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HYDRAZOBENZENE OXIDATION BY 2,6-DICHLOROPHENOL-INDOPHENOL IN A PHOTOREACTION CATALYZED BY SYSTEM I OF PHOTOSYNTHESIS

HYDRAZINE COMPOUNDS AS DONORS FOR PHOTOSYSTEM II*

JAAP HAVEMAN, LOUIS N. M. DUYSENS,
THEO C. M. VAN DER GEEST AND HANS J. VAN GORKOM
Biophysical Laboratory of the State University, Schelpenkade 14 A, Leiden (The Netherlands)

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SUMMARY

1. The kinetics of the photooxidation of hydrazobenzene with dichlorophenol (DCIP), methylviologen and $NADP^+$ as electron acceptors were studied in spinach chloroplasts, Tris-treated chloroplasts and System I and II particles, in the presence and absence of dichlorophenyldimethylurea (DCMU). The hydrazobenzene–DCIP photoreaction was much less inhibited by DCMU than the reactions with the other acceptors.

2. For chloroplasts the action spectrum of the quantum yield for the hydrazobenzene–DCIP redox reaction showed a maximum at 710 nm, indicating System I participation. Three or more electrons were transported per quantum absorbed at 700 nm.

3. It is concluded from these and other experiments that hydrazobenzene can be oxidized in at least two photoreactions. In the first place it acts as an efficient donor for System II. This reaction is inhibited by 5 μM DCMU. A second hydrazobenzene–DCIP reaction, which is not inhibited by DCMU, is presumably catalyzed *via* an oxidized component of the redox chain between Q and System I.

4. Effects of hydrazobenzene on fluorescence and luminescence of chlorophyll a_2 (the chlorophyll *a* of System II), carotenoid bleaching, and cytochrome b_{559} oxidation of System II particles can be explained by the efficient electron donation to System II.

5. *N,N'*-Phtaloylhydrazine and dichlorophenylhydrazine were found to be relatively efficient donors to System II, although less efficient than hydrazobenzene. The redox reactions with DCIP were inhibited by DCMU.

Abbreviations: CCCP, carbonylcyanide 3-chlorophenylhydrazone; DCIP, 2,6-dichlorophenolindophenol; DCMU, 3-(3',4'-dichlorophenyl)-1,1-dimethylurea; P700, pigment absorbing at about 700 nm, the primary photooxidant of System I; Q, "quencher", the primary electron acceptor of System II; Tricine, *N*-tris(hydroxymethyl)methylglycine.

* Dedicated to Professor Dr H. Veldstra on the occasion of his retirement from the chair of biochemistry of the University of Leiden.

INTRODUCTION

The following compounds are able to donate electrons to Photosystem II instead of water in a 3-(3',4'-dichlorophenyl-1,1-dimethylurea (DCMU)-sensitive way after destruction of the enzymic steps leading to oxygen evolution by heat or Tris treatment: *p*-phenylenediamine[1–3], hydroxylamine[4,5], semicarbazide[2,6,7], ascorbate[5,8], Mn^{2+} (refs 9–11), hydrazine[12,13], *sym*-diphenylcarbazide[6,7] and diketogulonate[14]. These artificial electron donors can be used to detect activity in subchloroplast particles (see *e.g.* Vernon and Shaw[6]). Haveman and Donze[15] reported that, amongst other substances with a hydrazine group in the molecule, hydrazobenzene was able to stimulate 2,6-dichlorophenolindophenol (DCIP) reduction in heat-treated chloroplasts and in System II particles prepared by means of digitonin. At a high hydrazobenzene concentration this reaction appeared to be DCMU insensitive, which could not be explained satisfactorily.

In this study we will give evidence that hydrazobenzene acts in a System I catalyzed DCIP reduction, which is not inhibited by DCMU and which takes place with the unexpectedly high quantum yield of more than 2 electrons per quantum absorbed. Evidence will also be given that hydrazobenzene is an efficient donor to Photosystem II; this reaction is inhibited by DCMU. Hydrazobenzene will be compared with some other hydrazine compounds, *e.g.* phtaloylhydrazine. The effect of hydrazobenzene on the photooxidation of cytochrome b_{559}, on pigment bleaching in System II particles, and on chlorophyll a_2 fluorescence and luminescence will also be described.

