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DISPROPORTIONATION OF 1,5-DIPHENYLCARBAZONE. A NEW REACTION CATALYSED BY PHOTOSYSTEM I

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SUMMARY

1. 1,5-Diphenylcarbazide (DPC) was shown to compete with water as an electron donor to photosystem II in untreated chloroplasts.

2. 1,5-Diphenylcarbazone (DPCN), a product of the above photooxidation, was shown to be disproportionated in a Photosystem I-dependent reaction. The products of the disproportionation reaction were DPC and a more oxidized form, probably diphenylcarbodiazone.

3. The disproportionation reaction is insensitive to 3-(3,4-dichlorophenyl)-1, 1-dimethylurea, does not require cytochrome 552 in Euglena chloroplasts, but shows a requirement for plastocyanin.

4. The disproportionation reaction required intensities about 100-fold lower than those required for a typical Hill reaction. The quantum requirement was determined as 0.07 quantum per molecule of DPCN at 715 nm. It is therefore suggested that the disproportionation reaction is catalyzed by a product of Photosystem I.

5. It is suggested that the reaction requires a smaller number of components around Photosystem I, than any other described to date.

INTRODUCTION

1,5-Diphenylcarbazide (DPC) was recently shown by VERNON AND SHAW[1] to serve as an electron donor to Photosystem II in subchloroplast particles of spinach, and in Tris-treated chloroplasts[2]. During attempts to take advantage of this property, we found that diphenylcarbazide can donate electrons also to untreated chloroplasts. In addition, we observed that the product of this reaction, 1,5-diphenylcarbazone (DPCN), rapidly disappeared in a 3-(3,4-dichlorophenyl)-1,1-dimethylurea (DCMU) insensitive, Photosystem I dependent reaction.

This communication describes the properties of the latter reaction, which we believe to be a new and very useful photoreaction in assessing the Photosystem I activity of various preparations.

Abbreviations: DPC, 1,5-diphenylcarbazide; DPCN, 1,5-diphenylcarbazone; DPC_{ox}, fully oxidized product of DPC or DPCN; DCIP, 2,6-dichlorophenolindophenol; DQ, 1,1'-ethylene-2,2'-dipyridillium dibromide; DCMU, 3-(3,4-dichlorophenyl)-1,1-dimethylurea.

MATERIALS AND METHODS

DPC was purchased from Eastman and was recrystallized from water–methanol. DPCN was purchased from British Drug Houses, Ltd. These two compounds were dissolved in methanol at a concentration of 50 mM.

Chloroplasts were isolated as previously described[3] from lettuce (*Lactuca sativa*) bought in the local market. Chloroplasts of Euglena were prepared according to SHNEYOUR AND AVRON[4]. Chromatophores of *Rhodospirillum rubrum* were prepared according to GROMET-ELHANAN[5]. Chlorophyll was determined according to ARNON[6]. Tris treatment was performed as described by YAMASHITA AND BUTLER[7]. Plastocyanin was prepared by a modification of the method of KATOH[8]. Cytochrome 552 of Euglena was prepared according to PERINI *et al.*[9]. Photophosphorylation experiments and assay of ATP were carried out according to AVRON[3].

Spectra were measured in a Cary 14 recording spectrophotometer. Chemical oxidation of the DPC, DPCN or their products was performed by adding the specified amounts of $K_3Fe(CN)_6$ in solution. The end of the oxidation was determined by following the appearance of absorption at 420 nm of excess ferricyanide. An appropriate amount of ferrocyanide, corresponding to the amount of ferricyanide which disappeared, was added to the reference cuvette in order to eliminate the absorbance due to the ferrocyanide formed in the sample cuvette. DPCN disappearance was measured at 485 nm using a millimolar extinction coefficient ($\varepsilon_{\mathrm{mM}}$) of 2.7 at pH 8.0. The oxidation of DPC was followed at 300 nm, using 5.4 as the millimolar extinction coefficient[2].

Photoinduced absorbance changes in chloroplast reactions were followed in a Cary-14 spectrophotometer equipped with a scatter-attachment. Actinic light was provided by a 500 W projector and filtered through a Corning c.s. 2-58 glass filter. In the case of chromatophores the actinic light was filtered through a Schott 882 nm interference filter.

