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Inhibition of Electron-Transport in Chloroplasts by a Quinone Analogue: Evidence for Two Sites of $DPIPH_2$ Oxidation*

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Abstract

A new inhibitor of photoreactions in chloroplasts, 2,3-dimethyl 5-dybroxy 6-phytol benzoquinone is shown to be an electron transfer inhibitor which blocks both cyclic and non-cyclic electron flow. Basal levels of electron transport from reduced dichlorophenol-indophenol to methyl viologen are not affected by the inhibitor, but uncoupler stimulated electron transport in the same system is inhibited. It is concluded that reduced dichlorophenol-indophenol can be oxidized by the photosynthetic electron transport chain in isolated chloroplasts at two sites: site I proximal to P_{700} and site II distal to P_{700}. Site I has a low affinity for the electron donor. Electron flow from this site to methyl viologen does not support ATP formation and it is resistant to inhibition by the quinone analogue. Electron donation at site II, located on the linear portion of the electron transport chain between the two photosystems, has a higher affinity for reduced dichlorophenol-indophenol and precedes a phosphorylation site. The electron flow from this site is stimulated by uncouplers and inhibited by the quinone analogue.

Introduction

Various treatments or compounds inhibit the photoinduced electron transport in the vicinity of system II causing an inhibition of the Hill reaction and its coupled ATP formation.[1,2] Under the same circumstances several photoreactions mediated by photosystem I, including electron flow from $DPIPH_2$,§ to a terminal acceptor or cyclic phosphorylation, are not affected.[1]

Both non-cyclic and cyclic phosphorylation can be inhibited by uncouplers or energy transfer inhibitors. Few compounds are known, however, which inhibit electron transport between the two photosystems. Salycil aldoxime, which acts at a point on the linear electron transport chain,[3] can be regarded as having such properties and also phenol and some phenol derivatives.[4]

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§ Abbreviations: DPIP, 2,6-dichlorophenol indophenol; MeV, methyl viologen; DCMU, 3-(3,4-dichlorophenyl-1,1-dimethylurea; CCP, *m*-chlorocyanocarbonyl phenylhydrazone; DTE, dithioerythritol; PMS, phenazine methosulfate; DMHPB, 2,3-dimethyl 5-hydroxy 6-phytol benzoquinone.

A new inhibitor of photoreactions in chloroplasts, 2,3-dimethyl 5-hydroxy 6-phytol benzoquinone* (DMHPB) is shown in this communication to be an electron transfer inhibitor which blocks both cyclic and non-cyclic electron flow. The obvious structural similarity between this compound and plastoquinone, which is thought to function as a carrier in the electron transport chain of chloroplasts,[1] may indicate that the inhibitor competes for a site(s) or cofactors(s) which normally interacts with endogenous plastoquinone. The results presented in this communication would therefore confirm previous work which suggests that plastoquinone is involved in non-cyclic electron flow[5] (and ATP formation) as well as cyclic electron flow catalyzed by PMS.[6]

Studies with this new inhibitor and other characteristic aspects of $DPIPH_2$ oxidation in DCMU treated chloroplasts strongly suggest that $DPIPH_2$ can donate electrons at two sites on the linear electron transport chain.

Materials and Methods

Chloroplasts were isolated from lettuce leaves *Lactuca satira* var. romaine. Fifty grams of leaves were homogenized for 10 sec in a Waring Blender in 130 ml grinding media of 0·4 M sorbital, 0·1 M tricine (pH 7·8), 0·07 M ascorbate, and bovine serum albumin (2 mg/ml final concentration). The homogenate was filtered through 4 and then 12 layers of cheesecloth and then centrifuged for 7 min at 1000 × *g*. The pellet obtained was washed in a solution of 0·4 M sorbital, 0·02 M tricine (pH 8·0), 0·01 M KCl and was centrifuged as before. The final chloroplast pellet was resuspended in the washing medium with bovine serum albumin added to a final concentration of 5 mg/ml.

Chlorophyll concentration was measured according to Arnon.[7] ATP formation was measured according to Avron.[8] Phosphorylating reaction mixtures were illuminated for 2 min at room temperature. Oxygen uptake was measured on a Gilson KM-C oxygraph with a YSI Clark electrode at 20°. Changes in pH were measured with Leeds and Northrup 124138 microelectrodes. A yellow Corning filter (#3-68) was placed between the light source and the sample to eliminate artifactual responses in both the O_2 and pH measurements.

