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The nature of photoinhibition in isolated thylakoids

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Isolated broken chloroplasts were exposed to irradiances up to 200 $W \cdot m^{-2}$ at temperatures between 0°C and 20°C. The degradation of their photochemical apparatus resembles that, induced in photosynthesizing cells by extremely high irradiances (photoinhibition) or by moderate irradiances in presence of chloramphenicol. Electron transport through Photosystem II and variable fluorescence decline in parallel, displaying irradiance-dependent biphasic kinetics (typical half-times of hours). The decay of Photosystem II activities is followed by a delay of several hours by disappearance of the pigment-protein complex CPa (the core of Photosystem II). Photosystem I activity and the corresponding pigment-protein complexes disappear much slower than those of Photosystem II, particularly in thylakoids exposed to light at lower temperatures (below 10°C). Exposures were made in the presence and absence of: electron acceptors, oxygen (vs. nitrogen flushing), ascorbate, catalase and DCMU; none of these agents caused a substantial difference in the rate of degradation. Bovine serum albumin increases nonspecifically the stability of all chloroplast activities both in light and dark. Our results agree with the proposed central role in the inhibition of the Q_B protein. The cause of its inactivation remains obscure. Hypotheses assuming PQ^-, $PQ^=$ or activated oxygen as the noxious species do not conform with some of our data. The primary step in Q_B protein inactivation need not be a damage to it; its modification serving regulatory purposes is an alternative possibility.

Introduction

Inhibition of protein synthesis on 70S ribosomes (e.g., by (D-)threochloramphenicol) in photosynthesizing cells results in a fast decline of electron transport through PS II accompanied by a parallel disappearance of variable fluorescence (F_v). The block in electron transport seems to be situated on the reducing side of PS II. PS I activity and constant fluorescence F_0 remain nearly unchanged during the period sufficient for a full suppression of PS II activities [1–5]. This response will be further referred to as the chloramphenicol effect. In various representatives of oxygenic photosynthesis (cyanobacteria, algae and higher plants) it is characterized by essentially similar features which are as follows [3,4]. The inhibition occurs only in light and its rate increases with increasing irradiance; it has a biphasic course with a half-time

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Abbreviations: BQ, *p*-benzoquinone; CPa, pigment-protein complex, the core of Photosystem II; DAB, 3,3′-diaminobenzidine; DCIP, 2,6-dichlorophenolindophenol; DCMU, 3(3,4-dichlorophenyl)-1,1-dimethylurea; DPC, 1,5-diphenylcarbazide; F_0, constant fluorescence; F_{max}, maximal fluorescence; F_v, variable fluorescence; FeCy, ferricyanide; LHCP, light-harvesting chlorophyll protein; Pheo, pheophytin; PS, Photosystem; PQ, plastoquinone; Q_A, primary electron acceptor of Photosystem II; Q_B, secondary electron acceptor of Photosystem II.

of the fast phase (at saturating irradiances) in the range of 30 min to 2 h. With a delay of 1–3 h the inhibition of electron transfer is followed by the degradation of the core of PS II detected as the selective disappearance of the CPa band in electrophoretograms [4]. The pigment-protein complexes constituting PS I become affected only after a considerable reduction of the amount of CPa occurred.

If photosynthetic cells not treated with chloramphenicol are exposed to excessively high irradiance an effect is induced which is well known as photoinhibition of photosynthesis (for a review, see Ref. 6). The events characterizing the early phases of photoinhibition resemble those encountered with the chloramphenicol effect [3,7].

Evidence has accumulated [8–10] that the common primary cause in both cases is inactivation, and subsequent removal from the thylakoid membrane of the 32 kDa rapidly turned over Q_B protein which mediates electron transfer between the primary (Q_A) and secondary (Q_B) electron acceptors of PS II and thus acts as the Q_A-plastoquinone oxidoreductase. This protein is identical with the 'proteinaceous shield of PS II' [11] and is also the target of PS-II-inhibiting herbicides [12]. Its properties and functions have been reviewed extensively [13]. The current hypothesis [5,13,14] is that the Q_B protein becomes inactivated by an irradiance-dependent reaction which inevitably accompanies the PS II function. It is digested by a specific protease and simultaneously resynthesized at a corresponding rate. Since the Q_B protein is being encoded and expressed in the chloroplast in the presence of chloramphenicol, its synthesis is suppressed and the net rate of its degradation becomes measurable.

Broken isolated chloroplasts do not possess the protein-synthesizing machinery. They also lose their photochemical activity more readily in the light than in the dark [6,15,16]. This paper provides evidence that the loss of activity in the early phases of exposure to light is predominantly due to processes operating in the chloramphenicol effect. Recently, light-dependent degradation of the Q_B protein in isolated pea thylakoids was demonstrated [26].

Materials and Methods

Leaves of manna grass (*Glyceria maxima*) were collected from plants growing on the shore of a pond near the laboratory. Leaves of sunflower and kohlrabi were taken from plants in the laboratory garden. Spinach and wheat were grown in an air-conditioned chamber (KTLK 1250, G.D.R.) under the following conditions: 10 h light (150 $W \cdot m^{-2}$ * from a mixture of incandescent lamps and high-pressure mercury discharge lamps) at 20°C and 14 h dark at 17°C. Spinach was used for experiments in the sixth week and wheat in the third week of growth.

