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DUROQUINOL AS AN ELECTRON DONOR FOR CHLOROPLAST ELECTRON TRANSFER REACTIONS

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Summary

Duroquinol (tetramethylhydroquinone) was found to function as an electron donor in chloroplasts. Non-cyclic electron transfer from duroquinol to electron acceptors such as oxygen proceeded at high rates, was insensitive to 3-(3,4-dichlorophenyl)-1,1-dimethylurea (DCMU) but was sensitive to the plastoquinone antagonist 2,5-dibromo-3-methyl-6-isopropyl-1,4-benzoquinone (DBMIB). The electron transport from duroquinol was coupled to the synthesis of ATP. Spectroscopic studies of chloroplast electron carriers in the dark indicated the high-potential "Rieske" iron-sulfur center, cytochrome *f*, plastocyanin and *P*-700 were all reduced by duroquinol. The dark reduction of the "Rieske" iron-sulfur center and cytochrome *f* were inhibited by DBMIB but not by DCMU. These results have been interpreted in terms of a linear sequence of electron carriers in the non-cyclic electron transport chain which includes plastoquinone, the "Rieske" iron-sulfur center, cytochrome *f*, plastocyanin and *P*-700.

Introduction

Duroquinone (tetramethylbenzoquinone) has been used extensively in studies of electron transfer reactions in mitochondria and submitochondrial particles [1—3]. The oxidized compound has been shown to function as an electron acceptor in the NADH dehydrogenase portion of the respiratory chain [1,2] while the reduced compound can serve as an electron donor for the cytochrome *b-c* region [3,4]. Reduced duroquinone (duroquinol) is also widely used as a reagent in the assay of mitochondrial Complex III which catalyzes the

Abbreviations: DCMU, 3-(3,4-dichlorophenyl)-1,1-dimethylurea; DBMIB, 2,5-dibromo-3-methyl-6 isopropyl-1,4-benzoquine.

transfer of electrons from this compound, as well as from other reduced quinones, to cytochrome *c* (see ref. 4 for a recent review).

In this communication we describe results of studies using duroquinol as an electron donor in spinach chloroplasts. Through the use of specific photosynthetic electron transfer inhibitors, it has been possible to show that duroquinol donates electrons to the plastoquinone pool in the non-cyclic electron transfer chain. It has also been possible to demonstrate electron transfer from duroquinol to terminal electron acceptors, such as oxygen, which is coupled to the synthesis of ATP and involves an electron transport chain which includes plastoquinone, the "Rieske" iron-sulfur center, cytochrome *f*, plastocyanin and *P*-700.

Materials and Methods

Chloroplasts were prepared from freshly picked greenhouse-grown spinach, as previously described [5,6]. The chloroplasts were washed once with 50 mM Tricine buffer (pH 8.2) containing 20 mM NaCl and resuspended in the same buffer. In some experiments it was necessary to add 2 mM potassium ferricyanide to the washing solution in order to obtain chloroplasts with the bound electron carriers in the oxidized state; such preparations were then resuspended in a final solution which contained buffer and NaCl but from which potassium ferricyanide was omitted.

Oxygen uptake was measured with a Rank oxygen electrode at 18° C. Air-saturated distilled water was used to calibrate the electrode. Samples (1.0 ml reaction mixture) were illuminated with red light (filtered through a Corning 2-64 glass filter) of intensity $5 \cdot 10^5$ ergs $\cdot$ cm$^{-2} \cdot$ s^{-1}. ATP determinations were carried out by the method of Hagihara and Lardy [7].

Cytochrome difference spectra were recorded at 25° C in an Aminco DW-2 spectrophotometer operating in the split beam mode. Spectra of chloroplast samples (containing 50—75 μg chlorophyll per ml) were recorded from 540 to 580 nm at a scan rate of 1 nm per s and a slit width of 1.5 nm.

Electron paramagnetic resonance (EPR) spectra were recorded, as previously described [5,6,8], at 15 K in a modified JEOL X-band spectrometer equipped with an Airco liquid helium cryostat. Samples (0.3 ml) were incubated at 4° C with duroquinol either in the presence or absence of inhibitors for 2 min in the dark prior to freezing to 77 K in calibrated 3-mm quartz EPR tubes. It was possible to monitor the oxidation-reduction state of several different chloroplast electron carriers (*P*-700, the "Rieske" iron-sulfur center, and plastocyanin) on the same chloroplast sample by choice of suitable instrument settings.

