Photoreduction and Photophosphorylation with Tris-Washed Chloroplasts[1]

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Abstract. The artificial electron donor compounds *p*-phenylenediamine (PD), *N, N, N', N'*-tetramethyl-p-phenylenediamine (TMPD), and 2,6-dichlorophenol-indophenol (DCPIP) restored the Hill reaction and photophosphorylation in chloroplasts that had been inhibited by washing with 0.8 M tris (hydroxymethyl)aminomethane (tris) buffer, pH 8.0. The tris-wash treatment inhibited the electron transport chain between water and photosystem II and electron donation occurred between the site of inhibition and photosystem II. Photoreduction of nicotinamide adenine dinucleotide phosphate (NADP) supported by 33 μM PD plus 330 μM ascorbate was largely inhibited by 1 μM 3-(3,4-dichlorophenyl)-1,1-dimethylurea (DCMU) while that supported by 33 μM TMPD or DCPIP plus ascorbate was relatively insensitive to DCMU. Experiments with the tris-washed chloroplasts indicated that electron donors preferentially donate electrons to photosystem II but in the presence of DCMU the donors (with the exception of PD at low concentrations) could also supply electrons after the DCMU block. The PD-supported photoreduction of NADP showed the relative inefficiency in far-red light characteristic of chloroplast reactions requiring photosystem II. With phosphorylating systems involving electron donors at low concentrations (33 μM donor plus 330 μM ascorbate) photophosphorylation, which occurred with P/e_2 ratios approaching unity, was completely inhibited by DCMU but with higher concentrations of the donor systems, photophosphorylation was only partially inhibited.

The demonstration that artificial electron donor systems such as DCPIP[2] plus ascorbate (1) or PMS plus ascorbate (2) could restore the photoreducing power of chloroplasts which had lost their capacity to evolve oxygen opened up a new method of exploring the photosynthetic electron transport system. A number of artificial electron donor compounds have been studied, often with the goal of determining the site of electron donation relative to the site of coupling of phosphorylation (3, 4, 5, 6). Any study of artificial electron donor systems for chloroplasts is firmly bound to the method of inhibiting normal electron transport from water. Most previous studies have used DCMU or similar acting inhibitors which block electron transport just after PS2 and thus preclude the study of electron donors acting between water and PS2. Yamashita and Horio (7) inhibited the Hill reaction by washing chloroplasts with 0.8 M tris-HCl (pH 8.0). They found that photophosphorylation and NADP photoreduction could be restored by adding DCPIP or TMPD with ascorbate. Cyclic photophosphorylation (no require-

ment for NADP) was obtained when the concentrations of ascorbate and DCPIP were about equal but non-cyclic photophosphorylation (strict requirement for NADP) predominated when an excess of ascorbate was present. The non-cyclic photophosphorylation with DCPIP was inhibited 50 % by DCMU or *o*-phenanthroline but that with TMPD was inhibited 100 %. The DCMU inhibition of the TMPD photoreaction suggested that the tris-wash treatment inhibited before the DCMU block and that TMPD could donate electrons prior to the DCMU block. Electron donation to the electron transport system prior to the DCMU block has been suggested previously by several laboratories (8, 9, 10) on the basis of experiments showing that the photooxidation of ascorbate by chloroplasts in the presence of autooxidizable quinones was inhibited by DCMU.

More direct measurements of electron donation prior to the DCMU block have been made recently. Katoh and San Pietro (11, 12) used ascorbate as an electron donor for the photoreduction of NADP with *Euglena* chloroplasts that had been inhibited by a heat treatment. The ascorbate-supported photoreduction of NADP was inhibited by DCMU. Yamashita and Butler (13) showed that NADP photoreduction in spinach chloroplasts inhibited with a tris-wash treatment could be restored with PD plus ascorbate as an electron donor system and that the electron transport from PD was inhibited by DCMU. The present paper is an extension of the previous one with measurements of both photoreduction and

[2] Abbreviations: PD, *p*-phenylenediamine; TMPD, *N,N,N',N'*-tetramethyl-*p*-phenylenediamine; DCPIP, 2,6-dichlorophenol-indophenol; DCMU, 3-(3,4-dichlorophenyl)-1,1-dimethylurea; PMS; phenazine methosulfate; PS1 and PS2-photosystem I and photosystem II.

photophosphorylation. The data demonstrate that the tris-wash treatment inhibits electron transport between water and PS2 and that electron donors can donate electrons to PS2.