The experiments were performed with crude preparations of isolated chloroplasts. Approx. 5 g freshly harvested leaves were washed with tap water, cut to small pieces and ground in a homogenizer Ultra-Turrax (Janke and Kunkel KG, F.R.G.) for 30 s with 25 ml of grinding medium (1 M mannitol/10 mM $MgCl_2$/7.5 mM KH_2PO_4/42.5 mM K_2HPO_4, giving pH 7.5, with or without 1% bovine serum albumin). After filtration through six layers of muslin the debris were removed by centrifugation at 2000 rpm for 2 min. Chloroplasts were spun down at 6000 rpm in 5 min. The pellet was resuspended in the exposure medium (medium E: 0.5 M mannitol/10 mM $MgCl_2$/50 mM potassium phosphate buffer (pH 7.5), with or without the addition of 0.5% bovine serum albumin) and diluted to a chlorophyll concentration of 15–30 $\mu g \cdot ml^{-1}$ as indicated.

200 ml of this suspension (exposure mixture) were then exposed to defined irradiances from incandescent lamps in plate-parallel cuvettes placed in a constant temperature bath. The path of the light through the cuvettes was 20 mm long. The irradiances indicated in the text were measured in the plane of the front wall of the cuvette. A photosynthetic irradiance meter constructed in this laboratory [17] with an energy proportional response between 400 and 700 nm was used to this purpose. The control was kept in the dark at the same temperature. The suspension was gently mixed by a slowly blowing air stream containing 2% CO_2.

* All irradiances throughout the paper refer to photosynthetically active radiation (λ = 400–700 nm).

Leaf discs were exposed to white light from incandescent lamps in the annular irradiation chamber [18]. During the exposure the discs were supplied with water through their cut edge, contacting a water-soaked polyurethane foam. In the inhibition experiments distilled water in the foam was replaced by the chloramphenicol solution (except for control sectors).

Oxygen exchange measured with a Clark-type oxygen sensor [19] was used in most cases to assess the rates of electron transport. Oxygen production with 2 mM benzoquinone or 2 mM ferricyanide as electron acceptors characterized the Hill reaction rate (electron transport through PS II). Oxygen consumption with 1 mM diaminobenzidine as electron donor and 200 μM methyl viologen as acceptor in the presence of 1 μM DCMU and 1 mM NaN_3 was used as a measure of PS I activity. All electron-transport rates were measured in the presence of 5 mM NH_4Cl to uncouple phosphorylation. Irradiance saturating the electron transport (150–300 $W \cdot m^{-2}$) was used in these measurements; light from a projector incandescent lamp passed the red filter RA 63 (Schott, Jena) with a cutting edge at $\lambda = 630$ nm.

The electron transport from H_2O or diphenylcarbazide (300 μM) to dichlorophenolindophenol (20 μM) was assayed by recording the decrease in absorbance at 580 nm. The phosphoroscope described below was used to this purpose using only the chopped analytical beam ($\lambda = 580$ nm) and unprotected photomultiplier to detect absorbance. The course of ferricyanide reduction was assessed by taking samples from the exposure cuvettes, settling the chloroplasts by centrifugation and measuring the absorbance of the supernatant at 421 nm from spectra recorded with Specord UV-VIS (Carl Zeiss, Jena).

All electron transport rates were measured at 20°C, irrespective of the exposure temperature. If chloroplasts were exposed to light in presence of DCMU the samples were washed twice with the exposure medium prior to electron transport measurements. In control experiments this washing restored always more than 90% of the initial activity. With chloroplasts exposed in the presence of ferricyanide or methylviologen Hill reaction rates were measured as oxygen evolution rates upon addition of 2 μM benzoquinone to the samples.

The constant and variable components of the fluorescence in presence of 5 μM DCMU were measured with a phosphoroscope built in the laboratory workshop and similar to that described in Ref. 20. Samples containing 5 $\mu g \cdot ml^{-1}$ of chlorophyll were stirred at 20°C in the phosphoroscope cuvette of cubic shape (volume, 3 ml) provided with a constant temperature jacket. The white actinic light from a 150 W halogen lamp was, as a rule, set at 100 $W \cdot m^{-2}$. The blue analytic beam (about 50 $mW \cdot m^{-2}$) was obtained by passing the light from a 30 W tungsten lamp through glass filters BG 12 and BG 28 (Schott, Jena). It was chopped at a frequency of 3 kHz. The modulated fluorescence response was detected by the photomultiplier EMI 9558 B protected by the cut-off Schott (Jena) filter RG 6 (edge at 670 nm) and the Schott interference filter SiF 700 ($\lambda_{max} = 700$ nm). The signal from the photomultiplier was amplified by the lock-in nanovoltmeter Unipan 232 B (Poland) and recorded on the pen recorder K201 (Carl Zeiss, Jena).

The course of fluorescence induction in the presence of 5 μM DCMU was measured on samples placed in the phosphoroscope cuvette. The cylindrical rotating screen was removed in this case and the samples were excited directly by the actinic beam (light from a 150 W halogen lamp passed through the filter combination BG 12 and BG 28). The irradiance of the cuvette was about 2 $W \cdot m^{-2}$ in this case and the lamp was fed from a stabilized DC source. Fluorescence was detected by the photomultiplier protected as above and viewing the cuvette perpendicularly to the direction of the exciting beam. The scattered light passing the protection filters was less than 5% of the F_0 value. The induction curve was recorded by the transient recorder Datalab DL 912 (Mitcham, U.K.) and was processed both numerically and graphically in the calculator HP-85. The half-rise time of variable fluorescence was calculated according to Ref. 21.