Duroquinol was prepared from the oxidized compound, duroquinone (K and K Chemicals, Inc.), by making a 50 mM solution of duroquinone in degassed methanol and reducing the solution with a small amount of solid $NaBH_4$. The excess borohydride was then removed by adjusting the pH to 6 with a small amount of HCl. In some cases, the reduced compound was used directly (duroquinol, K and K Chemicals, Inc.) by dissolving in degassed methanol. The final concentration of methanol added to the chloroplast suspension never exceeded 1%. 2,5-Dibromo-3-methyl-6-isopropyl-1,4-benzoquinone (DBMIB), a gift from Professor Achim Trebst, was dissolved in methanol.

Results

Duroquinol can serve as an electron donor for the photoreduction of oxygen. In common with other artificial electron donor systems known to donate electrons through Photosystem I [9], the reduction of oxygen with duroquinol as the electron donor was insensitive to 3-(3,4-dichlorophenyl)-1,1-dimethylurea (DCMU), the well-characterized inhibitor of chloroplast non-cyclic electron transfer. Even at concentrations of DCMU as high as 5 μM, no inhibition of the rate of oxygen uptake was observed when duroquinol served as the electron donor. In contrast to the insensitivity to DCMU, the plastoquinone antagonist, DBMIB, inhibited oxygen uptake at relatively low concentrations. As shown in Fig. 1, 50% inhibition was obtained at an inhibitor concentration of approx. 0.1 μM. The inhibition of electron transfer was, however, not complete in that a residual rate of oxygen uptake (10—20% of the control rate) was observed even at DBMIB concentrations as high as 5 μM. This concentration of DBMIB was sufficient to inhibit the non-cyclic transfer of electrons from water to $NADP^+$ or to oxygen by over 95%.

Electron donation from duroquinol to non-cyclic electron acceptors is coupled to the synthesis of ATP. As shown in Fig. 2, ATP synthesis is also sensitive to DBMIB and insensitive to DCMU when duroquinol is functioning as the electron donor. A comparison of DBMIB inhibition of ATP synthesis with duroquinol (in the presence of DCMU) and water as electron donors shows that the reaction with water as donor is approximately two to three times less sensi-

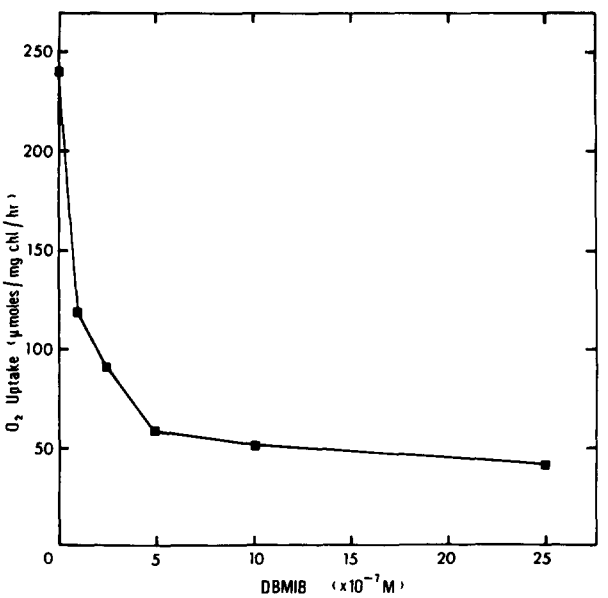


Fig. 1. Effect of DBMIB on light-dependent O_2 uptake with duroquinol as electron donor. The reaction mixture contained in a final volume of 1.0 ml: 100 mM Tricine buffer (pH 8.2), 1 μM DCMU, 3 mM NaN_3, 0.01 mM methyl viologen, 0.5 mM duroquinol, chloroplast fragments at a chlorophyll concentration of 50 μg per ml and, where present, the indicated concentration of DBMIB.