Results and Discussion

DPIP in the presence of ascorbate has been shown to relieve the CMU inhibition of NADP photoreduction.[9] This system is able to support ATP formation.[10] However, since it has been shown that ascorbate + DPIP are able to support phosphorylation even in the absence of an added electron acceptor, several workers concluded that ATP formation mediated by System I and $DPIPH_2$ is of a cyclic type even in the presence of the electron acceptor system of ferredoxin and NADP.[11–13] The stimulatory effect of the addition of ferredoxin + NADP upon ATP formation, has been interpreted as causing a proper oxidation-reduction "poise" for DPIP, in the light, which is required to support cyclic electron flow.[14, 15] Direct evidence for cyclic electron flow mediated by system I was also presented.[16] Supporters of the notion that ATP formed in the presence of $DPIPH_2$ is cyclic would maintain that the site of ATP formation is on the part of the

* The synthesis of the quinone and the initial observations on its biological activity will be reported separately by J. Boles, K. Folkers, and R. Dilley.

cyclic pathway which is not shared by the linear pathway of electron transport.[15] Our studies with the new inhibitor DMHPB are related to this question.

In Fig. 1 the effects of DMHPB on several photoreactions are shown. Both electron flow and the concomitant phosphorylation in a methyl viologen Hill reaction were

