Energy Conservation in Photoreductions by Photosystem I

Shuttles of Artificial Electron Donors for Photosystem I Across the Thylakoid Membrane

G. Hauska, W. Oettmeier, S. Reimer, and A. Trebst

Abteilung Biologie, Ruhr-Universität Bochum

(Z. Naturforsch. **30 c**, 37–45 [1975]; received October 31, 1974)

Photosynthesis, Energy Conservation, Phosphorylation, Uncoupler Mechanism

$NADP^+$ reduction in isolated chloroplasts of spinach by photosystem I at the expense of various artificial donor systems is not inhibited by the plastoquinone antagonist dibromothymoquinone. The coupled ATP formation in such photoreductions is attributed to an artificial energy conserving site, *i. e.* a proton liberation during oxidation of the donor at the inner surface of the thylakoid membrane.

Some donor systems for photosystem I are stimulated by uncouplers whereas others are not. The stimulation shows no correlation to the efficiency of the coupled photophosphorylation. Instead a correlation of the stimulation by uncouplers to the presence of an acidic OH-group in the donor molecule is seen. The uncoupler effect is therefore not explained by a release of electron transport control by the high energy state but rather by a pH-dependent distribution of the donor compound across the membrane. This is supported by the properties of donor systems in sonicated chloroplast particles with external oxidation sites of photosystem I.

Introduction

Recent results have strengthened the evidence for vectorial electron flow across the thylakoid membrane (s. review 1). This led to a reconsideration of the number of native energy conserving sites along the photosynthetic electron flow system from water to $NADP^+$ [1, 2]. Results with the plastoquinone antagonist dibromo-thymoquinone (DBMIB) * have given strong evidence for the existence of a second native energy conserving site connected with photosystem II and the water splitting reaction [3, 4] in addition to the previously noted site connected with plastoquinone [5, 6]. The experiments with DBMIB, furthermore, indicated that photophosphorylation of photosystem I reactions with artificial redox compounds, insensitive to this inhibitors, cannot involve the native energy conserving site with plastoquinone, because DBMIB reacts specifically at this site [7]. Therefore a concept of artificial energy conservation was developed in order to explain ATP formation in certain systems for cyclic photophosphorylation [1, 2, 7]. The same arguments also hold for coupled non-cyclic flow via photosystem I at the expense of artificial donor systems.

We wish to report here on the properties of photoreductions by photosystem I, particularly in relation to DBMIB sensitivity. The results provide further evidence that artificial donor systems carry reducing equivalents across the membrane to the oxidizing components of photosystem I. Their oxidation inside may be accompanied by the liberation of protons which in turn leads to artificial energy conservation. The stimulation of uncouplers in some but not in other donor systems is related to the chemical structure of the donor.

Part of this investigation has been presented at the 3rd Int. Congress on Photosynthesis Research [8].

Methods

Spinach chloroplasts were prepared according to McCarty and Racker [9]. Sonicated chloroplasts were prepared essentially as described earlier [10]. Plastocyanin was prepared according to Anderson and Mc Carty [11]. N,N,N′-Trimethyl-PD (N,N,N′-trimethyl-*p*-

Requests for reprints should be sent to Prof. Dr. A. Trebst, Ruhr-Universität Bochum, Abteilung Biologie, Lehrstuhl für Biochemie der Pflanzen, *D-4630 Bochum-Querenburg*, Postfach 2148.

* *Abbreviations:* DAD, 2,3,5,6-tetramethyl-*p*-phenylenediamine (diaminodurene); DBMIB, 2,5-dibromo-3-methyl-6-isopropyl-*p*-benzoquinone; DCMU, 3-(3′,4′-dichlorophenyl)-1,1-dimethylurea; DPIP, 2,6-dichloro-phenolindophenol; PD, *p*-phenylenediamine; TMPD, N,N,N′,N′-tetramethyl-*p*-phenylenediamine.

phenylenediamine) was synthesized from N,N-dimethyl-*p*-phenylenediamine as the starting material [12]. This compound was first converted into the 4-dimethylamino-*p*-tolylsulfonanilide, methylated by means of methyl iodide and the tolylsulfonyl protecting group was finally split off by acid hydrolysis [12]. Reduced phenolindophenol (4,4′-dihydroxydiphenylamine) was obtained by co-oxidation of *p*-aminophenol and phenol in alcaline solution by sodium hypochlorite and reduction of the dye by sodium dithionite [13]. Reduced N,N-dimethyl-indoaniline (4-hydroxy-4′-dimethylamino-diphenylamine) was prepared in a similar way by co-oxidation of N,N-dimethyl-*p*-phenylendiamine and phenol. The dye ("Phenol Blue") was again reduced by sodium dithionite [14]. A new procedure has been chosen for the synthesis of N-phenyl-N,N′,N′-trimethyl-PD (N-methyl-4-dimethyl-amino-diphenylamine), 4.2 g of 4-amino-diphenylamine, 5 ml methanol and 5 ml methyl iodide were heated in an autoclave at 160 °C for 7 hours. After cooling to room temperature, 8 ml of conc. ammonia were added and heating at 160 °C is continued for an additional 7 hours. After cooling to room temperature, the solid residue was washed with water, dissolved in a few ml of 6 N HCl, diluted to 400 ml and the amine was precipitated by conc. ammonia; yield 4.2 g (85%). For further purification, it was recrystallized twice from ethanol/water, m. p. 57 °C (Richter and Rothenberger [14] m. p. 57 °C). For reasons of better solubility, all compounds were converted into their hydrochlorides.

