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PROPERTIES OF PHOTOREDUCTIONS BY PHOTOSYSTEM II IN ISOLATED CHLOROPLASTS

III. THE EFFECT OF UNCOUPLERS ON PHENYLENEDIAMINE SHUTTLES ACROSS THE MEMBRANE IN THE PRESENCE OF DIBROMOTHYMOQUINONE

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SUMMARY

In the presence of the plastoquinone antagonist dibromothymoquinone the photoreduction of ferricyanide by isolated chloroplast membranes is attributed to Photosystem II. The reaction is stimulated by the addition of phenylenediamine or C-substituted phenylenediamines (which may form a diimine on oxidation) but not of N-substituted phenylenediamines (which form a stable radical on oxidation). Phenylenediamines also restore NADP reduction (and O_2 evolution) in 2,5-dibromo-3-methyl-6-isopropyl-*p*-benzoquinone (DBMIB)-treated chloroplasts. In this bypassing of the inhibition site, N-substituted phenylenediamines are very effective, whereas *p*-phenylenediamine and C-substituted phenylenediamines are inefficient. Uncouplers exhibit a surprising effect on these systems. Even under coupling conditions uncouplers inhibit electron flow to ferricyanide mediated by phenylenediamine in the pH range 7.3–8.0, whereas the restoration of the NADP system is stimulated.

For the interpretation of the results the side of the membrane involved is considered. It is proposed that in ferricyanide reduction by Photosystem II, a phenylenediimine/diamine shuttle operates which moves reducing equivalents from the inside to the outside across the membrane. This shuttle requires a pH gradient across the membrane because of different optimal ratios of diimine/diamine inside and outside. This pH difference is abolished by the uncoupler, accounting for the observed inhibition.

The restoration of electron flow from water to NADP in DBMIB-treated chloroplasts is assumed to be a bypass of the inhibition site inside the membrane *via* a phenylenediamine. Because the imine/amine ratio brought about by the pH gradient is not favorable for the inside oxidation an uncoupler stimulates NADP reduction even under coupling conditions.

Abbreviations: DBMIB, 2,5-dibromo-3-methyl-6-isopropyl-*p*-benzoquinone (dibromothymoquinone), DCIP, 2,6-dichlorophenolindophenol, MMPD, 2-methyl-5-methoxy-*p*-phenylenediamine, TMPD, *N,N,N′,N′*-tetramethyl-*p*-phenylenediamine

Also in photoreductions by Photosystem I, for example NADP reduction at the expense of *p*-phenylenediamine/ascorbate, a shuttle of reducing equivalents across the membrane occurs but this time from outside to inside.

INTRODUCTION

Dibromothymoquinone (DBMIB) is an antagonist of plastoquinone[1-3] It inhibits electron flow from Photosystem II to Photosystem I on the reducing side of plastoquinone[1-6]. In the presence of DBMIB Hill reactions, *i.e.* O_2 evolution and the reduction of an electron acceptor, are attributed to a photoreduction by Photosystem II[2,3,7]. Recently the properties of photoreductions by Photosystem II have been investigated in more detail by ourselves[7,11] and by Izawa and his colleagues[8-10]. It was concluded that photoreductions by Photosystem II include an energy conserving step with a P/e_2 ratio up to 0.4. We pointed out in addition that the side of the membrane involved in such photoreductions is of importance, lipophilic acceptors being reduced inside, polar acceptors outside[7,11]. We proposed that in the stimulation of ferricyanide reduction by phenylenediamines, as observed first by Saha *et al*[15], a phenylenediamine shuttle operates, which takes reducing equivalents from the inside to the outside[11]. This paper describes the effect of uncouplers on these systems which give further support for this view. The results indicate the importance of a pH gradient in facilitating the shuttle of reducing equivalents across the membrane in the reduction of ferricyanide by Photosystem II, *i e.* in the presence of DBMIB. Under these conditions and in a certain pH range uncouplers inhibit ferricyanide photoreduction by inhibiting this shuttle

METHODS

Spinach chloroplasts have been prepared according to Nelson *et al*[12] The particles were washed once in 5 mM Tricine buffer, pH 8.5. Photosynthetic activity was assayed with 0.2 mg chlorophyll at 15 °C under N_2 and illumination for 10 min with 30 000 lux. The medium contained in μmoles in a volume of 3 ml: $MgCl_2$, 10; ADP, 10, inorganic [^{32}P]phosphate, 10; and ferricyanide, 20 or NADP, 6 and ferredoxin, 0.01; respectively. At the different pH ranges 80 μmoles of the following buffers were used HEPES–NaOH, pH 7.0–8.6; MES–NaOH, pH 5.5–6.5, citric acid–NaOH, pH 4.0–5.5.

