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# Photosynthetic electron transport from water to $NADP^+$ driven by photosystem II in inside-out chloroplast vesicles

(phase partition/oxygenic photosystem/Z scheme)

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**ABSTRACT** **It is now widely held that the light-induced noncyclic (linear) electron transport from water to $NADP^+$ requires the collaboration in series of the two photosystems that operate in oxygen-evolving cells: photosystem II (PSII) photooxidizes water and transfers electrons to photosystem I (PSI); PSI photoreduces ferredoxin, which in turn reduces $NADP^+$ (the Z scheme). However, a recently described alternative scheme envisions that PSII drives the noncyclic electron transport from water to ferredoxin and $NADP^+$ without the collaboration of PSI, whose role is limited to cyclic electron transport [Arnon, D. I., Tsujimoto, H. Y. & Tang, G. M.-S. (1981) *Proc. Natl. Acad. Sci. USA* 78, 2942–2946]. Reported here are findings at variance with the Z scheme and consistent with the alternative scheme. Thylakoid membrane vesicles were isolated from spinach chloroplasts by the two-phase aqueous polymer partition method. Vesicles, originating mainly from appressed chloroplast membranes that are greatly enriched in PSII, were turned inside-out with respect to the original sidedness of the membrane. With added plastocyanin, ferredoxin, and ferredoxin–$NADP^+$ reductase, the inside-out vesicles enriched in PSII gave a significant photoreduction of $NADP^+$ with water as electron donor, under experimental conditions that appear to exclude the participation of PSI.**

It is now well established (1) that the photosynthetic apparatus of plants utilizes two photosystems, photosystem I (PSI) and photosystem II (PSII), for the conversion of the electromagnetic energy of sunlight into biologically useful chemical energy via two processes: cyclic photophosphorylation that generates only ATP (2, 3) and noncyclic photophosphorylation that concurrently generates ATP and reducing power, the carrier of which was identified as NADPH (4) and later as reduced ferredoxin (5, 6). Reduced ferredoxin is the versatile carrier of photosynthetically generated energy-rich electrons destined for many targets (7), including the enzymatic reduction of $NADP^+$ (8, 9) and the regulation of cyclic photophosphorylation (10).

The source of electrons needed for the light-induced reduction of ferredoxin is the photooxidation of water, a reaction that also produces molecular oxygen and contributes protons used to generate an electrochemical proton gradient (11, 12) responsible for the (noncyclic) ATP formation coupled to ferredoxin (6) or $NADP^+$ (4) reduction. There is wide agreement on two points: (*i*) photooxidation of water is driven by PSII (1) and (*ii*) cyclic photosphosphorylation is driven by PSI (1, 13). There is also agreement that PSI is capable of reducing ferredoxin and $NADP^+$ by artificial reductants (14), but there is disagreement as to whether PSI is responsible for the reduction of ferredoxin under physiological conditions—i.e., by electrons liberated in the photooxidation of water by PSII.

Most investigators favor the so-called Z scheme, according to which only PSI is competent to photoreduce ferredoxin, and a collaboration of PSII and PSI is therefore required for the complete noncyclic electron transport from water to ferredoxin (1, 14). However, a recently described alternative concept (15, 16) envisions that, under physiological conditions, PSII (renamed the oxygenic photosystem) drives the complete noncyclic electron transport from water to ferredoxin without the collaboration of PSI (renamed the anoxygenic photosystem), whose physiological role is limited to cyclic electron transport and its related phosphorylation. From this perspective, the ability of PSI to photoreduce $NADP^+$ with an artificial electron donor appears to be an experimental variant of cyclic electron transport.

