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Localization of Electron Transport Inhibition in Plastoquinone Reactions

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Abstract

Reduction kinetics of P700 following a short flash are measured in spinach chloroplasts after oxidation of the electron carriers between the two photoreactions by far-red light. Three features of the kinetics allow us to localize simultaneously inhibition at different sites between photoreaction II and the reducing site of plastoquinol. These are the initial lag, the halftime, and the area under the transient of the P700 absorbance change, which indicate the electron transfer time from photoreaction II to the reducing site of plastoquinol, the rate of plastoquinol oxidation, and the number of electrons transferred to the special plastoquinone B functioning as secondary electron acceptor of photosystem II, respectively. As an additional diagnostic parameter for inhibition before and after the plastoquinone pool, the area under the transient of the P700 absorbance change is used after long flashes. This area is proportional to the amount of reduced plastoquinone as shown by the absorbance change at 265 nm. The effects of 3-(3′,4′-dichlorophenyl)-1,1-dimethylurea (DCMU) and 2,5-dibromo-3-methyl-6-isopropyl-*p*-benzoquinone (DBMIB) are compared with those of 2-bromo-4-nitrothymol, 2,4-dinitrophenyl ether of 2-iodo-4-nitrothymol, and Illoxan as representatives for new classes of inhibitors. While 2-halogeno-4-nitrothymols inhibit the reduction of plastoquinone similarly to DCMU, their diphenyl ether derivatives inhibit selectively the oxidation of plastoquinol.

Key Words: Plastoquinone; P700; electron transport; inhibition; dinitrophenyl ether of iodo-nitrothymol; chloroplasts.

Introduction

Photosynthetic electron flow between the two photoreactions can be inhibited at various sites (for a review, see Trebst, 1980). For studies of proton transfer across the thylakoid membrane, inhibitors which block electron transfer reactions close to the plastoquinone pool are of particular interest. Inhibition

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before the plastoquinone pool between the primary, Q, and the secondary electron acceptor, B, of photosystem II is known in a large variety of compounds which include 3-(3′,4′-dichlorophenyl)-1,1-dimethylurea (DCMU)[2] (see Trebst, 1980). Recently alkyl-substituted 2-halogeno-4-nitrophenols with chemical parameters different from those of the other compounds have been shown to bind and inhibit at the same site as DCMU (Trebst, 1979). The diphenylether of these compounds are found to inhibit after the plastoquinone pool, as is known for 2,5-dibromo-3-methyl-6-isopropyl-benzoquinone (DBMIB) (Böhme et al., 1971). Additional inhibition by the diphenylethers before the plastoquinone pool has been suggested by some experiments (Trebst *et al.*, 1978). A variety of methods are standard for localizing inhibition sites, but there is none which will distinguish between several sites in just one experiment.

A localization of several inhibition sites is possible from the reduction kinetics of P700. (i) After a short flash the reduction of P700 was found to be delayed by 2–3 ms (Haehnel, 1976). This is caused by the electron transfer from photoreaction II to the site of plastoquinol oxidation. (ii) The oxidation of plastoquinol is rate-limiting for electron transfer from photosystem II to photosystem I and controls the reduction of P700 (Stiehl and Witt, 1969; Haehenl, 1973). (iii) The reduction of P700 after a flash is transitory at continuous far-red light. The area bound by the corresponding time course of the P700 absorbance change has been shown to indicate the activity of photosytem II (Rumberg, 1964) as well as the electrons accumulated by the pool of electron carriers between the two photoreactions (Malkin, 1968).

In the present study these properties of the P700 absorbance change are used to localize the inhibition of 2-halogeno-4-nitrophenols and their diphenyl ether derivatives.

Materials and Methods

Spinach chloroplasts were prepared as described previously (Nelson *et al.*, 1970). The reaction mixture contained these chloroplasts suspended at a chlorophyll concentration of 30 μM, 20 mM KCl, 3 mM $MgCl_2$, 20 mM Tricine-NaOH-buffer, pH 7.6, 0.1 mM anthraquinone-2-sulfonate as electron acceptor, 1.5 μM gramicidin D as uncoupler, and inhibitors as indicated. The temperature in the 1 × 1 cm cuvette was 20–22°C.