MATERIALS AND METHODS

Digitonin subchloroplast particles were prepared according to the method of Boardman and Anderson[16]. Chloroplasts in 50 mM phosphate buffer (pH 7.8), 10 mM KCl, 0.4 M sucrose, were incubated with digitonin (1 g per 60 mg chlorophyll, final concn 1 %) for 30 min at 4 °C. After removal of unbroken chloroplasts System II particles were sedimented at 25000 × *g* and System I particles at 144000 × *g*. These were resuspended in phosphate buffer and stored in liquid N_2. Before use the particles were diluted with phosphate buffer without sucrose. The System II particles had a chlorophyll *a*/*b* ratio of 2.0 to 2.3 and a P700 concentration as estimated from the absorption decrease at 705 nm (in the presence of 1 mM ascorbate and 20 μM DCIP) of 1 molecule to 800–1000 chlorophyll molecules. It was assumed that the molar extinction of P700 at 705 nm is the same as the one for chlorophyll *a in vivo* at 680 nm. The System I particles had a chlorophyll *a*/*b* ratio of 4.5–4.9 and a P700 concentration of 1 to 200–250 chlorophyll molecules.

Chloroplasts were prepared the same day in 50 mM *N*-tris(hydroxymethyl)-methylglycine (Tricine) buffer (pH 7.8), 10 mM KCl, 2 mM $MgCl_2$ and 0.4 M sucrose, and diluted in the same buffer without sucrose just before the experiments. In order to inhibit the O_2 evolving system, the chloroplasts were exposed for 5 min to a 0.2 M Tris buffer (pH 9.0), centrifuged and resuspended in the original buffer. Chlorophyll concentrations were estimated according to Arnon[17].

DCIP reduction, $NADP^+$ reduction, carotenoid bleaching[18,19] and absorption changes near 560 nm due to cytochrome b_{559} were measured with an Aminco–Chance

spectrophotometer (American Instrument Co., Silver Spring, Md., U.S.A.) in the split-beam mode, equipped with side illumination for one of the two 1 cm × 1 cm × 4 cm cuvettes. The device for the actinic side illumination was constructed in this laboratory. The actinic light passed a filter set which, except for the measurement of the quantum yields and action spectra, consisted of a Schott RG 645 glass filter and a Balzers Calflex C-1. These filters isolated a band between approx. 645 and 750 nm giving an intensity at the place of the cuvette of 25 $mW \cdot cm^{-2}$, as measured by an YSI–Kettering Model 65 radiometer (Yellow Springs Instrument Co., Ohio, U.S.A.) which saturated the photochemical reactions studied. The photomultiplier was protected from the actinic light by a Corning Cs 4-96 glass filter and/or an appropriate Balzers interference filter. For action spectra the actinic light was filtered by a Balzers interference filter and a Balzers Calflex C filter. The maxima of the interference filters used occurred at the following wavelengths in nm (the half band width is given in nm in brackets): 632 (12), 641 (11), 659 (12), 666 (12), 670 (12), 683 (11), 690 (14), 699 (12), 714 (11) and 724 (14). The intensity of the incident light was measured by a S1 vacuum phototube calibrated with the YSI–Kettering radiometer. The fraction absorbed by the chloroplasts or digitonin subchloroplast particles was measured for each filter and was expressed in $neinstein \cdot cm^{-2} \cdot s^{-1}$. For the estimation of quantum yields the molar extinction coefficient of DCIP (600 nm) was assumed to be $2.0 \cdot 10^4$ $cm^{-1} \cdot M^{-1}$. Hydrazobenzene, like hydrazine[13], caused a considerable dark reduction of DCIP: at 50 μM hydrazobenzene and 60 μM DCIP (in the presence of System II particles, 15 μg chlorophyll/ml) the absorption change was 0.5 per min. The rate of dark reduction was proportional to the concentration of hydrazobenzene at a fixed concentration of DCIP and was not altered after a period of illumination with actinic light, taking into account the decrease in hydrazobenzene concentration. The rate of the dark reduction was measured each time and the data given were corrected for it.

Delayed fluorescence was measured by means of a Becquerel phosphorescope, in principle similar to that described by Clayton[20]. The discs of the phosphorescope had 10 holes and rotated at 50 revolutions per s. The luminescence was measured about 1 ms after each flash. Prompt fluorescence was measured simultaneously by a second photomultiplier. The actinic light was filtered by a Corning Cs 4-96 and a Schott BG 18 glass filter giving an incident intensity of 5 $mW \cdot cm^{-2}$. The photomultipliers were provided with filter sets consisting of a Schott RG 665 glass filter and interference filters (Balzers or Schott) with maximum transmittance at 680 nm. The rate of $NADP^+$ reduction could also be determined by this apparatus by measuring the increase in fluorescence around 450 nm (ref. 21). The exciting light was provided by a mercury lamp and was filtered through a Schott UG 11 glass filter; actinic light was provided by a second incident beam and was filtered by a Schott RG 665 glass filter: the photomultiplier was protected from actinic light by means of a Schott GG 400, a Corning Cs 4-96 glass filter and a Balzers broadband "K3" interference filter.