The alternative concept is that, except for regulatory connections, the two photosystems are basically autonomous and operate not collaboratively in series but synchronously in parallel (15). A strong parallelism exists between this formulation and recent findings of spatial separation of the two photosystems in chloroplast membranes in a pattern that limits possibilities for intimate interactions between them as demanded by the Z scheme. PSI and PSII were found to be localized in large, separate membrane domains, about 0.5 $\mu$m in diameter (17). Chloroplast membranes are differentiated into regions of stacked (appressed) and unstacked (nonappressed) membranes. The margins and end membranes of the stacked region (grana) and the unstacked membranes (stroma lamellae) are exposed to the stroma, whereas the appressed membranes of the grana partitions have little contact with the stroma. PSII was found to be localized in the appressed membranes, whereas PSI was found to be localized in the stroma-exposed membranes (18–20).

The alternative scheme predicts that an isolated oxygenic photosystem (PSII) should be capable of using electrons from water to photoreduce $NADP^+$ without the collaboration of PSI, provided that the isolation was carried out by procedures gentle enough to protect the structural and functional integrity of chloroplast membranes. We report here that this prediction was to an appreciable degree verified by the use of membrane vesicles isolated by a two-phase partition method (17, 21). These vesicles are turned inside-out with respect to the original sidedness of the membrane (22, 23) and because they originate mainly from appressed chloroplast membranes, they are also greatly enriched in PSII (24). With added plastocyanin, ferredoxin, and ferredoxin–$NADP^+$ reductase, these vesicles gave a significant oxygenic photoreduction of $NADP^+$ under ex-

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Abbreviations: PSI, photosystem I; PSII, photosystem II; diuron (DCMU), 3-(3,4-dichlorophenyl)-1,1-dimethyl urea; DBMIB, 2,5-dibromo-3-methyl-6-isopropyl-*p*-benzoquinone (dibromothymoquinone); DNP-INT, dinitrophenyl ether of iodonitrothymol; DCIP and $DCIPH_2$, oxidized and reduced forms of 2,6-dichloroindophenol.

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perimental conditions that appear to exclude the participation of PSI.

## METHODS

Chloroplasts were isolated from freshly harvested spinach leaves (*Spinacia oleracea*, var. Marathon) grown in a greenhouse in nutrient solution (25). Previously described procedures were used for the estimation of chlorophyll (25), isolation and purification of ferredoxin (26), and determination of photoreduced $NADP^+$ (27). Photoreduction of 2,6-dichloroindophenol (DCIP) was measured by a decrease in absorption at 590 nm. Two kinds of plastocyanin were used interchangeably, one prepared by standard methods from spinach leaves and one isolated from green barley seedlings (by Nancy A. Crawford) in the course of thioredoxin preparation. An extinction coefficient of $4.9 \times 10^3$ $cm^{-1}$ $M^{-1}$ at the absorption peak of 597 nm for oxidized plastocyanin was used to determine plastocyanin concentration. 2,5-Dibromo-3-methyl-6-isopropyl-*p*-benzoquinone (DBMIB; dibromothymoquinone) and the dinitrophenyl ether of iodonitrothymol (DNT-INT) were added as methanol solutions.

**Preparation of Vesicles of Opposite Sidedness.** Inside-out vesicles enriched in PSII and right-side-out vesicles containing both PSII and PSI were prepared from thylakoid membranes by an aqueous polymer two-phase (dextran/polyethylene glycol) partition method (17, 22, 23). Several modifications were introduced. Spinach leaves were blended in 400 mM sucrose/50 mM sodium phosphate, pH 7.4/10 mM NaCl/1 mM sodium ascorbate. The slurry was filtered and centrifuged. The sedimented thylakoids were osmotically disrupted in 35 mM NaCl/1 mM sodium ascorbate, centrifuged, washed once in a "high-salt" solution (150 mM NaCl/50 mM sodium phosphate buffer, pH 7.4/1 mM sodium ascorbate) to maintain stacking conditions, and resuspended in the same solution. The suspension was passed twice through a Yeda press [1,450 pounds/$inch^2$ (10 MPa)]. The effluent from the press was centrifuged at 48,000 $\times$ *g* for 20 min and the pellet was treated as follows.

