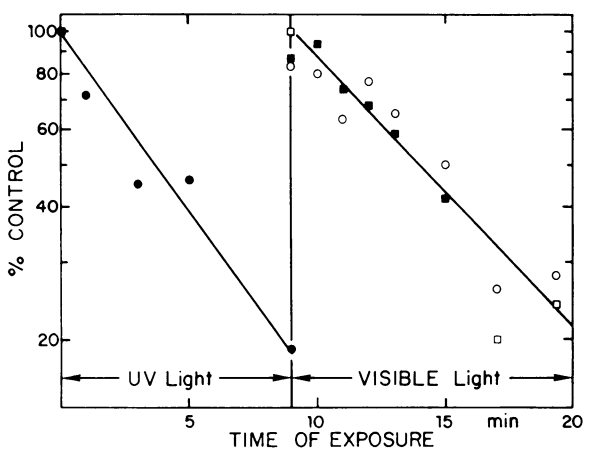

FIG. 3 (*left*). Decay of NADP reduction rate (full system) in UV light. (*Right*) Comparison of photoinhibition by red light of NADP reduction with (closed symbols) or without (open symbols) prior treatment with UV light. Full system: circles; donor system: squares. Assayed in limiting 690 mμ light as explained in Methods. Control rates: full system 25 μmoles NADP/mg Chl-hr; donor system: 21 μmoles NADP/mg Chl-hr. Light isolated from Xenon arc, UV = 230 − 410 mμ; visible, > 550 mμ. Irradiated in 3 ml quartz cuvette, assayed in 40 μl cuvette.

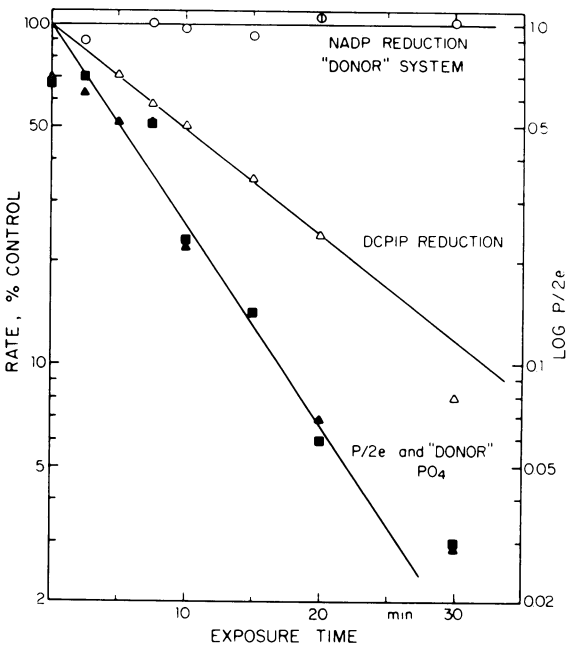

FIG. 4. Relative decay of rates in 253.7 mμ light of: ○, NADP reduction donor system, △, donor system phosphorylation; ■, $P/2_e$ ratio, △, DPIP reduction.

Reaction mixture for NADP reduction and phosphorylation contained in μmoles in 2 ml: 50, Tris-HCl; 2.5, K_2HPO_4; 15, MgCl; 0.5, P_{32} (cpm 26,000); 2, ADP; 1, NADP; 1, DCMU; 0.14, DPIP; 5, Na ascorbate; saturating PPNR and chloroplasts containing 50 μg chlorophyll. Reaction mixture was exposed to saturating light during 4 minutes and assayed for NADP reduction and phosphate incorporation. $P/2_e$ ratio assumed to be 1 in control. Donor system control rate: 57.8 μmoles NADP/NADP/mg Chl-hr.

DPIP reduction assayed in limiting light as in Methods. Control rate 31.5 μmoles DPIP/mg Chl-hr. Saturated rate 86.5 μmoles DPIP/mg Chl-hr.

reduction but not to photophosphorylation. Plausible explanations are that either 1) phosphorylation occurs at a locus in the electron transport chain which can be bypassed by $DPIPH_2$ without loss in the rate of electron transport, although the phosphorylating pathway is preferred, or 2) UV light does not inactivate the electron transport ability of the phosphorylation site but only its phosphorylating ability (uncoupling).