The compounds called "acridan" (compound X in Ref 16) and "dichloroacridan" were generously supplied by Dr. R. Hill and had been described by him previously [16].

Photosynthetic $NADP^+$ reduction and photophosphorylation was measured as previously described [7]. The reaction mixture was kept in Warburg vessels under argon in a final volume of 3 ml. It contained 30 mM Tris-HCl buffer (pH 8.0), 3 mM $MgCl_2$, 3 mM ADP, 3 mM P_i containing about 2×10^5 cpm ^{32}P, 2×10^{-5} M DCMU and chloroplasts corresponding to 200 μg chlorophyll. The electron donor compounds were usually added to 10^{-4} M together with 3 mM ascorbate as specified in the legends. Some of them, *e. g.* the indophenols and quinones, were added in the oxidized form, some in the reduced form, which in the presence of excess ascorbate does not make any difference. The samples were illuminated for 10 min at 15 °C with 35 000 lx of white light. NADPH formation was measured by its absorption at 340 nm from the difference before and after addition of N-methyl-phenazonium-methosulfate to the centrifuged sample after the illumination period.

Esterified $^{32}P_i$ was assayed according to [9].

The quench of 9-amino-acridine fluorescence by illuminated chloroplasts was measured as published [17] at an intensity of 2.5×10^5 erg/cm^2 per sec red light (RG 645 Schott, 2 mm) for illumination. The reaction mixture is given in the legend for Fig. 1. Corresponding phosphorylation and formation of NADPH were measured in the same cuvette after recording the fluorescence.

Gramicidin D was obtained from Serva.

Results

Photoreductions of $NADP^+$ by photosystem I at the expense of artificial donor systems are well known, some being coupled to ATP formation whereas others are not [18–20]. We have already reported that some such artificial donor systems for photosystem I in the presence of DCMU are insensitive to DBMIB [21]. Recent experience with cyclic photophosphorylation systems and the dependence of their DBMIB insensitivity on chemical properties [7] made it worthwhile to investigate non-cyclic donor systems in more detail.

Table I compiles the results for the photoreduction of $NADP^+$ in the presence of DCMU with some known and many new artificial electron donors, mainly from the indophenol- or phenylenediamine class. Rates of NADPH and ATP formation with the corresponding apparent P/e_2 ratios are given in the presence and absence of DBMIB. For comparison (and computation of overimposed cyclic flow) the last column contains the rates of ATP formation under conditions for cyclic electron flow. As seen from Table I only the photoreduction with reduced thymo-(hydro-)quinone, one of the few quinones being active in the absence of TMPD (s. ref. 7), was sensitive to DBMIB. This reaction thus seems to involve plastoquinone. All the other systems were insensitive to DBMIB, indicating that plastoquinone is not participating.

Table I, in its last column, shows that cyclic phosphorylation was always high if the P/e_2 ratio in the non-cyclic photoreduction was above 0.7. The extreme in this respect is held by dimethylindoaniline, if added in the same concentration as the other donors. Only at low concentration this compound exhibited low activity in cyclic electron flow.

The reactions with PD, indophenol and also DPIP showed a low P/e_2 ratio, the rate of cyclic

Table I. Photoreduction of $NADP^+$ by photosystem I with artificial mediators and their sensitivity to DBMIB. Conditions as described under Methods. DCMU at 2×10^{-5} M was always present. Rates are given in μmol formed per mg chlorophyll and hour. P/e_2 is the ratio of ATP over NADPH formed.

Donor [10^{-4} M]	Photoreduction			$+2 \times 10^{-6}$ M DBMIB			Cyclic photophosphorylation ATP
	NADPH	ATP	P/e_2	NADPH	ATP	P/e_2	
–	6	<5	–	–	–	–	–
PD	39	9	0.23	30	9	0.26	6
DAD	132	135	1.02	117	102	0.87	69
TMPD	84	<5	<0.1	90	21	0.23	<5
N,N-dimethyl-PD	96	<5	<0.1	96	9	0.1	<5
N,N,N′-trimethyl-PD	126	93	0.75	123	105	0.85	21
N-phenyl-PD	126	78	0.62	120	81	0.68	15
N-phenyl-N,N′-N′-trimethyl-PD	87	<5	<0.1	81	<5	<0.1	<5
N-phenyl-N,N′-N′-trimethyl-PD [10^{-6} M]	14	<5	–	13	<5	–	
phenolindophenol	48	18	0.37	39	18	0.46	24
DPIP	57	30	0.54	54	27	0.50	15
N,N-dimethyl-indoaniline	135	219	1.63	102	219	2.14	219
N,N-dimethyl-indoaniline [10^{-6} M]	78	45	0.58	78	45	0.58	26
"acridan"	57	57	1.0	54	63	1.16	78
"dichloro-acridan"	69	72	1.04	66	66	1.0	66
tetrachloroquinone	15	<5	–	12	<5	–	–
thymoquinone	18	9	0.5	0	<5	–	12
control without DCMU	123	108	0.88	3	<5	–	<5