To induce unstacking, the pellet was washed and suspended in a "low-salt" solution (5 mM NaCl/10 mM sodium phosphate buffer, pH 7.4/100 mM sucrose). The suspension was passed twice through the Yeda press (1,450 pounds/$inch^2$). The press effluent was then fractionated several times by phase partition, essentially as described (22, 23), into a bottom phase containing mainly inside-out vesicles enriched in PSII (fraction B4) and a top phase (fraction T3) with right-side-out vesicles (sidedness as in chloroplasts) containing both PSII and PSI.

After phase partition, the polymers were removed from the T3 fraction by adding $MgCl_2$ (to 10 mM) to aggregate the vesicles (28). After centrifugation at 48,000 $\times$ *g* for 10 min, the pellet was suspended in a solution of 100 mM sucrose and 20 mM NaCl. To remove the polymers from the B4 fraction, it was partitioned to a top phase by adding fresh top phase and water to decrease the polymer composition to 5.5% for each polymer. The B4 vesicles then in the top phase were freed from polymers and resuspended as described for T3 above.

The range of starting membrane material varied between 10 and 50 mg of chlorophyll per 80-ml phase system. At the highest concentration of membrane material, the yields of B4 and T3 were 5 and 7 mg of chlorophyll, respectively. The polymer composition, ionic composition, and temperature of the phase system are critical. For optimal separation, different batches of polymers may need different polymer concentrations within a range of 5.7–6% for each polymer. The concentration we used was 0.4% higher for both polymers than the minimum concentration (5.4% of each polymer in our case) at which the mixture turns from a one-phase to a two-phase system (21).

In one instance only (Fig. 3 *Inset*) a different preparation of inside-out vesicles was also included. Designated "randomized," these vesicles contained accessible PSII and PSI in the same proportions as in chloroplasts and were obtained by acid-induced membrane-pairing of thylakoids that had been destacked and randomized prior to fragmentation, as described elsewhere (24).

## RESULTS

The types of vesicles that can be formed from chloroplast membranes (24, 29) are shown in Fig. 1. Mechanical breakage under stacking conditions gives rise to inside-out vesicles derived from appressed membranes and right-side-out vesicles derived mainly from stroma-exposed membranes. The inside-out and the right-side-out vesicles are separated by phase partition under unstacking conditions. The right-side-out vesicles (types 1, 4, and 5 in Fig. 1), containing both PSI and PSII, partition into the top phase (polyethylene glycol). The inside-out vesicles (types 2 and 3 in Fig. 1) that originate from regions of appressed membranes and contain only PSII (type 2) or PSII fused with a small PSI segment (type 3) partition into the bottom phase (dextran). However, a certain contamination of inside-out vesicles in the top phase and right-side-out vesicles in the bottom phase may still be present even after repeated partitions (23).

Fig. 2 compares the PSI and PSII electron transport activities of the inside-out (fraction B4) and the right-side-out (fraction T3) vesicles. The right-side-out vesicles retained appreciable PSI and PSII electron transport activities. The inside-out vesicles retained a small and in some preparations (as shown here) not even measurable PSI electron transport activity but had a higher PSII electron transport activity than did the right-side-out vesicles (Fig. 2).

The enrichment of the B4 fraction in PSII was also reflected in their high ratios of PSII to PSI derived from measurements (made by A. Melis; cf. ref. 20) of the light-induced oxidation of P700 (reaction center chlorophyll of PSI) and the light-induced reduction of Q, the primary electron acceptor of PSII. The chlorophyll/P700 ratio of 2,520 and the chlorophyll/Q ratio of 386 are similar to the corresponding ratios reported by Andersson and Haehnel (19) and Anderson and Melis (20) for inside-out spinach chloroplast vesicle preparations and correspond to a PSII/PSI ratio of 6.5.