O_2 evolution was followed manometrically, NADPH was measured at 340 nm (and calculated from the difference in extinction before and after addition of PMS) and ATP was measured by the incorporation of ^{32}P according to the modification of Conover *et al.*[13].

DBMIB has been kindly synthetised by Dr W. Draber, Bayer Forschungszentrum, Wuppertal, according to ref. 1. The phenylenediamines, not commercially available, were a gift of Bayer-Leverkusen and recrystallized from 6 M HCl. Gramicidin D was purchased from Serva, Heidelberg. 2-*n*-Undecylbenzimidazol was synthetised according to ref. 14.

RESULTS

The rate of ferricyanide reduction by Photosystem II (*i.e.* in the presence of DBMIB) is low in intact membrane systems of chloroplasts[1-3]. It is stimulated by the addition of a benzoquinone[7] or of a phenylenediamine[10,11]. Saha *et al.*[15] have first described *p*-phenylenediamine as a superior acceptor for Photosystem II As reported recently phenylenediamine and C-substituted *p*-phenylenediamines are particularly active in stimulating the photoreduction of ferricyanide by Photosystem II in the presence of DBMIB, whereas N-substituted phenylenediamines are inactive[11]. This is indicated again in the results of Table I.

TABLE I

EFFECT OF GRAMICIDIN ON THE PHENYLENEDIAMINE-STIMULATED PHOTOREDUCTION OF FERRICYANIDE BY PHOTOSYSTEM II (*i e* IN THE PRESENCE OF 10^{-6} M DBMIB)

Conditions in the presence of ADP/P_i.

Additions (10^{-4} *M*)	*μequiv* O_2 *evolved/10 min light*			
	pH 6.0		*pH 8.0*	
	minus gramicidin	*plus gramicidin (5 μg/ml)*	*minus gramicidin*	*plus gramicidin (5 μg/ml)*
None	—	1.1	1.4	1.4
p-Phenylenediamine	1.6	4.4	6.1	2.4
2,6-Diaminotoluene	2.1	5.5	5.8	2.4
2,6-Diethyl-*p*-phenylenediamine	2.6	3.0	5.4	2.4
TMPD	1.4	2.6	1.4	1.4
N,N-Dimethyl-*p*-phenylenediamine	1.7	3.5	1.5	1.5

The stimulating effect of certain phenylenediamines on ferricyanide reduction by Photosystem II (*i e* in the presence of DBMIB) is dependent on the pH and, depending on the pH range used, on the absence or presence of an uncoupler. The effect of the uncoupler gramicidin on the system at pH 6.0 and pH 8.0 is shown in Table I At pH 6.0 gramicidin stimulates electron flow with all phenylenediamines. At pH 8.0, however, N-substituted phenylenediamines are practically inactive, whether gramicidin is added or not. More important the stimulating effect of *p*-phenylenediamine and C-substituted phenylenediamines is inhibited by gramicidin. It is important to note that the effect of gramicidin in Table I as well as in Fig. 1 is observed in the presence of ADP/P_i. Fig. 1 summarizes in more details the effect of pH and gramicidin on this ferricyanide photoreduction by Photosystem II, comparing *p*-phenylenediamine and 2,6-diethyl-*p*-phenylenediamine. At a high pH range gramicidin inhibits the rate of the reaction, while at a low pH range it is stimulating with both phenylenediamines. The pH at which these two effects of gramicidin are mutually cancelling depends, however, on the substitution of the phenylenediamine. This point, which shall be called here crossover point, is at pH 7.3 in the case of *p*-phenylenediamine and at pH 6.4 with diethyl-*p*-phenylenediamine. The pH of the maximum rate in the absence of gramicidin above this crossover point (called here