The low-temperature emission spectra were recorded by an instrument built in the laboratory and consisting of the high-pressure mercury lamp HBO 200, two monochromators SPM 2 (Carl Zeiss, Jena) and a photomultiplier EMI 9885 B. The first monochromator was used to select the line for excitation (436 nm in most cases). The leaf seg-

ments kept on a holder were plunged directly into liquid nitrogen in a Dewar flask. The signal from the photomultiplier was digitized in the transient recorder and the spectra were recorded for the spectral sensitivity of the detector in the calculator.

Polyacrylamide gel electrophoresis of the pigment protein complexes from sodium-dodecylsulfate-solubilized thylakoids was performed by a slightly modified method of Andersson and Anderson [22], as described in Ref. 23. The gels were scanned in a device constructed in the laboratory using the monochromator SPM 2 to obtain the scanning beam of the desired wavelength. The bands on the electrophoretograms are labelled according to the nomenclature introduced by Anderson et al. [24].

Results

The irradiance dependence of the Hill reaction photoinhibition in isolated chloroplasts of *Glyceria* is shown in Fig. 1A. The biphasic kinetics, characteristic of the chloramphenicol effect in algal cells (cf. Ref. 3) is well expressed in this case. This is not always so with isolated chloroplasts (see, e.g., Figs. 2, 4 and 7). With this proviso, courses of photoinhibition measured with isolated chloroplasts of wheat (Fig. 2), barley, spinach, sunflower and kohlrabi (data not shown) were rather similar to those presented in Fig. 1. The addition of bovine serum albumin to the isolation and exposure medium yields higher initial rates of the Hill reaction and is essential for maintaining stable activity of chloroplasts incubated in the dark. Photoinhibition also proceeds faster in the absence of albumin.

In Fig. 1B the decline of the relative yield of variable fluorescence (F_v/F_0) is illustrated for the same preparation of *Glyceria* chloroplasts. Its irradiance dependence and the protective effect of bovine serum albumin are similar as with the Hill reaction. Expressed in percentage of the initial value F_v/F_0 decreases always somewhat faster than the Hill reaction rate. In Fig. 1B this difference is characterized quantitatively by the ratio $(f_{ET} - f_{F_v})/f_{ET}$, where f_{ET} is the fraction of initial electron transport activity and f_{F_v} the fraction of initial variable fluorescence yield which remain

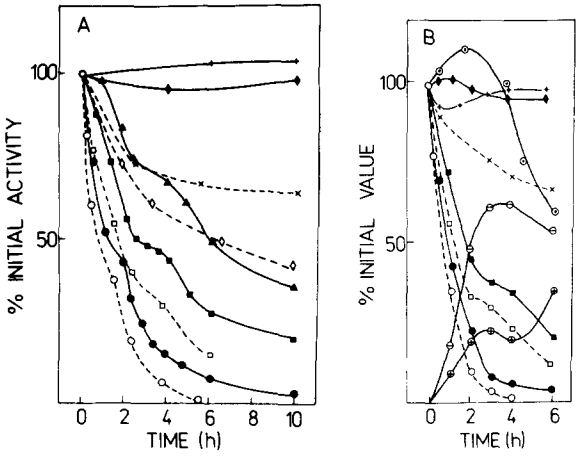

Fig. 1. The irradiance dependence of PS II photoinhibition in isolated *Glyceria* chloroplasts exposed to light at 20°C in a mixture containing 15 $\mu g \cdot ml^{-1}$ of chlorophyll. (A) Rates of electron transport expressed as percentages of initial activities which were as follows: Hill reaction ($H_2O \rightarrow BQ$) 150 and 105 μM O_2 per mg Chl per h for exposures with and without bovine serum albumin, respectively; PS I activity (DAB → methyl viologen) 335 and 280 μequiv. e^- per mg Chl per h with and without bovine serum albumin, respectively. The meaning of symbols: ◆ and ◇, PS I activity with and without bovine serum albumin, respectively, irradiance 75 $W \cdot m^{-2}$ in both cases. All other values are for Hill reaction rates: full symbols and + for exposures with bovine serum albumin, open symbols and × (dashed lines) for exposures without bovine serum albumin. Irradiances: + and ×, dark; ▲, 25 $W \cdot m^{-2}$; ■ and □, 75 $W \cdot m^{-2}$; ● and ○, 200 $W \cdot m^{-2}$. (B) Fluorescence characteristics in the same experiment; identical symbols are used for values from the same exposures in part A and B. ◆, F_0 value, irradiance 75$W \cdot cm^{-2}$, with bovine serum albumin. All other points are for F_v/F_0 ratio (initial value 1.6): + and ×, dark, ■ and □, 75 $W \cdot m^{-2}$, ● and ○ 200 $W \cdot m^{-2}$ with and without bovine serum albumin, respectively. The points ⊖ (200 $W \cdot m^{-2}$) and ⊕ (74 $W \cdot m^{-2}$) characterize the difference (see text for calculation) between corresponding values for percentage inhibition of Hill reaction rate (in A) and of F_v/F_0 (in B). The points ⊙ give the relative half-rise time of F_v in samples from the 200 $W \cdot m^{-2}$ exposure.