The absorbance change of P700 was monitored at 703 nm (FWHM = 2.2 nm) with a silicon photocell SGD 444 (EG&G), amplified (AM502,

[2]The abbreviations used are: BNT, 2-bromo-4-nitrothymol; DBMIB, 2,5-dibromo-3-methyl-6-isopropyl-*p*-benzoquinone; DCMU, 3-(3′,4′-dichlorophenyl)-1,1-dimethylurea; DNP-INT, 2-iodo-6-isopropyl-3-methyl-2′,4,4′-trinitrodiphenyl ether.

Tektronix), and stored in a signal averager TN-1500 (Tracor). The intensity of the measuring light of 703 nm (FWHM = 5 nm) was 1.5 W/m^2. Excitation with short flashes was with a xenon flash (t_{10-90} = 6 μs) passed through a Schott filter BG 23/6 mm. Long flashes were obtained from the light of a 250-W tungsten halide lamp passed through 7 cm of water, a Calflex C (Balzers), and a BG 23/6 mm (Schott) filter with an intensity of 340 W/m^2 and controlled by an electronic shutter (Prontor electronic m). Additional far-red light of 716 nm (FWHM = 11 nm) with an intensity of 4 W/m^2 was used in the experiments with long flashes. The electrical bandwidth was chosen in all experiments close to the distance between the addresses of the signal averager.

Results and Discussion

In order to localize inhibition sites, complementary measurements of P700 absorbance changes were carried out. In all experiments most of P700 was oxidized first by continuous far-red light. The subsequent time course of the P700 absorbance change induced either by a short or a long flash was monitored at 703 nm. A high intensity of the far-red monitoring light has the additional advantage of a high signal-to-noise ratio.

Figure 1 shows the kinetics of P700 induced by a short flash at three different time scales and illustrates the parameters investigated. These parameters are affected specifically by inhibitors and allow us to localize different inhibition sites in linear electron transport as detailed below. The short flash oxidizes residual P700 as indicated by the negative absorbance change in Fig. 1.

(A) The positive amplitude after the flash (Fig. 1) is caused by reduction of $P700^+$ by electrons from photosystem II (Kok and Hoch, 1961; Rumberg, 1964). At a constant intensity of the far-red light the area below the positive transient marked in Fig. 1A is proportional to the number of electrons produced by photosystem II (Malkin, 1968). Inhibition between the donors of P680 and the special plastoquinone B (cf. region marked with A in the scheme) will diminish this area.

(B) The half-time of $P700^+$ reduction shown in Fig. 1B is caused by the electron transfer via the rate-limiting oxidation of the plastoquinone pool (Stiehl and Witt, 1969; Marsho and Kok, 1970) by the cytochrome f/b_6 complex. Inhibition of this reaction will increase the half-time of the $P700^+$ reduction. Similar effects caused by low proton concentration inside the thylakoid (Rumberg and Siggel, 1969) were avoided by addition of the uncoupler gramicidin D.