O_2 consumption was measured by means of a Clark type electrode (Yellow Springs Instrument Co.). The reaction chamber was illuminated from one side by an Aldis projector fitted with a 500 W incandescent lamp. The light passed first a 0.1 M $CuSO_4$ solution (1 cm light path) and was filtered by means of a Schott RG 645 glass filter, providing a saturating intensity for the photochemical reactions studied.

Digitonin, 2,5-dichlorophenylhydrazine, *N*,*N'*-phtaloylhydrazine, semicarbazide

and *sym*-diphenylcarbazide were obtained from Fluka, Buchs, Switzerland; hydrazinoethanol from Schuchardt, München, Germany; DCMU from K and K Laboratories, U.S.A.; ferredoxin from Sigma Chemical Co., St. Louis, U.S.A.; nigericin from Eli Lilly and Co., Indianapolis, U.S.A.; NADP from Boehringer, Mannheim, Germany; CCCP was a gift from Dr P. G. Heytler (DuPont de Nemours); all other chemicals were obtained from E. Merck, Darmstadt, Germany or BDH Chemicals, Poole, England.

Hydrazobenzene, dichlorophenylhydrazine, phtaloylhydrazine diphenylcarbazide, nigericin, carbonylcyanide 3-chlorophenylhydrazone (CCCP) and DCMU were dissolved in ethanol and were used at a final concentration of less than 0.3 % ethanol.

RESULTS AND DISCUSSION

In Table I the effect of artificial electron donors on DCIP reduction with System II particles is shown. Without added donor the activity is rather low, apparently due to damage to the O_2 evolving mechanism caused by digitonin treatment. As in Tris-treated chloroplasts[2,7] artificial electron donors are able to enhance DCIP reduction, replacing water oxidation.

From the donors mentioned in Table I, hydrazobenzene attracts attention by its DCMU insensitivity. Haveman and Donze[15] reported that chloroplasts in saturating light the rates of DCIP reduction in μmoles·mg^{-1} chlorophyll·h^{-1} were 115 and 0, in the absence and presence of 5 μM DCMU, respectively. In the presence of 100 μM hydrazobenzene these rates were 264 and 235, showing that addition of hydrazobenzene practically removed DCMU inhibition.

In Fig. 1A the rates of DCIP photoreduction by System II particles is shown as a function of the hydrazobenzene concentration in a double reciprocal plot, in the presence and absence of DCMU. The straight lines crossing on the ordinate can be interpreted to indicate that hydrazobenzene removes the inhibition by displacing DCMU. The lines of Fig. 1B show that for phtaloylhydrazine the percentage of inhibition by DCMU does not increase with decreasing donor concentration.

The observation that the DCMU-inhibited non-cyclic photophosphorylation in chloroplasts with methylviologen as acceptor (in the presence of O_2) was not relieved by hydrazobenzene (L. N. M. Duysens and P. H. Lems, unpublished observations) indicated that the above interpretation that hydrazobenzene relieves inhibition by displacing DCMU was not correct; hydrazobenzene alone did not inhibit photophosphorylation.

Fig. 2 shows that in Tris-treated chloroplasts, with methylviologen as acceptor, and hydrazobenzene as donor, electron transport is largely inhibited by DCMU. This also indicated that hydrazobenzene acts mainly as a conventional System II donor. The hydrazobenzene-stimulated DCMU-insensitive reduction may be caused by electron donation by hydrazobenzene to the electron transport chain between Photoreactions I and II.

The same conclusions may be drawn from the experiments given in Table II. Here the effect of added hydrazobenzene on $NADP^+$ reduction in chloroplasts is shown. Under approximately the same conditions, O_2 evolution was inhibited by 100 μM hydrazobenzene by 40 %, presumably due to competition of hydrazobenzene with water as electron donor for Photosystem II. In the presence of DCMU, hydrazobenzene was able to restore $NADP^+$ reduction only to a small extent.