detectable in the preparation. As can be seen, the ratio increases with the progress of photoinhibition. F_0 remains approximately constant during the experiment. The half-rise of the fluorescence induction also does not change significantly during the first phases of photoinhibition. Comparable results to those shown in Fig. 1B were also obtained with chloroplasts of wheat (cf. Fig. 2), spinach and kohlrabi (data not shown). Thus the events illustrated in Fig. 1A and B can be assumed

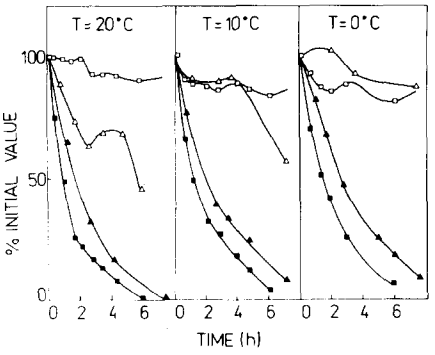

Fig. 2. The temperature dependence of photoinhibition by 100 $W \cdot m^{-2}$ in isolated wheat chloroplasts. The exposure mixture contained 15 $\mu g \cdot ml^{-1}$ of chlorophyll and 0.5% bovine serum albumin. Electron transport rates and fluorescence yields were measured at 20°C and are expressed as percentages of initial values which were: 118 μM O_2 per Chl per h for the Hill reaction ($H_2O \rightarrow BQ$), 230 μequiv.e^- per mg Chl per h for PS I activity (DAB → methyl viologen) and 1.45 for F_v/F_0. Meaning of the symbols: ▲, rate of Hill reaction, △, PS I activity; ■, F_v/F_0; □, F_0.

to occur in chloroplasts isolated from various plants.

It is characteristic of the chloramphenicol effect in photosynthetic cells that the PS I structure and function are little affected during the time period sufficient for considerable suppression of PS II activity (cf. Ref. 3). In the experiment illustrated in Fig. 1 the rate of electron transport through PS I (Fig. 1A) and the value of the constant component of fluorescence (Fig. 1B) also change little during the light exposure. With isolated chloroplasts, however, this picture is usually obtained only at lower exposure temperatures (less than 10°C) as illustrated for wheat chloroplasts in Fig. 2. Very similar results were obtained also with spinach chloroplasts (data not shown). In both cases the inhibition of electron transport through PS I is substantially accelerated with increasing temperature. F_0 does not show this strong temperature dependence: the structure responsible for this part of fluorescence is, apparently, less affected. The stability of PS I seems also to depend on conditions of growing the plant material. At 20°C the PS I was much more stable in chloroplasts isolated from *Glyceria* leaves harvested in a series of sunny summer days, as compared to leaves harvested in autumn or from plants grown in the air-conditioned chamber. Photoinhibition of the PS II activities is only moderately temperature dependent (Fig. 2).

If a sample withdrawn from the exposure cuvette is being illuminated in the measuring chamber no oxygen production is detected unless artificial electron acceptors are added. Endogenous acceptors are evidently absent in the exposure mixture. We have exposed chloroplast to moderate and saturating light with and without ferricyanide and methyl viologen (see Fig. 3). No significant difference in the rate of PS II inactivation was found to result from the presence of the acceptors. The course of ferricyanide reduction was followed spectrophotometrically and its concentration was restored when it falled below 1 mM (see Fig. 3). The availability of methyl viologen as electron acceptor during the exposure was controlled by taking samples of the exposure mixture and measuring the oxygen uptake upon addition of NaN_3 to block catalase present in the mixture. As a rule some light-depen-

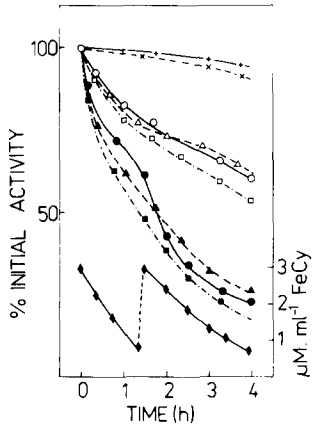

Fig. 3. The course of PS II photoinhibition in isolated *Glyceria* chloroplasts exposed at 10°C to 30 $W \cdot m^{-2}$ (open symbols and ×) and to 100 $W \cdot m^{-2}$ (full symbols and +) in the presence of electron acceptors. The exposure mixture contained 20 $\mu g \cdot ml^{-1}$ of chlorophyll and 0.5% bovine serum albumin. The 200 $W \cdot m^{-2}$ and 30 $W \cdot m^{-2}$ exposures were run in succession with the same preparation of chloroplasts. The initial rates of the Hill reaction ($H_2O \rightarrow BQ$) were 135 and 125 μM O_2 per mg Chl per h, respectively. Meaning of the symbols: ● and ○, exposure mixture without acceptor in the light and, + and ×, in the dark. ▲ and △ exposure mixture with 3 mM ferricyanide added at the start of the experiment and restored after 90 min; ◆, the course of ferricyanide reduction for the 100 $W \cdot m^{-2}$ exposure, scale on the right. ■, □, exposure mixture with 2 mM methyl viologen and 400 catalase units per ml added at the start of the experiment.

dent O_2 uptake was observed even without NaN_3 addition; this may result from oxidation of reduced methyl viologen by other cellular components present in the crude chloroplast preparation.

