Reinvestigation of the Effects of Disalicylidenepropanediamine (DSPD) and 2-Heptyl-4-hydroxyquinoline-N-oxide (HQNO) on Photosynthetic Electron Transport

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The effects of disalicylidenepropanediamine (DSPD) and 2-heptyl-4-hydroxyquinoline-N-oxide (HQNO) on photosynthetic electron transport have been reexamined.

The results confirm earlier observations that lower concentrations of DSPD (< 100 μM) block electron transport at the levels of ferredoxin and plastocyanin. High concentrations of DSPD even inhibit electron transport from $H_2O \rightarrow$ pBQ, suggesting that DSPD has an inhibitory site in PS II as well. Thermoluminescence curves of DSPD and DCMU treated chloroplasts were very similar, showing that the third inhibitory site of DSPD is similar to that of DCMU.

Both oxidized and reduced HQNO, (0.6–6 μM) blocked electron transport from $H_2O \rightarrow$ pBQ, $H_2O \rightarrow$ MV/FeCy to a similar extent. The effect of HQNO on thermoluminescence showed that its inhibitory site is probably located before that of DCMU. At higher concentration (> 6 μM), the $H_2O \rightarrow$ MV/FeCy reactions were more strongly inhibited by oxidized HQNO than those occuring from $H_2O \rightarrow$ pBQ, suggesting that a new site of inhibition must also be considered.

The dark decay of the P 700 signal was not influenced by the addition of oxidized HQNO which shows that the new inhibitory site of HQNO is located between plastoquinone and P 700.

The reduced form of HQNO did not inhibit non-cyclic electron transport around PS I. Indeed, at higher concentrations, reduced HQNO even accelerates electron flow from DCIP → MV and the dark reduction of P 700, thus suggesting that this compound has a "donor-mediator" function in PS I.

Introduction

As an inhibitor of photosynthetic electron transport DSPD was first introduced by Trebst and Burba [1]. It has long been known that DSPD is a specific inhibitor of PS I at the level of ferredoxin [1–5]. A recent study has indicated that DSPD is also an effective inhibitor of photosynthetic electron flow between PS II and PS I before or at PC [6].

HQNO blocks non-cyclic electron transport at a site similar to that inhibited by DCMU [7, 9], though Izawa *et al.* have suggested that the inhibitory sites of HQNO and DCMU are somewhat different [10]. Another site of inhibition of HQNO also exists. Hind and Olson have proposed that the inhibitor blocks electron flow between cytochrome b_6 and PS I [11].

The purpose of this work was to reinvestigate in detail the effects of DSPD and HQNO on photosynthetic electron transport by measurement of thermoluminescence and photosynthetic O_2 evolution and uptake. Evidence was obtained which indicates that DSPD inhibits electron flow not only at the level of ferredoxin and PC but also at a third inhibitory site in PS II. HQNO inhibits the acceptor side of PS II but the sites of action of these two inhibitors are not exactly the same. Moreover, HQNO has another inhibitory site, namely in the non-cyclic electron flow between PQ and P 700. In addition, reduced HQNO can act as a donor to PS I. According to our results the use of DSPD and HQNO as specific inhibitors of photosynthetic electron transport has to be questioned.

Abbreviations: asc, ascorbate; pBQ, p-benzoquinone; cyt f, cytochrome f; DCIP, 2,6-dichlorophenolindophenol; DCMU, 3-(3,4-dichlorophenyl)-1,1-dimethylurea; DSPD, disalicylidenepropanediamine; FeCy, ferricyanide; Fd, ferredoxin; HEPES, N-2-hydroxyethylpiperazine-N′-2-ethane sulphonic acid; HQNO, 2-heptyl-4-hydroxyquinoline-N-oxide; MV, Methylviologen; PC, plastoquinone; PS I, PS II, photosystem I and II; P 700, P 680, reaction center of PS I and II; Q, primary acceptor of PS II; R, secondary acceptor of PS II.