photophosphorylation being also low. But these systems also had a low electron flow rate. N,N,N′-trimethyl-PD and N-phenyl-PD, however, have the advantage of being poor mediators of cyclic electron transport, but still giving high rates of photoreduction. The P/e_2 ratios of 0.6 – 0.7 found with these compounds therefore might come close to the true stoichiometry of non-cyclic electron flow. The "acridan" derivatives recently introduced by Hill [16] where efficient electron donors for photoreduction as well as for cyclic photophosphorylation (Table I).

The P/e_2 ratio dropped practically to zero if the donor compound is not able to liberate protons upon oxidation, as in the case of the completely N-methylated compounds. This was first observed comparing the two phenylenediamines TMPD and DAD [19] and could recently be extended to indamine and its N-methyl-derivatives [22]. In Table I the same is shown for N-phenyl-PD versus N-phenyl-N,N′,N′-trimethyl-PD, photoreduction with the former being coupled, but not with the latter. Comparison of N-methylated PDs reveals that N-dimethyl-PD behaved like TMPD as already known [19], but N,N,N′-trimethyl-PD gave a coupled reaction. The latter compound might have a greater tendency to loose a proton from its oxidized form as is expected. Because of the additional methyl group on the second nitrogen the delocalisation of the radical free electron includes this nitrogen to a higher extent in trimethyl-PD leading to a higher probability of proton release.

The coupling of the donor system with trimethyl-PD is of importance, because it possibly offers an explanation for the observation [23] that high concentrations of TMPD catalyze coupled electron flow: We tend to attribute this to a contamination by some trimethyl-PD. Another possibility, however, is that TMPD at higher concentrations induces electron flow via plastoquinone (s. below).

TMPD and N,N′-dimethyl-PD form stable cation radicals, Wurster's blue and Wursters's red, respectively. Both, themselves unable to catalyze coupled photoreduction, have the striking property of stimulating coupled electron flow through photosystem I

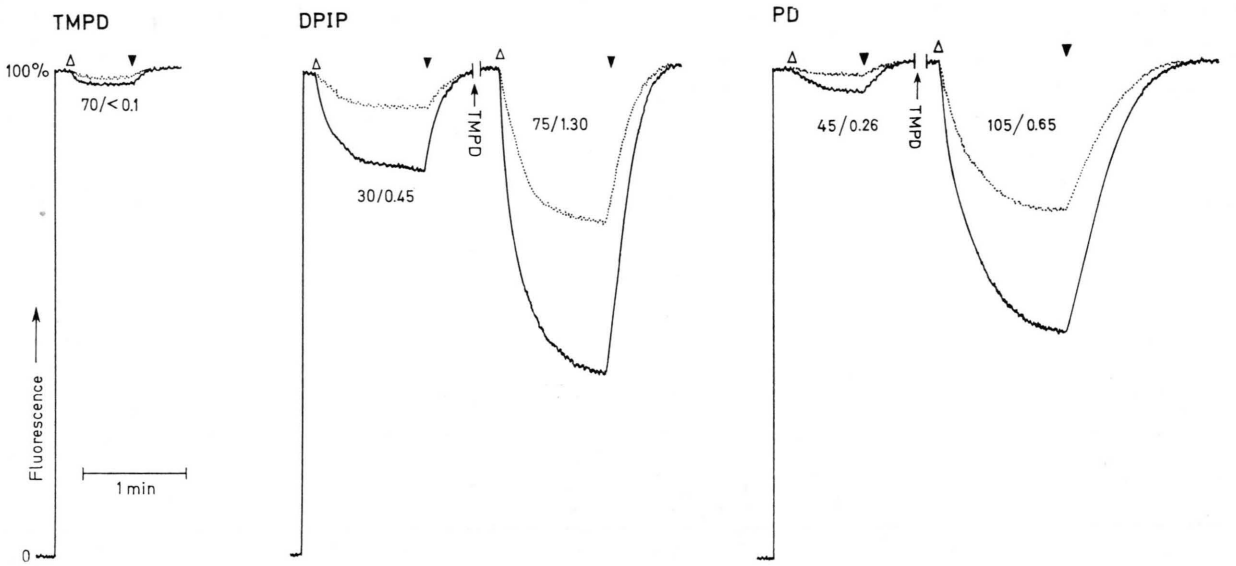

Fig. 1. Stimulation of the quench of 9-amino-acridine fluorescence by TMPD during photoreduction of $NADP^+$ by photosystem I with DPIP and PD as electron donors. — Fluorescence was measured as described previously [7]. The reaction mixture contained in a final volume of 3 ml, 50 mM NaCl, 50 mM tricine-NaOH, pH 8.0, 5 mM $MgCl_2$, 2×10^{-5} M DCMU, chloroplasts corresponding to 20 μg of chlorophyll, 3 mM ascorbate, 0.25 mM $NADP^+$, saturating amounts of ferredoxin and the donors at 10^{-4} M. The base line of fluorescence was recorded before addition of 9-amino-acridine to 5×10^{-6} M. The temperature was kept at 15 °C and the illuminating red light had an intensity of 2×10^5 ergs/cm^2 per sec. For simultaneous measurement of phosphorylation 3 mM ADP and 2 mM P_i containing about 10^6 cpm ^{32}P were included in the reaction mixture (dotted traces). The numbers represent the rate of NADPH formation, in μmol/mg chlorophyll and hour and the P/e_2 ratio. Open triangles stand for light on, closed triangles for light off.