Changes in O_2 concentration were followed with a Clark type oxygen electrode. Thin-layer chromatographic separations were performed on plates of Silica G (Riedel–De Haen, Hanover), using a mixture of chloroform–methanol–ethyl acetate (1.5:2:1, by vol.) for 30 min. To observe the position of the bands, parallel plates with fluorescin were run and inspected under ultraviolet light.

RESULTS

Diphenylcarbazide as an electron donor to photosystem II in untreated chloroplasts

The data of Table I illustrate three types of evidence which indicate that DPC can compete with water as an electron donor to Photosystem II in untreated chloroplasts. It inhibited O_2 evolution without inhibiting DCIP reduction; DPC was observed to be oxidized at a rate approximately equal to the diminution in the rate of O_2 evolution, and it increased the rate of oxygen uptake in the presence of diquat as an autooxidizable electron acceptor (see ref. 10). All these reactions were fully sensitive to DCMU. These results confirm and extend the data of VERNON AND SHAW[1,2].

During these studies we noticed that the product of the oxidation of DPC possessed a large absorption peak around 485 nm, and therefore attempted to follow its formation at this wavelength. As can be seen in Fig. 1, rather unusual results were obtained. The increase of absorption at 485 nm could be easily followed and was

TABLE I

DIPHENYLCARBAZIDE AS AN ELECTRON DONOR TO PHOTOSYSTEM II IN UNTREATED CHLOROPLASTS

The reaction mixture contained, in a total volume of 3.0 ml, in µmoles: Tricine (pH 8.0), 60; KCl, 60; DCIP, 0.15; and chloroplasts containing 27 µg chlorophyll. In the case of diquat, DCIP was replaced by diquat, 0.1 and NaN_3, 1.5. Where indicated DCMU was added at 1 µM. Other conditions as described under MATERIALS AND METHODS.

Reaction	*DPC (mM)*	*Effect followed*	*Reaction rate (µmoles electrons · mg chlorophyll^{-1} · h^{-1})*	
			−DCMU	*+DCMU*
H_2O to DCIP	0	Oxygen evolution	124	0
	0.5	Oxygen evolution	67	2
	0	DCIP reduction	150	0
	0.5	DCIP reduction	160	11
	0.5	DPC oxidation	70	0
H_2O to diquat	0	Oxygen uptake	48	0
	0.5	Oxygen uptake	74	15

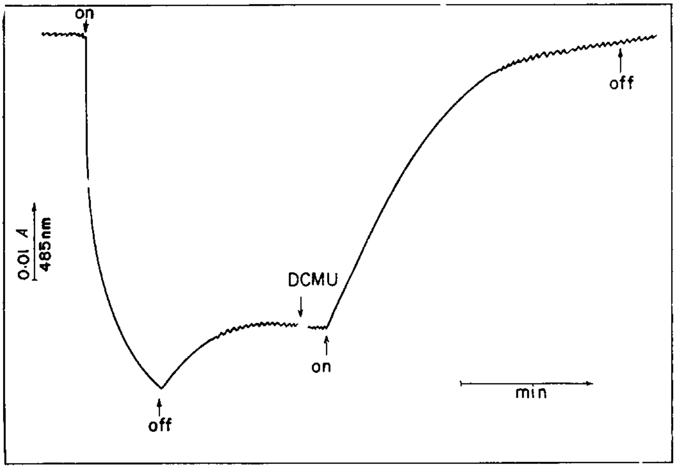


Fig. 1. Oxidation of DPC and disproportionation of DPCN. Reaction mixture contained in a total volume of 3.0 ml in µmoles: phosphate (pH 8.0), 150; NaCl, 40; DPC, 0.5; and lettuce chloroplasts containing 30 µg chlorophyll. Where indicated, 0.01 µmole of DCMU were added. Other conditions as described under MATERIALS AND METHODS.

completely sensitive to DCMU. However, the product formed disappeared rapidly, particularly in the presence of DCMU, in a light dependent reaction. As will be shown this product, DPCN is rapidly disproportionated in a new Photosystem I dependent reaction into DPC together with more highly oxidized product, not fully identified, which we shall refer to as DPC_{ox}.

Photosystem I induced disproportionation of diphenylcarbazone

Fig. 2 illustrates the spectra of DPC, DPCN, and DPC_{ox} at pH 8.3. The spectra of DPC and DPCN are of the recrystallyzed commercial samples, while that of the DPC_{ox} was obtained by oxidation of DPC or DPCN by ferricyanide. As can be seen in Fig. 3 the absorption of DPCN at 485 nm was highly pH dependent, with an apparent pK of 8.4. At pH 8.0 the molar extinction of DPCN at 485 nm was 2700.