Of special interest were the results pertaining to the ability of inside-out vesicles to photoreduce $NADP^+$ by electrons originating from water. No such oxygenic photoreduction of $NADP^+$ by inside-out vesicles enriched in PSII and depleted of PSI is encompassed by the Z scheme of photosynthetic electron transport. But, as shown in Fig. 3, inside-out vesicles exhibited an appreciable rate of reduction of $NADP^+$ when supplemented with catalytic amounts (*ca.* 2 $\mu$M) of plastocyanin, in addition to ferredoxin and ferredoxin-$NADP^+$ reductase. The marked effect of added plastocyanin on the oxygenic photoreduction was consistently observed in about 20 consecutive preparations of inside-out vesicles.

The possibility existed that the plastocyanin effect was an experimental artifact, an artificial electron transport pathway in which (*i*) plastocyanin was first reduced by PSII with water and (*ii*) reduced plastocyanin served as electron donor for the photoreduction of ferredoxin–$NADP^+$ by the small PSI component still remaining in the inside-out vesicles (see type 3, Fig. 1).

To test this possibility, PSII activity was totally inhibited by diuron and reduced plastocyanin was provided to the inside-out vesicles by the inclusion of both plastocyanin and ascorbate in the reaction mixture. Ascorbate readily reduces plastocyanin (30). Haehnel *et al.* (31) have shown that externally added plastocyanin, when reduced by ascorbate, is an effective electron