under suboptimal conditions with other donors as already reported for cyclic systems [7]. This is also true for non-cyclic donor systems especially with hydroquinones. The observed increase of ATP formation in the systems with TMPD, or N,N-dimethyl-PD (Table I), in the presence of DBMIB, which is at least partially reduced to the hydroquinone by ascorbate, can be attributed to such an effect. Fig. 1 shows this stimulation of energy conservation by TMPD also in photoreductions with DPIP and PD. Light-dependent quench of 9-amino-acridine was measured which was shown to reflect the pH gradient across the thylakoid membrane [24]. As previously described coupled phosphorylation decreased the fluorescence change considerably reflecting the drain from the high energy state in the steady state of the reaction [25]. DPIP and PD in photoreduction with photosystem I yielded a small light-induced quench which was drastically stimulated by the addition of TMPD. In accordance with this is that the rate of photoreduction of $NADP^+$ and even more the P/e_2 ratio (Fig. 1) were also stimulated by TMPD. Similarly the same effect has been shown previously for the photoreduction of O_2 using methyl viologen as electron acceptor [7]. In the case of DPIP the addition of TMPD increased the P/e_2 ratio from 0.45 to 1.30. This high P/e_2 implies that TMPD induced coupled cyclic electron flow on top of the rate of non-cyclic photoreduction. The stimulation by TMPD was higher at lower concentrations of the donor. This was especially true for DAD where the effect of TMPD was pronounced at 10^{-5} M DAD but negligible at 5×10^{-4} DAD [7].

A photoreduction at the expense of TMPD alone yielded a small quench of 9-amino-acridine fluorescence (Fig. 1), which it did not under conditions for cyclic electron flow, although also in the latter case a small amount of H^+-uptake was observed in light [7]. We would like to suggest the possibility that TMPD can reduce to a small extent endogenous plastoquinone which then pumps hydrogens across the membrane.

Like TMPD other fully N-methylated donors were expected to exert this stimulatory effect on coupled electron transport in photosystem I. However, we found inhibition of energy conservation with N-pentamethyl-indamine and N-phenyl-N,N′,N′-trimethyl-PD (Table II). This could have two reasons.

Table II. Inhibition of photophosphorylation coupled to photoreduction with DAD by methylated donors. The conditions are described under Methods and further details are found in the legend to Table I. DAD and the other donors were added to 10^{-4} M.

Additions to DAD	NADPH	ATP	P/e_2
—	102	105	1.03
N-phenyl-N,N′,N′-trimethyl-PD	93	45	0.47
N-tetramethyl-indamine (symmetric)	66	36	0.55
N-pentamethyl-indamine	72	15	0.21

Either these compounds react so fast with photosystem I attenuating instead of stimulating the reaction of the other donor compound, or they can act as uncouplers. The former is suggested by the observed decrease also of the electron transport rate, which might reflect competitive cyclic electron flow. The latter is getting some support from the data in Fig. 2 with N-tetramethyl-indamine, which

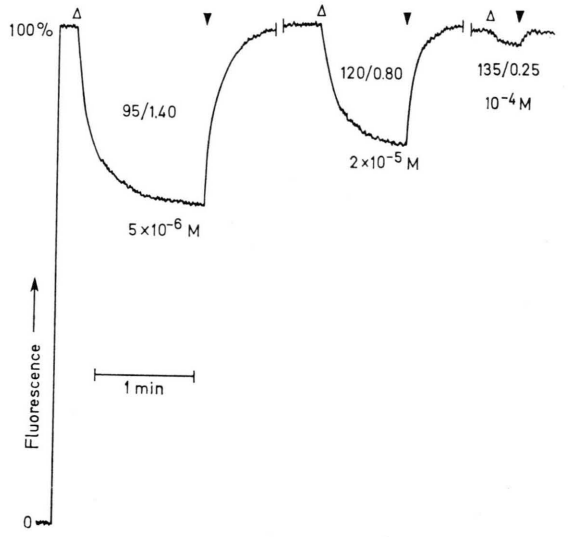

Fig. 2. Quench of 9-amino-acridine fluorescence during photoreduction by photosystem I with N-tetramethyl-indamine. — The conditions of the assay and the symbols are described in the legend for Fig. 1. Rates of photoreduction and the P/e_2 values are also given as in Fig. 1. N-tetramethyl-indamine was added to increasing concentrations as indicated below the trace.

has one —NH group [22]. At increasing concentrations it shows decreasing light-induced quench of 9-amino-acridine fluorescence with a fast decay in the dark. As shown previously [22] the P/e_2 ratio in non-cyclic flow also falls drastically with concentration. This looks like a self-uncoupling effect of the donor and should be expected whenever charged forms are membrane-permeable (s. Discussion). N-tetramethyl-indamine also inhibited coupled photophosphorylation with DAD (Table II).