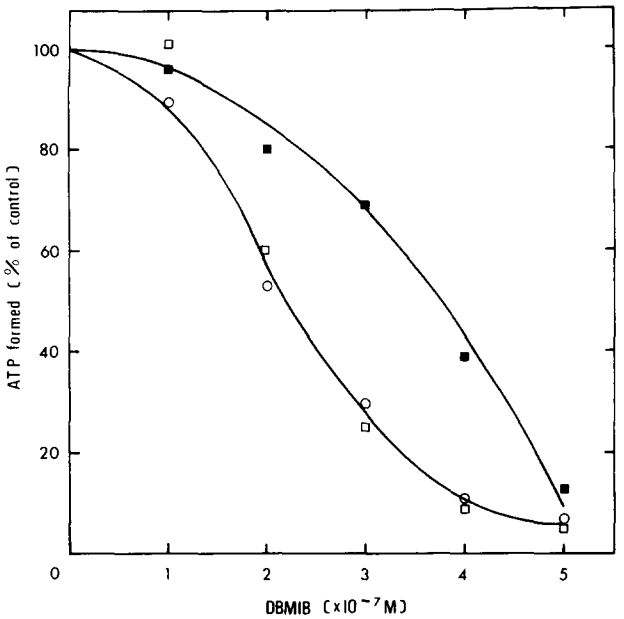


Fig. 2. Effect of DBMIB on non-cyclic ATP formation with water or duroquinol as electron donor. The reaction mixture contained in a final volume of 1.0 ml: chloroplast fragments at a chlorophyll concentration of 50 μg per ml, 100 mM Tricine buffer (pH 8.3), 5 mM $MgCl_2$, 5 mM ADP, 5 mM $K_2H^{32}PO_4$, 0.01 mM spinach ferredoxin and 5 mM $NADP^+$. Ascorbate, when added, was present at a concentration of 10 mM. When duroquinol (1.0 mM) was used as the electron donor, 10 μM DCMU was also added. The indicated amount of DBMIB was also present. Samples were illuminated for 10 min at 25°C with red light (Corning 2-58 filter, intensity of $2 \cdot 10^5$ ergs $\cdot$ $cm^{-2} \cdot s^{-1}$). Control rates of ATP formation were approx. 2 μmol ATP formed in 10 min with either electron donor. Electron donors: ■———■, H_2O; □———□, H_2O in the presence of ascorbate; ○———○, duroquinol.

tive to DBMIB than when duroquinol serves as the donor. When ascorbate is added to the system in which water serves as the electron donor, the DBMIB inhibition curve is shifted and is essentially the same as that obtained with duroquinol. The addition of ascorbate to the duroquinol system does not produce any significant effect.

In order to characterize the chloroplast electron transfer carriers involved in the transfer of electrons from duroquinol to electron acceptors, we have used optical and EPR spectroscopy to study these carriers in situ. In these experiments the effect of duroquinol on the reduction of carriers in the dark was examined and the effect of inhibitors on these dark reactions was studied. In such experiments, it was necessary to use chloroplast membrane fragments which had been washed with potassium ferricyanide in order to obtain carriers in the oxidized state in the dark prior to the addition of duroquinol. We have found that plastocyanin, cytochrome *f* and *P*-700 are in the reduced state in our chloroplast fragments and that the inclusion of 2 mM potassium ferricyanide to the washing solution is sufficient to oxidize all these carriers. The removal of ferricyanide from the fragments was tested for by measurements of oxygen evolution after washing and the demonstration of a ferricyanide requirement for this reaction in the washed fragments.

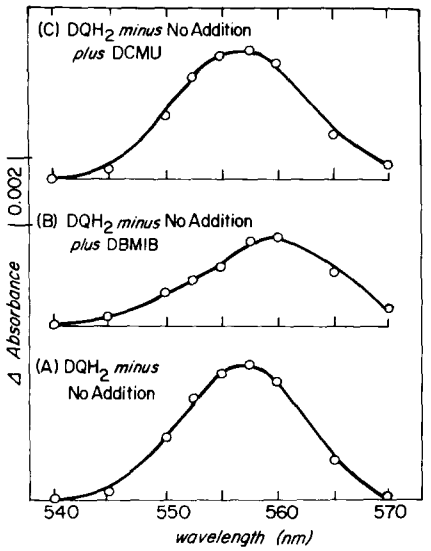


Fig. 3. Effect of duroquinol (DQH_2) and inhibitors on dark reduction of cytochromes. The reaction mixture contained in a final volume of 3.0 ml: 50 mM Tricine buffer (pH 8.2), 20 mM NaCl and ferricyanide-washed chloroplast fragments at a chlorophyll concentration of 50 μg per ml. Duroquinol (0.25 mM) was added to the sample cuvette in order to reduce the cytochromes. DBMIB or DCMU, when present, were added at concentrations of 2 and 1 μM, respectively.

