Energy Conservation in Photoreductions by Photosystem I

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

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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 reaction3,4 in addition to the previously noted site connected with plastoquinone5,6. 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 site7. Therefore a concept of artificial energy conservation was developed in order to explain ATP formation in certain systems for cyclic photophosphorylation1,2,7. 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 Research8.

Methods

Spinach chloroplasts were prepared according to McCarty and Racker9. Sonicated chloroplasts were prepared essentially as described earlier10. Plastocyanin was prepared according to Anderson and McCarty11. N,N,N',N'-Trimethyl-PD (N,N,N'-trimethyl-p-

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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-dimethyleurea; DPIP, 2,6-dichloro-phenoldiophenol; PD, phenylenediamine; TMDP, N,N,N',N'-tetramethyl-p-

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phenylenediamine) was synthesized from N,N-di-
methyl-p-phenylenediamine as the starting mate-
rial. This compound was first converted into the
4-dimethylamino-p-tolylsulfonanilide, methylated by
means of methyl iodide and the tolylsulfonyl pro-
tecting group was finally split off by acid hydro-
lysis. Reduced phenolindophenol (4,4'-dihydroxy-
diphenylamine) was obtained by co-oxidation of
p-aminophenol and phenol in alkaline solution by
sodium hypochlorite and reduction of the dye by
sodium dithionite. Reduced N,N-dimethyl-indo-
aniline (4-hydroxy-4'-dimethylamino-diphenylami-
e) was prepared in a similar way by co-oxidation
of N,N-dimethyl-p-phenylenediamine and phenol. The
dye ("Phenol Blue") was again reduced by sodium
dithionite. 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
HCl, diluted to 400 ml and the amine was precipi-
tated by conc. ammonia; yield 4.2 g (85%). For
further purification, it was recrystallized twice from
ethanol/water, m. p. 57 °C (Richter and Rothen-
berger, m. p. 57 °C). For reasons of better solubi-
lity, all compounds were converted into their hydro-
chlorides.

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.

Photosynthetic NADP+ reduction and photophos-
phorylation was measured as previously described.
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 MgCl₂,
3 mm ADP, 3 mm Pᵢ containing about 2 × 10⁵ cpm
³²P, 2 × 10⁻³ M DCMU and chloroplasts corre-
sponding 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 illumi-
nated for 10 min at 15 °C with 35 000 lx of white
light. NADPH formation was measured by its ab-
sorption at 340 nm from the difference before and
after addition of N-methyl-phenazonium-methosul-
fate to the centrifuged sample after the illumination
period.

Esterified ³²Pᵢ was assayed according to 9.

The quench of 9-amino-acridine fluorescence by
illuminated chloroplasts was measured as pub-
lished at an intensity of 2.5 × 10⁵ erg/cm² per
sec red light (RG 645 Schott, 2 mm) for illumina-
tion. The reaction mixture is given in the legend for
Fig. 1. Corresponding phosphorylation and forma-
tion 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. We have already re-
ported that some such artificial donor systems for
photosystem I in the presence of DCMU are insensi-
tive to DBMIB. Recent experience with cyclic
photophosphorylation systems and the dependence
of their DBMIB insensitivity on chemical proper-
ties made it worthwhile to investigate non-cyclic
donor systems in more detail.

Table I compiles the results for the photoreduc-
tion of NADP+ in the presence of DCMU with
some known and many new artificial electron
donors, mainly from the indophenol- or phenylen-
ediamine class. Rates of NADPH and ATP form a-
tion with the corresponding apparent Pᵢ/e₂ 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
(see 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 Pᵢ/e₂ ratio
in the non-cyclic photoreduction was above 0.7.
The extreme in this respect is held by dimethylindo-
aniline, if added in the same concentration as the
other donors. Only at low concentration this com-
ponent exhibited low activity in cyclic electron flow.
The reactions with PD, indophenol and also
DPIP showed a low Pᵢ/e₂ 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 $2 \times 10^{-5} \text{ M}$ was always present. Rates are given in μmol formed per mg chlorophyll and hour. P/e₂ is the ratio of ATP over NADPH formed.