The rate of ferricyanide reduction in Fig. 3 may appear low compared with the Hill reaction rates indicated in the legend to this figure and elsewhere in this paper. It has to be kept in mind, however, that the exposure was at 10°C, while the Hill reaction rates were measured at 20°C and with benzoquinone as electron acceptor. Rates of ferricyanide reduction assessed at 20°C with a comparable suspension of chloroplasts were slightly more than twice the rates at 10°C. Initial rates with 20 μg Chl · ml^{-1} were around 5 μmol FeCy · $ml^{-1} \cdot h^{-1}$ at 20°C as compared with 2 μmol FeCy $ml^{-1} \cdot h^{-1}$ in the experiment illustrated. The former value corresponds to an oxygen evolution rate of 62.5 μmol O_2 per mg Chl per h and, since oxygen evolution with benzoquinone as electron acceptor is faster by a factor of approx. 2, the Hill reaction rate around 130 μmol O_2 per mg Chl per h is obtained.

Inhibition of electron transport through PS II by DCMU also does not change the rate of photoinhibition (Fig. 4). Samples taken from the mixture incubated with DCMU were washed free of the inhibitor (see Methods) and assayed for Hill reaction. In the same experiment (Fig. 4) the effect of the following conditions during the light exposure of the chloroplasts was investigated: substantial reduction of oxygen partial pressure achieved by passing pure nitrogen through the mixture and decomposition by catalase of the potentially present H_2O_2 (resulting from pseudocyclic electron flow). Neither of these conditions caused a noticeable change in the rate of the photoinhibition as compared with the control mixture.

The localization of the block in PS II electron transport due to photoinhibition is still controversial (see Discussion). We have confirmed that if the rate of dichlorophenolinophenol reduction by PS II becomes photoinhibited in isolated chloroplasts this cannot be enhanced by addition of diphenylcarbazide (Fig. 5). Similarly, no increase in F_v could be produced by the same treatment (data not shown). These results do not exclude the possibility that inhibition of electron transport

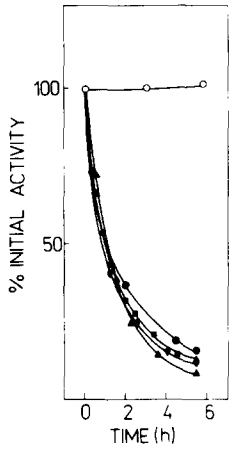

Fig. 4. The course of photoinhibition of the Hill reaction ($H_2O \rightarrow BQ$) in wheat chloroplasts exposed to 200 $W \cdot m^{-2}$ at 20°C under various conditions: ●, control mixture without additions flushed with air + 2% CO_2; ■, mixture flushed with N_2 + 2% CO_2; ◆, the same with 1 μM DCMU; ▲, the same with $4 \cdot 10^5$ units of catalase; ○, control mixture in the dark. The mixtures contained 25 $\mu g \cdot ml^{-1}$ of chlorophyll and 0.5% bovine serum albumin. The initial Hill reaction rate was 95 μM O_2 per mg Chl per h.

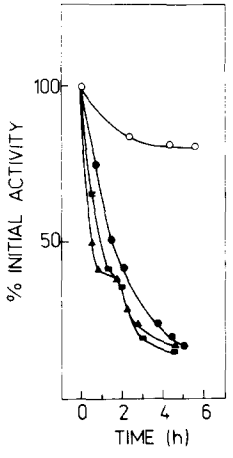

Fig. 5. The course of photoinhibition of the Hill reaction with water or diphenylcarbazide as electron donor to PS II. Glyceria chloroplasts, irradiance 150 $W \cdot m^{-2}$, temperature 20°C. The exposure mixture contained 18 $\mu g \cdot ml^{-1}$ of chlorophyll and 0.5% of bovine serum albumin. Initial rates of the Hill reaction were 96 μM O_2 per mg Chl per h for electron transport $H_2O \rightarrow BQ$, 245 μequiv.e^- per mg Chl per h for $H_2O \rightarrow$ DCIP and 112 μequiv. e^- per mg Chl per h for DPC → DCIP. The control initial rate for the transfer DPC → DCIP was obtained by heating the chloroplasts at 50°C for 2 min. Meaning of the symbols: ●, $H_2O \rightarrow BQ$; ▲, $H_2O \rightarrow$ DCIP; ■, DPC → DCIP; ○, $H_2O \rightarrow BQ$ for the mixture kept in the dark.

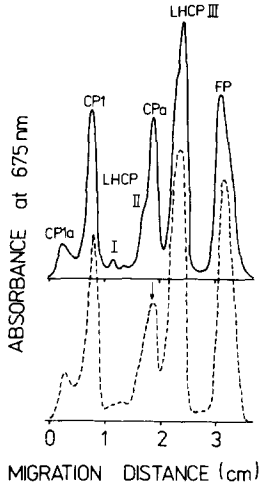

Fig. 6. Scans of gels showing electrophoretic separation of the pigment protein complexes from isolated *Glyceria* chloroplasts before (full line) and after (dashed line) 7 h of photoinhibitory treatment in the experiment illustrated also in Fig. 7. The rate of Hill reaction in the chloroplasts was reduced to less than 10% at the time of sampling, the amount of CPa to about 65%. The quantity of CPa was estimated by deconvolution of the merged peaks of CPa and LHCP II using electrophoretograms from later stages of photoinhibition.