Experimental Procedures

Chloroplasts were prepared by grinding 50 g of spinach leaves in a Waring Blendor for 20 sec in 150 ml of solution containing 0.4 M sucrose, 0.05 M tris-HCl (pH 7.8), and 0.01 M NaCl (abbreviated as STN solution). The supernatant from a 1-min centrifugation at 300g was centrifuged again at 600g for 7 min in 4 centrifuge tubes. The pellet from 2 tubes was suspended in 20 ml of 0.8 M tris-HCl (pH 8.0) to about 0.1 mg chl/ml (tris-washed chloroplasts). The pellet from the other 2 tubes was suspended in 20 ml of STN solution (intact chloroplasts). After standing for 10 min, heavy particles of both batches of chloroplasts were removed by centrifugation at 300g for 1 min. The remaining supernatants were centrifuged at 1500g for 7 min and the precipitates were resuspended in 2 ml of STN solution. PS1 particles were prepared from chloroplasts incubated in 0.5 % digitonin for 30 min by centrifugation at 50,000 to 144,000g for 30 min according to the method of Anderson and Boardman (14). Measurements were made with chloroplasts suspended in a standard reaction medium consisting of 15 mM tris-HCl, 4 mM KPO$_4$, 1 mM ADP, 4 mM MgCl$_2$, and 20 mM NaCl, pH 7.8.

Chlorophyll concentration was determined by the method of MacKinney (15). Ferredoxin was purified from spinach leaves; DCMU donated by Dr. P. G. Heytler was used as a methanolic solution. ADP and NADP were obtained from Calbiochem Company. p-Phenylenediamine and TMPD were the products of Eastman Organic Chemicals Company; DCPIP was obtained from the La Motte Chemical Products Company.

NADP photoreduction was measured under aerobic conditions with an Aminco-Chance dual-wavelength spectrophotometer (340 vs 370 nm) with an EMI 9524 phototube blocked by Corning filters 5840 and 5970. In anaerobic photophosphorylation experiments, NADPH was measured at 340 nm with a Unicam SP 800 spectrophotometer at the end of the irradiation. Rates of photoreduction in μmole NADPH formed/mg chl·hr averaged over 3 min of irradiation are indicated by the numbers in parentheses in figures 1, 2, 6, 7, and 8. Photooxidation of endogenous cytochrome f was measured with the double-beam instrument (554 vs 540 nm) with the blocking filters changed to 2 Corning 9788 filters and an Optics Technology 600 nm short pass cut-off filter. The fluorescence-yield measurements were made with an instrument similar to that used previously by Duysens and Sweers (16) and by Butler and Bishop (17). Fluorescence of the chloroplast suspension was excited by a weak monochromatic beam (650 nm, 50 ergs cm^{-2} sec^{-1}) chopped at a frequency of 300 cps. The phototube signal was measured with an EMI 9558 phototube and a PAR Lock-in Amplifier synchronized to the chopping frequency. A steady actinic beam (645 nm, 7.1 × 10^4 ergs cm^{-2} sec^{-1}) was used to irradiate the sample in a 1 cm cuvette from the side. Corning filters 9830 and 2030 placed directly in front of the phototube blocked the measuring and actinic beams but passed fluorescence of wavelengths longer than 680 nm. The lock-in amplifier responded only to the modulated fluorescence excited by the weak, chopped measuring beam but not to the steady fluorescence excited by the actinic beam. Changes in fluorescence yield due to the actinic irradiation were monitored by the measuring beam.

The red and far-red actinic light was obtained with a Unitron LKR microscope illuminator and Baird Atomic 645 and 715 nm interference filters (approximately 10 nm half band width), with additional infrared blocking filters.

Amounts of ATP formed from ADP and radioactive inorganic phosphate were detected as organic phosphate by the method of Nielsen and Lehninger (18) as modified by Avron (19). Photophosphorylation was carried out at 22° in 1 cm diameter Thunberg tubes or 1 cm pathlength Thunberg type cuvettes irradiated with white light (10^6 ergs cm^{-2} sec^{-1}). Anaerobic conditions were obtained by evacuating the tubes and refilling with argon gas 3 times. The reaction was stopped by addition of trichloroacetic acid to 7.5 % after the measurement of NADPH. The relationship between phosphorylation and electron transport is expressed as a P/e$_2$ ratio where e$_2$ represents the mole pairs of electrons transported.

Results

Electron Transport. Measurements of the photoreduction of NADP by tris-washed chloroplasts in the presence of added ferredoxin are shown in figure 1. Photoreduction of NADP in the absence of an artificial electron is low (5 μmoles NADPH/mg chl·hr vs 70 μmoles NADPH/mg chl·hr for intact chloroplasts). A substantial recovery of activity was regained by adding 33 μM PD and 330 μM ascorbate as an electron donor system. The coupling of photophosphorylation to electron transport was indicated by the decreased rate of photoreduction when ADP was left out of the reaction medium (−ADP) and by the increased rate when the uncoupler, NH$_4$Cl, was used in place of ADP (+NH$_4$Cl). The photoreduction of NADP from PD and ascorbate was inhibited 75 % by 1 μM DCMU (+DCMU).