TABLE I

ARTIFICIAL ELECTRON DONORS TO PHOTOSYSTEM II AS INHIBITORS OF THE CAROTENOID BLEACHING AND AS DONORS TO DCIP REDUCTION IN SYSTEM II PARTICLES

Carotenoid bleaching was measured at 490 nm in the presence of 5 μM CCCP and the initial slope of the kinetic curve was determined. DCIP reduction was measured at 600 nm with 60 μM DCIP present. Chlorophyll concentration in both cases: 15 μg/ml.

Donor	*Concn (mM)*	*% inhibition carotenoid bleaching*	*DCIP reduction (μmoles·mg chlorophyll^{-1}·h^{-1})*	
			Without DCMU	*With 5 μM DCMU*
	—		18.4	0.0
Semicarbazide	0.8	62		
	5		25.4	0.0
	8	89		
	20		43.2	0.0
Hydrazinoethanol	0.5	52		
	1	81		
	5	96	40.0	0.0
	20		54.4	0.0
Phtaloylhydrazine	0.1	64	41.3	0.0
	0.2	86		
	0.5	99	64.8	0.0
sym-Diphenylcarbazide	0.005	37		
	0.01	47		
	0.05	84		
	0.1		36.7	0.0
	0.5		59.2	0.0
Dichlorophenylhydrazine	0.0005	41		
	0.005	87		
	0.05	96		
	0.1		75.2	2.9
	0.5		91.2	6.1
Hydrazobenzene	0.0002	62		
	0.0005	88		
	0.001	94		
	0.05		115.2	46.3

If one assumes that DCMU inhibits at one location, then the foregoing experiments show that the DCMU-insensitive part of the light-driven redox reaction between hydrazobenzene and DCIP is not caused by Photoreaction II. It may be driven by light absorbed by pigment System I. Another possibility is that it is sensitized by pigments of System II, but not *via* the DCMU-sensitive Photoreaction II. In order to solve this question, action spectra of the quantum yield of DCIP reduction in chloroplasts were determined with water and with hydrazobenzene as donor. The quantum yield was determined by extrapolation to zero light intensity as described in Fig. 3. The action spectrum with hydrazobenzene as donor (open triangles of Fig. 4) deviates markedly from that of the normal DCIP reduction (solid circles) and shows that long wavelength absorbing chlorophyll contributed more effectively to the hydrazobenzene stimulated reaction than the short wavelength forms. The shape of

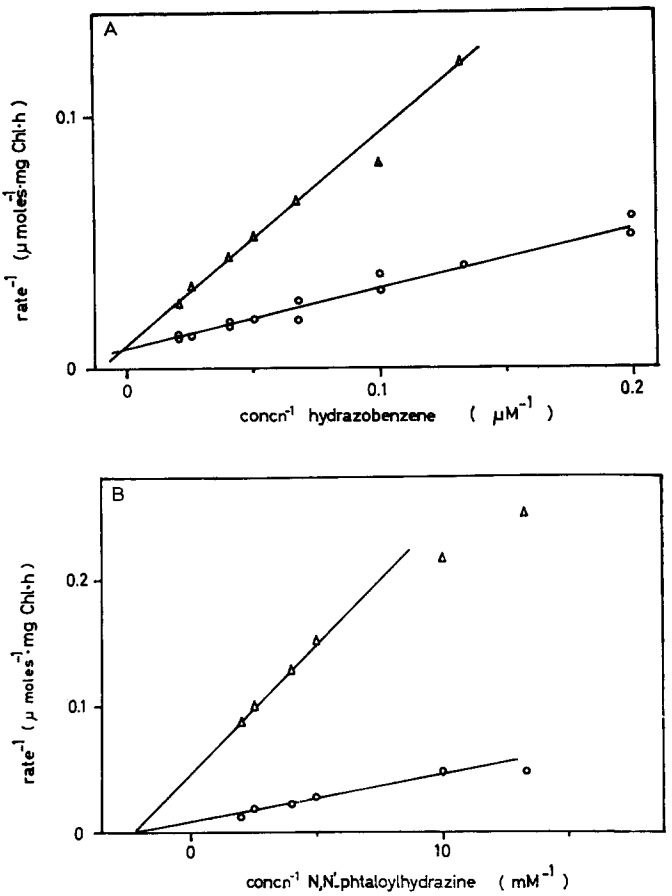

Fig. 1. Double reciprocal plots of the rate of DCIP reduction *versus* donor concentration for System II particles (15 μg chlorophyll/ml) which were largely devoid of O_2 evolution activity; 60 μM DCIP; ○—○, without DCMU, △—△, with 1 μM DCMU. (A) With hydrazobenzene. (B) With phtaloylhydrazine.