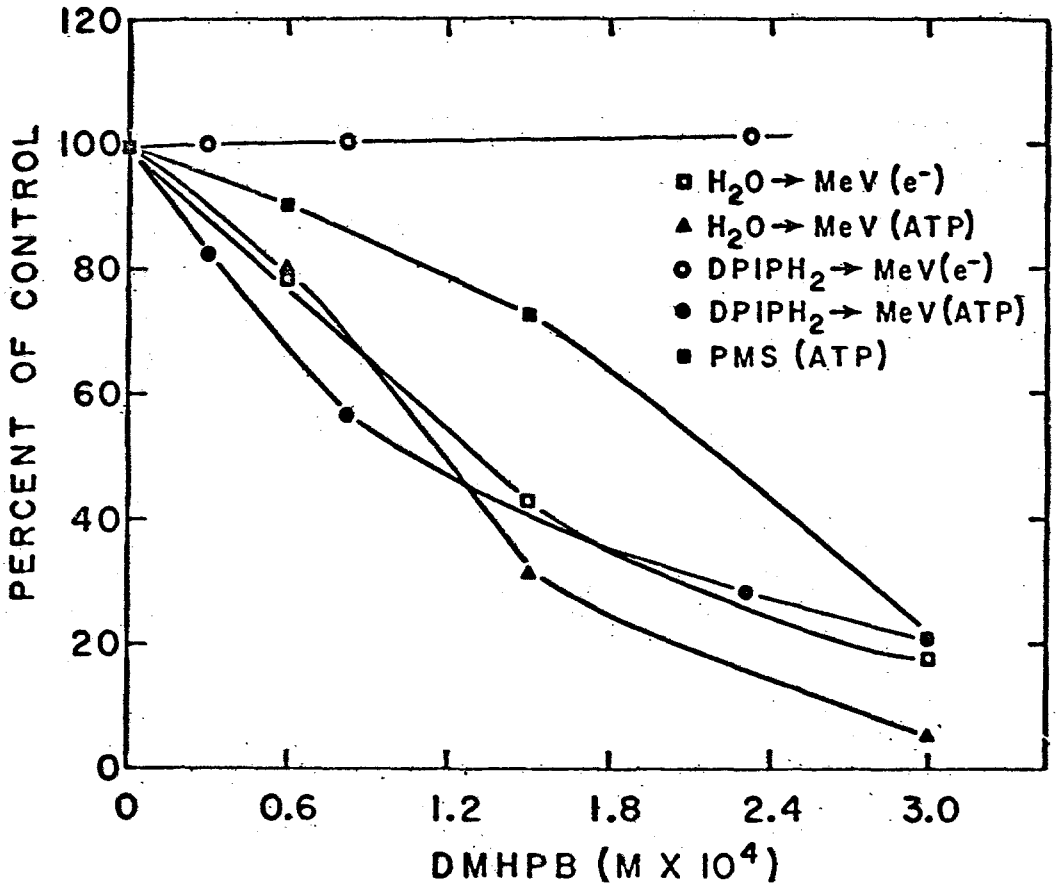

Figure 1. The effects of DMHPB on phosphorylation and electron transport. Reaction mixtures contained (in μmoles): Na-Tricine (pH 8·0), 25; $MgCl_2$, 5; ADP, 2; and NaPi, 5. The different reaction mixtures also contained the following (in μmoles): PMS phosphorylation assays—PMS, 0·075; DCMU, 0·03; and ascorbate, 5; H_2O to MeV assays—MeV 2·5 and NaN_3, 2·5; $DPIPH_2$ to MeV assays—DPIP, 0·81; ascorbate, 10; DCMU, 0·03; MeV, 2·5; and NaN_3, 2·5. Total reaction volumes were 1·5 ml. Chlorophyll concentrations were 30 μg/ml. Light intensities for sample illumination were 1×10^5 ergs/cm².sec for PMS phosphorylation and 6×10^5 ergs/cm².sec for all other reactions. (Lower light intensities were used for PMS phosphorylation to give activities which fall within the same range of phosphorylation as with the other systems.) Control rates of electron transport and phosphorylation (μmoles O_2 consumed or μmoles ATP formed per mg chl per h) were: PMS, (ATP) 202; H_2O to MeV, (ATP) 410, (e^-) 155; $DPIPH_2$ to MeV, (ATP) 105, (e^-) 436.

found to be inhibited (the latter appears to be a result of the former). The inhibition of electron flow was observed at pH 7·5, pH 8·0 and pH 8·25. In addition, PMS catalyzed ATP formation, and photophosphorylation supported by $DPIPH_2$ in the presence of methyl viologen and DCMU were all inhibited to about the same extent. One reaction, transfer of electrons from $DPIPH_2$ to MeV (measured by O_2 uptake), was not affected

by DMHPB to any great extent over this entire concentration range. It is interesting to note that Jones and Kok[17] observed a very similar pattern of inhibition of Hill reaction activity and $DPIPH_2$ supported phosphorylation but insensitivity of $DPIPH_2$ to NADP electron flow in ultraviolet light treated chloroplasts. Their suggestion that plastoquinone was the site of UV inhibition would be in agreement with the postulation that DMHPB is acting as a quinone analogue which inhibits the oxidation or reduction of the endogenous

