Diethyldithiocarbamate, a New Photosystem I Electron Donor of Mehler-Type Hill Reactions*

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Diethyldithiocarbamate, Photoreductant, Electron Donor, Ascorbate, Superoxide Dismutase

Diethyldithiocarbamate (DEDTC) does not accept electrons from the photosynthetic electron transport (PET) but can donate electrons to a photosystem I (PSI) Mehler reaction in the presence of the following PET inhibitors: DCMU, DBMIB, and bathophenanthroline. It cannot photoreduce PSI in the presence of cyanide, a PET inhibitor. These data indicate that the site of electron donation is after the plastoquinone pool. Ascorbate is not required for the ability of DEDTC to donate electrons to PSI. There is no photoreductant activity by DEDTC in ferredoxin/NADP Hill reactions. Superoxide dismutase inhibits DEDTC/DCMU or bathophenanthroline → methylviologen/O_2 Mehler reaction. Catalase does not recover the consumed O_2 from a DEDTC/DCMU → methylviologen/O_2 Mehler reaction, indicating O_2^- has not been dismutating into H_2O_2. These results indicate that superoxide is required for DEDTC ability to donate electrons, therefore DEDTC is limited only to Mehler-type reactions.

Introduction

The use of artificial electron donors and acceptors has been very beneficial in the study of the chloroplast electron transport system [1, 2]. They have been used in elucidating the sequence of electron transport components, the energy conserving steps, and the topography of the chloroplast membrane. These compounds are also used to locate sites of inhibition by xenobiotics and to develop assays for isolated chloroplasts components [1, 2].

The first artificial electron donor introduced was dichlorophenolindolephenol (DCPIP) [3], which donates electrons to photosystem I. Other suitable PSI electron donors include the substituted phenylenediamines [4–6], indamines [7], diaminobenzidine [8, 9], and durohydroquinone [10, 11]. With the exception of durohydroquinone, the above photoreductants require ascorbate as an electron reservoir, which is advantageous in that only catalytic amounts of the donors are required. However, ascorbate will enhance Mehler reactions through superoxide radical (O_2^-)-dependent oxidations of ascorbate [12–14]. Normally $2O_2^-$ will dismutate to $H_2O_2 + O_2$, but in the presence of ascorbate, $2O_2^-$ are reduced to $2H_2O_2$, therefore stimulating the rates. In addition, ascorbate will reduce catalytic amounts of transition metals (*i.e.* Fe^{3+}), thereby increasing the rate of O_2 uptake through a Fenton reaction: Fe^{3+} + Asc → Fe^{2+} + H_2O_2 → Fe^{3+} + OH^- + OH· [15]. The addition of superoxide dismutase (SOD) eliminates the side reactions of O_2^- with ascorbate.

However, SOD does not eliminate the stimulatory effects of transition metals, reduced by ascorbate, on Mehler type reactions. Therefore, it would be advantageous to use electron donors that do not require ascorbate when studying Mehler type reactions (reduction of $O_2 \rightarrow O_2^- \rightarrow H_2O_2$). Durohydroquinone is such a donor, but it is susceptible to air oxidation and is only stable for short term experiments (3–4 min) [10, 11]. It is also sensitive to oxidations by the O_2^- radical making the addition of SOD necessary [10].

It would be of interest to find a PSI electron donor for Mehler type reactions that does not require ascorbate and would not be impaired by O_2^-. A substituted carbamate compound (*p*-nitroacetophenoxime

Abbreviations: DEDTC, diethyldithiocarbamate; MV, methylviologen; DCMU, 3,4-dichlorophenyl-N,N′-dimethylurea; KFeCN, potassium ferricyanide; MES, [2-(N-morpholino)-ethanesulfonic acid]; HEPES, [N-2-hydroxyethyl piperazine-N′-2-ethane sulfonic acid]; DBMIB, dibromthymoquinone; HCN, cyanic acid; BPT, bathophenanthroline; PET, photosynthetic electron transport; PSI or PSII, photosystem I or II; Fd, ferredoxin; PCN, plastocyanin; cyt f, cytochrome f; Fe-S, iron-sulphur; PQ, plastoquinone; Chl, chlorophyll; Asc, ascorbate; SOD, superoxide dismutase; hv, light.