the top spectrum indicates that System I is largely responsible for the hydrazobenzene–DCIP photoreaction at low intensity. The quantum yield was not lowered by addition of DCMU: The initial parts of the rate *versus* intensities curves with and without DCMU coincided (Fig. 5). Around 710 nm the quantum yield (electrons per quantum) for the hydrazobenzene–DCIP reaction exceeded 2 for most preparations: *e.g.* the quantum yield at 711 nm calculated by extrapolation to zero intensity (Fig. 3) amounted to 5.5, and the quantum yield at the lowest intensity actually used was about 4. The high quantum yield around 710 nm indicates that the hydrazobenzene–DCIP reaction is not an electron transfer *via* Photoreaction I, but a redox reaction catalyzed by some intermediate or product of the Photosystem I reaction. The reaction resembles the disproportion of diphenylcarbazone described by Shneyour and Avron[22] for which very high quantum yields were also found. The quantum yield of the normal DCIP reduction in our preparations with water as donor was about 0.5 electron equivalents per absorbed quantum, which is the expected order of magnitude. In the

presence of 0.5 mM *sym*-diphenylcarbazide or of 0.5 mM phtaloylhydrazine both the quantum yields and the shapes of the action spectra were the same as in the presence of DCIP alone.

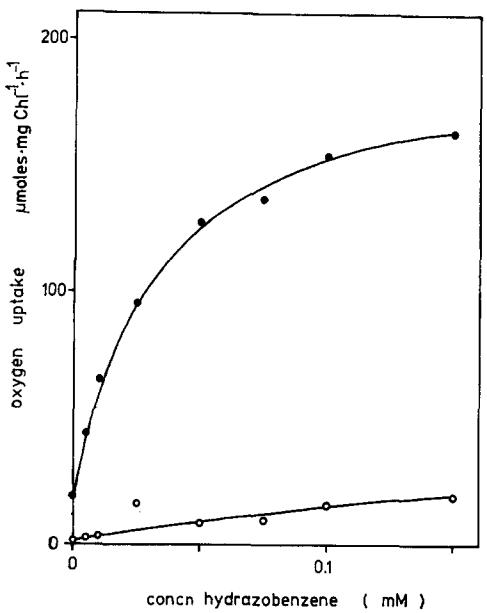

Fig. 2. Effect of hydrazobenzene on the methylviologen mediated O_2 uptake in Tris-washed chloroplasts. The reaction mixture contained: Tris-washed chloroplasts 10 μg chlorophyll/ml, 50 μM methylviologen, 0.5 mM methylamine, 0.5 mM NaN_3 and the concentrations of hydrazobenzene as indicated. ●—●, without DCMU, ○—○, with 5 μM DCMU.

TABLE II

INFLUENCE OF ADDED HYDRAZOBENZENE ON THE $NADP^+$ REDUCTION IN CHLOROPLASTS

Measured by means of the fluorescence of NADPH at 450 nm, the control rate being approx. 80 μmoles·mg chlorophyll^{-1}·h^{-1} as measured by the absorption change near 340 nm, assuming a molar extinction coefficient for NADPH of $6.2 \cdot 10^3$ cm^{-1}·M^{-1}. The reaction mixture contained chloroplasts in Tricine buffer, 30 μg chlorophyll/ml and in addition 8 μg/ml ferredoxin, 0.2 mM $NADP^+$ and where indicated 0.1 mM hydrazobenzene and/or 1 μM DCMU.

Additions	*Rate as percentage of control*
—	100
Hydrazobenzene	74
DCMU	5
Hydrazobenzene + DCMU	13

The following experiments provide additional evidence concerning the activity of hydrazobenzene and other hydrazine compounds as a donor for System II. System II particles and Tris-treated chloroplasts[18] show photobleaching of their pigments due to oxidation by the photooxidant of System II (ref. 19). We found that by adding 5 μM CCCP the rate of photobleaching of pigments was enhanced 2-fold. Table I