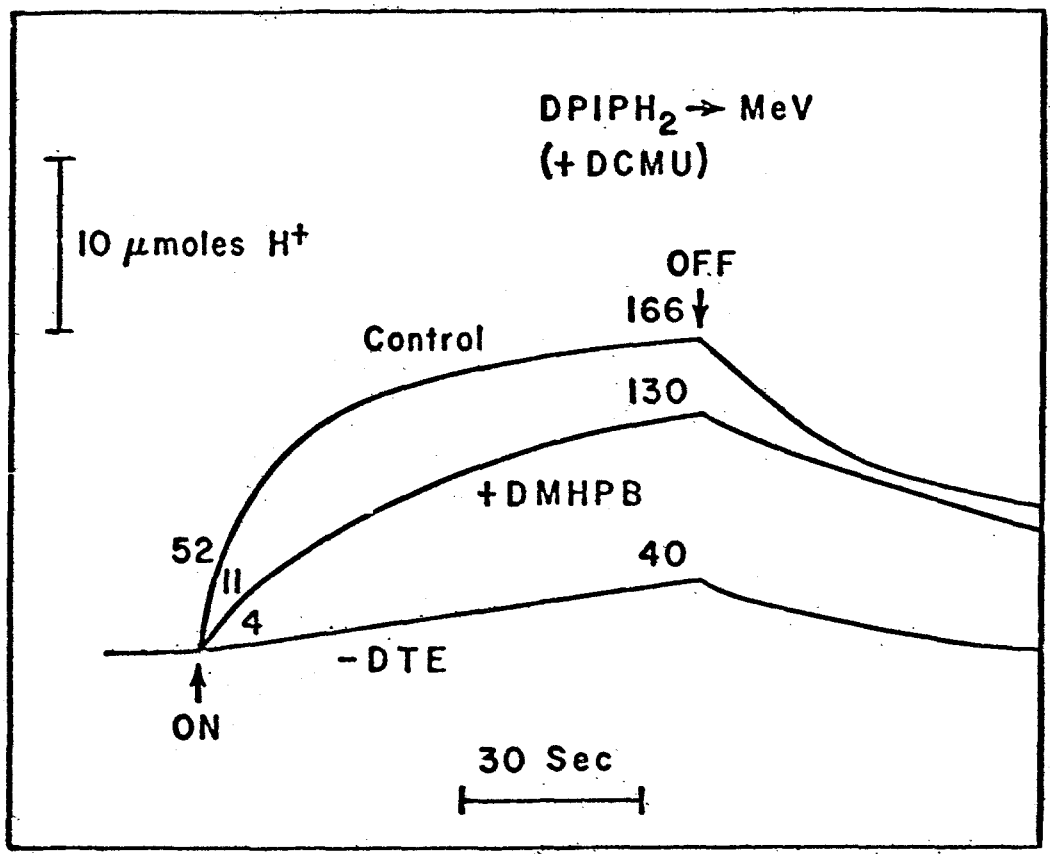

Figure 2. Light-induced pH changes catalyzed by electron flow from reduced DPIP to MeV in the presence of DCMU. Values for the initial rate: in μmoles H^+/mg chl per h, and the extent: in mμuoles/mg chl, are shown on the figure. Reaction mixtures contained (in μmoles) KCl, 50; MeV, 2·5; NaN_3, 2·5; DPIP, 0·81; DCMU, 0·03; DTE, 2 and 114 μg chlorophyll in 3 ml. DMHPB was added as indicated to give a final concentration of $1·5 \times 10^{-4}$ M. Reactions were carried out at pH 8·0 at 16° with a light intensity of 3×10^5 ergs/cm².sec.

plastoquinone. Bishop[18] has previously shown that UV irradiation causes plastoquinone destruction in chloroplasts. However, other effects of UV irradiation have been documented.[19]

Further evidence that DMHPB acts by blocking electron flow rather than at some terminal step in phosphorylation is indicated from its effect on light-induced pH changes. As shown in Fig. 2, proton uptake by illuminated chloroplasts was catalyzed by methyl viologen reduction using DPIP-dithioerythritol as the electron donor system. The re-

quirement for reduced DPIP is evident from the very low level of activity observed when dithioerythritol was omitted from the reaction mixture. (Ascorbate was not used as a reducing agent since dark oxidation gives rise to pH changes.) When DMHPB was added to the reaction mixture, the initial rate of proton uptake was inhibited by 80% and the extent of the pH gradient was reduced by 20%.

Evidence presented in Figs. 1 and 2 has indicated that phosphorylation and the closely related proton uptake catalyzed by $DPIPH_2$ is blocked by DMHPB while electron flow from $DPIPH_2$ to methyl viologen is unaffected. This might be explained by assuming that $DPIPH_2$ catalyzed phosphorylation proceeds via a cyclic electron pathway that is independent of the observed electron flow (O_2 uptake). Observations which are contradictory to this line of thought are shown in Fig. 3.

As was previously shown by Izawa *et al.*,[18] the uncoupler NH_4Cl stimulated the rate of electron flow from $DPIPH_2$ to methyl viologen (Fig. 3). Since this stimulation is of approximately the same extent over a wide range of DPIP concentrations (Fig. 3A, C, E), it is unlikely that the increase in electron flow is due to a "non-specific" increase in electron donor permeability caused by NH_4Cl (also note the effect of other uncouplers listed below). Moreover, DMHPB blocked this stimulated flow to about the same extent in all cases, causing a return to the basal level of electron transfer. On the other hand, the inhibitor does not greatly effect the basal electron flow (Fig. 3B, D, and F). However, in the presence of the inhibitor, NH_4Cl does not stimulate electron flow (Fig. 3B, D, and F). The concentration of DMHPB used in this experiment was selected to give maximal inhibition of uncoupled electron flow. This inhibition of the uncoupled electron flow and lack of inhibition of basal electron flow could be observed over a wide range of concentrations of DMHPB. To exclude the possibility that the inhibition is due to a non-specific interaction between uncoupler and inhibitor, the effect of DMHPB on electron flow from H_2O to methyl viologen is shown in Fig. 3G and H. In this case, both the basal and stimulated rates of electron flow were blocked by the inhibitor.

To show that DMHPB inhibition was not just a fortuitous result of NH_4Cl-inhibitor binding or inactivation, other uncouplers of phosphorylation were tested in the presence and absence of inhibitor. The results shown in Table I clearly indicate that DMHPB does not affect the basal rate of electron flow but does block the stimulation by nigericin, *m*-chlorocyanocarbonyl phenylhydrazone and gramicidin D.

Inhibition of the methyl viologen Hill reaction indicates that DMHPB acts at a carrier located on the linear electron transport pathway. In addition, since all the inhibited photoreactions are reduced to approximately the same extent over a variety of DMHPB concentrations, it would appear that there is probably only one inhibitory site. The electron transport carrier inhibited by DMHPB would be located in a region of the linear electron transport chain utilized by electron flow from H_2O to methyl viologen, and would also be involved in electron flow supporting PMS or $DPIPH_2$ phosphorylation. Assuming that DMHPB does inhibit electron flow at a carrier localized on the linear pathway between the two photosystems, it is difficult to visualize how such a carrier will not participate in electron transport from $DPIPH_2$ to methyl viologen (which is insensitive to the inhibitor), but will be involved in electron transport initiated by $DPIPH_2$ and leading to ATP formation (which is sensitive to DMHPB). This inconsistency can be resolved by hypothesizing that $DPIPH_2$ can donate electrons to the electron transport chain at two sites, as has been previously suggested for several donors.[1,21-24] The first