Table II illustrates that the disproportionation reaction of DPCN was completely DCMU insensitive and was not affected by Tris-treatment of the chloroplasts. Plastocyanin removal by sonication decreased the rate of the reaction, although longer periods of sonication were required than in the case of the ascorbate + DCIP

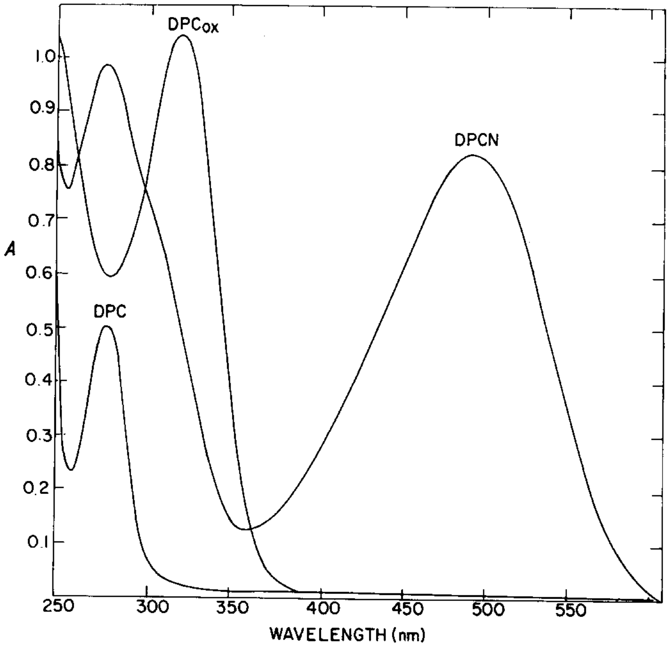


Fig. 2. Spectra of DPC, DPCN and their oxidation product. Medium contained in a total volume of 3.0 ml: phosphate (pH 8.3), 180 μmoles; and 0.6 μmole of the indicated compound. The spectrum indicated DPC$_{ox}$ was obtained by the addition of either approx. 2 equiv. of $K_3Fe(CN)_6$ to DPCN, or approx. 4 equiv. of $K_3Fe(CN)_6$ to DPC. Equivalent amount of $K_4Fe(CN)_6$ was added to the reference cuvette, to compensate for the absorption of the $K_4Fe(CN)_6$ formed during the oxidation. Other conditions as described under MATERIALS AND METHODS.

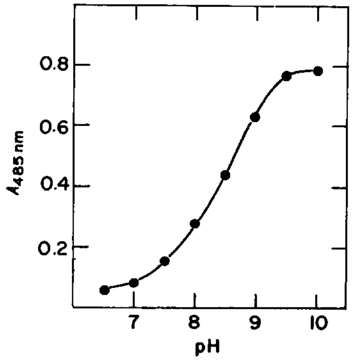


Fig. 3. pH dependence of the absorption of DPCN at 485 nm. Small volumes of conc. NaOH were added to a solution of 0.1 mM DPCN in 60 mM phosphate (pH 6.0) and the pH and optical density were followed.

TABLE II

DISPROPORTIONATION OF DIPHENYLCARBAZONE IN CHLOROPLASTS

Reaction mixture for DPCN disproportionation contained in a total volume of 3.0 ml in µmoles: phosphate (pH 8.0), 150; NaCl, 40; diphenylcarbazone, 0.5; and lettuce chloroplasts containing 30 µg of chlorophyll. The same reaction mixture was used for DCIP reduction except that 0.1 µmole DCIP replaced DPCN. Where indicated, DCMU was added at a final concentration of 25 µM. Other conditions as described under MATERIALS AND METHODS.

Type of chloroplasts	*Addition*	*Activity (μmoles·mg chlorophyll^{-1}·h^{-1})*	
		DCIP reduction	*DPCN disproportionation*
Untreated	—	128	466
	DCMU	0	468
Tris-treated	—	24	465
	DCMU	0	474

TABLE III

PLASTOCYANINE REQUIREMENT FOR THE DISPROPORTIONATION OF DIPHENYLCARBAZONE

Reaction mixture for the disproportionation of DPCN was as described under Table II. For the reduction of NADP$^+$, DPCN was replaced by ascorbate, 20 µmoles; DCIP, 0.05 µmole; NADP$^+$, 0.05 µmole; and a saturating amount of ferredoxin. Where indicated 4.8 nmoles of plastocyanin were added. Sonication was performed with a Branson sonifier with a microtip at 4 A at 0°. Chlorophyll concentration during sonication was 300 µg/ml. After sonication the chloroplasts were spun down at 12000 × *g* for 10 min and resuspended in the same medium. Other conditions as described under MATERIALS AND METHODS.