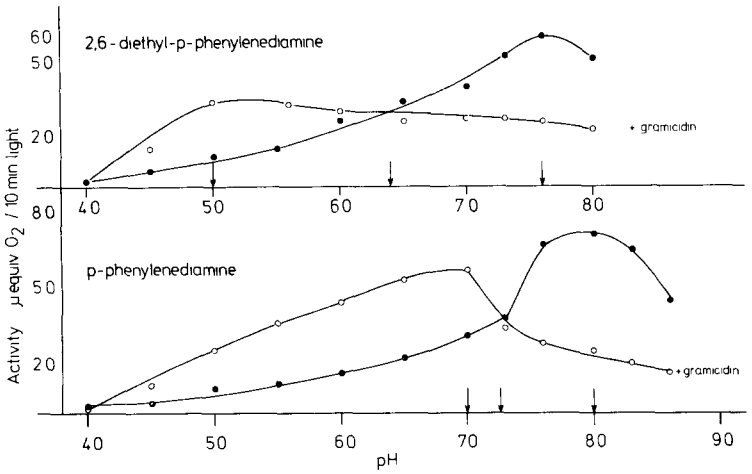

Fig 1 Influence of pH and gramicidin (5 μg/ml) on a phenylenediamine (10^{-4} M)-stimulated photoreduction of ferricyanide by Photosystem II (*i.e.* in the presence of 10^{-6} M DBMIB)

maximum rate of the shuttle of phenylenediamine across the membrane) is 8.0 and 7.6, respectively, while the maximum rate in the presence of gramicidin below this point (*i.e.* maximum rate of the electron transport chain) is 7.0 for *p*-phenylenediamine, but 5.0 for 2,6-diethyl-*p*-phenylenediamine. In Table II the values of these three points are given for several substituted phenylenediamines calculated from Fig. 1 or from corresponding curves for the other phenylenediamines. The general behaviour is the same for all phenylenediamines listed but there is a shift towards a lower pH range with increasing substitution with alkyl groups on the ring (and the lower the p*K* as discussed later). From Table I it seems that the rate of ferricyanide reduction in the presence of gramicidin at pH 8.0 is the same independent of the (C-substituted) phenylenediamine used. This indicates that the shuttle operating maximally only in the absence of gramicidin is dependent on the structure of the phenylenediamine. The *N,N,N',N'*-tetramethyl-*p*-phenylenediamine (TMPD) sys-

TABLE II

DEPENDENCE ON pH OF THE EFFECT OF GRAMICIDIN (5 μg/ml) ON THE STIMULATION BY PHENYLENEDIAMINES OF THE PHOTOREDUCTION OF FERRICYANIDE BY PHOTOSYSTEM II (*i.e.* IN THE PRESENCE OF 10^{-6} M DBMIB)

Additions (10^{-4} *M*)	*pH*		
	Maximum of electron transport (presence of uncoupler)	*Crossover point*	*Maximum of p-phenylenediamine shuttle (absence of uncoupler)*
p-Phenylenediamine	7.0	7.3	8.0
2,4-Diaminotoluene	6.0	7.0	8.0
2,6-Diethyl-*p*-phenylenediamine	5.0	6.4	7.6
Diaminodurene	5.0	6.2	7.6

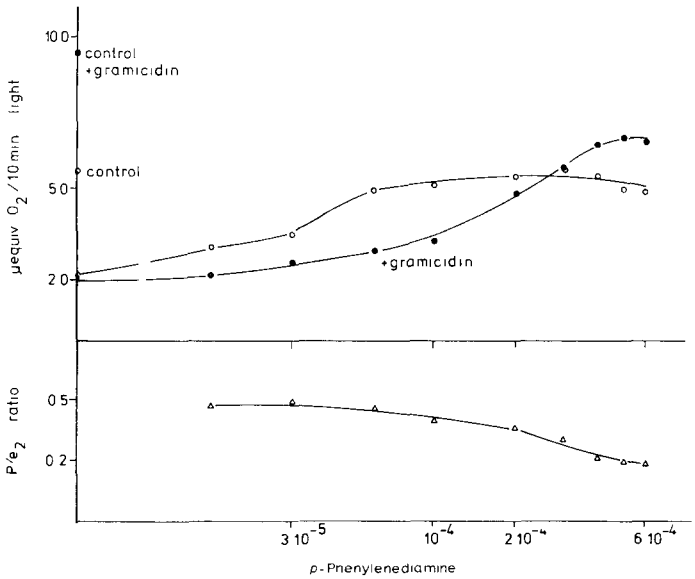

Fig. 2. Influence of the concentration of *p*-phenylenediamine on the photoreduction of ferricyanide by Photosystem II (*i.e.* in the presence of 10^{-6} M DBMIB) in the absence or presence of 5 µg/ml gramicidin.

tem which is practically inactive in stimulating ferricyanide photoreduction by Photosystem II at pH 8.0 is not effected by gramicidin at this pH. However, at low pH also the TMPD system as well as the other phenylenediamines is stimulated by an uncoupler (Table I).