In view of a possible self-uncoupling effect at the high concentration of 10^{-4} M N-pentamethyl-indamine or N-phenyl-N,N′,N′-trimethyl-PD it is necessary to emphasize that photoreduction with these donors was also not coupled at lower concentrations [22], where there is no uncoupling. Therefore the statement that complete N-methylation of electron donors prevents energy conservation can be maintained.

All electron donors used for photoreductions by photosystem I (Table I) are lipid soluble — DPIP-sulfonate is no electron donor for photosystem I [26]. This led to the conclusion that the oxidant of photosystem I is located beyond a permeability barrier inside the thylakoids [26]. During photoreduction electrons are shuttled by the donor compound from ascorbate outside to this oxidant inside. The old observation that uncouplers stimulate photoreduction with DPIP but not with DAD [20, 27, 28], although the latter shows the higher P/e_2 ratio, can be explained on the basis of such a shuttle (s. Discussion), as has been done for uncoupler effects in other systems of photosynthetic electron transport [29]. For support of the hypothesis the stimulation of electron flow by some, but not other donors was related to the chemical structure of the donor.

Table III. Stimulation of NADPH formation in photoreductions by photosystem I by the uncoupler gramicidin. The assay conditions and the reaction mixture are given under Methods. The various donors were added to a final concentration of 10^{-5} M only to diminish superimposed cyclic electron flow. Rates are given in μmol NADPH formed/mg chlorophyll per hour.

Donor [10^{-5} M]		+10^{-6} M Gramicidin	% Stimulation
DPIP	45	87	93
"acridan"	54	93	72
"dichloro-acridan"	45	99	122
phenolindophenol	33	48	45
p-dimethyl-aminophenol	27	36	33
tetrachloro-quinone	18	72	300
thymoquinone	18	36	100
PD	51	48	−6
DAD	90	90	○
N,N-dimethyl-indo-aniline [10^{-6} M]	33	33	○
TMPD	107	109	2
TMPD+DPIP	107	120	11

The data in Table III show that photoreductions with indophenols and most pronounced with tetrachloroquinone were stimulated by gramicidin. The reactions with phenylenediamines on the other hand were not. This observation suggests a correlation between the ability of the donors to form phenolate anions and the stimulation by addition of gramicidin. The photoreduction rate with DPIP when stimulated by TMPD was not further increased by gramicidin. Other uncouplers like NH_4Cl and carbonylcyanide-*m*-chlorophenyl-hydrazone gave the same results.

The reaction with thymoquinone which involves plastoquinone (s. Table I) might not fall into this correlation, because in this case a native energy conserving site is responsible for ATP formation.

If chloroplasts are fragmented by sonication or detergents the oxidant of photosystem I is rendered accessible from the outside, which is reflected by the oxidation of externally added plastocyanin (s [30]). Table IV demonstrates the dependence of photoreduction in drastically sonicated chloroplasts [10] on the addition of plastocyanin with various donors.

Table IV. Photoreductions by photosystem I in sonicated chloroplasts and its stimulation by plastocyanin. The conditions for the assay described under Methods. The numbers represent μmol NADPH formed/mg chlorophyll per hour.

Donor [10^{-4} M]	Sonicated chloroplasts		Untreated chloroplasts
		02 μM plastocyanin	
–	0	30	6
PD	12	39	48
DAD	21	48	132
TMPD	27	51	84
phenolindophenol	25	48	36
DPIP	75	117	57
"dichloro-acridan"	120	137	69

For comparison the rates in untreated chloroplasts are also given. The difference in reacting is obvious. Phenylenediamines seem to be much more dependent on addition of plastocyanin than indophenols of lower pK. Indophenols gave the highest rates in fragmented chloroplasts in the presence of plastocyanin while phenylene-diamines reacted optimally in untreated chloroplasts, as has been shown previously.