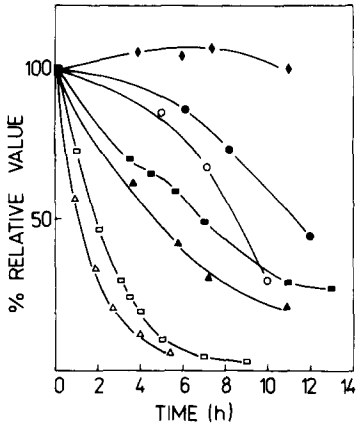

Fig. 7. A comparison of photoinhibition in isolated *Glyceria* chloroplasts (open symbols) and in chloramphenicol (5 mM) treated *Glyceria* leaf discs (full symbols). Both chloroplasts and leaf discs were exposed to 200 $W \cdot m^{-2}$ at 20°C. At the times indicated by the experimental points batches of discs were sampled and properties of chloroplasts isolated from them were determined. The photoinhibition was monitored by the Hill reaction rate (□, ■), by variable fluorescence (▲, △) and by the amount of the CPa complex (○, ●). The half-rise time of the variable fluorescence in chloroplasts from leaf discs is also indicated (◆). All quantities are indicated as percentages of initial values: Hill reaction rate ($H_2O \rightarrow BQ$) 120 μM O_2 per mg Chl per h and $F_v = 1.45$. The PS I activity decreased very little in both cases (data not shown).

occurs between the donation site of DPC and the PS II reaction center. We were unable to obtain unequivocal results for the Hill reaction with silicomolybdate as electron acceptor.

The electrophoretic separation of pigment-protein complexes revealed that CPA (the core of PS II) selectively disappears in the later phases of the photoinhibition (see Fig. 6). The course of its disappearance is similar as in the case of the chloramphenicol effect; its noticeable decline appears with a lag of approx. 3 h following the Hill reaction rate decline (Fig. 7). The CPa degradation shows a similar irradiance dependence as the inhibition of the Hill reaction: at low irradiances it is slow, and the error in exact quantitation of the complex from scans of the gels makes it difficult to characterize its decay precisely.

We made a direct comparison of the two forms of photoinhibition using chloramphenicol-treated discs from *Glyceria* leaves on the one hand and isolated *Glyceria* chloroplasts on the other (Fig. 7). In both cases qualitatively the same changes occur in the electron transport, fluorescence and pigment-protein complexes, but the rate is much slower in the leaf discs. We assume that this is due to the shielding effect exerted by the chloroplasts in the upper cell layers of the leaf tissue on the

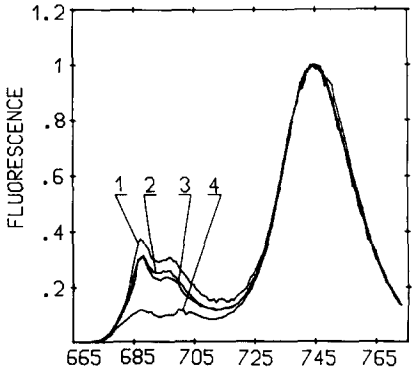

Fig. 8. A comparison of low temperature (77 K) spectra of the fluorescence emitted from the exposed (1, 4) and shaded (2, 3) surface of the chloramphenicol treated leaf discs before (1, 2) and after (3, 4) 4 h of strong actinic illumination (200 $W \cdot m^{-2}$). The discs were sampled from the experiment illustrated also in Fig. 7. The spectra were normalized to unity at the peak of the long-wavelength band.

chloroplasts in the bottom cell layers as described also by other authors [25]. The results illustrated in Fig. 8 support this assumption. In this figure, the low-temperature spectra are compared of the fluorescence emitted from the exposed and the shaded surfaces of the leaf discs which were exposed in the irradiance chamber in the presence of chloramphenicol. Changes in low temperature fluorescence spectra characteristic of the photoinhibition are much more pronounced on the upper surface of the discs than on their lower surface.

Discussion

Injury to the photosynthetic apparatus induced by exposure to light has been studied for a long time and many different effects have been described as photoinhibition [6]. In the present paper we concentrate on PS II inactivation which is most prominent among the short-term photoinhibition phenomena and is characterized by a parallel decline of electron transport through PS II and of variable fluorescence. In intact cells this inhibition has been shown to be tightly correlated with impairment of the Q_B protein [13] in the two situations: the photoinhibition under extremely high irradiances [5,7–9], and the effect of chloramphenicol under moderate irradiances [2–4,10]. The main point we wish to demonstrate by the results presented in this paper is that in isolated broken chloroplasts exposed to light qualitatively similar phenomena occur with comparable kinetics so that a common primary cause may be assumed. This makes sense, since the Q_B protein cannot be synthesized and integrated into the membrane in the preparations of isolated thylakoids. This was recently confirmed by a direct proof [26].

The crucial role played by the Q_B protein in all the above-mentioned variants of PS II photoinhibition can hardly be questioned. Several important points remain controversial, however, and these will now be discussed.