The inhibition by gramicidin of the ferricyanide system stimulated by a C-substituted phenylenediamine at pH 8.0 depends on the concentration of the phenylenediamine. As Fig. 2 shows for *p*-phenylenediamine the stimulation of ferricyanide photoreduction at pH 8 0 is already saturated at a concentration of about 10^{-4} M *p*-phenylenediamine. At this concentration gramicidin inhibits. The inhibition is overcome by adding more *p*-phenylenediamine. The P/e_2 ratios are lowered at high *p*-phenylenediamine concentrations.

Those phenylenediamines which are inactive in stimulating ferricyanide reduction by Photosystem II (*i.e.* in the presence of DBMIB) are active in restoring NADP reduction and *vice versa*. *p*-Phenylenediamine which is very active in stimulating the ferricyanide system rather weakly restores NADP reduction. On the other hand TMPD is very active in restoring NADPH formation in the presence of DBMIB[11]. This effect of different phenylenediamines on the bypassing of the inhibition site of DBMIB and thus restoring NADPH photoreduction (and stoichiometric O_2 evolution[11]) is summarized in Table III. Furthermore the effect of ADP/P_i and gramicidin is indicated in Table III The stimulatory effect of ADP/P_i, apparent in the two N-substituted phenylenediamine systems with high electron flow rates is indicative of the coupling of these systems to ATP formation. In the other systems no stimulation can be expected, because it is quite obvious that the *p*-phenylenediamine bypass is limiting As presented elsewhere[11] the P/e_2 ratios in these restored NADPH systems

TABLE III

EFFECT OF GRAMICIDIN ON THE RESTORATION OF NADP REDUCTION BY PHENYLENEDIAMINES IN DBMIB-TREATED CHLOROPLASTS (pH 8 0)

Additions to 10^{-6} M DBMIB	*μmoles NADPH formed/10 min light*		
	Absence of ADP/P_i (no gramicidin)	*Presence of ADP/P_i*	
		minus gramicidin	*plus gramicidin (5 μg/ml)*
Control without DBMIB	2 3	4 3	5 0
—	—	0 2	0 2
TMPD	3 8	4 3	4 4
N,N-Dimethyl-*p*-phenylenediamine	3 2	4 2	4 4
p-Phenylenediamine	0 9	1 1	2 2
2,4-Diaminotoluene	1 0	1.1	1 7
MMPD	1 9	1.6	1 0
2,6-Diethyl-*p*-phenylenediamine	1.1	1.2	1 1
Diaminodurene	1 0	0 6	0 4

are about 1. For the purpose of this paper the ADP/P_i column is only to illustrate that the large gramicidin effects even in the presence of ADP/P_i are not reflecting control *via* the coupling system. Whereas there is no or little effect of gramicidin in the TMPD system (which has already very high rates) the *p*-phenylenediamine and 2,4-diaminotoluene systems are stimulated. The pH dependence of this gramicidin stimulation in the *p*-phenylenediamine system is shown in Fig. 3. From this it is evident that under the condition in which gramicidin inhibits the phenylenediamine stimulation of ferricyanide photoreduction, it stimulates NADP reduction (compare Figs 1 and 3) As pointed out already[11] the chemical constitution of a phenylenediamine required for stimulating NADP reduction is just the opposite of the one for ferricyanide reduction and *vice versa*. This becomes very clear again from the experiments

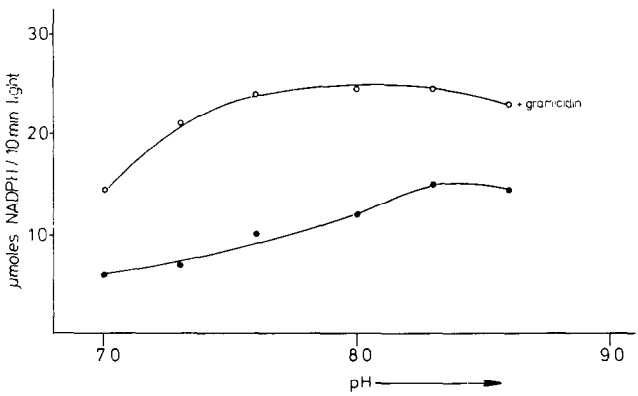

Fig. 3 Influence of pH and gramicidin (5 μg/ml) on the restoration of NADP reduction by 10^{-4} M *p*-phenylenediamine (in the presence of 10^{-6} M DBMIB)

TABLE IV

EFFECT OF UNCOUPLERS ON THE RESTORATION OF NADPH FORMATION OR STIMULATION OF FERRICYANIDE REDUCTION IN DBMIB-TREATED CHLOROPLASTS