Discussion

Photosynthetic electron transport in the chloroplast membrane allows for two energy conserving loops according to Mitchell ([31, 32], s. [1, 2], for review). The two photocenters are responsible for the electrogenic transport of electrons from inside to outside the thylakoid membrane. Subsequently hydrogens are carried into the thylakoid by plastoquinone in the first, by water in the second energy conserving loop. Both are oxidized inside, liberating protons and reducing the oxidants of the photocenters. We therefore deal with two energy conserving parts of the whole electron transport chain rather than two phosphorylation sites localized at one specific redox reaction each. The use of the inhibitor DBMIB allowed to study each energy conserving loop separately [3, 4]. However, it became unlikely that in DBMIB insensitive photoreductions by photosystem I a native proton translocating step is still operative. This is because DBMIB inhibits the oxidation of plastoquinone inside the thylakoid eliminating the native energy conserving loop, located at plastoquinone [5, 6]. We therefore refrain to speak about phosphorylation site I [33, 34] if we deal with DBMIB insensitive electron transport through photosystem I, although the native electrogenic step in the reaction center is still operative. Energy conservation in photosystem I insensitive to this inhibitor demanded further explanation. Systematically investigating many artificial redox mediators for their ability to catalyze energy conservation in photosystem I in cyclic and non-cyclic photophosphorylation system ([7, 22, 26, 35] and this paper) we arrive at the conclusion that in DBMIB-insensitive energy conservation in photosystem I reactions the hydrogen-carrier plastoquinone is replaced by the reduced mediator (Fig. 3). This is based mainly on two facts. The first is that only reasonably lipid soluble mediators are active; sulfonated derivatives were found to be inactive [35]. The second is that compounds, which do not liberate protons upon oxidation, but form cationic radicals, mediate electron flow, but no energy conservation [7, 19, 22]. The data in Table I substantiate the second point with a series of N-methylated phenylenediamines. Some of them, *e. g.* N-phenyl-N-N′,N′-trimethyl-PD and N-pentamethyl-indamine [22], had to be used at low concentration. At higher concentration they possibly act as uncouplers or more likely they catalyse

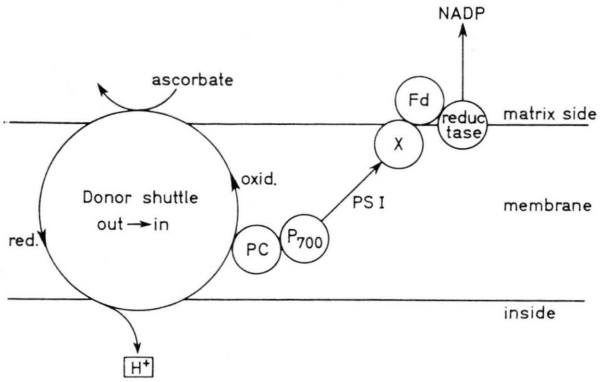

Fig. 3. Photosynthetic electron flow in photoreductions by photosystem I with an artificial electron donor shuttling electrons and protons across the thylakoid membrane. — PSI stands for photosystem I, PC for plastocyanin, Fd for ferredoxin and X for the primary reductant of photosystem I.

a competitively fast, not coupled cyclic electron flow (s. Table II).

According to the first argument above one should expect that lipid soluble hydroquinones would be good donors for photoreduction in photosystem I. This is not the case. Only a few show rather low activity, like reduced thymoquinone (Table I). Furthermore their reaction remains sensitive to DBMIB. Their reducing equivalents thus seem to intermingle with the pool of reduced plastoquinone. The same was observed in cyclic electron transport [7]. A feasible explanation is that hydroquinones are too insoluble in aqueous media for rapid reaction with the primary donor for photosystem I. This gap in the electron flow can be bridged by TMPD [7].

Tetrachloroquinone introduced by Izawa *et al.* [20] did not behave like other quinones in these respects; it behaved rather like the indophenols (see below).

TMPD also stimulates coupled photoreductions by photosystem I with indophenols and phenylenediamines, especially if they are used at suboptimal concentrations (Fig. 1; s. also Ref. 7), possibly by inducing a radical mechanism for the oxidation of these donors [7]. TMPD must facilitate the oxidation of the donor and not its reduction, since the stimulation is not inhibited by addition of a very active electron acceptor for photosystem I, like methylviologen [7]. TMPD increases the P/e_2 ratio in photoreductions of $NADP^+$ with the less potent electron acceptor ferredoxin (s. Table II). Presumably the increased concentration of the oxidized donor by TMPD in the steady state enhances cyclic electron flow.

The estimation of the true P/e_2 ratio of photoreductions by photosystem I is complicated by the possibility of superimposed cyclic electron flow [36]. Cyclic electron flow can be decreased by a more active electron acceptor if oxygen uptake instead of $NADP^+$ reduction is measured [37], but this in turn is obscured by induced radical reactions reflecting the action of superoxide radical anion [38]. Izawa *et al.* overcame the latter complication, but not the former, by the use of superoxide dismutase [39]. They found a ratio of 0.5 with DPIP as donor. Goffer and Neumann tried to overcome the complication using diaminobenzidine as donor, which is supposed to form an insoluble precipitate upon oxidation and therefore should be unable to cycle [40]. We tried to find a donor which is relatively inactive in cyclic photophosphorylation. This is the case with N-phenyl-PD and with N,N,N′-trimethyl-PD, these systems yield a P/e_2ratio of 0.6 to 0.7 (Table I) in non-cyclic photoreductions, which is therefore taken as the true stoichiometry. In agreement with Ort and Izawa [39] this value is notably lower than one. It agrees with the P/e_2 ratio in photoreductions by photosystem II, which is also in the order of 0.6 [3, 4]. This supports the notion that in non cyclic photophosphorylation from water to $NADP^+$ two energy conserving sites contributing each about 0.7 ATP to yield a total P/e_2 ratio of 1.33 [2].