(1) Is the degradation of the Q_B protein the only primary cause of effects accompanying PS II photoinhibition?

The only direct proof that in early phases of photoinhibition the PS II electron transport is blocked solely at the Q_B protein site was obtained by Arntzen and co-workers in *Chlamydomonas* [8] and by Edelman and co-workers for the chloramphenicol effect in *Spirodella* [12,14]. Electron transport from water to silicomolybdate was little affected when the Hill reaction with conventional electron acceptors was largely suppressed. The former authors admit, however, that with progress of the inhibition damage on the donor side of PS II occurs. In the cyanobacterium *Microcystis aeruginosa* [26] electron transfer from both water and diphenylcarbazide to silicomolybdate became photoinhibited to the same degree as the transfer from the respective donor to DCIP. We have found the results with silicomolybdate as Hill reaction acceptor to be rather variable (data not shown) and so we would hesitate to accept either result as a conclusive argument. On the other hand we find good agreement between data from various laboratories [8,16,27,28] including our own results (Fig. 5) that photoinhibited PS II electron transport cannot be restored by artificial PS II electron donors. Consequently, if some block occurs on the oxidizing side of PS II it is situated between the site where artificial donors feed electrons and the PS II reaction center.

The decisive role of the Q_B protein in PS II photoinactivation is further suggested by the parallelity of its damage and repair in the thylakoids on one hand and of the progress and retreat of photoinhibition [9,10] or the chloramphenicol effect [2–4] on the other hand. Since the rate of Q_B protein synthesis is light dependent and at high irradiances faster by at least an order of magnitude than the synthesis rate of any other known thylakoid polypeptide this evidence, even if indirect, is rather significant. It can be namely assumed that damage to or destruction of another thylakoid protein is responsible for photoinhibition effects only if it is plausible that its repair can be as fast as it is with the Q_B protein. In this respect extremely attractive field for speculations opens with the accumulating information concerning the so called D2 protein [13]. This has the same relative molecular mass as the Q_B protein, displays a high degree of homology with the latter and is assumed also to bind a quinone molecule that might act as the primary electron donor to P-680.

(2) What is the nature and the molecular mechanism of Q_B-protein inactivation?

In the various forms of photoinhibition the rate of PS II inactivation increases with increasing irradiance. Hence, the assumption readily occurred that the Q_B protein gets worn out proportionally to the rate of electron transfer mediated by it [2,3,7]. More specifically it was proposed [5,8] that either the singly reduced semiquinone radical or the doubly reduced quinone may cause damage to the active site of the Q_B protein which mediates their reduction and stabilizes the intermediary reduction products (see Ref. 13 for a review on possible mechanisms of the process). An alternative proposal ascribes the noxious effect to oxygen radicals formed through interaction of molecular oxygen with the quinone radicals in the immediate vicinity of the Q_B protein active site [9]. Supporting evidence for these hypotheses has been thought to come from the effect of PS-II-inhibiting herbicides on photoinhibition. It is well documented that compounds like DCMU or atrazine bind close to the active site of the Q_B protein and thus prevent the diverse quinone species from contacting it (see Ref. 29 for a review). It was found [8] that photoinhibition in *Chlamydomonas* monitored as the parallel decline of the Hill reaction rate and of variable fluorescence yield was retarded in atrazine-treated cells. On the other hand, prelabelled Q_B protein was seen to disappear from the thylakoids of illuminated *Spirodella* plants more slowly in the presence of DCMU [10].

We failed to find any relieving effect of DCMU or atrazine on the inhibition either in chloramphenicol-treated algae [3] or in isolated chloroplasts (Fig. 4). The unchanged course of photoinhibition in the nitrogen flushed exposure mixture (Fig. 4) is at variance with the activated oxygen hypothesis. In the DCMU-poisoned cells no oxygen should be generated photosynthetically. Finally, the actual concentration of the reduced quinone species should be lower in thylakoids incubated with an ample supply of electron acceptor as compared with thylakoids illuminated without acceptors. Nevertheless, we did not find any difference in the rate of photoinhibition under the two sets of conditions (Fig. 3). It is, of course, possible that the transfer of electrons to artificial acceptors represents the limiting step even under non-saturating irradiance (curves for 30 $W \cdot m^{-2}$ in Fig. 3) so that the PQ pool is largely reduced also in this case. Direct estimations of the reduced fraction in the PQ pool would be clearly desirable (cf. Ref. 30).

Thus, in our opinion, convincing evidence is still missing that the first step in Q_B protein inactivation is a damage to it, resulting from its function. As an alternative hypothesis we propose that this step consists in a detachment of the Q_B protein from its functional site. This could be related to a dephosphorylation event as indicated by the reports that phosphorylation of the Q_B protein is a precondition of its proper function [31] and by the very fast onset of photoinhibition in the presence of higher concentrations of uncouplers described in Ref. 3. The proposal does not fall in line, however, with the presently popular view that a restriction of PS II activity is, as a rule, related to a phosphorylation event. Thoroughly documented is the role of LHCP phosphorylation in State 1–State 2 transitions [32]. It has been also found that in vitro thylakoid phosphorylation brings about all the effects characteristic of photoinhibition although in a rather limited extent [33]. Further experimental evidence is needed to decide between the two hypotheses.