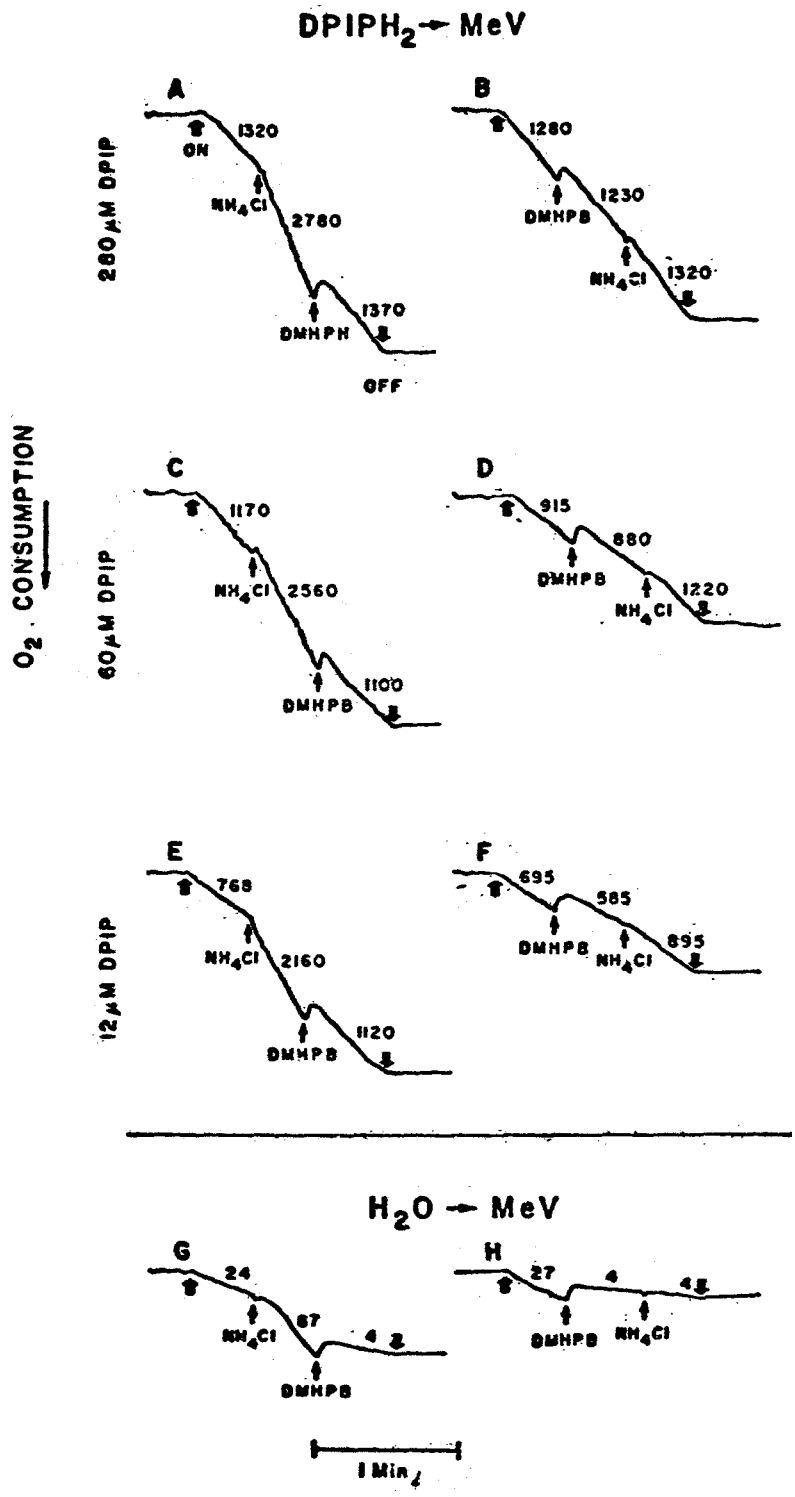
DPIPH2 → MeV
A
ON
1320
NH4Cl
2780
DMHPH
1370
OFF
280 μM DPIP
B
1280
DMHPB
1230
NH4Cl
1320
O2 CONSUMPTION
C
1170
NH4Cl
2560
DMHPB
1100
60 μM DPIP
D
915
DMHPB
880
NH4Cl
1220
E
768
NH4Cl
2160
DMHPB
1120
12 μM DPIP
F
695
DMHPB
585
NH4Cl
695
H2O → MeV
G
24
NH4Cl
67
DMHPB
H
27
4
DMHPB
NH4Cl
1 Min