Additions to 10^{-6} M DBMIB	*µequiv O_2 evolved/10 min light*	
	NADP system	*Ferricyanide system*
Control without DBMIB	4 5	4 6
—	0 1	1 4
10^{-4} M *p*-phenylenediamine	1 2	5 6
10^{-4} M *p*-phenylenediamine + gramicidin (5 µg/ml)	2 3	2 3
10^{-4} M *p*-phenylenediamine + 2 10^{-5} M undecylbenzimidazole	2 0	2 1
10^{-4} M *p*-phenylenediamine + 2 10^{-3} M NH_4Cl	2 2	2 3

discussed in this paper. Substituted phenylenediamines with more than one alkyl substituent (like MMPD, diethyl-*p*-phenylenediamine and diaminodurene) are slightly inhibited by gramicidin in the restoration of NADP photoreduction (Table III).

Other uncouplers show the same effect as gramicidin. Table IV indicates that the uncouplers NH_4Cl and undecylbenzimidazol[14] have the same influence as gramicidin, *i.e.* they inhibit the stimulation by *p*-phenylenediamine of the photoreduction of ferricyanide and stimulate the restoration of NADPH formation (all in the presence of DBMIB and of ADP and phosphate).

The interpretation of the results, as discussed later, is that in ferricyanide reduction a phenylenediamine shuttle across the membrane occurs, which carries electrons as well as hydrogens from the inside to the outside According to the chemiosmotic hypothesis this will lower the coupled ATP formation and the P/e_2 ratio as is actually observed in such photoreductions by Photosystem II[11]. We assume that the same phenylenediamine shuttle operates and hydrogens are removed from the inside also in the ferricyanide Hill reaction + *p*-phenylenediamine in the absence of DBMIB, *i.e.* under the original conditions of Saha *et al*[15] As Saha *et al.*[15] found by adding *p*-phenylenediamine to a ferricyanide Hill reaction by broken chloroplasts, the rate of electron flow is stimulated with a P/e_2 ratio of about 0 5. This was explained by Saha *et al.*[15] as a photoreduction by Photosystem II with the participation of just one ATP forming site. We quite agree with them[7,11] that photoreductions by Photosystem II are coupled to just one energy conserving step. But we would like to point out that it is quite possible that plastoquinone and the energy conserving step coupled to it, still participate in the phenylenediamine reduction by Photosystem II. But the *p*-phenylenediamine shuttle through the membrane will transport some of the hydrogens back outside. It is this shuttle which lowers the P/e_2 ratio In Fig. 4 the effect of *p*-phenylenediamine and gramicidin on a ferricyanide Hill reaction is described in the absence of DBMIB (these are the conditions of Saha *et al*[15]) It is apparent that indeed *p*-phenylenediamine acts as a kind of uncoupler. The rate of electron flow to ferricyanide is stimulated by either gramicidin or *p*-phenylenediamine. By adding *p*-phenylenediamine to a ferricyanide reduction system only the electron flow rate is increased but not the rate of ATP formation, *i.e.* the stimulated electron flow rate is not coupled. We would like to term this uncoupling

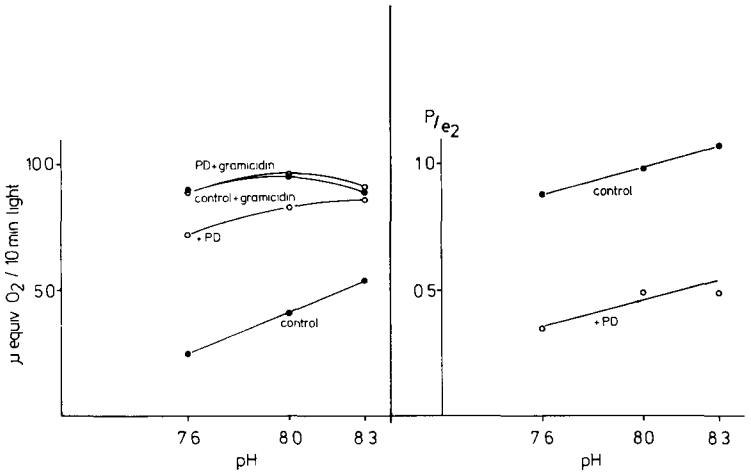

Fig 4. Stimulation of ferricyanide photoreduction by gramicidin (5 μg/ml) and/or 10^{-4} M *p*-phenylenediamine (PD) (in the absence of DBMIB).

effect of *p*-phenylenediamine a stoichiometric uncoupling, because the uncoupling effect of *p*-phenylenediamine is stoichiometric to the increased rate brought about by *p*-phenylenediamine.

