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Evidence from Chloroplast Fragments for Three Photosynthetic Light Reactions

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Abstract. Previous reports from this laboratory described a new concept of three light reactions in plant photosynthesis comprising two short-wavelength ($\lambda < 700$ nm) photoreactions belonging to Photosystem II and one long-wavelength ($\lambda > 700$ nm) photoreaction belonging to Photosystem I. Among the electron carriers assigned to Photosystem II were cytochrome b_{559} and plastocyanin and to Photosystem I, cytochrome *f*.

According to a widely held view, the light-induced reduction of NADP by water requires the collaboration of Photosystems I and II and involves specifically cytochrome *f* and P700 (a portion of chlorophyll *a* peculiar to Photosystem I). By contrast, the new concept ascribes the light-induced reduction of NADP by water solely to the two photoreactions of Photosystem II, without the participation of Photosystem I and its components, cytochrome *f* and P700.

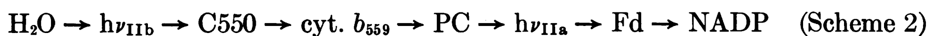
Further evidence in support of the new concept has now been obtained from chloroplast fragments. Two kinds of chloroplast fragments have been prepared: (*a*) one with Photosystem II activity, capable—in the presence of plastocyanin—of photoreducing NADP with water but lacking P700 and functional cytochrome *f* and (*b*) another having only Photosystem I activity, lacking plastocyanin, and enriched in P700.

Reducing power for CO_2 assimilation in photosynthesis is generated by a light-induced electron flow from water to ferredoxin (Fd) and thence, by an enzymic reaction independent of light, to NADP. The light-induced reduction of NADP by water—readily measured in isolated chloroplasts—proceeds most effectively in short-wavelength light ($\lambda < 700$ nm) associated with Photosystem II and falls off sharply in long-wavelength ($\lambda > 700$ nm) light associated with Photosystem I of plant photosynthesis.[1] Nevertheless, for reasons reviewed elsewhere,[2] it is widely held that the light-induced reduction of NADP by water requires the collaboration of both Photosystems I and II, operating in series and joined by an electron transport chain that includes a postulated fluorescence quenching substance (*Q*), cytochrome b_{559}, cytochrome *f*, and plastocyanin (PC), as shown in abbreviated form in Scheme 1 (ref. 2).

$$H_2O \rightarrow h\nu_{II} \rightarrow Q \rightarrow \text{cyt. } b_{559} \rightarrow \text{cyt. } f \rightarrow PC \rightarrow h\nu_I \rightarrow Fd \rightarrow NADP \quad \text{(Scheme 1)}$$

A different hypothesis, advanced in 1965,[3] attributed the (noncyclic) electron flow from water to ferredoxin-NADP solely to Photosystem II and limited

Photosystem I to a cyclic electron flow and its experimental variants, such as the light-induced reduction of NADP by a reduced dye [2,6-dichlorophenol indophenol (DPIP)]. This concept was further refined in the light of recent evidence that Photosystem II includes not one, but two, short-wavelength photoreactions (IIa and IIb); these operate in series and are joined by an electron transport chain that includes (but is not limited to) cytochrome b_{559}, plastocyanin, and C550, a newly discovered chloroplast component (distinct from cytochromes) that undergoes photoreduction in Photosystem II light.[4,5] According to this concept, the photoreduction of ferredoxin and NADP is accomplished solely by Photosystem II reactions as shown in Scheme 2.


$$H_2O \rightarrow h\nu_{IIb} \rightarrow C550 \rightarrow \text{cyt. } b_{559} \rightarrow PC \rightarrow h\nu_{IIa} \rightarrow Fd \rightarrow NADP \quad \text{(Scheme 2)}$$

The two photoreactions of Photosystem II and the one photoreaction of Photosystem I would thus constitute the three light reactions of plant photosynthesis.

The concept of three photoreactions arranged in two parallel photosystems would be greatly strengthened if chloroplasts could be subdivided into fragments with properties and activities corresponding to those ascribed here to Photosystem II and Photosystem I, respectively. This communication reports experiments with isolated chloroplast fragments having only Photosystem I activity ($DPIPH_2 \rightarrow NADP$) and with chloroplast fragments devoid of Photosystem I activity and free of P700 and functional cytochrome *f* but capable of photoreducing ferredoxin–NADP by water. (P700 represents a small component part of the total chlorophyll *a* and has *in situ* an absorbance peak at 700 nm; P700 is considered to act as the terminal trap for the light energy absorbed by the "bulk" chlorophyll of Photosystem I and to participate directly in photochemical reactions.[6]) Chloroplast fragments exhibiting only Photosystem I activity have been described previously.[7–9] The isolation of Photosystem II chloroplast fragments, free of P700 and functional cytochrome *f* and capable of photoreducing NADP with water, is conceivable according to Scheme 2 but is theoretically impossible according to Scheme 1.