(C) The initial lag in the reduction of $P700^+$ (Haehnel, 1976), as shown

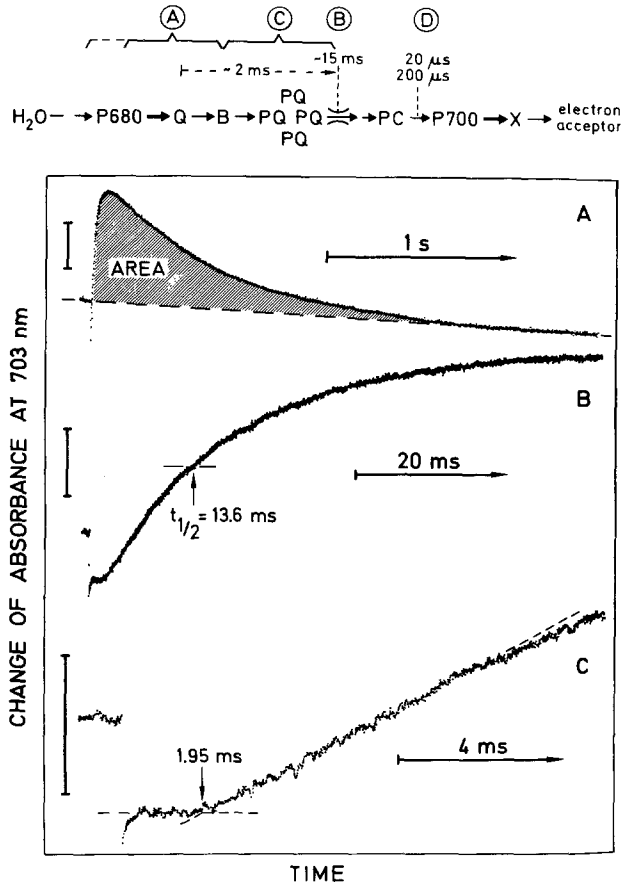

Fig. 1. Above: Simplified scheme of photosynthetic electron transfer reactions. The characteristic half-times and the site of the reactions which affect the $P700^+$ reduction are indicated. A, B, C, and D indicate respective sites of inhibitors with distinct effect on the P700 kinetics. Further details are given in the text in the paragraphs marked with the same letters. Below: Kinetics of P700 at 703 nm induced by a short flash in spinach chloroplasts. The vertical bars represent an absorbance change of $\Delta I/I = 10^{-3}$. (A) Complete time course of the reduction and the slow reoxidation by the monitoring light. The area below the positive transient is indicated. Ten signals induced at intervals of 3.5 s were averaged. (B) Reduction kinetics of $P700^+$. The half-time of 13.6 ms in this control experiment is indicated. Fifty signals induced at intervals of 5 s were averaged. The flash-induced fluorescence was subtracted without monitoring light. The dwell time was 17 µs/address. (C) Initial delay in the reduction kinetics of $P700^+$ shown in Fig. 1 B. A lag of 1.95 ms is found as the intercept of the tangent at the point of inflection. The small negative spike immediately after the flash is probably due to the absorbance changes of P680. Abbreviations: Q, primary acceptor of photosystem II; B, secondary acceptor of photosystem II; PQ, plastoquinone pool; PC, plastocyanin; X, complex of primary electron acceptors of photosystem I.

in Fig. 1C, results from the function of $P700^+$ as the terminal electron acceptor of the consecutive electron transfer reactions from photosystem II. The duration of about 2 ms of the lag is due to all electron transfer steps before the rate-limiting oxidation of plastoquinol (Haehnel, 1976). Therefore, an increase in the lag will indicate inhibition after B of the proton uptake and possibly of the mechanism of electron/proton transfer to the inside of the thylakoid and to the cytochrome f/b_6 complex. Inhibition at these sites is not easily detected by other techniques. Inhibitors acting either between P680 and B or directly on B will not cause an increase in the lag because each photosystem II functions separately in this region. The inhibitor would block the particular inhibition site but would not slow down reactions at active sites.

(D) A prerequisite for the kinetics shown in Fig. 1 is an oxidation of P700 as well as of its immediate electron donors before the flash. The reduced electron donors would reduce $P700^+$ with half-times of 20 and 200 μs (Haehnel *et al.*, 1980). It should be mentioned that inhibition of plastocyanin can be recognized from a decrease of these fast reduction kinetics, and inhibition of P700 itself or of its primary electron acceptors, from a decrease of the total flash-induced amplitude.

Complementary to a localization of inhibition sites from the kinetics of P700 after one flash is a localization of the amount of plastoquinol accumulated during a long flash. Inhibition of electron transport before the plastoquinone pool will decrease the amount of accumulated plastoquinol, while inhibition of the oxidation of plastoquinol will increase this amount. The area under the P700 absorbance change during oxidation by far-red light should be proportional to this amount of plastoquinol. In order to examine this relation, the area as a function of the number of flashes of a flash group is compared with the amplitude of the absorbance change of plastoquinol at 265 nm and with the amount of oxygen evolved.