TABLE 1. Effects of DMHPB on basal and uncoupled electron flow from $DPIPH_2$ to MeV

Additions	μMoles O_2 consumed per mg chl.h	% of Control
Exp. 1		
A Control	180	100
+CCP (6·7 μM)	327	183
+ DMHPB	223	123
B Control	195	100
+DMHPB	184	94
+CCP (6·7 μM)	254	130
C Control	156	100
+Gramicidin D (4 μM)	410	263
+DMHPB	230	145
D Control	223	100
+DMHPB	232	104
+Gramicidin D (4 μM)	265	118
Exp. 2		
E Control	78	100
+Nigericin (0·17 μM)	290	320
+DMHPB	105	135
F Control	90	100
+DMHPB	105	116
+Nigericin (0·17 μM)	135	150

The experimental procedure was the same as that shown in Fig. 3, except reaction mixtures contained 0·016 μmoles DPIP and 16 μgr chlorophyll per assay. In addition, nigericin reactions contained 50 μmoles KCl. Final concentration of DMHPB in all cases was 0·46 mM.

site (I) would be at a more positive redox potential (perhaps P_{700}) and would not be coupled to ATP formation. The second site (II) would be at a more negative potential and would support electron flow through a phosphorylating site. Since P/e ratios in the $DPIPH_2$ to methyl viologen system are very low[22] it can be concluded that under normal conditions most electrons enter the chain at site I. The marked stimulation of electron flow from $DPIPH_2$ to methyl viologen in the presence of uncouplers indicates that in the absence of uncouplers $DPIPH_2$ oxidation at site II is strongly limited by the coupling

Figure 3. The effect of DMHPB on basal and NH_4Cl stimulated electron transport from $DPIPH_2$ to MeV and H_2O to MeV. The individual figures shown are direct tracings of oxygraph data showing light-induced O_2 uptake. Heavy arrows indicate the onset (upward arrow) and termination (downward arrow) of illumination. The numbers shown give either the initial rate of O_2 consumption (μmoles O_2/mg chl per h) observed in the light or the rate after the addition of NH_4Cl or DMHPB. Concentrations of Tricine, MeV, and NaN_3 in the reaction mixtures were as in Fig. 1. In addition, concentrations of DPIP as indicated in the figure, 2·5 μmoles ascorbate, 0·03 μmoles DCMU, and 5 μg chlorophyll were included for $DPIPH_2$ to MeV electron flow. The MeV Hill reaction contained 82 μg chlorophyll. Light intensities as in Fig. 1. Final concentrations of NH_4Cl and DMHPB were 5·0 mM and 0·35 mM respectively.

process. Assuming that DMHPB blocks electron transport at a point prior to site I, the inhibitor would therefore not be expected to effect to a large extent the basal electron flow from $DPIPH_2$ to methyl viologen.

To further characterize the two electron donation sites by $DPIPH_2$, electron transport