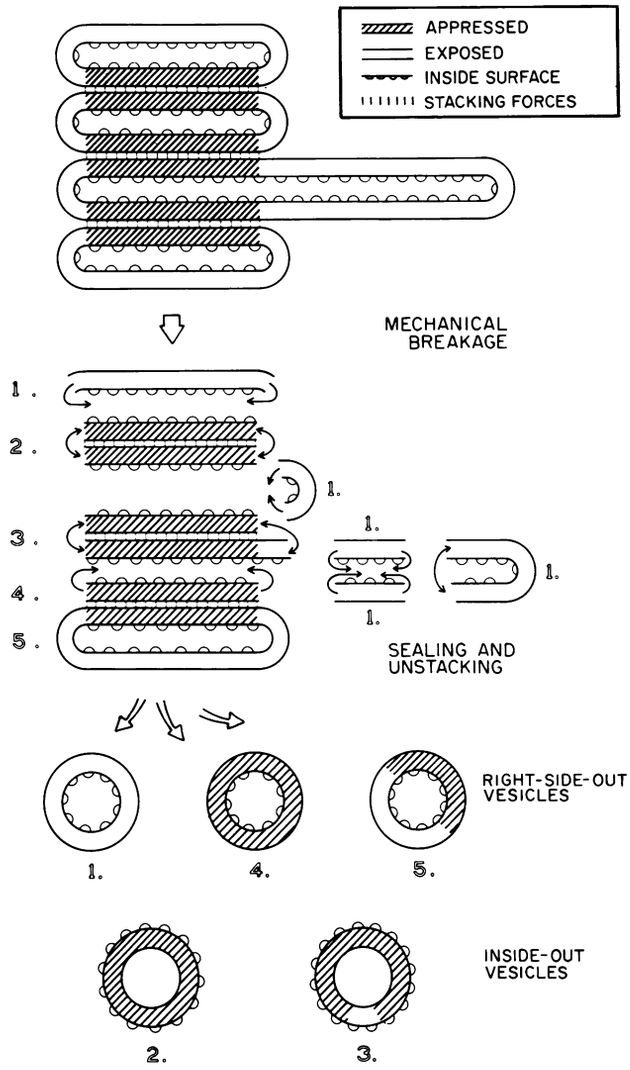


FIG. 1. Scheme for the formation of types of inside-out and right-side-out vesicles by mechanical disruption of chloroplast membranes. (*Top*) Diagram of stacked chloroplast membranes showing appressed (PSII) and stroma-exposed (PSI) membranes. Stacking conditions are maintained by the presence of cations (150 mM NaCl or 5 mM $MgCl_2$). (*Middle*) Mechanical breakage (Yeda press) ruptures membrane stacks at their exposed edges but the appressed membranes remain appressed. 1, Edges of stroma-exposed membrane fragments seal, giving rise to right-side-out vesicles enriched in PSI. 2, Fragments of appressed membranes fuse and their edges seal, giving rise to inside-out vesicles enriched in PSII. 3, Same as 2, except that a fragment of a stroma-exposed membrane is incorporated with the appressed membranes, introducing an inside-out PSI component into a predominantly inside-out PSII vesicle. 4, After unstacking, the edges of a single appressed membrane seal and give rise to a right-side-out PSII vesicle. 5, An undisrupted chloroplast membrane that gives rise to a right-side-out vesicle containing both PSII and PSI. (*Bottom*) After destacking (by low cation concentration), the right-side-out vesicles (types 1, 4, and 5) can be separated by phase partition into the upper phase and the inside-out vesicles (types 2 and 3) into the lower phase of a dextran/polyethylene glycol two-phase system.

donor to P700 when P700 is accessible. Thus, if the observed plastocyanin-stimulated $NADP^+$ reduction by water were due to PSII and PSI reactions acting in sequence, a comparable rate of $NADP^+$ reduction would be expected when ascorbate replaced PSII as the reductant for plastocyanin.

Fig. 3 shows that plastocyanin, maintained in the reduced state by an excess of ascorbate, did not induce significant $NADP^+$ reduction in the inside-out vesicles. This finding speaks against

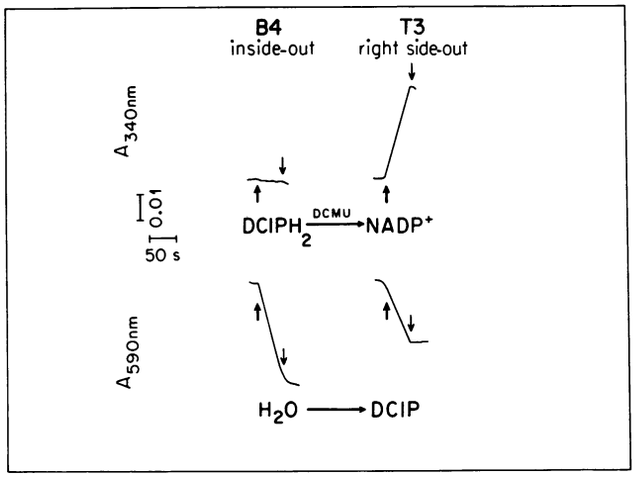


FIG. 2. Light-induced electron transport by PSI ($DCIPH_2 \rightarrow NADP^+$) and PSII ($H_2O \rightarrow$ DCIP) in inside-out (B4) and right-side-out (T3) thylakoid vesicles. All reaction mixtures contained B4 or T3 vesicles (50 $\mu$g of chlorophyll per ml), 50 mM 2(*N*-morpholino)ethanesulfonic acid (Mes) buffer at pH 6.7, 5 mM $MgCl_2$, 15 $\mu$g of gramicidin, and 100 $\mu$M DCIP. In addition, for $NADP^+$ reduction the following were included: 2 mM $NADP^+$, 10 $\mu$M ferredoxin, 10 mM sodium ascorbate, 10 $\mu$M 3-(3,4-dichlorophenyl)-1,1-dimethyl urea (diuron; DCMU) and a saturating amount of ferredoxin–$NADP^+$ reductase. The reaction mixtures were illuminated at room temperature in cuvettes (2-mm light path) by 650-nm light ($5 \times 10^4$ erg $cm^{-2}$) for $NADP^+$ reduction and 670-nm light for DCIP reduction. Arrow up, light on; arrow down, light off.

the participation of PSI in the plastocyanin-dependent electron transport from water to $NADP^+$. Thus, it appears that the accessible PSI segment that may have been present in the inside-out vesicles (type 3, Fig. 1) was negligible.