DISCUSSION

Rumberg's[23,24] as well as Avron's group[25,26] have pointed out that the internal rather than the external pH controls the rate of electron transport in chloroplast membranes The pH gradient generated in the light inside the membrane allows maximum electron transport even when the outside pH is high. Uncouplers shift the pH maximum of electron flow (in the medium) to lower pH values because now a lower pH inside than outside is not maintained Recently Bamberger *et al.*[27] suggested that the rate controlling site is embedded in the membrane and is a function of both internal and external pH. Their result in the light of the results presented here may be viewed rather as a manifestation of the optimal pH favoured inside (for oxidation) and outside (for reduction) for the native shuttle of reducing equivalents as well as hydrogens across the chloroplast membrane *via* plastohydroquinone/plastoquinone.

The results in this paper show how this optimal pH difference for electron flow, governed by the native shuttle, is shifted when the optimal pH difference of an artificial shuttle of reducing equivalents is superimposed in the presence of a plastoquinone antagonist. Such a shuttle in the opposite direction across the membrane occurs when in photoreductions by Photosystem II (electron flow to Photosystem I prevented by DBMIB) a phenylenediamine mediates an electron flow from plastohydroquinone inside the membrane towards the terminal acceptor, the polar ferricyanide, outside. In this shuttle the phenylenediimine is transported to the inside and the phenylenediamine to the outside (see the scheme in Fig 5). The properties of the reduced as well as the oxidized form, *i.e.* lipophilicity, charge and difference in

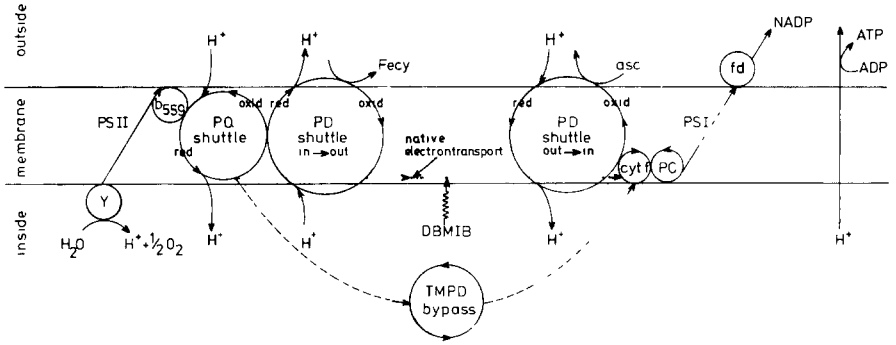

Fig 5. Scheme of photosynthetic electron transport in a zig-zag across the membrane The inhibition site of DBMIB, a phenylenediamine shuttle across the membrane in/out in the photoreduction of ferricyanide by Photosystem II, a phenylenediamine shuttle out/in for the photoreduction of NADP by Photosystem I and a bypass of the inhibition site by TMPD inside the membrane is indicated. No stoichiometry of protons released inside is given · , native electron flow in the absence of DBMIB.

pK influence the activity of a phenylenediamine in this shuttle. N-Substituted phenylenediamines are oxidized by ferricyanide outside to the polar radical, which cannot penetrate the membrane against the positive membrane potential inside. Therefore TMPD and *N*-dimethyl-*p*-phenylenediamine do not stimulate the photoreduction of ferricyanide by Photosystem II at pH 8.0. At pH 6 0 and in the presence of gramicidin TMPD does stimulate, possibly because the membrane potential is broken down. The imine form of *p*-phenylenediamine and C-substituted phenylenediamines have pK values around 3–4[28]. They are not protonated at high pH outside and, therefore, are able to penetrate the membrane. The amine form with pK values around 6[28] are protonated inside. In this way they are even acting as internal buffers[26]. This will lead to a low concentration of the free base of the amine inside but high outside of the membrane. Assuming that the free base is the species reacting in oxidation and reduction reactions, the pH gradient built up by the energy conserving sites of the electron flow system will lead to a distribution of imine and amine (as base) inside and outside which is favourable for the overall reaction with the reduction of the imine inside and oxidation of the amine outside, *i.e.* a high ratio reduced/oxidized outside and a high ratio oxidized/reduced (not protonated) inside

An uncoupler by breaking down the pH gradient inhibits this shuttle and this favourable ratio inside and outside because of the equilibration and, therefore, inhibits the stimulation of ferricyanide reduction by a lipophilic phenylenediamine at high outside pH. It should be kept in mind that this inhibition by an uncoupler at high outside pH occurs in the presence of ADP/P_i and, therefore, does not reflect an influence of the uncoupler on the coupling system. Increasing the absolute concentration of *p*-phenylenediamine in the system overcomes the inhibition of the uncoupler by increasing the concentration of free base inside. In a way *p*-phenylenediamine at high concentration is to be considered an uncoupler. Also in the presence of gramicidin in the high pH range no dependence on substitution (leave out the N-substitution) is observed indicating that the shuttle is limiting the overall rate.