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Materials and Methods

Isolation of chloroplasts

Intact chloroplasts were isolated from mesophyll protoplasts prepared by enzymatic digestion of the

first leaves of maize (*Zea mays* KSC 360) according to the method described previously [12]. Chloroplasts were resuspended in a medium containing 0.4 M d-sorbitol, 10 mM NaCl, 1 mM $MnCl_2$, 5 mM $MgCl_2$, 2 mM EDTA, 0.4% bovine serum albumin and 50 mM HEPES at pH 7.5 [13].

Measurement of photosynthetic oxygen evolution and uptake

The rate of photosynthetic oxygen evolution and uptake was measured by using a Clark-type electrode (Rank Brothers, Cambridge, U.K.) in a temperature controlled cuvette at + 25 °C under saturating white light. The assay medium contained 0.1 M d-sorbitol, 10 mM K_2HPO_4, 20 mM NaCl, 4 mM $MgCl_2$, 2 mM EDTA, 50 mM HEPES, pH 7.5 and chloroplasts carrying 50 µg chlorophyll in a final volume of 3.0 ml [13].

Different parts of the electron transport chain were studied by addition of electron acceptors and donors: 2 mM FeCy or 0.1 mM MV (PS II + PS I), 0.25 mM pBQ (PS II), 2 mM asc + 40 µM DCIP and 0.1 mM MV supplemented with 0.1 mM NaN_3 and 2 µM DCMU (PS I). The whole electron transport chain from $H_2O \rightarrow NADP^+$ was measured by adding 0.2 mg Fd and 0.25 mM $NADP^+$ to the sample.

Measurement of thermoluminescence

The chloroplast suspension was diluted to a final chlorophyll concentration of 170 µg/ml and it contained 65% glycerol. Aliquots (0.6 ml) of the suspension were used for measurement of thermoluminescence.

Measurement of glow curves was carried out in the temperature region from −80 °C to +80 °C using an apparatus similar to that described by Tatake *et al.* [14]. The light emission of the samples was measured by a red sensitive photomultiplier (EMI 9558 B) and the signal was amplified through a home-made differential amplifier and fed to a Philips PM 8120 X-Y recorder. The temperature of the sample holder was monitored using a platinum resistor thermometer placed below the samples. Samples were illuminated with white light from a NARVA halogen lamp of 650 W for 5 minutes during continuous cooling from +20 °C to −80 °C. The exciting light was passed through a heat-absorbing water filter (thickness 10 cm) and a Balzers neutral density filter giving an illumination intensity of 10 Wm^{-2}. For the best resolution of peaks the rate of heating was 10 °C per min, as used by Sane *et al.* [15].

Measurement of light induced absorbance change of P 700

The chloroplasts were slightly sonicated and diluted to a final chlorophyll concentration of 10 µg/ml.

Absorbance changes were measured at 700 nm with an AMINCO DW-2 UV-VIS spectrophotometer. Samples were illuminated with saturating blue light (9 Wm^{-2}).