There remained the possibility that the failure of ascorbate–plastocyanin to support $NADP^+$ reduction by PSI was due not

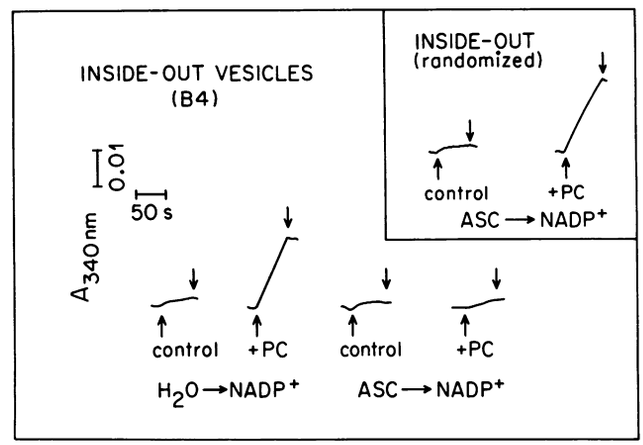


FIG. 3. Effect of plastocyanin (PC) on photoreduction of $NADP^+$ in inside-out (B4) thylakoid vesicles. Experimental conditions and reaction mixtures were as for Fig. 2 except that in $H_2O \rightarrow NADP^+$, DCIP, diuron, and ascorbate were omitted, and in ascorbate (ASC) $\rightarrow NADP^+$ only DCIP was omitted. Where indicated, 1.7 $\mu$M plastocyanin (PC) was included. In this experiment the rate of $NADP^+$ reduction by water in the presence of plastocyanin was 25 $\mu$mol per mg of chlorophyll per hr. The basal rate of $NADP^+$ reduction by water varied in different preparations but in every case the addition of plastocyanin gave an increase of several hundred percent. To illustrate, in a preparation that had a basal rate of $NADP^+$ reduction by water of 12 $\mu$mol per mg of chlorophyll per hr, the addition of plastocyanin increased the rate to 39, as compared with corresponding rates of 0 and 7 for the ascorbate $\rightarrow NADP^+$ system (diuron present). (*Inset*) Plastocyanin reduced by ascorbate is an effective donor for $NADP^+$ reduction by accessible PSI in "randomized" inside-out vesicles (see *Methods*).

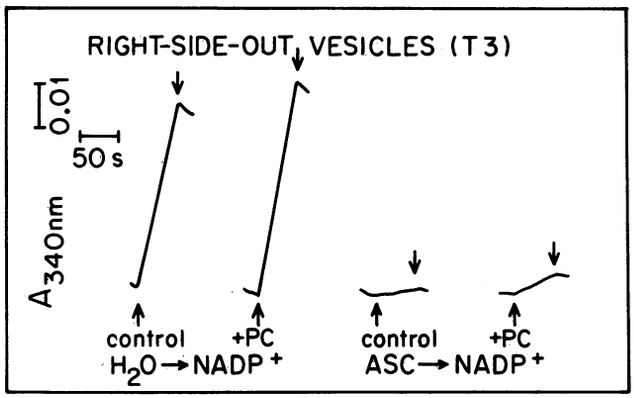


FIG. 4. Effect of plastocyanin (PC) on photoreduction of $NADP^+$ in right-side-out (T3) thylakoid vesicles. Experimental conditions and reaction mixtures were as for Fig. 3.

to the insufficiency of accessible PSI but to the sluggishness of ascorbate as an electron donor to plastocyanin. This possibility was rendered improbable when ascorbate in the presence of plastocyanin was found to be an effective donor for $NADP^+$ reduction by PSI when PSI was made accessible (Fig. 3 *Inset*).

As expected, there was little effect of added plastocyanin on $NADP^+$ reduction in the right-side-out vesicles with either water or ascorbate as electron donor (Fig. 4). Right-side-out vesicles retain appreciable plastocyanin and their P700 is not accessible to added plastocyanin (31). Thus, it is unlikely that the observed plastocyanin-stimulated photoreduction of $NADP^+$ by water (Fig. 3) was due to a contamination of the inside-out vesicles by right-side-out vesicles containing PSI.