A long observed, but puzzling and not satisfactorily explained result is the effect of uncouplers on donor systems for photosystem I. Keister was the first to show a stimulation of the DPIP donor system, which he took as evidence for energy coupling [27]. Later it was observed that some donor systems were stimulated by uncouplers, whereas others were not, without correlation between stimulation of electron flow by uncouplers and phosphorylating efficiency [20, 28]. The DAD system with a high P/e_2 ratio was not stimulated, but the DPIP system with a low P/e_2 ratio and low electron flow rate was. The recognition [26, 35] that in donor systems for photosystem I the mediator has to shuttle electrons across the membrane offers an explanation. From the results in Table IV it becomes obvious that the stimulating effect of uncouplers correlates with the chemistry of the mediator, in particular with the presence of a weakly acidic OH-group and Fig. 4 represents our view of why uncouplers stimulate photoreduction with donors, which may form phenolate anions, and do not with donors of

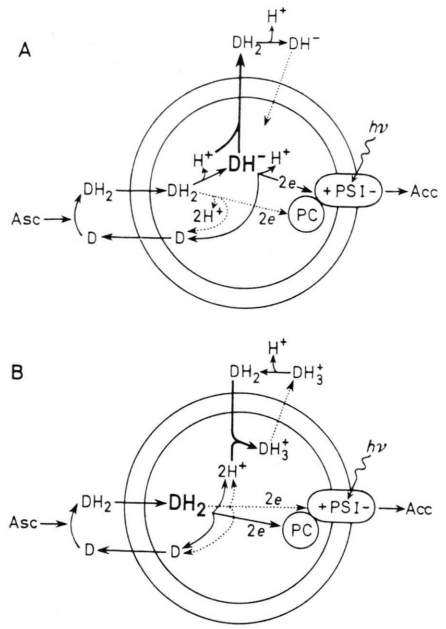

Fig. 4. Shuttles of acidic and basic electron donors for photosystem I across the thylakoid membrane. — Scheme A represents the reaction and distribution of a weakly acidic electron donor, like DPIP; scheme B shows the same for a weakly basic compound, like DAD. D stands for electron donor, Asc. for ascorbate, PS I for photosystem I and PC for plastocyanin. Active forms of the donors are printed in fat letters.

the phenylenediamine type. It is based first of all on the assumption that charged forms of the reduced donor — DH^- and DH_3^+ — are impermeable relative to the neutral form DH_2, (if charged forms are permeabel — dotted arrows through the membrane in Fig. 4 —, as in the case of some methylated donors, uncoupling by the donor itself does occur). Secondly it is assumed that phenolate anions donate electrons more rapidly to the positive charge in photosystem I than neutral forms; ammonium forms are thought to be inactive. Thirdly we assume that the neutral forms of phenylenediamines react more readily with plastocyanin than the neutral forms of the phenol type.

Equilibration of the donor through the membrane is brought about by the neutral form [41]; its concentration is the same on both sides of the membrane independent on pH. It forms charged species in the aqueous phases which is governed by the respective pH. A pH-gradient established by the oxidation of the donor will suppress formation of phenolate anions, but will increase formation of ammonium ions inside the vesicles. In other words it will force active phenolate forms out, especially if the pK of the donor falls between the pH values outside and inside. It will, on the other hand, cause accumulation of inactive ammonium forms of the donors. This is analog to the mechanism of uncoupling by NH_4Cl [42], but the concentration of the donor and the pK of aromatic amines are too low to cause uncoupling. It causes a buffering inside the thylakoids [43], which is also nicely observed by the lower rates of rise and decay of the pH-gradient, as measured by the quench of 9-amino-acridine fluorescence (compare the trace for PD with that for DPIP in Fig. 1; s. also ref. [17]). Uncouplers abolish the pH-gradient and therefore increase the concentration of phenolate forms of donors, but do not affect the distribution of neutral forms. Thus they increase the rate of photoreduction with the reactive phenolate anions, but have no effect with donors of the phenylenediamine type. In addition our results with sonicated chloroplasts and plastocyanin (Table III) suggest that phenolate forms react with the reaction center directly, while neutral forms preferentially with plastocyanin.

This mechanism for the stimulation of electron transport by uncouplers is more specified than the classical view of uncoupler action by release of electron transport control. It is another example of how a pH-gradient might control electron flow, this time not *via* the pH-optimum of rate limiting electron transport enzymes inside the thylakoid [44], but by controlling the distribution of active forms inside/outside the membrane of a mobile electron carrier. Also in photoreductions by photosystem II a surprising effect of uncouplers (*i. e.* inhibition of electron flow) is attributed to the side of the membrane involved and to the effect of the pH on the ratio reduced/oxidized acceptor inside the membrane [29].

The stimulation of electron flow in donor systems by uncouplers not due to the coupling system indicates that the shuttle of the mediator across the membrane is the limiting step. This is supported by the result that in sonicated particles with non-compartimented reaction sites significantly those donors have higher activities which are also stimulated by an uncoupler in the compartimented chloroplast system. This is particularly apparent with the "dichloroacridan" as donor. Because its reaction also seems to be plastocyanin independent, this compound may prove useful for studying further de-

tails of shuttle mechanism across the photosynthetic membrane.