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n-methylcarbamate) was recently reported to function as a PSI electron acceptor [17]. Electron acceptors can often be electron donors if they are in a reduced state [1]. The potential activity of the diethyldithiocarbamate (DEDTC) as an electron donor or acceptor in photosynthesis has not been studied. Diethyldithiocarbamic acid (DEDTC), a copper chelator, is often used for spectrophotometric analysis and separations of trace metals [16] and inhibition of copper containing-enzymes such as polyphenol oxidase [19]. The objectives of this study were to determine (a) whether DEDTC may serve as an electron donor to PSI, (b) whether mediation of electron transport at PSI by DEDTC requires the presence of ascorbate and (c) if O_2^- is detrimental to DEDTC activity, as an electron donor.

Materials and Methods

Peas (*Pisum sativum* cv 'Little Marvel') were grown in a soilless mixture of 1:2:2 of peat:vermiculite:weblite, plus a slow release fertilizer (Osmocote®). Plants were kept in a growth chamber with a 12 h light/dark cycle at temperatures of 20 °C day/17 °C night. The light intensity was varied during the day cycle, starting with 40 $\mu E \cdot m^{-2} \cdot sec^{-1}$ and gradually increasing for 3 h to a maximum intensity of 800 $\mu E \cdot m^{-2} \cdot sec^{-1}$, which was held constant for 1 h. Then this process was reversed. After 4 weeks of growth in this environment, pea plants were used for chloroplast isolation.

Chloroplasts were isolated as follows: leaves were gathered from 5–7 plants and macerated for 5 sec in a partially frozen extraction medium (75 ml) containing 330 mM sorbitol, 5 mM $MgCl_2$, 20 mM MES-NaOH/pH 6.5. The homogenate was filtered through one layer of Miracloth and centrifuged for 1 min at $2{,}000 \times g$ at 0 °C. The pellet was resuspended in a 1:20 dilution of the extraction medium (10 ml) then centrifuged for 1 min at $4{,}000 \times g$ at 0 °C. The pellet was resuspended in an assay medium containing 330 mM sorbitol, 50 mM HEPES-NaOH/pH 7.6, 2 mM $MgCl_2$, 1 mM NH_4Cl, and 2 mM EDTA.

Hill reaction rates were determined by monitoring changes in O_2 concentration as a function of time using a Gilson-Oxygraph Clark-type oxygen electrode. Assay volumes, light intensities at the surface of the reaction vessel and the assay temperature were 1.5 ml, 2000 $\mu E \cdot m^{-2} \cdot sec^{-1}$, and 20 °C, respectively. All chemicals were from Sigma Chemical Company, except for sorbitol and MES which were from Calbiochem. Chlorophyll concentrations were determined according to the methods of Arnon [18] and usually ranged from 35–50 μg Chl per assay.

Results and Discussion

Diethyldithiocarbamate (DEDTC) was found to be unable to accept electrons in a chloroplast preparation containing no exogenous electron acceptors. DEDTC reverses the inhibition of a methylviologen (MV)-mediated Mehler reaction caused by DCMU (Fig. 1, Table I), DBMIB (Table I), and BPT (Table I). The inhibition of a MV-mediated Mehler reaction by HCN is not alleviated by DEDTC (Table I). The photosynthetic electron transport (PET) inhibitor, DCMU, blocks electron flow at the Q_B binding site of PSII [20]. DBMIB inhibits PET at the oxidizing side of the plastoquinone pool [21, 22], while bathophenanthroline (BPT) inhibits electron

Table I. The effects of DEDTC on Mehler reaction rates in the presence of various PET inhibitors.

Control rates $H_2O \rightarrow MV/O_2$ [$-\mu mol\ O_2 \cdot min^{-1} \cdot mg\ Chl^{-1}$]	Inhibitor	Experimental rates DEDTC + Inhibitor $\rightarrow MV/O_2$ [$-\mu mol\ O_2 \cdot min^{-1} \cdot mg\ Chl^{-1}$]
4.54 ± 0.26	DCMU	3.82 ± 0.36
2.42 ± 0.51	HCN	0.29 ± 0.46
2.87 ± 0.13	DBMIB	1.11 ± 0.31
3.43 ± 0.14	BPT	3.68 ± 0.20

Experimental conditions were as described in Materials and Methods. Mehler reaction rates in the presence of the respective inhibitor alone (−DEDTC) were zero. Concentrations of DEDTC, MV, DCMU, HCN, DBMIB, and BPT were 10 mM, 100 μM, 10 μM, 50 mM, 10 μM, and 50 μM, respectively. Each datum represents an average of at least 3 replicates ± standard deviation.