Site of UV Photoinhibition. Chloroplasts contain many compounds with UV absorption spectra resembling the action spectrum of photoinhibition we have observed. Many of these are probably altered by exposure to UV light. Bishop (3) and Shavitz and Avron (14) proposed that plastoquinone is the responsible moiety and several arguments favor this hypothesis. Plastoquinone has been proposed (1,17) as being close to or identical with the primary reductant of photosystem II as well as the site of photophosphorylation. It was shown in several laboratories that heptane extraction of lyo-

phylized chloroplasts results in a loss of activity of both O_2 evolution and photophosphorylation, but not of system I activity. The activity can be restored by recondensation of plastoquinone (2, 3, 11, 16). Bishop (3) and recently Trebst and Pistorius (15) found upon UV irradiation of chloroplasts a loss of plastoquinone which paralleled the loss of Hill reaction activity. Our action spectra (7) show a maximum between 250 and 260 mμ, the same location as that of plastoquinone absorption in ethanol. The 280 mμ shoulder could possibly be due to light absorption by the reduced form in vivo; in ethanol solution this form absorbs maximally at about 290 mμ. Another argument rests upon the high quantum yield value for photoinhibition by UV light in the chloroplasts (7).

Direct proof of the involvement of plastoquinone in UV photoinhibition would be the restoration of activity of photoinhibited chloroplasts by the addition of exogenous plastoquinone, analogous to the extraction and readdition experiments of Krogman and Olivero (11). Such experiments have been done by Shavitz and Avron (14), and by Trebst and Pistorius (15), but yielded negative results. A considerable number of such experiments were made in this laboratory, none of which were successful. We also tried other approaches to test whether plastoquinone is the moiety responsible for UV photoinhibition. One such procedure was based upon the 9-fold difference in molar extinctions of the oxidized and the reduced forms at 253.7 mμ. The sensitivity of the chloroplasts to 253.7 mμ light should be up to 9 times greater if the chloroplasts are exposed under conditions where the quinone is oxidized, than under conditions where the quinone is reduced. The data of Amesz (1) (if applicable also to chloroplasts) predict that in the presence of DCMU and far-red light, the pool of photosynthetically active quinone remains oxidized, thus yielding maximum sensitivity to UV light. Conversely, irradiation with short-wave visible light in the absence of a Hill oxidant should keep the pool of active plastoquinone reduced and thus lower the sensitivity of the chloroplasts to UV light. As seen in experiment figure 5, the 2 exposure conditions yielded no difference in sensitivity or kinetics (a slightly lower activity of the DCMU-treated chloroplasts was probably due to the additional washing procedure).

Still another procedure to check the role of plastoquinones was the following: after the plastoquinone is extracted from the chloroplasts, the sensitivity toward UV light should reside in the extract and the extracted chloroplasts should be much less sensitive. We indeed found that irradiating a heptane extract with UV light renders it incapable of reactivating extracted chloroplasts. The exposure time needed for half inactivation of the extract was shorter than the exposure needed to half-inhibit a comparable chloroplast suspension as one would expect from the absence of other masking pigments

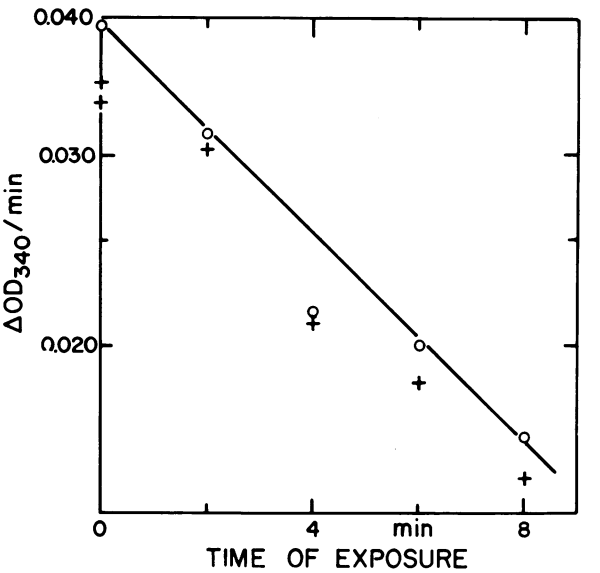

FIG. 5. Decay of DPIP reduction activity by UV irradiation of chloroplasts in the presence of DCMU (1.6×10^{-6} M) and far-red light (+), and in the presence of white light and the absence of DCMU (○) during UV irradiation. After exposure the samples containing DCMU were washed twice in suspending medium before the assay of activity in limiting light. Control rate; 18 μmoles DPIP/mg Chl-hr.