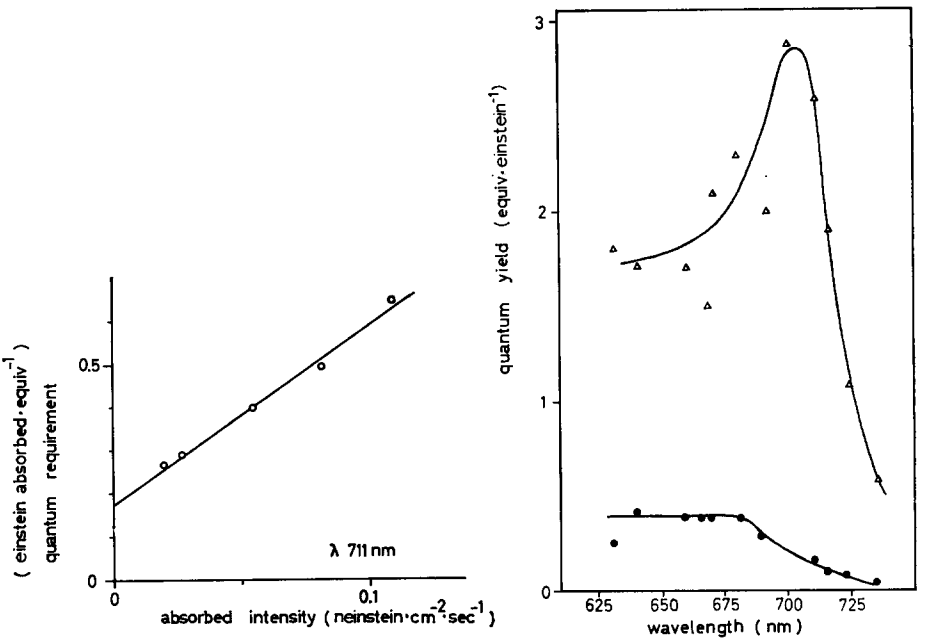

Fig. 3. Determination of the quantum yield of the DCIP reduction by chloroplasts at 711 nm with 20 μM hydrazobenzene, 20 μM DCIP and 5 μg chlorophyll/ml. The quantum requirement was found by linear extrapolation to zero intensity. The quantum yield is the inverse of this.

Fig. 4. Action spectra of the DCIP reduction. The reaction mixture contained 20 μM DCIP, 5 μg chlorophyll/ml chloroplasts. ●—●, with water as donor, △—△, with 20 μM hydrazobenzene as donor. The decrease in the quantum yield beyond 700 nm may well be an artifact because the scattering in this region may have been larger than estimated.

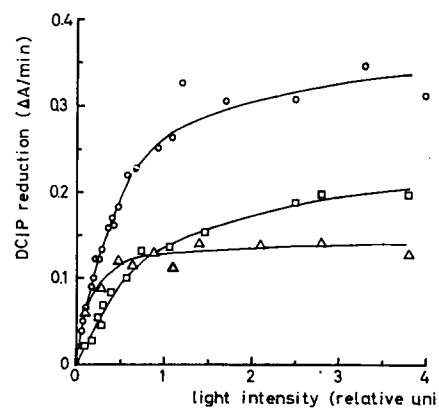

Fig. 5. Light intensity dependence of the DCIP reduction in chloroplasts. The reaction mixtures contained: 20 μM DCIP, 5 μg chlorophyll/ml. □—□, without further additions, ○—○, with 20 μM hydrazobenzene, △—△, with 20 μM hydrazobenzene and 5 μM DCMU. The actinic light was filtered by means of a Balzers "K6" interference filter and a Calflex C-1 giving a band from approx. 620–675 nm.

shows that artificial electron donors prevent the pigment bleaching, presumably by reducing the photooxidant. Hydrazobenzene prevents pigment bleaching at very low concentrations (down to 0.2 μM hydrazobenzene). These small amounts of hydrazobenzene are oxidized during the experiment as one can see in Fig. 6. During oxidation of hydrazobenzene an absorption increase occurred near 340 nm (maximum at 320 nm) which is presumably due to azobenzene: the absorption spectrum of azobenzene was the same as the oxidation product of hydrazobenzene. DCMU inhibits carotenoid bleaching apparently by inhibiting electron transport. As Fig. 6 shows it also retarded the hydrazobenzene consumption. In these experiments no electron acceptor was added. O_2 presumably acted as such. The low rate of electron transport (about 4 μmoles·mg chlorophyll^{-1}·h^{-1}) explains the fact that low concentrations of hydrazobenzene are not limiting and are thus effective.