(3) What is the cause of the decline of variable fluorescence?

With all diverse types of PS II photoinhibition it can be observed that the variable component of PS II fluorescence (F_v) declines in parallel with the Hill reaction rate and ultimately disappears. The constant component (F_0) remains unchanged or is only slightly affected (Fig. 1b and 2, cf. also Refs. 2,4,6,8,27,28,34,35,36 and 37). Concomitantly corresponding changes occur in the shape of low-temperature (77 K) emission spectra, i.e., gradual lowering of the F_{695} and F_{685} peaks is observed (Fig. 9, cf. also Refs. 4 and 25).

The simplest and straightforward explanation of this parallelity would be that photoinhibition interrupts the electron-donating chain on the oxidizing side of PS II. At present, this cannot be accepted in view of the direct and indirect evidence, showing that, at least in the first phases of photoinhibition, the only block in the PS II electron transport occurs at the Q_B protein site.

Kyle et al. [8] analyzed the problem from this point of view and they propose several mechanisms which could reconciliate the seemingly contradictory facts. They assume that in PS II reaction centers with an inactivated or detached Q_B protein one of the following processes may take place.

(a) A very efficient path for cyclic electron flow around PS II opens and brings about an extremely fast oxidation of reduced Q_A.

(b) The centers become entrapped in the nonfluorescent state with reduced pheophytin, i.e., P-680 · $Pheo^- \cdot Q_A^-$, similarly as it happens if thylakoids are illuminated in the presence of a strong reducing agent [38].

(c) A structural modification in PS II reaction centers increases the probability of thermal deexcitation of P-680* and, herewith, lowers the fluorescence yield.

We agree that these data are the three most important possibilities to be considered. We also find the first mechanism to be highly improbable both for reasons given in Ref. 8 and in view of some of our results (not presented in this paper). We cannot, however, endorse the choice of mechanism (c) as the most plausible one [8]. If thermal deexcitation of P-680* should become so efficient as to entirely quench the fluorescence, the yield of photochemistry should also drop considerably and the Hill reaction with silicomolybdate could not persist.

On the other hand we find the reduced pheophytin variant (b) to be most probable. The hypothesis that variable fluorescence in PS II is generated by a charge recombination between P-680^+ and $Pheo^-$ [38,39] is now generally accepted. In the original experiments by Karapetyan and Klimov [40], from which this idea developed, phenomena resembling the characteristics of PS II photoinactivation were observed: in broken isolated chloroplasts incubated in the presence of dithionite illumination produced an irreversible decrease of both F_v and Hill reaction rate which persisted after the thylakoids were washed and assayed in a medium without the reducing agent.

Dithionite has been assumed by most authors to have a dual role: to provide a reducing environment around Q_A^- and to increase the probability of P-680^+ reduction during the lifetime of charge separation. Recently, it was proposed [41,42] that the specific effect of dithionite consists in reducing Q_A not to Q_A^-, but to its quinol form, Q_AH_2. In contrast to Q_A^-, from which the electron may apparently drain off under any conditions, Q_AH_2 is relatively stable. If, further, pheophytin is burried deeply within the protein matrix of the reaction center and can equilibrate with the outer phase only through Q_A [41], it can be understood why $Pheo^-$ can accumulate only when Q_A is reduced to Q_AH_2. Moreover, it is conceivable that some modification of the Q_B protein or its detachment may change the environment of Q_A, so as to render it prone to this aberrant mode of reduction. It should be recalled in this connection that partial trypsin digestion of the Q_B protein in thylakoids also leads to gradual disappearance of F_v [43].

In considering the behaviour of fluorescence in photoinhibition it should be also kept in mind that F_v decline runs always slightly ahead of the Hill reaction inhibition. With the progress of photoinhibition an increasing fraction of PS II reaction centers that are still capable of photochemistry appears to be nonfluorescent. At present we are unable to suggest any explanation of this phenomenon.

(4) What is the relation between photoinhibition of PS II and of other steps in the photosynthetic electron transport?

In the present paper we try to show that in isolated broken chloroplasts the Q_B protein linked PS II inactivation is the most important component of photoinhibition in the first hours of chloroplast exposure to moderate irradiances. This is in line with results in Ref. 16 and with results of photoinhibition experiments (including the chloramphenicol effect) in algae and in cyanobacteria [3,4,7,8,27] as well as in leaves of higher plants [2,6,28,44]. Compared with intact cells, however, the inhibition of PS I activity (and, perhaps, of other steps in electron transport) proceeds relatively faster, or sets in earlier, in isolated chloroplasts illuminated at 20°C. PS I inactivation can be slowed down markedly by lowering the incubation temperature (Fig. 2) and its response to the protecting effect of bovine serum albumin is also more pronounced (Fig. 1A). We take this as an indication that there is no such intrinsic ground

for photoinactivation of PS I as it exists for PS II; PS I inhibition largely reflects the impairment of functional integrity of the thylakoidal membrane by diverse factors in the exposure medium.

Since the rate of PS I inactivation in isolated chloroplasts depends noticeably on conditions of the experiment it may be difficult to get a detailed insight into the relative importance of PS II vs. PS I inactivation in experiments with only one fixed time period of exposure to light [36]. It is also not surprising that different ratios for PS II/PS I inactivation were reported for the same type of chloroplasts under different conditions [16,45].

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