and light scattering. However, we observed at the same time that exposure to UV light of chloroplasts from which plastoquinone was extracted, rendered them incapable of reactivation by the readdition of plastoquinone or nonirradiated heptane extracts. These results could be explained either by incomplete extraction of the plastoquinone or by a multiplicity of the action of UV light.

In the heptane extract of chloroplasts, a decrease in absorption at 254 mμ is easily observed after exposure to 253.7 mμ light. A large concomitant decrease in absorption between 400 and 500 mμ is also brought about by the destruction of the carotenoids in the extract. Although the absorption of the carotenoids at 254 is relatively low, their molar extinction is similar to that of plastoquinone at this wavelength. Irradiation of pure, oxidized plastoquinone$_{45}$ in absolute ethanol causes the absorption spectrum to shift first to the absorption of the reduced form (peak at 290 mμ) plus a broad band between 400 and 500 mμ which persists for several minutes, giving the solution a pinkish cast. Longer periods of irradiation annihilate all structure in the spectra. Irradiation of the reduced form in ethanol, or of the oxidized or reduced form in heptane or CCl_4, causes a loss of absorption at all wavelengths without the formation of the 290 peak or the broad band between 400 and 500 mμ. Possibly upon irradiation in ethanol, the quinone forms a charge transfer complex with the solvent, ethanol being oxidized to acetaldehyde while the quinone becomes reduced.

Site(s) of Photoinhibition by Visible Light. NADP reduction with either water or ascorbate as electron donor is inactivated by strong visible light with identical rate constants. The photoreduction of DPIP, however, proved less sensitive, consistently showing ($\sim$ 2 times) slower rate of decay. Peculiarly, this same difference of sensitivity between the DPIP and the (full) NADP system was observed with UV photoinhibitory light (cf. fig 4). The sensitivity of NADP reduction clearly indicates inactivation of photoact I by strong visible light. On the other hand, the action spectra reported in (7) were typical for the pigments associated with photoact II both in spinach chloroplasts and *Anacystis* particles. These spectra were measured using DPIP reduction to assay activity. It seemed unlikely that pigments of photoact II would sensitize the photodestruction of photoact I and we thus wondered whether the inactivation of DPIP reduction could be sensitized by different pigments (system II) than NADP reduction (system I or both I and II). To answer this question, we compared the sensitivity of the O_2 evolving process to photoinhibition using either DPIP or NADP as an electron acceptor.

Two wavelengths were used: 646 mμ, which sensitizes both photoacts–system II somewhat in excess–and 703 mμ which sensitizes chiefly system I (cf. 12). Table I shows that the NADP system is about equally sensitive to both wavelengths. The DPIP system is less sensitive than the NADP system in 650 light ($\sim$ 1/2) and much less so in 703 light ($\sim$ 1/4). The simplest explanation of these data is that DPIP is reduced by system II only, or mainly, and that both photoacts are inactivated independently by strong visible light, system I being the more sensitive. This agrees with the observation that NADP reduction with ascorbate as a terminal electron donor is equally as sensitive as the O_2 evolving system: in both cases the efficiency of quantum conversion is limited by the efficiency of system I. The simplest interpretation is that the reaction center of each photosystem can be inactivated by a photon absorbed by its associated harvesting pigment.

Table I. *Comparison of the Sensitivity of DPIP and Full System NADP Reduction to 646 mμ and 703 mμ Preillumination*

Assays, light measurement and exposure method identical to those of table III in (7). Photoinhibitory light isolated from Xenon arc illumination by interference filters. 646 mμ light; 10 mμ half band-width, 703 mμ light: wide band interference filter plus RG-8 short wave cut off (band 690 to 740 with peak at 703 mμ). $t_{1/2}$ calculated from complete decay curves. Fractional absorption measured with double-beam white sphere instrument.