Time of sonication (min)	*Activity (μmoles·mg chlorophyll^{-1}·h^{-1})*			
	NADP$^+$ reduction		*DPCN disproportionation*	
	— Plastocyanin	*+ Plastocyanin*	*— Plastocyanin*	*+ Plastocyanin*
0	20	28	860	880
1	6	23	610	810
4	1	15	400	930
8	0	14	190	850

to NADP$^+$ reaction (Table III). Readdition of plastocyanin to the plastocyanin-depleted particles fully restored the rate of the disproportionation reaction.

We also tested whether, in Euglena chloroplasts, cytochrome 552 which is generally considered to function in the plastocyanin-requiring site[11–13] is required for the disproportionation reaction. Table IV illustrates that the addition of cytochrome 552 to Euglena chloroplasts was not essential. The reaction exhibited a similar heat-stability to that of the ascorbate + DCIP to NADP$^+$ reaction (Table V). It was independent of oxygen, and proceeded just as well under an atmosphere of argon. Chromatophores isolated from *R. rubrum* were unable to catalyse this photoreaction. No photophosphorylation was observed to be coupled to the disproportionation reaction.

From the above data we concluded that the reaction most probably involved

TABLE IV

LACK OF EFFECT OF CYTOCHROME 552 ON THE DISPROPORTIONATION REACTION

The reaction mixture for the disproportionation of DPCN was as described under Table II, except that Euglena chloroplasts containing 30 μg chlorophyll replaced the lettuce chloroplasts. For the diquat reaction DPCN was replaced by 20 μmoles ascorbate, 0.1 μmole DCIP, 0.1 μmole diquat, 3 μmoles NaN_3 and 0.01 μmole DCMU. Where indicated, 15 nmoles cytochrome 552 were added. Other conditions as described under MATERIALS AND METHODS.

	Ascorbate + DCIP to diquat (μmoles O_2·mg chlorophyll^{-1}·h^{-1})	*Disproportionation of DPCN (μmoles carbazone·mg chlorophyll^{-1}·h^{-1})*
Chloroplasts	15	211
Chloroplasts + cytochrome 552	49	211

TABLE V

HEAT STABILITY OF THE DISPROPORTIONATION REACTION

The reaction mixture for the disproportionation of DPCN was as described under Table II, and that for the diquat reaction as described under Table IV, except that 30 μg of lettuce chloroplasts replaced the Euglena chloroplasts. Heat treatment was given by immersing the suspension of chloroplasts at 2 mg chlorophyll/ml in small test tubes in a water bath maintained at 65° for the period indicated followed by immediate transfer into crushed ice. Control activities were 137 and 818 μmoles O_2 taken up or DPCN disproportionated per mg chlorophyll per h, respectively. Other conditions as described under MATERIALS AND METHODS.

Time at 65° (min)	*% of control*	
	Ascorbate + DCIP to diquat	*DPCN disproportionation*
0	(100)	(100)
1	93	98
2	25	40
10	7	12

a disproportionation of DPCN to produce DPC and DPC_{ox} in accordance with the following stoichiometry:

$$2\ DPCN \xrightarrow{\text{Photosystem I}} DPC + DPC_{ox}$$

In order to ascertain that this indeed was the reaction, we isolated the products of a disproportionation reaction run to completion. The spectrum of this mixture of products is shown as B in Fig. 4, and agreed with that of an approximately 1:1 mixture of DPC and DPC_{ox} (see Fig. 2). Addition of excess ferricyanide to the product mixture converted the spectrum to that of DPC_{ox} (A in Fig. 4). Separation of the product mixture by thin-layer chromatography yielded two major bands, the spectra of which are shown in Fig. 4 (C and D), and correspond to the spectra of DPC_{ox} and DPC, respectively. These results confirm the proposed stoichiometry of the reaction.