<table>
<thead>
<tr>
<th>Donor</th>
<th>Photoreduction</th>
<th>+2 × 10⁻⁶ M DBMIB</th>
<th>Cyclic photophosphorylation ATP</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>NADPH ATP P/e₂</td>
<td>NADPH ATP P/e₂</td>
<td>ATP</td>
</tr>
<tr>
<td>---</td>
<td>---</td>
<td>---</td>
<td>---</td>
</tr>
<tr>
<td>---</td>
<td>---</td>
<td>---</td>
<td>---</td>
</tr>
<tr>
<td>PD</td>
<td>39 9 0.23</td>
<td>30 9 0.26</td>
<td>6</td>
</tr>
<tr>
<td>DAD</td>
<td>132 135 1.02</td>
<td>117 102 0.87</td>
<td>69</td>
</tr>
<tr>
<td>TMPD</td>
<td>84 &lt;5 &lt;0.1</td>
<td>90 21 0.23</td>
<td>&lt;5</td>
</tr>
<tr>
<td>N,N-dimethyl-PD</td>
<td>96 &lt;5 &lt;0.1</td>
<td>96 9 0.1</td>
<td>&lt;5</td>
</tr>
<tr>
<td>N,N,N'-trimethyl-PD</td>
<td>126 93 0.75</td>
<td>123 105 0.85</td>
<td>21</td>
</tr>
<tr>
<td>N-phenyl-PD</td>
<td>126 78 0.62</td>
<td>120 81 0.68</td>
<td>15</td>
</tr>
<tr>
<td>N-phenyl-N,N'-trimethyl-PD</td>
<td>87 &lt;5 &lt;0.1</td>
<td>81 &lt;5 &lt;0.1</td>
<td>&lt;5</td>
</tr>
<tr>
<td>N,N,N'-trimethyl-PD [10⁻⁶ M]</td>
<td>14 &lt;5</td>
<td>13 &lt;5</td>
<td>---</td>
</tr>
<tr>
<td>phenolindophenol</td>
<td>48 18 0.37</td>
<td>39 18 0.46</td>
<td>24</td>
</tr>
<tr>
<td>DPIN</td>
<td>57 30 0.54</td>
<td>54 27 0.50</td>
<td>15</td>
</tr>
<tr>
<td>N,N-dimethyl-indoaniline</td>
<td>135 219 1.63</td>
<td>102 219 2.14</td>
<td>219</td>
</tr>
<tr>
<td>N,N-dimethyl-indoaniline [10⁻⁶ M]</td>
<td>78 45 0.58</td>
<td>78 45 0.58</td>
<td>26</td>
</tr>
<tr>
<td>&quot;acridan&quot;</td>
<td>57 57 1.0</td>
<td>54 63 1.16</td>
<td>78</td>
</tr>
<tr>
<td>&quot;dichloro-acridan&quot;</td>
<td>69 72 1.04</td>
<td>66 66 1.0</td>
<td>66</td>
</tr>
<tr>
<td>tetrachloroquinone</td>
<td>15 &lt;5</td>
<td>12 &lt;5</td>
<td>---</td>
</tr>
<tr>
<td>thymoquinone</td>
<td>18 9 0.5</td>
<td>0 &lt;5</td>
<td>12</td>
</tr>
<tr>
<td>control without DCMU</td>
<td>123 108 0.88</td>
<td>3 &lt;5</td>
<td>&lt;5</td>
</tr>
</tbody>
</table>

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/e₂ 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/e₂ 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 Wurster'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 \(\times\) \(10^{-5}\) m DCMU, chloroplasts corresponding to 20 \(\mu\)g 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 \(5 \times 10^{-8}\) m. The temperature was kept at 15 °C and the illuminating red light had an intensity of \(2 \times 10^5\) ergs/cm\(^2\) per sec. For simultaneous measurement of phosphorylation 3 mm ADP and 2 mm P\(_1\) containing about \(10^6\) cpm \(^32\)P were included in the reaction mixture (dotted traces). The numbers represent the rate of NADPH formation, in \(\mu\)mol/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-di-methyl-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 \(5 \times 10^{-4}\) 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.