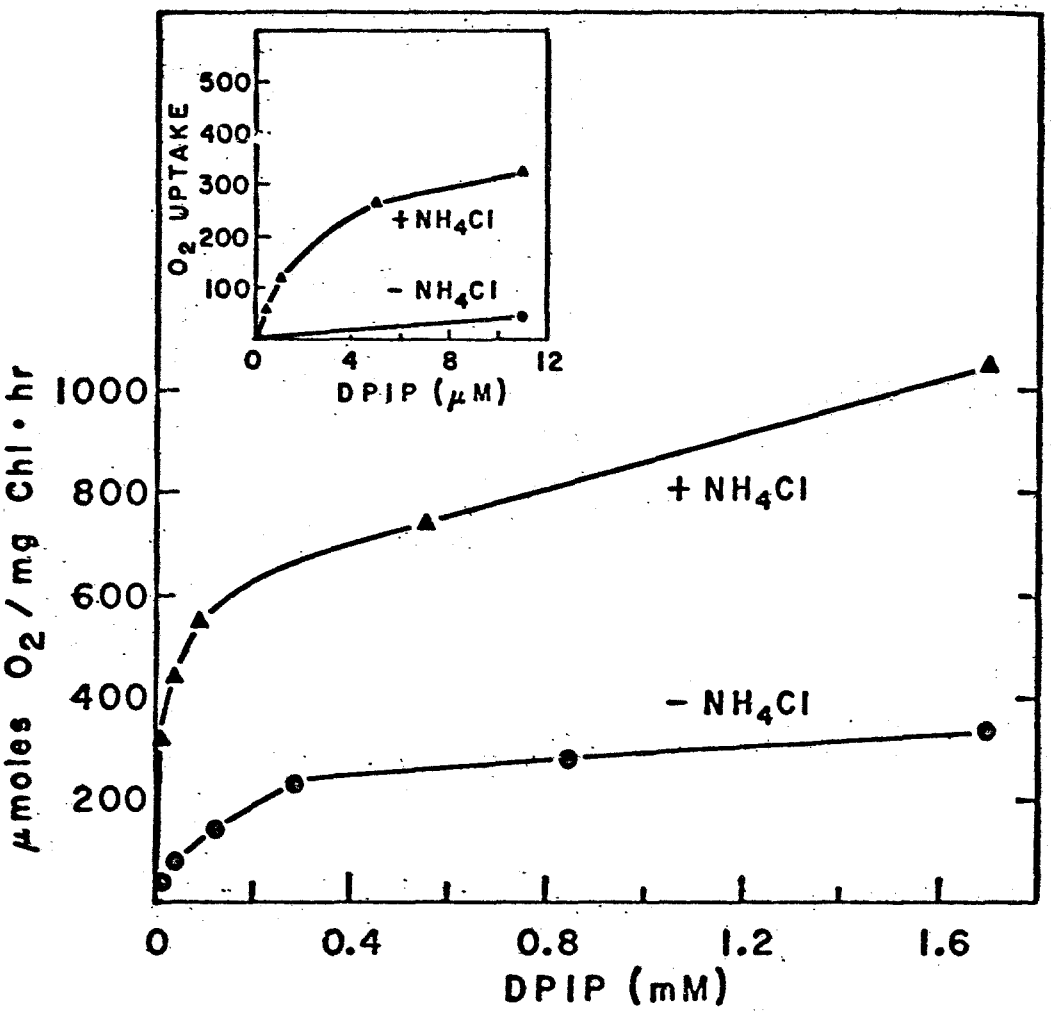

Figure 4. The effect of increasing concentrations of DPIP on electron flow from $DPIPH_2$ to MeV in presence or absence of NH_4Cl. Reaction conditions were as in Fig. 3 except 5 μM ascorbate and 32 μgr chlorophyll were used in each assay.

activity was measured as a function of $DPIPH_2$ concentration in the presence and absence of NH_4Cl. As is shown in Fig. 4, the rate of O_2 uptake was minimal at low DPIP concentrations in the absence of an uncoupler (primarily electron donation via site I). In the presence of NH_4Cl, however, rapid electron flow was catalyzed even by very low levels of $DPIPH_2$ (see inset in Fig. 4). This indicates that site II has a greater affinity for