Fig. 7 shows that chlorophyll a_2 luminescence by System II particles is diminished 3–4 times by the addition of 1 μM hydrazobenzene. When a sufficiently

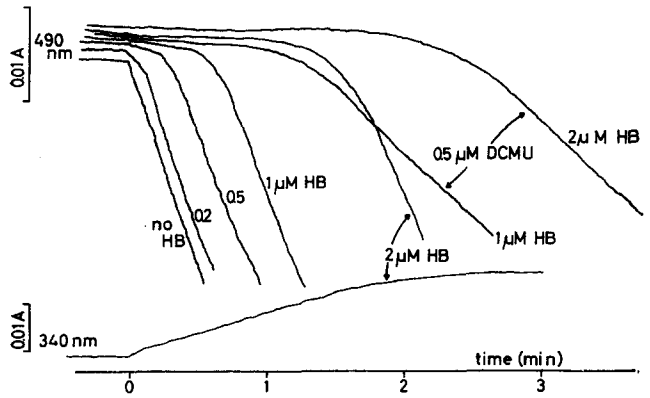

Fig. 6. Recorder tracings of carotenoid bleaching at 490 nm and hydrazobenzene (HB) photooxidation at 340 nm in System II particles, with hydrazobenzene and DCMU added at the concentrations indicated; 15 μg chlorophyll/ml; 5 mM CCCP is added to stimulate carotenoid bleaching. The inhibition by DCMU of carotenoid bleaching in the absence of hydrazobenzene was the same as observed after exhaustion of hydrazobenzene (see right hand side of the figure).

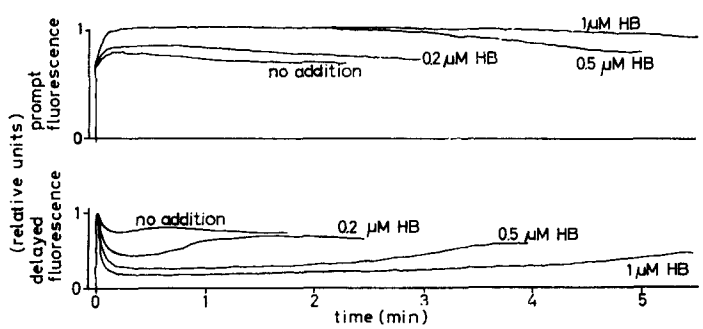

Fig. 7. The effect of hydrazobenzene (HB) on prompt and delayed chlorophyll fluorescence in System II particles. The prompt and delayed fluorescence are measured simultaneously at each concentration of hydrazobenzene 15 μg chlorophyll/ml. Illumination and measurement on the same side of the 1 mm cuvette.

low hydrazobenzene concentration was chosen, *e.g.* 0.2 μM, the delayed fluorescence returned after a while to the level of the control, apparently due to oxidation of hydrazobenzene as in the experiment with pigment bleaching. Hydrazobenzene apparently reduces the photooxidized component of System II, which in the absence of hydrazobenzene causes luminescence in a back reaction with the reduced primary acceptor Q^- (ref. 23). The stimulation of the (prompt) fluorescence yield by hydrazobenzene (same figure) is also to be expected since photoreduction of Q, which causes the increase in fluorescence, requires a reduced primary electron donor. Reduction of this donor by water is largely inhibited in these System II particles. Yamashita and Butler[2] made similar observations on fluorescence with other donors. Also phtaloylhydrazine lowered the steady-state level of the delayed fluorescence emission and stimulated the fluorescence. No reversal of the effect was observed after a short time since the donor was not exhausted because of its high concentration.

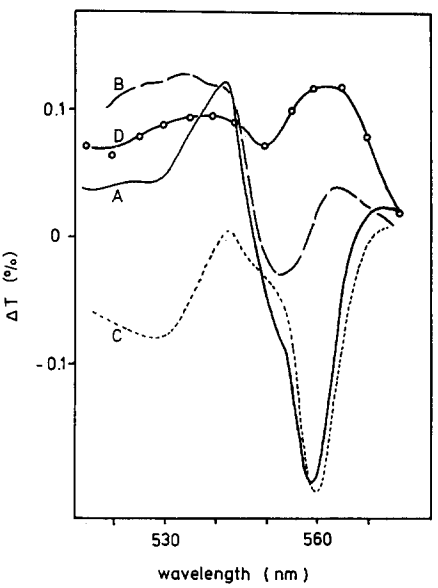

Fig. 8. Light *minus* dark difference spectra of System II particles. 50 μg chlorophyll/ml, in the presence of 0.5 μM nigericin, 1 cm light path. Curve A without further additions, Curve B with 10 μM hydrazobenzene, Curve C is Curve A *minus* Curve B, Curve D with 1 μM CCCP. The half band width was about 2.5 nm. The noise level was about 0.01 % of the transmitted light.