As shown in Fig. 3A, the addition of duroquinol to chloroplasts results in the appearance of a reduced cytochrome absorbance band at approx. 556 nm. This peak originates from the superposition of the α-band maxima of chloroplast cytochrome *f* (α-band at 554 nm) and the high-potential form of cytochrome *b*-559 (α-band at 559 nm). Both cytochromes are normally in the reduced state in untreated chloroplasts and the ferricyanide washing procedure converts them to the oxidized state. The spectrum in Fig. 3A indicates both cytochromes are reduced in the dark by duroquinol. The effect of DBMIB on the duroquinol-induced reduction of cytochromes *f* and *b*-559 is shown in Fig. 3B. The reduction of cytochrome *f* is markedly inhibited by DBMIB as evidenced by the shift in the absorbance maximum to approx. 559 nm, indicating that the major absorbing species in the presence of duroquinol and DBMIB is cytochrome *b*-559. A quantitative evaluation of the extent of DBMIB inhibition of duroquinol-induced cytochrome *f* reduction is difficult from this figure because of spectral interference by cytochrome *b*-559. In several experiments, complete inhibition of cytochrome *f* reduction was observed in the presence of DBMIB and duroquinol as evidenced by the absence of absorption in the 550—540 nm region where there is little interference from the *b*-type cytochrome. In contrast to the results with DBMIB, DCMU addition had no effect on the reduction of cytochrome *f* by duroquinol (Fig. 3C).

The remaining electron carriers we examined were detected by their characteristic EPR signals at cryogenic temperatures after incubation of chloroplast samples with duroquinol at physiological temperatures. A study of the effect of

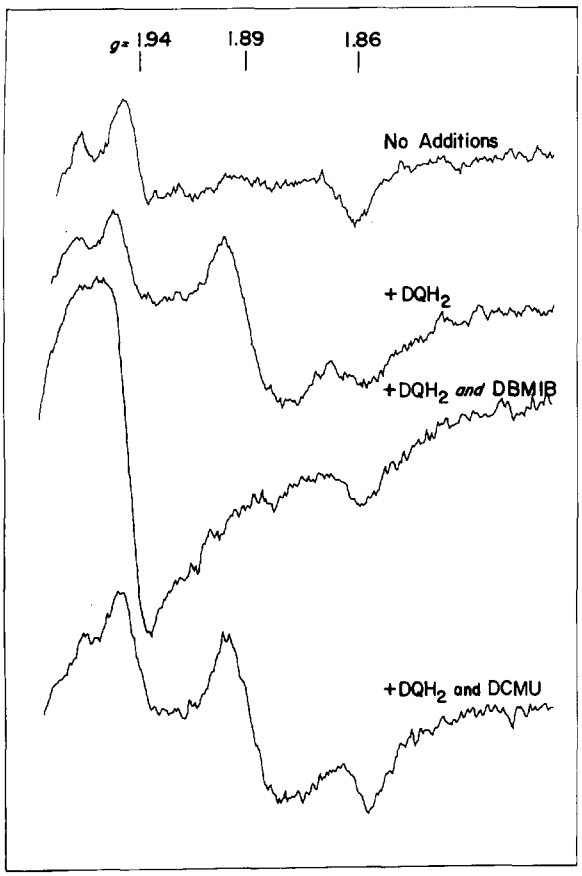


Fig. 4. Effect of duroquinol (DQH_2) and non-cyclic electron transfer inhibitors on the reduction of the $g = 1.89$ "Rieske" iron-sulfur center. The reaction mixture contained 0.5 ml of ferricyanide-washed chloroplast fragments at a chlorophyll concentration of 1 mg per ml. 0.25 μmol of duroquinol were added to the chloroplasts and the sample incubated in the dark for 2 min at 4°C prior to freezing to 77 K. Where present, DBMIB (0.05 μmol) or DCMU (0.04 μmol) were added prior to the duroquinol. EPR conditions: field setting, 3500 ± 250 G; modulation amplitude, 10 G; microwave power, 10 mW; temperature, 15 K; amplifier gain, 200.