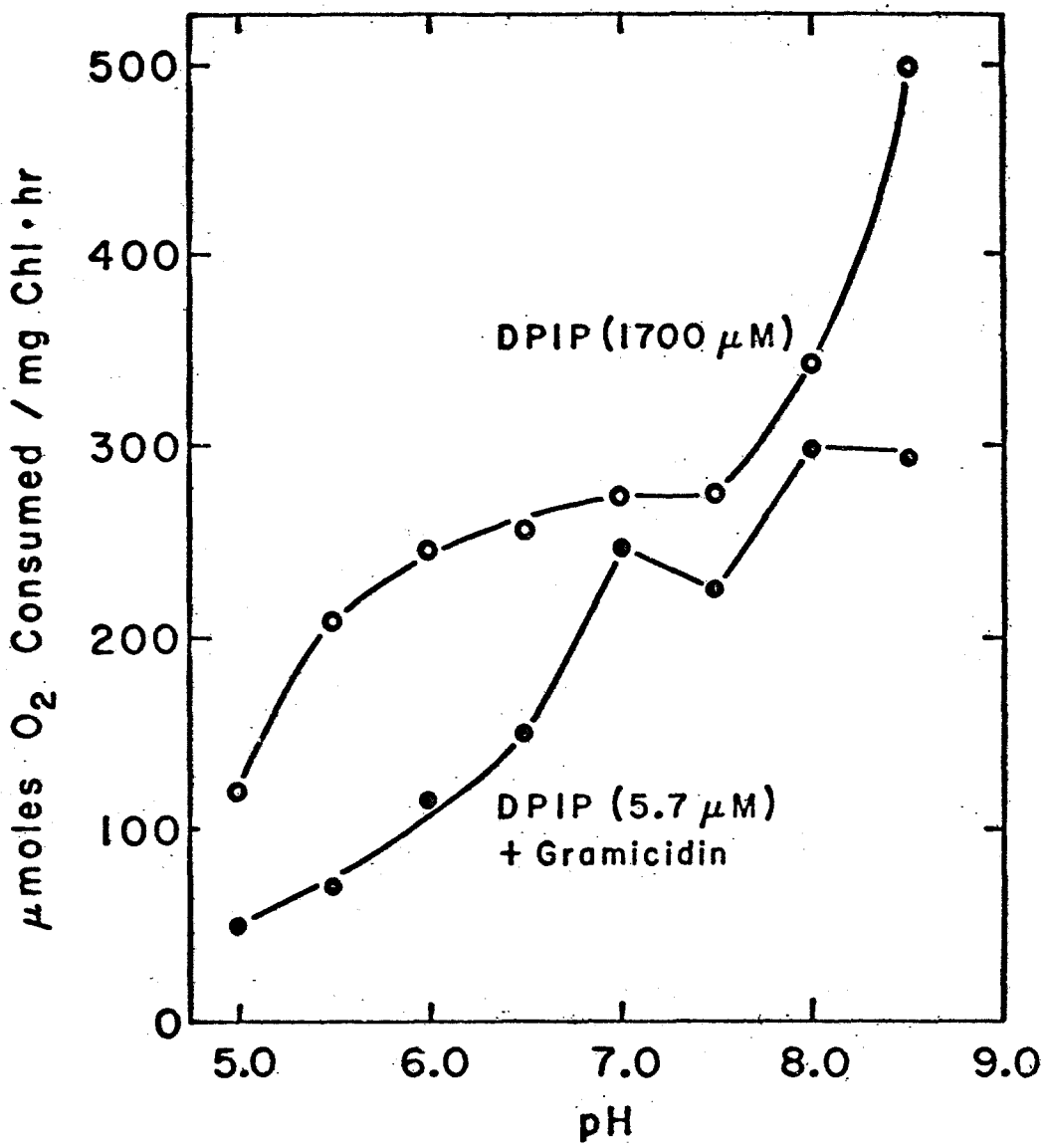

Figure 5. The effect of pH on electron transport from $DPIPH_2$ to MeV under different reaction conditions. In the presence of low DPIP concentrations plus an uncoupler of phosphorylation (primarily electron donation at site #2—see text), the pH profile is shifted from that observed with high levels of DPIP (electron donation mainly at site #1). Reaction mixtures contained, MeV, NaN_3, DCMU, and ascorbate as in Fig. 1, plus 50 μM Tricine-maleate buffer, 40 μgr chlorophyll, and DPIP and 0·036 μM gramicidin as indicated. Other conditions as in Fig. 1.

$DPIPH_2$. However, as discussed above, the rate limiting step of $DPIPH_2$ oxidation at site II is the coupling process.

Since the above data indicate that electron donation from low levels of $DPIPH_2$ in the presence of NH_4Cl is primarily via site II and donation at high levels of $DPIPH_2$ with no uncoupler present is primarily via site I, it is possible to compare the rates of

the two reactions over a range of pH. The results of such an experiment are given in Fig. 5. Again, evidence for two sites of donation is supported by the observed shift in the pH curves. Since there is probably some contribution to electron flow from each site at all $DPIPH_2$ concentrations, the absolute donation at each site cannot be determined. This overlapping pattern of donation may account for some of the erratic pattern of the pH profile.

One other experimental difficulty which has limited some further experimentation is the fact that DMHPB, being a quinone analogue, can act as an electron donor to the electron transport chain by itself. High concentrations of DMHPB, in the presence of a reducing agent such as ascorbate, gave measurable rates of O_2 uptake with methyl viologen as the electron acceptor. This does not alter any of the observations reported in this communication however, since low levels of the inhibitor were used and its contribution to electron flow in the presence of $DPIPH_2$ is negligible except under conditions of very low rates.

Conclusions

Several workers have suggested multiple sites for the donation of electrons to the photosynthetic electron transport chain by $DPIPH_2$[1,21] and by other electron donors.[21-24] This postulate is strongly supported by our results obtained with the electron transport inhibitor DMHPB which selectively inhibits electron flow from site II of $DPIPH_2$ electron donation. Since DMHPB also blocks $DPIPH_2$ supported ATP formation, our data indicate that a site of phosphorylation coupled to electron transport from $DPIPH_2$ to methyl viologen (in the presence of DCMU) is located on a portion of the linear electron transport pathway between the two sites of electron donation by $DPIPH_2$. Additional work is in progress to substantiate this idea.

Acknowledgements

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