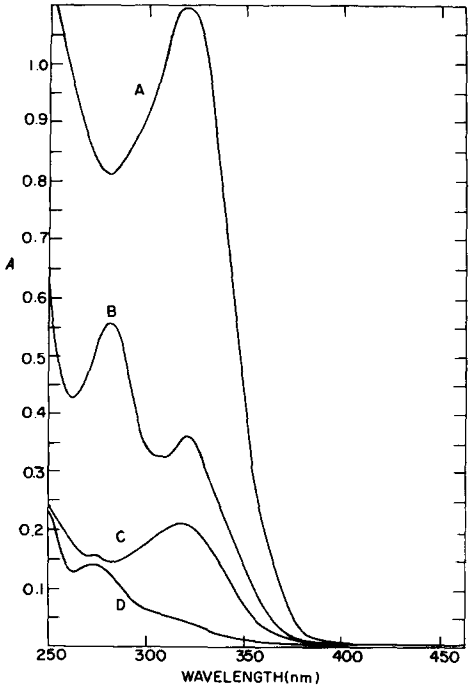


Fig. 4. Spectra of the products of the disproportionation of DPCN. A, spectrum of product mixture after addition of ferricyanide, as described under Fig. 2; B, spectrum of product-mixture; C, spectrum of the faster moving band after separation on thin-layer chromatography as described under MATERIALS AND METHODS; D, spectrum of the slower moving band after separation on thin-layer chromatography as described under MATERIALS AND METHODS. All spectra were measured in 60 mM phosphate (pH 8.3).

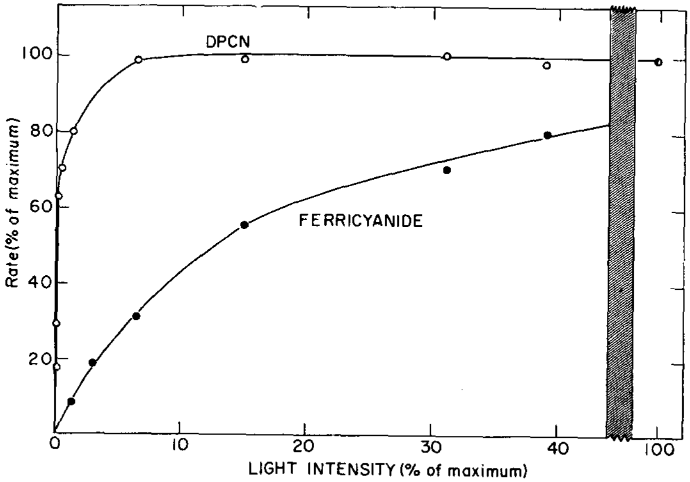


Fig. 5. Light intensity dependence of the disproportionation of DPCN. Reaction mixture for the disproportionation of DPCN contained in a total volume of 3.0 ml in µmoles: Tricine (pH 8.0), 60; KCl, 60; DPCN, 0.6; DCMU, 0.003, and chloroplasts containing 31 µg of chlorophyll. For the ferricyanide reaction, 1.5 µmoles ferricyanide replaced DPCN and DCMU in the reaction mixture. Light was passed through a Schott RG 645 filter and its intensity varied by the insertion of calibrated screens in the light path. Other conditions as described under MATERIALS AND METHODS.

Light requirement of the disproportionation reaction

On comparing the light intensity requirements of the DPCN disproportionation reaction with that of a classical Hill reaction, it became clear that saturation of the disproportionation occurred at much lower intensities (Fig. 5). Half maximal activity of the disproportionation reaction was observed at an intensity more than 100-fold lower than that required to give half maximal activity in the photoreduction of ferricyanide.

Since it was previously shown that the photoreduction of ferricyanide in this system proceeds with a reasonably low quantum requirement[14], it was of considerable interest to determine the quantum requirement for the DPCN disproportionation reaction. In Fig. 6 the quantum requirement for the DPCN disproportionation reaction extrapolated to zero intensity is compared with that of another Photosystem I dependent reaction, the photoreduction of $NADP^+$ by ascorbate + DCIP in the presence of DCMU. As can be seen, extremely low values were observed. Around 0.07 quantum were required for the disproportionation of one molecule of DPCN.