Fig. 5 shows that the photoreduction of $NADP^+$ by water in inside-out vesicles was predictably inhibited by diuron.

Less predictable was inhibition by two inhibitors of plastoquinone function in chloroplasts, DBMIB (32, 33) and DNP-INT (33, 34), that inhibit $NADP^+$ reduction by water in unfractionated chloroplast membranes. According to the Z scheme these inhibitors act by blocking plastoquinone-mediated electron transfer from PSII to PSI (33). Thus, when only PSII function is involved, as in the case of inside-out vesicles, no inhibitory effect of DBMIB or DNP-INT would be expected. However, an inhibitory effect would be expected according to the alternative scheme (15), which assigns to plastoquinone an essential role in the oxygenic photoreduction of ferredoxin by PSII, quite apart from any intersystem electron transfer.

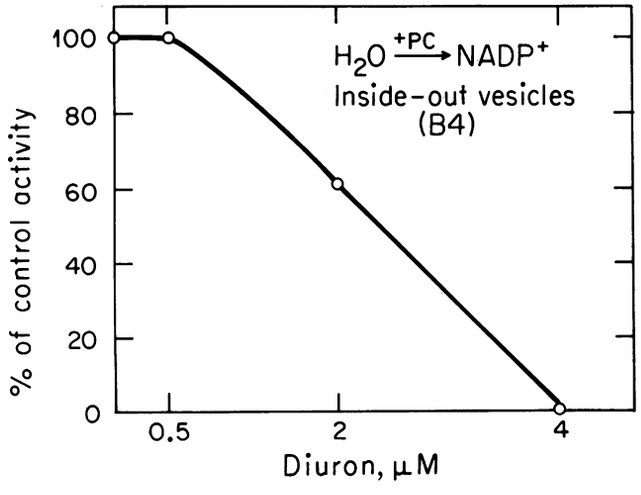


FIG. 5. Diuron inhibition of plastocyanin (PC)-dependent photoreduction of $NADP^+$ by water in inside-out (B4) thylakoid vesicles. Conditions (aside from inhibitor) as for Fig. 3 for $H_2O \rightarrow NADP^+$ in the presence of plastocyanin.

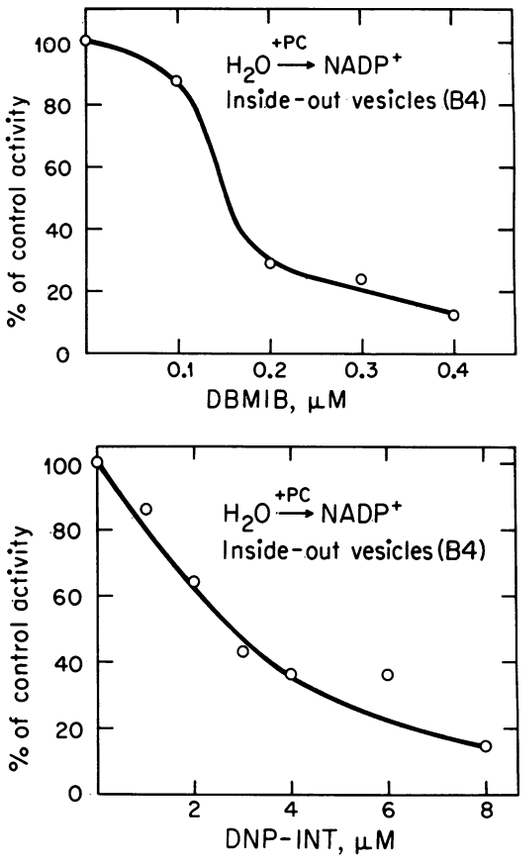


FIG. 6. Inhibition by DBMIB (*Upper*) and DNP-INT (*Lower*) of plastocyanin (PC)-dependent photoreduction of $NADP^+$ by water in inside-out (B4) thylakoid vesicles. Conditions (aside from inhibitor) as for Fig. 5.