Assay	Incident μEin/min	Absorbed μEin/min	Quanta Chl-min	$t_{1/2}$ (min)	Quanta/Chl for $t_{1/2}$
646 mμ preillumination					
Dye	3.25	0.819	740	1.9	1410
NADP	3.22	0.812	730	1.15	840
703 mμ preillumination					
Dye	11.5	0.885	800	3.7	2960
NADP	11.1	0.855	780	1.0	780

Chloroplasts from spinach grown in the winter (either from the green house or market) were 2 to 3 times more sensitive to photoinhibition by visible light than chloroplasts from spinach grown in the summer. Summer grown chloroplasts therefore were more difficult to work with. They also showed a smaller difference in sensitivity between dye and NADP reduction. The lower light intensities available for growth during the winter season probably cause the production of chloroplasts with an increased amount of harvesting pigment per active photosynthetic unit (i.e. shade plants). As a result, each individual reaction center will receive a larger fraction of the absorbed quanta which will increase the chance of its inactivation. This agrees with the observations of Kok et al. (10) that chloroplasts prepared from greenhouse spinach grown in winter had a lower capacity of cytochrome c photooxidation (photoact I) than summer market spinach. It remains to be elucidated whether such a seasonal variation of the photosynthetic unit is less for system II, as our data seem to imply.

Discussion

Radiation between 230 and 750 mμ has a multiplicity of deleterious effects upon photosynthesis. The inhibition by UV light of photosystem II and photophosphorylation and the inhibition by visible light of photosystem I, and photosystem II precede a photo-destruction of chlorophylls and carotenoids.

As described in the preceeding paper (7) the mode of action of inhibitory light was quite similar, regardless of wavelength. In all cases we observed validity of the I $\times$ t law, absence of an (O_2) or temperature effect and a decay of quantum yield of the affected process. Thus, although we could distinguish 3 types of photoinhibition, with different quantum yields and sites of action, the primary mechanisms might be much alike.

The actual nature of the photoinhibitory effects is far from clear. Ultraviolet light probably affects several cell components, one of which, plastoquinone, is generally assumed to function at the reducing side of photosystem II. Surveying our results (e. g. fig 4) and those of Shavitz and Avron (19), and Trebst and Pistorius (15), the various chloroplasts reactions can be arranged in order of decreasing sensitivity to UV light: 1) phosphorylation coupled to NADP reduction (either full system or with ascorbate as electron donor) 2) O_2 evolution with concomitant NADP reduction 3) O_2 evolution concomitant with DPIP reduction 4) phosphorylation mediated by PMS 5) NADP reduction with ascorbate as electron donor. Whereas the latter is entirely unaffected, the 4 processes do not differ

greatly in sensitivity (2, at most 4-fold). Noteworthy in this connection are the similar subtle differences between these 3 processes in their response to lyophylization, extraction and readdition of P.Q. or other chloroplast quinones (see Ogren et al. 13).

Concerning the inhibition by visible light, the present data (e. g. table I) seem to fit best with the assumption that 2 sensitizations and 2 sites of action are involved: one affecting system II and one destroying system I. White, and especially long wave light, affects the latter system more strongly than the first. It was previously reported from this laboratory (9) that the photoinhibition of system I activity was accompanied by a loss of detectable P700, its reaction center. The actual mechanism of this inactivation is as yet unclear.

It is even more difficult to pinpoint the site of inactivation of photosystem II by visible light. However, we should point out that in respect to the damage to system II, UV and visible light act alike in many aspects and are distinctly different than any of the presently known chemical inhibitors of this system (8, 9). For instance, Malkin and Jones (to be published) observed a linear relation between the decay of the O_2 evolution activity and of the variable fluorescence component, regardless whether UV or visible light had been used to inactivate the sample. Thus, until a more precise analysis is available, one might adhere to the speculation that the inactivated site is identical in both cases. Conceivably, this site could be destroyed either by a (UV) photon absorbed by itself, a high quantum yield process, or by a chlorophyll sensitized excitation, a low quantum yield process.

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