duroquinol on the "Rieske" iron-sulfur center is shown in Fig. 4. This high-potential iron-sulfur center (E_m = +290 mV) is characterized by an EPR signal at $g = 1.89$ in the reduced state and has no signal in its oxidized state (ref. 10). Duroquinol is able to reduce the "Rieske" center in the dark as evidenced by the appearance of a signal at $g = 1.89$. The addition of DBMIB prior to duroquinol inhibits the reduction of the "Rieske" center although a new EPR signal at $g = 1.94$ does appear. This signal, which is of yet undetermined nature, appears after the addition of DBMIB to chloroplasts even in the absence of duroquinol, and further characterization of the interaction of DBMIB with chloroplasts is required before conclusions can be drawn as to its origin. Also shown in Fig. 4 is the finding that DCMU, in contrast to DBMIB, does not inhibit the reduction of the "Rieske" center by duroquinol. Thus, the pattern of behavior of the "Rieske" center in relation to duroquinol reduction is similar to that of cyto-

chrome *f*: the reduction of the "Rieske" center is sensitive to DBMIB but not to DCMU.

The oxidation-reduction state of plastocyanin in situ was monitored by following changes in the $g = 2.05$ EPR signal of oxidized plastocyanin (ref. 5). As shown in fig. 5, plastocyanin was oxidized before any additions because of the previous ferricyanide washing procedure. Duroquinol reduces plastocyanin as indicated by the disappearance of the $g = 2.05$ EPR signal. Also shown in Fig. 5 are the effects of DCMU and DBMIB: neither of these inhibitors affected the reduction of plastocyanin by duroquinol. Decreasing the duroquinol concentration by a factor of ten did not affect these results in that plastocyanin reduction remained insensitive to DCMU and DBMIB.

The remaining electron carrier which was monitored was *P*-700, the Photosystem I reaction center chlorophyll. This carrier is characterized in the oxidized state by a free-radical signal (Signal I) with a *g* value of 2.0026 and a linewidth of approx. 8 G (ref. 11). As shown in Fig. 6, in ferricyanide-washed chloroplasts a complex EPR signal in the $g = 2.00$ region is present in the dark. This signal has been shown to originate from two different chloroplast components: Signal I from oxidized *P*-700 and Signal II (*g* value of 2.0046; linewidth of approx. 20 G). The latter signal has been proposed to originate from a carrier which functions on the oxidizing side of Photosystem II (ref. 12). The addition of