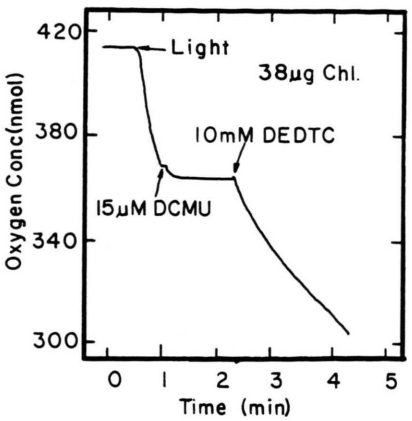

Fig. 1. Oxygen electrode trace of a DEDTC/DCMU → MV/O_2 Mehler reaction. Experimental conditions were as described in Materials and Methods. Concentration of MV was 100 µM. This experiment was repeated at least three times, giving the same results.

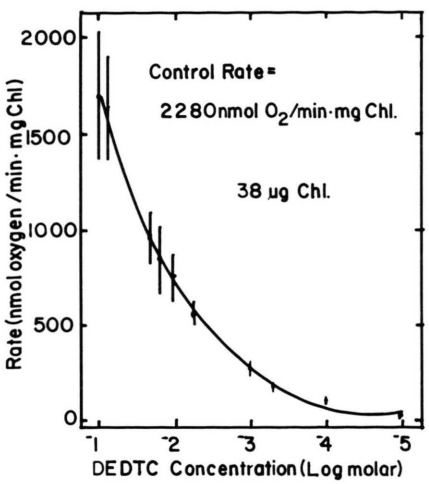

Fig. 2. The effects of various DEDTC concentrations on DEDTC/DCMU → MV/O_2 Mehler reaction rates. Experimental conditions were as described in Materials and Methods. Each datum represents an average of three replicates. The error bars are the standard deviation of the three replicates. Concentrations of DEDTC, MV, and DCMU were 10 mM, 100 µM, and 15 µM, respectively.

flow at Cyt *f* or the "Rieske" Fe-S center [23, 24]. HCN blocks electron flow at plastocyanin (PCN) [25, 26]. The reversal of inhibitions at the Q_B binding site, Cyt *f*, and oxidizing side of PQ but not PCN with the addition of DEDTC, indicates that DEDTC donates electrons after the PQ pool. The rate of a DEDTC/DBMIB → MV/O_2 Mehler reaction is less than reactions containing DCMU or BPT (Table I). Thio-compounds are known to interfere with DBMIB inhibition of PET [27], which means DBMIB could interfere with the activity of thio-compounds such as DEDTC. Ascorbate was not required and DEDTC was stable for many days. This makes DEDTC valuable when electron transport studies of a Mehler reaction must be done in the absence of ascorbate.

The apparent PET rate should double when H_2O is replaced with DEDTC since two electrons from H_2O will cause the consumption of ½ O_2 into H_2O_2 [13], while two electrons from DEDTC will have a net consumption of 1 O_2 into H_2O_2. However, when DEDTC replaces H_2O as an electron donor, the rate does not double at 100 mM DEDTC (Table I). The concentration of DEDTC in Table I has been found to be saturating for these experiments. An exponential increase in the rate of the Mehler reaction as a function of the DEDTC concentrations was observed (Fig. 2). This biphasic response suggests that a second factor may be involved in the photoreduction of PSI by DEDTC, particularly at lower concentrations.

One such factor may be O_2^-. Durohydroquinone, a PSI photoreductant is an example of a donor which requires no ascorbate but is sensitive to the O_2^- radical [10]. Superoxide inhibits the overall durohydroquinone → MV/O_2 reaction, and SOD reverses the inhibition. When SOD is added to a DEDTC/DCMU → MV/O_2 or DEDTC/BPT → MV/O_2 reaction (data not shown), the reaction was unexpectedly inhibited

Table II. The effects of SOD and copper on DEDTC + DCMU → MV/O_2 Mehler reactions.