Methods. Chloroplast fragments having only Photosystem II activity were prepared by minor modifications of the method described by Huzisige *et al.*[10] However, since our chloroplast fragments (DTS-III) prepared in this way had properties different from those observed by Huzisige *et al.*, the procedure we used will be described in detail.

1 kg of cooled, washed spinach leaves was blended for 2 min at full speed in a Waring Blendor, Model CB-5, with a mixture of 17.0 ml of 1 M Tris·HCl buffer, pH 7.3–500 ml cold distilled water and made to 1 liter with distilled-water ice. The slurry was filtered through two layers of cheesecloth and centrifuged 3 min at 2000 × *g;* the pellet was discarded. The supernatant suspension was then centrifuged for 30 min at 13,000 × *g* and the supernatant suspension discarded. The pellet was resuspended with 17 mM Tris·HCl buffer, pH 7.3 (used throughout) to give a chlorophyll concentration of 1.25 mg/ml (in about 400 ml). An equal volume of 0.5% digitonin in Tris buffer was added to this suspension. The mixture was incubated at 0°C for 40 min with gentle stirring and disrupted by 30 sec of sonication at power setting no. 8 (Branson Sonifier, Model 125), then centrifuged for 20 min at 2000 × *g*. The pellet was discarded and the supernatant suspension was recentrifuged for 30 min at 12,000 × *g*. The pellet was resuspended in Tris buffer to give a chlorophyll concentration of 1 mg/ml (in about 180 ml).

At successive 20-min intervals, a 60-ml aliquot of this suspension was treated with an equal volume of 0.4% Triton X-100 in Tris buffer; each mixture was incubated for 20 min

at 0°C with gentle stirring and then sonicated with a microtip for 15 min, at power setting no. 3, with a magnetic stirrer and an ice-salt water bath to maintain the temperature during sonication between 2 and 5°C. After sonication, each mixture was immediately diluted 10 times with Tris buffer and centrifuged 30 min at 12,000 × *g*, and the supernatant suspension was discarded. The pellet was resuspended in 15 ml Tris buffer; 5 ml was layered on each of three tubes containing 25.5 ml of a 15–50% linear sucrose density gradient and centrifuged for 60 min at 70,000 × *g* in a Spinco SW 25.1 rotor. The centrifugation yielded three distinct green bands and a pellet. The lowest green band, just above the pellet, was collected and diluted 10 times with Tris buffer and centrifuged at 34,000 × *g* for 10 min. The resulting pellet was resuspended to give a suspension containing 0.5 mg of chlorophyll per ml, designated DTS-III and used as the System II fragment. The chlorophyll *a*/chlorophyll *b* ratio of DTS-III fragments varied from 1.4 to 1.8. An aliquot of the chloroplast suspension taken just prior to the digitonin treatment was used as a control.

The P144 chloroplast fragments were prepared as described by Sane, Goodchild, and Park[11] (by the nondetergent method introduced by Michel and Michel[12]) except that our preparation included the fraction obtained by centrifuging between 40,000 and 144,000 × *g* (essentially equivalent to the 160K fraction of Sane *et al.*). The chlorophyll *a*/chlorophyll *b* ratio of the P144 fraction varied from 4.0 to 5.5. An aliquot of the chloroplast suspension taken just prior to the French press treatment was used as a control.

Photoreduction of NADP and DPIP were measured under air at room temperature with a Gilford spectrophotometer as described by McSwain and Arnon,[13] using 664 nm actinic illumination of 3.0×10^4 ergs/cm^2/sec. Light-induced cytochrome changes were measured with a dual-wavelength spectrophotometer[5,14] and oxygen evolution, polarigraphically by a modified[15] Hagihara's closed-cell method.[16] The P700 content was determined from the ascorbate minus ferricyanide difference spectra measured with a Cary Model 14 spectrophotometer.[9] Cytochrome *f* was measured at 550 nm (minus 540 nm reference) as the ascorbate minus ferricyanide difference ($\epsilon_{550-540}$ = 15.5 mM^{-1} cm^{-1}); similarly, cytochrome b_{559} at 562 nm (minus 570 nm reference) ($\epsilon_{562-570}$ = 17.0 mM^{-1} cm^{-1}). Chlorophyll was determined as described by Arnon.[17] Plastocyanin was prepared and estimated by a modification of the method[9,18] of Katoh *et al.*;[19] ferredoxin and ferredoxin-NADP reductase were prepared as described.[20,21]