The optimal or near optimal concentration of PD was chosen at 33 μM. Doubling the concentration of PD increased the rate of photoreduction only slightly. Lower concentrations could be used; 2 to 3 μM PD gave 50 % of the rate obtained with 33 μM PD. DCPIP and TMPD plus ascorbate could also serve as electron donor systems in the tris-washed

NADP PHOTOREDUCTION WITH ELECTRON DONOR PD

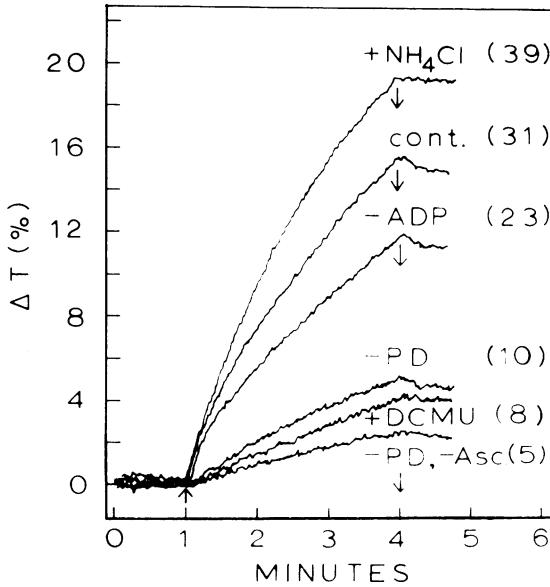FIG. 1.   Photoreduction of NADP by tris-washed chloroplasts (12 μg chl/ml) in 3 ml standard reaction medium with 330 μM NADP, 3 μM ferredoxin, 33 μM PD, and 330 μM ascorbate (complete).   In other curves, ADP, PD, and PD plus ascorbate were deleted, 1 μM DCMU was added and ADP was replaced by 1 mM NH$_4$Cl where indicated.   Red actinic light (6.6 $\times$ 10^4 ergs cm^{-2} sec^{-1}).   Numbers in parentheses indicate rates of photoreduction in μmoles NADPH/mg chl·hr averaged over 3 min.

PHOTOREDUCTION OF NADP WITH THREE DIFFERENT ELECTORON DONOR SYSTEMS

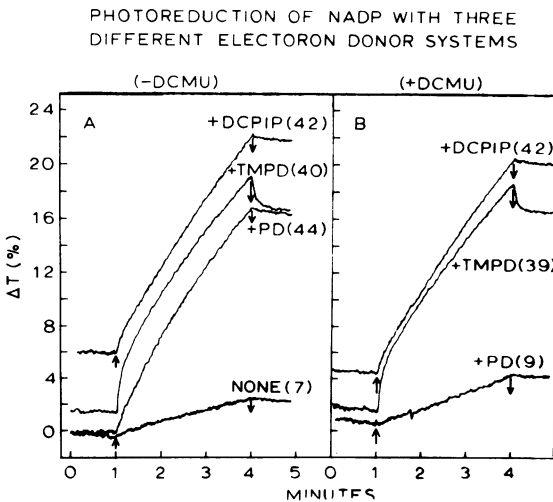
FIG. 2.   Photoreduction of NADP by tris-washed chloroplasts (11 μg chl/ml) in 3 ml of standard reaction medium with 330 μM NADP and 3 μM ferredoxin.   33 μM DCPIP, TMPD, or PD was added in combination with 330 μM ascorbate as the electron donor system where indicated.   Irradiation with red light (6.6 $\times$ 10^4 ergs cm^{-2} sec^{-1} on and off at arrows).   A) In the absence of DCMU.   B) In the presence of 1 μM DCMU.

chloroplasts to give approximately the same rates of NADP photoreduction as PD but these donor systems showed no inhibition by DCMU (fig 2).   The DCMU inhibition of electron transport from PD, indicating electron donation to PS2 but not to PS1, proved useful in analyzing artificial electron donor systems.

Electron transport reactions in the neighborhood of PS2 and the DCMU block have been studied by fluorescence-yield measurements.   The fluorescence-yield changes of chlorophyll in photosynthetic systems have been related to the redox state of the hypothetical primary electron acceptor of PS2, denoted Q (16).   The oxidized form of Q quenches fluorescence while the reduced form does not.   DCMU is thought to block the transfer of electrons from Q to the next member of the electron transport system.   These concepts have proved useful in analyzing the tris-washed chloroplasts.   In contrast to normal chloroplasts, the tris-washed chloroplasts showed very little increase of fluorescence yield when irradiated with actinic light.   Irradiation of normal chloroplasts in the absence of an electron acceptor increased the fluorescence yield 300 % (fig 3A) while irradiation of the tris-washed chloroplasts increased the yield only 20 % (fig 3B).   The fluorescence yield