The results in Fig. 2 show that the amount of reduced plastoquinone indicated by the absorbance change at 265 nm is proportional to the area under the P700 absorbance change. This is consistent with previous measurements of the fluorescence induction and the P700 oxidation kinetics (Malkin, 1968). The relative area seems to be slightly larger than the relative amplitude of the plastoquinol absorbance change after a group with many flashes, possibly because some plastosemiquinone, Q^- and B^-, is in equilibrium with the reduced plastoquinone pool. After more than two flashes of a group the relative oxygen yield exceeds the amount of plastoquinol. This is due to the electron transfer during the flash group from plastoquinol to the electron carriers of photosystem I with a half-time of about 10 ms (Stiehl and Witt, 1969).

The approach to a steady state of the plastoquinol concentration in the

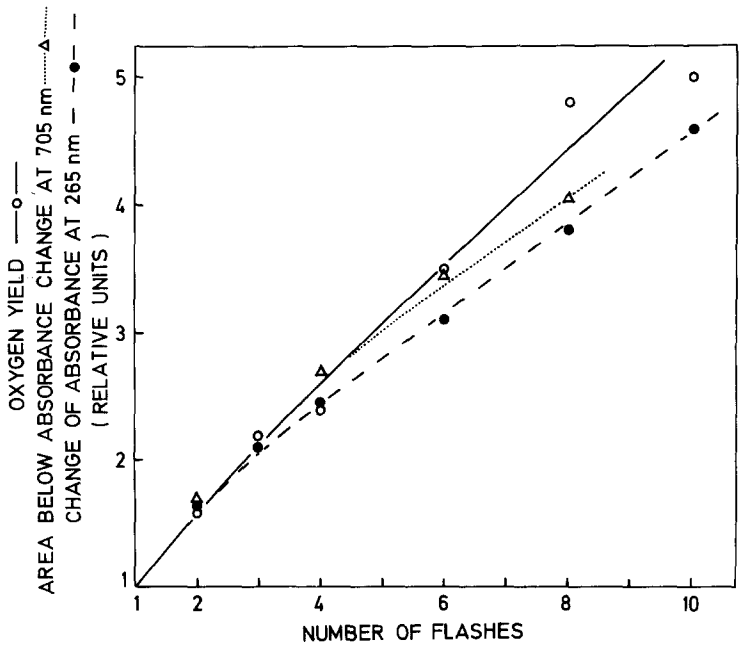

Fig. 2. Oxygen yield (open circles, solid line), amplitude of the absorbance change of plastoquinol at 265 nm (filled circles, dashed line), and area under the positive absorbance change of P700 at 705 nm (triangles, dotted line), at continuous illumination with far-red light as a function of the number of flashes of a flash group. The values are normalized to the value found after one flash. The distance between the individual flashes of saturating intensity was 1.6 ms. The repetition rate of the flash groups was 0.2 Hz. The intensity of the far-red light of 720 nm was 10 W/m^2. The absorbance changes were measured as described (Haehnel, 1973). The oxygen yields were determined by Dr. G. Renger under conditions almost identical to those of the measurements of the absorbance changes.

pool during long flashes of increasing duration was measured as the increasing area under the time course of P700 oxidation and is shown in Fig. 3. From this curve a flash duration of 33 ms was chosen for the inhibitor studies because maximal effect of both inhibition sites before and after the plastoquinone pool on the amount of plastoquinol is expected at partial reduction of the pool.