At lower pH outside, the inhibition by an uncoupler disappears and the usual

stimulation of an electron transport chain by an uncoupler becomes apparent because now the coupling system limits electron flow.

Even at pH 5.0 and below high electron transport rates are observed. This depends on the effectiveness of the phenylenediamine shuttle. Highly C-substituted phenylenediamines, which will have higher lipophilicity and also lower pK values (because of steric shielding of the amino group) are better electron acceptors for Photosystem II at lower pH than the unsubstituted *p*-phenylenediamine. The points chosen in Table II to illustrate this are, of course, of no significance as such because at any pH the system is complex.

The stimulation by phenylenediamines of the bypassing of the DBMIB inhibition site in the NADP system is visualized to occur inside. Both the reduction and oxidation of the *p*-phenylenediamine used occurs inside and no shuttle across the membrane is required. Arguments for this have also been discussed elsewhere[11] The inhibition of the shuttle by gramicidin as observed in the ferricyanide system (Table I) has been attributed above to an increase in the amount and ratio of the reduced (not protonated) to the oxidized form of the phenylenediamine reduced inside (see above) This ratio is favourable for a reduction of the donor side of Photosystem I and, therefore, uncouplers stimulate the NADP system.

The shuttle of the phenylenediimine/phenylenediamine in the ferricyanide system will also carry hydrogens across the membrane, because hydrogens are required for reduction of the diimine (not to be confused with the protonisation of the amine). According to the chemiosmotic theory this will lower the amount of protons available for the ATP-forming system and the P/e_2 ratio of the complete systems The P/e_2 ratio of the photoreduction of ferricyanide by Photosystem II, therefore, does not necessarily indicate that only one energy conserving site is participating, but rather that some of the hydrogens pumped inside across the membrane are transported back outside stoichiometrically to the electron flow. This we would like to term stoichiometric uncoupling.

Stoichiometric uncoupling also occurs, when *p*-phenylenediamine is added to a ferricyanide system in the absence of DBMIB, *i e.* the conditions of Saha *et al* [15]. Therefore, the conclusion that only one energy conserving site is operating in photoreductions by Photosystem II is correct only if the overall stoichiometry is considered.

To account for the coupling to ATP formation of photoreductions by Photosystem II with about half the stoichiometry (P/e_2 ratio) of non-cyclic electron flow[7-11], we would like to accept the scheme of Witt and his colleagues[16] According to Junge and Witt and co-workers[16,29,30] the water-splitting reaction occurs inside the membrane The protons released in the water-splitting reaction contribute together with the protons pumped across the membrane in a plastoquinone shuttle[31,32] to the pH gradient, which in turn gives rise to ATP formation (with a P/e_2 ratio of 1.33 in non-cyclic electron flow on the assumption of 3 H^+/ATP[33]). The protons released in the water-splitting reaction inside are responsible for the energy conserving site in photoreduction by Photosystem II (Fig 5).

Presumably in a phenylenediamine shuttle two protons per 2 e are moved back outside. This would then lead to an expected P/e_2 ratio of about 0.66 The observed value in photoreduction by Photosystem II as reported is in the order of 0 3–0 5[7-11] Because in the restoration of electron flow from water to NADP (bypass of the DBMIB inhibition site) *via* TMPD inside (Fig. 5) no hydrogens are lost

the P/e_2 ratio is again 1[11].

In photoreductions by Photosystem I at the expense of an electron donor system like various phenylenediamines/ascorbate or DCIP/ascorbate a shuttle of reducing equivalents also occurs, but this time from the outside to the inside because the polar ascorbate will not penetrate through the membrane[21]. If a lipophilic, hydrogen carrying phenylenediamine is used, the system is coupled to ATP formation[17,19]. This is again because the shuttle not only carries reducing equivalents inside but also releases protons inside[21]. The stimulation of the DCIP/system by NH_4Cl which has been observed[17-19,22] but not yet satisfactorily explained, is possibly due to a stimulation of this shuttle.