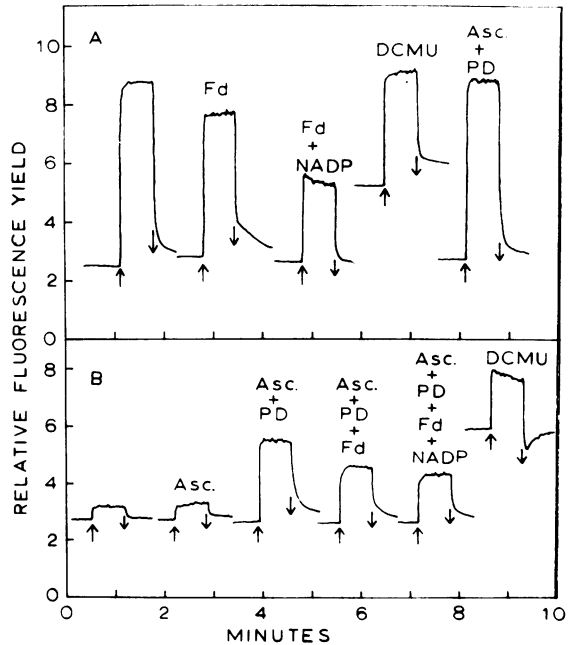## FLUORESCENCE YIELD OF INTACT AND TRIS WASHED CHLOROPLASTS

FIG. 3.   Fluorescence yield of chloroplasts (11 μg chl/ml) in 3 ml of standard reaction medium.   330 μM ascorbate, 33 μM PD, 3 μM ferredoxin, 330 μM NADP and 1 μM DCMU were added where indicated.   Red actinic light (7.0 $\times$ 10^4 ergs cm^{-2} sec^{-1}) on at upward arrow, off at downward arrow.   A) With intact chloroplasts.   B) With tris-washed chloroplasts.

in the dark (*i.e.*, in weak measuring light) was the same in both cases. The absence of the light-induced fluorescence-yield increase suggested a lack of electrons for the reduction of Q. Addition of PD and ascorbate as an artificial electron donor system to the tris-washed chloroplasts restored a large part of the fluorescence-yield increase. Addition of the donor system to intact chloroplasts had no effect. Addition of an electron acceptor (NADP and ferredoxin) to either the intact chloroplasts or to the tris-washed chloroplasts with PD and ascorbate had the expected effect of lowering the fluorescence yield during irradiation because of the turnover of Q during sustained electron transport. Addition of DCMU to the tris-washed chloroplasts in the absence of the donor system also resulted in a high fluorescence yield in actinic light (fig 3B). Apparently the tris-washed chloroplasts contain enough of an endogenous electron donor to reduce Q if the electron flow out of Q is blocked by DCMU. The fluorescence-yield measurements with the tris-washed chloroplasts also indicate that PD donates electrons to the electron transport system before the DCMU block, presumably on the oxygen side of PS2.

FLUORESCENCE YIELD OF TRIS-WASHED CHLOROPLASTS
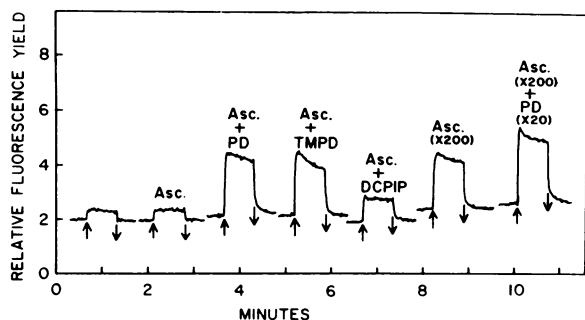WITH VARIOUS ELECTRON DONORS

FIG. 4. Fluorescence yield of tris-washed chloroplasts (10 μg chl/ml) in 3 ml of standard reaction medium. 330 μM ascorbate, 33 μM PD, 33 μM TMPD, 33 μM DCPIP, 67 mM ascorbate, and 670 μM PD were added where indicated. Red actinic light (7.0 × 10⁴ ergs cm⁻² sec⁻¹) on at upward arrows, off at downward arrows.

Light-induced fluorescence-yield changes with ascorbate and ascorbate plus PD, TMPD or DCPIP are shown in figure 4. Ascorbate alone at 330 μM had essentially no effect. At low concentration (33 μM), PD was the most effective of the electron donors tried. However, even DCPIP which is noted as an electron donor for PS1 could restore a part of the fluorescence of variable yield. The yield of fluorescence during irradiation depends on the relative rates of the photoreduction of Q and the dark oxidation of QH. The lower yield obtained with DCPIP may reflect a slower rate of electron donation by this donor. At high concentration, ascorbate (67 mM) could also donate electrons for the photoreduction of Q. Electron donation by 670 μM PD and 67 mM ascorbate was also examined because this

donor system will also supply electrons for photoreduction and photophosphorylation in the presence of DCMU.