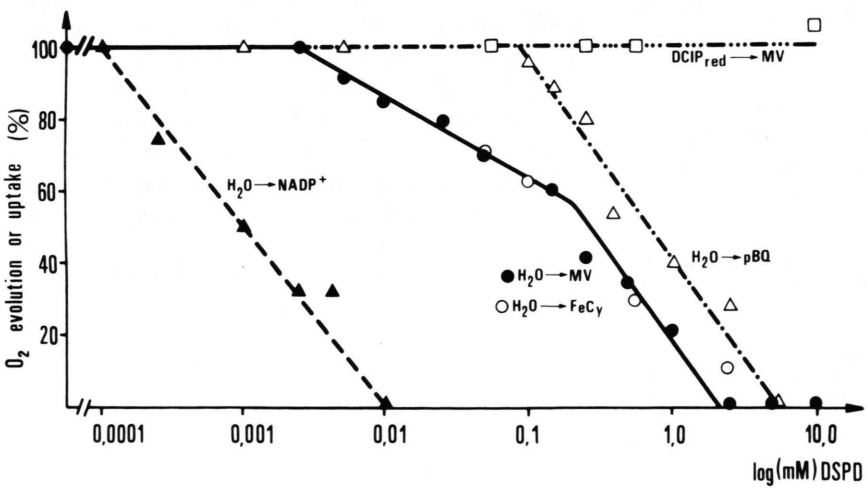

Fig. 1. Effects of DSPD on photosynthetic electron transport. The rate of O_2 evolution and uptake versus the concentration of DSPD is given as a percentage of the values obtained by the untreated controls.

Results and Discussion

It has been shown previously that DSPD inhibits potosynthetic electron transport at the level of ferredoxin [1–5]. In agreement with these observations we found that DSPD strongly inhibited electron flow from $H_2O \rightarrow NADP^+$ in the concentration range from 0.1 to 10 μM. However, electron flow from $H_2O \rightarrow$ MV/FeCy was practically unaffected by the same range of inhibitor concentrations (Fig. 1).

Higher concentrations of DSPD inhibited electron flow from $H_2O \rightarrow$ MV/FeCy but had no effect on electron transport from $H_2O \rightarrow$ pBQ. These results suggest that DSPD has a second inhibitory site between the two photosystems and confirm the recent work of Laasch *et al.*, who showed that DSPD is an effective inhibitor of electron transport before or at PC [6].

Electron flow from $DCIP_{red} \rightarrow$ MV was not inhibited, even by very high DSPD concentrations. Since the redox potential of DCIP is more negative than that of P 700, it can donate electrons directly to P 700 bypassing PC [16, 17]. This fact explains why electron flow from $DCIP_{red} \rightarrow$ MV was not inhibited by DSPD. Increasing the concentration of DSPD to over 0.1 mM caused inhibition of electron flow from $H_2O \rightarrow$ pBQ with a concomitant inhibition of the reaction from $H_2O \rightarrow$ MV/FeCy. The inhibition of the reaction from $H_2O \rightarrow$ pBQ suggests that DSPD also blocks electron transport somewhere in PS II.

Depending on its redox state HQNO has two different modes of action. The inhibition of electron flow in PS II by HQNO is illustrated in Fig. 2. At low concentrations (0.6–6 μM), both oxidized and reduced HQNO inhibited electron flow from $H_2O \rightarrow$ pBQ and $H_2O \rightarrow$ MV/FeCy. This is in accordance with earlier results demonstrating that HQNO blocks electron transport in PS II and that its site of action is similar to that of DCMU [7–9]. On the other hand Izawa *et al.* have reported that the sites of action of DCMU and HQNO are different [10]. They proposed that HQNO blocks electron transport behind the site of action of DCMU.

Since both DSPD and HQNO inhibited electron transport in PS II, we tried to localize their sites of action by thermoluminescence measurements. Thermoluminescence originates from PS II [18–21] and the bands of the glow curve can be related to the different components of the electron transport chain. This method offers a good opportunity to localize the sites of these inhibitors of PS II. DCMU treated chloroplasts exhibit a main thermoluminescence band at about +10 °C [21]. In the presence of 65%