In Fig. 8 the light-induced absorbance changes in the α- and β-band region of cytochromes of the System II particles are shown. They were determined by scanning spectra before and during illumination and subtracting them (Curves A and B), or by measuring the absorption change upon illumination at a fixed wavelength using a new sample for each point (Curve D). Nigericin was added to minimize contributions of the 515-nm change which affect the spectrum up to 545 nm. A photooxidation of cytochrome b_{559} is evident; the other changes will not be discussed.

The photooxidation was completely inhibited by 5 μM DCMU (not shown) indicating that this photooxidation is due to Photosystem II activity. The photooxidation was largely prevented by 10 μM hydrazobenzene (Curve B). Addition of hydrazo-

benzene did not change the baseline in the dark and did not accelerate the (very) slow dark reduction of cytochrome b_{559} in the dark after illumination. These results indicate that cytochrome b_{559} photooxidation can be observed, if the photooxidant of System II is not reduced by the water splitting system (which is largely absent in these System II particles) or by hydrazobenzene. Knaff and Arnon[24] using Tris-treated chloroplasts and Erixon and Butler[25] using untreated chloroplasts at liquid N_2 temperature presumably could observe cytochrome b_{559} for the same reason.

Hydrazobenzene efficiently competes with cytochrome b_{559} as a donor to Photosystem II. Phtaloylhydrazine at a concentration of 0.5 mM prevented pigment bleaching but did not appreciably prevent cytochrome b_{559} oxidation, which suggests different points of entry for hydrazobenzene and phtaloylhydrazine, or a less efficient reduction by the latter compound.

Curve D suggests that in the presence of 1 μM CCCP some photoreduction occurred, which was inhibited by 5 μM DCMU. In the presence of CCCP presumably cytochrome b_{559} shifts from its high potential form to its low potential form (see Cramer and Böhme[26]).

CONCLUSIONS

We have established two (perhaps three) different photoreactions driving the hydrazobenzene–DCIP redox reaction.

(1) A conventional DCMU-inhibited redox reaction driven by System II (Fig. 2).

(2) A DCMU-insensitive redox reaction probably catalyzed by System I, which occurs with anomalously high quantum yields of 3–5 (Fig. 4), which shows that this reaction does not proceed *via* the normal electron transport reaction, which has a quantum yield of about 0.5.

(3) Hydrazobenzene presumably donates also electrons directly to the redox chain between Photoreactions I and II. The maximum rate of this reaction, which we will not discuss further, is low (Fig. 2, open circles).

At high light intensity (Fig. 5) the rate of the second reaction (represented by the rates measured in the presence of DCMU and 20 μM hydrazobenzene) is limited by the relatively low hydrazobenzene concentration, as is indicated by the light saturation. Fig. 5 also shows that in the presence of hydrazobenzene alone at high intensity the reaction rate is about equal to the sum of the rate of the second reaction and that of the "normal" Hill reaction with DCIP.

At low light intensity, however, addition of DCMU to the chloroplasts with hydrazobenzene did not decrease the reaction rate, at least not by the amount of the normal Hill reaction. The same is true at high light intensity, if the concentration of hydrazobenzene is high, as reported by Haveman and Donze[15]. The explanation of this phenomenon may be that the System I reaction is stimulated by the addition of DCMU, so that the loss due to inhibition of the normal Hill reaction is approximately compensated by an increase in the rate of the System I catalyzed reaction. This only occurs if the hydrazobenzene concentration is not limiting the System I reaction. In the presence of DCMU the components of the redox chain between Q and System I tend to get oxidized and one of these components or a substance oxidized by it, may thus be responsible for catalyzing the hydrazobenzene–DCIP reaction.

Besides hydrazobenzene, dichlorophenylhydrazine and phtaloylhydrazine ap-

peared to be donors to Photosystem II. DCIP reduction with these donors was inhibited by DCMU. Although less efficient than hydrazobenzene, these donors were more efficient than the already known donors semicarbazide and *sym*-diphenylcarbazide.

In System II particles hydrazobenzene had striking effects on fluorescence and luminescence of chlorophyll a_2, and prevented cytochrome b_{559} oxidation and carotenoid and chlorophyll bleaching. These phenomena could be satisfactorily explained by the rapid reduction of the photooxidized electron donor(s) of Photoreaction II by hydrazobenzene.

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