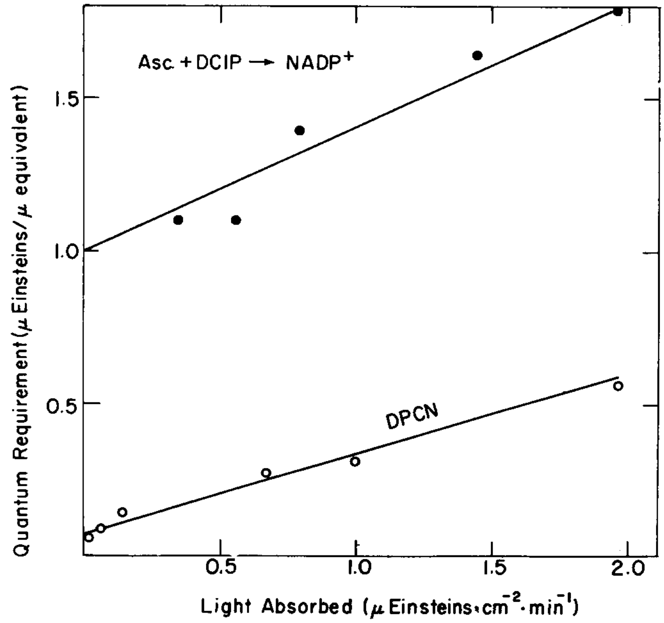
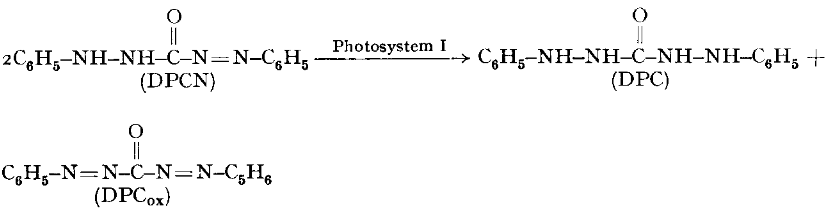


Fig. 6. Quantum requirement of the disproportionation of DPCN. Reaction mixture for the disproportionation reaction was as described under Fig. 5, except that the chloroplasts contained 43 μg of chlorophyll. For the ascorbate + DCIP to $NADP^+$ reaction, DPCN was replaced by $NADP^+$, 0.5 μmole; DCIP, 0.1 μmole; ascorbate, 20 μmoles; and a saturating amount of ferredoxin. Light was passed through a Baird-Atomic 715 nm interference filter of 15 nm half band width, and varied with metal screens. It was measured with a Yellow Springs Radiometer Model 65. The amount of light absorbed by the chloroplasts was taken from a previously given calibration (Fig. 1 in ref. 14). Other conditions as described under MATERIALS AND METHODS.

DISCUSSION

The data described support the conclusion that we have observed a new Photosystem I dependent reaction in which two molecules of DPCN are disproportionated to a more oxidized and more reduced product; it is clear that the more reduced product is DPC. The identity of the more oxidized product, DPC_{ox}, has not been fully established, but it would be reasonable to assume that it is 1,5-diphenylcarbodiazone



$$\underset{\text{(DPCN)}}{2C_6H_5\text{-}NH\text{-}NH\text{-}\overset{O}{\overset{\|}{C}}\text{-}N{=}N\text{-}C_6H_5} \xrightarrow{\text{Photosystem I}} \underset{\text{(DPC)}}{C_6H_5\text{-}NH\text{-}NH\text{-}\overset{O}{\overset{\|}{C}}\text{-}NH\text{-}NH\text{-}C_6H_5} + \underset{(DPC_{ox})}{C_6H_5\text{-}N{=}N\text{-}\overset{O}{\overset{\|}{C}}\text{-}N{=}N\text{-}C_5H_6}$$

The fact that in Euglena chloroplasts cytochrome 552 is not required for the reaction, seems to indicate that, contrary to previous opinion[11-13], the function of plastocyanin is not always replaced by cytochrome 552 in these chloroplasts.

The very low quantum requirement, of around 0.07 quantum per molecule DPCN observed, seems to indicate that we are not dealing here with a direct electron transfer reaction catalysed by Photosystem I. The most reasonable interpretation would seem to be a catalysis of the DPCN disproportionation by a product of the Photosystem I reaction. This interpretation is supported by the often observed phenomena (not shown) that the reaction does not terminate immediately on turning the light off, but seems to continue for a few seconds before ceasing. Thus, whatever catalyses the reaction has a lifetime of a few seconds in the dark.

The reaction may serve as a useful tool for further studies of the mechanism of electron transport in photosynthetic systems, since it seems to require a smaller number of intact components around Photosystem I than any hitherto studied reaction.

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