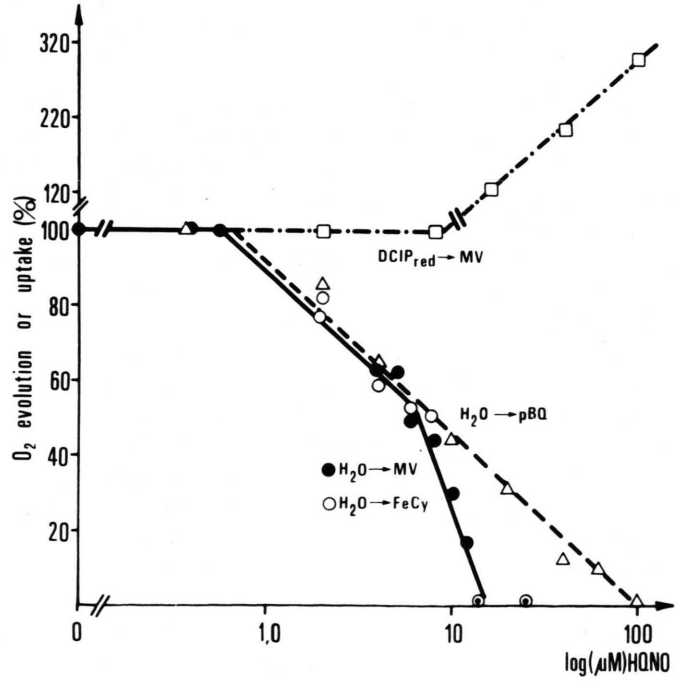

Fig. 2. Effects of HQNO on photosynthetic electron transport. Measuring conditions as in Fig. 1.

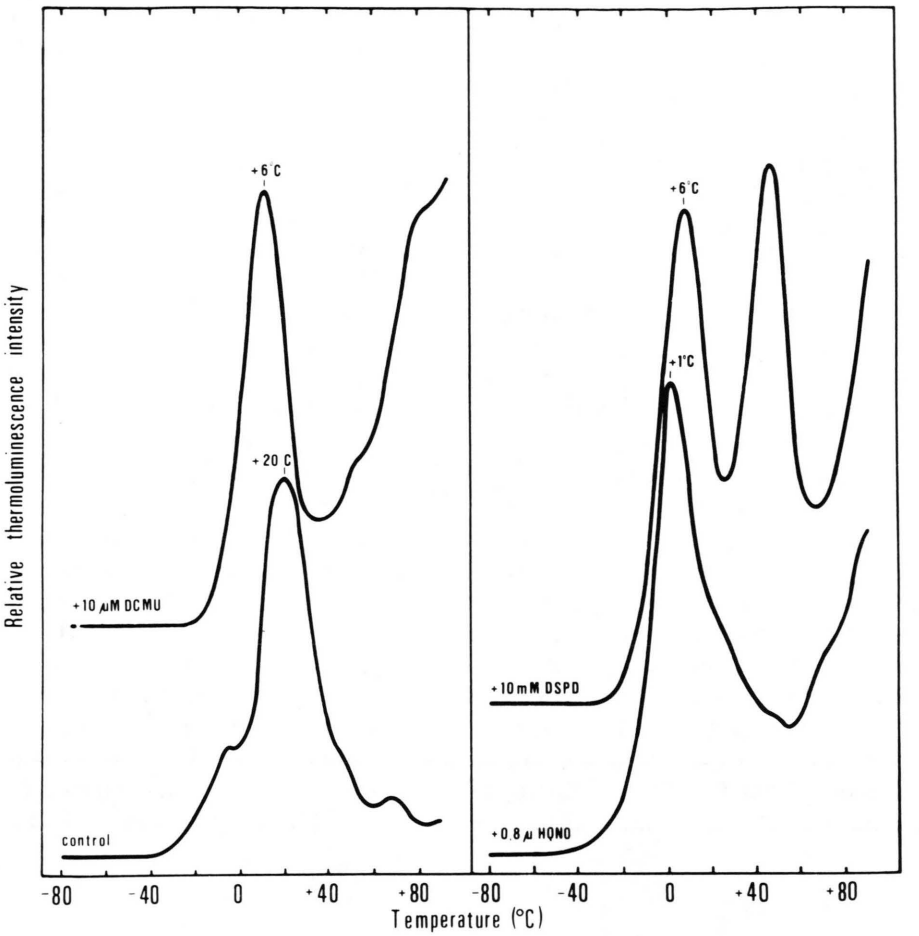

Fig. 3. Effect of DCMU, DSPD and HQNO on the thermoluminescence of isolated chloroplasts. Details of measurements are given in Materials and Methods.