DEDTC Conc. [mM]	Rate [$-\mu mol\ O_2 \cdot min^{-1} \cdot mg\ Chl^{-1}$]	
	−SOD	+SOD
0	4.30 ± 0.64	4.30 ± 0.64
10	0.36 ± 0.04	0.00 ± 0.00
100	2.93 ± 0.21	1.52 ± 0.18
	$-CuCl_2$	$+CuCl_2$
10	0.84 ± 0.10	1.16 ± 0.19
	$-CuSO_4$	$+CuSO_4$
10	0.91 ± 0.24	0.80 ± 0.22

Experimental conditions were as described in Materials and Methods. Concentrations of SOD, MV, DCMU, $CuCl_2$ and $CuSO_4$ were 500 units, 100 µM, 10 µM, 100 µM and 100 µM, respectively. Each datum represents the average of 3 replicates ± standard deviation.

(Table II). This shows that O_2^- was not inhibiting these reactions, and does not explain the reduction in the predicted rate. In contrast, it appears that O_2^- is essential for DEDTC photoreduction abilities. The possibility that DEDTC chelates with the copper from SOD and the Cu-DEDTC chelate becomes ineffective was considered. Data in Table II show that additions of copper salts ($CuCl_2$ & $CuSO_4$) did not affect DEDTC's efficacy to donate electrons to PSI, ruling out the above hypothesis.

If O_2^- is essential for the efficacy of DEDTC to donate electrons to PSI, one can predict that DEDTC will not donate electrons to a $H_2O \rightarrow$ Fd/NADP reaction, since no superoxide is produced in this reaction. DEDTC did not reverse the inhibitory effects of DCMU on a $H_2O \rightarrow$ Fd/NADP reaction (Fig. 3). This supports further the hypothesis that O_2^- is essential for DEDTC's ability to donate electrons. The uptake of oxygen, exhibited in Fig. 3 after the addition of DEDTC is due to the ability of the chloroplasts to reduce oxygen in the absence of an exogenous electron acceptor (Fig. 4) and DEDTC can reverse the inhibition of this reaction by DCMU. The assay chamber was washed several times with 10% Micro-cleaner (International Products Corp.), to assure that MV contamination was not a reason for the results observed in Fig. 4.

If O_2^- is required for the reduction of DEDTC, very little dismutation of O_2^- to H_2O can take place. The use of catalase can determine how much H_2O_2 is present. In a $H_2O \rightarrow MV/O_2$ Mehler reaction, catalase liberated 100% of the O_2 incorporated into H_2O_2. While in a DEDTC $\rightarrow MV/O_2$ Mehler reaction, only 14 ± 7% of the O_2 was recovered. This indicates that H_2O_2 is not being formed and is probably the result of O_2^- donating electrons to DEDTC as expressed in Eqn. (1) and (2).

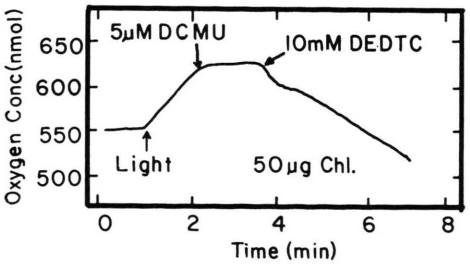

Fig. 3. Oxygen electrode trace of a DEDTC/DCMU → Fd/NADP Hill reaction. Experimental conditions were as described in Materials and Methods. Concentrations of Fd and NADP were 50 μM and 1 mM, respectively. This experiment was repeated three times, giving the same results.

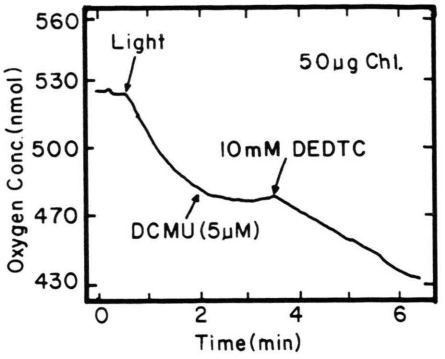

Fig. 4. Oxygen electrode trace of a chloroplast preparation that contained no exogenous electron acceptors. Experimental conditions were as described in Materials and Methods. This experiment was repeated three times, giving the same results.

$$h\nu + DEDTC_{red} \rightarrow DEDTC_{ox} + 2e^- \quad (1)$$

$$DEDTC_{ox} + 2O_2^- \rightarrow DEDTC_{red} \quad (2)$$

At lower concentrations of DEDTC, it becomes essential that O_2^- reduces the oxidized DEDTC, while at higher concentrations an excess of reduced DEDTC minimizes the need for O_2^-. This allows O_2^- to undergo normal dismutation which gives a greater apparent electron transport rate as observed in Fig. 2. The implications of Eqn. (1) and (2) suggest that the redox state of DEDTC is dependent on the presence or absence of O_2^-.