The photooxidation of the endogenous cytochrome *f* in tris-washed chloroplasts by red and far-red light is shown in figure 5. In the absence of the electron donor system, irradiation with either red or far-red light caused the cytochrome *f* to go fully oxidized. In the presence of PD and ascorbate, however, the red light was less oxidizing, indicating that electrons were driven by PS2 from PD to cytochrome *f*. The action of far-red light, which primarily activates PS1 was not altered by PD. Addition of DCMU blocked the supply of electrons from PD so that red light again fully oxidized cytochrome *f*. Difference spectra for the red light-induced absorbancy changes with and without DCMU confirmed that absorbancy changes at 554 nm were due to cytochrome *f*.
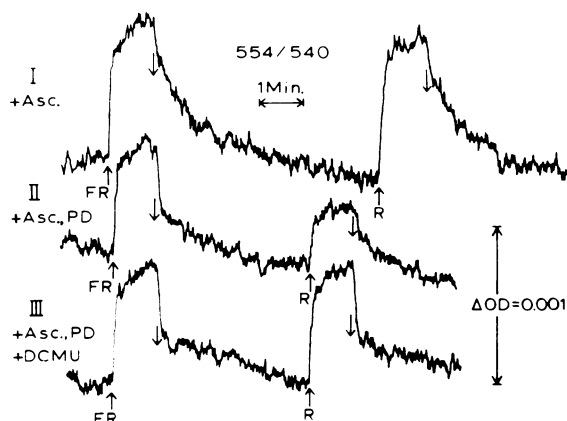
PHOTOOXIDATION OF CYTOCHROME-f

FIG. 5. Photooxidation of cytochrome *f* in tris-washed chloroplasts (32 μg chl/ml) in 3 ml of standard reaction medium. Far-red (1.5 × 10⁴ ergs cm⁻² sec⁻¹) and red (1.2 × 10⁴ ergs cm⁻² sec⁻¹) actinic light where indicated. Further addition I., 330 μM ascorbate. II., 330 μM ascorbate and 33 μM PD. III., 330 μM ascorbate, 33 μM PD and 1 μM DCMU.

The functioning of PS2 in the PD-supported NADP photoreduction by tris-washed chloroplasts was also shown by comparing the relative effectiveness of red and far-red light for the photoreduction of NADP with either PD or DCPIP as the electron donor (see fig 6). DCMU was added to the DCPIP plus ascorbate electron donor system to insure that only PS1 was functioning. For the PS1 activity, the red and far-red actinic sources were equally effective (fig 6B). In the case of electron donation from PD plus ascorbate (no DCMU added), however, the same red irradiation was twice as effective as the far-red irradiation (fig 6A) indicating that both PS2 and PS1 functioned in the photoreduction of NADP from PD.

The tris-washed chloroplasts were compared with PS1-active, PS2-inactive chloroplast fragments obtained from digitonin-treated chloroplasts by the

EFFECT OF RED AND FAR RED LIGHT ON
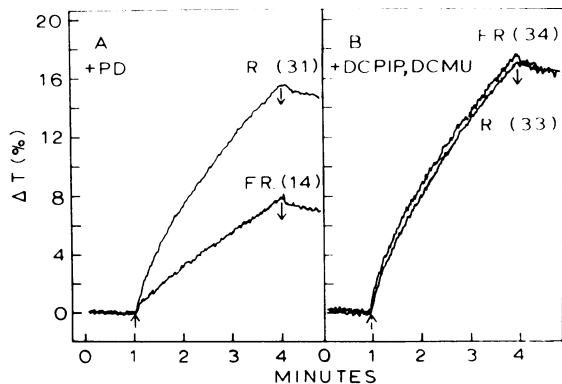PHOTOREDUCTION OF NADP

FIG. 6. Photoreduction of NADP by tris-washed chloroplasts (12 μg chl/ml) in 3 ml standard reaction medium with 330 μM NADP, 3 μM ferredoxin plus electron donor system. A) 33 μM PD plus 330 μM ascorbate. B) 200 μM DCPIP plus 3.3 mM ascorbate and 1 μM DCMU. Irradiation with red (6.6 × 10⁴ ergs cm⁻² sec⁻¹) and far-red (6.9 × 10⁴ ergs cm⁻² sec⁻¹) light.

method of Anderson and Boardman (14) (fig 7). DCPIP (200 μM) could donate electrons to the PS1 fragments for the photoreduction of NADP but PD (33 μM) could not.

The above experiments show that PD (at 33 μM) donates electrons almost exclusively to PS2. In contrast, previous studies by Arnon et al. (20) showed that PD plus ascorbate could serve as an electron donor system in chloroplasts which had been