glycerol (to prevent the sample from solid-liquid phase transition) this single band appears at +6 °C (Fig. 3). High concentrations of DSPD produced a similar effect on thermoluminescence (one main band at +6 °C), indicating that one of its sites of inhibition is located in PS II, probably at the same site also inhibited by DCMU. The other main thermoluminescence band at +45 °C might be due to a secondary reaction caused by the autooxidazible character of DSPD [6, 22]. The shape of the glow curve of HQNO treated chloroplasts was similar to that of DCMU treated chloroplasts, though there was a shift in the peak position of the main band from +6 °C to +1 °C. At constant half-band width, a shift in the peak position of a thermoluminescence band to lower temperatures represents a lowering of activation energy of the band [19]. The decrease of the activation energy of a single band can be explained in two different ways: a) HQNO forms a complex with the "R" component of the electron transport chain [23] which is different from that formed between DCMU and "R" or b) the action site of HQNO is closer to the reaction center of PS II than the inhibitory site of DCMU. Therefore, in contrast to the proposal of Izawa *et al.* [10], we conclude that the inhibitory site of HQNO is probably located before the action site of DCMU.

Higher concentrations of oxidized HQNO inhibited electron flow from $H_2O \rightarrow$ MV/FeCy more strongly than electron flow from $H_2O \rightarrow$ pBQ (Fig. 2) thus suggesting that another inhibitory site of HQNO may exist after PQ in the non-cyclic electron transport chain. The dark decay of the P 700 signal was not influenced by the addition of oxidized HQNO. Comparing the effects of oxidized HQNO as studied by oxygen evolution/uptake, and P 700 measurements, we conclude that the new inhibitory site of HQNO is located between PQ and P 700.

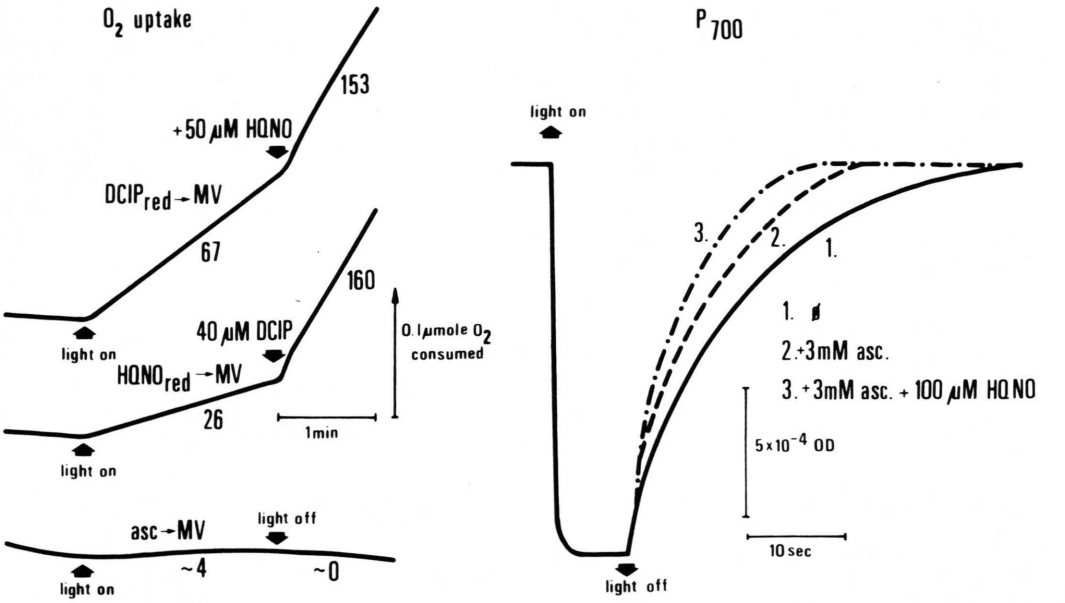

Fig. 4. Effect of reduced HQNO on the linear electron transport around PS I and the light induced P 700 signal. Numbers on the traces of oxygen uptake measurements represent electron transport activity in μmol O_2 consumed/mg Chl/h. In all measurements of PS I reactions 2 μM DCMU was added to the samples.