<table>
<thead>
<tr>
<th>Additions to DAD</th>
<th>NADPH</th>
<th>ATP</th>
<th>$P/e_2$</th>
</tr>
</thead>
<tbody>
<tr>
<td>N-phenyl-N,N',N'-trimethyl-PD</td>
<td>93</td>
<td>45</td>
<td>0.47</td>
</tr>
<tr>
<td>(symmetric) N-tetramethyl-indamine</td>
<td>66</td>
<td>36</td>
<td>0.55</td>
</tr>
<tr>
<td>N-pentamethyl-indamine</td>
<td>72</td>
<td>15</td>
<td>0.21</td>
</tr>
</tbody>
</table>

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 has one — NH group. At increasing concentrations it shows decreasing light-induced quench of 9-amino-acridine fluorescence with a fast decay in the dark. As shown previously 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, 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. This led to the conclusion that the oxidant of photosystem I is located beyond a permeability barrier inside the thylakoids. 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, 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. 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 $\mu$mol NADPH formed/mg chlorophyll per hour.

<table>
<thead>
<tr>
<th>Donor $[10^{-5}$ M]</th>
<th>+10$^{-6}$ M</th>
</tr>
</thead>
<tbody>
<tr>
<td>DPIP</td>
<td>45</td>
</tr>
<tr>
<td>“acridan”</td>
<td>54</td>
</tr>
<tr>
<td>“dichloro-acridan”</td>
<td>45</td>
</tr>
<tr>
<td>phenolindophenol</td>
<td>33</td>
</tr>
<tr>
<td>$p$-dimethyl-aminophenol</td>
<td>27</td>
</tr>
<tr>
<td>tetrachloro-quinone</td>
<td>18</td>
</tr>
<tr>
<td>thymoquinone</td>
<td>18</td>
</tr>
<tr>
<td>PD</td>
<td>51</td>
</tr>
<tr>
<td>DAD</td>
<td>90</td>
</tr>
<tr>
<td>N,N-dimethyl-indoaniline $[10^{-6}$ M]</td>
<td>33</td>
</tr>
<tr>
<td>TMPD</td>
<td>107</td>
</tr>
<tr>
<td>TMPD + DPIP</td>
<td>107</td>
</tr>
</tbody>
</table>

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.
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$_4$Cl 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 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.

<table>
<thead>
<tr>
<th>Donor [10$^{-4}$ M]</th>
<th>Sonicated chloroplasts 02 µM plastocyanin</th>
<th>Untreated chloroplasts</th>
</tr>
</thead>
<tbody>
<tr>
<td>PD</td>
<td>12</td>
<td>39</td>
</tr>
<tr>
<td>DAD</td>
<td>21</td>
<td>48</td>
</tr>
<tr>
<td>TMPD</td>
<td>27</td>
<td>51</td>
</tr>
<tr>
<td>phenolindophenol</td>
<td>25</td>
<td>48</td>
</tr>
<tr>
<td>DPIP</td>
<td>75</td>
<td>117</td>
</tr>
<tr>
<td>“dichloro-acridan”</td>
<td>120</td>
<td>137</td>
</tr>
</tbody>
</table>

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$^{31, 32}$ and Mitchell$^{1, 2}$, 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
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 \(\text{et al.}^20\) 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 \text{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\(^\prime\)-trimethyl-PD, these systems yield a P\(e_2\) ratio 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 — \( \text{DH}^- \) and \( \text{DH}_3^+ \) — are impermeable relative to the neutral form \( \text{DH}_2 \), (if charged forms are permeable — 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 \( \text{DH}_2 \); 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 analogous to the mechanism of uncoupling by \( \text{NH}_4\text{Cl} \), 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, 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; see 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, 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-compartmented reaction sites significantly those donors have higher activities which are also stimulated by an uncoupler in the compartmented chloroplast system. This is particularly apparent with the “di-chloroacridan” 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.

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