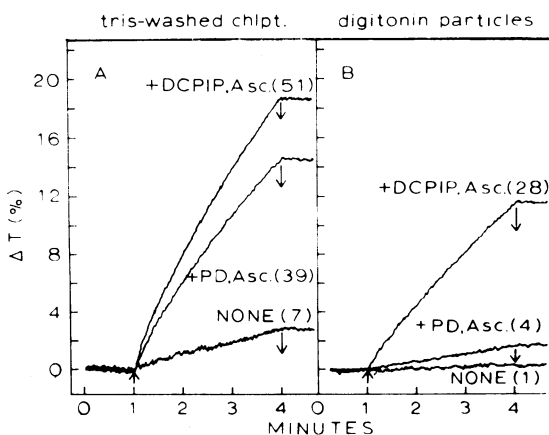DIFFERENT EFFECT OF PD ON NADP
PHOTOREDUCTION ACTIVITY

FIG. 7. Photoreduction of NADP by tris-washed chloroplasts (9 μg chl/ml) and digitonin-treated chloroplast fragments (10 μg chl/ml) in 3 ml of standard reaction medium with 330 μM NADP, 3 μM ferredoxin. 200 μM DCPIP plus 3.3 mM ascorbate or 33 μM PD and 330 μM ascorbate was added where indicated. Red actinic light (6.6 × 10⁴ ergs cm⁻² sec⁻¹).

inhibited by DCMU. They used much higher concentrations of the donor components, however, 670 μM PD and 67 mM ascorbate. Figure 8 compares the DCMU sensitivity of the photoreduction of NADP by tris-washed chloroplasts with the low (33 μM PD, 330 μM asc) and high (670 μM PD, 67 mM asc) concentrations of donor system. The data confirmed that PD at the higher concentration could donate electrons to the electron transport system beyond the site of the DCMU inhibition. Ascorbate alone at the higher concentration also supported an appreciable rate of photoreduction which was not sensitive to DCMU. The fluorescence-yield measurements in figure 4 indicated that in the absence of DCMU the high concentration of PD and ascorbate or ascorbate alone could also donate electrons to PS2.

EFFECT OF DCMU ON NADP PHOTOREDUCTION
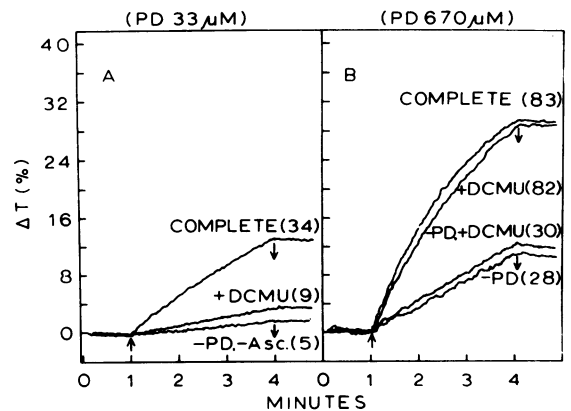IN THE PRESENCE OF PD

FIG. 8. Photoreduction of NADP by tris-washed chloroplasts in 3 ml of standard reaction medium with 330 μM NADP and 3 μM ferredoxin. Control curves A with 33 μM PD plus 330 μM ascorbate and B with 670 μM PD plus 67 mM ascorbate. Addition of 1 μM DCMU and deletion of PD and ascorbate where indicated. Red actinic light (6.6 × 10⁴ ergs cm⁻² sec⁻¹).

In the above experiments, inhibition of the Hill reaction was achieved by washing the chloroplasts with 0.8 M tris buffer at pH 8.0. The same degree of inhibition could also be obtained by washing the chloroplasts with 0.05 M tris buffer at pH 9.1. In this case, as well, a DCMU-sensitive photoreduction of NADP was restored with 33 μM PD plus ascorbate. The inhibition at pH 9.1 depended on the presence of tris-HCl. Washing with 0.05 M tricine buffer at pH 9.1 resulted in essentially no inhibition. In all cases, the chloroplasts were suspended in the standard STN medium pH 7.8 after the wash treatment.

Photophosphorylation. Rates of NADP photoreduction and photophosphorylation by tris-washed chloroplasts were measured with the different electron donor systems. The measurements were made

Table I. *Photophosphorylation by Tris-washed Chloroplasts (30 μg chl/ml) in 3 ml of Standard Reaction Medium With 330 μM NADP, 3 μM Ferredoxin, 33 μM PD and 330 μM Ascorbate*

Addition of 1 μM DCMU and deletions of PD, NADP, and ferredoxin where indicated.

Addition	μmoles ATP formed mg chl·hr
Complete	32
—PD	5
—NADP	6
—Ferredoxin	6
—NADP, Ferredoxin	5
+DCMU	0

under anaerobic conditions to prevent oxygen from acting as an electron acceptor. Table I shows that photophosphorylation with the tris-wash chloroplasts required both an electron donor (PD and ascorbate) and an electron acceptor (ferredoxin and NADP) and was inhibited by DCMU.

Table II compares 2 electron donor systems, 33 μM PD and 330 μM ascorbate, which we have shown donates electrons primarily to PS2, and 200 μM DCPIP and 6.7 mM ascorbate, which is commonly used as an electron donor for PS1 in DCMU-inhibited chloroplasts. DCMU inhibited the photoreduction of NADP with PD as the electron donor but not with DCPIP. (The same result was shown

in fig 2 under aerobic conditions). Concommitantly, phosphorylation with PD as the electron donor was completely inhibited while that with DCPIP was only 50 % inhibited.