Results and Discussion. Huzisige *et al.*[10] described the preparation of chloroplast fragments (by a combination of digitonin, Triton X-100, sonication, and sucrose density gradient treatments) that were capable of photoreducing either *p*-

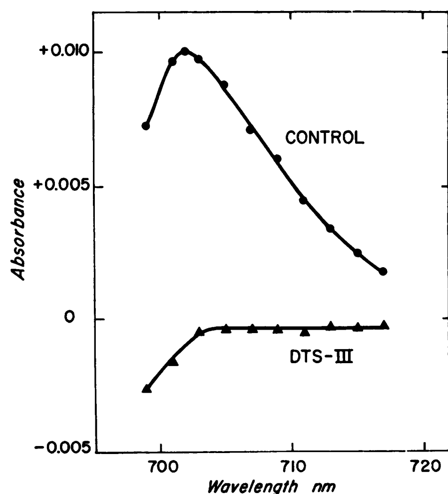


FIG. 1. P700 content of DTS-III chloroplast fragments. The reaction mixture contained (per 1.0 ml) control chloroplasts or DTS-III fragments (equivalent to 100 μg chlorophyll) and 50 μmol of MES [2-(*N*-morpholino)ethanesulfonic acid] buffer (pH 7.2). The spectra were obtained by adding 10 μmol of sodium ascorbate to the sample cuvette and 10 μM potassium ferricyanide to the reference cuvette. Light path, 1 cm; gas phase, air.

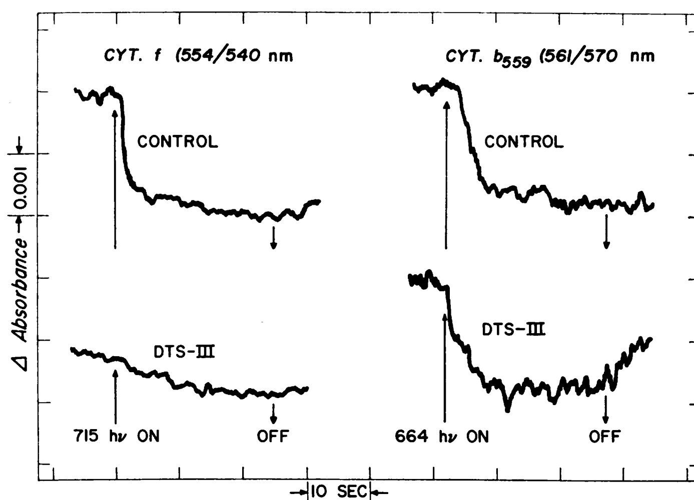


FIG. 2. Cytochrome photooxidations in DTS-III chloroplast fragments. The reaction mixture for the cytochrome b_{559} photooxidation contained (per 1.0 ml) control chloroplasts or DTS-III fragments (equivalent to 75 μg chlorophyll) and the following in micromoles: glycine buffer (pH 9.5), 33.3; K_2HPO_4, 5; $MgCl_2$, 2; ascorbate, 1; ferredoxin, 0.01, NADP, 1; and ferredoxin-NADP reductase, equivalent to an absorbance at 456 nm of 0.008. The reaction mixture for the DTS-III particles also contained 0.01 μmol plastocyanin. The reaction mixture for the cytochrome f photooxidation contained (per 1.0 ml) control chloroplasts or DTS-III fragments (equivalent to 75 μg chlorophyll) and the following in micromoles: MES buffer (pH 7.2), 33.3; K_2HPO_4, 5; $MgCl_2$, 2; ascorbate, 1; ferredoxin, 0.01; NADP, 1; and ferredoxin-NADP reductase, equivalent to an absorbance at 456 nm of 0.008. Gas phase, nitrogen. The 664 nm monochromatic light beam had an intensity of approximately 1.5×10^4 ergs/cm^2/sec and the 715 nm beam, an intensity of approximately 0.9×10^4 ergs/cm^2/sec. Light path, 1 cm.

benzoquinone or DPIP by water but were incapable of photoreducing ferredoxin-NADP either via Photosystem II (with water as electron donor) or via Photosystem I (with ascorbate-DPIP as electron donor). Using their procedure, we isolated spinach chloroplast fragments which were free of measurable plastocyanin, had little, and in some preparations no, P700 (Fig. 1) and were unable to photooxidize cytochrome f but showed no impairment in ability to photooxidize cytochrome b_{559} in the presence of added plastocyanin (Fig. 2).