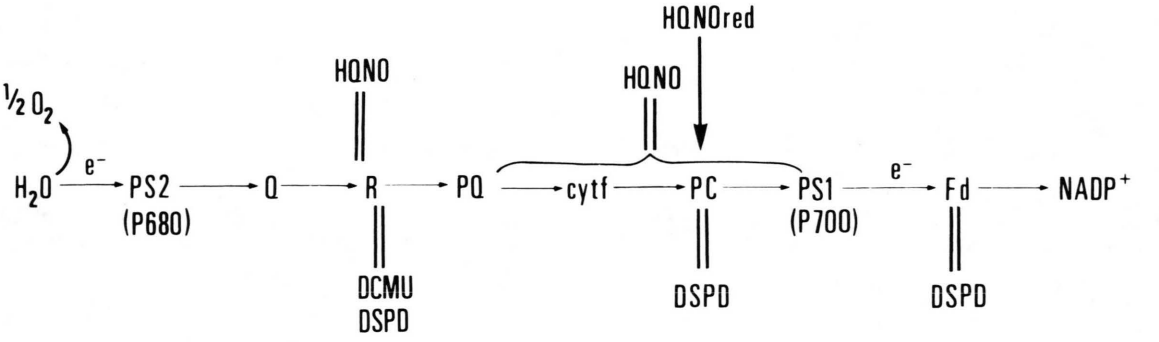

Fig. 5. Possible sites of action of DSPD and HQNO in the photosynthetic electron transport chain.

Oxidized HQNO (14 μM) completely inhibited electron transport from H_2O → FeCy (Fig. 2). However, 30% of the electron transport activity remained (as measured by the H_2O → MV reaction – not shown in Fig. 2) after addition of reduced HQNO at the same concentration. This difference shows that the remaining 30% activity in the reaction from H_2O → MV was probably due to a Mehler type reaction driven by PS I. High concentrations of reduced HQNO increased the rate of electron flow from DCIP + asc → MV (Fig. 2).

This acceleration of electron flow in PS I by reduced HQNO was further investigated as shown in Fig. 4. In the presence of DCMU, HQNO, reduced by ascorbate or dithionite, was able to donate electrons to MV, but the rate of electron flow was relatively low compared to the rate of electron transport from $DCIP_{red}$ → MV. Ascorbate alone is only a very weak electron donor to PS I [24]. This suggests that the reduced HQNO itself was the effective agent in the PS I reaction. This observation was confirmed by the measurement of the light induced P 700

signal (Fig. 4). Dark decay of the signal was more markedly accelerated by addition of reduced HQNO than by addition of asc alone. The results obtained by measurement of both oxygen uptake and P 700 suggest that reduced HQNO has a "donor-mediator" function in electron transport before P 700. Since reduced HQNO inhibited electron flow from $H_2O \rightarrow$ pBQ we conclude that the site receiving electrons from HQNO is located between PQ and P 700.

Summarizing our results a scheme is presented in Fig. 5 which illustrates the possible sites of action of both DSPD and HQNO in the photosynthetic electron transport chain. According to this scheme, DSPD has three inhibitory sites at the levels of Fd, PC and the R component of PS II, respectively. HQNO has two inhibitory sites in the non-cyclic electron transport chain. In PS II, its site of action is different from but close to that of DCMU. The other inhibitory site is located between PQ and P 700. The reduced form of HQNO can act as an electron donor to PS I.

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