Similar results are shown in table III where a low concentration (33 μM PD and 330 μM asc) and high concentration (670 μM PD and 67 mM asc) of the PD donor system were compared. The high concentration of the PD donor system acted very much like the DCPIP donor system in table I. Swartz (21) has demonstrated that high concentration of TMPD will also support photophosphorylation in DCMU-inhibited chloroplasts.

Table IV compares electron transport and photophosphorylation with 3 electron donors, PD, TMPD, and DCPIP, all at 33 μM with 330 μM ascorbate. Photophosphorylation was completely blocked with PD and TMPD and almost completely with DCPIP. Overall electron transport, however, from DCPIP and TMPD was only 30 % inhibited by DCMU while that from PD was 80 % inhibited.

Ascorbate alone, particularly at the higher concentrations, could also support electron transport and photophosphorylation in the tris-washed chloroplasts (tables II and III). In this case phosphorylation, but not electron transport, was completely inhibited by DCMU. The fluorescence-yield data in figure 4 had also indicated that a high concentration of ascorbate (67 mM) could supply electrons to PS2.

Table II *Photoreduction of NADP and Photophosphorylation by Tris-washed Chloroplasts (30 μg chl/ml) in 3 ml of Standard Reaction Medium With 330 μM NADP, 3 μM Ferredoxin Plus the Addition of the Electron Donor and DCMU as Indicated*

Addition (μM)	μmoles formed mg chl·hr		$\dfrac{P}{e_2}$
	NADPH	ATP	
PD (33), Asc. (330)	83	55	0.66
PD (33), Asc. (330), DCMU (1)	19	0	0.00
Asc. (330)	32	5	0.16
Asc. (330), DCMU (1)	3	0	0.00
DCPIP (200), Asc. (6700)	96	80	0.83
DCPIP (200), Asc. (6700), DCMU (1)	96	36	0.38
Asc. (6700)	47	14	0.30
Asc. (6700), DCMU (1)	20	0	0.00

Table III. *Photoreduction of NADP and Photophosphorylation by Tris-washed Chloroplasts (30 μg chl/ml) in 3 ml of Standard Reaction Medium With 330 μM NADP, 3 μM Ferredoxin Plus the Addition of the Electron Donor and DCMU as Indicated*

Addition (μM)	μmoles formed mg chl·hr		$\dfrac{P}{e_2}$
	NADPH	ATP	
PD (33), Asc. (330)	76	72	0.95
PD (33), Asc. (330), DCMU (1)	16	2	0.13
Asc. (330)	29	8	0.28
Asc. (330), DCMU (1)	4	3	0.75
PD (670), Asc. (67000)	97	80	0.83
PD (670), Asc. (67000), DCMU (1)	87	19	0.22
Asc. (67000)	71	31	0.44
Asc. (67000), DCMU (1)	59	0	0.00

Table IV. *A) Photoreduction of NADP and Photophosphorylation by Tris-washed Chloroplasts (30 μg chl/ml) in 3 ml of Standard Reaction Medium with 330 μM NADP, 3 μM Ferredoxin Plus the Addition of the Electron Donor and DCMU as Indicated*

B) Normal chloroplasts were used instead of tris-washed chloroplasts.

A) Tris-washed chloroplasts Addition (μM)	μmoles formed mg chl·hr		$\dfrac{P}{e_2}$
	$NADPH$	ATP	
Asc. (330)	24	3	0.13
PD (33), Asc. (330)	64	45	0.70
PD (33), Asc. (330), DCMU (1)	11	0	0.00
TMPD (33), Asc. (330)	56	49	0.88
TMPD (33), Asc. (330), DCMU (1)	40	0	0.00
DCPIP (33), Asc. (330)	45	35	0.78
DCPIP (33), Asc. (330), DCMU (1)	31	5	0.16
B) Normal chloroplasts			
None	156	105	0.67
DCMU (1)	13	2	0.15

The comparison of the P/e_2 ratios is complicated by electron transport not coupled to phosphorylation and by electron donation directly from the ascorbate. If it is assumed that electron donation by the primary donor and by the ascorbate are strictly additive, the ascorbate contribution can be subtracted. If such a correction is made, the P/e_2 ratios for 200 μM DCPIP in table I decrease from 1.35 to 0.47 on addition of DCMU and the ratios for 670 μM PD in table II decrease from 1.75 to 0.68. However, the assumption of strict additivity is probably not valid, particularly at high concentrations of the donor components. Ascorbate can donate electrons to different sites and the relative rates of donation to the different sites may well be altered by the presence of a companion electron donor.