Localization of Inhibition Sites of Various Inhibitors

Figure 4 shows on the left-hand side the effects of DCMU on electron transport as control experiment. A 50% inhibition of photosystem II is found from the area under the P700 absorbance change after a single flash at a concentration of 25 nM DCMU, in agreement with previous measurements of the oxygen yield (Siggel *et al.*, 1972). The twofold higher concentrations

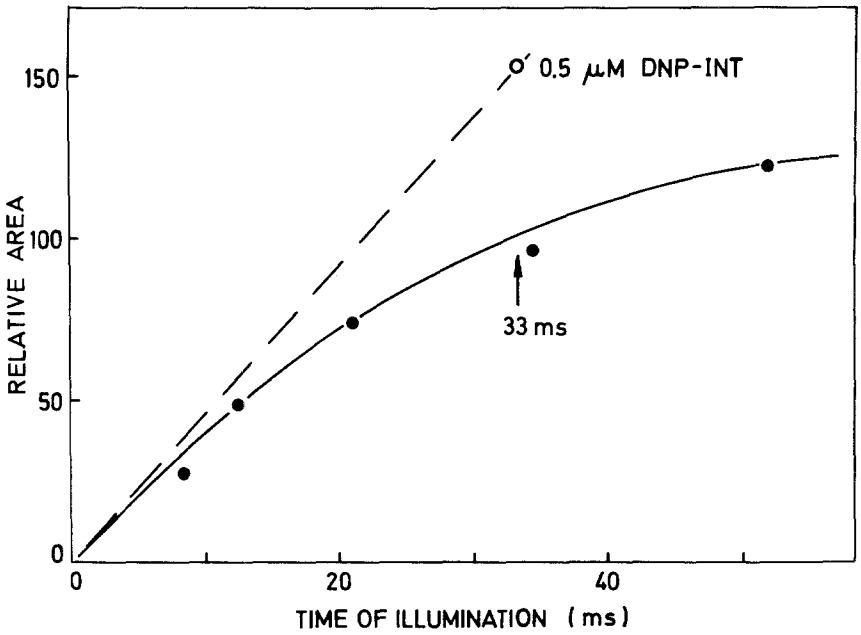

Fig. 3. Area below the absorbance change at 703 nm after illumination with long flashes of increasing duration. The area was estimated as shown in Fig. 1A. Ten signals were averaged with a repetition rate of 0.18 Hz. The arrow at 33 ms indicates the flash duration used in the inhibitor studies. The open circle gives the area found in the presence of 0.5 μM DNP-INT. For further details see text.

necessary for 50% inhibition in a long flash (Fig. 4, bottom) compared to that in a short flash is in agreement with the results of Siggel *et al.* (1972). This is caused by the cooperation of several photosystems II via a common pool of plastoquinone with several photosystems I. The constant half-time of the $P700^+$ reduction allows us to exclude any effect of DCMU on the oxidation of plastoquinol.

The phenolic inhibitor BNT has been shown by functional studies as well as by displacement experiments (Trebst, 1979) to inhibit in a similar way as DCMU. Its effects on the electron transport measured via the P700 kinetics are the same as those of DCMU, except for a slight additional inhibition of the oxidation of plastoquinol of about 30% at 200 nM BNT, which has also been found by other measurements (Trebst, 1981). A 50% inhibition of photosystem II was found at 60 nM BNT, in agreement with previous results (Trebst, 1979).

Illoxan is a diphenylether inhibiting electron transport which does not replace inhibitors of the DCMU type (Trebst, 1979). Nevertheless, it does inhibit 50% of photosystem II at 100 μM (Fig. 4, top). But the rate-limiting oxidation of plastoquinol is also inhibited to 50% at the slightly higher