Absorption spectra of reduced and oxidized DEDTC are given in Fig. 5. At a wavelength of 235 nm, a large change in absorbance is observed between the oxidized and reduced states of DEDTC. Therefore, the effects of O_2^- on the redox state of DEDTC can be determined by monitoring absorbance changes at 235 nm. In a DEDTC/DCMU → MV/O_2 reaction, O_2^- is produced and DEDTC, according to Eqn. (2), will predominantly be in the reduced state. The addition of SOD to this reaction will remove the O_2^- and the oxidized DEDTC will remain in this redox state (Eqn. (1)).

A DEDTC/DCMU → MV/O_2 reaction was conducted in the absence and presence of SOD for seven minutes. Following the reaction, the chloroplasts were centrifuged out of the assay medium. An aliquot of the supernatant was taken for an absorbance measurement at 235 nm. In the absence and pres-

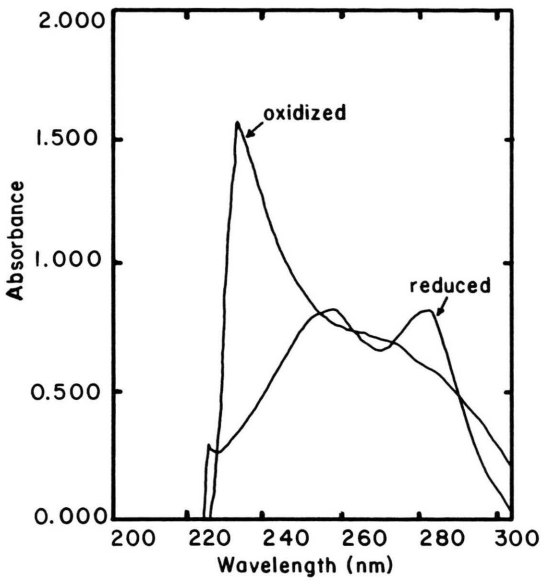

Fig. 5. Absorption spectra of reduced and oxidized DEDTC. The reduced DEDTC was gased with nitrogen for 20 minutes and the oxidized state of DEDTC was maintained with excess KFeCN. A Beckman Du-6 spectrophotometer was used. Blanks contained the assay medium (+ KFeCN for oxidized DEDTC curve). Concentrations of DEDTC and KFeCN were 100 μM and 150 μM, respectively. This experiment was repeated three times, giving the same results.

ence of SOD, an absorbance of 0.036 and 0.152 was obtained, respectively. This increase of absorbance in the absence of O_2^- (presence of SOD) indicates the oxidation of DEDTC as predicted in Eqn. (1). The absorbance of the supernatant of the control (DEDTC added to chloroplasts in the dark) was 0.063. Therefore, the absorbance reading of 0.036 which was from chloroplasts reacted in the presence of O_2^- (absence of SOD) indicated that the DEDTC was in the reduced state as predicted in Eqn. (2). The above experiments were repeated and absorbance readings were taken at 255 nm. At 255 nm there should be no change in absorbance between the oxidized and reduced states of DEDTC (Fig. 5). The resulting absorbance measurements at 255 nm were 0.079, 0.080, 0.079 for the control, −SOD, +SOD, respectively. The DEDTC purchased from Sigma Chemical Co. was 99% pure. The 1% impurities were mainly inorganic compounds. This makes it highly unlikely that an impurity is responsible for the observed responses in Table I and Figs. 1 and 2.

DEDTC will not replace or become more important than existing PSI reductants but it should be of use in many photochemical studies with chloroplasts particularly in experiments that require the absence of ascorbate. Furthermore, DEDTC is a stable molecule and could facilitate chloroplast experiments which require a more extensive time period than can be accomplished with durohydroquinone.

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