Fig. 6 shows that $NADP^+$ reduction by water in inside-out vesicles was inhibited by DBMIB and DNP-INT. It appears that plastoquinone function is essential for the oxygenic reduction of ferredoxin–$NADP^+$ by PSII under conditions in which the participation of PSI is not likely.

## DISCUSSION

The present findings are at variance with the Z scheme on two points. They exclude a role of PSI in the reduction of $NADP^+$ by water and they point to a role for plastocyanin in electron transport involving only PSII. The Z scheme limits the role of plastocyanin to electron donation to P700, the reaction center chlorophyll of PSI (14, 33). However, the alternative concept that guided this work assigns a dual role to plastocyanin, one as a donor to P700 in the cyclic electron pathway of PSI (the anoxygenic photosystem) and another as a donor to P680, the reaction center chlorophyll of PSII (15).

The proposed link of plastocyanin to PSII was not entirely hypothetical. Plastocyanin was found to be required for the photooxidation of cytochrome *b*-559, a chloroplast component not included by the Z scheme in intersystem electron transport between PSII and PSI (35) but one consistently present, even enriched, in PSII chloroplast preparations, whether made with detergents (36–39) or by phase partition (40–42). Cytochrome *b*-559 appears to be structurally bound in close proximity to the reaction center of PSII as evidenced by its preferential photooxidation by PSII at 77 K (43–45).

It is noteworthy that plastocyanin stimulated the photoreduction of $NADP^+$ but not that of DCIP (data not shown). Our tentative explanation is that only strongly electronegative acceptors require a plastocyanin-dependent coupling of the two photoacts postulated for PSII (15), a fragile coupling that is readily disrupted by loss of plastocyanin, by membrane artifacts induced by preparative procedures, or by both.

The photoreduction of ferredoxin–$NADP^+$ by PSII in inside-out vesicles was unexpected because ferredoxin (a hydrophilic, negatively charged protein) is thought to be reduced on the outer sides of the thylakoid membranes (reviews in refs. 14 and 33) that become inaccessible in sealed inside-out vesicles. Further study is needed of this matter, including the possibility of leaks in the vesicles or of the existence of a lipophilic election carrier such as pheophytin that is able to reduce ferredoxin on either side of the membrane.

The possibility, for which there is as yet no evidence, that pheophytin may be an electron donor to ferredoxin is very attractive on thermodynamic grounds. Entrenched in textbook discussions of the Z scheme is the conclusion that PSII generates a strong oxidant and a weak reductant inadequate for ferredoxin reduction, whereas PSI generates a weak oxidant and a strong reductant capable of reducing ferredoxin and other strongly electronegative carriers associated with PSI. However, this thermodynamic argument against the ability of PSII to photoreduce ferredoxin lost its force when it was recently demonstrated that PSII photoreduces pheophytin (46), whose redox potential ($E_m = -610$ mV) is much more electronegative than that of ferredoxin ($E_m = -420$ mV) (5).

In an apparent contradiction of the findings reported here with inside-out vesicles prepared by phase partition are reconstitution experiments of the photosynthetic electron transport to $NADP^+$ with PSII and PSI fractions isolated from chloroplasts with detergents (47–49). Common to the experiments with detergent-made preparations was an obligatory dependence of $NADP^+$ reduction on the inclusion of both PSII and PSI complexes when water (49) or a substitute PSII donor (diphenyl carbazide) was used (47, 48).

These reconstitution experiments record the electron transport capacity of recombined chloroplast fractions but, as noted by Lam and Malkin (49), the identity of such a reconstituted pathway with the *in vivo* pathway remains to be determined. Detergents modify membrane structure by releasing and exchanging with lipids and lipid-soluble components (50). Native links between components may be lost and new links established. For these reasons the reconstitution experiments with PSII and PSI fractions made with detergents are not necessarily in conflict with findings obtained with inside-out membrane vesicles enriched in PSII and prepared by phase partition, a method more likely to protect membrane integrity (21).

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