POSTSCRIPT

When we were preparing the manuscript of this paper we received a communication from Dr Good's laboratory describing similar experiments. We seem to agree in some basic experimental data but arrive at different explanations.

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REFERENCES

1 Trebst, A., Harth, E and Draber, W. (1970) *Z Naturforsch.* 25b, 1157–1159
2 Bohme, H., Reimer, S and Trebst, A (1970) *Z Naturforsch* 26b, 341–352
3 Trebst, A (1972) *Proc 2nd Int. Cong. Photosynthesis Res. Stresa*, Vol I, p 399–417 (Forti, G , Avron, M. and Melandri, M., eds) Junk, The Hague
4 Bohme, H and Cramer, W A. (1971) *FEBS Lett* 15, 349–351
5 Lozier, H. and Butler, W L (1972) *FEBS Lett* 26, 161–164
6 Bauer, R (1973) Dissertation, Aachen
7 Trebst, A and Reimer, S (1973) *Biochim Biophys Acta* 305, 129–139
8 Ort, D R , Izawa, S , Good, N E. and Krogmann, D. W (1973) *FEBS Lett* 31, 119–122
9 Ouitrakul, R. and Izawa, S. (1973) *Biochim Biophys Acta* 305, 105–118
10 Izawa, S , Gould, J M , Ort, D R., Felker, P and Good, N E (1973) *Biochim Biophys Acta* 305, 119–128
11 Trebst, A and Reimer, S. *Z Naturforsch* in the press
12 Nelson, N ,Drechsler, Z and Neumann, J. (1970) *J Biol Chem.* 245, 143–151
13 Conover, T E., Prairie, R L and Racker, E (1963) *J. Biol Chem* 238, 2831–2837
14 Trebst, A , Pistorius, E and Buchel, K H (1966) *Z Naturforsch* 21b, 667–672
15 Saha, S , Ouitrakul, R , Izawa, S. and Good, N E (1971) *J. Biol Chem* 246, 3204–3209
16 Witt, H T , Rumberg, B. and Junge, W. (1968) in *Biochemie des Sauerstoffs* (Hess, B and Staudinger, Hj , eds), Springer Verlag, Berlin
17 Trebst, A. and Pistorius, E. (1965) *Z Naturforsch* 20b, 143–147
18 Trebst, A. and Pistorius, E. (1967) *Biochim Biophys Acta* 131, 580–582
19 Izawa, S , Connolly, T. N , Winget, G D and Good, N E (1966) *Brookhaven Symp* No 19, 169–187
20 Hauska, G , McCarty, R. E , Berzborn, R and Racker, E (1971) *J. Biol Chem* 246, 3524–3531
21 Hauska, G., Trebst, A. and Draber, W (1973) *Biochim Biophys. Acta* 305, 632–641
22 Keister, D. L (1965) *J. Biol. Chem* 240, 2673–2677
23 Rumberg, B , Reinwald, E , Schroder, H and Sigel, U (1968) *Naturwissenschaften* 55, 77–79
24 Rumberg, B., Reinwald, E , Schroder, H and Sigel, U (1969) *Prog in Photosynthesis Res* (Metzner, H , ed) Vol III, pp 1374–1382, Verlag Lichtenstern, Tubingen

25 Rottenberg, H., Grunwald, T. and Avron, M. (1971) *FEBS Lett.* 13, 41–44
26 Rottenberg, H., Grunwald, T. and Avron, M. (1972) *Eur. J. Biochem.* 25, 54–63
27 Bamberger, E. J., Rottenberg, H. and Avron, M. (1973) *Eur. J. Biochem.* 34, 557–563
28 Clark, W. M. (1960) *Oxidation reduction potentials of organic systems*, Williams and Wilkins, Baltimore
29 Junge, W. and Witt, H. T. (1968) *Z. Naturforsch.* 23b, 244–254
30 Schliephake, W., Junge, W. and Witt, H. T. (1968) *Z. Naturforsch.* 23b, 1571–1578
31 Reinwald, E., Stiehl, H. H. and Rumberg, B. (1968) *Z. Naturforsch.* 23b, 1616–1617
32 Bohme, H. and Cramer, W. A. (1972) *Biochemistry* 11, 1155–1160
33 Schroder, H., Muhle, H. and Rumberg, B. (1972) *Proc. 2nd Int. Congr. Photosynthesis Res., Stresa*, Vol. 2, p. 919–930 (Forti, G., Avron, M. and Melandri, M., eds) Junk, The Hague