Discussion

The tris-wash treatment clearly inhibits the electron transport system between water and PS2. Low concentrations of reduced PD restore the photoreducing capacity of the chloroplasts but electron transport from PD to an electron acceptor such as NADP is largely blocked by DCMU. DCMU appears to act at the primary electron acceptor of PS2, Q, so that any DCMU-sensitive electron donor implies donation prior to PS2. The fluorescence-yield data, which presumably reflect the redox state of Q, also indicate that the sites of tris-wash inhibition and PD electron donation are between water and PS2. The tris-wash treatment greatly reduces light-induced fluorescence-yield increase because the source of electrons for the photoreduction of Q has been blocked but the electron donation by PD restores the fluorescence of variable yield. The experiments on the relative effectiveness of red and far-red light provide more direct evidence for the participation of PS2 in PD-supported electron transport. PD supplies electrons for the photoreduction of cytochrome f by red but not far-red light. The

classical test for PS2 activity, i.e., the "red drop phenomenon", was also demonstrated for electron transport from PD by comparing the relative effectiveness of red and far-red light for the PD-supported photoreduction of NADP with that for a known PS1-mediated reaction, i.e., the DCPIP-supported photoreduction of NADP in the presence of DCMU.

The low concentration of PD was different from the other electron donor systems in terms of its specificity for donating electrons prior to PS2. The other donors could also donate electrons to PS2 in the absence of DCMU as evidenced by their ability to partially restore the light-induced fluorescence-yield changes but these donors were able to support the photoreduction of NADP in the presence of DCMU to a much greater extent than was 33 μM PD. When electron flow from the electron donation site prior to PS2 was blocked by DCMU, other sites for electron donation beyond the DCMU block were available but not so available to low concentrations of PD as to the other donors.

Phosphorylation was coupled to electron transport in the tris-washed chloroplasts. Even low concentrations (33 μM), of PD, TMPD, and DCPIP supported photophosphorylation with P/e_2 ratios comparable to those obtained with intact chloroplasts. The DCMU-sensitivity of the photophosphorylation with low donor concentration showed that the electron transport was initiated prior to PS2. In the presence of DCMU, 33 μM TMPD or DCPIP also donated electrons to a site after PS2 but the electron transport in this case was not coupled to phosphorylation. With higher concentrations of the electron donor systems (200 μM DCPIP or 670 μM PD) a part of the phosphorylation was insensitive to DCMU inhibition.

These data suggest 3 sites for electron donation to the photosynthetic electron transport system at the regions indicated in figure 9. The fluorescence-yield measurements and the phosphorylation experiments indicated that all of the donor systems tested could
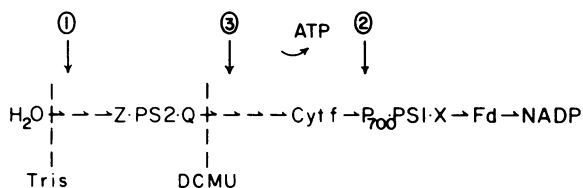
FIG. 9. Sites for electron donation to photosynthetic electron transport chain. Q and X are hypothetical primary electron acceptors for PS2 and PS1 respectively. Z is the hypothetical primary electron donor for PS2.

donate electrons to PS2 of the tris-washed chloroplasts via donor site [1][3]. In the presence of DCMU which blocks electron flow through PS2, donor site [2] is available to low concentrations (33 μM) of DCPIP and TMPD but electron flow from this site to NADP does not involve phosphorylation. At higher concentration, these donors can supply electrons through site [3] which does encompass a coupling site for phosphorylation. The order of preference for electron donation appears to be correlated to the redox potential of the site to which donation occurs, the more positive sites being most ready to accept electrons. The partial inhibition of phosphorylation by DCMU with high concentrations of the donor systems suggests all or most of the electron transport is via site [1] in the absence of DCMU and is shared by sites [2] and [3] in the presence of DCMU. Equal electron flow through these latter 2 sites would result in a 50 % decrease in the P/e$_2$ ratio.

Any such scheme for the sites of electron donation by artificial donor systems will depend upon the kind of electron transport system envisioned. Izawa and Good (22) have made strong arguments that chloroplasts contain 2 kinds of electron transport chains: those that are uncoupled, perhaps by the isolation procedures, and yield no ATP and those that are coupled but with 2 sites of phosphorylation in the linear electron transport chain between water and NADP. Our data are also consistent with their scheme. The electron transport ascribed to donor site [2] could be to sites in the uncoupled chains. The electron transport supported by the low concentration of ascorbate (330 μM) alone (see tables II and III) which is poorly coupled to phosphorylation is inhibited by DCMU suggesting the possibility of electron donation to PS2 of uncoupled chains. The 50 % inhibition of phosphorylation by DCMU would also be consistent with 2 sites of phosphorylation in the coupled chains, 1 coupling site being between donor sites [1] and [3] and, therefore, sensitive to DCMU and the other site being after donor site [3] and insensitive to DCMU.

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[3] Site numbers 1, 2, and 3, in brackets, refer to circled numbers in figure 9.

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