The photochemical activity of DTS-III fragments is shown in Table 1. They

TABLE 1. *Photochemical activity of chloroplast fragments DTS-III.*

	Control (Q)*	DTS-III (Q)*
$H_2O \rightarrow$ DPIP	100	31
$H_2O \rightarrow$ NADP	74	0
$H_2O \xrightarrow{+\text{ plastocyanin}}$ NADP	87	41
Ascorbate + DPIP $\rightarrow$ NADP	120	0

* Q = μmoles acceptor reduced per mg chlorophyll per hr.

The reaction mixture contained (per 1.0 ml) control chloroplasts or DTS-III fragments (equivalent to 50 μg chlorophyll), and 100 μmol buffer, pH 7.2 (see Fig. 1). For the $H_2O \rightarrow$ DPIP reaction, 0.25 μmol DPIP was added. For NADP reduction, ferredoxin, 10 nmol, NADP, 2 μmol, and ferredoxin-NADP reductase, equivalent to an absorbance at 456 nm of 0.0080, were added; in addition, where indicated, ascorbate, 10 μmol, DPIP, 0.10 μmol, and plastocyanin, 20 nmol were added. Where ascorbate was used, 10^{-6} M DCMU was present.

were free of Photosystem I activity, as measured by their inability to photoreduce NADP in the presence of 3-(3′,4′-dichlorophenyl)-1,1-dimethyl urea (DCMU) with ascorbate-DPIP as the electron donor. The DTS-III particles, however, retained appreciable Photosystem II activity. With water as the electron donor, they were able to photoreduce DPIP and, in the presence of plastocyanin, NADP. Confirmation that water was the electron donor in NADP reduction was obtained by parallel measurements of oxygen evolution with an oxygen electrode. Both oxygen evolution and NADP reduction were completely inhibited by 1×10^{-6} M DCMU—a further confirmation that the activity measured was that of Photosystem II.

In accordance with Scheme 2, the requirement of plastocyanin for NADP reduction points to the involvement of both photoreactions IIb and IIa, whereas the dispensability of plastocyanin in DPIP reduction suggests the involvement of only photoreaction IIb in that reaction. In sum, the photochemical activity of DTS-III chloroplast fragments is consistent with the concept that the photoreduction of NADP by water depends only on the two photoreactions of Photosystem II and is independent of P700, cytochrome *f*, or any other component of Photosystem I.

The P144 preparation consisted of chloroplast fragments which had only Photosystem I activity. In agreement with the findings of Sane, Goodchild, and Park,[11] the P144 fragments were, on a chlorophyll basis, greatly enriched in P700, unchanged in cytochrome *f* content, and free of plastocyanin (Table 2).

TABLE 2. *Selected components of P144 chloroplast fragments.*

	Cyt. *f*		Plastocyanin		P700	
	Chl*/cyt. *f*	Relative abundance	Chl/PC	Relative abundance	Chl/P700	Relative abundance
Control	775	100	550	100	570	100
P144	775	100	0	0	130	440

* Chl, chlorophyll; PC, plastocyanin.

TABLE 3. *Photochemical activity of chloroplast fragments P144.*

	Control (Q)*	P144 (Q)
$H_2O \rightarrow$ NADP	52	0
$H_2O \xrightarrow{+\text{ plastocyanin}}$ NADP	52	0
Ascorbate → NADP	20	0
Ascorbate + DPIP → NADP	96	208
Ascorbate + PC → NADP	28	74
Ascorbate + PC + DPIP → NADP	105	261

* Experimental details were as described in Table 1, except that P144 fragments and corresponding control chloroplasts were used. PC, plastocyanin.

Table 3 shows that the P144 fragments had no Photosystem II activity, as measured by the photoreduction of ferredoxin–NADP by water, with or without added plastocyanin. Of special interest was the finding that adding plastocyanin had only a slight effect on the Photosystem I activity (reduced DPIP → NADP) of the P144 fragments—a finding consistent with earlier observations that plastocyanin was not essential for Photosystem I reduction of NADP by $DPIPH_2$.[9,22]

Abbreviations: DPIP and $DPIPH_2$, oxidized and reduced forms of 2,6-dichlorophenol indophenol; DTS III, a preparation of chloroplast fragments; DCMU, 3-(3',4'-dichlorophenyl)-1,1-dimethyl urea.

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