The work has been supported by Bundesministerium für Forschung und Technologie.

[1] 1. A. Trebst, Ann. Rev. Plant Physiol. **25**, 423 [1974].
[2] A. Trebst and G. Hauska, Naturwissenschaften **61**, 308 [1974].
[3] A. Trebst and S. Reimer, Biochim. Biophys. Acta **305**, 129 [1973].
[4] S. Izawa, J. M. Gould, D. R. Ort, P. Felker, and N. E. Good, Biochim. Biophys. Acta **305**, 119 [1973].
[5] H. Böhme and W. A. Cramer, Biochemistry **11**, 1155 [1972].
[6] E. Reinwald, H. H. Stiehl, and B. Rumberg, Z. Naturforsch. **23 b**, 1626 [1968].
[7] G. Hauska, S. Reimer, and A. Trebst, Biochim. Biophys. Acta **357**, 1 [1974].
[8] G. Hauska, 3rd Int. Congr. on Photosynthesis Res., Rehovot, Israel, 1974.
[9] R. E. McCarty and E. Racker, J. Biol. Chem. **242**, 3435 [1967].
[10] E. F. Elstner, E. Pistorius, P. Böger, and A. Trebst, Planta **79**, 146 [1968].
[11] M. M. Anderson and R. E. McCarty, Biochim. Biophys. Acta **189**, 193 [1967].
[12] J. E. LuValle, D. B. Glass, and A. Weissberger, J. Amer. Chem. Soc. **70**, 2223–2233 [1948].
[13] G. Heller, Liebigs Ann. Chem. **392**, 16–48 [1912].
[14] L. F. Fieser and H. T. Thompson, J. Amer. Chem. Soc. **61**, 376–383 [1939].
[15] F. Richter and E. Rothenberger, Helv. Chim. Acta **5**, 166–181 [1922].
[16] R. Hill, J. Bioenergetics **4**, 229 [1973].
[17] G. Hauska and R. C. Prince, FEBS-Letters **41**, 35 [1974].
[18] J. S. C. Wessels, Biochim. Biophys. Acta **79**, 640 [1964].
[19] A. Trebst and E. Pistorius, Z. Naturforsch. **20 b**, 143 [1965].
[20] S. Izawa, T. N. Connolly, G. D. Winget, and N. E. Good, Brookhaven Symp. Biol. **19**, 169 [1966].
[21] H. Böhme, S. Reimer, and A. Trebst, Z. Naturforsch. **26 b**, 341 [1971].
[22] W. Oettmeier, S. Reimer, and A. Trebst, Plant Science Letters **2**, 267 [1974].
[23] M. Schwartz, Biochim. Biophys. Acta **112**, 204 [1966].
[24] S. Schuldiner, H. Rottenberg, and M. Avron, Eur. J. Biochem. **25**, 64 [1972].
[25] U. Pick, H. Rottenberg, and M. Avron, FEBS-Letters **32**, 91 [1973].
[26] G. A. Hauska, FEBS-Letters **28**, 217 [1972].
[27] D. L. Keister, J. Biol. Chem. **240**, 2673 [1965].
[28] A. Trebst and E. Pistorius, Biochim. Biophys. Acta **131**, 580 [1967].
[29] A. Trebst and S. Reimer, Biochim. Biophys. Acta **325**, 546 [1973].
[30] P. V. Sane and G. Hauska, Z. Naturforsch. **27 b**, 932 [1972].
[31] P. Mitchell, Biol. Rev. **41**, 445 [1966].
[32] W. Junge and H. T. Witt, Z. Naturforsch. **23 b**, 244 [1968].
[33] J. M. Gould and S. Izawa, Biochim. Biophys. Acta **314**, 211 [1973].
[34] J. M. Gould and D. R. Ort, Biochim. Biophys. Acta **325**, 157 [1973].
[35] G. Hauska, A. Trebst, and W. Draber, Biochim. Biophys. Acta **305**, 632 [1973].
[36] Z. Gromet-Elhanan, Progr. in Photosynthesis Res., Freudenstadt, **vol. III**, p. 1197, (H. Metzger, ed.), Tübingen 1969.
[37] G. Hauska, R. E. McCarty, and E. Racker, Biochim. Biophys. Acta **197**, 206 [1970].
[38] E. Elstner and R. Kramer, Biochim. Biophys. Acta **314**, 340 [1973].
[39] D. R. Ort and S. Izawa, Plant Physiol. **53**, 370 [1974].
[40] J. Goffer and J. Neumann, FEBS-Letters **36**, 61 [1973].
[41] L. Packer, D. W. Deamer, and A. R. Crofts, Brookh. Symp. Biol. **19**, 281 [1966].
[42] A. R. Crofts, J. Biol. Chem. **242**, 3352 [1967].
[43] M. Avron, Proc. 2nd Int. Congr. Photosynth. Res., Stresa, **vol. II**, p. 861 (G. Forti, M. Avron, and B. A. Melandri, eds.), Dr. W. Junk publisher, The Hague 1971.
[44] E. S. Bamberger, H. Rottenberg, and M. Avron, Eur. J. Biochem. **34**, 557 [1973].