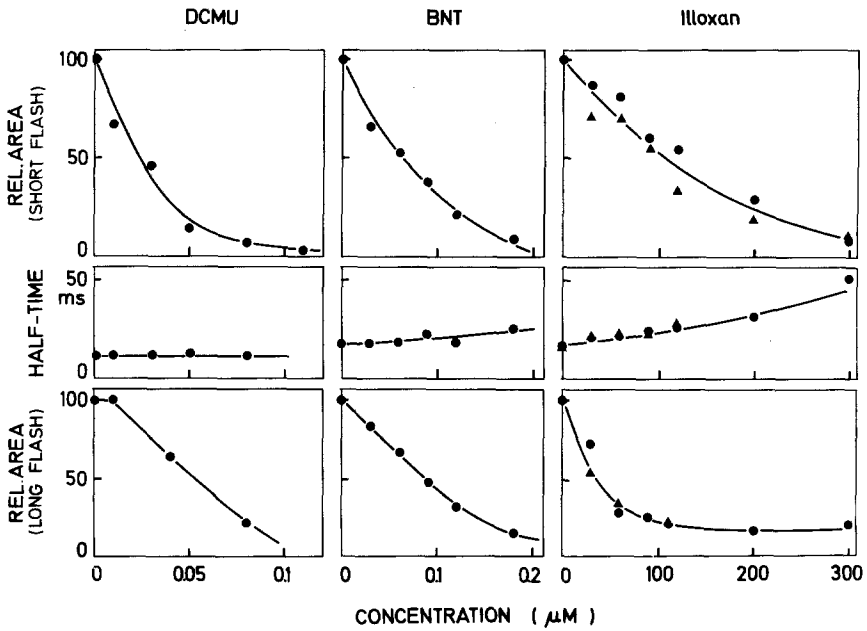

Fig. 4. Inhibition of electron transfer steps between the two light reactions by DCMU (left-hand side), BNT (middle), and Illoxan (right-hand side) as measured by the absorbance change of P700 at 703 nm. Top, relative area after a short flash, determined as in Fig. 1A. Middle, half-time of the electron transfer via the rate-limiting step, determined as in Fig. 1B. Bottom, relative area below the positive transient after a long flash of 33 ms (cf. Fig. 3). Illoxan is a trademark of Hoechst, Federal Republic of Germany.

concentration of 180 μM (Fig. 4, middle). Therefore, this inhibitor acts on both the reduction and the oxidation of the plastoquinone pool.

DBMIB is well known as a specific inhibitor of the oxidation of the plastoquinone pool, and inhibition at the DCMU site can be excluded in the low concentration range investigated (Trebst, 1980). The decrease in the area below the P700 transient after a short flash in Fig. 5, top, has to be ascribed to the function of DBMIB as an electron acceptor between the photoreactions.

When DBMIB inhibits the rate of plastoquinol oxidation (cf. Fig. 5, middle) one should expect an increase of the area after a long flash. Because of the electron acceptor property of DBMIB, this is not observed (Fig. 5, bottom). Direct measurements of plastoquinone have shown an increase in reduced plastoquinone during a long flash when DBMIB is added (Haehnel, 1977). Addition of 0.1 μM DBMIB increases the half-time of P700 reduction twofold, in agreement with the value found for the inhibition of linear electron transport (Böhme *et al.*, 1971).

The results with DNP-INT in Fig. 5 indicate its selective action as an inhibitor at the site of plastoquinol oxidation. The effect of 1 μM DNP-INT

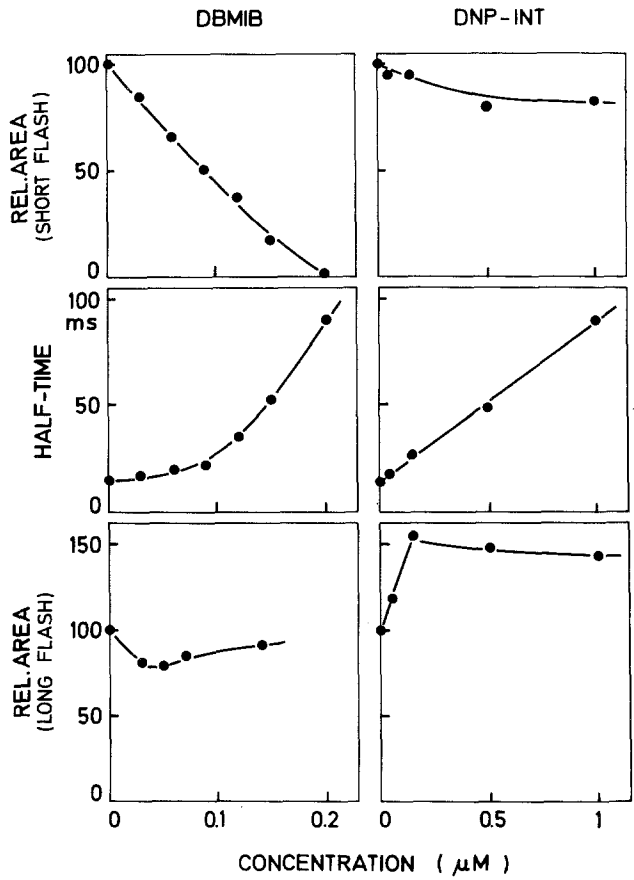

Fig. 5. Inhibition of electron transfer steps between the two light reactions by DBMIB (left-hand side) and DNP-INT (right-hand side). Other details as in Fig. 4.

on the amount of electrons transferred from photosystem II to P700 after a short flash is not greater than 20%.