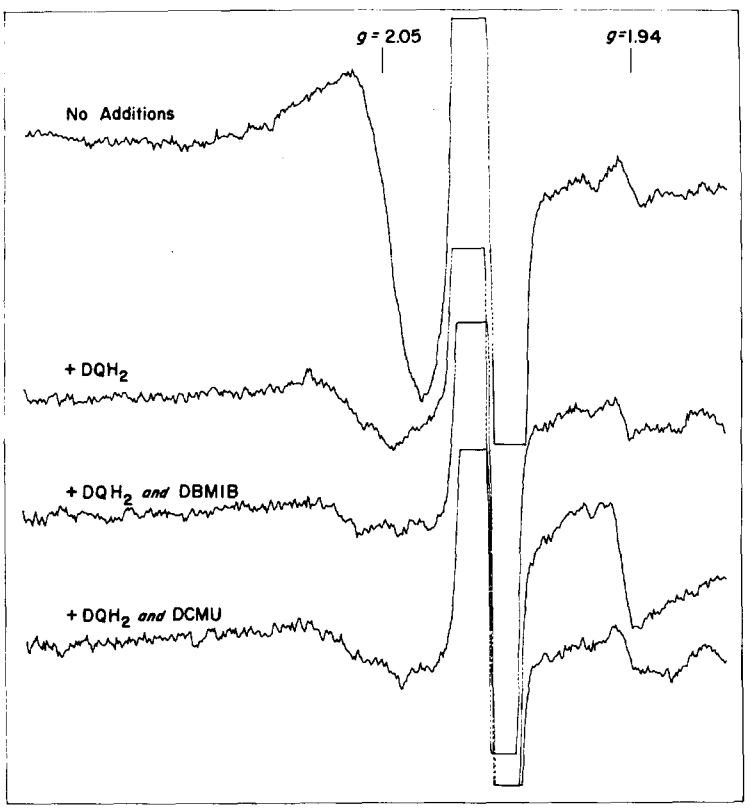


Fig. 5. Effect of duroquinol (DQH_2) and non-cyclic electron transfer inhibitors on the reduction of plastocyanin. The reaction conditions were as in Fig. 4. EPR conditions: field setting, 3300 ± 250 G; modulation amplitude, 10 G; microwave power, 10 mW; temperature, 15 K; amplifier gain, 120.

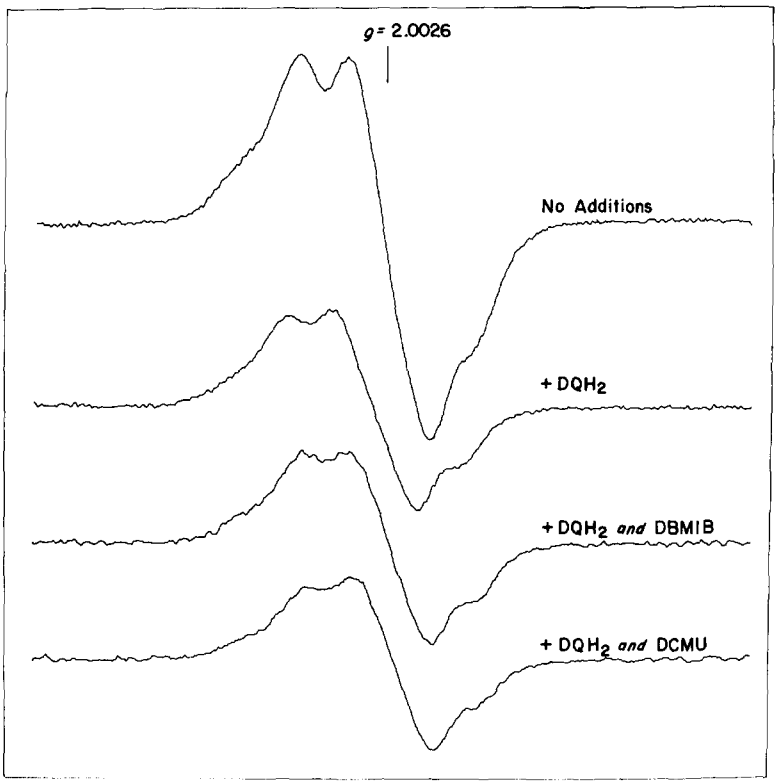


Fig. 6. Effect of duroquinol (DQH_2) and non-cyclic electron transfer inhibitors on the reduction of *P*-700. The reaction conditions were as in Fig. 4. EPR conditions: field setting, 3285 ± 50 G; modulation amplitude, 4 G; microwave power, 1 mW; temperature, 15 K; amplifier gain, 45.

duroquinol results in a reduction of the oxidized *P*-700 signal at $g = 2.0026$ and there is also a smaller effect on Signal II since there is some decrease in intensity of the low-field peak which arises mostly from Signal II. As in the case of plastocyanin, neither DCMU nor DBMIB inhibit the reduction of oxidized *P*-700 by duroquinol.

Discussion

In common with many other artificial electron donor systems known to function with chloroplasts, the light-induced transfer of electrons from duroquinol to electron acceptors is insensitive to DCMU. This finding indicates that the site of duroquinol electron donation is beyond the primary electron acceptor of Photosystem II since most evidence supports a site of action of DCMU between the Photosystem II primary electron acceptor and the secondary acceptor pool which is presumably plastoquinone [13,14]. In contrast to other electron donors which are also DCMU sensitive, such as reduced dichlorophenolindophenol or reduced diaminodurene, electron donation by duroquinol is sensitive to DBMIB. The only other reported donors which are sensitive to DBMIB while being insensitive to DCMU are thymoquinone [15] and plastoquinol [16] while other donor systems are insensitive to DBMIB [17]. The rates of electron trans-

fer with thymoquinone are quite low [15], being less than 10% of those we have found with duroquinol, and the reactions of the former have not been studied in detail. Plastoquinol has been used in chloroplast fragments treated with digitonin [16] and has been employed to characterize a plastoquinone-plastocyanin reductase system which may involve some of the electron carriers which we have considered in this work.