In contrast to DBMIB, DNP-INT cannot function as an electron acceptor. The half-time of $P700^+$ reduction increases linearly with the concentration of the inhibitor and is doubled in the presence of 0.2 μM DNP-INT. If the total amount of the added inhibitor is presumed to be bound specifically, this indicates one inhibition site per 300 chlorophyll molecules or approximately one inhibition site per electron transport chain (cf. Trebst *et al.*, 1978). The amount of plastoquinol accumulated during the long flash is increased to a maximal amount of 150% at 0.2 μM and higher concentrations of DNP-INT. Under these conditions the rate of plastoquinol oxidation should

be negligible compared to the rate of plastoquinone reduction. This is indicated in Fig. 3 by the amount of plastoquinol accumulated in the presence of 0.5 μM DNP-INT, which is reached during the flash of 33 ms if the reduction occurs with the maximal initial rate without loss of electrons. Compared to DBMIB the advantage of DNP-INT is that it is not a redox compound and will therefore neither accept electrons nor show interfering absorbance changes.

None of the inhibitors increases the lag of 2.0 ($\pm$0.2) ms of the $P700^+$ reduction after a short flash (Fig. 1C) (Illoxan was not tested). DCMU seems to shorten the lag slightly to 1.6 ms. Thus, inhibition of intermediate steps in the plastoquinone pool could not be detected.

Acknowledgments

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References

Böhme, H., Reimer, S., and Trebst, A. (1971). *Z. Naturforsch. Teil B* **26,** 341–351.
Haehnel, W. (1973). *Biochim. Biophys. Acta* **305,** 618–631.
Haehnel, W. (1976). *Biochim. Biophys. Acta* **440,** 506–521.
Haehnel, W. (1977). In *Bioenergetics of Membranes* (Packer, L., Papageorgiou, G. C., and Trebst, A., eds.), Elsevier, Amsterdam, pp. 317–328.
Haehnel, W., Pröpper, A., and Krause, H. (1980). *Biochim. Biophys. Acta* **593,** 384–399.
Kok, B., and Hoch, G. (1961). In *Light and Life* (McElroy, W. D., and Glass, B., eds.), Johns Hopkins Press, Baltimore, p. 197.
Malkin, S. (1968). *Biochim. Biophys. Acta* **162,** 392–401.
Marsho, T. V., and Kok, B. (1970). *Biochim. Biophys. Acta* **227,** 240–250.
Nelson, N., Drechsler, Z., and Neumann, J. (1970). *J. Biol. Chem.* **245,** 143–151.
Rumberg, B. (1964). *Z. Naturforsch. Teil B* **19,** 707–716.
Rumberg, B., and Siggel, U. (1969). *Naturwissenschaften* **56,** 130–132.
Siggel, U., Renger, G., Stiehl, H. H., and Rumberg, B. (1972). *Biochim. Biophys. Acta* **256,** 338–345.
Stiehl, H. H., and Witt, H. T. (1969). *Z. Naturforsch. Teil B* **24,** 1588–1598.
Trebst, A. (1979). *Z. Naturforsch. Teil C* **34,** 986–991.
Trebst, A. (1980). *Methods Enzymol.* **69,** 675–715.
Trebst, A. (1981). In *Quinones in Electron Transport* (Trumpower, B. L., ed.), Academic Press, New York, in press.
Trebst, A., Wietoska, H., Draber, W., and Knops, H. J. (1978). *Z. Naturforsch. Teil C* **33,** 919–927.