Our finding that duroquinol can donate electrons in untreated chloroplasts in a reaction which is insensitive to DCMU and sensitive to DBMIB indicates this donor donates electrons to plastoquinone. Such a conclusion is consistent with the reported oxidation-reduction midpoint potentials of duroquinol (E_m = +5 mV, pH 7.0, ref. 18) and plastoquinone (E_m = +80 mV, pH 7.0, ref. 19) which indicate that duroquinol is competent to reduce plastoquinone on the basis of its thermodynamic properties.

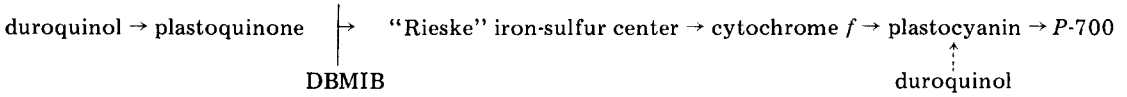
Electron transfer from duroquinol to terminal electron acceptors, such as oxygen, is coupled to the synthesis of ATP, and the coupled phosphorylation is sensitive to DBMIB. This suggests that, in the terminology of Trebs and co-workers [9,15], a "native" chloroplast coupling site is involved in energy transduction as opposed to an "artificial" energy-conserving site which involves proton-transporting electron donors, such as reduced diaminodurene [9,15]. However, since duroquinol itself can function as a proton carrier, this conclusion must be considered as tentative and further studies on the relationship between ATP synthesis and duroquinol oxidation are being undertaken.

In comparing the DBMIB sensitivity of non-cyclic electron transfer reactions, we have consistently observed that the duroquinol reactions are at least two times more sensitive to this inhibitor than are reactions with water as electron donor. The inhibition curves are similar, however, when ascorbate is present. This result may be related to the finding that under reducing conditions, DBMIB has been reported to become a more effective inhibitor of various electron transfer processes [20—22]. Under our conditions duroquinol would then keep DBMIB in the reduced state and increase its efficiency as an inhibitor of non-cyclic electron transfer processes. The similarity of the inhibition curves with duroquinol and water (in the presence of ascorbate) as electron donors suggests that both donors are utilizing a common portion of the electron transport chain and duroquinol does not utilize any alternate pathway involving plastoquinone.

In the spectroscopic studies on the effect of duroquinol on the reduction of in situ chloroplast electron carriers, we observe that the reduction of both cytochrome *f* and the "Rieske" iron-sulfur center are inhibited by DBMIB. This pattern suggests that electrons from duroquinol which reduce these carriers pass through the chloroplast plastoquinone pool. On the basis of their midpoint oxidation-reduction potentials, one might expect the "Rieske" iron-sulfur center (E_m = +290 mV, ref. 10) to function on the reducing side of cytochrome *f* ($E_m \approx$ +350—380 mV, refs. 23—25). The results on duroquinol reduction of the "Rieske" iron-sulfur center would be the first indication that this carrier functions in the chloroplast non-cyclic electron transfer chain.

The reduction of plastocyanin and *P*-700 by duroquinol has been found to be insensitive to DBMIB. It would appear that a direct reduction of these car-

riers can occur which does not involve plastoquinone. This view would be consistent with the residual rate of DBMIB-insensitive electron transfer from duroquinol to oxygen which has been observed (see Fig. 1). Accordingly, plastocyanin and *P*-700 can interact with duroquinol directly in the presence of DBMIB while cytochrome *f* and the "Rieske" iron-sulfur center can only accept electrons from duroquinol through the plastoquinone pool. On the basis of these results, a linear electron transport chain, as shown below, can be proposed:



The finding that duroquinol can directly reduce plastocyanin while the reduction of cytochrome *f* and the "Rieske" iron-sulfur center involve plastoquinone suggests that the accessibility of plastocyanin in the chloroplast membrane to electron donors is greater than that of the other electron carriers. There are presently some conflicting results on the location of plastocyanin in the chloroplast membrane [26—28], and our results on its interaction with duroquinol would be consistent with at least a partial accessibility of this carrier at the chloroplast membrane surface.

Acknowledgements

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